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Gastrointestinal Antigen Processing and its Relevance to Enteric Vaccine Delivery in Rainbow Trout, *Oncorhynchus mykiss* (Walbaum, 1792)

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**Gastrointestinal Antigen Processing and its Relevance to
Enteric Vaccine Delivery in Rainbow Trout,
Oncorhynchus mykiss (Walbaum, 1792)**

by

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A thesis submitted to the University of Plymouth in
partial fulfilment of the requirements for the degree of.

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ABSTRACT

An investigation of antigen processing in the rainbow trout gastrointestinal tract was carried out to provide a rational basis for the design of oral delivery systems for protein antigens. Using *in vitro* systems involving isolated luminal enzymes and gut cell suspensions the degradation of human gamma globulin (HGG) and bovine serum albumin (BSA) was analysed by Western blotting and laser densitometry. Proteolysis by luminal enzymes was dependant on pH and temperature and serine proteases were found to be partly responsible for antigen degradation in the intestine. The extent of intracellular proteolysis depended on the antigen used and on the gut region from which the cells were isolated. To test the predictive value of results obtained from the *in vitro* studies, the processing of HGG in the digestive tract after oral administration was investigated. The findings indicated that different regions of the gut perform distinct but complementary roles in proteolysis. Measurement of the uptake of HGG into the bloodstream of these fish by enzyme linked immunosorbent assay (ELISA) and Western blotting indicated that the nature of proteins absorbed from the gut could be influenced by altering the conditions in the gastrointestinal tract. After parenteral and oral immunisation of HGG the antibody response was investigated in plasma and in mucosal and biliary secretions and found that a fragment of HGG produced by partial digestion with intestinal enzymes was highly antigenic in trout. The methods developed to study antigen processing in the gut were applied to assess the potential value of modern enteric delivery systems in teleosts. Encapsulation of HGG in poly lactide-co-glycolide (PLG) microparticles partially protected HGG from degradation in the gut and increased its absorption into the bloodstream. A live attenuated strain of *Aeromonas salmonicida* was shown to adhere to and invade isolated trout enterocytes and Atlantic Salmon tissue culture cells using a range of light - and electron microscopical techniques. These results indicate that an investigation into antigen processing by the gut is a valuable preliminary step in the formulation of oral delivery systems for teleosts.

All experimental work involving animals was carried out under personal Home Office Licence number 30/02757

LIST OF CONTENTS

Copyright statement	1
Title page	2
Abstract	3
Licence	4
List of contents	5
List of Figures	12
List of Tables	16
Acknowledgement	22
Declaration	23
List of abbreviations	25
CHAPTER 1: GENERAL INTRODUCTION	28
CHAPTER 2: LITERATURE REVIEW	35
2.1: Non-specific gastrointestinal defenses in mammals.. . . .	36
2.1.1: Non-specific protection associated with normal gastrointestinal physiology . . .	36
2.1.2: Gastrointestinal barriers of non-epithelial origin	37
2.1.3: Non-specific humoral defense in the mammalian gut	38
2.1.4: The microenvironment overlying the gastrointestinal epithelium	39
2.1.5: Lumenal barriers of epithelial origin	41
2.1.6: Non-specific cellular barriers in the gastrointestinal	43
2.1.7: Non-specific mucosal defense in teleosts	45
2.1.8: The gut microflora of teleosts	47
2.2: Absorption of materials across the gastrointestinal	48
2.2.1: Uptake of macromolecules by the gut	48
2.2.2: Transcellular absorption pathways	49
2.2.3: Uptake of inert particulate materials across the digestive tract	51
2.3: Specific defense in mammalian mucosa-associated lymphoid tissue (MALT)	52

2.3.1: Morphological and cellular aspects of MALT	53
2.3.2: Specific secretory IgA (S-IgA) mediated humoral defense at the mucosae	58
2.3.3: Antibody isotypes other than IgA in MALT	62
2.3.4: The role of cytotoxic T cells in MALT	63
2.3.5: Regulation of specific immune responses in MALT	67
2.4: The nature and functions of MALT in teleosts	69
2.4.1: Morphological and cellular aspects of teleost MALT	69
2.4.2: Evidence for a common mucosal immune system in teleosts	73
2.5: Immunological roles of epithelial cells in the gut	75
2.6: Oral tolerance	77
2.7: Oral vaccination	83

CHAPTER 3: IN VITRO ANALYSIS OF SOLUBLE PROTEIN ANTIGEN

PROCESSING BY RAINBOW TROUT LUMENAL ENZYMES.	86
3.1: INTRODUCTION	87
3.2: MATERIALS AND METHODS	91
3.2.1: Animals	91
3.2.2: Isolation and preparation of lumenal enzyme samples.	91
3.2.3: Determination of protein content of enzyme solutions.	91
3.2.4: Optimisation of conditions for analysing proteolysis.	92
3.2.5: Time course of antigen degradation by proteases.	92
3.2.6: Effect of temperature on antigen degradation.	92
3.2.7: Effect of pH on antigen degradation.	93
3.2.8: Influence of a range of potential inhibitors on antigen degradation.	93
3.2.9: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS - PAGE).	93
3.2.10: Gel staining for total protein.	94
3.2.11: SDS - PAGE with protein substrate incorporated.	94
3.2.12: Western blotting.	95

3.2.13	<i>Laser densitometry.</i>	95
3.3:	RESULTS	97
3.3.1:	<i>Estimation of protein content and molecular weights of enzymes present in gastric and intestinal secretions.</i>	97
3.3.2:	<i>Densitometric analysis of the dynamics of antigen processing by luminal enzymes.</i>	97
3.3.3:	<i>Time courses of antigen degradation by gastrointestinal enzymes.</i>	97
3.3.4:	<i>Temperature dependence of antigen proteolysis.</i>	98
3.3.5:	<i>pH dependence of degradation.</i>	99
3.3.6:	<i>Effects of inhibitors on antigen degradation.</i>	99
3.4:	DISCUSSION	121
CHAPTER 4: CELLULAR PROCESSING OF SOLUBLE PROTEIN ANTIGENS BY ISOLATED RAINBOW TROUT INTESTINAL CELLS IN VITRO.		129
4.1:	INTRODUCTION	130
4.2:	MATERIALS AND METHODS	133
4.2.1:	<i>Animals</i>	133
4.2.2:	<i>Isolation and preparation of intestinal tissue.</i>	133
4.2.3:	<i>Isolation of intestinal cells.</i>	133
4.2.4:	<i>Assessment of cell viability.</i>	134
4.2.5:	<i>Microscopic analysis of intestinal cells.</i>	134
4.2.6:	<i>Determination of protein concentration.</i>	134
4.2.7:	<i>Analysis of antigen processing by intestinal cells.</i>	134
4.2.8:	<i>Analysis of the influence of specific inhibitors on antigen processing.</i>	134
4.2.9:	<i>Analysis of antigen processing by cells from individual regions of the gut.</i>	135
4.2.10:	<i>SDS-PAGE, Western blotting and densitometry.</i>	135
4.3:	RESULTS	136
4.3.1:	<i>Viability of isolated intestinal cells.</i>	136

4.3.2:	<i>Intracellular processing of soluble protein antigens by isolated intestinal cells</i>	136
4.3.3:	<i>Analysis of supernatants from cells after incubation with HGG or BSA.</i>	136
4.3.4:	<i>Comparative analysis of intracellular processing of BSA and HGG by cells isolated from various gut regions.</i>	136
4.3.5:	<i>Comparative analysis of supernatants from cells isolated from various gut regions after incubation with BSA or HGG.</i>	137
4.3.6:	<i>Analysis of the effects of a range of specific inhibitors on intracellular processing of BSA and HGG.</i>	137
4.3.7:	<i>Analysis of the effects of a range of potential inhibitors on the nature of BSA and HGG detected in supernatants.</i>	138
4.4:	DISCUSSION	153

CHAPTER 5: AN INVESTIGATION INTO THE VALUE OF GASTRIC INHIBITORS

	<i>IN THE ORAL DELIVERY OF SOLUBLE PROTEIN ANTIGENS TO TELEOSTS</i>	162
5.1:	INTRODUCTION	163
5.2:	MATERIALS AND METHODS	166
5.2.1:	<i>Animals</i>	166
5.2.2:	<i>Sample collection</i>	166
5.2.3:	<i>Establishment of optimal doses of HGG and gastric inhibitors for oral administration</i>	167
5.2.4:	<i>Investigation of the effects of gastric inhibitors on the time course of HGG absorption</i>	168
5.2.5:	<i>Enzyme linked immunosorbent assay (ELISA) for HGG detection.</i>	168
5.2.6:	<i>Modified Western blotting methods for detection of HGG.</i>	169
5.2.7:	<i>Image analysis of immunoblots</i>	170
5.2.8:	<i>Statistical analysis of results from ELISA analyses</i>	170
5.3:	RESULTS	173

5.3.1: Dose response to HGG	173
5.3.2: Dose effects of gastric inhibitors on gastrointestinal pH and HGG uptake . . .	173
5.3.3: Effects of gastric inhibitors on a range of parameters over a time course <i>after antigen administration</i>	175

5.4: DISCUSSION	208
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CHAPTER 6: THE NATURE AND KINETICS OF THE SYSTEMIC AND MUCOSAL ANTIBODY PRODUCED TO HGG FOLLOWING ORAL AND PARENTERAL ADMINISTRATION 119

6.1: INTRODUCTION	220
6.2: MATERIALS AND METHODS	224
6.2.1: Animals	224
6.2.2: Immunisation protocol	224
6.2.3: Procedure for the collection of blood and secretions from fish.	224
6.2.4: Production of rabbit antiserum to rainbow trout IgM	226
6.2.5: Determination of the specificity of the rabbit anti-trout IgM antiserum <i>and of the susceptibility of trout IgM to degradation by rainbow trout intestinal</i> <i>proteases.</i>	226
6.2.6: Indirect ELISA for detection of antibody to HGG.	227
6.2.7: Determination of the specificity of rainbow trout antisera in plasma and <i>secretions.</i>	228
6.2.8: Expression of antibody titres and statistical comparison of groups of immunised <i>fish</i>	228
6.3: RESULTS	230
6.3.1 Specificity of the polyclonal rabbit anti-rainbow trout IgM antiserum and <i>susceptibility of trout IgM to rainbow trout intestinal proteases.</i>	230
6.3.2 Detection of antibody to HGG in plasma, bile and mucus	231
6.4: DISCUSSION	249

CHAPTER 7 A PRELIMINARY INVESTIGATION INTO THE USE OF BIODEGRADABLE (POLY-LACTIDE-CO -GLYCOLIDE) MICROPARTICLES FOR THE ORAL IMMUNISATION OF TELEOST FISH.	256
7.1: INTRODUCTION	257
7.2: MATERIALS AND METHODS	261
7.2.1: Animals	261
7.2.2: Preparation and characterisation of PLG (Poly-lactide-co-glycolide) microparticles incorporating HGG.	261
7.2.3: Antigen delivery and collection of samples.	262
7.2.4: Analysis of antigen proteolysis, antigen uptake and antibody responses to HGG.	262
7.3: RESULTS	263
7.3.1: Influence of encapsulation on the retention time and fate of HGG in the gastric region of the trout digestive tract after oral delivery.	263
7.3.2: Effect of encapsulation on the form of HGG detected in the intestines of fish after oral delivery.	263
7.3.3: Effect of encapsulation on the form of HGG detected in the plasma of fish after oral delivery.	264
7.3.4: Antibody responses to soluble and encapsulated HGG in plasma and secretions	264
7.4: DISCUSSION	272
 CHAPTER 8: THE ADHERENCE TO AND INVASION OF SALMONID EPITHELIAL CELLS BY WILD TYPE AND ATTENUATED (ΔaroA) STRAINS OF <i>Aeromonas salmonicida</i>.	 279
8.1: INTRODUCTION	280
8.2: MATERIALS AND METHODS	286
8.2.1: Bacterial strains	286
8.2.2: Bacteriological media and growth conditions	286
8.2.3: Isolation and culture of primary intestinal cells and tissue culture cells	287

8.2.4: Infection of cells with bacteria	287
8.2.5: Transmission electron microscopy (TEM).	288
8.2.6: Scanning electron microscopy (SEM)	288
8.2.7: Light microscopy- Giemsa staining	289
8.2.8: Acridine orange/ crystal violet fluorescence microscopy	289
8.2.9: Specific immunostaining for actin.	290
8.3: RESULTS	291
8.3.1: Interaction of <i>Aeromonas salmonicida</i> strains with rainbow trout enterocytes	
8.3.2: Determination of the viability of intracellular bacteria	291.
8.3.3 The role of actin in the internalisation of bacteria.	292
8.4 DISCUSSION	301
CHAPTER 9: GENERAL DISCUSSION	315
BIBLIOGRAPHY	322
APPENDICES	399

LIST OF FIGURES

CHAPTER 3

3.1: <i>Coomassie Blue stained 11% PAGE gels displaying the time course of HGG degradation by rainbow trout intestinal enzymes.</i>	101
3.2: <i>Densitometric analysis of the intestinal enzyme proteolysis of HGG over a range of temperatures.</i>	102
3.3: <i>Densitometric analysis of the pH dependence of gastric enzyme degradation of HGG</i>	103
3.4: <i>Time course of the proteolysis of HGG by gastric enzymes.</i>	104
3.5: <i>Time course of the proteolysis of BSA by gastric enzymes.</i>	105
3.6: <i>Time course of the proteolysis of HGG by intestinal enzymes.</i>	106
3.7: <i>Time course of the proteolysis of BSA by intestinal enzymes.</i>	107
3.8: <i>Temperature dependence of the proteolysis of HGG by gastric enzymes.</i>	108
3.9: <i>Temperature dependence of the proteolysis of BSA by gastric enzymes.</i>	109
3.10: <i>Temperature dependence of the proteolysis of HGG by intestinal enzymes.</i>	110
3.11: <i>Temperature dependence of the proteolysis of BSA by intestinal enzymes.</i>	111
3.12: <i>pH dependence of the proteolysis of HGG by gastric enzymes.</i>	112
3.13: <i>pH dependence of the proteolysis of BSA by gastric enzymes.</i>	113
3.14: <i>pH dependence of the proteolysis of HGG by intestinal enzymes.</i>	114
3.15: <i>pH dependence of the proteolysis of BSA by intestinal enzymes.</i>	115
3.16: <i>Effects of specific inhibitors on the proteolysis of HGG by gastric enzymes.</i>	116
3.17: <i>Effects of specific inhibitors on the proteolysis of BSA by gastric enzymes.</i>	117
3.18: <i>Effects of specific inhibitors on the proteolysis of HGG by intestinal enzymes.</i>	118
3.19: <i>Effects of specific inhibitors on the proteolysis of BSA by intestinal enzymes.</i>	119
3.20: <i>Western blots demonstrating the effects of various factors on gastric and intestinal enzyme proteolysis of BSA and HGG.</i>	120

CHAPTER 4

4.1: <i>Relationship between cell viability of intestinal cells isolated by collagenase</i>	
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<i>digestion in Leibovitz L-15 medium and time after isolation.</i>	<i>139</i>
4.2: <i>Time course of intracellular processing of HGG by isolated intestinal cells. . . .</i>	<i>140</i>
4.3: <i>Time course of intracellular processing of BSA by isolated intestinal cells. . . .</i>	<i>141</i>
4.4: <i>Analysis of supernatants from cells incubated with HGG over a 2hr time course. . . .</i>	<i>142</i>
4.5: <i>Analysis of supernatants from cells incubated with BSA over a 2hr time course. . . .</i>	<i>143</i>
4.6: <i>Intracellular processing of HGG by cells isolated from various gut regions. . . .</i>	<i>144</i>
4.7: <i>Intracellular processing of BSA by cells isolated from various gut regions. . . .</i>	<i>145</i>
4.8: <i>Analysis of supernatants from cells isolated from various gut regions after a 1hr incubation with HGG.</i>	<i>146</i>
4.9: <i>Analysis of supernatants from cells isolated from various gut regions after a 1hr incubation with BSA.</i>	<i>147</i>
4.10: <i>Influence of a range of inhibitors on intracellular processing of HGG by isolated intestinal cells.</i>	<i>148</i>
4.11: <i>Influence of a range of inhibitors on intracellular processing of BSA by isolated intestinal cells.</i>	<i>149</i>
4.12: <i>Influence of a range of inhibitors on the nature of HGG detected in supernatants from intestinal cells after incubation.</i>	<i>150</i>
4.13: <i>Influence of a range of inhibitors on the nature of BSA detected in supernatants from intestinal cells after incubation.</i>	<i>151</i>
4.14: <i>Western blots showing processing of HGG and BSA by isolated intestinal cells. . . .</i>	<i>152</i>

CHAPTER 5

5.1: <i>Experimental procedures used in the investigation of antigen proteolysis and absorption.</i>	<i>167</i>
5.2.(a): <i>Example of the layout of an ELISA microtitre plate for assessment of plasma HGG levels.</i>	<i>171</i>
(b): <i>Photograph of a representative ELISA microtitre plate demonstrating the presence of HGG in rainbow trout plasma samples.</i>	<i>171</i>

5.3: Derivation of semi - quantitative data from immunoblots by image analysis . . .	172
5.4: HGG dose response measured 1 hr after antigen administration	181
5.5.(a): Dose effect of sodium bicarbonate on gastrointestinal pH	182
(b): Bicarbonate dose response	182
5.6.(a): Dose effect of cimetidine on gastrointestinal pH.	184
(b): Cimetidine dose response	184
5.7: Time course of HGG absorption into plasma	186
5.8.(a): Coomassie Blue stained PAGE gel showing the presence of proteins in plasma samples collected from fish which were orally intubated with 35 mg of sodium bicarbonate 1 hr before administration of 35 mg HGG.	188
(b): Corresponding immunoblot to the PAGE gel in (a) showing the presence of HGG in the plasma.	188
5.9.(a): Effect of bicarbonate (35mg) on gastrointestinal pH	189
(b): Effect of bicarbonate (35mg) on HGG absorption into plasma.	189
5.10: Photographic representations of immunoblots demonstrating the presence of HGG in regions of the rainbow trout gut at various times after the administration of HGG and prior administration of cimetidine	193
5.11.(a): Effect of cimetidine (1mg) on gastrointestinal pH	194
(b): Effect of cimetidine (1mg) on absorption of HGG into plasma	194
5.12: Effect of cimetidine (10mg) on absorption of HGG into plasma	
5.13.(a): Effect of cimetidine (50mg) on gastrointestinal pH	202
(b): Effect of cimetidine (50mg) on absorption of HGG into plasma	202

CHAPTER 6

6.1: Procedure used to screen partially digested HGG with a range of antisera from fish on Western blots.	234
6.2: Specificity of the polyclonal rabbit-anti-trout IgM antiserum and susceptibility of rainbow trout IgM to digestion by intestinal proteases.	235

6.3: <i>Dilution profiles of plasma samples from fish orally or intra-peritoneally immunised with HGG</i>	236
6.4: <i>Western blot showing recognition of HGG fragments produced by intestinal enzyme degradation by antisera from i.p. immunised trout.</i>	237
6.5(a): <i>Anti-HGG antibody titres in plasma from fish immunised orally or i.p with 35 mg HGG.</i>	238
(b): <i>Comparison of anti-HGG titres determined by ELISA with the recognition of a 52 kDa HGG fragment on Western blots</i>	238
6.6: <i>Anti-HGG antibody titres in cutaneous mucus of fish immunised orally or i.p. with 35 mg HGG</i>	240
6.7: <i>Anti-HGG antibody titres in bile of fish immunised orally or i.p. with 35 mg HGG.</i>	242
6.8: <i>Anti-HGG antibody titres in intestinal mucus from fish immunised orally or i.p. with 35 mg HGG.</i>	244
6.9: <i>Anti-HGG antibody titres in gastric mucus from fish immunised orally or i.p. with 35 mg HGG</i>	246

CHAPTER 7

7.1: <i>Scanning electron micrographs of PLG microparticles incorporating HGG.</i> . . .	266
7.2. (a): <i>Western blot demonstrating the presence of HGG in gastric scrapings of rainbow trout over a time course after administration of 5 mg HGG in 0.2 ml PBS.</i> .	267
7.2. (b): <i>Western blot demonstrating the presence of HGG in gastric scrapings of rainbow trout over a time course after administration of 58.4 mg of PLG - encapsulated HGG (containing 5mg antigen)</i>	267
7.3: <i>Effect of microencapsulation on retention time of HGG in the stomach</i>	268
7.4: <i>Antibody levels in plasma after oral immunisation of fish with soluble or encapsulated HGG</i>	271

CHAPTER 8

8.1: Scanning electron micrographs of the interactions between <i>A. salmonicida</i> (Δ aro A) and isolated rainbow trout enterocytes.	294
8.2: Transmission electron micrographs demonstrating the adherence of <i>A. salmonicida</i> (Δ aro A) to isolated rainbow trout enterocytes.	295
8.3: Transmission electron micrographs demonstrating stages in the invasion of isolated rainbow trout enterocytes by <i>Aeromonas salmonicida</i> (Δ aro A).	296
8.4: Transmission electron micrographs showing the presence of internalised <i>A. salmonicida</i> (Δ aro A) bacteria.	297
8.5: Giemsa stained smears of cells after 30 min incubation with bacteria	298
8.6: Smears of enterocytes stained with acridine orange and counterstained with crystal violet after a 15 min incubation with <i>A. salmonicida</i> (Δ aro A).	299
8.7: Indirect immunofluorescent staining of enterocytes before and after infection with <i>A. salmonicida</i> (Δ aro A).	300

LIST OF TABLES

CHAPTER 3

3.1: Proteases found in the gastrointestinal of some teleosts.	90
3.2: Inhibitory/ modulatory agents used in the study of luminal proteases.	96

CHAPTER 4

4.1 : Substances used in analysis of antigen processing by isolated intestinal cells. . .	135
--	-----

CHAPTER 5

5.1: Overview of experimental protocols applied in the study of gastric inhibitors. . .	166
5.2: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in the plasma of fish orally intubated with 35 mg of HGG 1 hr after intubation with various doses of sodium bicarbonate.	183
5.3: Apparent molecular weights (kDa) and relative percentages of HGG bands detected	

<i>in the plasma of fish orally intubated with 35 mg of HGG 1 hr after intubation with various doses of cimetidine.</i>	185
5.4: <i>Apparent molecular weights (kDa) and relative percentages of HGG bands detected in the plasma of fish orally intubated with 35 mg of HGG 1 hr after intubation with control PBS.</i>	187
5.5: <i>Apparent molecular weights (kDa) and relative percentages of HGG bands detected in the plasma of fish orally intubated with 35 mg HGG 1 hr after intubation with 35 mg sodium bicarbonate.</i>	190
5.6: <i>Apparent molecular weights (kDa) and relative percentages of HGG bands detected in gastric scrapings of fish orally intubated with 35 mg HGG 1 hr after 35 mg bicarbonate.</i>	191
5.7: <i>Apparent molecular weights (kDa) and relative percentages of HGG bands detected in scrapings from the intestine of fish orally intubated with 35 mg HGG 1 hr after intubation with 35 mg bicarbonate.</i>	192
5.8: <i>Apparent molecular (kDa) weights and relative percentages of HGG bands detected in the plasma of fish orally intubated with 35 mg HGG 1 hr after intubation with 1 mg cimetidine.</i>	195
5.9: <i>Apparent molecular weights (kDa) and relative percentages of HGG bands detected in gastric scrapings of fish orally intubated with 35 mg HGG 1 hr after intubation with 1 mg cimetidine.</i>	196
5.10: <i>Apparent molecular weights (kDa) and relative percentages of HGG bands detected in scrapings from the pyloric caecae of fish orally intubated with 35 mg HGG 1 hr after intubation with 1 mg cimetidine.</i>	197
5.11: <i>Apparent molecular weights (kDa) and relative percentages of HGG bands detected in the anterior portion of the intestine of fish orally intubated with 35 mg HGG 1 hr after intubation with 1 mg cimetidine.</i>	198
5.12: <i>Apparent molecular weights (kDa) and relative percentages of HGG bands detected in scrapings from the posterior portion of the intestine of fish orally intubated with</i>	

<i>35 mg HGG 1 hr after intubation with 1 mg cimetidine.</i>	<i>199</i>
<i>5.13: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in the plasma of fish orally intubated with 35 mg HGG 1 hr after intubation with 10 mg cimetidine.</i>	<i>201</i>
<i>5.14: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in plasma of fish orally intubated with 35 mg HGG 1 hr after intubation with 50 mg cimetidine.</i>	<i>203</i>
<i>5.15: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in gastric scrapings of fish orally intubated with 35 mg HGG 1 hr after intubation with 50 mg cimetidine.</i>	<i>204</i>
<i>5.16: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in scrapings from pyloric caecae of fish orally intubated with 35 mg HGG 1 hr after 50 mg cimetidine.</i>	<i>205</i>
<i>5.17: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in scrapings from the anterior portion of intestine of fish orally intubated with 35 mg HGG 1 hr after intubation with 50 mg cimetidine.</i>	<i>206</i>
<i>5.18: Apparent molecular weights (kDa) and relative percentages of HGG scrapings from the posterior portion of the intestines of fish orally intubated with 35 mg HGG 1 hr after intubation with 50 mg cimetidine.</i>	<i>207</i>

CHAPTER 6

<i>6.1: HGG fragments produced by partial digestion of HGG by rainbow trout intestinal enzymes which are recognised by antibodies in trout plasma from fish immunised orally or i.p. with 35 mg HGG.</i>	<i>239</i>
<i>6.2: HGG fragments produced by partial digestion of HGG by rainbow trout intestinal enzymes recognised by antibody in cutaneous mucus from fish which were immunised orally or i.p. with 35 mg HGG.</i>	<i>241</i>
<i>6.3: HGG fragments produced by partial digestion of HGG by rainbow trout intestinal enzymes recognised by antibody in bile from fish which were immunised orally or i.p.</i>	

<i>with 35 mg HGG.</i>	243
6.4: <i>HGG fragments produced by partial digestion of HGG by rainbow trout intestinal enzymes recognised by antibody in intestinal mucus from fish which were immunised orally or i.p. with 35 mg HGG.</i>	245
6.5: <i>HGG fragments produced by partial digestion of HGG by rainbow trout intestinal enzymes recognised by antibody in gastric mucus from fish which were immunised orally or i.p. with 35 mg HGG.</i>	247
 CHAPTER 7	
7.1: <i>Apparent molecular weights and relative percentages of HGG bands detected in intestinal scrapings from fish administered either soluble HGG or HGG encapsulated in PLG microparticles.</i>	269
7.2: <i>Apparent molecular weights and relative percentages of HGG bands detected in plasma from fish administered either soluble HGG or HGG encapsulated in PLG microparticles.</i>	270
Table 7.3 <i>Antibody titres measured in intestinal mucus of fish after boosting with soluble HGG.</i>	271

CHAPTER 8

8.1: <i>Overview of methodologies used and results obtained in the investigation of the invasiveness of bacterial species.</i>	293
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I dedicate this thesis to my parents, Eddie and Maureen Lavelle for their love, support and tolerance over the past 25 years.

God guard me from those thoughts men think

In the mind alone;

He that sings a lasting song

Thinks in a marrow-bone

W.B Yeats.

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. . . . *Ní fheicfid a leitheid arts.*

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List of publications and conference contributions

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Lavelle, E.C. and Harris, J. E. (1993). The value of *in vitro* models in the design of oral delivery systems for teleost fish. A paper presented at the first annual congress of the British Society for Immunology. Brighton. England.

Lavelle, E. C., Jenkins, P. G., Collingwood, N. D., Harris, J. E. and Moate, R. M. (1992). Degradation of orally administered protein antigens by gastrointestinal cells and secretions of rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) Submitted for publication in *Advances in Mucosal Immunology*.

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Jenkins, P. G., Lavelle, E. C., Harris, J. E. and Pulsford, A. L. (1992). Aspects of the mucosal immunology of teleosts. Submitted for publication in *Advances in mucosal immunology*

Jenkins, P. G., Barton, R. N., Harris, J. E., Wrathmell, A. B. and Lavelle, E. C. (1992). Enhanced delivery systems for enteric antigen uptake in teleosts. A poster presented at the Vaccines for Enteric Diseases Conference. Cambridge. England.

Barton, R. N., Jenkins, P. G., Harris, J. E., Wrathmell, A. B., Lavelle, E. C., Lauer, S. and New, R. R. C. (1991). The liposomal targeting of enterically delivered antigens in *Oncorhynchus mykiss*. A paper presented at the 5th meeting of the European Association of Fish Pathologists. Budapest. Hungary.

Jenkins, P. G., Barton, R. N., Harris, J. E., Wrathmell, A. B., Lavelle, E. C., Lauer, S. and New, R. R. C. (1991). The oral and intraperitoneal delivery of liposomally encapsulated antigens to rainbow trout *Oncorhynchus mykiss*. A poster presented at the 5th meeting of the European Association of Fish Pathologists. Budapest. Hungary.

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ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
ANOVA	analysis of variance
BHI	brain heart infusion
BSA	bovine serum albumin
°C	degrees centigrade
cAMP	cyclic adenosine monophosphate
CBB	Coomassie Brilliant Blue
CD	cellular determinant
conA	concanavalin A
CT	cholera toxin
CTB	cholera toxin B subunit
DAB	3,3'-diaminobenzidine
CTL	cytotoxic T lymphocyte
DNA	deoxyribonucleic acid
DTH	delayed type hypersensitivity
ECP	extracellular product
EDTA	ethylene diamino tetraacetic acid
EGC	eosinophilic granulocyte
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EIEC	enteroinvasive <i>Escherichia coli</i>
EPEC	enteropathogenic <i>Escherichia coli</i>
ETEC	enterotoxigenic <i>Escherichia coli</i>
ELISA	enzyme linked immunosorbent assay
F _{ab}	antibody binding fragment of immunoglobulin
FAE	follicle associated epithelium
F _c	crystallisable fragment of immunoglobulin
FCA	Freund's complete adjuvant
FCS	foetal calf serum
FIA	Freund's incomplete adjuvant
FITC	fluorescein isothiocyanate
g	grams
GALT	gut associated lymphoid tissue
GCAT	glycerophospholipid: cholesterol acyltransferase
GM1	GM1 monosialoganglioside receptor on enterocyte membrane
GM-CSF	granulocyte-macrophage colony-stimulating factor

H chain	immunoglobulin heavy chain
HEV	high endothelial venule
HGG	human gamma globulin
hr	hours
HRP	horseradish peroxidase
Ig	immunoglobulin
IEL	intraepithelial lymphocyte
ipa	invasion plasmid antigen
IFN γ	interferon gamma
IL	interleukin
i.p.	intraperitoneal
ISCOM	immune stimulatory complex
J chain	joining chain
kDa	kilodaltons
HCl	hydrochloric acid
KLH	keyhole limpet haemocyanin
KV	kilovolts
L chain	immunoglobulin light chain
L-15	Leibovitz L-15 tissue culture medium
LM	light microscopy
LPL	lamina propria lymphocyte
LPS	lipopolysaccharide
M	molar
MALT	mucosa associated lymphoid tissue
MDP	muramyl dipeptide
mg	milligrams
min	minutes
MLN	mesenteric lymph node
ml	millilitres
mRNA	messenger RNA
MHC	major histocompatibility complex
Na ₂ HCO ₃	sodium hydrogen carbonate
ng	nanograms
NK cell	natural killer cell
OD	optical density
OPD	O-Phenylenediamine
OVA	ovalbumin

PAS	periodic acid - Schiff
PBS	phosphate buffered saline
PCS	photon correlation spectroscopy
PLG	poly lactide-co-glycolide
PMA	phorbol myristate acetate
PMSF	phenylmethane sulphonate
pVC	polyvinyl chloride
Quil-A	extract from <i>Quillaja saponaria</i> [molina]
RNA	ribonucleic acid
SBTI	soybean trypsin inhibitor
sc	subcutaneous
SC	secretory component
SCID	severe combined immunodeficiency disease
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sIgA	surface IgA
S-IgA	secretory IgA
S-IgM	secretory IgM
SE	standard error of the mean
SEM	scanning electron microscopy
SFB	segmented filamentous bacteria
TCR	T cell receptor
TEM	transmission electron microscopy
TGF α	transforming growth factor alpha
TGF β	transforming growth factor beta
Th	T helper cell
Tris	Tris(hydroxymethyl) aminomethane
Ts	suppressor T cell
TSA	trypticase soy agar
TSB	trypticase soy broth
TSM	Tris-saline + non-fat dried milk
Tsw	switch T cell
UV	ultraviolet
o/w	oil-in-water
w/o/w	water-in-oil-in-water
μ g	micrograms
μ l	microlitres

CHAPTER 1

GENERAL INTRODUCTION

The vertebrate gastrointestinal tract represents the interface between nutrients and other extraneous substances and the interior *milieu* of the animal thus playing a critical role in the maintenance of homeostasis. In addition to its role in nutrient digestion and absorption the digestive tract occupies a vital defensive position responding to incessant immunological challenges via a number of specific and non-specific factors (Walker, 1982). The function of the epithelium is highly complex, being influenced by endocrine, neurocrine, stromal and immune elements (McKay and Perdue, 1993). The previously held belief that macromolecules were completely reduced to their component monomers in the gut prior to uptake into the body has been challenged by numerous studies which have demonstrated macromolecular absorption through the gut in immunologically significant quantities (Owen and Ermak, 1990; Jenkins *et al.*, 1991). Macromolecules absorbed in such fashion in mammals have been found to interact with immunologically responsive cells in the gut, part of an immune machinery which in conjunction with lymphoid tissues at the other mucosae comprise a common mucosal immune system (Kraehenbuhl and Neutra, 1992; McGhee and Kiyono, 1993). The capacity for intact antigen uptake and for inducing immunological responsiveness via the gut indicates the feasibility of enteric vaccination against pathogens.

The significance of a protective local immune system in the mammalian gut was first suggested by the demonstration of protection against fatal dysentery in rabbits after oral immunization with killed 'Shiga' bacillus regardless of serum antibody titre (Besredka, 1919). Oral immunization was introduced in the early part of the 20th century as a means to protect against intestinal infections (Besredka, 1927) and was first applied successfully in immunisation against poliovirus (Ogra *et al.*, 1968). The identification of secretory IgA - a unique antibody isotype in external body fluids (Tomasi and Zigelbaum, 1963) with characteristics ideally suited to its defensive role in the gut environment led to great interest in the role of gut-associated lymphoid tissue (GALT) in immunological protection. The secretory IgA (S-IgA) isotype constitutes more than 80 % of all antibody produced in mucosa associated lymphoid tissue (MALT) and is induced, transported and regulated by mechanisms distinct from those involved in systemic antibody responses (Hanson and Brandtzaeg, 1989).

It is becoming increasingly appreciated in mammals at least, that many of the dogmas set for peripheral immune responses may not hold for mucosal immunity (James *et al.*, 1988; Mayer *et al.*, 1991a). This may relate to some extent to antigen handling in the gastrointestinal tract as a result of pre - processing of antigens by digestive enzymes (Bland and Whiting, 1990). GALT in mammals consists, in addition to organised lymphoid tissue, of a diffuse collection of lymphocytes and plasma cells in the lamina propria and in the epithelial layer. Antigens induce initial sensitization and differentiation principally through organised lymphoid tissues while the diffuse lymphoid collections in the lamina propria and intra - epithelium are efferent lymphoid areas where antigen interaction with cells leads to terminal differentiation resulting in IgA production, immunoregulatory or cytotoxic reactions (McGhee and Kiyono, 1993). These two compartments are linked by a "homing system" (Gowans and Knight, 1964). Separating the organised lymphoid tissues from the intestinal lumen is a lympho-epithelium comprising a single layer of specialised microvillous/ membranous 'M' cells (Jepson *et al.*, 1993a) and epithelial cells rich in microvilli resembling villous epithelium (Owen and Jones, 1974). M cells sample antigenic material from the lumen via pinocytosis and transport it to cells bearing MHC class II determinants potentially capable of antigen presentation (Nagura *et al.*, 1991). Beneath this dome area is a follicular area and germinal core where B cells differentiate into mature IgA producing plasma cells (McGhee *et al.*, 1992). The critical role of secretory IgA in antibody mediated defense at the mucosal surface is now firmly established (Nagura and Sumi, 1988). In addition to humoral factors, distinctive cell mediated immune mechanisms also exist in the intestinal mucosa (Ganguly and Waldman, 1978), it is thus apparent that a complex immunological network involving both humoral and cellular arms exists in the gut. This complexity is evidenced by the wide variety of immunologic events initiated by the mucosae ranging from the production of secretory IgA and cytotoxic responses to the induction of hypo-responsiveness and systemic interactions (Kagnoff, 1987; McGhee *et al.*, 1992). Serum immune responses in mammals have often been found to be of little value as indicators of local intestinal immunity (Forrest *et al.*, 1992) so the investigation of mucosal immunity is essential to assess enteric vaccine efficacy.

Recent work indicates that considerable interaction between T lymphocytes and intestinal epithelial cells occurs which affects the immune responses elicited to luminal antigens (Brandtzaeg *et al.*, 1991; Panya *et al.*, 1993). In addition to specific immunological mechanisms a number of non specific factors such as highly glycosylated mucin glycoproteins, lysozyme, lactoferrin and proteinase inhibitors reside in the mucous coat which acts as an "unstirred layer" for these agents, increasing their effectiveness by preventing loss by peristalsis (Russel and Walker, 1990; Wallace and Bell, 1993). This mucous coat acts independently of or in conjunction with specific immunological factors to prevent adhesion and uptake of dietary macromolecules and microorganisms from the digestive tract, thus forming a mucosal barrier (Walker, 1982).

The presence of immunologically responsive lymphoid cells at the mucosae of teleosts is well documented (Hart *et al.*, 1988; Rombout *et al.*, 1993a). The suggestion by Ellis (1985) that the skin, gills and gut of fish may provide portals for entry of certain pathogens and that local immunity at these sites might be important, suggests that the induction of specific immune responses at the teleost mucosae would be beneficial in protecting fish against infectious organisms. Application of oral vaccines against infectious diseases in teleost fish has however met with little success to date. In contrast to higher vertebrates teleosts do not possess highly organised mucosal lymphoid tissues, rather the lymphoid cells of the teleost mucosae reside as single cells or small aggregates in diffuse tissues in the gastrointestinal (Georgopoulou and Vernier, 1986; Rombout *et al.*, 1993a), gills (Goldes *et al.*, 1986) and skin epidermis (Peleteiro and Richards, 1985). The existence in teleost mucosal secretions of immunoglobulin is firmly established, antibody being detected in the bile (Lobb and Clem, 1981a; Rombout *et al.*, 1986), cutaneous mucus (Lobb, 1987; Rombout *et al.*, 1993b) and occasionally in the intestinal mucus (Fletcher and White, 1973; Rombout *et al.*, 1986a). In contrast to higher vertebrates however, only a single immunoglobulin type has been detected in teleosts, a tetrameric IgM - like molecule. Significant heterology in its structure has been reported however (Glynn and Pulsford, 1990; Sanchez *et al.*, 1993) and the existence of a J chain (Sanchez *et al.*, 1989) and of a secretory component type structure (Lobb and Clem, 1981b) has been proposed.

The capacity for macromolecular protein absorption from the gut has been documented in a number of teleost species (Georgopoulou *et al.*, 1988; Jenkins *et al.*, 1992; Doggett *et al.*, 1993) and reviewed by McLean and Donaldson (1990). Enterically administered antigen has been observed in contact with lymphocytes in the epithelium and lamina propria (Rombout *et al.*, 1989b; Georgopoulou and Vernier, 1986). Specific antibody to such antigens has been detected in serum and external secretions (Jenkins, 1992; Rombout *et al.*, 1993b) so the mechanistic requirements necessary for the induction of immune responses via enteric antigen administration appear to exist in the teleost intestine and the existence of a common mucosal immune system in teleosts has been proposed (Lobb, 1987; Rombout *et al.*, 1993a). Oral administration was in fact the first mass immunization method employed to vaccinate fish against furunculosis (Duff, 1942) and vibriosis (Fryer *et al.*, 1978), reviewed by Klontz and Anderson (1970). Unfortunately the early indications of high protective efficacy have not materialised, the oral administration of bacterin vaccines in field trials indicated that vaccine consumption was higher and level of protection lower than for injection, immersion and spray methods (Amend and Johnson, 1981; Ellis, 1988). Studies have indicated that immunisation with bacterins via the anal rather than the oral route resulted in a higher degree of protection upon challenge suggesting that secretions of the gastric and upper intestinal regions pose a major barrier to the oral delivery of antigens (Johnson and Amend, 1983a, b). To circumvent problems associated with gastric destruction of antigens and the poor immune responses often produced recent studies have centred on the development of strategies to reduce gastrointestinal degradation of antigen and to adjuvant vaccine preparations (Ellis, 1988; Jenkins *et al.*, 1992; Wong *et al.*, 1992). Oral vaccination is however regarded as the optimal means of disease prophylaxis since it alleviates the stress associated with fish handling, enables repeated boosting and is easily incorporated into routine fish husbandry practices (Ellis, 1988).

Presently, in mammals all widely used vaccines except the Sabin trivalent oral polio vaccine are administered by systemic routes and while being effective in inducing cell mediated and systemic antibody responses they are poor at inducing mucosal immunity in humans who have not had a previous mucosal infection by the causative organism (McGhee and Kiyono, 1993). The

development of strategies for enhancement of immune responses elicited to orally administered antigens is presently an expanding sphere of mammalian research and initial indications suggest that similar strategies may be applicable in fish vaccination. A common approach involves avoidance or modification of gastrointestinal secretions by the use of gastric inhibitors (Clemens *et al.*, 1986) anti-proteases (Udall *et al.*, 1984) acid resistant films (Lehmann and Drehner, 1981) or encapsulation (Morris *et al.*, 1994). A range of other substances such as penetration enhancers, surfactants, detergents and a range of adjuvants have also been applied with variable degrees of efficacy and have been reviewed in detail previously (Jenkins, 1992). Soluble antigens tend to elicit poor responses when delivered orally, however, a number of elegant strategies have been devised to increase their efficacy as mucosal immunogens. Incorporation of antigens into liposomes or microparticles protects them from harmful digestive secretions and thus allows the use of lower doses than is the case when soluble antigen is administered. The use of poly - lactide - co glycolide (PLG) biodegradable microparticles whose formulation may be modified to effect controlled release and allow targeting to specific tissues (Aguado and Lambert, 1992) is an approach showing promise as an enteric delivery system for soluble protein antigens. Cholera toxin is a potent enteric immunogen and exerts strong adjuvant effects on gut immune responses to unrelated antigens when presented concurrently (Holmgren *et al.*, 1993; Vajdy and Lycke, 1993). Another way to increase the efficacy of orally administered vaccines involves incorporation into immuno-stimulatory complexes (ISCOMS) (Mowat and Donachie, 1991). ISCOMS confer immunogenicity on proteins delivered by the oral route and very low amounts of antigen in such structures are immunogenic. Quil-A saponin, a constituent of ISCOMS itself acts as an adjuvant when given orally (Campbell and Bede, 1989). Oral live vaccines yield higher antibody titres in remote site secretions and in serum than do oral killed vaccines and research is now focusing on the use of attenuated live organisms both as oral vaccines and as carrier vehicles for enteric delivery of heterologous antigen (Turner *et al.*, 1993; Offit *et al.*, 1994).

While the above strategies have been widely applied in mammalian studies their use to date in fish research has been limited. Anti - proteases and detergents have been used to enhance the absorption of orally administered soluble proteins (McLean and Ash, 1990). Similarly, gastric

inhibitors have been shown to increase the effectiveness of oral delivery of protein hormones (Solar *et al.*, 1990). Additionally Wong *et al.* (1992) found that protection of antigens by enteric coating resulted in increased antibody responses to an orally delivered *Vibriosis* vaccine. The use of Quil-A saponin alone or incorporated into micelles or ISCOMS led to enhanced immune response and antigen uptake in tilapia (Jenkins, 1992). Recently an attenuated live aromatic amino acid dependent *Aeromonas salmonicida* vaccine has been developed (Vaughan *et al.*, 1993) and found to be effective as a parenterally delivered vaccine. This indicates the potential for application of a new generation of vaccines and strategies in fish farming. In addition to these approaches the opportunity to devise novel strategies for fish due to their aquatic habitat also exists. An example of this is bioencapsulation - the enveloping of vaccines in living feed organisms (Campbell *et al.*, 1993), this approach has been successfully applied in the plankton - mediated oral delivery of *Vibrio anguillarum* to juvenile ayu (Kawai, *et al.*, 1989) and may be of particular use for oral vaccination of larval and juvenile fish species.

The objective of the present study was to investigate in detail the nature of the gastrointestinal enzymic barriers to the enteric delivery of protein antigens in rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792). Rainbow trout were selected as the experimental animal since salmonids are among the most widely farmed and economically important fish species. Additionally, a number of studies have investigated the infectious agents responsible for salmonid disease and their immunological responses to vaccination. It was intended to develop methods to analyse antigen processing by secretions and cells in the trout gut so strategies to reduce the extent of antigen degradation in the digestive tract could be devised. These methods could then be applied to investigate the potential value of modern oral delivery systems such as encapsulation in biodegradable microparticles and the use of live recombinant bacteria as carriers of heterologous antigens. It was further hoped to investigate the antibody response to orally delivered antigen to determine the immunological consequences of antigen degradation in the gut.

CHAPTER 2

***A COMPARATIVE REVIEW OF THE LITERATURE ON MAMMALIAN AND TELEOST
MUCOSAL IMMUNOLOGY AND THE SCOPE FOR ENTERIC VACCINE DEVELOPMENT.***

Of the available external surface area for microbial colonisation and invasion in vertebrates the mucosae comprise the greatest part, leading to the evolution of a complex immune system to defend these sites. The mucosal environment possesses many features uniquely adapted to its role in allowing for the diverse requirements of nutrient digestion, maintenance of a stable microflora, macromolecule absorption and the induction of immunological reactivity. This review is intended to survey the literature on mammalian and teleost specific and non - specific mucosal defense systems and to discuss the means by which enteric presentation of antigens may be used to activate such systems to induce immunological protection.

2.1 Non-specific gastrointestinal defenses in mammals.

2.1.1 *Non-specific protection associated with normal gastrointestinal physiology.*

The digestive tract by virtue of its role in macromolecule degradation provides a hostile environment to microorganisms and their products. Features such as gastric acidity, the mucus layer, peristalsis, the gut microflora and luminal proteases may therefore be regarded as the first line of non- specific luminal defense in vertebrates. This barrier function may be subdivided into anatomical/ physiological, biochemical and immunological components although *in vivo* these factors act in concert to provide a comprehensive defense to infection.

Within the luminal environment two phases may be distinguished - the "bulk phase" of the luminal contents and the microenvironment immediately overlying the epithelium (Ecknauer, 1981). A dynamic environment is maintained in the digestive tract by intestinal motility shunting luminal contents along the gut (Sarna and Otterson, 1993) this enhances enzyme interaction with food and associated substances and is a deterrent to microbial colonisation of the intestine (Walker and Owen, 1990). Peristalsis is thus regarded as an important defense mechanism against colonisation by foreign microorganisms, indeed it has been found that bacterial translocation (passage of bacteria from gut lumen to regional lymph nodes and systemic organs) in mammals is promoted by prolonging intestinal transit, this translocation is secondary to overgrowth of enteric bacteria in the intestinal lumen (Runkel *et al.*, 1993). Other mechanical phenomena such

as the pumping movements of villi and contraction of microvilli may also be important in the maintenance of the mucosal barrier (reviewed by Ecknauer, 1981).

2.1.2 Gastrointestinal barriers of non-epithelial origin.

A harsh environment in the digestive tract is maintained by the presence of an acidic gastric pH (Schubert, 1993), bile salts (Ecrantz and Sjoval, 1957; Sung *et al.*, 1993), proteolytic enzymes (Borgstrom *et al.*, 1957) and very low iron concentrations as a result of lactoferrin activity (Payne and Finkelstein, 1978). The gastric barrier is an efficient protection against invading pathogens since the low pH maintained (in most vertebrates) is lethal for the majority of ingested organisms. Gastric juice drastically reduces the numbers of ingested bacteria (Giannella *et al.*, 1994) and begins the digestion of proteins (chapter 3). The important sterilising role of low gastric pH in establishing a gastric barrier to infection has long been recognised (Drasar *et al.*, 1969). Bacteria in the small intestine are not normally pathogenic but lack of gastric acid and small intestinal hypomotility and stasis may lead to ordinarily nonpathogenic bacteria colonizing the small intestine and contributing to malabsorption (Drasar *et al.*, 1969). Bacteria have been found to contribute to the pathogenesis of tropical sprue and blind loop syndrome, diseases frequently associated with achlorhydria (Ecknauer, 1981). The large differences in infective doses of enteropathogens ranging from the 10 - 500 *Shigella* species necessary to cause dysentery in healthy adults (DuPont *et al.*, 1972) to the 10⁸ *Vibrio cholerae* organisms required to induce cholera in similar studies (Cash *et al.*, 1974) may at least partly be attributed to differences in acid sensitivity. A comparative study found that 75 % of *Shigella* species tested were acid resistant (> 10% of inoculum survived exposure to pH 2.5 for 2 hr) contrasting with 66% of enteroinvasive *E. coli* strains and only 0.001 % of *Salmonella* isolates (Gorden and Small, 1993). The capacity of *Shigella flexneri* for survival at low pH was found in the latter study to be dependent on time and growth phase - the acid resistance phenotype not being expressed until the late exponential growth phase. The infectious dose of *Vibrio cholerae* in human volunteer experiments was reduced from 10⁸ to 10⁴ organisms by the administration of sodium bicarbonate with the inoculum (Cash *et al.*, 1974) and intestinal colonisation of oral shigella vaccine strains was similarly enhanced by

concurrent administration of sodium bicarbonate (DuPont *et al.*, 1972; Herrington *et al.*, 1990). Epidemiologic data has also suggested an association between achlorhydria and salmonellosis (Drasar *et al.*, 1969) indicating an important role for gastric acidity in protection against this species. The recognition that the immunologically important toxin constituents of *Vibrio cholera* and *Escherichia coli* vaccine preparations are acid labile has led to routine treatment with sodium bicarbonate prior to vaccine delivery (Clemens *et al.*, 1986). In addition to acidity the stomach possesses proteolytic activity, the principal gastric endopeptidase being pepsin - an endoprotease active at low pH which is likely to commence the degradation of labile pathogen-associated proteins (section 3.1).

A number of potent hydrolytic enzymes are found in the intestine of both mammals and fish which function in the breakdown of large nutrient polymers (after initial gastric cleavage) to their component monomers or to short polymer chains (section 3.1). Proteolysis by pancreatic enzymes in mammals limits penetration by bacterial toxins (Walker and Owen, 1990) and interference with the proteolytic activity of digestive secretions has been found to increase the quantity of orally administered protein reaching the bloodstream. Studies utilising the protease inhibitors, soybean trypsin inhibitor and aprotinin to reduce the activity of intestinal serine proteases in both teleosts (McLean and Ash, 1990) and in rats (Udall *et al.*, 1984) have reported increased absorption of soluble proteins into the bloodstream. Bile salts in mammals have been shown to have cytotoxic and bacteriostatic properties (Sung *et al.*, 1993). The delicate balance which prevails in the gastrointestinal tract is revealed by the observations that where bacterial overgrowth occurs bile acids are metabolically transformed by such processes as deconjugation and dehydroxylation to products which damage the intestinal mucosa (Ecknauer *et al.*, 1981).

2.1.3 *Non-specific humoral defense in the mammalian gut.*

A number of constitutive bacteriostatic and bactericidal humoral factors are present in the digestive tract of mammalian species. Antimicrobial agents have been described in gastric (Smith, 1966) and pancreatic juice (Pierzynowski *et al.*, 1993); the latter study suggesting that such factors may be of importance in regulation of the small bowel microbiota and in maintenance of bacterial

homeostasis. Lactoferrin is an iron binding protein commonly present in external secretions with a bacteriostatic effect potentiated by antibody (Bullen *et al.*, 1972). The role of antibody in this process appears to be in preventing the synthesis, secretion or activity of bacterial iron-chelating compounds (Bullen *et al.*, 1974) so lactoferrin can bind iron which is therefore not available as a bacterial growth factor. Lactoferrin in mammals has been implicated in antibacterial activity, promotion of lymphocyte growth and in stimulating the activity of macrophages, granulocytes and neutrophil leucocytes (Sakai *et al.*, 1993). Lysozyme (muramidase) has also been found in a number of secretions and is active against the cell wall of gram positive bacteria, lysozyme is involved in a number of defense mechanisms including bacteriolysis, opsonization, immune response potentiation and possibly in restricted anti - viral activity (Jollés and Jollés, 1984; Lie and Syed, 1986). It has also been found that lactoperoxidase present in external secretions plays a role in defense against infection (Gothefors and Markerlund, 1975; Moldoveanu *et al.*, 1983). Secretory IgA has been shown to enhance the effect of the lactoperoxidase system by a mechanism independent of its antibody specificity (Tenovno *et al.*, 1982; Moldoveanu *et al.*, 1983). Interferon may also play a role in mucosal defense, its synthesis being stimulated by infection or topical application of viral vaccines and has been detected in nasal secretions within 24 hours of infection/ stimulation (Danielescu *et al.*, 1975). Analogues of receptors for bacteria may also be present in exocrine secretions which can prevent bacterial attachment (Andersson *et al.*, 1985; Andersson *et al.*, 1986). Indeed, it has been hypothesised that parts of the glycocalyx shed from cells may bind to potentially harmful lectins present in the lumen, thus preventing these compounds reaching the epithelial cells (Ecknauer, 1981). Desensitization of the adenylate cyclase has been observed after enteric exposure of mice to cholera toxin (Lönroth and Lange, 1981). Treatment of rats with cholera toxin resulted in the induction of a 25 kDa hormone-like factor implicated in desensitisation which was detectable in bile and milk but not in the serum (Lange and Lönroth, 1986).

2.1.4 The microenvironment overlying the gastrointestinal epithelium.

This region appears to be very stable, its composition depending on secretions of the intestinal

mucosa, gut motility, the glycocalyx and its adsorbed components (Ecknauer, 1981). Studies have been carried out in higher vertebrates on the bacterial composition (van der Waaij, 1992) and the pH on the epithelium surface while other work has focused on the effects of the "unstirred layer" - a component of this microenvironment, on absorption kinetics (Ecknauer, 1981). The indigenous microflora is partly determined by the composition of this microenvironment however it tends to be relatively stable for a given individual and resists colonisation by "new" bacterial species (Van der Waaij *et al.*, 1971). Where luminal barriers fail to control bacterial populations in the gut bacterial overgrowth may occur with metabolic and clinical consequences (Runkel *et al.*, 1993), the final result of this may be bacterial invasion of the internal organs but earlier in the process a reduction in the number of intraepithelial lymphocytes and disturbances in goblet cell mucus secretion have been described (Ecknauer, 1981). The normal human colonic microflora contains a relatively stable population of more than 300 distinct bacterial species with a biomass of 10^{13} - 10^{14} bacteria (Holdeman *et al.*, 1976). The participation of a range of bacteria with different roles in mucin degradation appears to contribute to this diversity (Hoskins, 1993). Van der Waaij *et al.* (1971, 1972) first recognised the importance of the barrier formed by the relatively benign indigenous flora by using poorly absorbable antibiotics that suppressed the facultative flora but left the anaerobic flora intact. Disruption of the indigenous intestinal microflora was associated with overgrowth by resistant microorganisms which may have resulted in their translocation to the internal organs (Van der Waaij, 1992). The anaerobic flora in particular appears to interfere with colonisation by opportunistic pathogens, Wells *et al.* (1987) finding that the elimination of anaerobes led to significant rates of dissemination of intestinal bacteria into mesenteric lymph nodes. Antimicrobial metabolites produced by normal enteric flora, especially anaerobic or 'colonisation-resisting flora,' significantly control non - indigenous flora (Walker and Owen, 1990). The protective effect of gut colonisation by the endogenous anaerobic fraction of the intestinal microflora has been termed 'Colonisation resistance' (Van der Waaij *et al.*, 1971) and the anaerobic flora responsible called 'Colonisation resistance factor' (Van der Waaij *et al.*, 1977). The mucous membranes in addition to the immune system of the gut may contribute to the stability and maintenance of the endogenous flora (Van der Waaij, 1989).

The autochthonous gut microbiota or components of it can enhance both specific and non-specific immunity at systemic and/ or mucosal levels. Lipopolysaccharide (LPS) produced by gram negative bacteria in the intestine constantly stimulates host immunity through interaction with intestinal phagocytes or after transport into Peyer's patches (Walker and Owen, 1990). Oral administration of *Lactobacillus casei* for example was shown to increase non-specific immunity in mice by increasing the activity of peritoneal macrophages (Perdigon *et al.*, 1986). Development of the intestinal microbiota in mice resulted in an increase in the number of duodenal IgA plasma cells (Moreau *et al.*, 1982) and intestinal bacteria have also been implicated in the regulation of peripheral T cell activity (Takeuchi *et al.*, 1993). In mice, gut colonization by the normal intestinal flora greatly increased the number of $\alpha\beta$ T cell receptor ($\alpha\beta$ TCR) bearing T lymphocytes but had little effect on the number of $\gamma\delta$ T cell receptor ($\gamma\delta$ TCR) bearing cells present (Bandiera *et al.*, 1990) suggesting an important role in antigen driven T cell expansion in the gut. A recent study suggested that segmented filamentous bacteria (apathogenic autochthonous bacteria found in the distal small intestine of various animal species) stimulate the mucosal immune system of mice (Klassen *et al.*, 1993). A comparison between mice mono-associated with segmented filamentous bacteria (SFB) and germ free mice found that SFB's resulted in increases in the numbers of lymphoid cells in the lamina propria of the ileal and caecal mucosae, the numbers of IgA secreting cells in the intestinal mucosa, the IgA titres in serum and intestinal secretions and in the Concanavalin A (Con A) induced proliferative responses of mesenteric lymph node cells. The effects of colonisation by a specific pathogen free flora were similar but less pronounced. The means by which these organisms exert their effects is uncertain, some workers suggesting that SFB's competitively exclude pathogens from the distal small intestine thus contributing to gastrointestinal colonization resistance (Garland *et al.*, 1982) while others propose that SFB's enhance host resistance by directly influencing GALT (Glick *et al.*, 1978).

2.1.5 Lumenal barriers of epithelial origin.

In mammals a mucus gel forms a barrier over the epithelial surface with which most intestinal microbes are associated (Lamont, 1992). Mucus produced by goblet cells along the gut acts as

a physical barrier by impeding diffusion and by making the tract more slippery (Forstner *et al.*, 1981). This substance may protect the epithelium by covering glycolipid and glycoprotein receptors on the cell surface or can mimic cellular receptors to which bacteria attach or may physically entrap microbes (Walker and Owen, 1990). Immune complexes especially those incorporating IgA may contribute to the release of goblet cell mucus onto the intestinal surface (Walker *et al.*, 1982) thus preventing interaction of immune complexes with the microvillous surface. It has been suggested that the ability of guinea pig mucus to inhibit the invasion of mammalian tissue culture cells by *Shigella flexneri* was associated with the animals relatively high resistance to developing dysentery (Dinari *et al.*, 1986). In contrast, mucus from monkeys did not prevent invasion of the bacteria *in vitro* and these animals were more susceptible to infection. Mucus\ mucin has also been shown to prevent the adhesion of virulent *Yersinia enterocolitica* to epithelial cell membranes (Mattle *et al.*, 1989). Deterioration of the murine mucus barrier as a result of ionising radiation has been found to increase the susceptibility of mice to oral challenge with *Pseudomonas aeruginosa* (Walker *et al.*, 1985). There is evidence that it is the combination of specific IgA and mucus which is effective in specific pathogen exclusion in the gut and not mucus or antibody alone (Burr *et al.*, 1988). The principal component of mucus and that responsible for its characteristic visco - elastic properties is mucin - a high molecular weight glycoprotein (Neutra and Forstner, 1987). Mucus/ mucin can protect against infection with enteric pathogens by entrapping them in the gut lumen, inhibiting their attachment to epithelial cells and assisting in their removal from the body. This contention is based on findings in mammals that pathogens (Khavkin *et al.*, 1980) and their secretory products particularly enterotoxins (Forstner *et al.*, 1981) cause a marked increase in mucin secretion. On some occasions however the possession of mucus receptors by bacteria may be regarded as a pathogenicity factor, piglet ileal mucus for instance contains protein and glycolipid receptors specific for *Escherichia coli* K88 fimbriae (Blomberg *et al.*, 1993) and adhesion of *Yersinia enterocolitica* to purified rabbit and human intestinal mucin is associated with the possession of the virulence plasmid (Mantle and Husar, 1993). Therefore the possession of receptors for mucins may in certain cases facilitate colonisation and in others the removal of microorganisms.

The role of the immune system in the control of goblet cell activity is not fully defined but mucus release in mammals is affected by anaphylactic antigen challenge (Walker *et al.*, 1982), mediators of inflammation (Tao and Wilson, 1984) and possibly T cells (Miller *et al.*, 1979). Intestinal mucus production during Salmonellosis has been found to be regulated by Tumour necrosis factor α (TNF α) (Arnold *et al.*, 1993). It has been suggested that immune reactions occurring at epithelial surfaces may stimulate mucus release from goblet cells increasing the effectiveness of the mucus barrier (Walker *et al.*, 1982). Using animal models it has been demonstrated that goblet cell function may be dependent on functional T lymphocytes (Ahlstedt and Kristofferson, 1982) and this may also be the case in man (Karlsson *et al.*, 1985). In addition to the physical barrier posed by mucus it has been demonstrated that antigens trapped within the mucous layer in immune complexes are more rapidly degraded by intestinal proteases (Walker *et al.*, 1975).

Enzymes produced by intestinal epithelial cells can be found in the lumen include peptidases, disaccharidases and enterokinase (chapters 3&4). In addition, the brush border membranes of the adult villus has been found to be rich in hydrolytic enzymes (Quaroni, 1985). Indeed Holmes and Loble (1989) reported the presence of 22 constitutive brush border digestive enzymes in mammals. Digestive enzymes in the apical cell membrane or in the cell interior such as carbohydrases, peptide-hydrolases and lysosomal enzymes can also degrade macromolecules and enterokinase plays a crucial role in the intestine by activating trypsinogen to produce trypsin which initiates protein digestion in the small intestine (sections 3.1 and 4.1).

2.1.6 Non-specific cellular barriers in the gastrointestinal.

Epithelial cells, mesenchyme - derived cells and extracellular matrix molecules comprise the three elements of an integrated functional unit which is influenced by lymphocytes, hormones and cytokines (Louvard *et al.*, 1992). Columnar epithelial cells and their precursors form approximately 90% of the cell population of the small intestine, these cells actively secrete the glycocalyx forming a pre-epithelial barrier, particularly to diffusion of molecules with negatively charged groups (Ecknauer, 1981; Walker and Owen, 1990). Antibodies and pancreatic proteases

adsorb to this glycocalyx (Walker *et al.*, 1985) and these adsorbed proteases are implicated in the digestion of complexes formed by local secretory antibody with bacteria and other antigens (Walker *et al.*, 1975). Thus, the glycocalyx provides the close physical proximity necessary for the function of luminal/ mucosal components. T cell factors have been shown to be capable of modulating such epithelial functions as tight junction permeability (Madara and Stafford, 1989), ion secretion (Holmgren *et al.*, 1989a), proliferation of small intestinal crypt cells and mucosal morphology (Marsh and Cummins, 1993). After crossing the glycocalyx the next barrier to antigens is the apical cell membrane which forms characteristic microvilli and is "loaded" with enzymes, carriers, binding sites and pores (Louvard *et al.*, 1992) playing roles both in communication and in separation between the glycocalyx and epithelial layers. Its communication function is referred to as the "digestive absorptive function" (Ecknauer, 1981). The apical epithelial membrane acts as a selective barrier against large, hydrophilic and charged molecules. In addition to the discrete apical surface architecture of cells the barrier function of the epithelium also depends on the characteristics of spaces between the cells. The cytoskeleton of the intestinal brush border can be divided into two distinct areas - the microvilli and the terminal web, the latter is a cytoskeleton-rich area in the apical cytoplasm, the structural organisation of the vertebrate brush border cytoskeleton is highly conserved (Louvard *et al.*, 1992). Intestinal epithelial cells are effectively 'welded' together by an intricate system of cell - cell connections whose order from the apical cell pole to the basement membrane is zonula occludentes (tight junctions), zonula adherentes (belt desmosomes), macula adherentes (spot desmosomes) and macula occludentes (gap junctions) (Ecknauer, 1981; Louvard *et al.*, 1992). It is believed that the tightness of the epithelium is represented by the tight junctions, epithelium tightness is higher in the villi than in the crypts and increases along the intestine, being highest in the colon (Ecknauer, 1981). The role of tight junctions in preventing uptake of macromolecules is evidenced by their increased uptake in both mammals, and in teleosts in the presence of Quil - A saponin which is reported to result in 'loosening' of these junctions (Campbell and Bede, 1989; Jenkins, 1992).

2.1.7 Non-specific mucosal defense in teleosts.

Non-specific defenses in fish have been the subject of a number of comprehensive reviews (Ingram, 1980; Ellis, 1981; Fletcher, 1982; Alexander and Ingram, 1992). The mucosal non specific defenses in teleosts however have received considerably less attention than their mammalian counterparts. It may be assumed that such factors as gut motility and the structural integrity of the epithelial layers in teleosts play a similar role in teleost mucosal defense as those described for mammals.

The epithelial surfaces of fish and their mucous secretions provide a physical barrier between the animal and environmental microorganisms (Fletcher, 1978; Pickering and Richards, 1980; Roberts and Bullock, 1980). Particularly important in this regard may be the scales covering the epidermal surface of most species, the secretion of copious amounts of mucus by fish lacking scales, it has been suggested may be a compensatory mechanism for the absence of this physical barrier to infection by aquatic microorganisms (Al-Hassar *et al.*, 1985). Piscine surfaces are covered by a layer of mucus secreted as in higher vertebrates by goblet cells in the epidermal layers (Harris *et al.*, 1973; Pickering, 1974), similar in structure to mucins in other animals. The chemical composition of fish mucins have been described (Harris *et al.*, 1973; Pickering, 1976; Wold and Selset, 1977). A role of this layer in providing a defense against colonisation by pathogens has been appreciated for some time (Jakowska, 1963). In addition to the presence of mucopolysaccharide constituents, mucus is also a source of natural (non-specific) agglutinins (Kamiya *et al.*, 1988), lysins (Suzuki, 1985; Al - Lahham *et al.*, 1987) and specific immunoglobulin (chapter 6). Additionally a C reactive protein (CRP)- like substance has been detected in higher concentrations in the epidermal mucus of tilapia than in the serum after induced stress (Ramos and Smith, 1978). The presence of substances inhibitory to the growth of a number of organisms including fungal and bacterial species in cutaneous and gut mucus has also been reported (Harrell *et al.*, 1976; Takahashi *et al.*, 1987; Austin and McIntosh, 1988; Lie *et al.*, 1989). Antibacterial activity against a number of gram positive and gram negative pathogens was demonstrated in the skin mucus of turbot (Fouz *et al.*, 1990), the inhibition being most extensive against *Aeromonas*

salmonicida. The latter authors suggested that the antibacterial component responsible was a glycoprotein. Rose (1990) however found that Atlantic salmon and rainbow trout mucus did not exert an inhibitory effect on the growth of *Aeromonas salmonicida*. It has been suggested that complement activity is present in fish mucus (Harrell *et al.*, 1976) in addition to complement-independent defensive toxins (Cameron and Endean, 1973). Lactoferrin activity has not been identified in teleosts to date but Sakai *et al.* (1993) found that oral administration of bovine lactoferrin lead to increased protection against *V. anguillarum* challenge. The presence of haemolysins in some fish species has also been demonstrated (Suzuki, 1985; Al - Lahham *et al.*, 1987), but the latter study suggested that they were not involved in defense.

It has been suggested that proteases present in the mucosal secretions of teleosts play a role in defense (Hjelmeland and Raa, 1982; Hjelmeland *et al.*, 1983; Braun *et al.*, 1990). Hjelmeland *et al.* (1983) suggested that the Vibriolytic activity present in skin mucus could be accounted for by its trypsin activity. This author found the enzyme to be indistinguishable to its pyloric caecae counterpart so one may assume that in the intestine this enzyme and the barrage of other proteolytic enzymes present (section 3.1) pose a considerable barrier to colonisation by bacteria, particularly against secreted products and susceptible surface components. Chitinase has also been found to be present in the digestive tract of fish (Jeuniaux, 1961; Micha *et al.*, 1973; Lindsay, 1984), but it is uncertain if it plays a role in defense against chitinous organisms (Alexander and Ingram, 1992). Lysozyme has been identified in fish mucus (Fletcher and White, 1973; Takahashi *et al.*, 1986). In rainbow trout, lysozyme was found to be most abundant at sites where the risk of bacterial invasion was greatest such as in the mucus and gills, consistent with a function in host defense (Lindsay, 1983; Murray and Fletcher, 1976). Indeed Lindsay (1984) reasoned that since the presence of gastric lysozyme in fish was not associated with species which ingest a significant proportion of bacteria in the diet it was likely that rainbow trout oesophageal and gastric lysozyme plays a role in defense rather than digestion, a contention supported by the findings of Lie *et al.* (1989) who also indicated a defensive role for lysozyme in the teleost gut. A role for digestive enzymes in degrading foreign protein antigens in the teleost lumen is discussed in chapters 3 and

2.1.8 *The gut microflora of teleosts.*

The gut microflora of teleost species has not received the same attention as that of mammals although some studies have reported that it is less stable and more dependent on the environmental microflora. There have been a number of reports of high bacterial numbers in the intestines of fish (Trust and Sparrow, 1974; Yoshimizu *et al.*, 1976; Kamei *et al.*, 1985). Yoshimizu *et al.* (1976) found that the numbers of such bacteria fluctuate seasonally and Horsley (1977) proposed that the commensal bacterial flora of the piscine gastrointestinal tends to be similar to that of the skin and gills but is often characterised by high numbers of *Vibrio* species and strict anaerobes. Wood (1967) however suggested that there didn't appear to be a specific bacterial flora within the piscine digestive tract, a belief which may find support in the findings of Shewan (1961) and Seki (1969) that the bacterial genera in the piscine intestine reflected those present in the ingested food. This contention was crystallised in the suggestion of Horsley (1977) that the digestive tracts of marine fish rather than possessing an autochthonous microflora act as enrichment vessels for ingested bacteria. Lesel (1979) suggested that the trout microflora present at an ambient temperature of 17°C was small compared to that of homeotherms and that it was a specialised variant of the environmental microflora. Yoshimizu and Kimura (1976) and Hamid *et al.* (1978) however found the piscine gut microflora was different from the ambient microflora, the latter study reporting that among 10 species of marine fish in no case was the intestinal flora similar to the ambient flora. Yoshimizu *et al.* (1980) suggested that the microflora in early salmonid fry is similar to the diet and does not assume a distinct character (*Aeromonad* dominated) typical of adults until the advanced fry or fingerling stage. Findings that bacteria do not persist in the intestine of fasting fish (Margolis, 1953; Liston, 1957) led to the remarkable suggestion that the digestive tract becomes effectively sterile soon after emptying (Ruby and Morin, 1979). Trust (1975) and Lesel (1979) in contrast found that in salmonids the microflora was still detectable in the digestive tract after prolonged starvation.

Anaerobic bacteria have been reported in the gut of salmonid fish (Trust and Sparrow, 1974;

Austin and Al-Zahran, 1988), the main species described being *Bacteroides* and *Clostridia* (Sakata *et al.*, 1981; Kamei *et al.*, 1985). Trust *et al.* (1979) however could not isolate significant numbers of strict anaerobes from the rainbow trout gastrointestinal tract and postulated that difficulties in obtaining significant counts of strict anaerobes in salmonid fish may be a result of the low ambient temperatures pertaining at which the generation time for anaerobic bacteria is too slow to allow the organisms to reach a population level sufficient to establish a gut microflora before peristaltic washout occurs. Large numbers of aerobic heterotrophic bacteria have also been found in the piscine gut (Sakata *et al.*, 1978; Austin and Al - Zahran, 1988), in freshwater salmonid fish the main species isolated were *Enterobacteriaceae*, *Aeromonas* and *Acinetobacter* (Trust and Sparrow, 1974; Niento *et al.*, 1984). It has been proposed that *Aeromonas* and *Enterobacteriaceae* are the principal aerobic species in the intestines of freshwater fish whilst *Vibrio* and *Pseudomonas* are dominant in marine fish (Aiso *et al.*, 1968; Kamei *et al.*, 1985). Studies on anadromous species suggested that salinity plays a pivotal role in determining the genera constituting the piscine microflora (Yoshimizu and Kimura, 1976; Sugita *et al.*, 1982). In conclusion, the microflora of the teleost gastrointestinal tract appears less stable and less numerous and diverse than that of mammals so one may surmise it may pose less of a barrier to colonisation than is the case with mammals.

2.2 Absorption of materials from the gastrointestinal tract.

2.2.1 Uptake of macromolecules by the gut.

Macromolecule absorption is a process of alimentation which is the sole means of nutrient procurement in evolutionary simple organisms and has traditionally been regarded as a feature of such animals (Georgopoulou *et al.*, 1985). The importance of macromolecular uptake and intracellular digestion apparently declines as the capacity for extracellular digestion increases and macromolecular absorption is considered to be of little importance in higher organisms (Georgopoulou *et al.*, 1985). Three possible pathways exist in mammals for movement of macromolecules across the intestinal barrier - transcellular movement through the enterocytes, uptake by specialised 'M' cells and paracellular movement between cells and via tight junctions

(McKay and Perdue, 1993). There have additionally been suggestions that 'solvent drag' of small molecules including oligopeptides (< 1900 Da) may occur across the guinea pig epithelium in conjunction with glucose absorption (Pappenheimer and Reiss, 1987; Atisook and Madara, 1991). Direct penetration of bacterial and plant toxins into cells has also been described but this route does not appear to apply to molecules such as protein hormones (Goldstein *et al.*, 1979). The advent of modern immunological techniques has enabled the demonstration of macromolecular uptake in both mammalian and in a number of teleost species (section 4.1). The general features of macromolecule uptake and subsequent processing appear to be similar in mammalian and piscine species so the processes will be discussed simultaneously. One pivotal difference however is that while only absorptive intestinal enterocytes are implicated in transcellular macromolecule absorption in teleosts, enterocytes (Mayer *et al.*, 1990; Panya *et al.*, 1993), 'M' cells (Wolf and Bye, 1987) and Paneth cells (Erlandson and Chase, 1972) appear to be involved in such processes in mammals - M cell mediated uptake apparently being dominant.

2.2.2 Transcellular absorption pathways.

Macromolecular uptake pathways may be divided into transcellular pathways where macromolecules are transported across cells and paracellular pathways where macromolecules pass between cells. The details of transcellular transport depend on whether macromolecules are living or nonliving, soluble or particulate and are described in detail for uptake of macromolecules in teleosts in section 4.1. The process of soluble antigen uptake is termed pinocytosis while phagocytosis is used to describe uptake of particulates, in addition to this dichotomy the mechanics of uptake depend on whether or not specific receptors for the molecule are present on the cell surface. The process of receptor - mediated endocytosis is initiated by specific ligand binding to receptors on infoldings of the apical surface of the plasma membrane (Udall and Walker, 1987) resulting in clustering of receptors in the coated pits areas of the cells allowing increased binding. This clustering is followed by invagination and internalisation of the coated pit to form a coated vesicle, the ligand being still attached to the receptor may be protected from intracellular degradation (Rodewald and Abrahamson, 1982). A second ligand may also be present which

allows macromolecule internalisation (McLean, 1987). This process involves cells ingesting extracellular materials by trapping them within inward foldings of the plasma membrane which 'pinch off' from the surface to form intracellular vesicles. The contents of the coated vesicle may then be discharged into the lateral intercellular space and the vesicle receptors re - utilised through recycling (Goldstein *et al.*, 1979). Conversely in the case of fluid - phase endocytosis macromolecules bind non-specifically to the apical cell surface (Nicklin, 1987; Udall and Walker, 1987) and are internalised into vesicles in the cytoplasm.

It has been demonstrated in teleost species that changes in membrane fluidity occur upon adsorption of soluble proteins after which macromolecules are internalised (McLean, 1987; Georgopoulou *et al.*, 1988; Doggett, 1989; Jenkins *et al.*, 1992). Vesicle formation ensues to envelope the absorbed material (Silverstein *et al.*, 1977; Goldstein *et al.*, 1979). The precise details of vesicle size and the mechanics of uptake differ depending on the macromolecule in question (Georgopoulou *et al.*, 1988; Rombout and van den Berg, 1989). This process either results in vesicle fragmentation or multi - vesicular coalescence (Iida and Yamamoto, 1985; Georgopoulou *et al.*, 1988) progressing to produce supranuclear vacuoles (Georgopoulou *et al.*, 1988). Secondary lysosomes are formed upon coalescence of supranuclear vacuoles with cellular lysosomes (Watanabe, 1982). This is not always the outcome however - either lysosomal merger or intercellular exocytosis may occur depending on the pathway involved (Abrahamson and Rodewald, 1981). Distinct vesicle populations may be involved in determining whether selective degradation or escape of proteins from lysosomes occurs (McLean, 1987). Cytoplasmic tubules are also implicated in intracellular transport of macromolecules, these tubules can form complex extensions linking endocytic vesicles with the basolateral membrane where antigen has been demonstrated in studies of protein uptake in teleosts (Iida and Yamamoto, 1985). Based on ultrastructural features of macromolecule uptake in the posterior gut/ second gut segment the uptake of macromolecules by the teleost gut has been compared to that in newborn mammals (Watanabe, 1981). Mehrazar *et al.* (1993) found up to 30% of anti-bovine gamma globulin antibody fed to 3/5 day old piglets was absorbed, this nonselective and massive uptake in

milligram quantities was followed by 'gut closure' after which a second stage characterised by selective absorption of immunoglobulin in microgram quantities (0.02 - 0.01 % of dose) ensued. Gut closure is a characteristic of some neonatal mammals which prevents further transfer of large quantities of macromolecules from the gut of neonates. The quantities of absorption found in some teleosts (see section 5.4) are however extremely small and are thus somewhat in conflict with the view that the uptake is somehow analogous to that of mammalian neonates, however as outlined in section 5.4 the extent of such absorption varies considerably between teleost species.

2.2.3 Uptake of inert particulate materials across the digestive tract

Uptake of particulate material across the intact gastrointestinal tract into the blood and lymph was first reported 150 years ago by Herbst (1844) but for some time such findings were regarded as a pathophysiological accident (Herbst, 1844; Voit and Bauer, 1869; Verzar, 1911). Later studies by Thompson *et al.* (1960) and Sanders and Ashworth (1961) again reported uptake of solid particles followed by the even-now highly contentious studies of Volkheimer and Schultz (1968) and Volkheimer (1975) claiming uptake of orally administered 7 - 100 μm starch granules into venous blood and postulating that passage of particulate matter across the intestinal barrier resulted from the activity of the muscularis mucosa layers of the gut kneading particles between cells at the desquamation zones of the intestinal villi, a paracellular process the author termed 'persorption'. Renewed interest in this area and the advent of sophisticated analytical technology has resulted in a number of workers reporting particulate uptake using polystyrene and biodegradable particles in the nanometre - micrometre size range (Eldridge *et al.*, 1989; Pappo and Ermak, 1989; Eldridge *et al.*, 1990; Jepson *et al.*, 1993b). Three possible routes for gastrointestinal uptake of small particles exist; - intracellular uptake by enterocytes, intercellular/paracellular uptake or uptake via the M cells of Peyer's patches (Kreuter, 1991). The dominant opinion appears to suggest that particulate uptake in mammals is principally via the M cells of Peyer's patches (LeFevre and Joel, 1984; Sass *et al.*, 1990; Jepson *et al.*, 1993a, b; Scherer *et al.*, 1993) and although uptake by enterocytes in the villous part of the gastrointestinal tract, (Sanders and Ashworth, 1961; Matsumo *et al.*, 1983; Kataoka *et al.*, 1989; Jani *et al.*, 1992), and

absorption by paracellular means (Aprahamin *et al.*, 1987; Jani *et al.*, 1992) has been demonstrated, these routes probably only play a minor role in particulate uptake (Eldridge *et al.*, 1990). Jani *et al.* (1992) suggested that the upper size limit for absorption by intestinal enterocytes and the paracellular route may be 100 μm . The extent of particulate uptake depends on the size (Eldridge *et al.*, 1990; Sass *et al.*, 1990; Jani *et al.*, 1992), hydrophobicity (Eldridge *et al.*, 1990), charge (Jani *et al.*, 1989, 1992), and polymeric composition (Eldridge *et al.*, 1990) of the particles. Accurate modern techniques for the measurement of fluorescent microparticle absorption indicate that levels of uptake constitute only a small fraction of the administered dose and indicate that earlier reports of high absorption levels were exaggerated (Davis, 1992; Jenkins *et al.*, 1994). The capacity for particulate uptake by the teleost gut has been the subject of very little study and is discussed in chapter 7 in relation to the use of biodegradable microparticles in oral vaccination. A different mode of particle internalisation - gastrointestinal uptake/ invasion of epithelia by live bacteria is discussed in chapter 8.

2.3 Specific defense in mammalian mucosa-associated lymphoid tissue (MALT).

This review thus far has discussed nonspecific constitutive components of the mucosal barrier, a very sophisticated specific inducible immune system is also present in the vertebrate gastrointestinal which possesses many unique features and acts somewhat independently of the systemic immune system. The process of induction of immune B and T cells in MALT followed by their migration to effector sites for the development of mucosal immune responses is termed the common mucosal immune system (Mestecky, 1987), GALT is a major component of this interconnected network. Evidence for the concept of a common mucosal immune system initially came from antibody induction studies (Goldblum *et al.*, 1975), cell migration experiments (Gowans and Knight, 1964) and morphologic studies including the demonstration of specialised antigen-trapping cells in the gut (Owen, 1977). Specialised lymphoreticular tissues in the gastrointestinal and upper respiratory tract possess the capacity to take up environmental antigens (Craig and Cebra, 1971; Rudzik *et al.*, 1975) and oral delivery of antigens in mice and humans leading to stimulation of IgA precursor B cells can result in dissemination of B and T cells to mucosal effector sites such as

lamina propria regions of the intestinal, respiratory and genitourinary tracts and to secretory glands for antigen specific secretory IgA responses (Mestecky, 1987; Phillips - Quagliata and Lamm, 1988; Scicchitano *et al.*, 1988). It is the induction of this local immune system which in most cases is the target of oral vaccination. The mammalian mucosal immune system may be divided into two separate interconnected compartments both functionally and anatomically (McGhee *et al.*, 1992; McGhee and Kiyono, 1993), sites where antigens are encountered and where initial responses are induced and sites where IgA plasma cells are found and where production of secretory IgA leading to local immune protection occurs. Mucosal inductive regions include GALT and are the sites where environmental antigens are encountered. Stimulation of T helper cells and IgA precursor B cells in GALT, particularly in Peyer's patches with orally delivered antigen leads to dissemination of B and T helper cells to mucosal effector sites such as the lamina propria of the gastrointestinal tract and to secretory glands leading to antigen specific secretory IgA antibody responses. Mucosal effector tissues consist mainly of T cells, particularly CD4+ cells of memory/ effector phenotype, they are enriched for B cells and plasma cells (IgA) and are covered by epithelial cells expressing secretory component (SC) which transports polymeric IgA into external secretions.

2.3.1 *Morphological and cellular aspects of MALT.*

GALT in higher vertebrates is composed of the Peyer's patches, appendix, small solitary lymphoid nodules, mesenteric lymph nodes and isolated lymphoid cells in the intraepithelium and lamina propria (McGhee *et al.*, 1992; McGhee and Kiyono, 1993). The Peyer's patches possess a "dome" region enriched with lymphocytes and macrophages and some plasma cells which is covered by a unique epithelium rich in specialised antigen sampling M cells showing thin extensions around lymphoid cells (Owen and Jones, 1974; Wolf and Bye, 1984). These M cells possess short microvilli, small cytoplasmic vesicles and few lysosomes and are specialised for endocytosis and transport of both protein antigens (Bockman and Cooper, 1973; Owen, 1977) inert particles (section 2.2.3) and microorganisms including bacteria, viruses and protozoans (Russell and Walker, 1990) into GALT. M cells transport antigen from the surface luminal membrane

to the pocket membrane with no degradation or chemical alteration (Inman and Cantey, 1983). Peyer's patches have a reduced number of mucus - secreting goblet cells and are therefore more accessible for microorganisms than other epithelial surfaces (Walker and Owen, 1990). Particle binding to the apical membrane of M cells leads to rapid internalisation and shuttling to pocket lymphocytes and to mucosal immune inductive regions (Pappo and Ermak, 1989). Distinct follicles are found under the dome region of the Peyer's patch containing germinal centres where significant B cell division occurs. It is in these germinal centres that the processes of B cell switching to IgA production and affinity maturation occur, indeed these sites contain the majority of surface IgA + (sIgA+) B cells (Lebman *et al.*, 1977; Butcher *et al.*, 1982), in contrast, few plasma cells are found in these regions. It has been shown that a very high percentage (30 - 40 %) of Peyer's patch B cells are in cell cycle due to the constant environmental stimulation of GALT cells. As a result of this stimulation these cells have been used in studies on cytokine production without adding the B cell mitogen LPS in contrast with splenic B cells (Beagley *et al.*, 1988). T cell dependent areas are located adjacent to the follicles; Peyer's patch T cells are mature containing a T cell receptor - CD3 complex and more than 95 % of these cells display the $\alpha\beta$ form of T cell receptor. Additionally, approximately 60% of Peyer's patch T cells are CD3+ CD4+ CD8- demonstrating properties of T helper cells including the provision of support for IgA responses (Hanson and Brandtzeag, 1989). Functional cytotoxic T lymphocytes (CTLs) can also be induced in the Peyer's patch - around 25 % of Peyer's patch T cells having a CD3+ CD4- CD8+ phenotype (London *et al.*, 1987; Hanson and Brandtzeag, 1989). It is thus apparent that all the necessary cells for the induction of mucosal immune responses are present at the MALT inductive sites including CD4+ T helper cells, CD8+ cytotoxic T lymphocytes, sIgA+ B cells and accessory cells. T helper cells (CD3+ CD4+ CD8-) are induced to epitopes presented in conjunction with MHC class II molecules on antigen presenting cells (Marrack and Kappler, 1986; Bierer *et al.*, 1989). Cells present in GALT which may function in antigen presentation include dendritic cells (Steinman, 1991), macrophages (Unanue, 1984), B cells (Chesnut and Grey, 1985), activated T cells (Kabelitz *et al.*, 1987), endothelial cells (Nunez *et al.*, 1983) enterocytes (Mayer and Schlien, 1987) and possibly M cells (Finzi *et al.*, 1993). Liu and MacPherson (1993) found

that orally delivered antigen was effectively presented by intestinal dendritic cells but not by other cells present in the draining lymph. Moreover, antigen-bearing dendritic cells in the lymph were found to prime naive T cells directly *in vivo* while T cells or macrophages could not. Acid phosphatase-positive macrophages were found to process particulate antigens which were transported by M cells from the gut lumen to the dome region of Peyer's patches (Van Reece *et al.*, 1988). A function of macrophages in antigen presentation at this site agrees with the finding that in contrast to dendritic and B cells, macrophages are especially equipped to process and present particulate antigen (Van Rooijen, 1990). Studies on murine species have however demonstrated that mucosa - associated (alveolar and intestinal lamina propria) macrophages can actually have a suppressive effect on immune responses *in vitro* and *in vivo* (Fireman *et al.*, 1988; Thepen *et al.*, 1989; Pavli *et al.*, 1990). Data from a comparative study utilising human cells suggested that intestinal macrophages played no role in stimulation of mixed leucocyte response stimulation despite a high level of class II MHC antigen expression in contrast with dendritic cells which were highly effective in antigen presentation (Pavli *et al.*, 1993). This study suggested that dendritic cells are the major cell population capable of inducing a mixed leucocyte response in the human colonic lamina propria. It has been proposed that the dendritic cell endocytic apparatus is specialised for antigen presentation (Inaba *et al.*, 1993) while the bulk of phagocytic activity in macrophages may result in degradation (Steinman and Cohn, 1972). Overall, dendritic cells are regarded as the major antigen presenting cell population in the Peyer's patch dome and in the T cell region where these cells are referred to as interdigitating cells (Biewenga *et al.*, 1993).

After antigen entry into the Peyer's patch and presentation to B and T lymphocytes, activated B and T cells depart the patches via efferent lymphatics eventually reaching the systemic circulation via the thoracic duct. Transported systemically, these lymphocytes arrive at the mucosal effector sites (lamina propria of respiratory, gastrointestinal and reproductive tracts and glandular tissues) where they are selectively retained (Kraehenbuhl and Neutra, 1992; McGhee and Kiyono, 1993;). Lymphocytes constantly circulate from the bloodstream into lymph nodes and lymph ducts travelling via the lymphatics to re - enter the bloodstream (Yednock and Rosen, 1989), a process

which enables antigen specific B and T cells to survey all the sites where pathogens might appear. Factors controlling the localisation and persistence of lamina propria and other mucosal cells are poorly understood. Antigen was initially believed to be the signal determining the localisation of IgA committed B cells in mucosal sites (Husband, 1982), it is now recognised however that antigen alone is insufficient for B cell localisation and additional T-cell signals also appear necessary (Dunkley and Husband, 1991). Therefore, in addition to their role in providing cognate help for precursors of IgA-producing B cells in Peyer's patches (section 2.3.5) T cells may also provide differentiation signals to incoming plasma cell precursors in lamina propria effector sites (Dunkley and Husband, 1991). The process of lymphocyte escape from blood (extravasation) is facilitated by the differentiation in the endothelial lining of blood vessels in lymph nodes where capillaries widen into postcapillary venules. This endothelium of postcapillary venules (high endothelium) in lymph nodes and related organs is thicker, has a distinctive cell phenotype and is specialised for trapping lymphocytes. Specific migration patterns of lymphocytes activated in peripheral lymph nodes and Peyer's patches respectively are dictated by receptor/ ligand interactions between lymphocyte adhesion molecules 'homing receptors' (Shimizu *et al.*, 1992) with site - specific 'addressins' on high endothelial venules (Jalkanen *et al.*, 1986; Picker and Butcher, 1992). The receptor molecule on lymphocytes which mediates initial recognition and adherence of lymphocytes to high endothelium has been demonstrated to be a member of the 'selectin' family of adhesion molecules (addressins) (Stoolman, 1989). These molecules are named on the basis of the cell type on which they were first demonstrated - L selectin being the peripheral lymph node homing receptor. A mucin - like molecule, Gly CAM-1, identified on high endothelium venule cells has been proposed as an important component of the structure recognised by L selectin on high endothelial venules (HEV), it is uncertain whether this interaction involves protein - protein or lectin - carbohydrate interactions or a combination of both (Seed, 1992). Another group of important adhesion molecules on lymphocytes are the integrins which bind to extracellular matrix proteins or to proteins on the surface of neighbouring cells, a range of such molecules has been identified both in mice and in humans (Hollander, 1993). Under the tripartite influence of antigen, T cells and cytokines B cells undergo clonal expansion to form mature IgA

producing plasma cells (Mestecky and McGhee, 1987; McGhee *et al.*, 1989). In the lamina propria effector region the cellular repertoire includes 20 - 40 % B cells (including mainly IgA+ plasma cells), $\approx$ 10 % macrophages, $\approx$ 5 % eosinophils, $\approx$ 1 - 3 % mucosal mast cells and 40 - 60 % T cells making T cells the most abundant cell type present in these areas (Bull and Bookman, 1977; Kanof *et al.*, 1988). The majority of these T cells are CD3+ CD4+ CD8- demonstrating helper function (Kanof *et al.*, 1988), but $\approx$ one third are CD3+, CD4-, CD8+ and may possess cytotoxic T lymphocyte or suppressor functions. The previously held belief that gastrointestinal plasma cells were all derived from Peyer's patch B cells has been challenged by the recognition that a significant number of murine gastrointestinal IgA plasma cells can derive from a self renewing Ly - 1+ (CD5+) B cell population resident in the peritoneal cavity (Solvason *et al.*, 1991). Results indicate that this B cell lineage may differ from B cells from bone marrow since they are derived from the fetal omentum (Kroese *et al.*, 1988) and demonstrate unique phenotypic and localisation characteristics (Herzenberg *et al.*, 1986; Solvason *et al.*, 1991). Peyer's patches are markedly deficient in Ly - 1+ B cells (Hayakawa *et al.*, 1984). The peritoneum may be seen as an additional induction site for mucosal IgA responses since up to half of the plasma cells in the lamina propria are derived from peritoneal B - 1 cells (Ly - 1+ B cells) (Biewenga *et al.*, 1993). Additionally there has been a suggestion in humans at least that omental 'milky spots' and related lymphoid tissue are implicated in development of abdominal and gastrointestinal immunity particularly during infancy (Koten and Otter, 1991). These omental 'milky spots' which are present in young children gradually transform into fatty tissue only reappearing upon intraperitoneal infection. Omental lymphatic tissue constitutes a discrete lymphatic unit closely associated with the spleen and haemal lymph nodes which surround the branches of the portosplenic venous tree (Dux, 1988; Shimotsuma, 1991). It has been proposed that the function of these spots may be related to food ingestion since after a meal there is a greatly increased blood flow and pooling of blood in the portal venous system resulting in contact between blood which has passed through the gut and the lymphatic tissue associated with the portal system. Therefore in infancy, in a process akin to thymic self/non - self recognition, the omental lymphatic system may adapt so it does not respond to incompletely digested antigenic material transported by the

portal blood (Kotten and Den Otter, 1991). McGhee *et al.* (1992) suggested therefore that both Peyer's patches and the peritoneum may represent sites for B cell development capable of repopulating mucosal tissues.

There is evidence to suggest that there is an element of compartmentalisation in the common mucosal immune system, possibly even within the gastrointestinal tract (McGhee and Kiyono, 1993), therefore considerable caution must be exercised in the design of vaccines and delivery systems so that the immune response is generated at the appropriate site. In addition to the antigen presenting cells described above other phagocytic cells are also present including basophils, eosinophils and neutrophils (McKay and Perdue, 1993), the number of polymorphonuclear leucocytes being elevated in the intestine after helminth infections (Capron *et al.*, 1986). A role for mucosal mast cells in defense against parasitic infection has also been described (Arizono and Nikao, 1988), these cells may produce a range of mediators which influence the epithelium and may induce or exacerbate inflammatory reactions (McKay and Perdue, 1993).

2.3.2 *Specific secretory IgA (S-IgA) mediated humoral defense at the mucosae.*

In the mucosae of mammals, secretory IgA may be regarded as the first line of specific immunological defense. In terms of the quantity produced, the IgA isotype predominates in humans, representing more than 60% of all antibody isotypes generated (Conley and Delacroix, 1987; Mega *et al.*, 1992). Indeed it has been shown that a normal individual produces in excess of 5 g of IgA daily. In mammals, most IgA is produced from plasma cells in mucosal effector regions, particularly in the lamina propria of the gastrointestinal tract (McGhee *et al.*, 1992). IgA may be divided into two subclasses, IgA1 and IgA2 which differ in their distribution in serum and in various external secretions (Mestecky and McGhee, 1987). More than 90% of serum IgA is of the IgA1 subclass and is mainly monomeric. Various secretions contain 50 - 70 % IgA1 and 30 - 50 % IgA2, a higher percentage being found in external secretions. Serum IgA is mainly monomeric, only approximately 10 % being detected in the form of dimers and larger polymers (Delacroix *et al.*, 1982). Secretory IgA is heterologous, being composed principally of dimers,

the proportion of larger polymers may amount to 5 - 20 % of the dimeric fraction however (Delacroix *et al.*, 1982). In external secretions, IgA is found principally in dimeric and tetrameric forms, therefore possessing 4 or 8 binding sites and demonstrating greater avidity than monomeric Ig - the functional significance of this being shown by the observation that polymeric IgA neutralizes viruses more effectively than its monomeric counterpart (Dimmock, 1984; Taylor *et al.*, 1987). This multivalence also enables IgA to neutralise biologically active antigens such as toxins and enzymes and to agglutinate bacteria more effectively than monomers (Killian *et al.*, 1988). S-IgA consists in addition to heavy and light chains, of a 15 kDa joining (J) chain polypeptide (also found in polymeric IgM) (Halpern and Koshland, 1970) disulphide linked to a cysteine moiety on heavy chains (Mestecky, 1976). Immunocytochemical evidence however indicated that there may have been 2 J chains present per IgA polymer which existed as a dimer in the molecular complex (Brandtzaeg, 1985a, b). It is currently believed that regardless of polymer size a single J chain acting as a 'clasp' binds only two subunits while the remaining polymer subunits (in polymers greater than dimeric) are connected by inter - heavy chain disulphide bridges (Garcia - Pardo *et al.*, 1981). S-IgA differs from serum polymeric IgA by its association with an epithelially derived glycoprotein - secretory piece or secretory component (SC). Therefore the secretory polymer consists of an IgA dimer, 1 or 2 J chains and a secretory component with a total molecular weight of 375 kDa (Hanson and Brandtzaeg, 1989). J chain appears to be essential for the association of IgA with SC. Among the principal features allowing S-IgA a selective advantage to fulfil its biological functions in the harsh environment of the gut is its innate proteolytic resistance and its association with secretory component (Mestecky and McGhee, 1987; Killian *et al.*, 1988). The proteolytic resistance of S-IgA appears to result from the dimeric structure *per se* and to the incorporation of SC (Lindh, 1975), the J chain however is very prone to proteolytic degradation. A deletion of 13 amino acids in the hinge region of the $\alpha 2$ heavy chain in IgA2 contributes to the molecule's high degree of resistance to proteolytic enzymes (Mestecky and McGhee, 1987; Killian *et al.*, 1988). It was demonstrated that *Escherichia coli* produces a protease capable of degrading colostral IgA (Moore *et al.*, 1964) but S-IgA can also exhibit antibody activity against some microbial enzymes thus neutralising their activity (Gilbert

et al., 1983). Indeed, S-IgA has been found to be resistant to most IgA proteases as a result of antibody-mediated neutralisation of the enzymes (Kobayashi *et al.*, 1987). Proteases from a number of oral and enteric bacteria are directed against IgA1 (Kilian and Reinholdt, 1986) but while serum IgA is comprised of $\approx 80\%$ IgA1 S-IgA is 30 - 40 % IgA2 (Delacroix *et al.*, 1982). A protease has been isolated from *Clostridia* species capable of degrading both IgA1 and IgA2 of the A2m(1) allotype (Fujiyama *et al.*, 1985) and the yeast species *Torulopsis* and *Candida* also produce proteases capable of degrading IgA1, IgA2 and S-IgA (Reinholdt *et al.*, 1987). It may be surmised therefore that *in vivo* S-IgA is not likely to be totally resistant to proteolysis, indeed it was found that only 20 - 80 % of milk S-IgA was left undegraded in the stool of breast fed babies (Davidson and Lönnerdal, 1987) this study did not however take into account IgA adsorbed onto microbes in the stool. Secretory IgA has been shown to function in immune exclusion in the gut, prior exposure to a foreign antigen leading to diminished absorption of that but not of unrelated antigens due to the presence of specific S-IgA (Andre *et al.*, 1974; Killian *et al.*, 1988). This property of S-IgA limits absorption of undigested antigenic material and the resultant formation of potentially harmful circulating immune (mainly IgG) complexes, a phenomenon seen in IgA deficient individuals (Cunningham - Rundles *et al.*, 1979). A novel method enabling production of IgA hybridomas from Peyer's patches and delivery of monoclonal IgA into intestinal secretions via the transepithelial transport mechanism has demonstrated that monoclonal S-IgA directed against single surface antigens on *Vibrio cholerae* (Winner *et al.*, 1991) and *Salmonella typhimurium* (Michetti *et al.*, 1992) is protective against oral challenge with the homologous organism. Human serum or secretory IgA when complexed with antigen, unlike IgG is unable to activate complement by either the classical or alternate pathways, thus precluding the formation of C3 and C5 cleavage products and consequent induction of local inflammatory reactions such as influx of polymorphonuclear leucocytes and release of mediators leading to tissue damage and increased permeability of mucosal membranes (McGhee *et al.*, 1992). IgA can also prevent against anaphylactic and arthus type hypersensitivity reactions mediated by IgE and IgG isotypes (Russell - Jones *et al.*, 1981). These studies indicate an important role for IgA in the abrogation of inflammatory side effects in the gut due to other effector mechanisms. Secretory IgA also

functions in an anti-bacterial capacity at the mucosae by inhibiting the adherence of microbes to host mucosal epithelial cells (Wold *et al.*, 1990). The inhibition of adherence, whether involving nonspecific hydrophobic interactions or specific bacterial factors and corresponding cell surface receptors is more effectively achieved by S-IgA than IgG of the same specificity due its unique charge, extensive glycosylation and proteolytic resistance (Magnusson and Stjernström, 1982). In addition to this specific antibody function S-IgA can inhibit adherence of a broad range of *Enterobacteriaceae* via terminal mannose containing oligosaccharide side groups on the heavy chain (especially on IgA2 molecules) recognised by mannose specific lectins which are present on type 1 fimbriae. SIgA can also render bacteria mucophilic (Magnusson and Stjernström, 1982) thus enhancing the barrier formed by the mucous layer. Bacteria selectively disadvantaged by specific S-IgA appear to be more effectively displaced by other bacteria in the competitive gut environment, the possession of IgA proteases by certain bacteria (see above) however may be regarded as virulence determinants (Kilian *et al.*, 1988). IgA can also enhance the anti - microbial effects of some innate anti - bacterial factors in external secretions such as lactoferrin and lactoperoxidase (section 2.1.3). A particularly important interaction of IgA may be that with mucosal phagocytic cells and lymphocytes involved in antibody dependent cellular cytotoxicity (ADCC) since *in vitro*, S-IgA can enhance the antibacterial activity of monocytes and lymphocytes of mucosal or peripheral origin (Tagliabue, 1989). The CD3+, CD4+, Leu8+ T lymphocyte subset can bind IgA via Fc receptors and effectively kill *Salmonella* and *Shigella* bacterial species, oral immunization with the *Salmonella typhi* Ty21a vaccine resulting in increased numbers of T cells participating in IgA-mediated ADCC (Tagliabue, 1989). S - IgA is also implicated in the process of virus neutralisation (Ogra *et al.*, 1989), binding of a single antibody molecule to a virus can result in its inactivation (Dimmock, 1984) and secretory IgA has been shown to be particularly effective in this process. S-IgA shows broader specificity in viral neutralisation than comparable serum antibodies (Shvartman *et al.*, 1977) which may be of advantage in contending with antigenic drift of organisms. In the mucus layer, S-IgA can prevent viral attachment to epithelial cells and subsequent penetration (Gartner *et al.*, 1986). The biological importance of the polymeric IgA structure in defense against viral infection was evidenced by the finding that while rat biliary IgA

anti-influenza haemagglutinin prevented viral attachment, both its dissociated monomeric form and IgG allowed attachment, penetration and viral genome accumulation in the cell nucleus (Taylor *et al.*, 1987) indicating that it resulted in a steric inhibition of haemagglutinin attachment to cells. In addition to the well accepted role of secretory IgA as an immune barrier, preventing adherence and absorption of antigens in the gut recent studies utilising polarised epithelial monolayers have suggested additional roles for IgA (Mazanec *et al.*, 1993). It has been proposed that IgA may neutralise intracellular microbial pathogens such as viruses directly within epithelial cells (Mazanec *et al.*, 1992) in the intraepithelial compartment and may bind absorbed antigens in the mucosal lamina propria and excrete them through the adjacent epithelium into the lumen thus ridding the body of locally formed immune complexes and decreasing their access to the systemic circulation (Kaetzel *et al.*, 1991; Mazanec *et al.*, 1991). Thus IgA may provide a comprehensive humoral mucosal defense by operating at the lumen, epithelium and lamina propria to inhibit surface spreading of infection along the mucosa.

2.3.3 Antibody isotypes other than IgA in MALT.

Antibody isotypes other than IgA have also been described in external secretions. Secretory IgM (S-IgM) has been found in small quantities in the gut of adult humans but in greater amounts in young infants and in patients with selective IgA deficiency (Mellander *et al.*, 1986). Secretory IgM (S-IgM) in secretions is found associated with SC, in fact pentameric IgM shows higher affinity for SC and binds it with stronger covalent forces than does dimeric IgA (Brandtzaeg, 1985a). In contrast to S-IgA, S-IgM is sensitive to proteolytic degradation (Haneberg, 1974). Some patients with selective IgA deficiency (at least in countries with good hygiene) live healthy lives, an observation which may be partly explained by a compensating appearance of IgM in external secretions (Hanson *et al.*, 1988). SIgM can exhibit antibody activity (Mellander *et al.*, 1986), IgM antibodies being highly effective in promoting phagocytosis and complement mediated bacteriolysis and in protection against intestinal infections (Hedde and Rowley, 1975). IgM derived from human duodenal fluid was found to enhance neutrophil phagocytosis of *E coli*, an effect which was enhanced by complement (Girard and Kalbertmatten, 1970). Small traces of IgG,

IgE and IgD have been detected in most normal human secretions. Transfer of IgG appears to be a passive process and in contrast with SIgA and SIgM is not associated with SC (Brandtzaeg *et al.*, 1971a). More than 90% of IgG in nasal fluid is of serum origin (Mygind *et al.*, 1975) and this 'leakage' of IgG is enhanced during inflammation (Brandtzaeg *et al.*, 1971). There have been suggestions that transmitted IgG differs in terms of size and antigenicity from serum IgG (Rossen *et al.*, 1966), an observation which may be explicable by preferential local synthesis of some IgG subclasses (Keller *et al.*, 1983). IgG in external secretions has the capacity of complement - dependent bactericidal and opsonizing activities (Eddie *et al.*, 1971) and of immune exclusion of soluble antigens (Stokes *et al.*, 1975), IgG however appears to be rapidly degraded in the gut (Haneberg and Endresen, 1976). IgE in secretions is not associated with SC (Bennick and Johansson, 1971), and relative enrichment in IgE levels in secretions compared to serum are believed to result from passive diffusion through the epithelium (Nakajima *et al.*, 1975) and partly from locally produced IgE by plasma cells but principally by mast cells armed with IgE in regional lymph nodes (Brandtzaeg, 1985). The biological significance of IgE in secretions is unknown and the isotype is rapidly degraded in intestinal fluid (Brown *et al.*, 1975). IgE is believed to be of importance in mucosal membranes for defense against parasites by arming macrophages, platelets and eosinophils (Capron *et al.*, 1987). IgD has been found in trace amounts in colostrum and saliva but not in intestinal juice (Sewell *et al.*, 1979) indicating the possibility of local secretion. IgD producing plasma cells are also absent from the gastrointestinal tract unlike other secretory sites (Brandtzaeg, 1983). Exocrine IgD can demonstrate antibody activity (Keller *et al.*, 1985) but its biological activity is unknown. In some patients with IgA deficiency an increase in IgD immunocytes in the nasal mucosa was noted.

2.3.4 The role of cytotoxic T cells in MALT.

The second major aspect of cell - mediated immune responses in MALT is the activity of cytotoxic T lymphocytes. Virus - infected cells in general are lysed by CD3+, CD4-, CD8+ cytotoxic T lymphocytes which to be induced must recognise a complex of virus - derived peptide and MHC class I molecule expressed on the surface of infected target cells (Marrack and Kappler, 1986;

Bierer *et al.*, 1989). Investigations on the mucosal tissues of both humans and experimental animals have revealed the occurrence of cell mediated cytotoxicity, antibody dependent cytotoxicity and natural killer (NK) activity (Davies and Parrott, 1981; Ernst *et al.*, 1985). Major differences exist in humans between lymphocyte populations in GALT and peripheral blood lymphocytes, for example many of the CD4- CD8+ intraepithelial and lamina propria lymphocytes do not express CD5 - a cell surface marker present on nearly all peripheral blood T lymphocytes and thymocytes (Selby *et al.*, 1983). Intraepithelial (IEL) and lamina propria (LPL) lymphocytes reside in different areas and are significantly different in terms of phenotype, function and morphology (Brandtzaeg *et al.*, 1988). Studies of intraepithelial lymphocytes in many species indicate that most express CD8, exhibit cytotoxic activity and a variable percentage contain cytoplasmic granules (Dobbins, 1986). Lamina propria and intraepithelial lymphocytes are mainly CD45RO+, a marker associated with previously activated or memory T cells (Brandtzaeg *et al.*, 1989). Within CD3+ lymphocytes there are two distinct subpopulations defined by expression of either the $\alpha\beta$ (Marrack and Kappler, 1986) or $\gamma\delta$ (Bell and Bell, 1994) heterodimeric T cell receptor (TCR). IEL's are a unique population of lymphocytes found individually interspersed within the intestinal epithelium (Ernst *et al.*, 1985). They are a very heterogeneous cell population distinguished by their TCR expression, granularity and expression of several activation and differentiation markers. The majority of T lymphocytes in the peripheral blood express the $\alpha\beta$ TCR which recognises antigen in association with classic MHC class I and II molecules. The population of $\gamma\delta$ T cells in peripheral circulation and peripheral lymph nodes is small ($\approx 5\%$), mainly CD4-CD8- (Groh *et al.*, 1989) and recognises MHC in addition to non - MHC encoded ligands (Farstad *et al.*, 1993). Lymphocytes resident in the intraepithelium possess unique features and may be important in the induction and regulation of mucosal immune responses. Intraepithelial T cells may be delineated on the basis of cell surface markers into 4 subsets; CD4-, CD8+ ($\approx 75\%$), CD4+, CD8- ($\approx 7.5\%$), CD4-, CD8- ($\approx 7.5\%$) and CD4+, CD8+ ($\approx 10\%$), (Klein and Kagnoff, 1987). In mice, small intestine - derived IEL's can be subdivided into two populations:- a population of thymus - dependent, antigen - dependent CD3+ CD8+, Thy - 1+ cells most of which use the $\alpha\beta$ form of T cell receptor ($\alpha\beta$ TcR) and a population of thymus -

independent, antigen - independent CD3+ CD8+ Thy - 1- cells most of which use the $\gamma\sigma$ form of TCR (Guy-Grand *et al.*, 1991). While thymus-dependent Thy - 1+, $\alpha\beta$ TCR+ intraepithelial lymphocytes are recruited to the gut after antigenic stimulation by the normal gut flora the thymus - independent Thy - 1-, $\gamma\sigma$ TCR+ population can appear in the absence of antigenic stimulation (Bandiera *et al.*, 1990). This observation and the recognition of a lack of $\alpha\beta$ TCR+ IEL but not $\gamma\sigma$ TCR+ IEL in athymic and germ - free mice suggests that the factors necessary for expansion\ maturation of the populations are likely to differ (Bandiera *et al.*, 1991). Although it was believed that only the Thy - 1- IEL subset was extrathymically derived subsequent work suggested that some Thy - 1+ cells may also be of extrathymic origin (Yoshikai *et al.*, 1991). Murine $\gamma\delta$ T lymphocytes preferentially localise to epithelial surfaces such as the gut, skin and reproductive tract where they constitute a major lymphocyte subset (this population is much smaller in the human intestine), this epithelial predominance has led to suggestions that such cells may engage in immunological surveillance at these sites (Van Kerckhove *et al.*, 1991). $\gamma\delta$ intraepithelial lymphocytes are the first lymphoid cells to appear in the intestine and may play roles in control of oral tolerance, bacterial colonisation and in elimination of damaged epithelial cells (Dunon *et al.*, 1993; Bell and Bell, 1994). It has been noted that in mice there is a greater heterology among $\gamma\delta$ T cells in the intestine (relative to other epithelia) in terms of junctional diversity and the germline gene segments utilised suggesting perhaps a role as foreign antigen - specific cells (Takagaki *et al.*, 1989). Murine studies have indicated the presence in addition to $\gamma\delta$ cells of thymic origin (Dunon *et al.*, 1993) of a thymus-independent subset of $\gamma\delta$ TCR+ intraepithelial lymphocytes (Bandiera *et al.*, 1991) and evidence also exists to suggest that two populations of $\alpha\beta$ TCR IEL's exist, one of which expresses CD8 molecules composed of CD8 α chains only (as opposed to the usual CD8 $\alpha\beta$ heterodimer) which appears to be of extrathymic origin (Rocha *et al.*, 1991). Among subsets of intraepithelial and lamina propria lymphocytes functional cytotoxic T lymphocyte activity has been demonstrated (Ernst *et al.*, 1985). Unlike other primary T cells, freshly isolated murine IEL's are constitutively cytolytic (Lefrancois and Goodman, 1989), an activity associated with the presence of the cytolytic granule markers perforin and granzyme A (Guy - Grand *et al.*, 1991). There is some evidence to suggest that IEL's are activated 'in situ'

by the presence of antigen in the intestinal lumen (Lefrancois and Goodman, 1989). IEL's cannot be stimulated *in vitro* with conventional T cell mitogens, interleukins or TCR specific antibodies (Mosley *et al.*, 1991) and it is not known from where these cells originate and where their selection and maturation occur. There is evidence that IEL's mature extrathymically (LeFrancois, 1991) and after injection into SCID mice it was found that these cells preferentially repopulated the intestinal epithelium. The possible existence of a precursor type cell that preferentially regenerates cells of its own population was proposed by Sydora *et al.* (1993a). There is convincing evidence for the presence of large numbers of extrathymically derived IEL's in the murine intestine (Lynch *et al.*, 1993; Sawyerr *et al.*, 1993) a recent study however suggested that the thymus does influence the development of these cells (Lin *et al.*, 1993). In addition to the recognition of antigen specific CTL's in the intra epithelial compartment a cell subset with natural killer function exhibiting spontaneous cytotoxic activity has been demonstrated (Ernst *et al.*, 1985). Antigen and alloantigen specific CTL's have been induced respectively by oral antigen administration in murine Peyer's patches (Kagnoff, 1978) and intraepithelium (Klein *et al.*, 1985). The latter CTL clones could be subdivided into two subsets - a subset with classic CD8+ cytotoxic T cell characteristics in relation to their proliferative and cytolytic activity, and a subset with the unique property that high concentrations of IL-2 led to their activation to exhibit an antigen non-specific lytic capability including NK activity. IELs were thought to be an immunocompromised population since they responded poorly to T cells mitogens, however a study where Thy-1 enriched and Thy-1 depleted lymphocytes from murine intestinal epithelium were studied separately found that Thy-1 enriched IEL are functionally competent T cells capable of proliferation and lymphokine (IL-3) secretion with T cell mitogens (Con A and PMA) and anti CD3 monoclonal antibody whereas the majority of Thy-1 depleted IEL did not proliferate and secreted minimal levels of lymphokines (Viney and McDonald, 1992). Sydora *et al.* (1993b) found intraepithelial lymphocytes are activated and cytolytic but do not proliferate as well as other T cells in response to mitogens. Among murine CD3+ intraepithelial T cells 50 - 60% express the $\gamma\delta$ form of T cell receptor (Fujihashi *et al.*, 1990) not seen in other organised lymphoid tissues. The capacity for induction of virus specific CTL's in GALT and in other mucosa-associated lymphoid

tissues by oral administration has been demonstrated. In rats, oral administration of vaccinia virus led to the production of virus-specific CTL's in Peyer's patches and mesenteric lymph nodes (Issekutz, 1984). Within 1 week of administration vaccinia specific CD4-, CD8+ CTL's were demonstrated in mesenteric lymph nodes indicating that antigen induced CTL's were transported lymphatically from Peyer's patches to the MLN's. Induction of virus specific CTL's in MALT has also been shown by oral administration of reovirus and rotavirus (London *et al.*, 1987; Offit and Dudzik, 1989) where in addition to the induction of virus specific CTL's in IgA inductive tissues within 6 days of oral administration, antigen specific CTL's were also demonstrated in systemic tissues such as the spleen.

2.3.5 Regulation of specific immune responses in MALT.

In mammals the mucosal immune system has been shown to be regulated by T cells and cytokines. Induction of effective immunity to pathogenic bacteria and viruses requires participation of both helper and cytotoxic T lymphocytes. IgA responses in MALT are subject to control by T cells, indeed there are indications that a separate T cell subset enriched in Peyer's patches but not present in spleen can selectively induce IgA synthesis while suppressing synthesis of IgM and IgG (Kawanishi *et al.*, 1982, 1983). It was found that these murine Peyer's patch switch T cells (Tsw) could selectively induce increased numbers of sIgA+ B cells but did not result in their terminal differentiation into plasma cells. A subset of Peyer's patch T helper cells expressing the Fc α receptor however selectively interacted with sIgA+ B cells resulting in their induction to IgA producing cells (Kiyono *et al.*, 1983). It has been determined on the basis of differential cytokine production that murine CD4+ T helper cells may be subdivided into T helper 1 and T helper 2 cells (Mosmann and Coffman, 1989; Xu-Amano *et al.*, 1993). While Th 1 cells upon activation by foreign antigen, alloantigen or concanavavin - A produce interleukin-2, interferon γ and lymphotoxin (tumour necrosis factor β) Th 2 cells produce interleukins 4, 5, 6 and 10 (Mossmann and Coffman, 1989; Xu-Amano *et al.*, 1993). Interleukins 4, 5 and 6 play roles in B cell responses and Ig synthesis (Beagley *et al.*, 1989) so the higher expression of Th2 cells in MALT may be related to IgA responses at these sites (Taguchi *et al.*, 1990). Th2 cytokines especially

IL - 4, 5, 6 and 10 induce IgA - isotype committed IgA B cells to differentiate into IgA secreting cells (Beagley *et al.*, 1992). Oral immunisation with tetanus toxoid as antigen and cholera toxin adjuvant selectively induced antigen specific Th2 type response thus this may represent the major helper type phenotype involved in the mucosal IgA response in the gut (Xu-Amano *et al.*, 1993). Dunkley *et al.* (1990) found that in addition to the presence of specific cytokines cognate T cell help is required for stimulation of IgA+ B cells in Peyer's patches. It was determined that lymphocyte populations from all mucosa-associated lymphoid tissues contained higher numbers of IL-1 and IFN γ secreting cells than those from spleen (Taguchi *et al.*, 1990). In GALT IgA effector sites were found to possess higher numbers of IL-5 producing Th2 cells than IFN γ producing Th1 cells while an equal distribution of Th1 and Th2 cells was found in IgA inductive sites. Functionally the Th1 cells are responsible for the early inductive phase of B - cell responses to antigen (Giedlin *et al.*, 1986) and Th2 are memory cells which can provide help for secondary IgA committed B cells (Kawakani and Parker, 1992). The intraepithelial lymphocyte T cell fraction is mostly ($\approx$ 80 %) CD8+ (Taguchi *et al.*, 1990). CD8+ cells in this compartment were found to possess the capacity to secrete both IL-5 and IFN γ but very few splenic or Peyer's patch CD8+ T cells secreted these cytokines.

Therefore in MALT, cytokines are involved in controlling IgA responses via their roles in influencing isotype switching to IgA and in the induction of IgA+ B cells to terminally differentiate into IgA plasma cells. Interleukins 4, 5 and 6 appear to constitute the protagonists in regulation of IgA responses in GALT. Recombinant interleukins 5 and 6 but not interleukin 4 have been shown to result in significant increases in IgA production in Peyer's patch B cell cultures (Beagley *et al.*, 1989). Interleukins 5 and 6 are the most effective in inducing IgA synthesis (Beagley *et al.*, 1989; McGhee *et al.*, 1989) and this induction has been shown to be selective for IgA (Beagley *et al.*, 1988; Lebman and Coffman, 1988) at least in the case of IL-5, since IL 6 is produced by most cell types and is involved in providing help for both B and T cell responses (McGhee *et al.*, 1992). Indeed it has been shown that addition of recombinant IL - 5 to Peyer's patch B cell cultures while increasing IgA synthesis had little effect on synthesis of IgM

or IgG (Beagley *et al.*, 1988). Studies on LPS induced splenic B cell cultures indicated an enhancement of IgA production by purified IL - 5, an effect which was augmented by IL - 4 (Murray *et al.*, 1987). IL - 5 produced by murine Th2 cell clones increased IgA production by LPS induced B cell cultures (Coffman *et al.*, 1987). It was determined however that IL - 5 can induce IgA production in LPS stimulated IgA + but not IgA - B cells (Harriman *et al.*, 1988). IL - 5 appears to act specifically in inducing the terminal differentiation of IgA committed B cells to secrete IgA in a means similar to its action on other B cell isotypes. The initial switching to IgA and increased expression of IL - 5 receptors may be induced by LPS so IL-5 could subsequently direct such cells to secrete IgA. IL 6 also appears to induce terminal differentiation of B cells activated with antigen or mitogen and was 2/ 3 times more potent than IL - 5 but in addition to its effect in inducing IgA production by large blast B cells also induced IgA production in small resting B cell cultures (Beagley *et al.*, 1989). Cells in the human lamina propria constitutively produce IL - 1 and granulocyte - macrophage colony - stimulatory factor (GM - CSF) (Pullman *et al.*, 1992) mediators which *in vitro* result in the stimulation of dendritic or epidermal Langerhans cells to mature into potent stimulators of the mixed lymphocyte response (Heufler *et al.*, 1988). A role for transforming growth factor β (TGF β) in mucosal immunity has also been suggested (McGee *et al.*, 1992), this cytokine may be important in such processes as induction of surface IgM+ B cells to commit to IgA and in promoting the maturation of intestinal epithelial cells (Coffman *et al.*, 1989; Sonoda *et al.*, 1989). TGF β is a multifunctional cytokine produced by a range of cell types including macrophages, lymphocytes and intestinal epithelial cells, it inhibits proliferation of cell types including B cells, T cells and intestinal epithelial cells.

2.4. The nature and functions of MALT in teleosts.

2.4.1 Morphological and cellular aspects of teleost MALT.

The structure of MALT in fish has been the subject of a considerable amount of study but its function is still relatively poorly understood. The apparently organised lymphoid tissue in the gut of elasmobranchs (Tomonaga *et al.*, 1986; Hart *et al.*, 1987a,b) and cyclostomes (Linna *et al.*, 1975; Ostberg *et al.*, 1976) associated with the spiral valve are absent from the gut in teleosts.

Instead the MALT of teleosts resides as isolated single cells or small accumulations in the mucosae of the gastrointestinal (Davina *et al.*, 1980; Rombout *et al.*, 1986; Georgopoulou and Vernier, 1986; Doggett, 1989; Rombout *et al.*, 1989a; Davidson, 1991; Rombout *et al.*, 1993a), gills (Smith, 1982; Goldes *et al.*, 1986) and skin epidermis (Lobb, 1987; Peleteiro and Richards, 1985, 1988, 1990; Davidson, 1991).

The GALT of teleosts has been described in a number of species, (Krementz and Chapman, 1975; Weinberg, 1975; Temkin and MacMillan, 1986; Doggett, 1989; Davidson, 1991; Rombout *et al.*, 1993a). Doggett (1989) found GALT in all regions of the tilapia gut but lymphoid cells were most numerous in the intestine. Among these cells were found lymphocytes, plasma cells, macrophages and granulocytes, lymphocytes and plasma cells were found in both the lamina propria and intra epithelial compartments. In contrast, macrophages were present mainly in the lamina propria and of three granulocyte types found in the mucosa two were found in the lamina propria which were not detectable in tilapia peripheral blood. Large and small intestinal macrophages have been described in the rainbow trout (Davidson, 1991) and carp (Rombout *et al.*, 1985; Rombout *et al.*, 1989b), in the latter studies the small cells being found in the lamina propria and capillaries and the larger cells between epithelial cells. Such phagocytes have been demonstrated to take up both soluble (Rombout *et al.*, 1985; Georgopoulou *et al.*, 1988; Rombout and van den Berg, 1989) and particulate (Davina *et al.*, 1982; Rombout *et al.*, 1986) antigens. It has been suggested that the ability of intestinal macrophages to take up antigen and to express antigenic determinants on the cell surface may reflect an antigen presentation capacity (Georgopoulou *et al.*, 1988; Rombout and van den Berg, 1989). Neutrophils have been described in the digestive tract mucosa of carp (Rombout *et al.*, 1989b) and rainbow trout (Sharp, 1990). The presence of macrophages and neutrophils in the piscine cutaneous mucosa has also been demonstrated (Mittal *et al.*, 1980; Peleteiro and Richards, 1985; Davidson, 1991). Macrophages can act as accessory cells in fish producing cytokines which can lead to lymphocyte activation (Miller *et al.*, 1985; Vallejo *et al.*, 1990). Granulocytic cells have also been demonstrated in teleost mucosae (Doggett, 1989; Vallejo and Ellis, 1989; Davidson, 1991). Eosinophilic granular cells (EGC) are found in the salmonid

gut (Vallejo and Ellis, 1989; Powell *et al.*, 1993) and have been likened to mammalian mast cells. In a recent study Dorin *et al.* (1993) found that after enteric administration of homologous and heterologous (bovine) somatotropin to rainbow trout, both proteins were detected inside intestinal macrophages whilst only the heterologous protein was found in ECG's. This may indicate a specific defensive role for these cells in the salmonid gut. ECG's do not appear to be present in the salmonid cutaneous mucosa (Davidson, 1991). The existence of Langerhans - like cells in teleost mucosae has been proposed (Mittal *et al.*, 1980; Davidson, 1991). The lymphocytes identified in the epithelium of tilapia in contrast to those in the peripheral blood were not periodic acid schiff stain (PAS) positive (Doggett, 1989). All cell types except lymphocytes were present in greater numbers in the lamina propria, lymphocytes being dominant in the intraepithelium. Among the number of studies listed above differences in the distribution of lymphoid cells are apparent but in accordance with the findings of Doggett (1989) greater numbers of lymphocytes were identified in the epithelium than in the lamina propria of goldfish (Weinberg, 1975) and rosy barb (Davina *et al.*, 1980) respectively. Davina *et al.* (1980, 1982) found that the number of leucocytes in the intestine of cyprinids increased considerably after antigenic stimulation and Davidson (1991) suggested that the finding in rainbow trout of large and small lymphocytes in the intestine might reflect the presence of cells in a constant state of stimulation as is the case with mammals due to incessant interactions with luminal antigens. That lymphocytes in fish act as effector immune cells has been well demonstrated with cells from the systemic compartment (Graham and Secombes, 1988; Vallejo *et al.*, 1991; Vallejo *et al.*, 1993). Davidson (1991) found that lymphocyte - like cells were much less frequent in the cutaneous compared to the intestinal mucosa possibly suggesting the greater exposure to antigen via the latter route. The presence of antibody - containing cells in the intestinal (Rombout *et al.*, 1989b) and cutaneous mucosa (Peleteiro and Richards, 1988) has also been demonstrated in addition to antibody-binding, antibody secreting (Georgopoulou and Vernier, 1986) and plasma cells in the intestine (Doggett, 1989).

The large numbers of lymphocytes present in the teleost epithelium suggests a similarity with the

mammalian GALT, however an analogy requires a more careful delineation of the nature and functions of the cells present. Unfortunately, there is little definitive information available on the exact nature of the lymphocytes in teleost GALT. Weinberg (1975) suggested that putative T cells were present in the goldfish intestine and Davina *et al.* (1980) indicated the existence of thymus-derived cells in the cyprinid intestine. Studies which demonstrated an increase in the numbers of IEL's after enteric administration of antigen indicated that these cells were responsive to antigenic stimuli (Weinberg, 1975; Davina *et al.*, 1980; 1982). The most convincing evidence to date that the lymphocyte distribution in the teleost GALT (in the lamina propria and intraepithelium) is analogous to that in mammals comes from a recent study by Rombout *et al.* (1993a). Using monoclonal antibodies directed against carp immunoglobulin or carp leucocytes the authors found that the cell population of the intestinal epithelium consisted mainly of Ig - negative lymphoid cells whereas in the lamina propria numerous Ig-positive lymphoid cells were found. Granulocytes and Ig-negative cells were found in the vicinity of the Ig-positive cells in the lamina propria. The authors suggested that this cell distribution may indicate the presence of putative T cells or natural killer cells in the intraepithelium and B cells and/ or plasma cells in the lamina propria. If this postulate proves correct it indicates a considerable degree of homology with the diffuse GALT of mammals.

The role of cytokines in regulating immune responses in GALT has not been investigated in great detail to date. Davidson (1991) investigated the cell-mediated immune functions of isolated intestinal cells in rainbow trout *in vitro*, finding that gut cells released powerful chemoattractants. Interestingly, the author found that intestinal leucocytes appeared unable to migrate upon treatment with these chemoattractants, to which head kidney cells did respond, and suggested that the accumulation of leucocytes in the gut may be a result of active migration of cells to this site rather than these cells having originated in the gut. The same author demonstrated that intestinal cells were responsive to the T cell mitogen, phytohaemagglutinin (PHA). Additionally, a factor capable of up - regulating bactericidal pathways in macrophages was secreted by intestinal cells upon stimulation with concanavalin A (Con A) or phorbol myristate acetate (PMA). Davidson (1991) thus postulated that the ability of rainbow trout intestinal cells to activate and sequester cells from

other branches of the immune system might be greater than their capacity to act as effector cells.

2.4.2 Evidence for a common mucosal immune system in teleosts.

Suggestions that a common mucosal immune system exists in teleosts have been based primarily on the finding of specific secretory antibody at mucosal sites remote from that of enteric antigen application (Kawai *et al.*, 1981; Kawai and Kusuda, 1983; Rombout *et al.*, 1985; 1989a; 1993b). The secretory antibody responses produced to enterically delivered antigen are discussed in detail in chapter 6 and will only be outlined here. After enteric delivery of particulate antigens specific antibodies have been detected in cutaneous mucus and bile but rarely in the serum (Rombout *et al.*, 1989a; Fletcher and White, 1973; Kawai *et al.*, 1981). Other factors such as the uptake of antigens in the second gut segment (section 4.1), its transport to large intraepithelial macrophages and the demonstration of antigenic determinants on their surface suggesting an antigen presenting function (Rombout *et al.*, 1985; 1986; 1989b) indicated that the GALT of teleosts could initiate immune responses.

Before any analogy can be drawn between the common mucosal immune system and the local immune mechanisms of teleosts a number of points must be considered. Firstly the enterocytes of the second gut segment/ posterior gut of teleosts which are implicated in macromolecule uptake appear dissimilar to M cells in that they appear to actively degrade antigen (section 4.1) unlike M cells which shuttle macromolecules undegraded to the pocket region of the Peyer's patch (section 2.3.1). There have been some indications that M cells may degrade certain absorbed antigens and partake in antigen presentation (McGhee and Kiyono, 1993) but it is widely accepted that these cells shuttle macromolecules intact across the epithelium. Second, there appear to be no organised germinal centres or T cell regions present beneath the absorptive enterocytes in this second gut segment, at most only a few lymphoid cells have been observed interacting with absorbed antigen unlike Peyer's patches where a highly organised inductive environment exists. Third, there is presently no evidence for specific recirculation of GALT derived lymphocytes back

to gut effector regions and indeed no evidence for a functional separation between inductive and effector regions in teleost GALT. To establish whether a common mucosal immune system akin to that in mammals exists a great deal more fundamental work on such aspects as the functional heterogeneity of lymphoid cells in teleost GALT, the capacity of lymphocytes, activated in GALT to 're-seed' these regions and the origin and function of secretory immunoglobulins in teleost secretions. Presently it appears plausible to suggest that teleost GALT merely represents an ancestor of its mammalian successor. GALT is recognised as the most ancient lymphoid organ in vertebrates (DuPasquier, 1993). It is thus conceivable that the presence of lymphoid cells in the intraepithelium and lamina propria was an early adaptation in vertebrates to provide a first line of specific defense. The suggestion by DuPasquier (1993) that the evolution of 'classic' secondary antibody responses replete with the characteristic of affinity maturation in birds and mammals coincided with the appearance of germinal centres, reliant on follicular dendritic cells as their organisational unit if correct may be of value in elucidating the phylogenetic position of teleost GALT. The appearance of germinal centres in Peyer's patches may have been required to facilitate the potent antibody responses, albeit difficult to induce, produced in mammals to some enterically presented antigens. If mammalian GALT for its organisational and functional (inductive and effector sites) integrity depends on the Peyer's patches and particularly on a type of dendritic cell and if these components are absent in the GALT of teleosts then any direct analogy appears flawed. There have been a number of suggestions that the presence of T and B cells of unconventional phenotype and origin (i.e. extrathymic or extra - bursa respectively) in the mammalian gut may indicate that they derived from an ancient cell lineage (Sawyerr *et al.*, 1993; Bell and Bell, 1994; Ohtsuka *et al.*, 1994). These cells have been shown to be immunologically responsive to antigen but at present are still very poorly understood. With increased understanding of the nature of the diffuse GALT in mammals it may become apparent that the teleost mucosal immune system is its homologue and thus the elucidation of the function of the diffuse GALT in mammals may cast light on its function in the teleost or vice versa. Therefore there may well be an alternative analogy, between the teleost GALT and the diffuse GALT of mammals.

2.5 Immunological roles of epithelial cells in the gut

There is accumulating evidence to suggest that intestinal epithelial cells play a more pro - active role in immunological responses in the gut than was formerly believed (Panya *et al.*, 1993). The importance of enterocytes in immune responses in the gut is highly contentious (Stenson and Alpers, 1994). Intestinal epithelial cells have been shown to express class II MHC antigens and to present antigen to CD8⁺ T cells in both rats and humans (Bland and Warren, 1986; Mayer and Shlien, 1987; Kaiserlien *et al.*, 1989; Mayer *et al.*, 1991; Olivier *et al.*, 1994). In contrast to typical non - professional antigen presenting cells enterocytes, particularly in the small intestine constitutively express class II molecules *in vivo* (Mayer *et al.*, 1991b). The distribution and intensity of class II molecule expression on gut epithelial cells can be altered by immune parameters (Gilhar *et al.*, 1993). The ability of these cells to take up and present large peptides to primed T cells *in vitro* suggests they may play an important immunological role, possibly relating to the generally suppressed tone of the gastrointestinal tract. In addition to the expression of class II antigen there is evidence that CD1d - a class I - like molecule expressed on murine and human intestinal epithelial cells may be important in intestinal epithelial cell - T cell interaction (Panya *et al.*, 1993). Class II antigen expression on enterocytes can be upregulated following enteric parasitic infection probably via cytokine release from T cells (Masson and Perdue, 1990). The finding reported by Kaiserlian (1991) that the class II antigens expressed by enterocytes are antigenically distinct from those on conventional antigen presenting cells may also have implications with regard to the consequences of enterocyte-mediated antigen processing. It has been found that class II antigen expression on enterocytes is regulated by cytokines, particularly IFN γ (Hughes *et al.*, 1991). Treatment of colonic epithelial cell lines with IFN γ and TNF α resulted in a pattern of class II antigen expression similar to that observed in the intestinal epithelium *in vivo* (Brandtzaeg *et al.*, 1992). IEL can secrete IFN γ and IL - 5 after antigen challenge (Fujihashi *et al.*, 1992) and IFN γ , IL-3 and IL - 2 after stimulation with parasite antigen (Dillon and MacDonald, 1986) which may increase class II expression on enterocytes. A number of inflammatory mediators can also activate epithelial cells (McKay and Perdue, 1993) and the epithelial cells can up - or down-regulate immune cell activity (Shanahan *et al.*, 1988). The

recognition of receptors for TGF β on intestinal epithelial cells in addition to its secretion by such cells suggests that the cytokine may act as an autocrine regulatory factor for intestinal epithelial cells (Coffey *et al.*, 1987; Barnard *et al.*, 1989). Functional IL-2 receptors have also been reported on intestinal epithelial cells and it was suggested that this cytokine may serve to integrate epithelial and lymphocyte responses in the intestinal mucosa (Ciacci *et al.*, 1992). Therefore in addition to the role of intestinal epithelial cells in the transport of locally produced polymeric IgA to the intestinal lumen (Mestecky and McGhee, 1987) these cells are also implicated both as immune regulatory and effector cells in GALT. IEC's can produce and may express receptors for IL-6 (Shirota *et al.*, 1990) and after LPS stimulation have also been shown to contain mRNA for IL-1 (Mayer *et al.*, 1990), IL-1 and IL-6 are produced by most antigen presenting cells and provide help for both B and T cell responses. TGF β can enhance expression of secretory component and MHC class I antigen on an epithelial cell line (McGee *et al.*, 1991). Utilising an adenocarcinoma cell line both IFN γ (an effect enhanced by IL-4) and TNF α enhanced SC expression (Sollid *et al.*, 1987; Kvale *et al.*, 1988) and IFN γ also enhanced MHC class II antigen expression on these cells (Phillips *et al.*, 1990). TGF β results in enhanced IL-6 secretion by an intestinal epithelial cell line in dose dependent fashion (McGee *et al.*, 1992). LPS activated M cells also produce IL-1 in Peyer's patches (Pappo and Mahlman, 1993). In addition to this role of intestinal enterocytes Peyer's patch M cells also produce IL-1 upon activation by LPS (Pappo and Mahlman, 1993) and since M cells recognise and translocate gram negative bacteria *in vivo* (Owen *et al.*, 1986) the interaction of M cells with luminal LPS may result in the delivery both of antigen and IL-1 as signals for the induction of mucosal immune responses. In contrast to these reports of positive immunological effects for enterocytes, Llana and Bell (1993) identified a factor produced by rat enterocytes which inhibited lymphocyte responses to con A and IL-2. The authors suggested that this agent may contribute to the low levels of intestinal T cell reactivity observed *in vivo*.

Very little is known of any immunological function for enterocytes in the gut of fish. Tomonaga *et al.* (1986) described structural modifications of absorptive enterocytes over discrete lymphoid

aggregations in the elasmobranch gut but similar modifications have not been described in teleosts. It has been suggested that IFN γ is present in fish (Graham and Secombes, 1990), if this is confirmed it would be interesting to examine its effects, if any on enterocyte processing of antigen. Fish lymphocytes respond to mammalian interleukin 1 (Hamby *et al.*, 1986) and similar factor(s) are produced by fish epithelial cells (Siegel *et al.*, 1986) possibly indicating an immunoregulatory function.

2.6 Oral tolerance

That the effector, accessory and regulatory components necessary for the induction of specific immunity via enteric presentation of antigen exist is now widely accepted; in many cases however oral delivery of antigen results not in an immune response but in the induction of specific tolerance to that antigen. Indeed it is believed that under normal conditions a state of oral tolerance to intestinal antigens is actively established (Tlaskalova - Hogenova and Mandel, 1992; Manganaro *et al.*, 1994). Oral administration of allergens, foreign proteins or cell - bound antigens can induce systemic suppression of subsequent humoral and cell - mediated immune responses - a phenomenon termed 'oral tolerance' (van Hoogstraten *et al.*, 1993). Immune responses subject to this form of unresponsiveness include IgG, IgM and IgE antibody production, antigen - specific T cell proliferation and delayed skin reactions (Kagnoff, 1978, 1987; Elson, 1985; Revillard *et al.*, 1992). Observations on oral tolerance have normally been made by the study of antibody production and delayed type hypersensitivity (DTH) but recent evidence indicates differences in the patterns of lymphokines produced after priming and tolerising regimes (Hayne *et al.*, 1993).

It was determined that short feeding periods with the non - cross reactive contact allergens nickel and chromium resulted in a dose dependent and metal - specific suppression of subsequently induced allergic contact hypersensitivity (van Hoogstraten *et al.*, 1993). This study found that with orally treated guinea pigs cutaneous contacts with the allergens provided boosting tolerogenic signals indicating that oral tolerance resulted not from clonal deletion but from active antigen

specific immunosuppression and additionally reported that unresponsiveness to cutaneous immunization was transferable by lymphoid cells from guinea pigs in metal-specific fashion. It is however the potential induction of oral tolerance to protein antigens (or rather its avoidance) which is of most importance to enteric vaccine (particularly protein/ peptide vaccines) design and formulation of oral immunization regimes.

Ovalbumin (OVA) has frequently been shown to result in oral tolerance and has been used to investigate the nature of the phenomenon. Suppression of the systemic antibody response to OVA may be transferred between animals with cells derived from the Peyer's patches or spleen (Ngan and Kind, 1978; Richman *et al.*, 1978). Bruce and Ferguson (1986a) found that serum collected from mice 1 hr after oral exposure to ovalbumin contained a factor which could induce systemic suppression of cell mediated immunity in mice when administered i.p. The factor was absent from serum of parenterally exposed mice and the authors suggested that after absorption from the gut into the serum the immunological properties of the molecule are altered. Subtle alterations to the native protein as a result of intestinal processing of the molecule could thus result in exposure of suppressor determinants on the molecule or the loss of helper determinants or both (Bruce and Ferguson, 1986b). An alternative explanation for these findings might have been the release of a factor by cells in the gut after contact with the antigen which down-regulated subsequent responses. Kay and Ferguson (1989a, b) suggested that serum factor(s) present 1 hr after feeding and suppressor cells found in mesenteric lymph nodes and spleen 1 week after feeding contributed to mediating oral tolerance of cell mediated immunity in mice orally exposed to cholera toxin. It was proposed that a radiation sensitive 'afferent' suppressor T cell population with a Ly 1- 2+ 3+ surface phenotype active in early inductive stages of the immune response was responsible. That these cells were initially stimulated in GALT and thereafter migrated to other lymphoid tissues was indicated by the observation that suppressor T cells were found in Peyer's patches three days after feeding and in spleen only after seven days (Richman *et al.*, 1981). A primary role for T suppressor cells in inducing oral tolerance was suggested by a number of authors (Richman *et al.*, 1981; Lamont *et al.*, 1987; Cowdery and Johlin, 1984). It has been postulated

that other mechanisms such as clonal inhibition might be implicated, indeed in mice fed HGG, in addition to the demonstration of suppressor cells indirect evidence for inhibition of HGG-specific B cell clones was reported (Vives *et al.*, 1980). MacDonald (1982) however provided strong evidence for a role of suppressor T cells in the immunoregulation of oral tolerance and it was suggested (in line with previous data) that suppressor T cells generated in Peyer's patches migrate to mesenteric lymph nodes and inhibit systemic immune responsiveness (MacDonald, 1983). Development of DTH to nickel was suppressed in dose dependent fashion and the hyporesponsiveness could be transferred by CD8+ cells (Van Hoogstraten *et al.*, 1993). Recently Melamed and Friedman (1993) found evidence for specific T lymphocyte anergy after a single oral administration of ovalbumin and excluded the involvement of bystander or specific suppression in this process, this direct evidence for oral tolerance resulting from T cell anergy is in contrast to the general view that the process is a result of suppression. Hayne *et al.* (1994) have reported differences in epitopes recognised by T cells during oral tolerance and priming which suggests that the initial antigen presentation event may be pivotal in determining the immunological outcome of enteric antigen administration.

The whole concept of T cell-mediated immune suppression is presently a highly contentious field, so much so in fact that in a recent article Green and Webb (1993) stated "There is little doubt that the 'S' word is the nearest thing to a dirty word we have in cellular immunology". Much of this scepticism has resulted from the absence of distinct phenotypic markers for such cells. The findings that the cells implicated in suppression do not express the cell marker, CD45RA in contrast with cytotoxic T lymphocytes (Inoue *et al.*, 1993) and the production of candidate monoclonal antibodies for both human and murine T suppressor cells (Torimoto *et al.*, 1992) may indicate an imminent acceptance of a function role for these cells. T suppressor cells may suppress immune responses via the inhibitory effects of cytokines released in response to the presentation of specific antigenic peptides - production of TGF β after antigen challenge for example (Miller *et al.*, 1992). This occurs in response to a specific antigen by T cells which were originally activated by oral administration of small amounts of antigen such as myelin basic protein

or type II collagen, re-exposure to the antigen by injection results in the inhibition not only of those immune responses directed to the same antigen but also those directed at accompanying antigenic determinants on the same or different molecules. Possible roles have been suggested for other cytokines in addition to TGF β such as IL-4, IL-10, and IL-2 and a number of other factors as the means of action of suppressor T cells (Green and Webb, 1993).

The requirements for induction of T suppressor cells have been reported to be distinct from those for induction of T helper cells. 10 - fold fewer antigen presenting cells are required for induction of T suppressor than T helper cells. It has been suggested that the macrophage is the most efficient antigen presenting cell in the induction of suppressor cells and these macrophages may be distinguished from those which induce T helper cells by the criteria of UV and cyclophosphamide sensitivity. MHC class II molecule expression on antigen presenting cells is necessary for T suppressor cell induction and interferon γ which induces MHC class II expression enhances the capacity of macrophages to generate T suppressor cells. Induction of T helper and suppressor cell responses can be mediated by the same MHC class II - bearing macrophage cloned cell lines (Jayaraman *et al.*, 1991; Simon *et al.*, 1991). Macrophages or related cells residing in privileged sites such as the brain selectively induce tolerance via murine T suppressor cell responses thus making these sites less prone to immunological attack (Wilbanks *et al.*, 1992). Phenotypic analyses of T suppressor cell populations indicate that both CD4+ and CD8+ subsets are required for suppression. CD4+ MHC class II - restricted inducer T suppressor cells are necessary early in an immune response but do not usually directly mediate suppression. Barone and Michael (1994) recently found that CD4+ but not CD8+ T cells were required for the induction and maintenance of high-dose oral tolerance. The regulation of oral tolerance induction appears to be very complex and depends on a range of interdependent factors. A recent study found that oral exposure to OVA in mice could result either in oral tolerance or in the induction of circulating anti OVA antibodies (Faria *et al.*, 1993). This study found that the principal deciding factor was the age of mice; strains of mice susceptible to induction of oral tolerance at 8 weeks of age became refractive at 24 weeks. It has been reported that both B cell (Doken *et al.*,

1980) and T cell populations (Dekkruyff *et al.*, 1980) in 24/ 28 week old mice are more refractory to tolerance induction than 6/ 8 week old mice. In concert with the decrease in susceptibility to oral tolerance induction in 24/ 44 week old mice is a parallel increase in the capacity to induce specific serum antibody after oral antigen administration (Kawanishi and Kiely, 1987). It has been proposed that it is in the period between the achievement of immunological maturity and before full adulthood that oral tolerance is most likely to occur (Hanson, 1981). Brandtzaeg *et al.* (1989) suggested that intestinal tolerance could be abrogated in the presence of enhanced class II MHC molecule expression on epithelial cells; normally with only minor levels of expression of such molecules induction of T suppressor cells is predominant, increased expression of class II molecules may result in general overstimulation of helper T cells with the production of specific antibodies and effector T cell clones (Pawalec *et al.*, 1988). Non - T cells may also participate in the induction of class II molecules presumably by producing cytokines such as interferon γ (Gilhar *et al.*, 1993).

The dose of and intervals between antigen doses are also critical determining factors in induction of oral tolerance (Nicklin and Miller, 1987). The extent and duration of unresponsiveness to OVA depends partly on dose but a single bolus feeding could lead to partial unresponsiveness lasting for months (Elson, 1985). In a previous study it was found that with 24 week old mice (Faria *et al.*, 1993) gavage administration of OVA resulted in an immune response, while voluntary ingestion of the antigen did not. Stokes *et al.* (1982) suggested that oral immunisation may require intermittent and rapid antigen uptake whereas oral tolerance induction needs gradual and continuous antigen administration, the most effective means of inducing tolerance being continuous administration of antigen in small doses. An interesting recent study however (Fitzpatrick *et al.*, 1992) found that feeding of OVA daily for 2 weeks to adult ponies led to significantly greater serum anti OVA IgG and antigen specific lymphocyte responses after parenteral injection with OVA in saponin. This study suggested that rather than becoming tolerant and despite the absence of B - or T - cell activation in peripheral blood during OVA feeding the animals were actually primed for an active secondary immune response and indicated that the equine gastrointestinal tract may

exhibit novel food antigen handling properties.

The form of antigen presented to the gut appears to of great importance, Enomoto *et al.* (1993) found that feeding of milk whey protein as a constituent of the diet led to oral tolerance and a systemic humoral response but heat - denatured whey protein led only to oral tolerance. An important role antigen fragments produced by digestive enzymes in the induction of oral tolerance has been proposed (Michael, 1989; Michael, 1993; Hachimura *et al.*, 1993; Brown *et al.*, 1994). Michael (1989) found that a non - immunogenic peptic digest of BSA was immunosuppressive when administered orally or injected into the mouse ileum whereas untreated BSA was only tolerogenic when administered orally but immunogenic following ileal administration. The author proposed that degradation of orally administered proteins by digestive enzymes of the gastrointestinal tract was necessary to acquire tolerogenic properties. A subsequent study (Michael, 1992) followed the logic that if digestive processing is implicated in the induction of oral tolerance, its abrogation would lead to the induction of immune reactivity. This study found that OVA dissolved in water and administered orally resulted in unresponsiveness to subsequent intraperitoneal challenge while oral administration of OVA made resistant to proteolytic digestion induced a vigorous humoral (in blood and intestinal secretions) and cellular immune response. It was further noted that oral administration of this proteolysis resistant antigen broke the orally induced tolerance to systemic challenge.

There have also been suggestions in teleosts of reduced responses to parenteral immunisation with vaccine after prior or concurrent oral immunisation (Udey and Fryer, 1978; Rombout *et al.*, 1989; Davidson *et al.*, 1993). Davidson *et al.* (1994) found that pre-exposure of rainbow trout to HGG for up to ten days had no effect on the subsequent antibody response to parenteral immunisation but when the antigen was administered simultaneously via the oral/anal and i.p routes the serum antibody response was significantly delayed. The suppression was almost complete when antigen was administered simultaneously via the anal and i.p routes. The authors also reported that no such effect resulted when *Aeromonas salmonicida* was used as the antigen which may indicate as

in mammals that oral tolerance in teleosts is more difficult to induce with particulate antigens.

2.7 Oral vaccination

Strategies for the oral immunisation of mammals and teleosts has been reviewed in detail previously (Jenkins, 1992) so only a brief overview is presented here. The literature on oral vaccination against enteric disease and experimental studies on mucosal responsiveness is beset by variable efficacy and is frequently difficult to interpret. Certain rules appear to apply however; live microorganisms provide much better antigens than killed bacterial or viral antigens possibly as a result of their capacity to adhere to mucosal surfaces (McGhee *et al.*, 1992). Most soluble antigens are less effective in inducing mucosal responses than particulate antigens (Biewenga *et al.*, 1993). This is thought to result from different routes of entry and subsequent differences in the cell types involved in antigen processing. Uptake of particulates into Peyer's patches may lead to presentation by dendritic cells and macrophages leading to the induction of immunity while uptake of soluble antigens by Peyer's patches is less efficient and antigen is taken up mainly across the villi and processed by macrophages in the lamina propria which may have a suppressive effect on immune responses (Soesatyo, 1992). Not all soluble proteins are poor mucosal immunogens however - some proteins/ glycoproteins such as cholera toxin (Walker, 1994) ricin and influenza virus haemagglutinin (Bergman *et al.*, 1986; Bergman and Waldman, 1989) and others (Mestecky and McGhee, 1989) can effectively induce antibody responses in serum and secretions of orally immunised subjects. An enhancement in the systemic and mucosal immune responses produced to orally administered BSA as a result of encapsulation in liposomes has been reported (Fujii *et al.*, 1993). MDP, liposomes and recombinant gram negative bacteria exhibit adjuvant activity when given orally (Michalek *et al.*, 1989). Aizpurna and Russel-Jones (1988) found proteins with lectin/ lectin - like binding activity are good mucosal immunogens whereas those lacking such activity are ineffective or suppressive. A number of problems hamper the development and delivery of oral vaccines however. Much higher and more frequently administered antigen doses are generally needed for oral compared to systemic immunization (McGhee *et al.*, 1992). It is believed that the poor responses elicited to orally delivered antigens is a result of gastrointestinal

enzymic degradation and low absorption levels resulting in little immunogenic antigen reaching the GALT (Hemmings, 1978).

In general live replicating antigens which multiply in the gut lumen or ideally in the GALT more effectively stimulate local and generalised systemic and secretory immunity (Mestecky, 1987; Mestecky and McGhee, 1989). Basic strategies have been applied to enhance the response to orally delivered antigens such as the administration of sodium bicarbonate to protect antigens from gastric acidity and proteolysis (Black et al, 1983; Mestecky, 1987). A wide range of other strategies such as the use of prills, immune stimulating complexes (ISCOMS), saponins and adjuvants for oral immunisation are also the subject of investigation (Jenkins, 1992). The use of gastric inhibitors (chapter 5), anti - proteases (chapter 3), microparticles (chapter 7) and live recombinant vectors (chapter 8) are discussed later in this thesis. Only cholera toxin will be discussed here as an example of an effective mucosal immunogen and adjuvant for heterologous antigens.

Cholera toxin (CT) is an enterotoxin produced by *Vibrio cholerae* which exhibits a very high affinity for many nucleated cells including intestinal enterocytes via a specific GM1 ganglioside receptor (Cuatrecasas, 1973). CT comprises a toxigenic A subunit which acts in ADP - ribosylation of the adenylate cyclase regulatory G proteins and a B subunit of five non - covalently associated subunits which acts as a carrier for the toxic A subunit (Betley *et al.*, 1986) and binds to the GM1 monosialoganglioside (Cuatrecasas, 1973). CT is a very potent mucosal immunogen resulting in specific S - IgA response and a plasma IgG response after oral administration (Lebman, Griffin and Cebra, 1977). This is not a universal phenomenon however, the S - IgA and plasma IgG responses to CT are restricted by the I - A subregion of the H - 2 MHC locus (Elson and Ealading, 1985) indicating that knowledge of the genetic status of subjects is necessary prior to use of CT as an adjuvant. Two immunological features of the immune response to CT make it particularly suitable as a mucosal adjuvant. The antibody response to CT appears to regionalise, the plasma cell response being most abundant at sites directly exposed to CT (Pierce and Cray, 1982), this suggests that by judicious local mucosal - oral combined immunisation the

IgA response may be induced at desired intestinal or non - intestinal sites. A prolonged systemic and/or mucosal memory response to CT itself and to unrelated antigens when administered simultaneously has been demonstrated after oral immunisation in mammals (Lycke and Holmgren, 1986; Vajdy and Lycke, 1992, 1993a, b; DeVos and Dick, 1993). CT and CTB are among the few proteins that do not induce oral tolerance (Elson and Ealting, 1984). The use of cholera toxin (CT) or its non-toxic B subunit (CTB) as an adjuvant in oral delivery systems has indicated that CT and CTB can prevent the induction of oral tolerance (Pierre *et al.*, 1992). Oral delivery of OVA with CT or CTB to mice prevented hyporesponsiveness to a subsequent parenteral injection of OVA. Enteric immunization of CT\ CTB with OVA actually appeared to prime the immune response leading to a stronger response to subsequent parenteral injection of OVA with FCA than in mice primed with OVA alone or saline controls Challacombe *et al.*, 1992.

The consequences of enteric antigen delivery to teleosts are less well understood but can result in systemic and mucosal immune responses in fish which may involve humoral or cellular factors (Davina *et al.*, 1980; Johnson and Amend, 1983a, b; Ellis, 1988; Rombout *et al.*, 1989b; McLean and Donaldson, 1990; Davidson, 1991; Jenkins, 1992). Oral immunisation with bacterins has been shown to induce protective immunity to bacterial challenge (Kawai *et al.*, 1981; Rombout *et al.*, 1989b). As yet effective oral delivery systems for immunisation of teleosts are not available and potent mucosal immunogens have not been identified.

CHAPTER 3

*IN VITRO ANALYSIS OF SOLUBLE PROTEIN ANTIGEN PROCESSING BY RAINBOW TROUT
LUMENAL ENZYMES.*

3.1 INTRODUCTION

The principal role of the vertebrate gastrointestinal tract has been regarded as the digestion of nutrient macromolecules to fundamental bioavailable units, their absorption into the tissues and subsequent excretion of indigestible components and waste products, thus precluding the absorption of bioactive molecules (Gardner, 1988). Recognition of the role of the gut in immunological protection and of the capacity for macromolecular absorption from the lumen has revealed a much greater complexity in gut physiology (Udall and Walker, 1982; McGhee and Kiyono, 1993). The modification of orally administered vaccines and macromolecules - particularly in the case of soluble proteins by gut digestive secretions will influence the functional nature and absorption of these agents and their consequent biological action. An understanding of the susceptibility of test antigens to modification in the gut is therefore a valuable, if not essential, initial step in the design of oral delivery systems.

Gastrointestinal proteolysis and gastric acid secretion have been recognised as obstacles to the oral delivery of vaccines in teleosts (Wong *et al.*, 1992) and in higher vertebrates (Morris *et al.*, 1994). Indeed mammalian studies indicate that the collective action of luminal proteases can remove orally delivered peptides and proteins within minutes of administration (Bunnett *et al.*, 1985). Proteolytic enzymes may be classed into endopeptidases and exopeptidases (Bergmann, 1942). Endopeptidases, such as pepsin and trypsin, hydrolyse proteins by splitting central and terminal peptide bonds to yield a mixture of peptides and amino acids. Exopeptidases cleave peptide bonds adjacent to a free terminal amino or carboxyl group and include aminopeptidases and carboxypeptidases. Extensive studies on proteolytic enzymes in fish have been undertaken (Kapoor *et al.*, 1975; Sastry, 1977; Hofer, 1979) and in Table 3.1 the wide range of proteases so far recognised in teleosts is outlined. In discussing proteolytic degradation in teleost fish, a clear distinction must be drawn between gastric (those possessing a functional stomach) and agastric (those without a functional stomach) species (Fänge and Grove, 1979). In both groups food is passed rapidly through the buccal cavity and pharynx to the post - pharyngeal part of the tract, generally termed the gut, where digestion occurs (Barrington, 1957). In most studies, enzymes

have not been detected in the buccal cavity and although protease activity in the oesophagus and pharynx of teleosts has been reported this may be attributable to regurgitation of intestinal or stomach enzymes (Fänge and Grove, 1979). Nonetheless, Sabapathy and Teo (1993) have suggested a more active role for the oesophagus in protein digestion in the rabbitfish, *Siganus canaliculatus*. In gastric teleosts as in higher vertebrates proteolysis normally begins in the stomach where pepsin at acidic pH hydrolyses proteins to a mixture of peptones, peptides and amino acids with the final hydrolysis of peptides to amino acids occurring in the intestine (Dawson and Holdsworth, 1962). The stomach of gastric teleosts secretes both hydrochloric acid and pepsinogen (Tarr, 1972), a pepsin precursor from which active pepsin is produced by acidic hydrolysis and subsequently by autocatalysis in the optimal peptic environment of the stomach (Twining *et al.*, 1983). The optimal proteolytic activity in the fish stomach has been reported at various pH values including pH 2, pH 3, pH 5 and pH 8 (Creac'h, 1963), perhaps indicating that the gastric fluid contains several types of protease. Many studies indicate that fish pepsins are less acidic proteases than their mammalian counterparts and some results suggest that they have higher activity, although inhibitor specificities appear similar for pepsins from fish and mammalian species (Norris and Mathies, 1953; Noda and Muramiki, 1981). The large number of proteases identified in the pyloric caecae and intestine of teleosts (Table 3.1) are pancreatic in origin (Croston, 1960; Bergoti, 1979) and are secreted into the anterior intestine as inactive zymogens. The pancreatic tissue of rainbow trout is diffuse and is located in the fat and mesentery surrounding the pyloric caeca (Weinreb and Bilstad, 1955). Pyloric caecae are not a general feature of all teleosts however, and are absent in a number of gastric and in all agastric species studied (Fänge and Grove, 1979). Pancreatic trypsinogen is cleaved by enterokinase (from the intestine) to form active trypsin which is consequently capable of autocatalysis (Uchida *et al.*, 1973) and activates the precursor forms of other pancreatic proteases such as chymotrypsin and elastase (Cohen *et al.*, 1981a, b). Most enzymes tend to show a sharp decline in activity towards the posterior intestine and rectum (Barrington, 1962) and there is some evidence for resorption of digestive enzymes in the posterior region of the teleost intestinal tract (Hofer and Schiemer, 1981). Although there have been few investigations of the entire luminal protease repertoire of individual

species, comprehensive studies of catfish enzymes indicate that a range of pancreatic proteases of differing specificities are present in the intestine (Yoshikana *et al.*, 1977, 1981, 1984a, b, 1985a, b). While protein digestion in teleosts is a sequential process involving a number of stages, luminal degradation is the first and most extensive stage and thus warrants investigation as a prerequisite to further studies on protein absorption.

In vivo models of antigen absorption have revealed the capacity for macromolecular uptake in fish and proposed a role for luminal enzymes in antigen degradation in the gut (McLean and Ash, 1990; Jenkins *et al.*, 1992) but these are not amenable to the investigation of the specific role of luminal proteases in antigen degradation prior to absorption. In this study an experimental *in vitro* system was utilised to investigate the role of luminal proteases in isolation which enabled strict control of environmental conditions and of the volumes and concentrations of enzymes and antigens. Teleosts as ectothermic aquatic vertebrates must adapt their physiology to a wide range of environmental conditions, particularly temperature, which influence the rate and efficiency of protease action and consequently the modification of enterically administered antigens. The objective of this study was to investigate the proteolysis of two antigens commonly employed in immunological studies, human gamma globulin (HGG) and bovine serum albumin (BSA) by enzyme preparations from both gastric and intestinal regions of the rainbow trout. The influence of time, temperature, pH and a range of inhibitors and modulators were investigated to assist in developing strategies for the oral administration of macromolecules to rainbow trout and other species.

Table 3.1: Proteases found in the gastrointestinal of some teleosts.

Enzyme name	Protease class	M _r (kDa) species and source	pH optimum	Reference
Trypsin	Serine protease	Catfish pancreas	Not determined	McLeese and Stevens, 1982.
Chymotrypsin	Serine protease	I :28.2 kDa II :28.8 kDa Rainbow trout	9.0	Krisjansson and Nielsen, 1992.
Metalloprotease	Metalloprotease	Not determined	7.5	Yoshinaka <i>et al.</i> , 1985a.
Elastase	Serine protease	26.0 kDa catfish pancreas (Elastase B)	8.0	Yoshinaka <i>et al.</i> , 1985a.
Elastolytic metalloprotease	Metalloprotease	24.0 kDa catfish pancreas	Not determined	Yoshinaka <i>et al.</i> , 1984a.
Collagenase	Serine protease	29.5 kDa catfish pancreas	7.5	Yoshinaka, <i>et al.</i> , 1986.
Carboxypeptidase A	Metalloprotease	34.0 kDa catfish pancreas	7.5	Yoshinaka <i>et al.</i> , 1985c.
Carboxypeptidase B	Metalloprotease	33.0 kDa catfish pancreas	7.5	Yoshinaka <i>et al.</i> , 1984b.
Aminopeptidases	Not determined	Not determined	Not determined	Ash, 1980.
Pepsin	Aspartic protease	I : 32.0 kDa II :27.0 kDa <i>Oncorhynchus keta</i>	3.0	Sanchez - Chiang <i>et al.</i> , 1986.

3.2 MATERIALS AND METHODS

3.2.1 *Animals*

Adult rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) 100-200 g of both sexes were maintained in a freshwater recirculating system at $14 \pm 1^\circ\text{C}$ and fed to satiation once daily on a commercial trout pellet preparation.

3.2.2 *Isolation and preparation of lumenal enzyme samples.*

The procedure was a modification of that used in an *in vitro* analysis of protein digestibility in rainbow trout (Grabner and Hofer, 1985). Fish were deprived of food for 24 hours, killed by a sharp blow to the head and dissected to expose the gut. The gut from the oesophagus immediately anterior to the stomach, to the anus was dissected out and divided carefully into gastric and intestinal regions which were opened longitudinally to reveal the lumen. Contents were collected by flushing with 0.5 M Tris-HCl buffer, pH 7.75 (intestinal samples) or 0.5 M Citrate-NaOH buffer, pH 3.8 (stomach samples) and gentle scraping with a spatula into plastic containers on ice. Pooled samples were centrifuged at $12,000 \times g$ for 20 min at 2°C and supernatants collected and defatted by shaking with an equal volume of chloroform on ice. The upper aqueous layer was removed, frozen at -70°C for 48 hr and lyophilised for 24 hr. Samples were resuspended overnight at 10 % of the original volume in a HCl solution, pH 3.0 (stomach samples) or 0.5 M Tris-HCl solution, pH 9.0 (intestine samples) and dialysed against large volumes of the corresponding buffers for 48 hr. After dialysis, samples were centrifuged at $6,500 \times g$ for 10 minutes, frozen at -70°C for 48 hr, lyophilised for 24 hr, reconstituted to a volume approximating that present initially in Citrate-NaOH, pH 3.8 (stomach samples) or Tris-HCl, pH 7.75 (intestine samples) and finally aliquoted and stored at -70°C .

3.2.3 *Determination of protein content of enzyme solutions.*

Protein content was estimated by a Bradford protein dye binding assay (Bradford, 1976) with a commercial Biorad (Watford, UK) assay kit. Solutions of BSA at a range of concentrations were used to construct a standard curve.

3.2.4 Optimisation of conditions for analysing proteolysis.

Enzyme solutions were added to antigens in corresponding buffers at a range of concentrations from $10\ \mu\text{g ml}^{-1}$ to $10\ \text{mg ml}^{-1}$ of either BSA (Fraction V, Sigma, Poole, Dorset) or HGG (Cohn fraction II, III, Sigma) for 1 hr. Reactions were terminated by snap freezing at -70°C and samples were subsequently subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (section 3.2.9). Optimum antigen concentrations of $4\ \text{mg ml}^{-1}$ were used in all experiments and enzyme solutions were mixed 1:1 with antigens for incubations. Initially freezing at -70°C was used to terminate reactions but this was found unsatisfactory since it did not stop proteolysis immediately. Using substrate SDS - PAGE (section 3.2.11) it was determined that the optimal method for terminating reactions was by adding an equivalent volume of non-reducing electrophoresis sample buffer and boiling for 3 minutes. Boiling was necessary since the addition of reducing or non-reducing sample buffer alone did not fully abrogate proteolysis. Using sample buffer served a dual function, in terminating enzymolysis and in preparing samples for electrophoresis.

3.2.5 Time course of antigen degradation by proteases.

All incubations were performed in Eppendorf tubes at 15°C , antigens ($4\ \text{mg ml}^{-1}$) and enzyme samples (preincubated at 15°C for 1 hour) were mixed and incubated over a range of times from 1 min to 15 hr or from 1 min to 11.5 hr, the *in vivo* food retention times in rainbow trout stomach and intestine respectively (Grabner and Hofer, 1985). The reactions were terminated by adding sample buffer and boiling. For each experiment both positive (antigen + buffer at corresponding pH) and negative controls (enzyme solution + buffer at corresponding pH) were included.

3.2.6 Effect of temperature on antigen degradation.

Corresponding antigen ($4\ \text{mg ml}^{-1}$) and enzyme solutions were preincubated independently for 1 hour at a range of strictly controlled temperatures from 2°C to 30°C before mixing and subsequent incubation at equivalent temperatures for 2 hours. An additional control was used for this investigation in which the enzyme solution was added to antigen immediately before boiling to

ensure that any degradation occurring prior to enzyme denaturation was made apparent.

3.2.7 Effect of pH on antigen degradation.

Incubations were allowed to proceed for 2 hours in each case. Corresponding antigen (4 mg ml⁻¹) and enzyme solutions were adjusted to pH values from 2-9 in Tris-HCl or Citrate-NaOH buffers, monitored by means of a microprobe (Aldrich Chemical Co, Poole, Dorset, U.K). Following the 2 hour incubation the pH in each tube was readjusted to 7.0 and reactions were terminated.

3.2.8 Influence of a range of potential inhibitors on antigen degradation.

The various compounds used and their concentrations are listed in Table 3.2. Enzyme solutions were incubated with each substance for 30 min prior to addition of antigen (1 mg ml⁻¹) and incubations allowed to proceed for a further 2 hr before termination.

3.2.9 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS - PAGE).

Samples derived from experimental incubations were subjected to discontinuous SDS - PAGE (Laemmli, 1970) to assess the extent of antigen proteolysis. Reagents for sample preparation, gel formulation and electrophoresis are outlined in Appendix A. To determine optimal electrophoretic conditions, samples were initially separated on 7% and 11% gels but for subsequent analyses non-reducing conditions with 11% gels were used for both BSA and HGG as this enabled optimal visualisation and analysis of the wide range of peptides liberated by proteolysis.

Gel constituents were de-gassed for 20 min after which ammonium persulphate was added and the resultant solution poured between glass electrophoresis plates (separated by 0.5 mm spacers) on a level casting stand. Gels were overlaid with isobutanol to exclude air and left for 45 minutes at room temperature to polymerise. Isobutanol was then removed by rinsing with distilled water and residual liquid removed by blotting with tissue paper. To prepare stacking gels, constituents were mixed and de-gassed prior to addition of ammonium persulphate. Stacking gel solution was then overlaid onto the separating gel and 10 well polystyrene combs inserted to produce wells.

After polymerisation, combs were removed, wells were rinsed with distilled water and all liquid removed with a syringe to dispose of unpolymerised acrylamide. Samples were applied to the wells and on each gel, molecular weight markers (Appendix A) were included. Two electrophoresis systems were utilised - a small scale mini Protean II system (Bio Rad, Herts) with which sample volumes of 10 μ l were electrophoresed at a constant voltage of 200V for 40 minutes and a larger scale LKB 2001 system (LKB Bromma, Sweden) with which 50 μ l sample volumes were electrophoresed at 8mA for 18 hours and which enabled better resolution of protein bands. In most cases only the Mini Protean system was used. After electrophoresis, gels were either stained directly or electrotransferred for specific immunostaining (section 3.2.10).

3.2.10 Gel staining for total protein.

Three stains were compared - amido black, Coomassie Brilliant Blue R.250 (CBB) and neutral silver stain. CBB appeared to be more sensitive than amido black and although less sensitive than neutral silver was chosen for routine application due to its ease of use. For CBB staining, Brilliant blue reagent (Appendix A) was added to gels which were incubated overnight at room temperature. Gels were destained in fixative (Appendix A) until bands were clearly visible. Molecular weight markers (Appendix A) were included on each PAGE gel to enable calculation of apparent molecular weights of protein bands after electrophoresis and staining.

3.2.11 SDS - PAGE with protein substrate incorporated.

To determine optimal conditions for terminating enzymic reactions a modification of the procedure of Heussen and Dowdle (1983) was applied. Enzyme samples were mixed 1:1 with sample buffer (reducing or non - reducing) and either left at room temperature for 1 hour or boiled for 3 minutes. Samples were applied to wells of the substrate gel and electrophoresed as described above. Subsequently gels were incubated in 2.5% Triton X - 100 (Sigma) for 2 hr to remove SDS and further incubated at 15°C for 4 hr in CitrateNaOH, pH 3.8 or Tris-HCl, pH 7.75 for gastric and intestinal enzymes respectively, to allow for enzymic degradation of the substrate. Gels were then stained with CBB and destained as described in section 3.2.9. Zones of clearance on gels

indicated enzyme activity.

3.2.12 Western blotting.

For immunoblotting (Western blotting) and staining for specific antigen detection an adaptation of the procedure of Towbin *et al.* (1979) was used. Subsequent to electrophoresis, proteins were electrotransferred to 0.45 μ m nitrocellulose membranes (by a "wet" blotting procedure) in transfer buffer (Appendix B) at 30 mA constant current for 18 hours using a Trans-Blot™ cell (Biorad). Blots were stained for total protein with Ponceau S (Appendix B) for 5 minutes and destained through several washes in PBS, pH 7.2, prior to specific immunostaining. After electrophoresis blots were washed in 0.5 M Tris - saline pH 7.5 for 20 min. A blocking step was then performed for 1 hr in Tris - saline + 3% non-fat dried milk pH 7.5 (TSM) (the blocking agent and diluent for all antisera for HGG immunoblotting) or 0.8 % gelatin, 1% Tween 20, in Tris-saline pH 7.5 (the blocking agent and diluent for all antisera for BSA immunoblotting). Blots were incubated in primary antisera (rabbit anti-BSA at 1:1000 dilution or rabbit anti-HGG at 1:500 dilution) for 6 hr. After washing 3 times in Tris-saline and twice in TSM, secondary antiserum (swine anti-rabbit IgG peroxidase conjugate at 1:1000 (Dako Ltd., High Wycombe, U.K.) was added for a further hour. Blots were washed in Tris - saline and developed in a 3,3'-diaminobenzidine (DAB) chromagen solution (Appendix B) for 1-5 min. Reactions were terminated by thorough washing in Tris-saline.

3.2.13 Laser densitometry.

To quantify the relative amounts of peptides present on immunoblots, an LKB ULTROSAN XL densitometer with a helium neon laser beam scanning at 633 nm was applied. By correlation of densitometry data with approximate molecular weights the relative abundances of individual fragments could be determined. Densitometry data was derived in two ways -

A: By scanning entire lanes of immunoblots the relative percentages of fragments as a function of the total amount of protein present was determined.

B: By scanning immunoblots laterally the relative percentages of individual fragments as a function

of the total amount of that fragment on the blot was determined.

Table 3.2 : Inhibitory/ modulatory agents used in the study of luminal proteases.

Inhibitor/ modulator	Target protease/mode of action	Concentration used	Reference
Phenylmethane sulphonate (PMSF)	All serine proteases. Cysteine proteases.	1 mM	James, 1978.
Soybean trypsin inhibitor (SBTI)	Serine proteases	10 mM	Birk, 1985.
Leupeptin	Trypsin - like serine and some cysteine proteases	100 μ M	Umezawa, 1976.
Pepstatin	Some aspartic proteases	10 μ M	Saino et al., 1982.
Ethylenediaminetetraacetic acid (EDTA)	Metalloproteases, Metal activated proteases	10 mM	Rich <i>et al.</i> , 1985.
Quillaja Saponaria (QUIL- A)	Potential serine and aspartic proteases	1 mM	Ishaaya and Birk, 1965.
Sodium Bicarbonate	Gastric acidity	35 mg ml ⁻¹	Clements <i>et al.</i> , 1986.
Cimetidine	Gastric acidity	10 mg ml ⁻¹	Dima <i>et al.</i> , 1992.

3.3 RESULTS

3.3.1 *Estimation of protein content and molecular weights of enzymes present in gastric and intestinal secretions.*

The protein concentrations in gastric and intestinal enzyme samples as determined by Bradford assay were 0.27 mg/ml and 1.03 mg/ml respectively. In Fig 3.1 (a,b) enzyme bands are visible in the negative control lanes (arrowed) and at corresponding positions in each lane containing digested HGG. By reference to molecular weight standards, approximate molecular weights were calculated for enzyme samples electrophoresed under reducing and non-reducing conditions and stained with Coomassie Blue. In intestinal samples run under non-reducing conditions only 2 bands were observed of 18.6 kDa and 25.7 kDa molecular weight (arrowed in Fig 3.1(a)). In the same samples run under reducing conditions 7 bands were detected of molecular weight 14.8 kDa, 21.6 kDa, 26.3 kDa, 28.1 kDa, 34.1 kDa, 35.0 kDa, and 36.0 kDa (not all visible in Fig 3.1(b)).

In gastric samples 3 bands were detected in reduced preparations of molecular weight 19.5 kDa, 21.5 kDa and 26.9 kDa while under non-reducing conditions only a single band of 25.4 kDa was found.

3.3.2 *Densitometric analysis of the dynamics of antigen processing by lumenal enzymes.*

In Fig 3.2 a representative densitometric analysis of the course of an enzyme mediated reaction is presented. This shows the progressive changes in the patterns and relative abundances of fragments produced by the degradation of HGG with intestinal enzymes at various temperatures. The data was derived from laser scans of entire lanes of immunoblots (Fig 3.10 displays this data in graphical form) thus representing the total range of antigen fragments present at a particular temperature. The densitograms presented in Fig. 3.3 show the stages involved in the derivation of data from individual bands on immunoblots.

3.3.3 *Time courses of antigen degradation by gastrointestinal enzymes.*

The breakdown patterns for both HGG (Fig 3.4) and BSA (Fig 3.5) by gastric enzymes were similar, proteolysis progressed rapidly until approximately 2 hours - post incubation with antigen

(leading to a reduction in the relative percentage of intact antigen to 43 % and 31 % for HGG and BSA respectively). After 2 hrs, the patterns of specified fragment groups were found to remain relatively constant. From Figs 3.4(c) and 3.5(c) it is apparent that the time course of changes in the relative percentages of discrete fragments reflect those of fragment groups (Figs 3.4(b) and 3.5(b)), increasing until approximately 2 hours post incubation and subsequently remaining relatively constant.

The time course patterns for HGG and BSA degradation by intestinal enzymes (Fig 3.6 and 3.7) were also similar. Degradation occurred very rapidly, the relative percentage of intact antigen decreasing by 57.6% and 66.6% within 1 minute and by a further 71% and 63.4% after 11.5 hours incubation, for HGG and BSA respectively (Figs 3.6(a) and 3.7(a)). Both antigens were degraded to low molecular weight peptides the patterns of which changed with time. Some fragments were detectable at high levels throughout the time course while others were apparently degraded over time. The relative percentage of the 13.7 kDa HGG fragment for example increased until after 6 hr post-incubation after which it decreased and was undetectable at 11.5 hr..

3.3.4 *Temperature dependence of antigen proteolysis.*

Little degradation of either HGG or BSA by gastric enzymes occurred at temperatures less than 5°C, but at 35°C, proteolysis amounting to 32.8% and 16.6% of the intact antigen for HGG and BSA respectively was observed (Figs 3.8 and 3.9). The relative percentages of HGG and BSA fragments released by proteolysis increased with increasing temperature up to 35°C (Fig 3.8(c) and 3.9(c)).

The degradation of both antigens by intestinal enzymes increased with increasing temperature up to 35° C (Fig 3.10 and 3.11), this effect was particularly marked in the case of HGG. There was a marked increase in the relative percentages of HGG fragments less than 50 kDa in molecular weight with increasing temperature. It was found that less than 45% of the antigen remained intact in the controls to which enzyme was added immediately before boiling (Fig 3.10(a)) indicating that

a considerable amount of proteolysis occurred before the enzymes were denatured which highlighted the rapid rate of proteolysis by intestinal enzymes.

3.3.5 pH dependence of degradation.

There appeared to be two pH optima for the degradation of HGG and BSA by gastric enzymes, one at pH 4-5 and the other at pH 7-8 with a clear intermediate point at pH 6.0 where relatively little proteolysis occurred. These optima were apparent both with respect to the relative percentages of the intact antigens present and the percentages of fragments released by degradation (Fig 3.12 and 3.13). In the case of BSA the effect of pH was most apparent in changes in the relative percentages of the 17.5 and 10.6 kDa fragments.

Intestinal enzyme degradation of antigens demonstrates a clear monophasic relationship with pH within the experimental pH range studied. The breakdown of intact HGG and BSA and accumulation of fragments steadily increased up to pH 9.0 (Figs 5.14 and 5.15). Analysis of the influence of pH alone on antigen degradation indicated that both antigens were stable between pH 2.0 - 9.0 suggesting that this breakdown was due solely to enzymic action (data not presented).

3.3.6 Effects of inhibitors on antigen degradation.

Breakdown of both BSA and HGG by gastric enzymes was partially inhibited by PMSF while pepstatin and leupeptin led to almost total abrogation of proteolysis (Figs 3.16 and 3.17).

The effects of leupeptin and pepstatin on proteolysis of BSA were apparent in terms of both an increase in the relative percentage of the intact antigen and as a reduction in the relative percentages of the 8.4, 16.3 and 18.4 kDa fragments present. Similarly in the case of HGG these inhibitors resulted in a reduction in the relative percentages of the 40.2, 14.9 and 10.4 kDa fragments.

The effects of inhibitors on intestinal enzyme proteolysis of HGG and BSA are presented in Figs 3.18 and 3.19. Only the serine protease inhibitors, PMSF, SBTI and leupeptin led to a reduction in degradation of BSA and HGG, the effect of PMSF being most marked. These serine protease inhibitors did not prevent the degradation of intact antigen but instead resulted in the accumulation

of a range of peptides of differing nature and relative abundances depending on the inhibitor used. Proteolysis in the absence of inhibitors was almost complete (Figs 3.18, 3.19 and 3.20 (c)), so while the densitometry data yields information on the relative amounts of the various fragments of each antigen present there is no indication of the low overall quantity of antigen remaining after incubation with intestinal enzymes, particularly in the case of BSA. Fig 3.20 also presents photographic information on the effects of time and pH on gastrointestinal proteolysis.

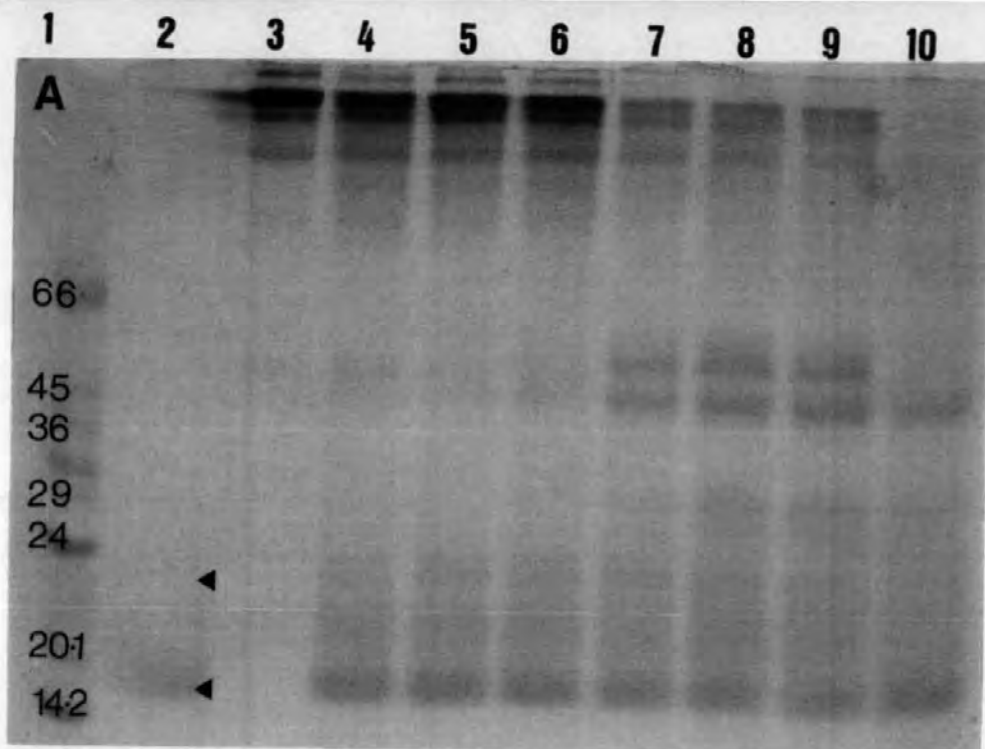


Fig 3.1. (a): Coomassie Blue stained 11% PAGE gel displaying the time course of HGG degradation by rainbow trout intestinal enzymes. Samples were run under non-reducing conditions. Loading order: Lane 1: 7H molecular weight markers (apparent molecular weights in kDa). Lane 2: negative control (enzyme solution only). Lane 3: positive control (2 mg/ml HGG). Lanes 4-10: HGG samples exposed to intestinal secretions for 1 min, 5 min, 15 min, 30 min, 1 hr, 2 hr and 5 hr respectively. Protein bands present in the enzyme solution are highlighted.

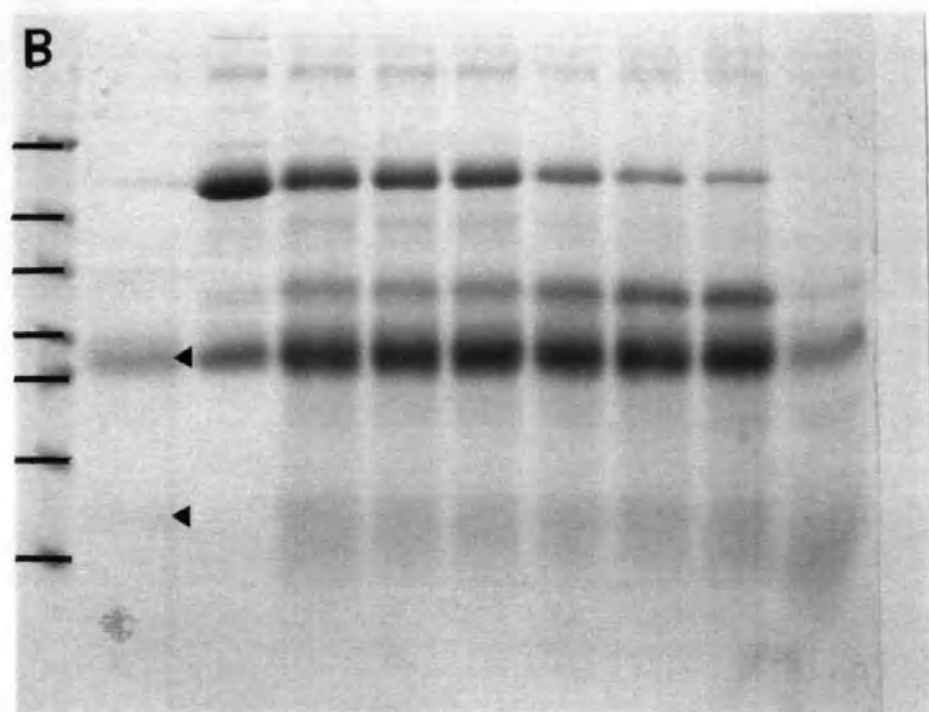


Fig 3.1. (b): Coomassie Blue stained 11% PAGE gel displaying the time course of HGG degradation by rainbow trout intestinal enzymes. This gel is identical to Fig 3.1 (A) above except that the samples were electrophoresed under reducing conditions. Bars represent apparent molecular weights of prestained molecular weight markers as in lane 1 of Fig.3.1(a).

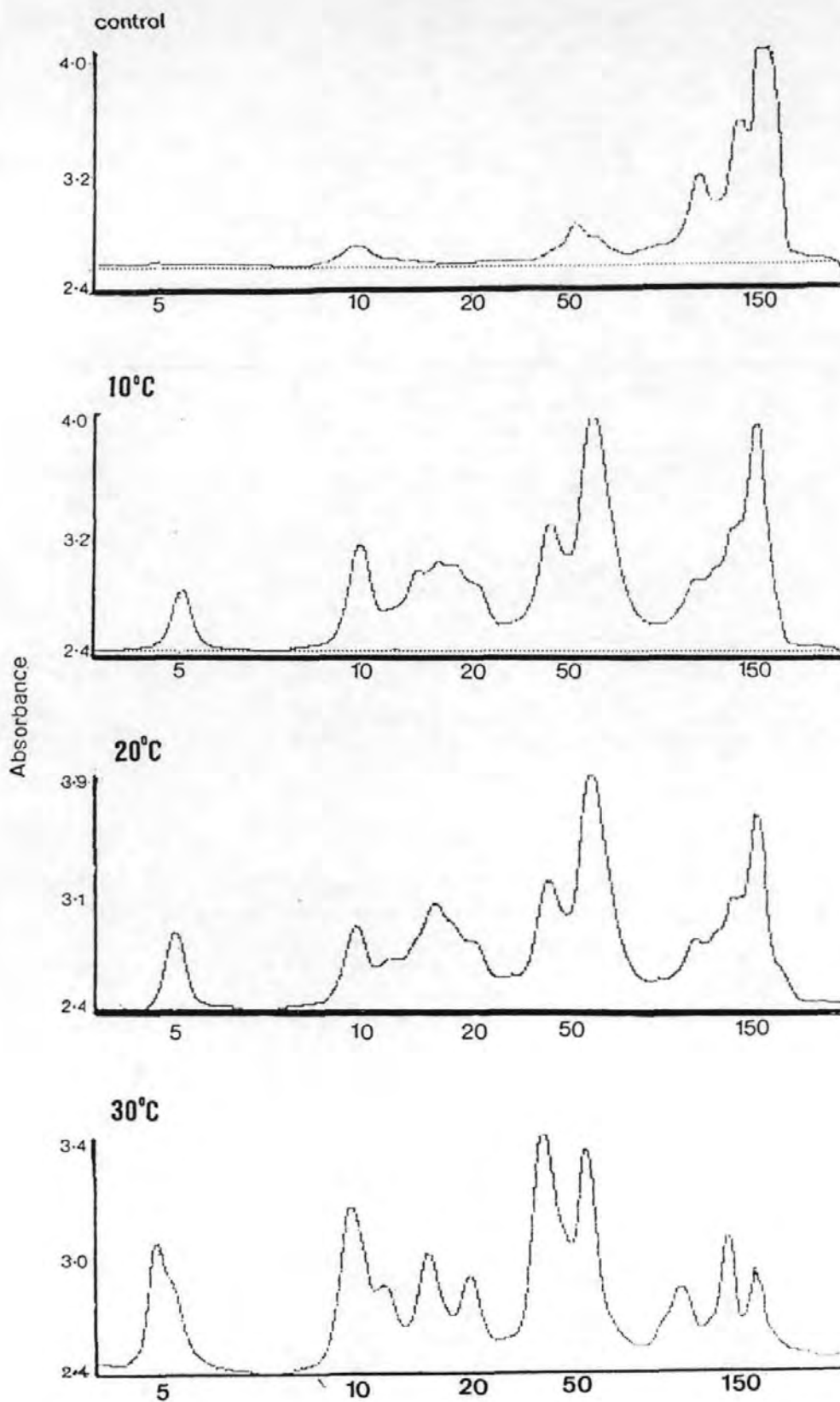


Fig 3.2. Densitometric analysis of the intestinal enzyme proteolysis of HGG over a range of temperatures. Data was derived from laser scans of entire lanes of an immunoblot. The apparent molecular weights of HGG bands are on the x axis and absorbance units (arbitrary) derived from the laser densitometer on the y axis.

Fig 3.3. *Densitometric analysis of the pH dependence of gastric enzyme degradation of HGG. Data was derived from laser scans of individual lanes on immunoblot. The graphs demonstrate the relative abundances of two HGG fragments at various pH values.*

Fig 3.3 (a). *Lanes of immunoblot from which densitometry data was derived. The loading order on the immunoblot strips for both fragments from left to right corresponds with the pH values on the profile in Fig 3.3(B) below*

Fig 3.3 (b). *Laser scans of the bands above demonstrating the relationship between band density and pH of incubation medium.*

Fig 3.3 (c). *Tabulated numeric values derived from densitometer software for the 17.5 kDa fragment.*

a

10.6kDa

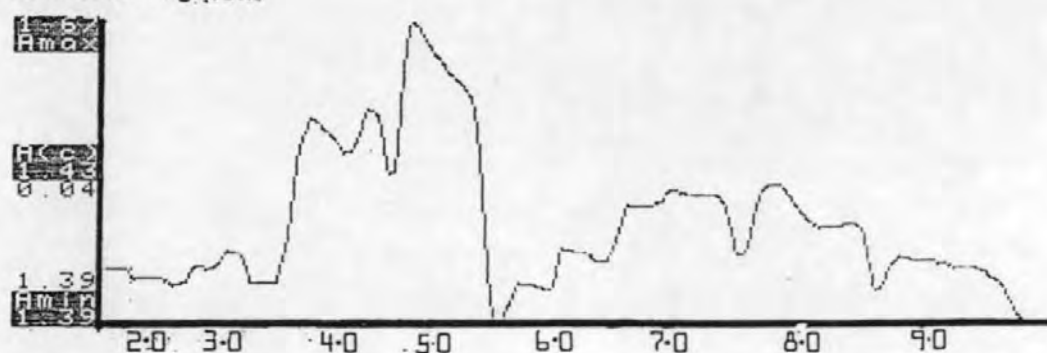


17.5kDa

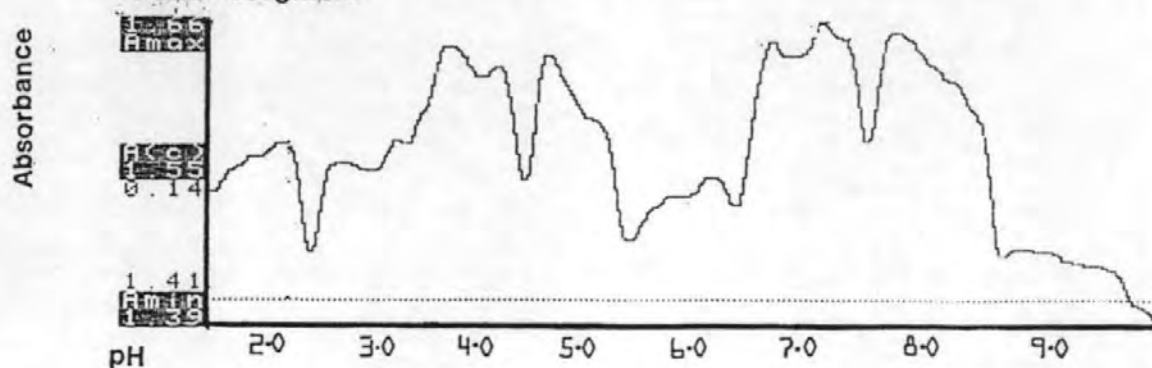


b

10.6kDa Fragment



17.5kDa Fragment



c

PEAK	POSITION	HEIGHT	AREA	REL. AREA	PEAK	POSITION	HEIGHT	AREA	REL. AREA
£	mm	AU	AU*mm	%	£	mm	AU	AU*mm	%
1	60.96	0.05	0.248	3.96	9	100.64	0.12	0.338	5.39
2	68.84	0.06	0.281	4.49	10	101.08	0.12	0.073	1.16
3	74.56	0.19	0.802	12.79	11	101.72	0.12	0.165	2.64
4	78.56	0.20	0.589	9.40	12	103.84	0.12	0.266	4.24
5	81.56	0.28	1.561	24.91	13	108.28	0.13	0.674	10.75
6	90.20	0.03	0.082	1.31	14	113.88	0.09	0.286	4.57
7	92.80	0.07	0.245	3.92	15	119.64	0.06	0.418	6.67
8	97.44	0.11	0.238	3.80					

Fig. 3.4: *Time course of the proteolysis of HGG by gastric enzymes.*

Fig. 3.4 (a): *Antigen degradation represented in terms of changes in the relative amount of intact HGG present with time. Data derived from laser scan of entire lanes of immunoblot.*

Fig. 3.4 (b): *Antigen degradation represented in terms of changes in the relative amounts of fragment groups (defined on the basis of relative molecular weight) present with time. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 3.4 (c): *Antigen degradation represented in terms of changes in the relative percentage of discrete antigen fragments present with time. Data derived from laser scans of individual bands on immunoblot.*

Fig.3.4(a):Gastric enzyme degradation of HGG.
Time course of proteolysis

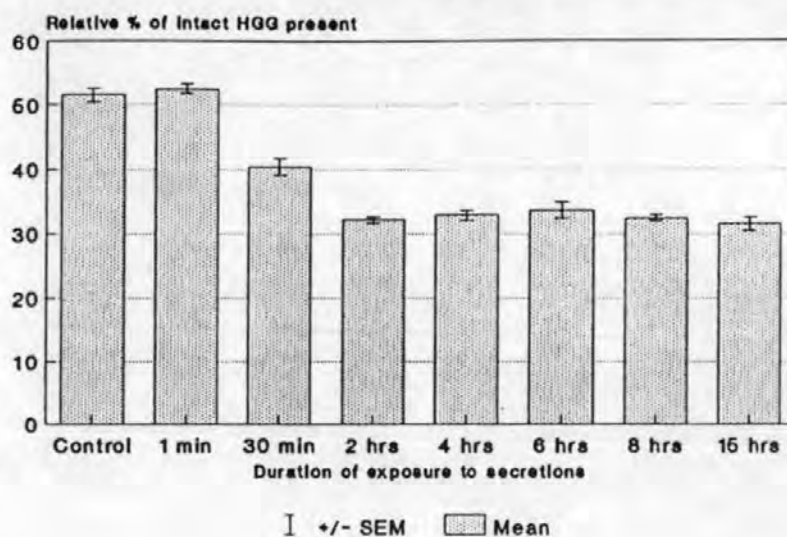


Fig.3.4(b): Gastric enzyme degradation of HGG.
Time course of proteolysis

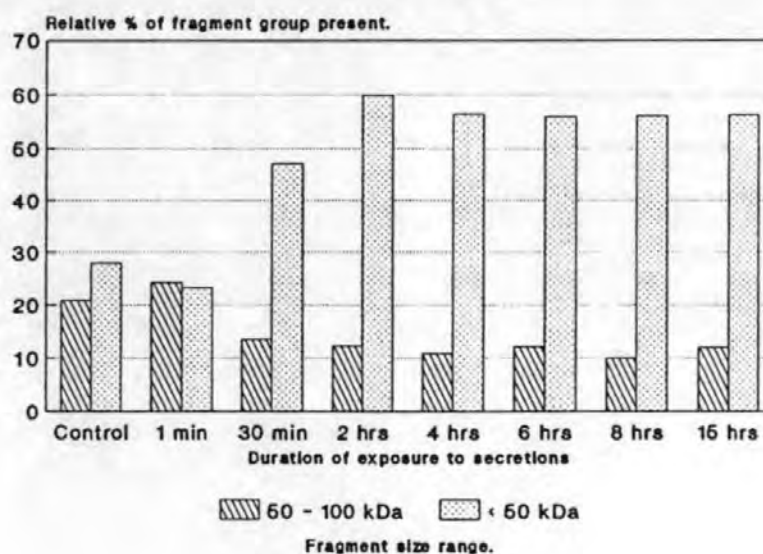


Fig.3.4(c): Gastric enzyme degradation of HGG.
Time course of proteolysis

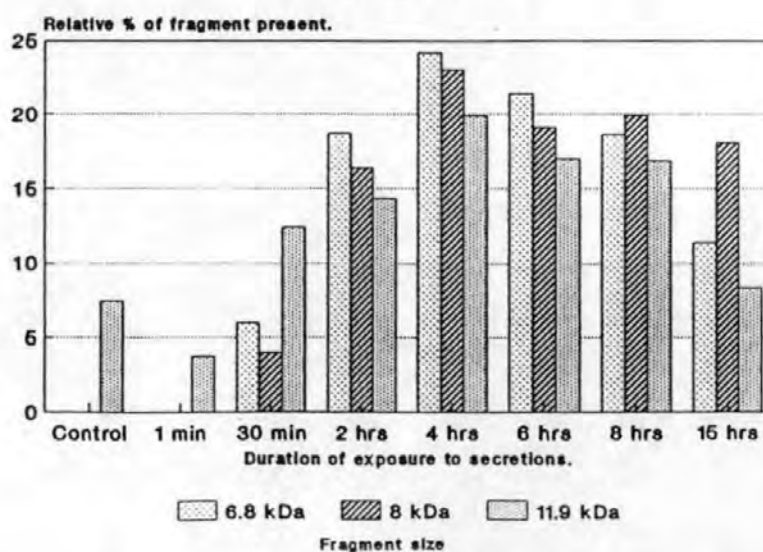


Fig. 3.5: *Time course of the proteolysis of BSA by gastric enzymes.*

Fig. 3.5 (a): *Antigen degradation represented in terms of changes in the relative amount of intact BSA (including multimers) present with time. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 3.5 (b): *Antigen degradation represented in terms of changes in the relative amounts of fragment groups present with time. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 3.5 (c): *Antigen degradation represented in terms of changes in the relative percentages of discrete fragments with time. Data derived from laser scans of individual bands on immunoblot.*

Fig.3.5(a): Gastric enzyme degradation of BSA.
Time course of proteolysis

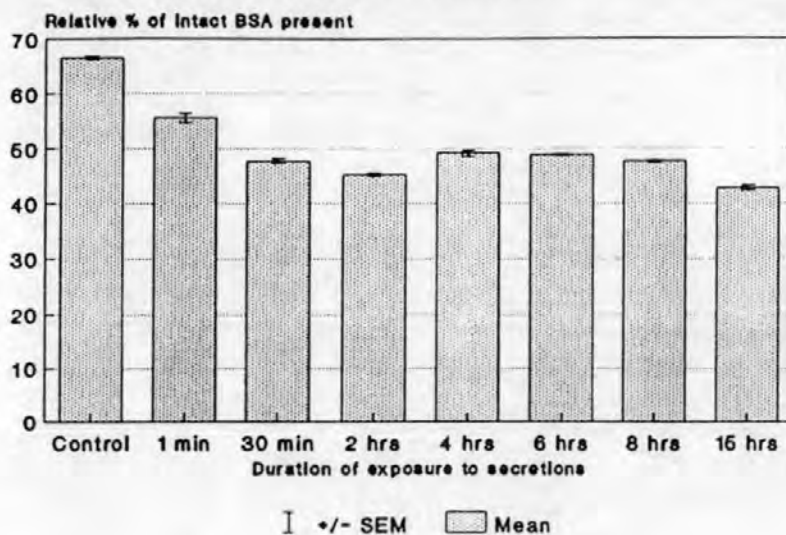


Fig.3.5(b): Gastric enzyme degradation of BSA.
Time course of proteolysis

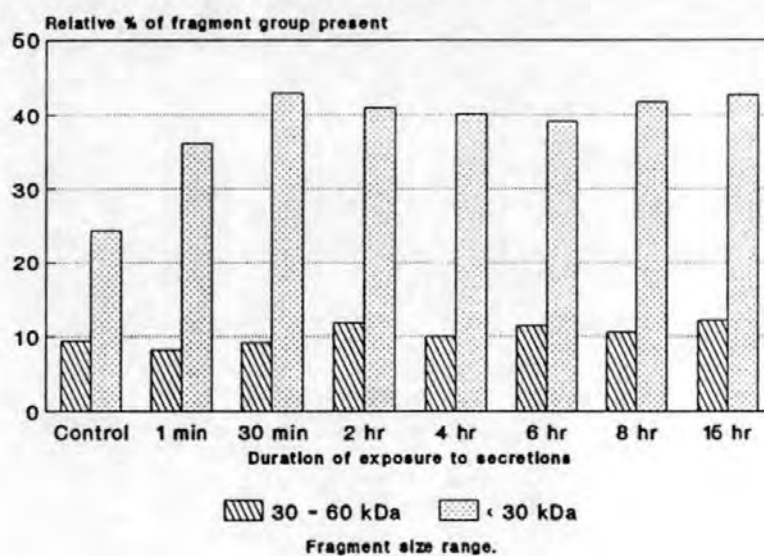


Fig.3.5(c): Gastric enzyme degradation of BSA.
Time course of proteolysis

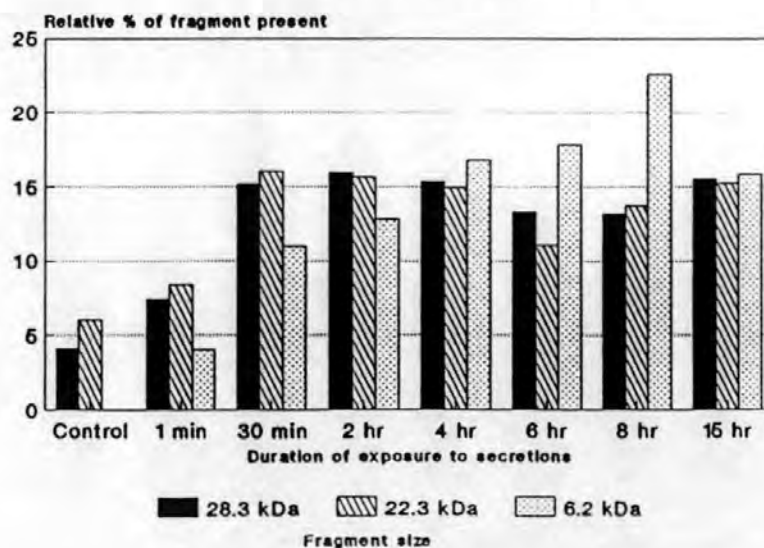


Fig. 3.6: *Time course of the proteolysis of HGG by intestinal enzymes.*

Fig. 3.6 (a): *Antigen degradation represented in terms of changes in the relative amount of intact HGG present with time. Data derived from laser scan of entire lanes of immunoblot.*

Fig. 3.6 (b): *Antigen degradation represented in terms of changes in the relative amounts of fragment groups present with time. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 3.6 (c): *Antigen degradation represented in terms of changes in the relative percentage of discrete fragments present with time. Data derived from laser scans of entire lanes of immunoblot.*

Fig.3.6(a): Intestinal enzyme degradation of HGG.
Time course of proteolysis

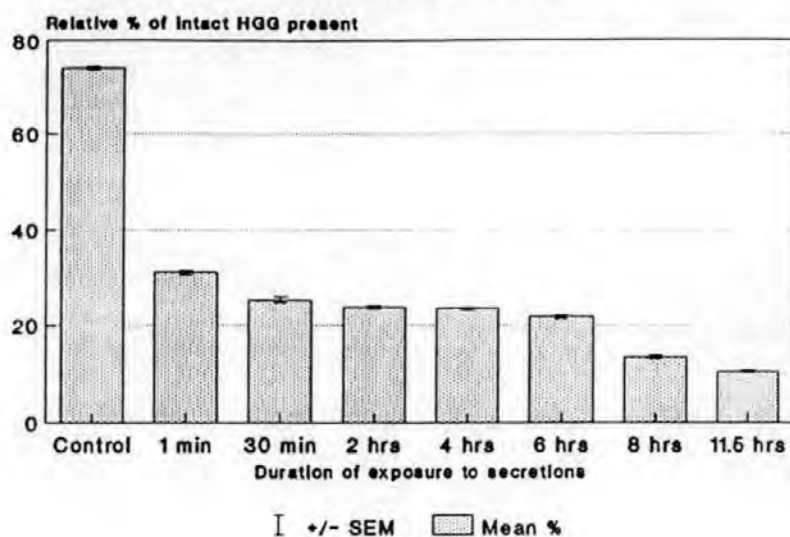


Fig.3.6(b): Intestinal enzyme degradation of HGG.
Time course of proteolysis

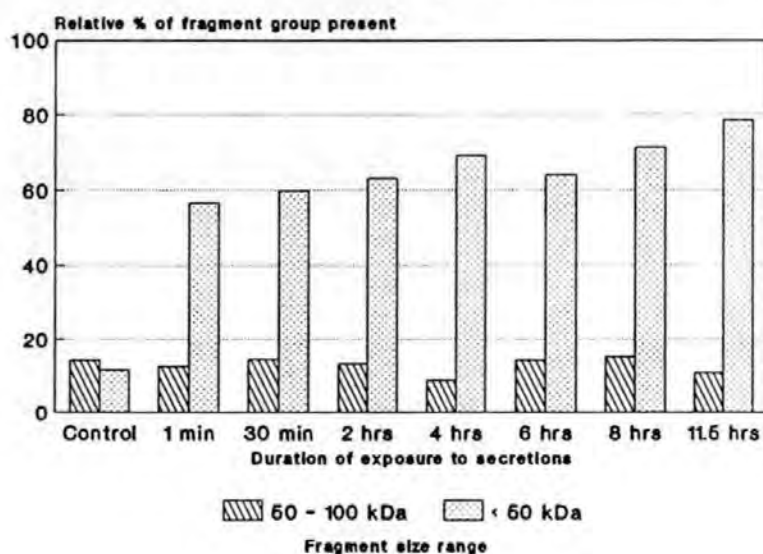


Fig.3.6(c): Intestinal enzyme degradation of HGG.
Time course of proteolysis

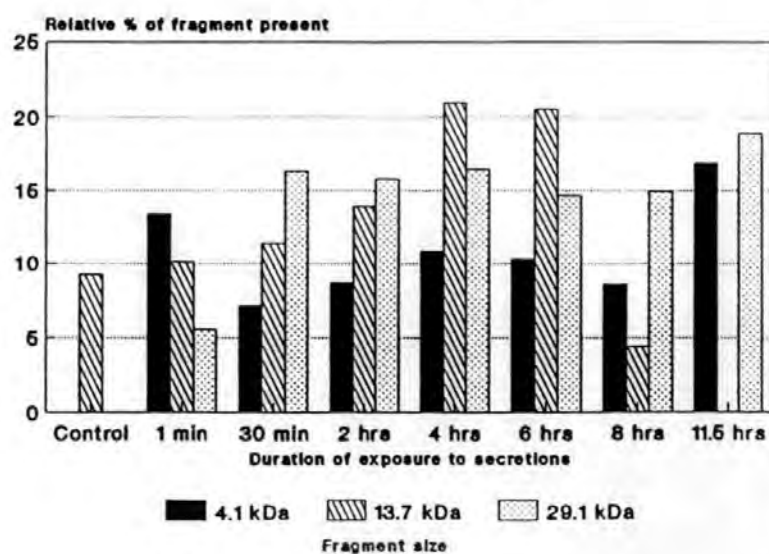


Fig. 3.7: *Time course of the proteolysis of BSA by intestinal enzymes.*

Fig. 3.7 (a): *Antigen degradation represented in terms of the relative amount of intact BSA (including multimers) present with time. Data derived from laser scan of entire lanes of immunoblot.*

Fig. 3.7 (b): *Antigen degradation represented in terms of changes in the relative amounts of fragment groups present with time. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 3.7 (c): *Antigen degradation represented in terms of changes in the relative percentages of discrete fragments with time. Data derived from laser scans of entire lanes of immunoblot.*

Fig.3.7(a): Intestinal enzyme degradation of BSA.
Time course of proteolysis

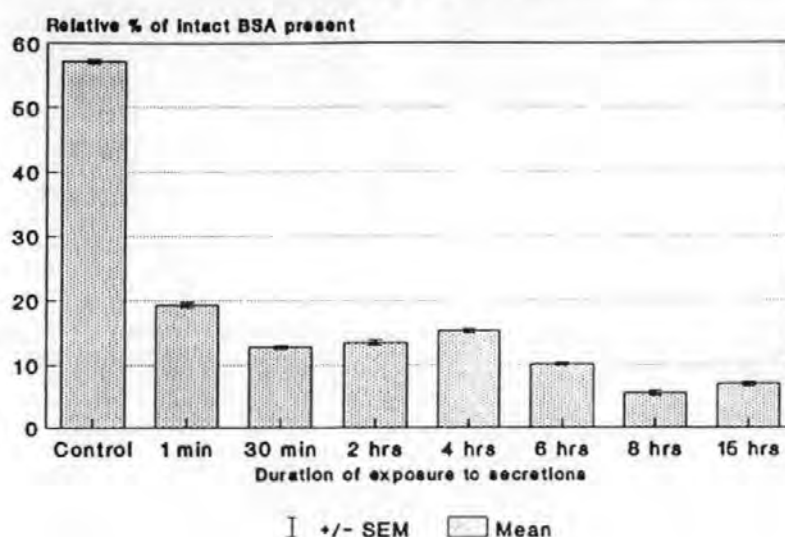


Fig.3.7(b): Intestinal enzyme degradation of BSA.
Time course of proteolysis

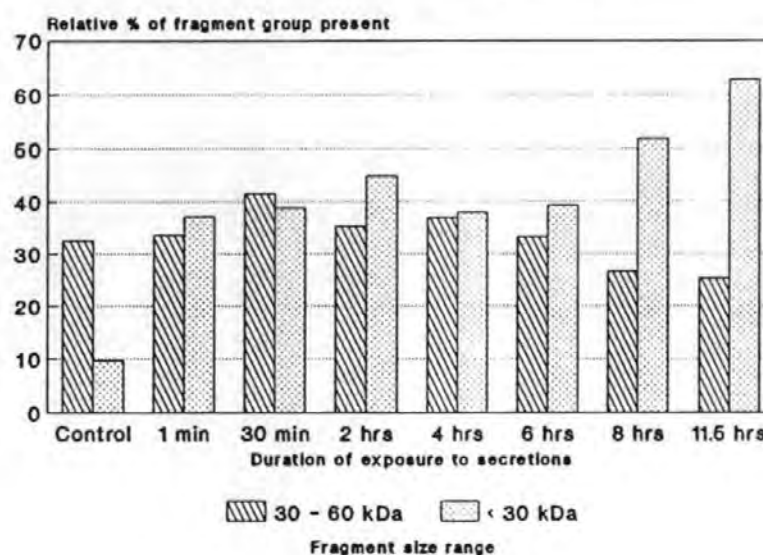


Fig.3.7(c): Intestinal enzyme degradation of BSA.
Time course of proteolysis

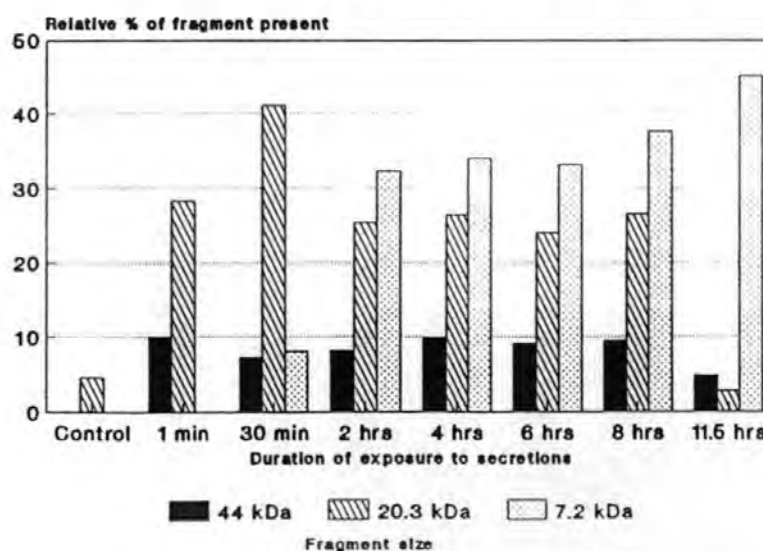


Fig. 3.8: *Temperature dependence of the proteolysis of HGG by gastric enzymes.*

Fig. 3.8 (a): *Antigen degradation represented in terms of changes in the relative amount of intact HGG present with increasing temperature. Data derived from laser scan of entire lanes of immunoblot. (Reactions allowed to proceed for 2 hours in each case).*

Fig. 3.8 (b): *Antigen degradation represented in terms of changes in the relative amounts of fragment groups (defined on the basis of molecular weight) present with increasing temperature. Data derived from laser scan of entire lanes of immunoblot.*

Fig. 3.8 (c): *Antigen degradation represented in terms of changes in the relative percentages of discrete fragments present with increasing temperature. Data derived from laser scans of individual bands on immunoblot.*

Fig.3.8(a): Gastric enzyme degradation of HGG.
Temperature dependence of proteolysis

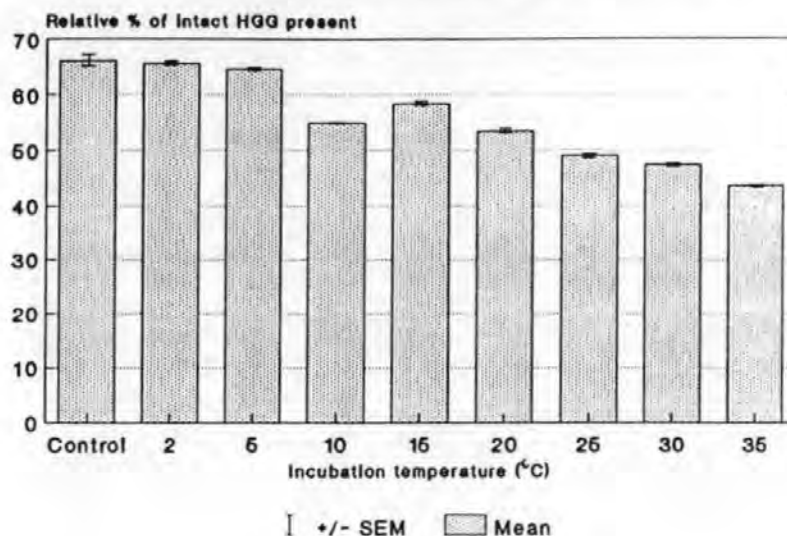


Fig.3.8(b): Gastric enzyme degradation of HGG.
Temperature dependence of degradation.

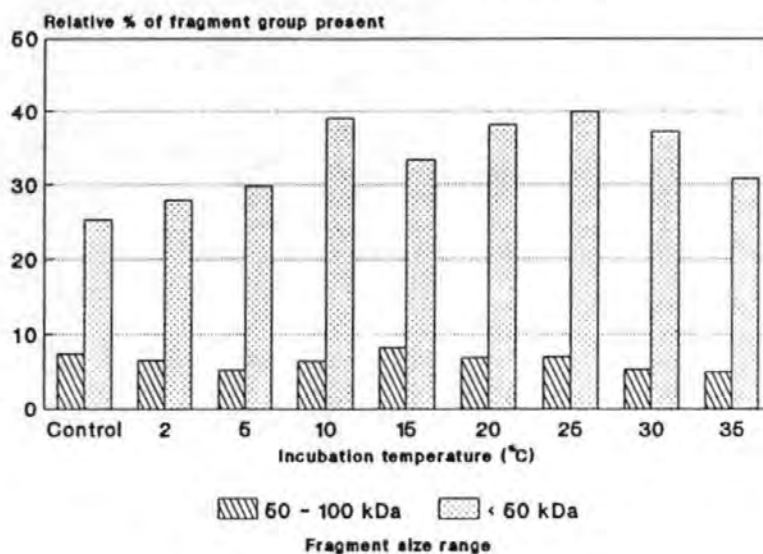


Fig.3.8(c): Gastric enzyme degradation of HGG.
Temperature dependence of proteolysis

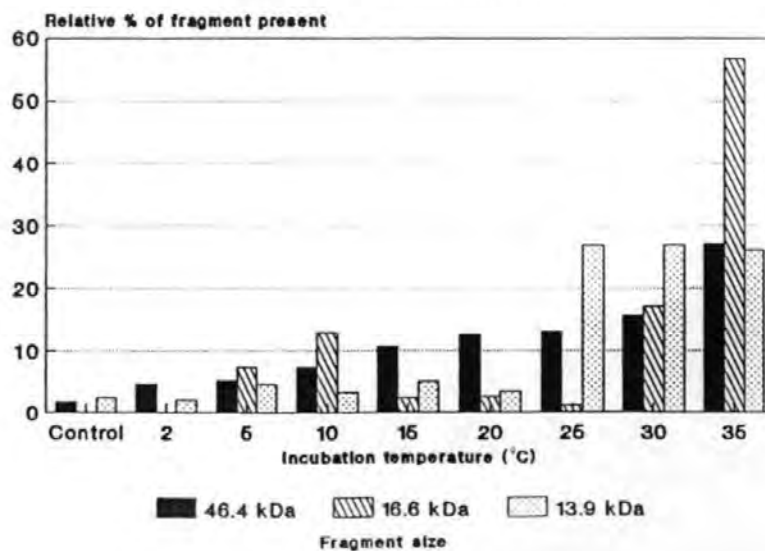


Fig. 3.9: *Temperature dependence of the proteolysis of BSA by gastric enzymes.*

Fig. 3.9 (a): *Antigen degradation represented in terms of changes in the relative amount of intact BSA (including multimers) present with time. Data derived from laser scan of entire lanes of immunoblot. (Reactions allowed to proceed for 2 hours in each case).*

Fig. 3.9 (b): *Antigen degradation represented in terms of changes in the relative amounts of fragment groups present with increasing temperature. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 3.9 (c): *Antigen degradation represented in terms of changes in the relative percentage of discrete fragments present with increasing temperature. Data derived from laser scans of individual bands on immunoblot.*

Fig.3.9(a): Gastric enzyme degradation of BSA.
 Temperature dependence of proteolysis

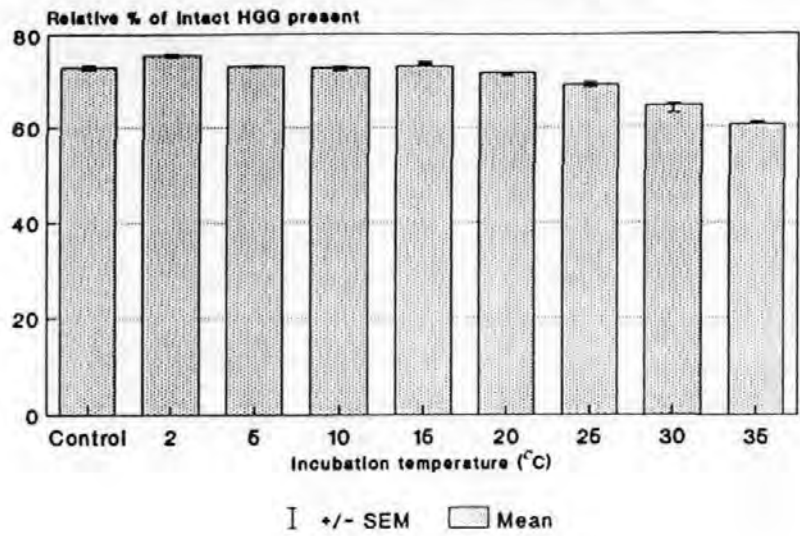


Fig.3.9(b): Gastric enzyme degradation of BSA.
 Temperature dependence of proteolysis

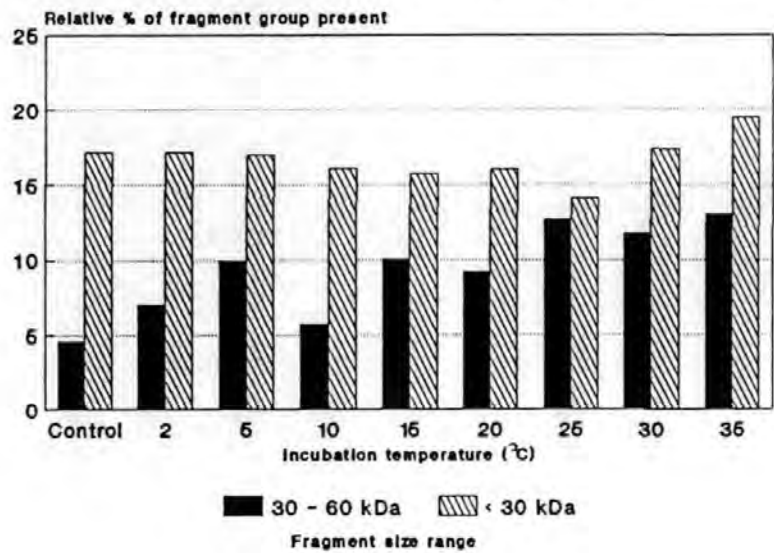


Fig.3.9(c): Gastric enzyme degradation of BSA.
 Temperature dependence of proteolysis

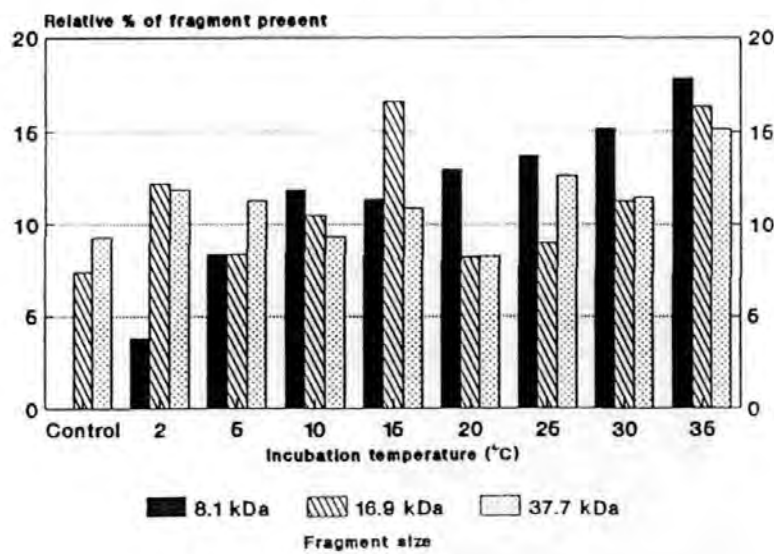


Fig. 3.10: *Temperature dependence of the proteolysis of HGG by intestinal enzymes.*

Fig. 3.10 (a): *Antigen degradation represented in terms of changes in the relative amount of intact HGG present with increasing temperature. Data derived from laser scans of entire lanes of immunoblot (Reactions allowed to proceed for 2 hours in each case).*

Fig. 3.10 (b): *Antigen degradation represented in terms of changes in the relative amounts of fragment groups present with increasing temperature. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 3.10 (c): *Antigen degradation represented in terms of changes in the relative percentages of discrete fragments present with increasing temperature. Data derived from laser scans of entire lanes of immunoblot.*

Fig.3.10(a): Intestinal enzyme degradation of HGG.
Temperature dependence of proteolysis

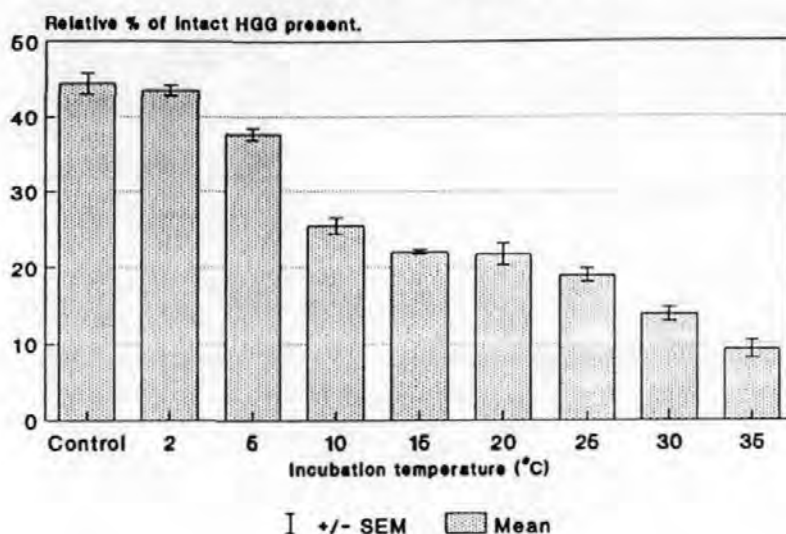


Fig.3.10(b): Intestinal enzyme degradation of HGG.
Temperature dependence of proteolysis.

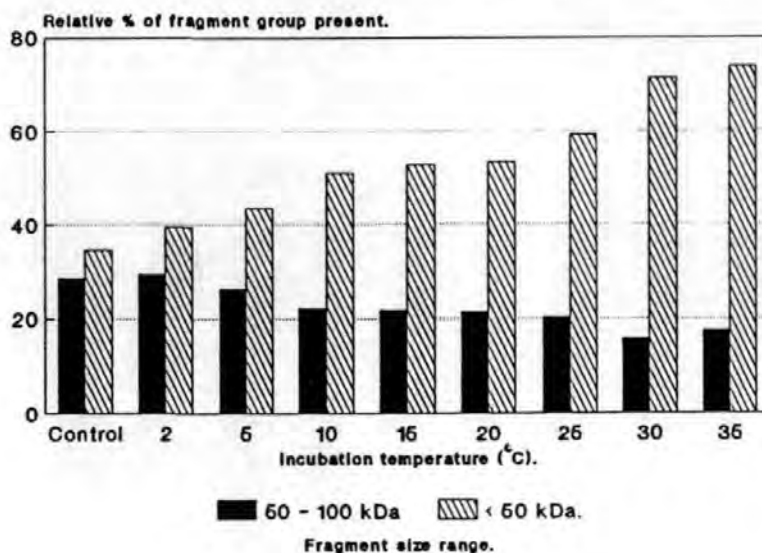


Fig.3.10(c): Intestinal enzyme degradation of HGG.
Temperature dependence of proteolysis

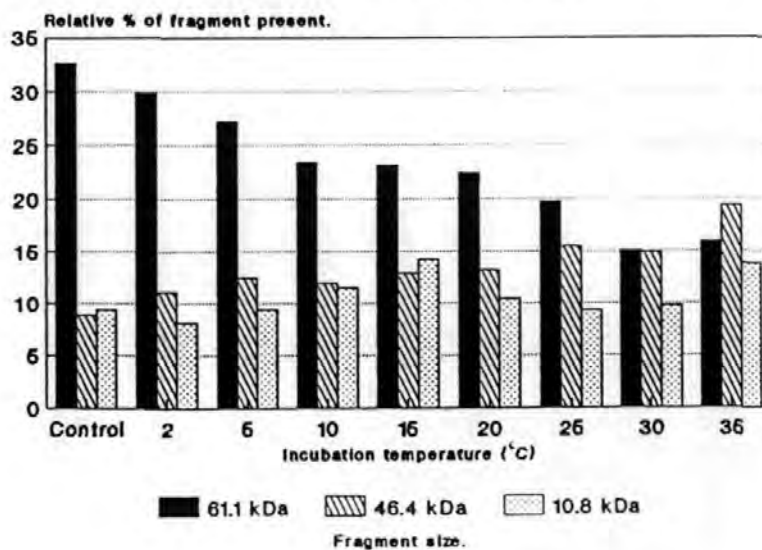


Fig. 3.11: *Temperature dependence of the proteolysis of BSA by intestinal enzymes.*

Fig. 3.11 (a): *Antigen degradation represented in terms of changes in the relative amount of intact BSA (including multimers) present with increasing temperature. Data derived from laser scans of entire lanes of immunoblot (Reactions allowed to proceed for 2 hours in each case).*

Fig. 3.11 (b): *Antigen degradation represented in terms of changes in the relative amounts of fragment groups present with increasing temperature. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 3.11 (c): *Antigen degradation represented in terms of changes in the relative percentages of discrete fragments present with increasing temperature. Data derived from laser scans of entire lanes of immunoblot.*

Fig.3.11(a): Intestinal enzyme degradation of BSA.
Temperature dependence of proteolysis.

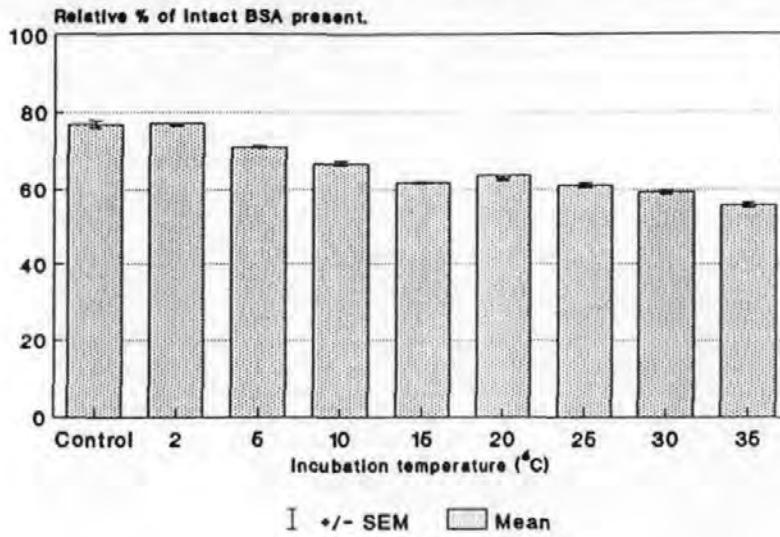


Fig.3.11(b): Intestinal enzyme degradation of BSA.
Temperature dependence of proteolysis.

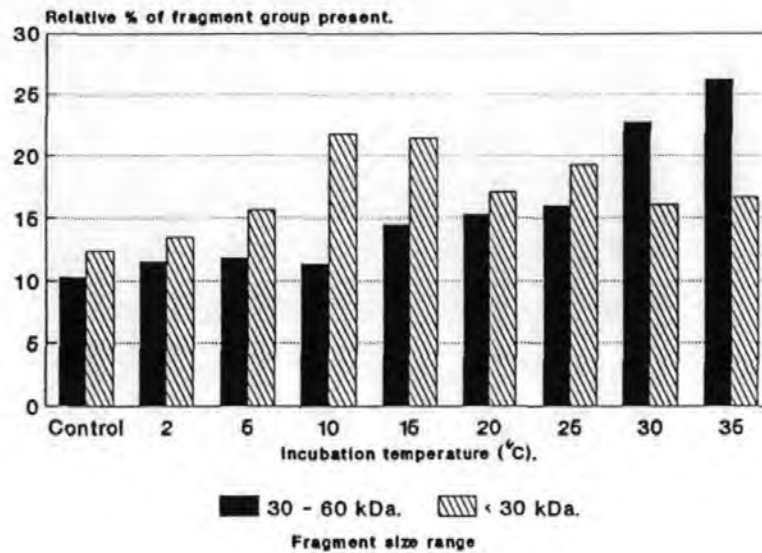


Fig.3.11(c): Intestinal enzyme degradation of BSA.
Temperature dependence of proteolysis.

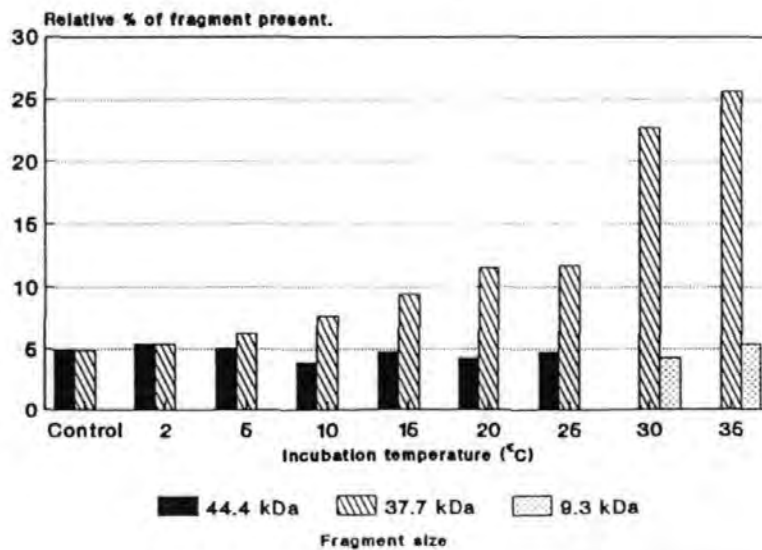


Fig. 3.12: *pH dependence of the proteolysis of HGG by gastric enzymes.*

Fig. 3.12 (a): *Antigen degradation represented in terms of changes in the relative amount of intact HGG present with increasing pH. Data derived from laser scans of entire lanes of immunoblot (Reactions allowed to proceed for 2 hours in each case).*

Fig. 3.12 (b): *Antigen degradation represented in terms of changes in the relative amounts of fragment groups present with increasing pH. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 3.12 (c): *Antigen degradation represented in terms of changes in the relative percentages of discrete fragments present with increasing pH. Data derived from laser scans of individual bands on immunoblot.*

Fig.3.12(a): Gastric enzyme degradation of HGG.
pH dependence of proteolysis.

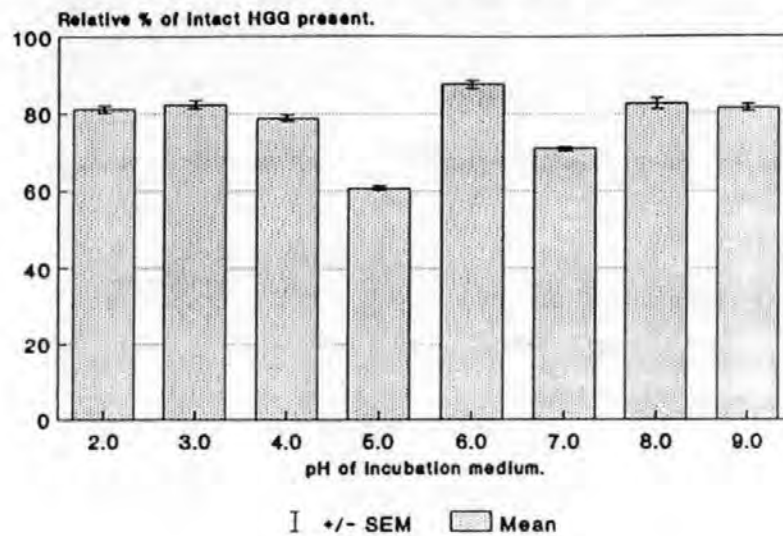


Fig.3.12(b): Gastric enzyme degradation of HGG.
pH dependence of proteolysis.

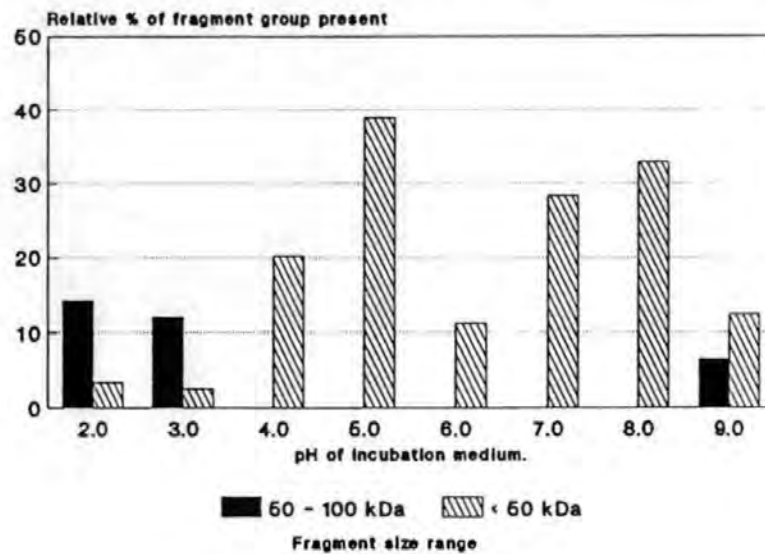


Fig.3.12(c): Gastric enzyme degradation of HGG.
pH dependence of proteolysis.

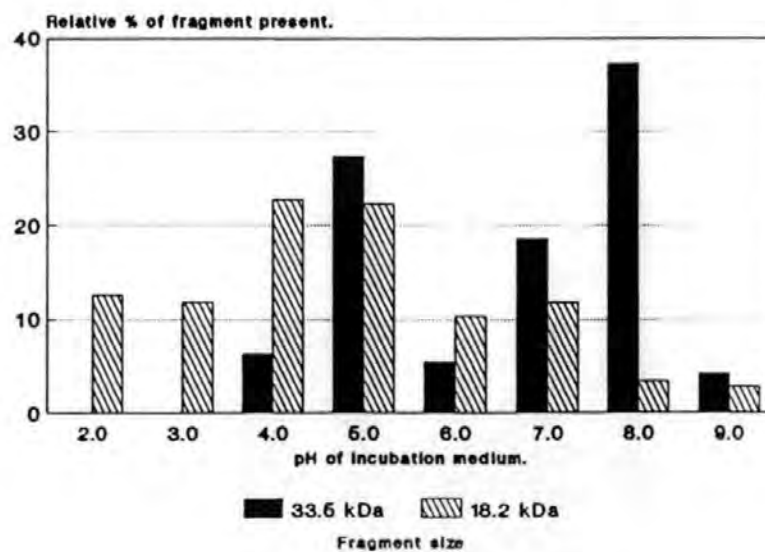


Fig 3.13: *pH dependence of the proteolysis of BSA by gastric enzymes.*

Fig. 3.13 (a): *Antigen degradation represented in terms of changes in the relative amount of intact BSA (including multimers) present with increasing pH. Data derived from laser scans of entire lanes of immunoblot (Reactions allowed to proceed for 2 hr in each case).*

Fig. 3.13 (b): *Antigen degradation represented in terms of changes in the relative amounts of fragment groups present with increasing Ph. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 3.13 (c): *Antigen degradation represented in terms of changes in the relative percentages of discrete fragments present with increasing pH. Data derived from laser scans of individual bands on immunoblot.*

Fig.3.13(a): Gastric enzyme degradation of BSA.
pH dependence of proteolysis.

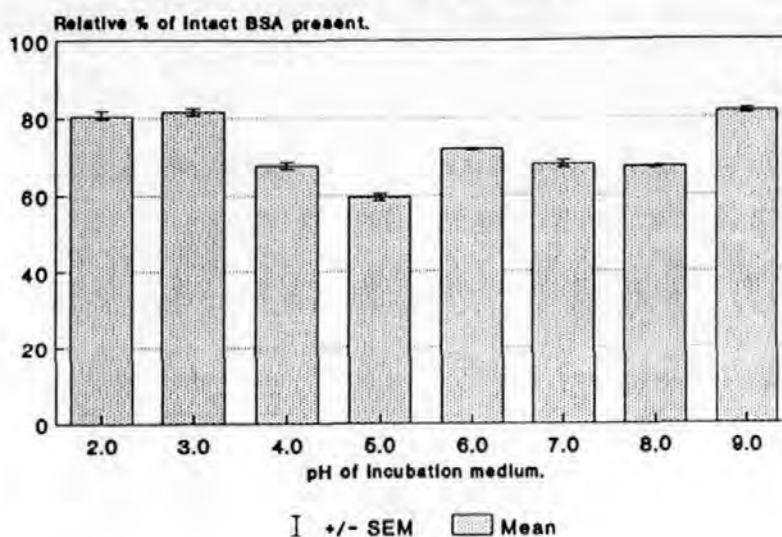


Fig.3.13(b): Gastric enzyme degradation of BSA.
pH dependence of proteolysis.

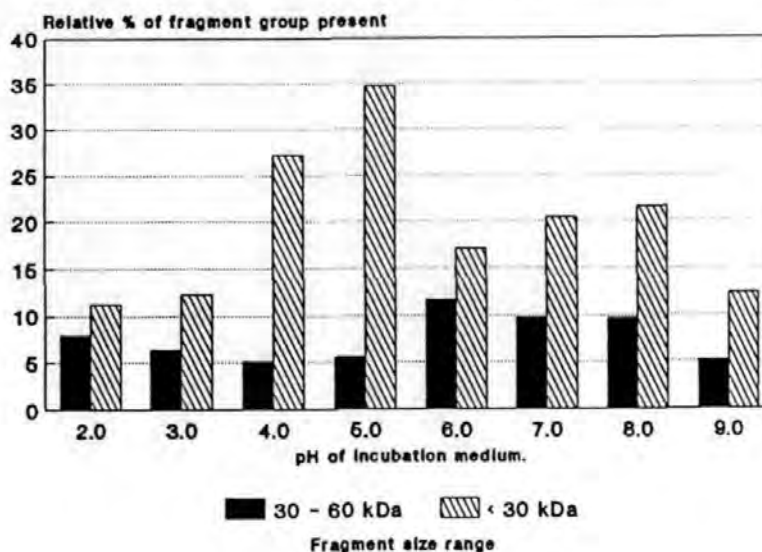


Fig.3.13(c): Gastric enzyme degradation of BSA.
pH dependence of proteolysis.

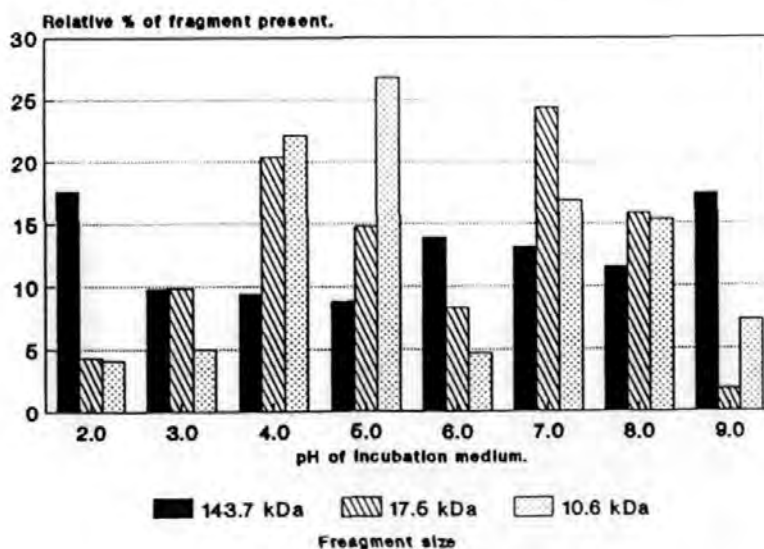


Fig. 3.14. *pH dependence of the proteolysis of HGG by intestinal enzymes.*

Fig. 3.14 (a): *Antigen degradation represented in terms of changes in the relative amount of intact HGG present with increasing pH. Data derived from laser scan of entire lanes on immunoblot (Reactions allowed to proceed for 2 hours in each case).*

Fig. 3.14 (b): *Antigen degradation represented in terms of changes in the relative amounts of fragment groups present with increasing pH. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 3.14 (c): *Antigen degradation represented in terms of changes in the relative percentages of discrete fragments present with increasing pH. Data derived from laser scans of entire lanes of immunoblot.*

Fig.3.14(a): Intestinal enzyme degradation of HGG.
pH dependence of proteolysis.

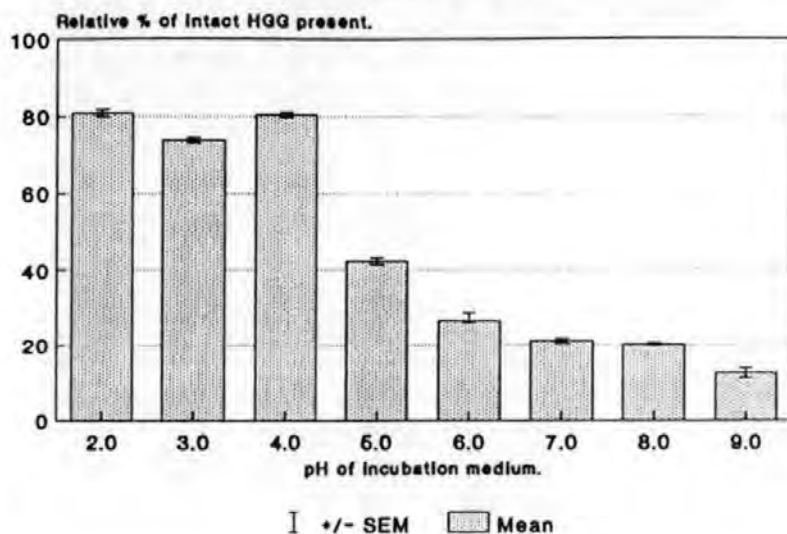


Fig.3.14(b): Intestinal enzyme degradation of HGG.
pH dependence of proteolysis.

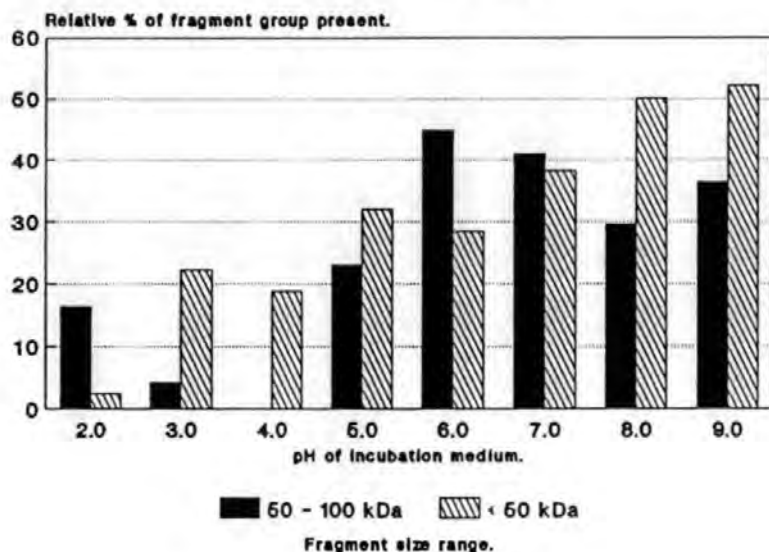


Fig.3.14(c): Intestinal enzyme degradation of HGG.
pH dependence of proteolysis.

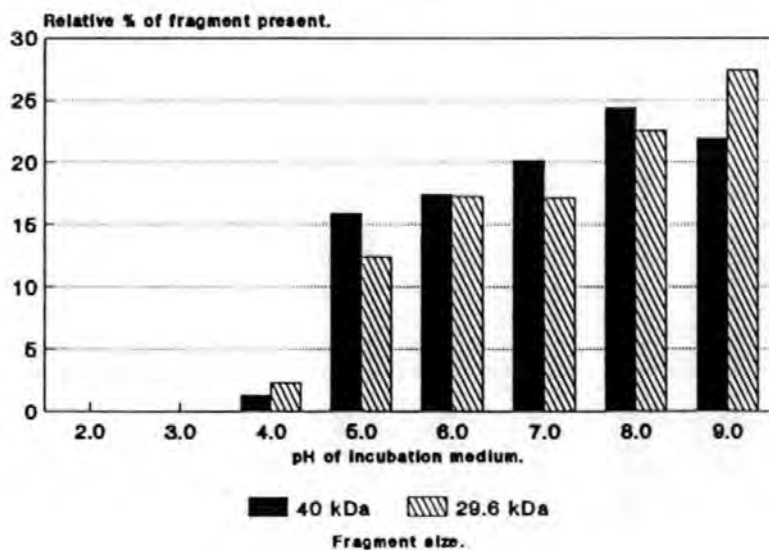


Fig. 3.15: *pH dependence of the proteolysis of BSA by intestinal enzymes.*

Fig. 3.15 (a): *Antigen degradation represented in terms of changes in the relative amount of intact BSA (including multimers) present with increasing pH. Data derived from laser scans of entire lanes of immunoblot (Reactions allowed to proceed for 2 hours in each case).*

Fig. 3.15 (b): *Antigen degradation represented in terms of changes in the relative amounts of fragment groups present with increasing pH. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 3.15 (c): *Antigen degradation represented in terms of changes in the relative percentages of discrete fragments present with increasing pH. Data derived from laser scans of entire lanes of immunoblot.*

Fig.3.15(a): Intestinal enzyme degradation of BSA.
pH dependence of proteolysis.

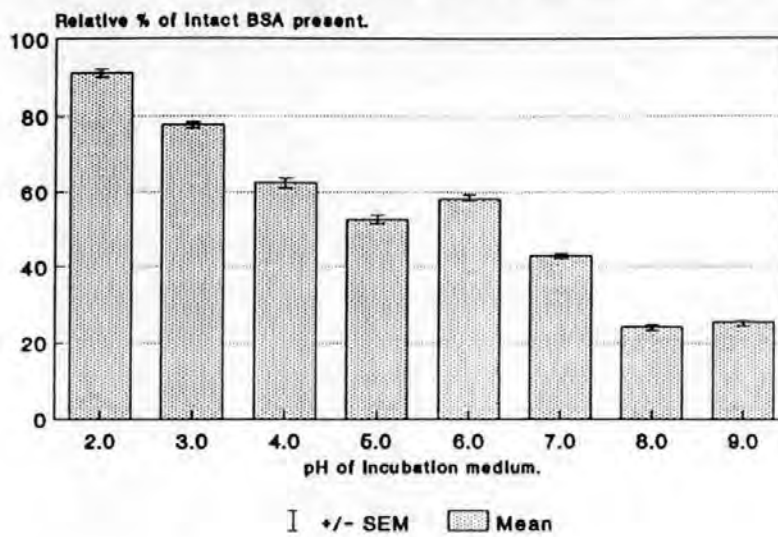


Fig.3.15(b): Intestinal enzyme degradation of BSA.
pH dependence of proteolysis.

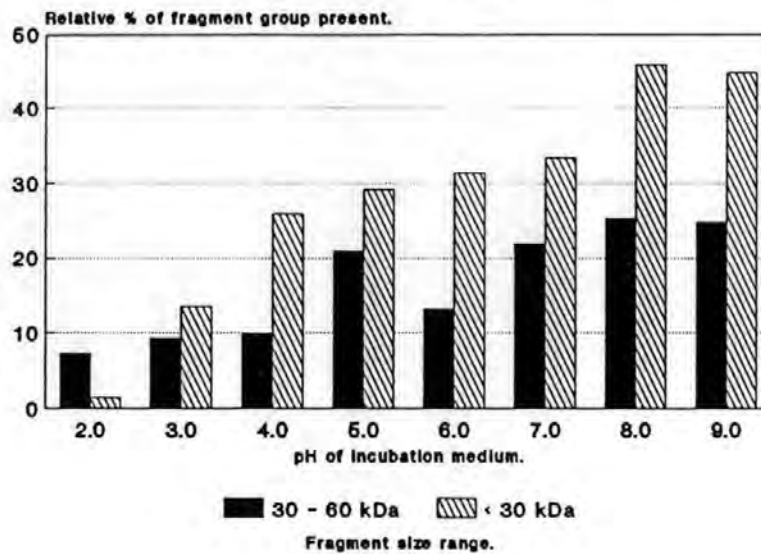


Fig.3.15(c): Intestinal enzyme degradation of HGG.
pH dependence of proteolysis.

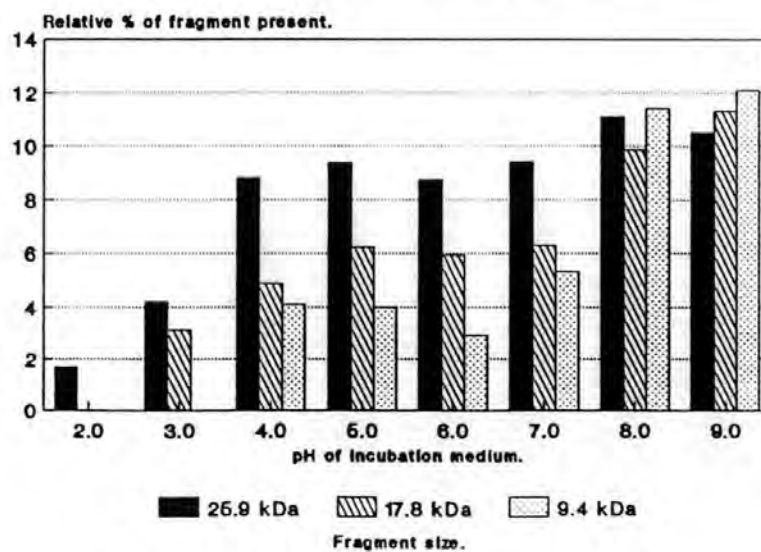


Fig. 3.16: *Effects of specific inhibitors on the proteolysis of HGG by gastric enzymes.*

Fig. 3.16 (a): *Antigen degradation represented in terms of changes in the relative amount of intact HGG present under various conditions. Data derived from laser scan of entire lanes of immunoblot (Reactions allowed to proceed for 2 hours in all cases in the presence or absence of inhibitors).*

Fig. 3.16 (b): *Antigen degradation represented in terms of changes in the relative amounts of fragment groups present under various conditions. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 3.16 (c): *Antigen degradation represented in terms of changes in the relative percentages of discrete fragments present under various conditions. Data derived from laser scans of individual bands on immunoblot.*

Descriptions of abbreviations used on barcharts opposite.

C = Control

2hr = 2 hour digest

SB = " + *SBTI*.

PM = " + *PMSF*.

Leu = " + *Leupeptin*.

Pep = " + *Pepstatin*.

QA = " + *Quil - A saponin*.

Cim = " + *Cimetidine*.

Bic = " + *Bicarbonate*

Fig.3.16(a): Gastric enzyme degradation of HGG.
Effect of inhibitors on proteolysis.

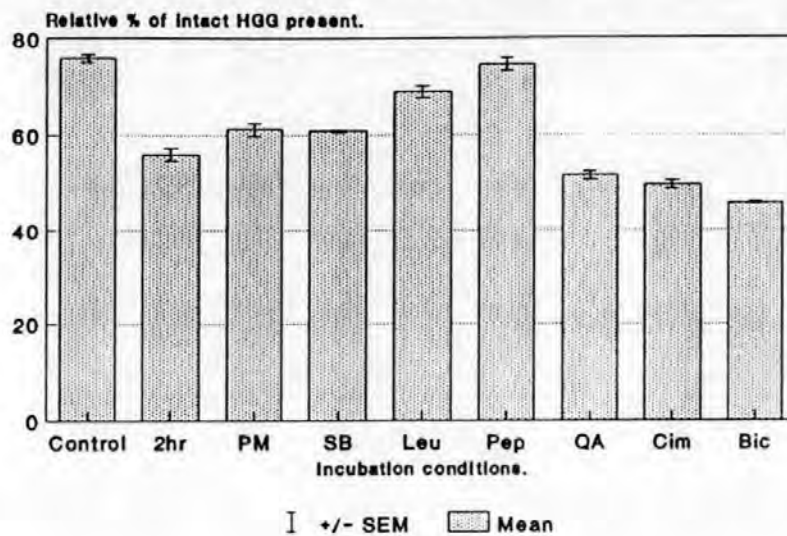


Fig.3.16(b): Gastric enzyme degradation of HGG.
Effect of inhibitors on proteolysis.

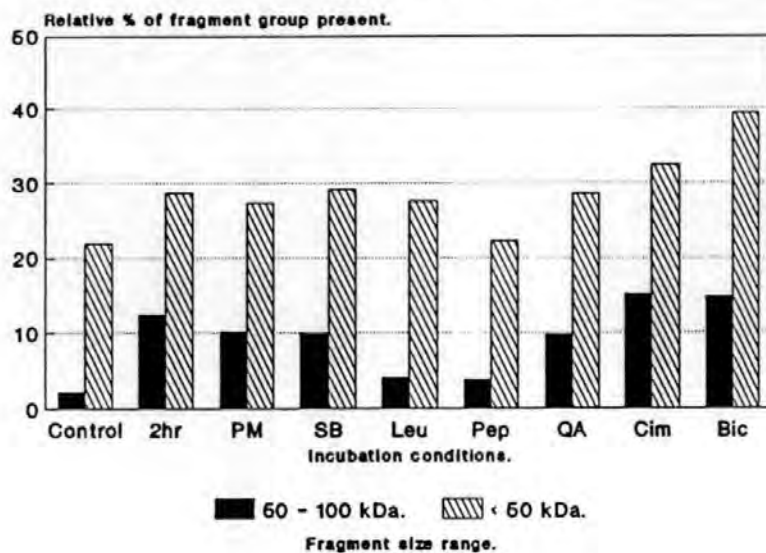


Fig.3.16(c): Gastric enzyme degradation of HGG.
Effect of inhibitors on proteolysis.

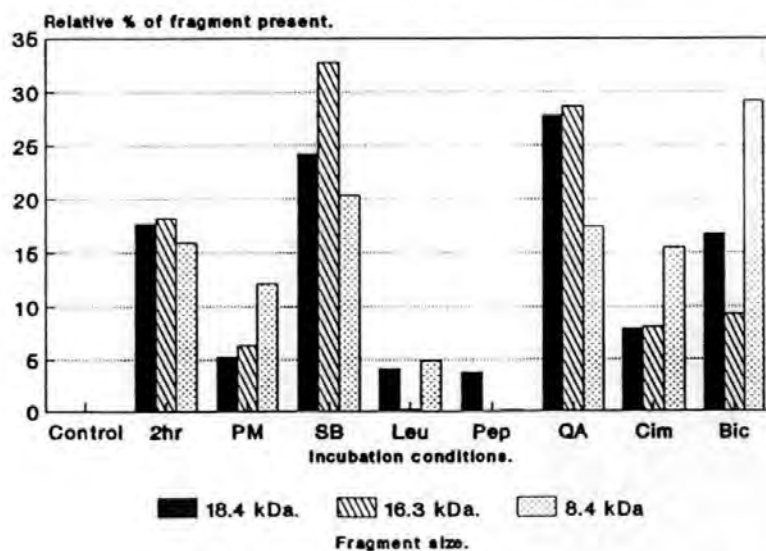


Fig. 3.17: *Effects of specific inhibitors on the proteolysis of BSA by gastric enzymes.*

Fig. 3.17 (a): *Antigen degradation represented in terms of the relative amount of intact BSA (including multimers) present after 2 hours incubation. Data derived from laser scans of entire lanes of immunoblot (Reactions were allowed to proceed for 2 hours in each case in the presence or absence of inhibitors).*

Fig. 3.17 (b): *Antigen degradation represented as a function of the relative amounts of fragment groups present after 2 hours incubation. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 3.17 (c): *Antigen degradation represented in terms of the relative percentages of discrete fragments present after 2 hours incubation. Data derived from laser scans of individual bands on immunoblot.*

Descriptions of abbreviations used on barcharts opposite.

C = Control

2hr = 2 hour digest

SB = " + *SBTI*.

PM = " + *PMSF*.

Leu = " + *Leupeptin*

Pep = " + *Pepstatin*.

QA = " + *Quil - A saponin*.

Cim = " + *Cimetidine*.

Bic = " + *Bicarbonate*

Fig.3.17(a): Gastric enzyme degradation of BSA.
Effect of inhibitors on proteolysis.

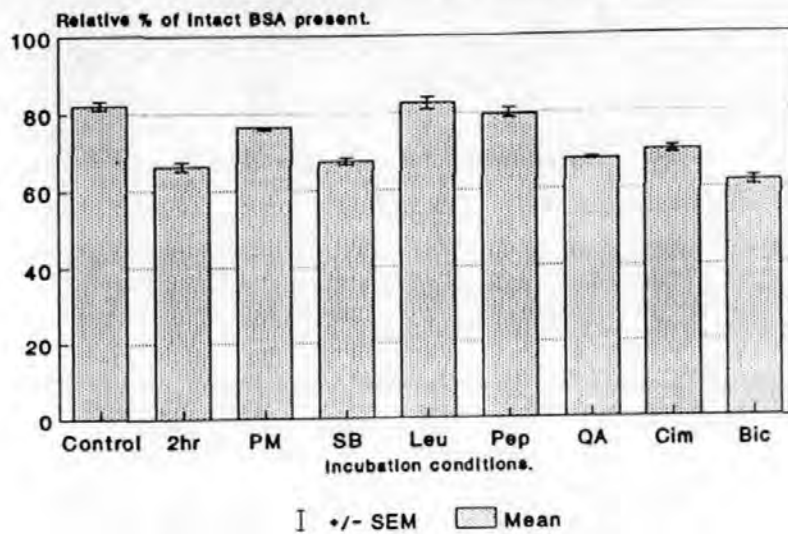


Fig.3.17(b): Gastric enzyme degradation of BSA.
Effect of inhibitors on proteolysis.

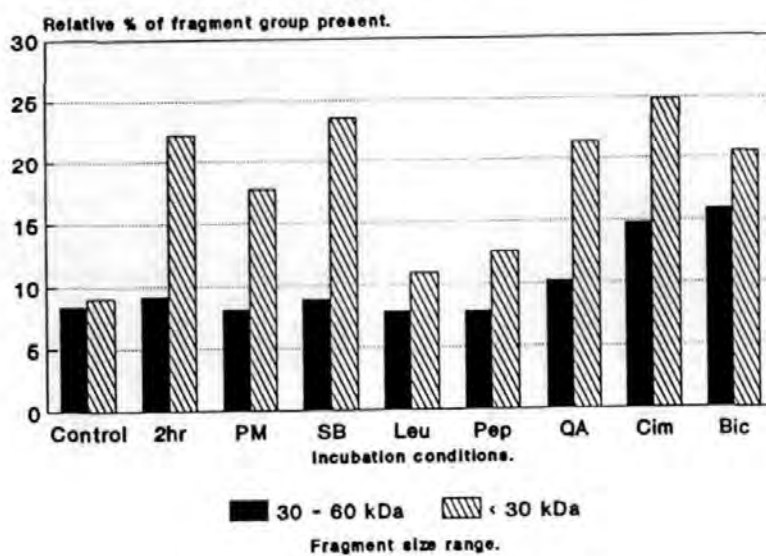


Fig.3.17(c): Gastric enzyme degradation of BSA.
Effect of inhibitors on proteolysis.

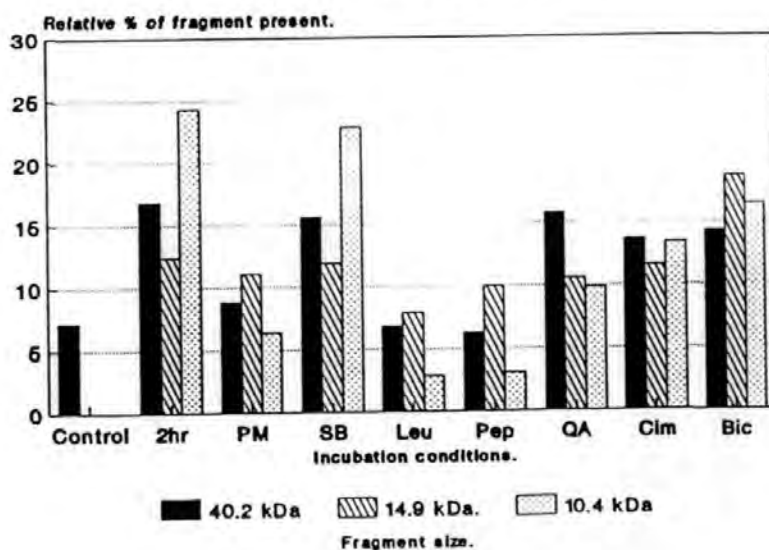


Fig. 3.18: *Effects of specific inhibitors on the proteolysis of HGG by intestinal enzymes.*

Fig. 3.18 (a): *Antigen degradation represented in terms of the relative amount of intact HGG present after 2 hours incubation. Data derived from laser scans of entire lanes of immunoblot (Reactions allowed to proceed for 2 hours in each case in the presence or absence of inhibitors).*

Fig. 3.18 (b): *Antigen degradation represented in terms of the relative amounts of fragment groups present after 2 hours incubation. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 3.18 (c): *Antigen degradation represented in terms of the relative percentages of discrete fragments present after 2 hours incubation. Data derived from laser scans of entire lanes of immunoblot.*

Descriptions of abbreviations used on barcharts opposite.

C = Control

2hr = 2 hour digest

SB = " + *SBTI*.

PM = " + *PMSF*.

Leu = " + *Leupeptin*

Pep = " + *Pepstatin*.

QA = " + *Quil - A saponin*.

Cim = " + *Cimetidine*.

Bic = " + *Bicarbonate*

Fig.3.18(a): Intestinal enzyme degradation of HGG.
Effect of inhibitors on proteolysis.

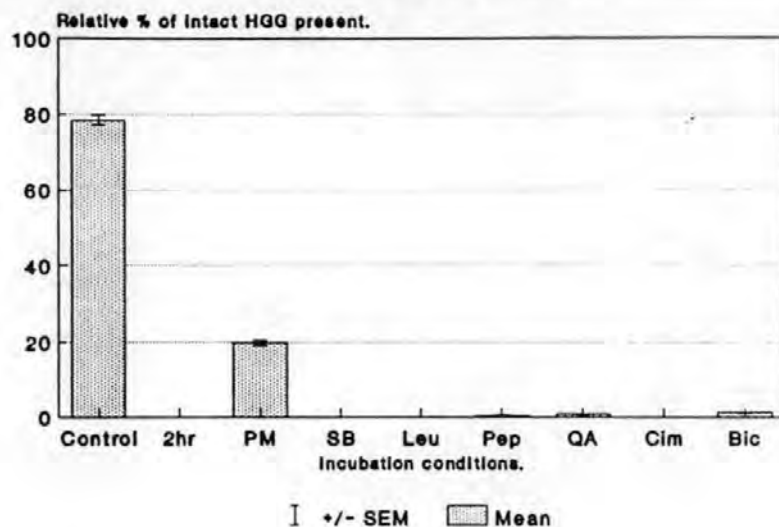


Fig.3.18(b): Intestinal enzyme degradation of HGG.
Effect of inhibitors on proteolysis.

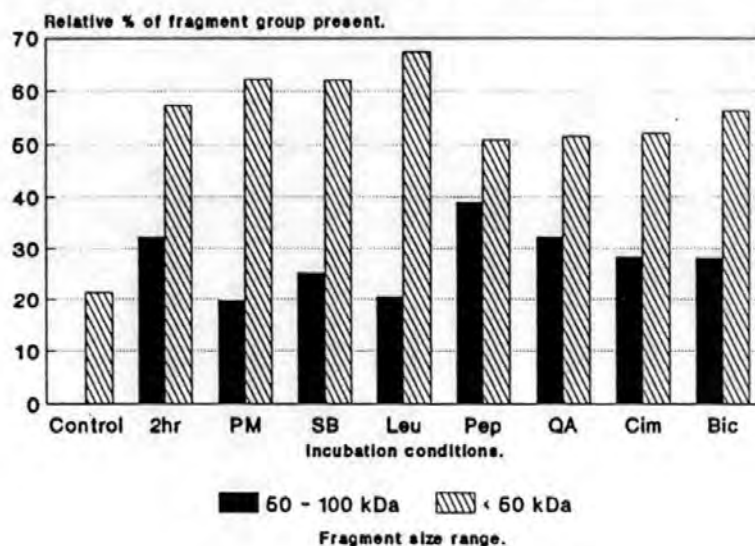


Fig.3.18(c): Intestinal enzyme degradation of HGG.
Effect of inhibitors on proteolysis.

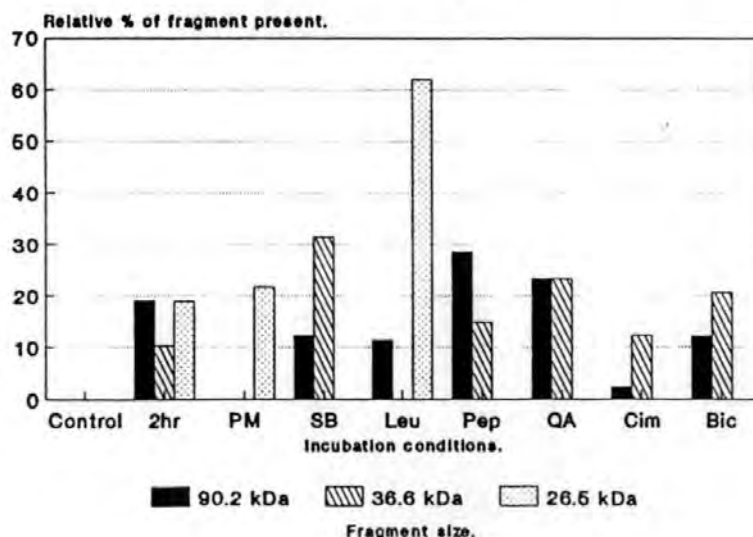


Fig. 3.19: *Effects of specific inhibitors on the proteolysis of BSA by intestinal enzymes.*

Fig. 3.19 (a): *Antigen degradation represented in terms of the relative amount of intact BSA (including multimers) present after 2 hours incubation. Data derived from laser scans of entire lanes of immunoblot (Reactions allowed to proceed for 2 hours in all cases in the presence or absence of inhibitors).*

Fig. 3.19 (b): *Antigen degradation represented in terms of the relative amounts of fragment groups present after 2 hours incubation. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 3.19 (c): *Antigen degradation represented in terms of the relative percentages of discrete fragments present after 2 hours incubation. Data derived from laser scans of entire lanes of immunoblot.*

Descriptions of abbreviations used on barcharts opposite.

C = Control

2hr = 2 hour digest

SB = " + *SBTI*.

PM = " + *PMSF*.

Leu = " + *Leupeptin*

Pep = " + *Pepstatin*.

QA = " + *Quil - A saponin*.

Cim = " + *Cimetidine*.

Bic = " + *Bicarbonate*

Fig.3.19(a): Intestinal enzyme degradation of BSA.
Effect of inhibitors on proteolysis.

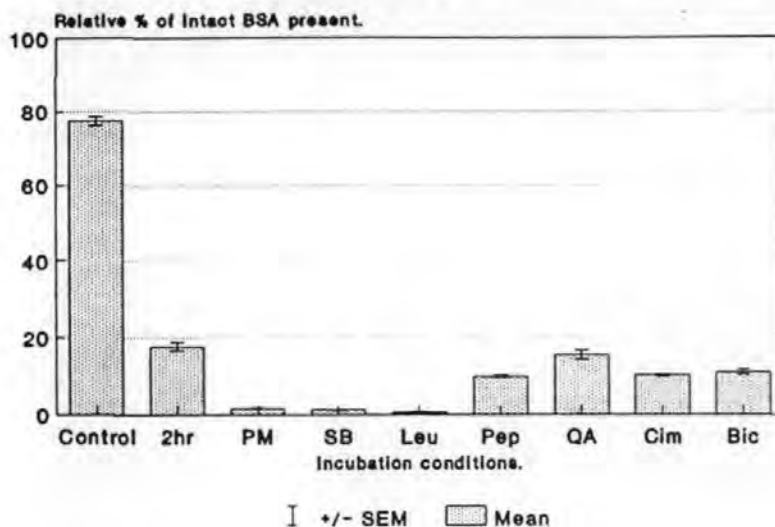


Fig.3.19(b): Intestinal enzyme degradation of BSA.
Effect of inhibitors on proteolysis.

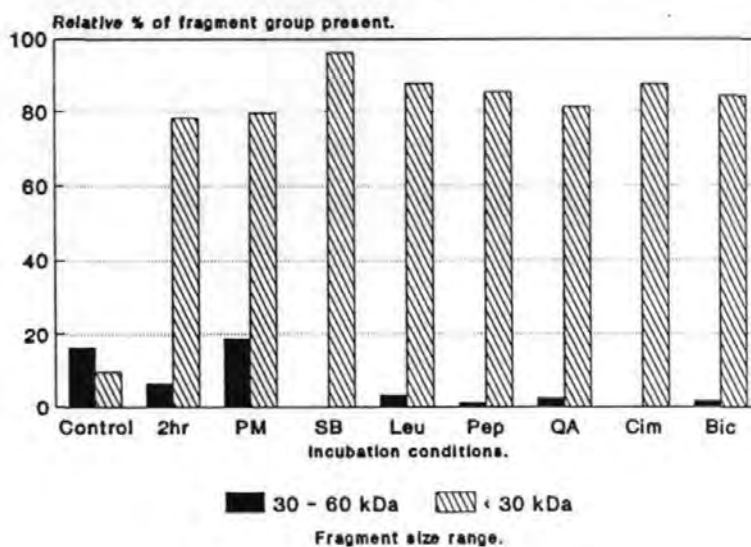


Fig.3.19(c): Intestinal enzyme degradation of BSA.
Effect of inhibitors on proteolysis.

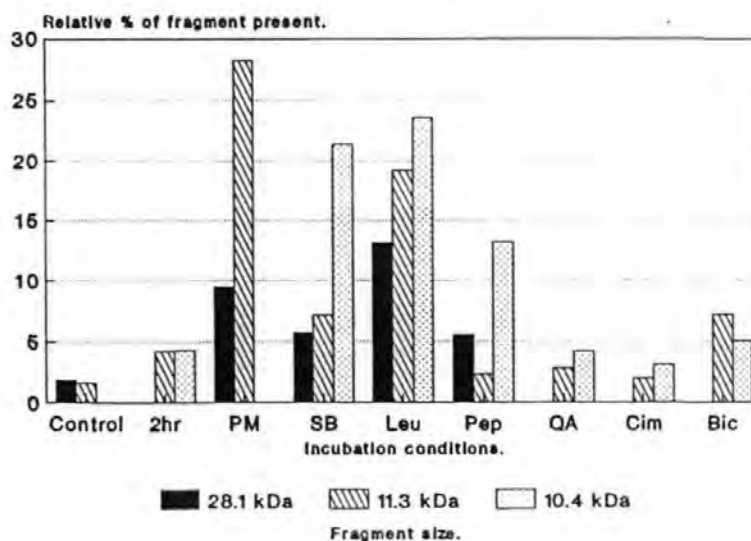
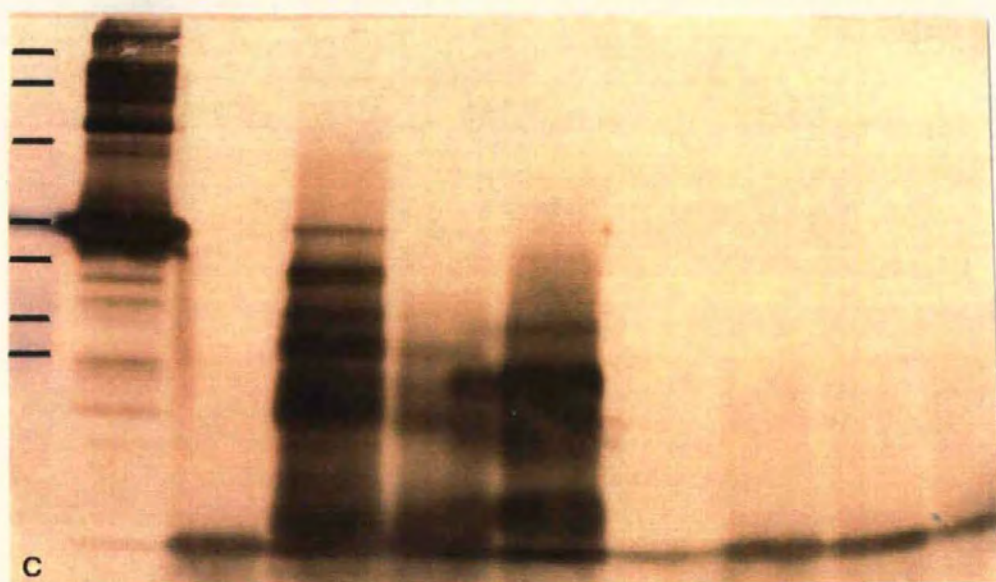
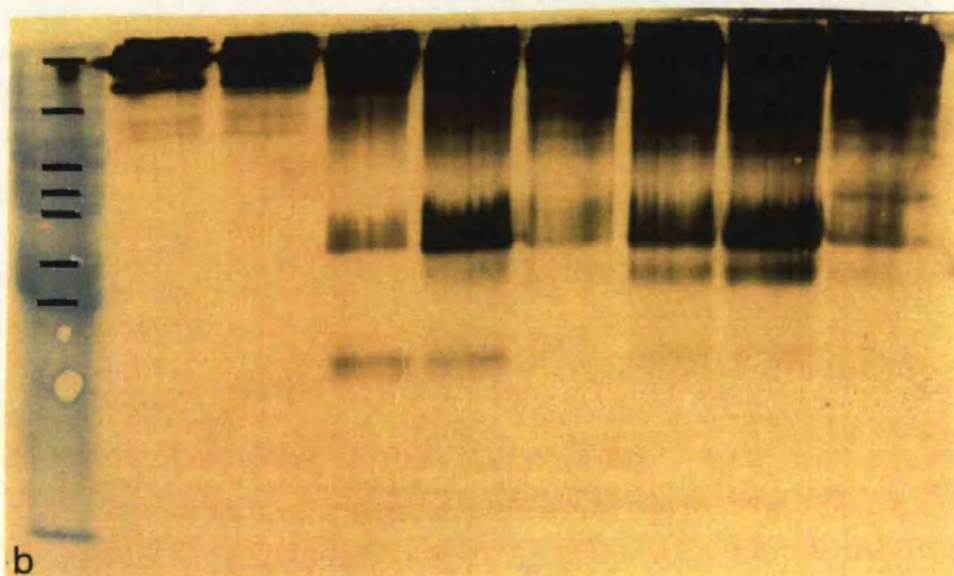
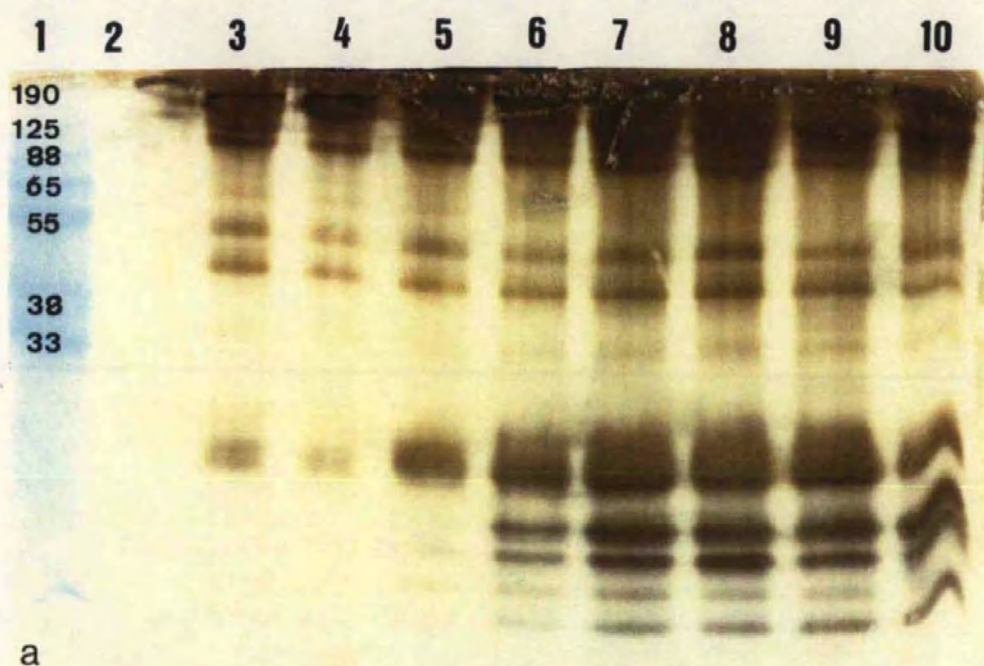


Fig 3.20. *Western blots demonstrating the effects of various factors on gastric and intestinal enzyme proteolysis of BSA and HGG. Apparent molecular weights of protein markers are labelled in Fig 3.20(a) and bars representing these markers are displayed on Fig 20 (b) and Fig 20(c).*

Fig 3.20(a). *Time course of gastric enzyme degradation of HGG (10 μ g protein loaded per lane). Loading order. Lane 1: Prestained protein standards. Lane 2: Negative control (enzyme solution only). Lane 3: Positive control (HGG only). Lanes 4 - 10: HGG samples exposed to gastric enzymes for 1 min, 30 min, 2 hr, 4 hr, 6 hr, 8 hr and 15 hr respectively.*

Fig 3.20(b). *pH dependence of gastric enzyme degradation of HGG (10 μ g protein loaded per lane). Loading order. Lane 1: Prestained protein standards. Lanes 2 - 10: HGG samples incubated with gastric enzymes at pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 respectively.*

Fig 3.20(c). *Effects of inhibitors on intestinal enzyme degradation of BSA (10 μ g protein loaded per lane). Loading order. Lane 1: Prestained protein standards. Lane 2: Positive control (BSA only). Lane 3: 2 hr digest of BSA by intestinal enzymes. Lanes 4 - 10: 2 hr digest of BSA by intestinal enzymes in the presence of PMSF, SBTI, leupeptin, pepstatin, Quil - A, cimetidine and bicarbonate respectively.*



3.4 DISCUSSION

In vitro analyses have previously been used in biochemical studies of teleost proteases (Ferraris and Ahern, 1984; Gildberg, 1988; Kolodzeiskaya *et al.*, 1988; Simpson *et al.*, 1989) and in nutritional studies of feed utilisation (Grabner and Hofer, 1985) to simulate gut conditions. In this study an *in vitro* system was devised to analyse protein antigen breakdown by luminal proteases. The use of SDS - PAGE, immunoblotting and laser densitometry analyses in this study has enabled a detailed investigation of the dynamics of antigen degradation and of the fragments released. The capacity of this method to detect and analyse individual antigen fragments is particularly valuable from an immunological viewpoint since mammalian studies suggest that enzymic release of immunogenic or tolerogenic epitopes may play a critical role in determination of the immune response to enterically presented antigens (Michael, 1989; Hachimura *et al.*, 1993).

Evidence exists from both mammalian (Russell and Walker, 1989) and teleost (Jenkins *et al.*, 1992) studies that the gastrointestinal mucosal barrier does not exclude the capacity to absorb macromolecules in a bioactive form. However, gastric and pancreatic secretions are recognised as major barriers to the absorption of intact protein macromolecules by the teleost gut (Johnson and Amend, 1983a, b; Jenkins *et al.*, 1992). The current study demonstrated that degradation of intact soluble protein antigens in the rainbow trout stomach resulted in partial proteolysis to a range of peptides. A large proportion of antigen was also found apparently intact even after 15 hours, the estimated gastric retention time in rainbow trout held at 15°C (Grabner, 1985). This supports previous findings on rainbow trout (Dabrowski *et al.*, 1986), cod, *Gadus morhua* (Lied and Solbakken, 1984) and other species (Sabapathy and Teo, 1993) and suggests that the stomach of gastric teleosts plays a primary role in the digestion of proteins to peptides prior to exposure to the intestinal peptidases and proteases (Glass *et al.*, 1989). Only very small amounts of free amino acids were found to be released from proteins in the stomach (Dabrowski *et al.*, 1986; Lied and Solbakken, 1984). The former study found that the stomach peptide content was only 28.4 % of the total protein present 10 hr after a meal while peptide amino acids accounted for up to 89% of total protein in the pyloric caecae region. The role of gastric secretions in protein

digestion is influenced by various factors such as food retention time, stomach distensibility, food bulk, the degree of food penetration by gastric secretions and the specific activity of pepsin (Ash, 1985). Although the trout stomach may play a limited part in antigen degradation, the preparation of protein for further digestion in the intestine and in the potential release of immunogenic fragments may be of greater importance. In this study only intact antigens were used as a substrate and the effect of gastric exposure on the rate of subsequent intestinal proteolysis was not assessed. Results presented later in this thesis (sections 5.3 and 7.3) indicate that the dose of soluble protein antigen orally administered to rainbow trout *in vivo* can play a critical role in determining the extent of degradation. Smaller doses are more rapidly and extensively degraded in the stomach which suggests that the large amounts of intact antigen which were found remaining after incubation with gastric enzymes in the present investigation may reflect an 'overloading' of the proteolytic capacity of the isolated proteases.

Intestinal enzymes from rainbow trout were found to rapidly degrade both BSA and HGG in this study suggesting that highly active proteases were present. Both HGG and BSA were rapidly converted to fragments which were themselves further degraded and after 11.5 hours incubation (the estimated intestinal retention time in rainbow trout at 15° C) (Grabner, 1985) very little antigen in any form was detectable. This rapid and extensive proteolysis is in accordance with previous studies on rainbow trout (Grabner, 1985; Dabrowski *et al.*, 1986) and cod (Lied and Solbakken, 1984) which have demonstrated that the intestinal proteases of these gastric teleosts are highly effective in digesting proteins to small peptides and amino acids. The final products of intestinal enzyme degradation were not detectable in this study due to the experimental limits of gel pore size in SDS-PAGE. A comparative study on pancreatic proteases from mammalian, avian and piscine species found that trypsin and chymotrypsin derived from trout were more active than those from all other species tested (Krogdahl and Holm, 1983). Trout trypsin and chymotrypsin when compared with the corresponding human enzymes were more than 10 and 5 times as effective, respectively, in hydrolysing the synthetic substrates N - benzoyl - L - arginine - p - nitro - analide and N - benzoyl - L - tyrosine - ethyl ester. The speed and extent of antigen degradation

by rainbow trout intestinal enzymes indicates that degradation of unprotected protein antigens could be expected to commence immediately after entry into the intestine. The release of a large number of fragments which may subsequently be absorbed could result in difficulty in predicting and controlling the immune response to orally administered protein antigens. Many workers concur that macromolecular uptake in gastric teleosts occurs principally in the second intestinal segment/hind gut and that absorption at this point is necessary for the induction of an immune response (Georgopoulou *et al.*, 1988; Rombout *et al.*, 1993a). If this is indeed the case then some degree of antigen protection from luminal secretions may be necessary. There is also evidence that antigen is absorbed in other regions of the gastrointestinal tract including the gastric region and that immunologically competent cells are present at these sites, albeit in low numbers (Doggett, 1989; Jenkins, 1992). In these anterior gut regions a greater proportion of protein antigens could be predicted to remain intact and if absorbed may be of immunological significance.

A clear increase in antigen degradation by gastric and intestinal enzymes with increasing temperature (up to 35°C) was noted in this study. Very little degradation was apparent at temperatures less than 5°C which is in accordance with other studies which indicate that activity at low temperatures is reduced in most species (Kitimikado and Tachino, 1960; Simpson and Haard, 1987). Under optimal conditions most fish pepsins have a temperature optimum around 40°C (Gildberg, 1988). Krogdahl and Holm (1983) found that the activity of rainbow trout pancreatic proteinases was roughly halved when the temperature was reduced from 37°C to 10°C and proposed that at low water temperatures the hydrolytic efficiency of trout pancreatic proteases would be relatively low. Certain fish species appear to display the characteristic of temperature adaptation of digestive enzymes to enable digestion at lower temperatures. This may be achieved by increased enzyme secretion or by the possession of enzymes with lower activation energies enabling relatively high activity at low temperatures (Gildberg, 1988). Owen and Wiggs (1971) found that pepsin activity was 30% greater in cold acclimated brook trout *Salvelinus fontinalis* than in warm acclimated fish when assayed at the same temperature but a similar effect was not apparent in the case of trypsin and chymotrypsins from rainbow trout (McLeese and Stevens,

1982). Nonetheless, degradation increases considerably with temperature up to and beyond the optimal trout habitation temperature (15°C) suggesting the possibility that oral vaccination at lower temperatures may reduce the degree of antigen proteolysis and thus improve the efficacy of this method of administration. Additionally, a lowering of temperature would be expected to result in a general slowing of metabolic processes thus leading to reduced secretion of enzymes further reducing proteolysis. However, low temperatures result in an increase in gastric retention times (Jones, 1974) and this increased exposure to less active enzymes may achieve an equivalent effect. Also the suppressive effect of low temperatures on piscine immune responses (Zapata *et al.*, 1992) may abrogate any advantage gained in terms of the quantity of intact antigen reaching the absorptive regions of the gastrointestinal tract.

The activity of rainbow trout luminal proteases was also shown in this study to be pH dependent. Gastric enzymes were optimally active at two distinct points, pH 4.0-5.0 and pH 7.0-8.0, with little proteolysis apparent at other pH values. A wide range of hydrolases including proteases have been recorded in the teleost stomach (Fänge and Grove, 1979). Pepsin has been reported as the dominant enzyme in this region with an optimal activity around pH 3.0 (Gildberg, 1988) although proteases with optima at pH 5.0 and pH 7.0 have also been described (Fänge and Grove, 1979). Furthermore, Fish (1960) demonstrated the presence of an "alkaline" protease in the stomach of perch and tilapia. In a study of the gastric proteases of cod by Squires *et al.* (1986) three enzymes were detected which were optimally active at pH 3.0-5.0. One of the proteases was highly unstable at pH values greater than 5.0 while the other two exhibited additional peaks of activity at pH 7.0. In the case of one of these proteases, activity at pH 7 was almost two thirds as great as at pH 4.0. This pattern is remarkably similar to that observed in the present study suggesting that a number of gastric proteases may be produced by rainbow trout. Alternatively, it is possible that a single enzyme may exhibit multiple activities which are pH dependant. Gildberg and Raa (1983) found that the optimal pH for pepsin from Arctic capelin *Mallotus villosus* varied according to the substrate used and Lindsay (1983) reported marked differences in the effects of pH on rainbow trout gastric chitinase activity which were also substrate dependant.

A study on Dover sole, *Solea solea* found three separate peaks of activity for gastric proteases acting on casein at pH 1.7, pH 6.0 and pH 10.0 (Clark *et al.*, 1985). Similarly, a study on gastric enzymes from capelin identified a pepsin which demonstrated greatly different optima, from pH 3.7 with cod sarcoplasmic protein as the substrate to a pH greater than 5.5 with cod myofibrillar protein as substrate (Gildberg and Raa, 1983). It is also possible that the second pH optimum, observed at 7.0-8.0 in this study was a result of contamination during the isolation procedure by pancreatic proteases. This is unlikely since as outlined below intestinal enzymes displayed sequentially increasing proteolytic activity up to pH 9.0 whereas the gastric enzymes were minimally active at this pH. Therefore it is proposed that either more than a single type of protease is present in the trout stomach or that the enzymes present can exhibit differential activity which is pH dependant. Since rainbow trout are opportunistic carnivores which 'gorge' themselves when food is available it is likely that the low pH necessary for "classical" pepsin-like activity is not maintained at all times, particularly after heavy feeding when acid present in the stomach would be considerably diluted leading to a pH closer to neutrality. Under these conditions it may be advantageous to possess an additional enzymic activity which operates at higher pH values until acidification of the contents is achieved in the trout stomach.

In contrast, intestinal enzymes demonstrated a clear increase in proteolytic activity with increasing pH from 2.0 to 9.0, in accordance with previous studies on rainbow trout which have demonstrated an optimal activity around pH 9 (Kitamikado and Tachino, 1960; Grabner, 1985). The optimal pH for two chymotrypsins from the rainbow trout pyloric caecae was reported to be 9.0 and such enzymes were unstable at pH values less than 5 (Kristjansson and Nielsen, 1992). It is likely that the range of intestinal proteases present in the rainbow trout intestine have different pH optima so the results in this study likely reflect the variable contributions of a range of enzymes depending on the pH.

For the purposes of oral vaccination the most obvious point at which pH (and possibly proteolysis) may be altered is the stomach. The use of gastric inhibitors has been suggested as a potential

strategy to enhance intact macromolecular absorption in fish, (McLean and Ash, 1987) and in mammals sodium bicarbonate is routinely administered prior to *Vibrio cholerae* vaccines in order to protect the acid labile cholera toxin and prior to *Salmonella typhi* Ty 21a vaccine in field trials (Clemens *et al.*, 1986). Use of such a system would appear from this study to be of limited value in enhancing uptake of BSA and HGG in rainbow trout. However, in cases where antigens are particularly susceptible to low pH this may be of considerable value. Additionally, if the putative "pH window" observed in gastric proteolysis of BSA and HGG in this study in the trout stomach is confirmed *in vivo*, adjustment of stomach contents to pH 6 may reduce antigen degradation, and possibly enhance the immune response to such orally delivered antigens.

An effort was made in this study to determine which protease class, or classes were responsible for protein antigen degradation in the gut lumen. Gastric proteases were partially inhibited by PMSF and almost totally inhibited by pepstatin and leupeptin. The inhibition by pepstatin was in accordance with previous work on salmonid pepsins (Cisternas *et al.*, 1983; Gildberg and Raa, 1983; Sanchez-Chiang *et al.*, 1986) indicating an aspartil protease activity. The inhibition by the serine protease inhibitors, PMSF and leupeptin was not expected as serine proteases have not previously been reported in the trout stomach. The degree of inhibition of gastric proteolysis by pepstatin and leupeptin was almost identical and it is possible that leupeptin can inhibit pepsin-like activity in the trout. If serine proteases were involved in gastric proteolysis, pepstatin would not have been expected to efficiently prevent proteolysis. In contrast with leupeptin, SBTI which is also a serine protease inhibitor, had little effect on gastric enzyme proteolysis further suggesting that PMSF and leupeptin may inhibit gastric enzymes by a means not related to their function as serine protease inhibitors. Alternatively since SBTI is not acid stable (Krogdahl and Holm, 1983) it is possible that the conditions applied in this study altered the substance and reduced its activity.

Analysis of the effects of inhibitors on intestinal enzyme proteolysis of HGG and BSA indicated that serine proteases were principally responsible, PMSF, SBTI and leupeptin all abrogated intestinal enzyme degradation to some degree. PMSF was most effective, but even with this broad

range serine protease inhibitor, significant degradation was noted suggesting that additional protease classes may be in operation. Krogdahl and Holm (1983) found that among a range of animal species the caseinolytic activity in extracts of pancreatic tissue from trout was more sensitive to inhibition by SBTI than that from all other species, hydrolysis of casein by trout pancreatic proteases in the latter study was almost halved by addition of SBTI. Other studies (Reece, 1988) have found that PMSF and SBTI only partially inhibited alkaline proteases from salmon viscera. Cohen *et al.* (1981) in a study on carp found that SBTI only inhibited chymotrypsin activity to a small extent and did not inhibit elastase activity. The greater inhibition due to PMSF observed in this study may, therefore, reflect the inhibition of chymotrypsin or elastase which was not observed when SBTI was used. Use of the metalloprotease inhibitor, EDTA appeared to have little effect on intestinal enzyme proteolysis (results not presented) and it is possible that the effect of metalloprotease inhibition was not apparent due to overwhelming serine protease activity. Therefore, it may only be possible to detect metalloprotease activity in the absence of serine proteases. Various studies have utilised protease inhibitors to enhance intact antigen uptake in both mammals and teleosts. Use of soybean trypsin inhibitor (SBTI) led to an increased uptake of BSA in mice (Udall *et al.*, 1984) and of HRP in rainbow trout (McLean and Ash, 1990). However, studies on mammals suggest that due to the multiple potential cleavage sites on most proteins and the widespread presence of a range of proteolytic enzymes at various points between the site of entry and that of action that protection of proteins/peptides from degradation in this way may not necessarily lead to a marked increase in stability or in the amount of the substance reaching the site of action (Lee *et al.*, 1986). Furthermore, there are differences in the effects of proteinase inhibitors on enzymes from different animal species (Mallory and Travis, 1975; Combs and Poston, 1978; Krogdahl and Holm, 1983) so the use of inhibitors for the enhanced oral delivery of proteins would need to be tailored for each individual species utilised.

A number of other approaches to modify teleost physiology in order to enhance intact macromolecule absorption are also possible. Teleost enzyme activity has in some cases been

shown to be highly malleable to starvation (Pederson and Hjelmeland, 1988), seasonal changes (Ananichev, 1959), temperature acclimation (Hochachka and Clayton-Hochachka, 1973; Tsukuda and Ohsawa, 1974; Tsukuda, 1975) and feed composition (Hofer, 1979; Reimer, 1982). In addition, some studies have correlated various enzyme activities with the levels of proteins, carbohydrates and lipids in the diet (Phadate and Srikar, 1988) suggesting that judicious manipulation of diets prior to antigen delivery may enhance the uptake of intact antigen. Fänge (1973) found that high dietary protein and low cellulose content can enhance proteolytic activity in rainbow trout and that elevated starch levels can reduce such activity. Similarly, Kawai and Ikeda (1972) found that increased levels of dietary protein led to increased protease activity, although no such effect was noted by McLeese and Stevens (1982) in rainbow trout. Compensatory growth is a phase of rapid growth greater than control or normal growth which occurs upon adequate refeeding after a period of undernutrition (Quinton and Blake, 1990). This novel physiological adaptation may lend itself to enhanced delivery of antigens. It may be hypothesised that in order to maintain/ gain weight in conditions of limiting nutrient levels a facility for enhanced uptake exists which may be utilised in oral vaccination regimes.

In conclusion this *in vitro* investigation indicates that rainbow trout possess considerable luminal enzymic barriers to orally delivered intact protein macromolecules and that some form of protection for antigens through this environment may increase the efficacy of immunisation/via the oral route. The method used may provide a useful model to investigate the susceptibility of vaccine antigens to degradation by digestive tract secretions from rainbow trout

CHAPTER 4

CELLULAR PROCESSING OF SOLUBLE PROTEIN ANTIGENS BY ISOLATED RAINBOW TROUT INTESTINAL CELLS IN VITRO.

4.1 INTRODUCTION

Orally administered protein antigens encounter a strongly proteolytic environment in the digestive tract. Those antigens which remain intact must penetrate the epithelial cells to gain access to the body tissues prior to stimulating gastrointestinal immune mechanisms. In mammals, although enterocytes are involved in macromolecular absorption antigen uptake is primarily a function of specialised microfold (M) cells located in the epithelium overlying the Peyer's patches (Owen, 1977; Wolf and Bye, 1984). Macromolecular absorption in teleosts however appears to occur principally via the absorptive enterocytes of the intestine (Noaillac-Depeyre and Gas, 1973). A number of studies have investigated the morphological and histological characteristics of enterocytes in teleost species such as carp, *Cyprinus carpio*, (Iwai, 1969; Noaillac-Depeyre and Gas, 1973); tench, *Tinca tinca*, (Noaillac-Depeyre and Gas, 1976); goldfish, *Carassius auratus*, (Yamamoto, 1966; Gauthier and Landis, 1972); tilapia, *Oreochromis mossambicus* (Doggett, 1989) and rainbow trout, *Oncorhynchus mykiss* (Yamamoto, 1965; Bergot, 1976). Similarities have been noted between the absorptive enterocytes of the teleost intestine and those of neonatal mammals which are specially adapted for antigen absorption (Walker, 1982). This circumstantial evidence suggests a role for teleost enterocytes in antigen uptake (Noaillac-Depeyre and Gas, 1973, 1979; Rombout *et al.*, 1985; Georgopoulou and Vernier, 1986). Initial studies of antigen absorption in mammals used direct microscopic visualisation of enzymatically active antigens, such as applied by Graham and Karnovsky (1966), which involved the detection of horseradish peroxidase in tissues on the basis of its enzymatic activity. This technique was first applied to demonstrate macromolecular uptake in mouse renal tubules (Graham and Karnovsky, 1966) and subsequently in the mouse and rat intestinal mucosa (Owen, 1977) and has also been successfully used to demonstrate macromolecule endocytosis by human absorptive epithelial cells (Blok *et al.*, 1981). Adaptation of this method to piscine studies revealed the capacity for exogenous protein uptake in the intestine of agastric cyprinids (Noaillac-Depeyre and Gas, 1973, 1976) and subsequently in a range of gastric species including rainbow trout (Bergot, 1976), perch (Noaillac-Depeyre and Gas, 1979), and catfish (Stroband and Kroon, 1981). The disadvantages of this method include the presence of endogenous peroxidase activity in normal tissues and the need for

functionally intact antigen for microscopic visualisation (Larssen, 1988). Furthermore, fragmented antigens may possess intact epitopes of immunological significance which are not detected using this technique, a factor which complicates the detailed analysis of antigen processing in the gut. Subsequent studies have applied immunocytochemical techniques for the detection of intact as well as processed antigen (Rombout and van den Berg, 1989). These studies have yielded much information on the nature and dynamics of antigen absorption by intestinal enterocytes of both agastric (Rombout *et al.*, 1986) and gastric teleosts (Georgopoulou *et al.*, 1986; Jenkins *et al.*, 1992). In addition, the application of the enzyme linked immunosorbent assay (ELISA) and also of Western blotting has clearly shown the capacity for macromolecular uptake by teleost enterocytes and the occurrence of antigen processing prior to detection in the tissues or serum (McLean and Ash, 1987; Doggett, 1989; Jenkins *et al.*, 1992). Most of the above studies used *in vivo* models of antigen uptake which provided fundamental information on the dynamics of macromolecular absorption by the gut. These studies did not however permit a detailed analysis of antigen modification following administration and preceding detection in the serum of the animal. *In vitro* systems for the study of macromolecular uptake have received relatively less attention in piscine investigations to date although everted gut sacs (Georgopoulou *et al.*, 1986), intestinal segments in organ culture (Iida and Yamamoto, 1985; Iida *et al.*, 1986) and isolated enterocytes (Ash and Mason, 1983) have been utilised to study the role of gut cells in the uptake and processing of antigens. The rationale for the use of *in vitro* models for the analysis of intact antigen uptake and processing in the present study was based on piscine studies which have indicated the retention of functional activity by isolated intestinal cells (Ash and Mason, 1983; Davidson, 1991). Mammalian studies using *in vitro* methods have revealed a role for intestinal enterocytes in antigen processing and presentation to immunologically responsive cells in the gut (section 2.5). Antigen presentation by intestinal epithelial cells can lead to selective induction of suppressor T cells which may play a role in the induction of immune hyporesponsiveness to some orally delivered antigens (Bland and Warren, 1986). In addition, evidence points to intimate interactions between enterocytes and lymphocytes in the follicle associated epithelium of mammals and the possibility exists that intraepithelial lymphocytes modify the normal process of enterocyte

development in their immediate vicinity (Cremaschi *et al.*, 1989). It has been hypothesised that heterogeneity in the structure of the epithelial brush border in mammals could arise as a result of the transformation of mature enterocytes by lymphocytes (Bhalla and Owen, 1982; McKay and Perdue, 1993). An understanding of these interactions may be of importance in elucidating the nature of antigen uptake and immune responses in the gut.

The present investigation used an *in vitro* system in order to study the processing of soluble protein antigen by isolated intestinal cells from rainbow trout and to help determine the origin of antigen fragments detected in the trout circulation after *in vivo* antigen administration. The method for the isolation of rainbow trout intestinal cells was that described by Davidson (1991). It was hoped to formulate a system which reflects *in vivo* antigen processing by teleost intestinal cells and which could be applied as a fundamental step in the analysis of macromolecular uptake and cellular interactions in the teleost gut. This system was used to compare antigen processing by cells isolated from different regions of the rainbow trout gut and to investigate the nature of the enzymes involved in cellular processing of soluble protein antigen.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Fish were maintained as described in section 3.2.1.

4.2.2 Isolation and preparation of intestinal tissue.

Fish were killed by a sharp blow to the head and the digestive tract from the pyloric caecae to the rectum was excised (unless otherwise stated). This tissue was placed in an ice cold petri dish with 0.15 M PBS, pH 7.2 and any adherent connective and vascular tissue was dissected away. A longitudinal cut was made and luminal contents were removed by rinsing thoroughly with PBS. Tissue was then cut into 0.5 cm² sections.

4.2.3 Isolation of intestinal cells.

(A) EDTA - chelation method

This procedure was performed as described by Ray *et al.* (1990). Tissue sections were incubated in EDTA buffer (2 ml buffer per piece of tissue) for 5 minutes at 37°C with gentle agitation and immediately transferred into ice cold Eagles minimal essential medium (MEM) (Gibco, Paisley, Scotland). Epithelial tissue was released using a wide bore Pasteur pipette and the resultant suspension was centrifuged at 100 x g for 1 min, washed 3 times in MEM and resuspended in fresh MEM + 0.5 % D - mannose.

(B) Isolation by the collagenase digestion method (after Davidson, 1991).

Tissue pieces in 25 ml PBS, pH 7.2 containing 0.37 mg ml⁻¹ EDTA and 0.145 mg ml⁻¹ dithiothreitol to prevent cell aggregation were incubated at 18°C in a shaking water bath for 10 - 15 min. The supernatant was discarded and tissue pieces were removed and washed in Leibovitz L-15 medium containing 50 mM L - glutamine, 100 units ml⁻¹ penicillin, 0.1 mg ml⁻¹ deoxyribonuclease and 100 µg ml⁻¹ streptomycin adjusted to pH 7.2. The tissue was then incubated for 2 hours at 18°C in this medium + 0.15 mg ml⁻¹ collagenase with shaking, pressed through a 100 µm nylon gauze using a spatula and flushed through with the supernatant and medium into a petri dish on ice. The resultant suspension was washed 3 times by centrifugation, resuspended in a 5 ml tissue culture medium and refiltered through a 1 mm gauze.

4.2.4 Assessment of cell viability.

Cells were counted using a haemocytometer and the viability established by trypan blue exclusion (Appendix D).

4.2.5 Microscopic analysis of intestinal cells.

A sample of the cell suspension produced by collagenase digestion was centrifuged at 500 x g for 5 min. The resultant pellet was fixed overnight in 4% glutaraldehyde, dehydrated through a series of alcohols, post - fixed in 1 % osmium tetroxide and embedded in Spurr's resin. Sections (100 nm) were stained with uranyl acetate and lead citrate and observed under a JEOL transmission electron microscope.

4.2.6 Determination of protein concentration.

Cells isolated by collagenase digestion were counted and lysed by sonication on a 50% cycle with 15 second bursts (Heat Systems Ultrasonics, Farmingdale, New York) for 5 min. Protein concentration in these samples was determined as outlined in section 3.2.3.

4.2.7 Analysis of antigen processing by intestinal cells.

Cells isolated by collagenase digestion were incubated in L-15 medium containing either bovine serum albumin (BSA) or human gamma globulin (HGG) at 10 mg ml⁻¹ for a period of 1 min to 2 hr. After incubation, cells were immediately washed twice in L-15 medium and resuspended in 0.5 ml culture medium. Supernatants were retained and both cells and supernatants were snap frozen in liquid nitrogen and stored in 0.5 ml aliquots until required. Cells were lysed by incubating 1.5 ml of cell suspension with a lysis buffer (1 mM EDTA, 1% Tween-20, 1% SDS, 1 mM PMSF) at 0°C for 30 min (Bland and Whiting, 1990).

4.2.8 Analysis of the influence of specific inhibitors on antigen processing.

The substances at the concentrations outlined in Table 4.1 were added to cells for 30 min prior to incubation with antigens for 1 hr.

4.2.9 Analysis of antigen processing by cells from individual regions of the gut.

For this investigation the entire digestive tract from the oesophagus to the rectum was dissected from fish. The gut was divided into 5 regions; stomach, pyloric caeca, anterior intestine, mid intestine and posterior intestine. From each region, cells were isolated by collagenase digestion (section 4.2.3) and an equal number of viable cells from each region (10^6) incubated with either HGG or BSA (10 mg ml^{-1}) for 1 hour and snap frozen in liquid nitrogen.

4.2.10 SDS-PAGE, Western blotting and densitometry.

Samples derived from the various experiments described above were electrophoresed under both reducing and non-reducing conditions, electrotransferred to nitrocellulose sheets, immunoblotted and analysed by laser densitometry as described in sections 3.2.9, 3.2.12 and 3.2.13. Densitometric analysis was performed three times and the standard deviations and standard errors of means were calculated.

Table. 4.1 : Substances used in analysis of antigen processing by isolated intestinal cells.

Substance used	Mode of action	Reference
Ammonium chloride	Increases lysosomal pH, inhibits pinocytosis, non specific immunostimulator	Lindmark <i>et al.</i> , 1994.
Cimetidine	Inhibits gastric acid secretion by binding H2 receptors, also has immunological effects	Plouffe <i>et al.</i> , 1986.
Pepstatin	Aspartic protease inhibitor. Inhibits lysosomal cathepsins.	Umezawa <i>et al.</i> , 1976
Leupeptin	Serine protease inhibitor. Inhibits lysosomal cathepsins.	Umezawa, 1976
Quil - A	Protease inhibitor.	Ishaya and Birk, 1965.

4.3 RESULTS

4.3.1 *Viability of isolated intestinal cells.*

Immediately after isolation up to 75% of intestinal cells were found to be viable. A slow but progressive decline in cell viability with time was subsequently noted. However, even after 2 hr 60% of the cells remained viable (Fig 4.1).

4.3.2 *Intracellular processing of soluble protein antigens by isolated intestinal cells*

HGG detected in cell lysates was largely intact up to 15 min post-incubation. From 15-30 min after incubation considerable fragmentation was noted, amounting to a 14.3% reduction in the relative percentage of HGG after 30 min but at subsequent time points little breakdown of HGG was found to have occurred (Figs 4.2 and 4.14(a)). Higher levels of the 10.6 and 17.7 kDa fragments were found in lysates after a 15 min incubation than at all other times. In contrast, BSA was fragmented to a greater extent, amounting to a 31.5% reduction in the relative percentage of the intact antigen following a 1 min incubation and the fragmentation pattern did not change greatly over a subsequent 2 hr period (Fig 4.3).

4.3.3 *Analysis of supernatants from cells after incubation with HGG or BSA.*

Under non-reducing conditions, the HGG detected in supernatants appeared to be largely intact.

However, when samples were reduced prior to electrophoresis a modification of the antigen was apparent (Fig 4.4). This was reflected in changes in the relative percentages of the 37.1 and 26.5 kDa fragments present over the time course of incubation. BSA detected in supernatants was more substantially fragmented, the effect being apparent as a 29% decrease (at 2 hr) in the relative percentage of intact antigen present, accompanied by an increase in the relative percentages of the 32.2 and 44.4 kDa fragments present (Fig 4.5). The relative percentage of the 20.5 kDa fragment however declined from 1 min to 2 hr.

4.3.4 *Comparative analysis of intracellular processing of BSA and HGG by cells isolated from various gut regions.*

When electrophoresed under non-reducing conditions, HGG appeared intact (results not presented). However under reducing conditions differences in the relative percentages of the 118.5, 55.8 and 27.2 kDa fragments produced in cells isolated from individual regions of the gut were apparent (Fig 4.6). Intracellular processing of BSA was also highly variable depending on the origin of the cells. Fragmentation of this protein was most extensive in cells isolated from the pyloric caecae (85.5% reduction in the relative % of intact HGG) and mid-intestinal region while little fragmentation was apparent in BSA detected after incubation with cells isolated from the gastric and anterior intestinal regions (Figs 4.7 and 4.14(b)). A greater amount of BSA appeared to be absorbed by stomach cells than by cells isolated from other gut regions and this antigen appeared to be intact (Fig.4.13(b)).

4.3.5 Comparative analysis of supernatants from cells isolated from various gut regions after incubation with BSA or HGG.

Differences were found in the relative percentages of the 71.1, 55.2 and 25.4 kDa HGG fragments detected in supernatants from cells isolated from different gut regions when electrophoresed under reducing conditions (Fig 4.8) while under non-reducing conditions there was little evidence of processing (results not presented). Fragmentation of BSA was also noted in supernatants after incubation of antigen with cells from all regions of the gut. Processing was least extensive in supernatants from cells isolated from the posterior intestinal region (Figs 4.9 and 4.14(c)).

4.3.6 Analysis of the effects of a range of specific inhibitors on intracellular processing of BSA and HGG.

Fragmentation of HGG was not apparent in samples when electrophoresed under non-reducing conditions. After the treatment of cells with specific inhibitors differences in the relative percentages of the 122.5, 79.9 and 52.4 kDa fragments in lysates were found when samples were electrophoresed under reducing conditions (Fig 4.10). Intracellular processing of BSA was partially inhibited by cimetidine and pepstatin and was completely abrogated by leupeptin (Fig 4.11) resulting in the detection only of apparently intact antigen. The reduction in the extent of

degradation after treatment with inhibitors was most apparent as a reduction in the relative percentages of the 22.4 and 29.7 kDa fragments.

4.3.7 Analysis of the effects of a range of potential inhibitors on the nature of BSA and HGG detected in supernatants.

Under non-reducing electrophoresis conditions differences were found in the relative percentages of the 37.5, 31.6 and 12 kDa HGG fragments present in supernatants from cells previously treated with inhibitors (Fig 4.12). Leupeptin, pepstatin and Quil-A appeared to limit processing to the greatest extent, leading to a reduction in the relative percentages of the 37.5 and 12 kDa fragments. Leupeptin had the greatest effect in reducing the extent of fragmentation of BSA, leading to a reduction in the relative percentages of the 18.7 and 25.2 kDa BSA fragments to control levels. Quil - A saponin and pepstatin also reduced degradation of BSA in supernatants but to a lesser extent (Fig 4.13).

Fig. 4.1: Influence of time on viability of intestinal cells isolated by collagenase digestion.

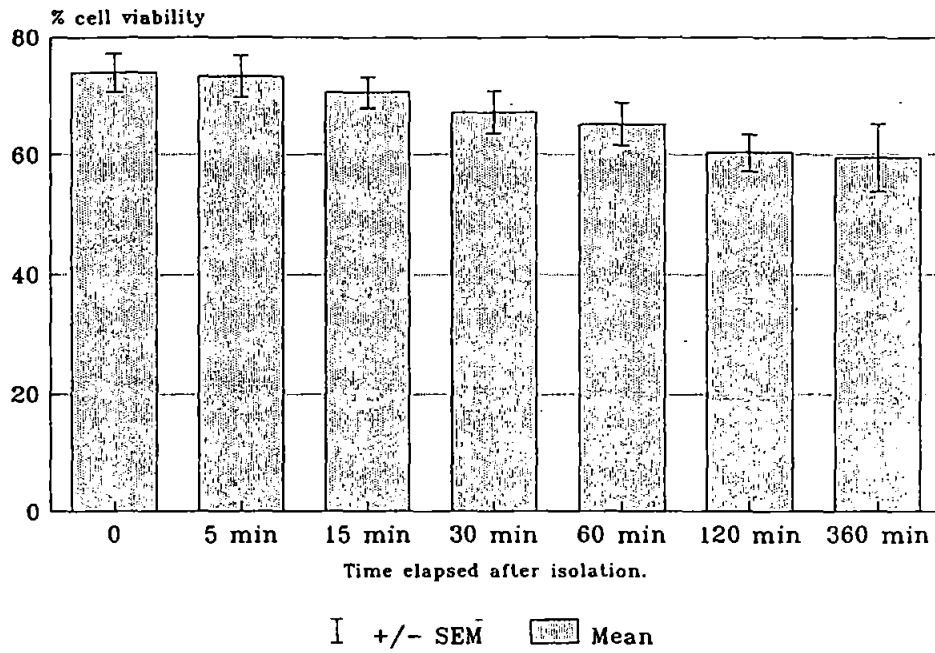


Fig. 4.1: Relationship between cell viability of intestinal cells isolated by collagenase digestion in Leibovitz L-15 medium and time after isolation.

Fig. 4.2: *Time course of intracellular processing of HGG by isolated intestinal cells. Analysis was carried out on non-reduced protein samples.*

Fig. 4.2(a): *Processing represented in terms of the relative % of intact HGG present. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 4.2(b): *Processing represented in terms of the relative % of discrete fragments present. Data derived from laser scans of individual bands on immunoblot.*

Fig.4.2(a): Time course of intracellular processing of HGG

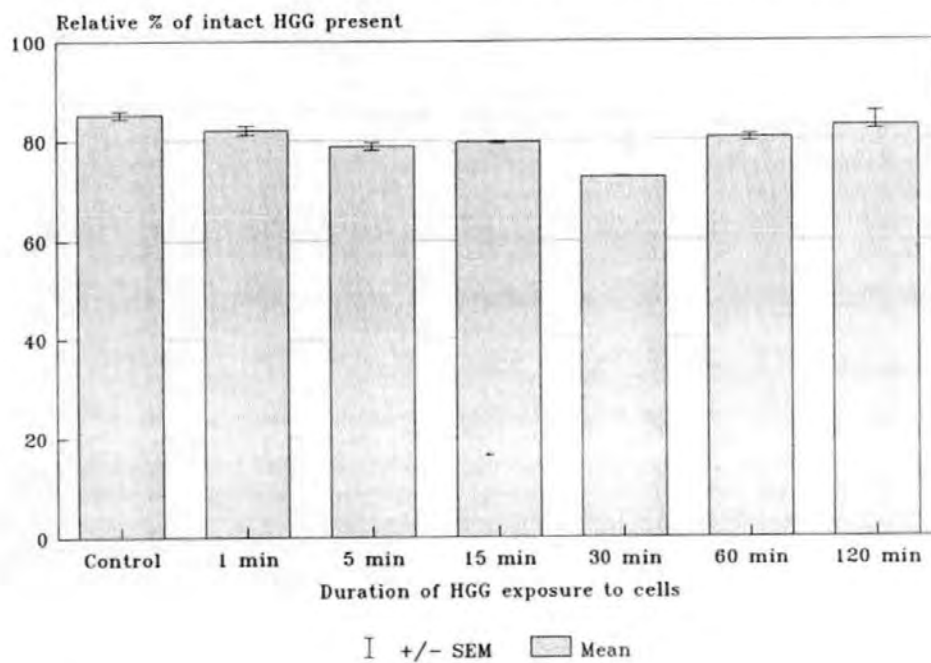


Fig. 4.2(b): Time course of intracellular processing of HGG.

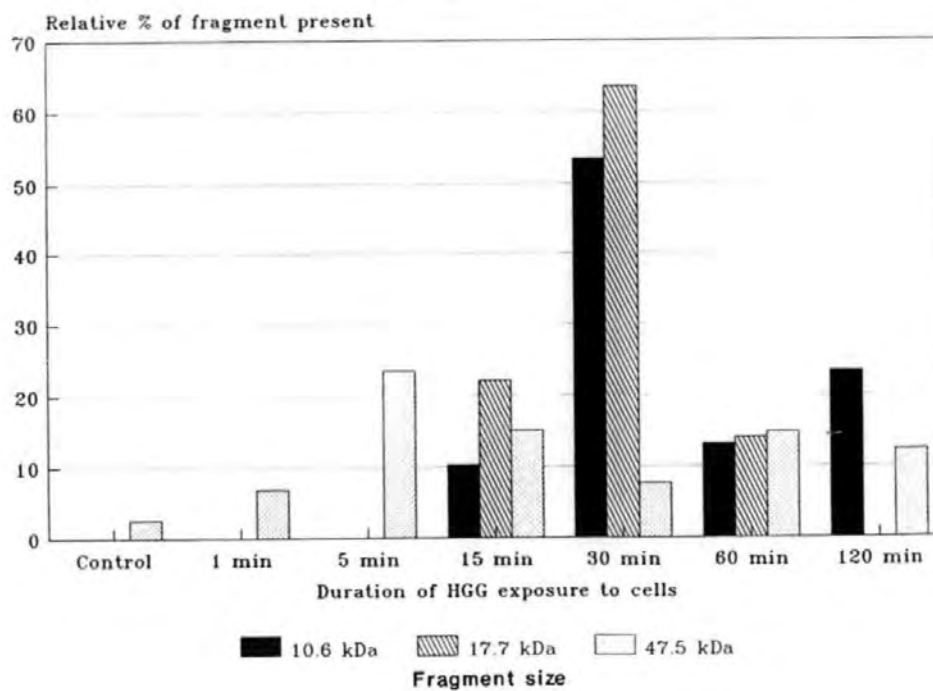


Fig. 4.3: *Time course of intracellular processing of BSA by isolated intestinal cells. Analysis was carried out on non-reduced protein samples.*

Fig. 4.3(a): *Processing represented in terms of the relative % of intact BSA present. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 4.3(b): *Processing represented in terms of the relative % of individual BSA fragments present. Data derived from laser scans of individual bands on immunoblot.*

Fig. 4.3(a): Time course of intracellular processing of BSA.

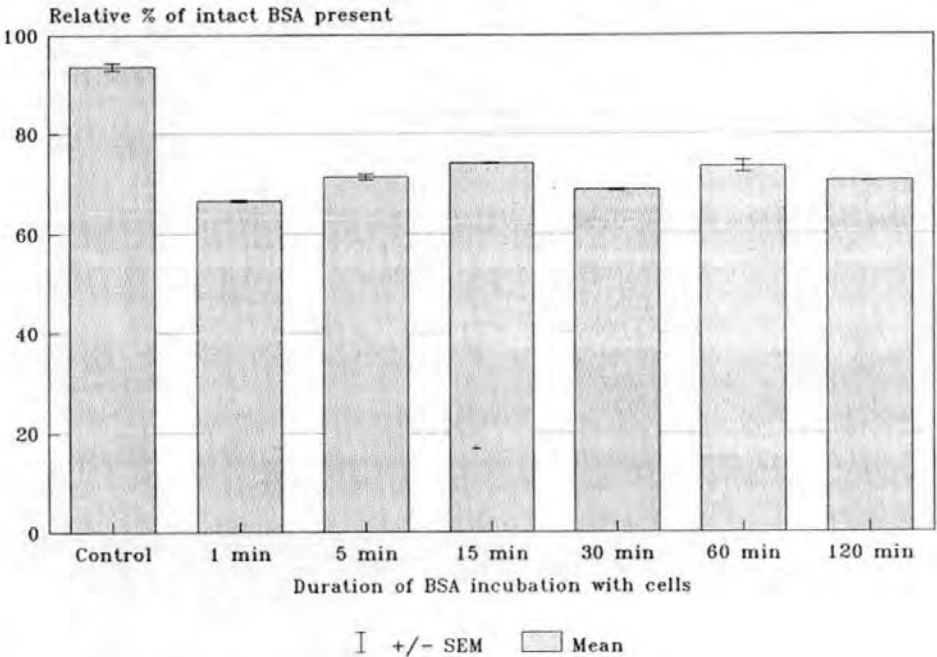


Fig. 4.3(b): Time course of intracellular processing of BSA.

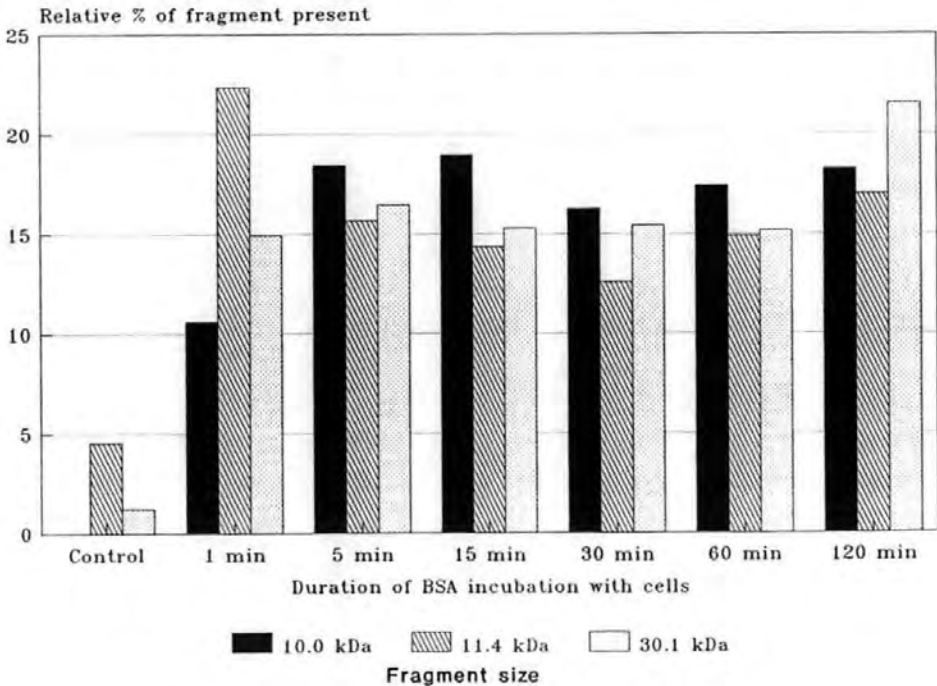


Fig. 4.4: *Analysis of supernatants from cells incubated with HGG over a 2hr time course. Analysis was carried out on reduced protein samples.*

Fig. 4.4(a): *Processing represented in terms of the relative % of the 56.6 kDa fragment present. Data derived from laser scans of entire lanes of immunoblots.*

Fig. 4.4(b): *Processing represented in terms of the relative % of the 37.1 kDa fragment present. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 4.4(c): *Processing represented in terms of the relative % of the 26.5 kDa fragment present. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 4.4(a): Analysis of supernatants from intestinal cells incubated with HGG over 2 hours

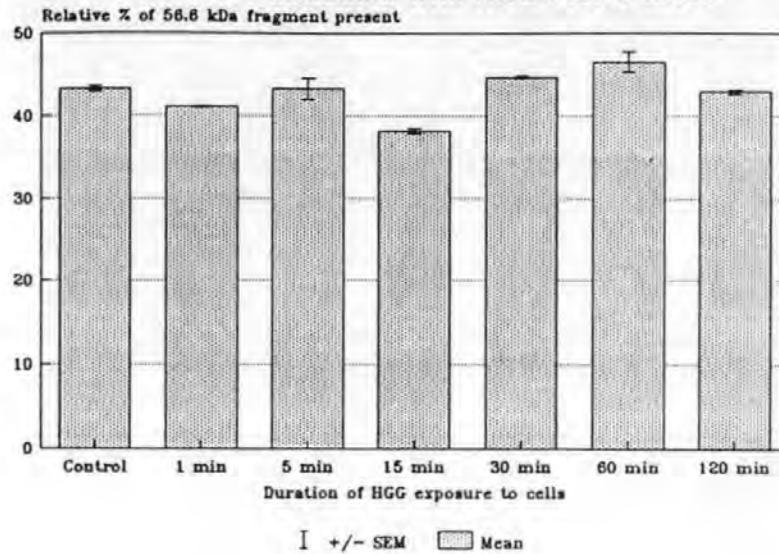


Fig. 4.4(b): Analysis of supernatants from intestinal cells incubated with HGG over 2 hours.

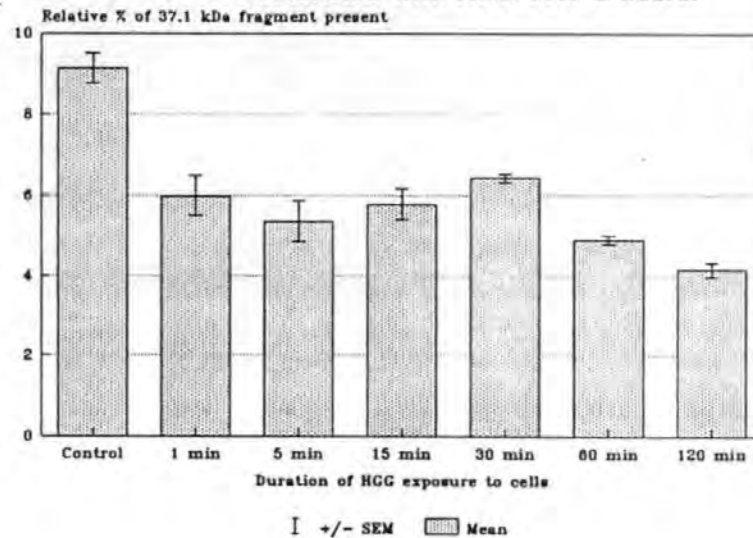


Fig. 4.4(c): Analysis of supernatants from intestinal cells incubated with HGG over 2 hours.

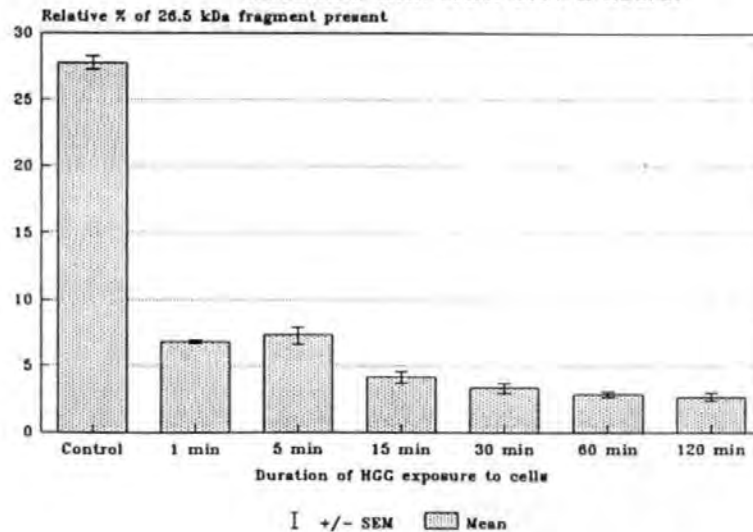


Fig. 4.5: *Analysis of supernatants from cells incubated with BSA over a 2hr time course. Analysis was carried out on non-reduced protein samples.*

Fig. 4.5(a): *Processing represented in terms of the relative % of intact BSA present. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 4.5(b): *Processing represented in terms of the relative % of individual BSA fragments present. Data derived from laser scans of individual bands on immunoblot.*

Fig. 4.5(a): Analysis of supernatants from cells incubated with BSA over 2 hours

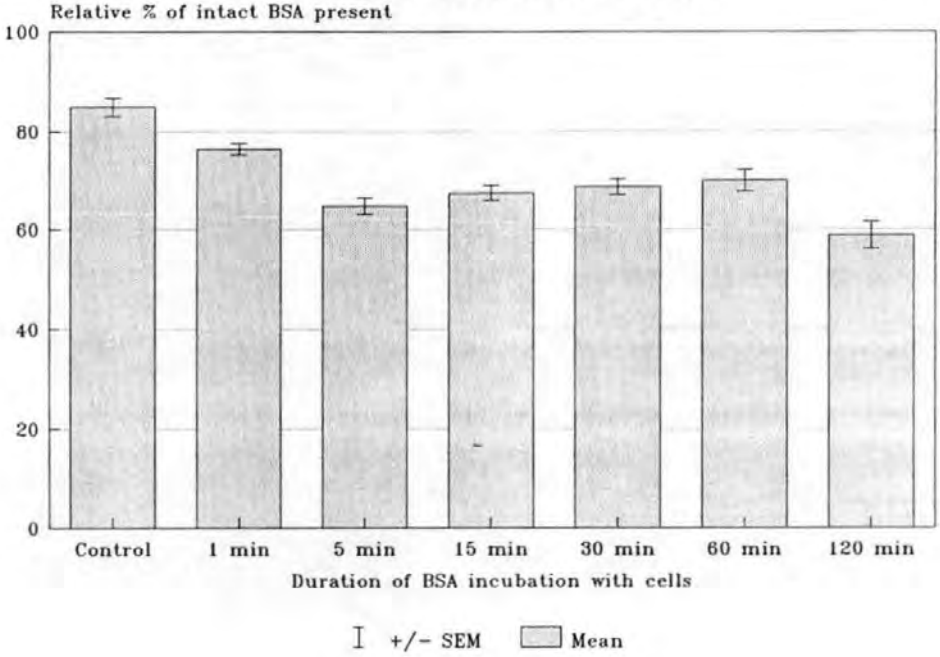


Fig. 4.5(b): Analysis of supernatants from cells incubated with BSA over 2 hours

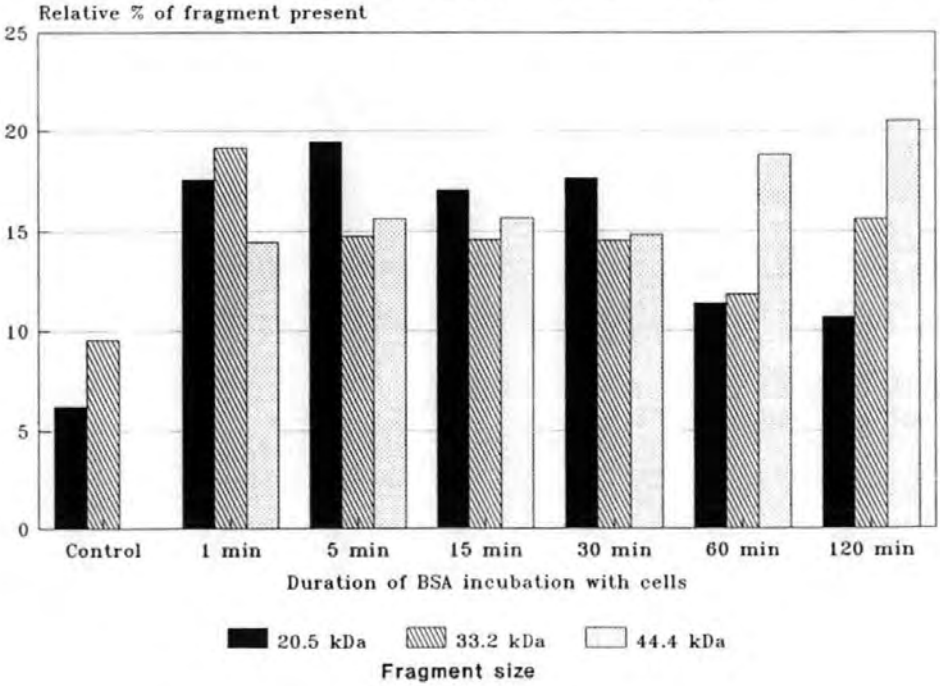


Fig. 4.6: *Intracellular processing of HGG by cells isolated from various gut regions. Incubations were terminated after 1 hr in each case and analysis was carried out on reduced protein samples.*

Fig. 4.6(a): *Processing represented in terms of the relative % of the 118.5 kDa band present. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 4.6(b): *Processing represented in terms of the relative % of the 55.8 kDa band present. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 4.6(c): *Processing represented in terms of the relative % of the 27.2 kDa band present. Data derived from laser scans of entire lanes of immunoblot.*

Key: *Pyl cae* = Pyloric caecae.

Ant.int = Anterior intestine.

Mid.int = Mid intestine.

Post.int = Posterior intestine.

Fig. 4.6(a): Intracellular processing of HGG by cells isolated from various gut regions

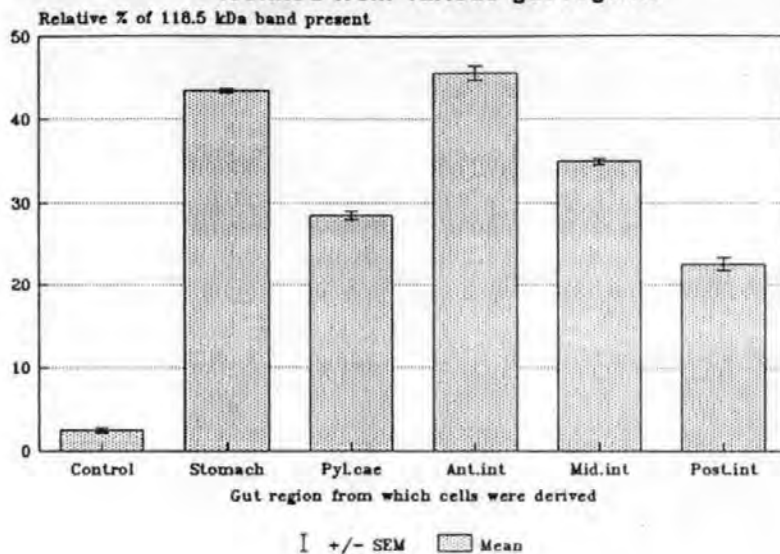


Fig. 4.6(b): Intracellular processing of HGG by cells isolated from various gut regions

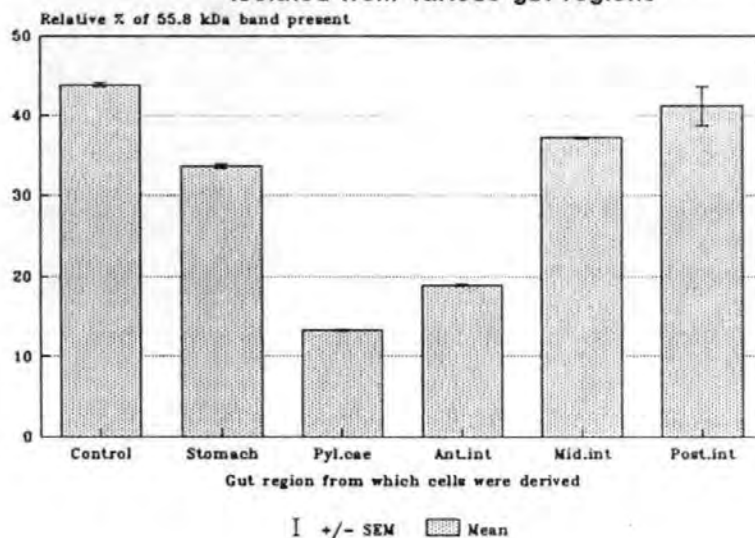


Fig. 4.6(c): Intracellular processing of HGG by cells isolated from various gut regions

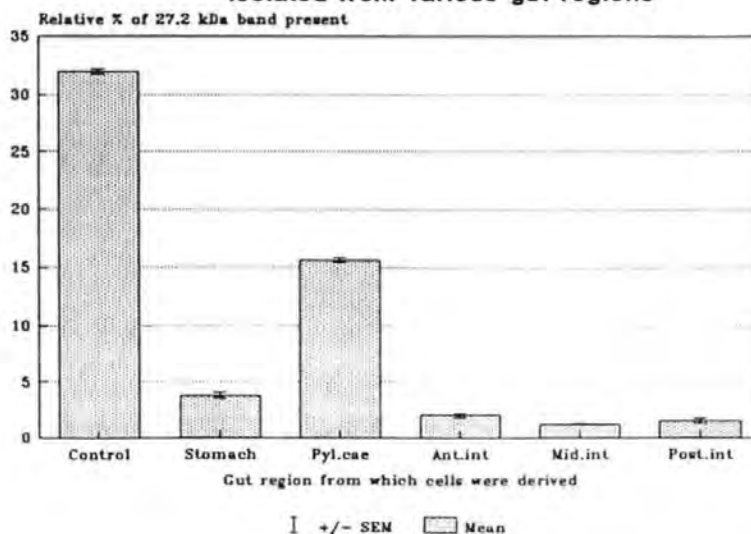


Fig. 4.7: *Intracellular processing of BSA by cells isolated from various gut regions. Incubations were terminated after 1 hr in each case and analysis carried out on non-reduced protein samples.*

Fig. 4.7(a): *Processing represented in terms of the relative % of intact BSA present. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 4.7(b): *Processing represented in terms of the relative % of individual BSA fragments present. Data derived from laser scans of individual bands of immunoblot.*

Key: *Pyl cae = Pyloric caecae.*

Ant.int = Anterior intestine.

Mid.int = Mid intestine.

Post.int = Posterior intestine.

Fig.4.7(a): Intracellular processing of BSA by cells isolated from various gut regions

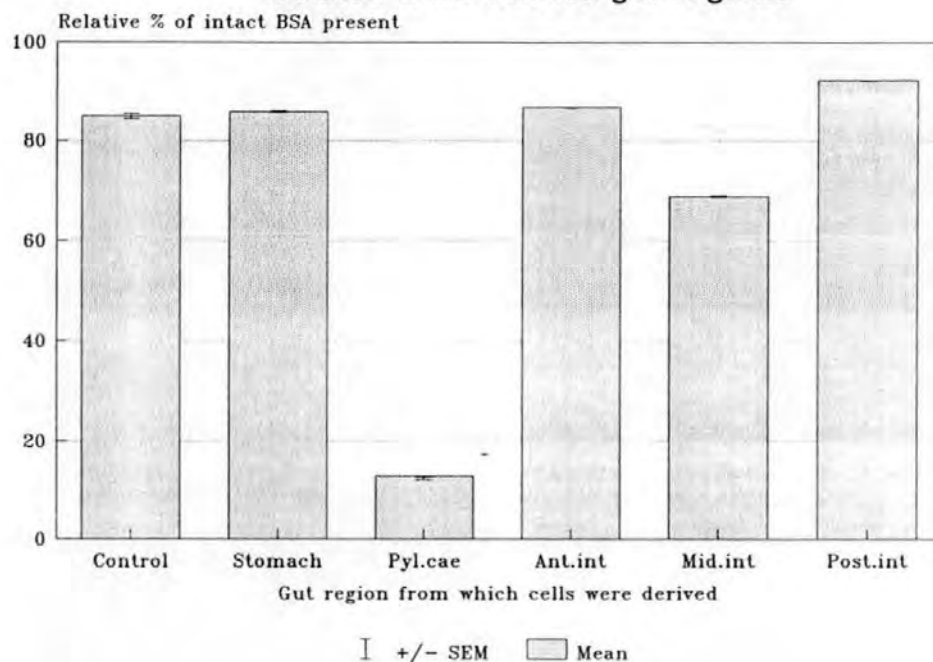


Fig.4.7(b): Intracellular processing of BSA by cells isolated from various gut regions

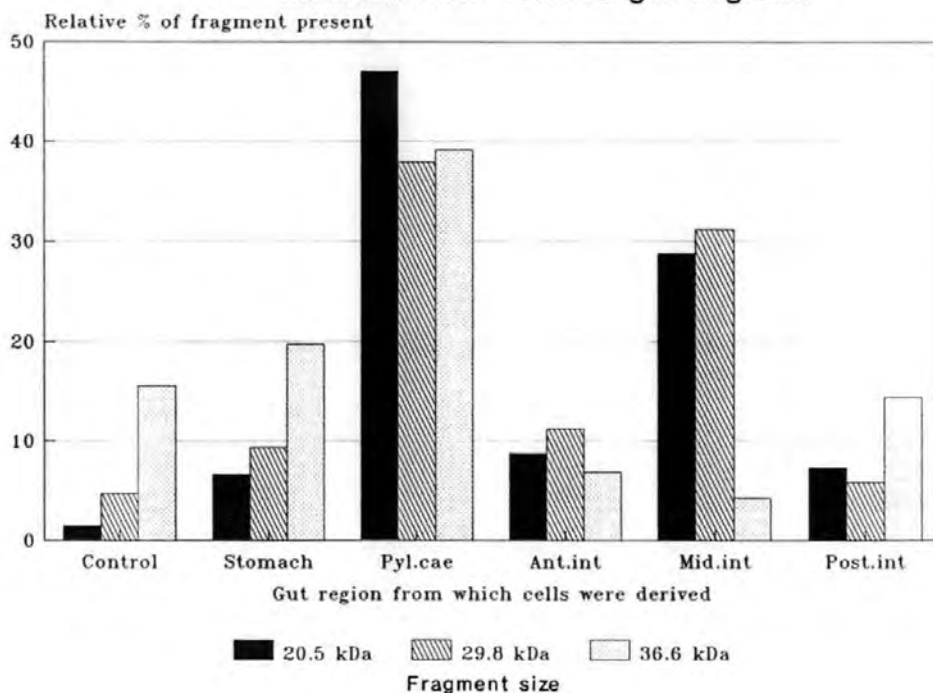


Fig. 4.8: *Analysis of supernatants from cells isolated from various gut regions after a 1hr incubation with HGG. Analysis was carried out on reduced protein samples.*

Fig. 4.8(a): *Processing represented in terms of the relative % of the 71.1 kDa fragment present. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 4.8(b): *Processing represented in terms of the relative % of the 55.2 kDa fragment present. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 4.8(c): *Processing represented in terms of the relative % of the 25.4 kDa fragment present. Data derived from laser scans of entire lanes of immunoblot.*

Key: *Pyl cae = Pyloric caecae.*

Ant.int = Anterior intestine.

Mid.int = Mid intestine.

Post.int = Posterior intestine.

Fig. 4.8(a): Analysis of supernatants from cells isolated from various gut regions after incubation with HGG.

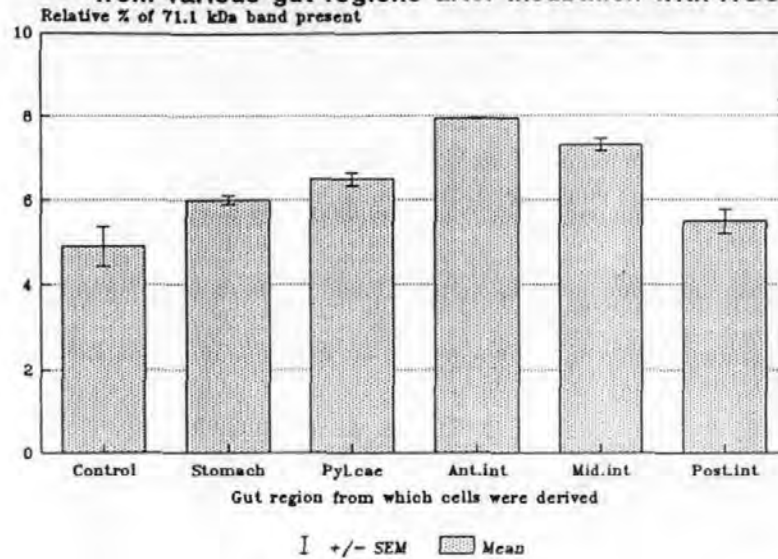


Fig. 4.8(b): Analysis of supernatants from cells isolated from various gut regions after incubation with HGG

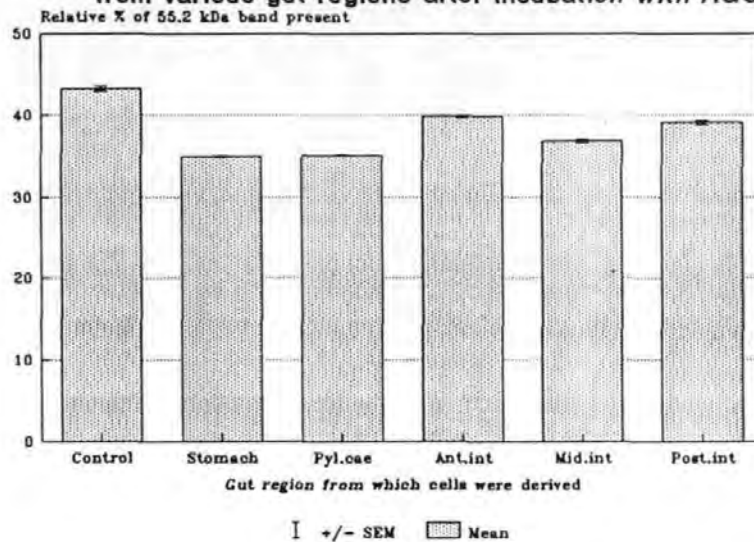


Fig. 4.8(c): Analysis of supernatants from cells isolated from various gut regions after incubation with HGG

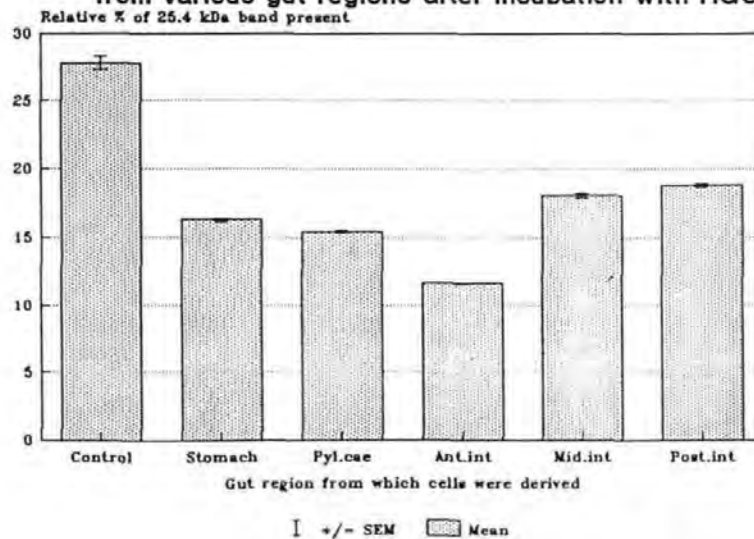


Fig. 4.9: Analysis of supernatants from cells isolated from various gut regions after a 1hr incubation with BSA. Incubations were terminated after 1 hr in each case and analysis carried out on non-reduced protein samples.

Fig. 4.9(a): Processing represented in terms of the relative % of intact BSA present. Data derived from laser scans of entire lanes of immunoblot.

Fig. 4.9(b): Processing represented in terms of the relative % of individual BSA fragments present. Data derived from laser scans of individual bands on immunoblot.

Key: *Pyl cae* = Pyloric caecae.

Ant.int = Anterior intestine.

Mid.int = Mid intestine.

Post.int = Posterior intestine.

Fig.4.9(a): Analysis of supernatants from cells isolated from various gut regions after incubation with BSA

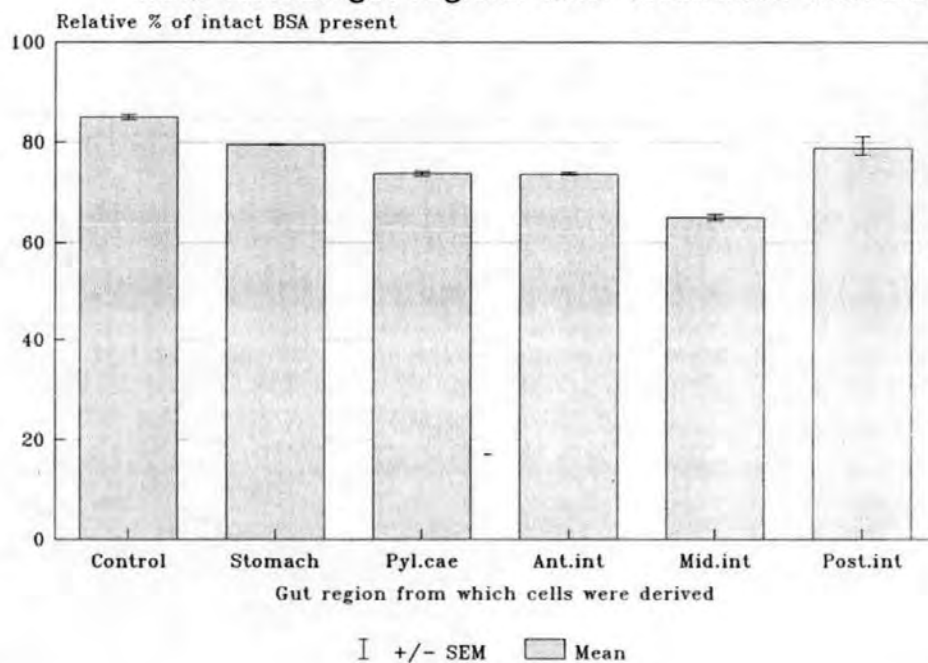


Fig. 4.9(b): Analysis of supernatants from cells isolated from various gut regions after incubation with BSA

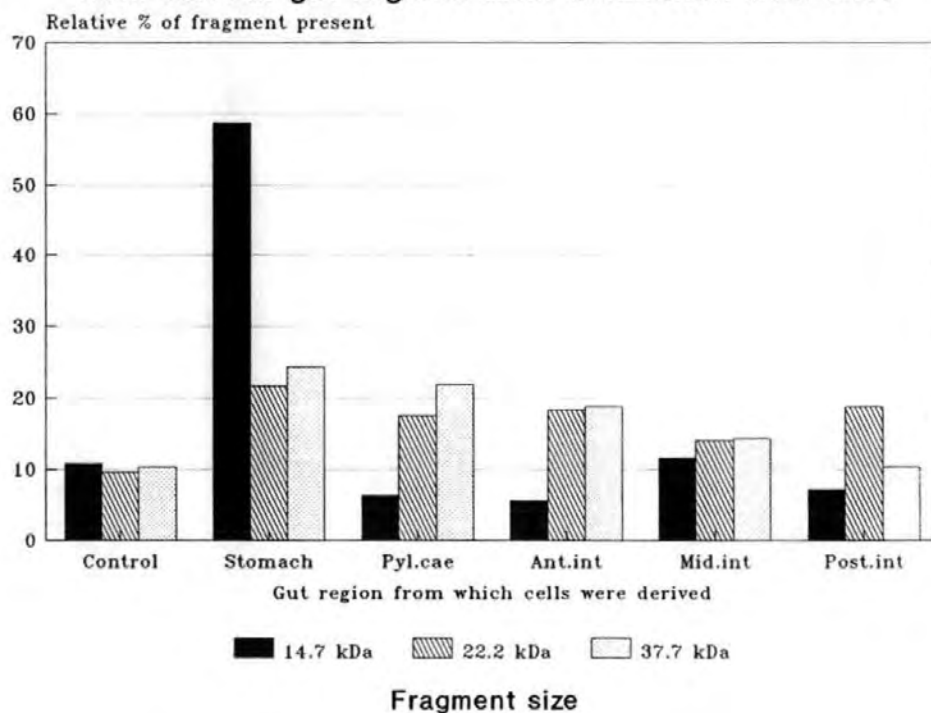


Fig. 4.10: *Influence of a range of inhibitors on intracellular processing of HGG by isolated intestinal cells. Reactions were allowed to proceed for 1 hr in each case and analysis carried out on reduced protein samples.*

Fig. 4.10(a): *Processing represented in terms of the relative % of the 122.5 kDa fragment present. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 4.10(b): *Processing represented in terms of the relative % of the 79.9 kDa fragment present. Data derived from laser scans of entire lanes of immunoblot.*

Fig 4.10(c): *Processing represented in terms of the relative % of the 52.4 kDa fragment present. Data derived from laser scans of entire lanes of immunoblot.*

Key : *AC = Ammonium chloride.*

Cim = Cimetidine.

Pep = Pepstatin.

QA = Quil-A.

Leu = Leupeptin.

Fig.4.10(a): Effect of potential inhibitors on intracellular processing of HGG

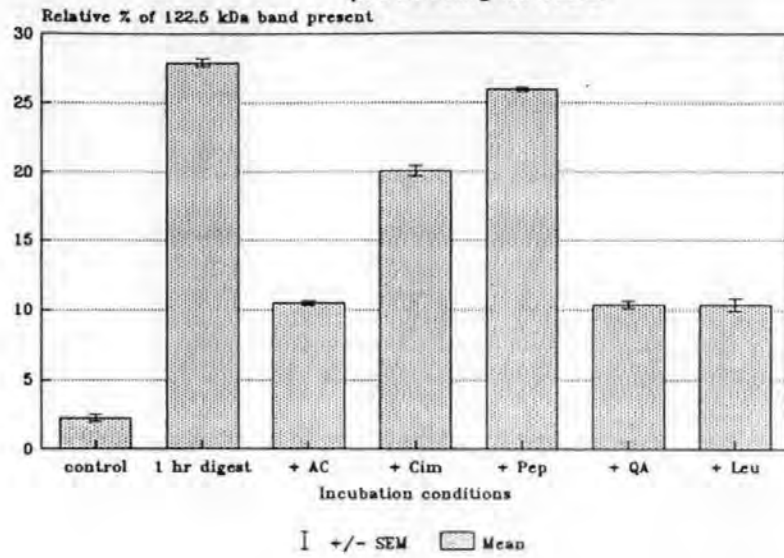


Fig.4.10(b): Effects of potential inhibitors on intracellular processing of HGG

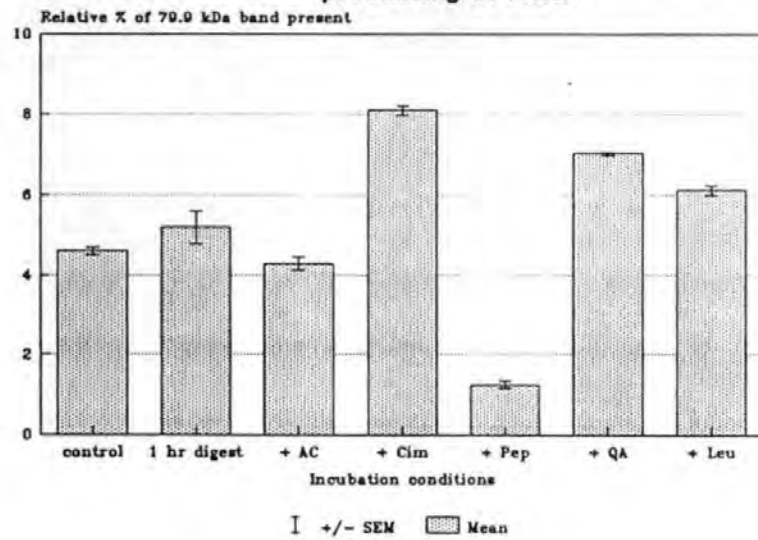


Fig.4.10(c): Effects of potential inhibitors on intracellular processing of HGG

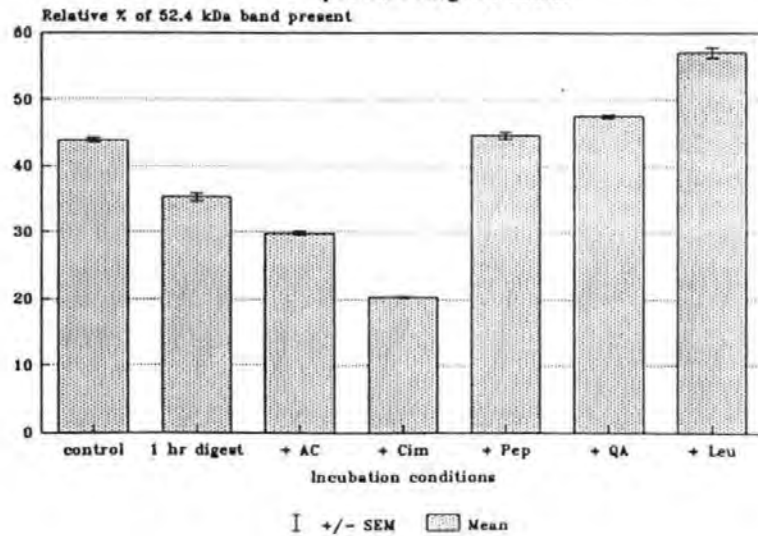


Fig. 4.11: *Influence of a range of inhibitors on intracellular processing of BSA by isolated intestinal cells. Reactions were terminated after 1 hr in each case and analysis carried out on non-reduced protein samples.*

Fig. 4.11(a): *Processing represented in terms of the relative % of intact BSA present. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 4.11(b): *Processing represented in terms of the relative % of discrete fragments present. Data derived from laser scans of individual bands on immunoblot.*

Key : *AC = Ammonium chloride.*

Cim = Cimetidine.

Pep = Pepstatin.

QA = Quil-A.

Leu = Leupeptin.

Fig.4.11(a):Effects of potential inhibitors on intracellular processing of BSA

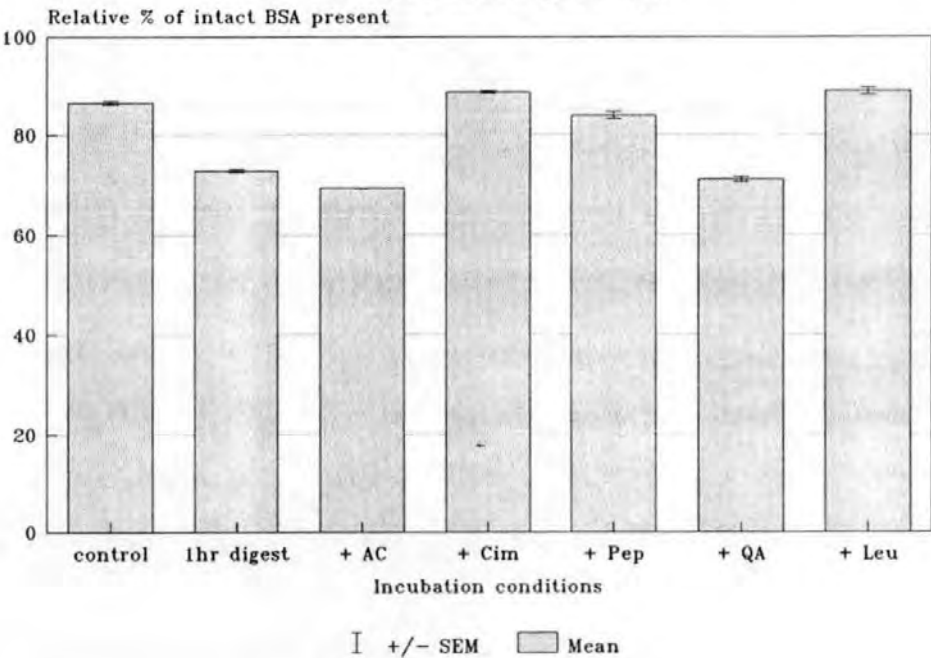


Fig.4.11(b):Effects of potential inhibitors on intracellular processing of BSA

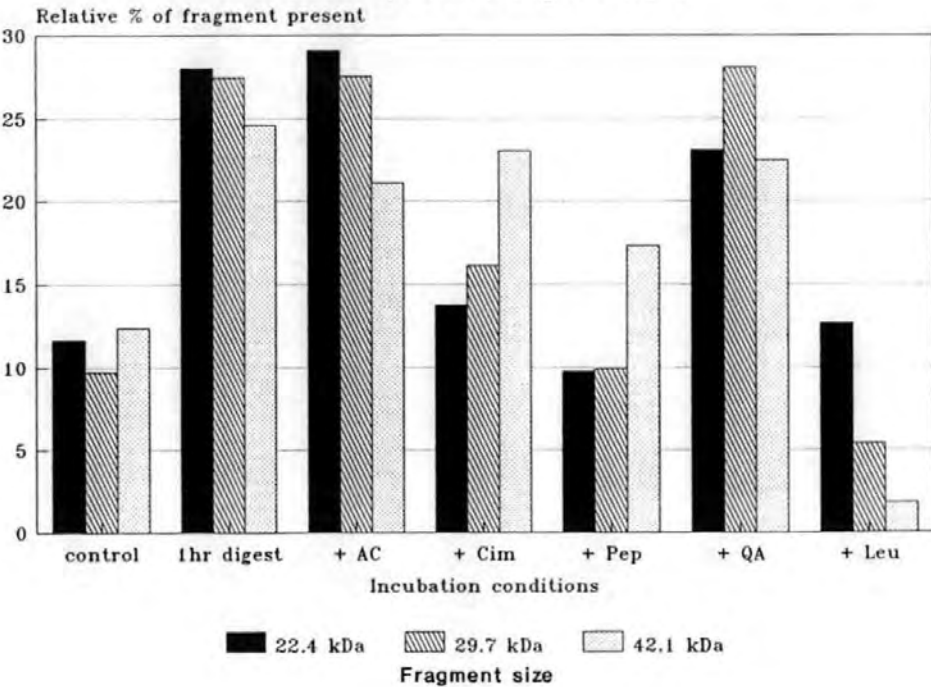


Fig. 4.12: *Influence of a range of inhibitors on the nature of HGG detected in supernatants from intestinal cells after incubation. Reactions were allowed to proceed for 1 hr in each case and analysis was carried out on non-reduced protein samples.*

Fig. 4.12(a): *Processing represented in terms of the relative % of intact HGG present. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 4.12(b): *Processing represented in terms of the relative % of discrete fragments present. Data derived from laser scans of entire lanes of immunoblot.*

Key : *AC = Ammonium chloride.*

Cim = Cimetidine.

Pep = Pepstatin.

QA = Quil-A

Leu = Leupeptin.

Fig.4.12(a):Effect of potential inhibitors on form of HGG detected in supernatants from cells after incubation

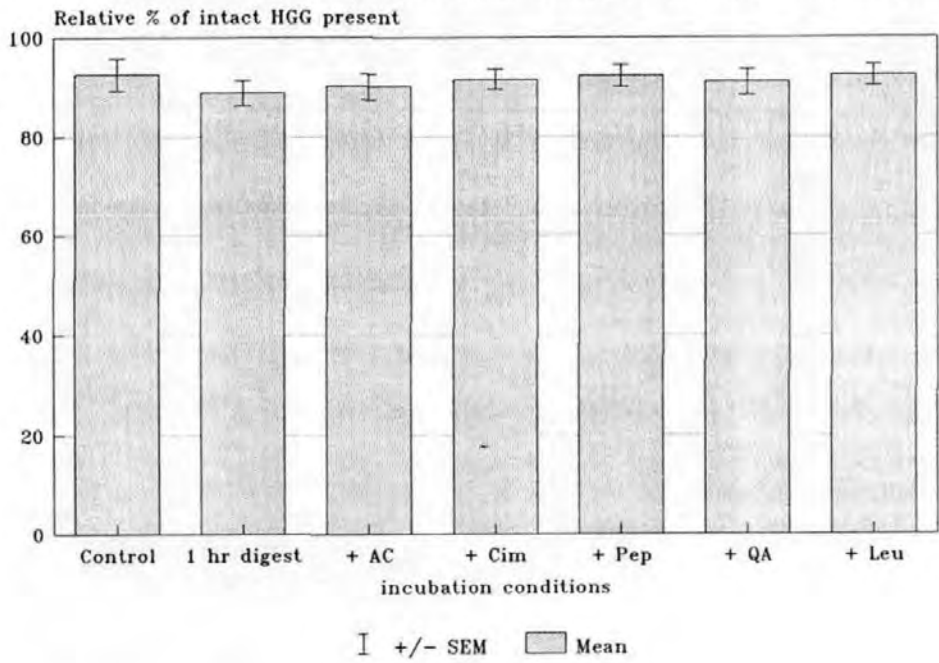


Fig.4.12(b):Effects of potential inhibitors on form of HGG detected in supernatants from cells after incubation

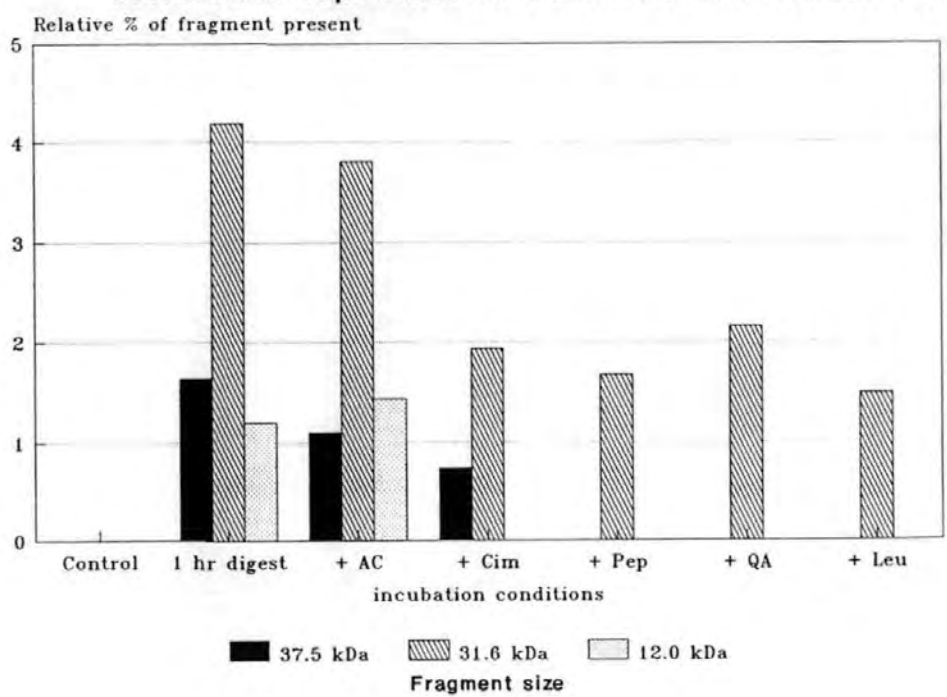


Fig. 4.13: *Influence of a range of inhibitors on the nature of BSA detected in supernatants from intestinal cells after incubation. Incubations were terminated after 1 hr in each case and analysis was carried out on non-reduced protein samples.*

Fig. 4.13(a): *Processing represented in terms of the relative % of intact BSA present. Data derived from laser scans of entire lanes of immunoblot.*

Fig. 4.13(b): *Processing represented in terms of the relative % of discrete fragments present. Data derived from laser scans of individual bands of immunoblot.*

Key : *AC = Ammonium chloride.*

Cim = Cimetidine.

Pep = Pepstatin.

QA = Quil-A.

Leu = Leupeptin.

Fig.4.13(a): Effects of potential inhibitors on form of BSA detected in supernatants from cells after incubation

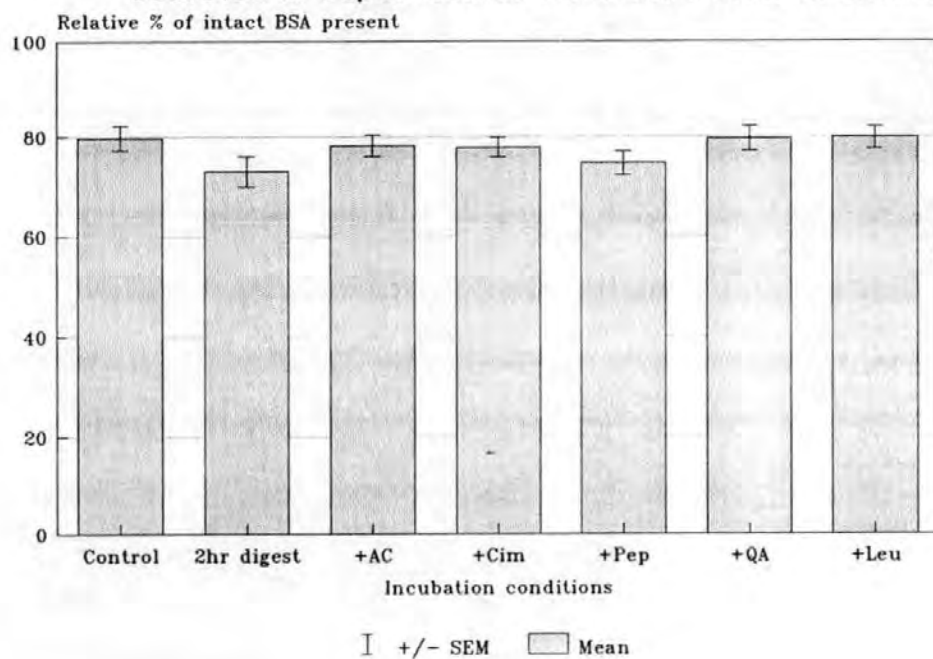


Fig.4.13(b): Effects of potential inhibitors on form of BSA detected in supernatants from cells after incubation

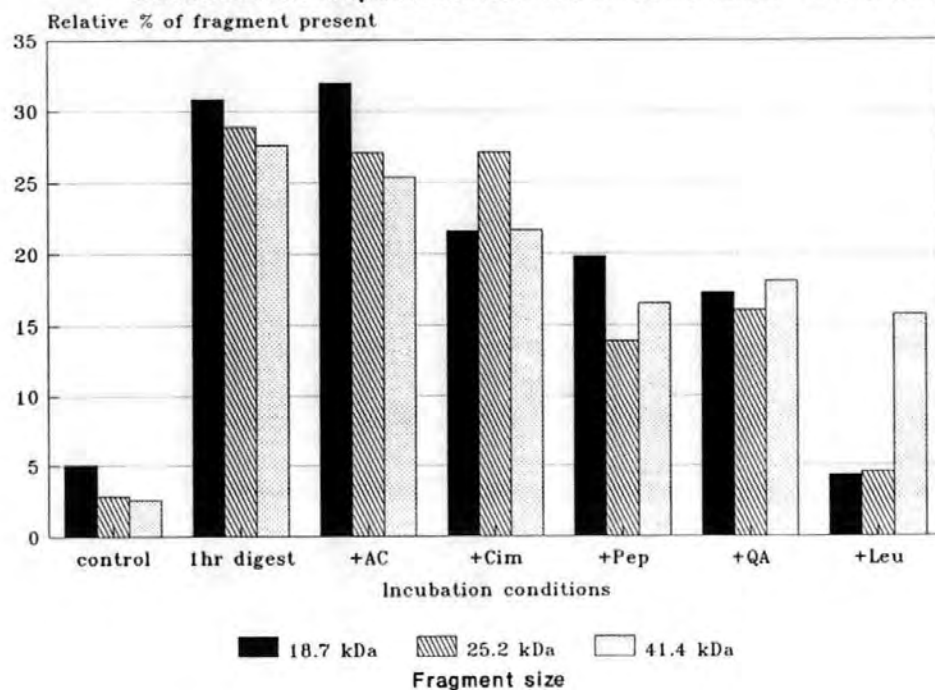
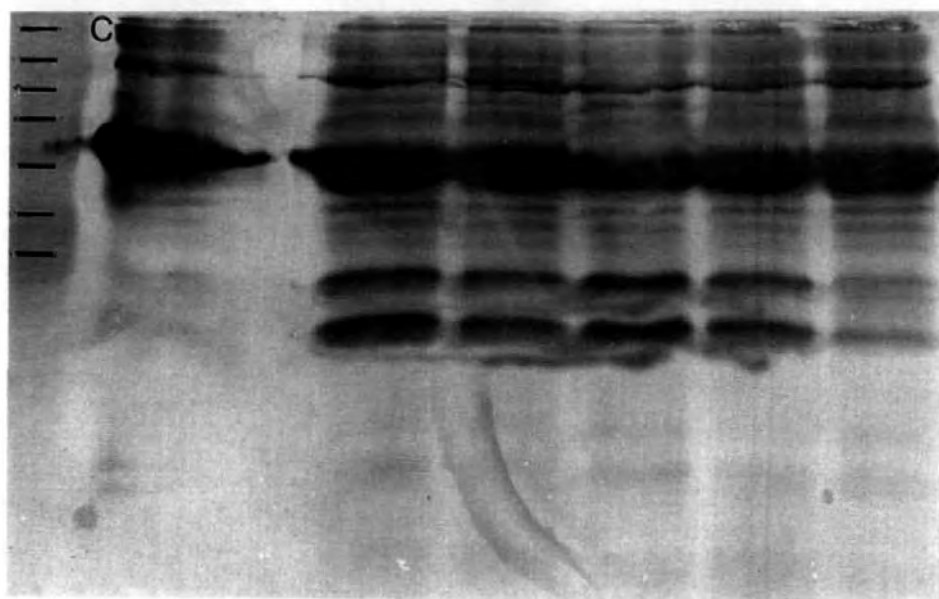
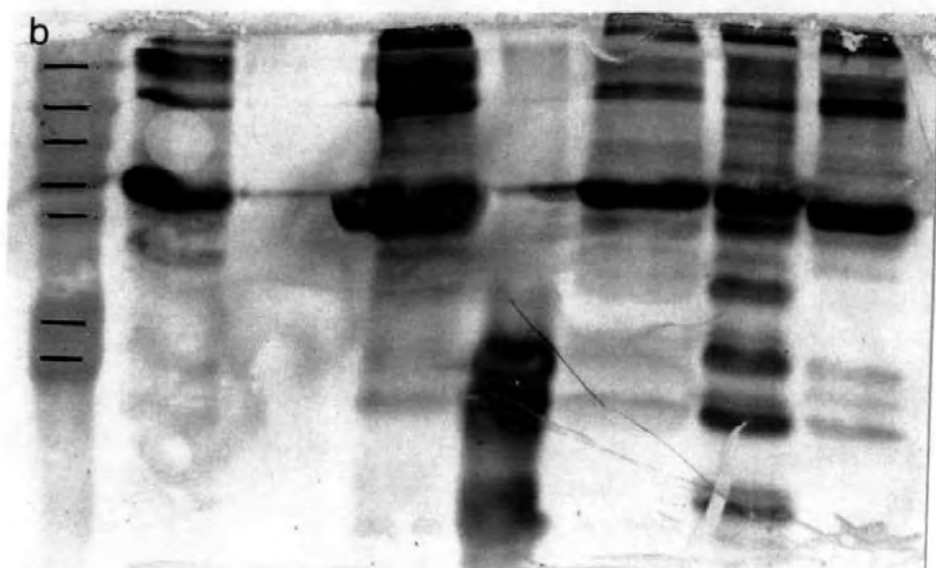
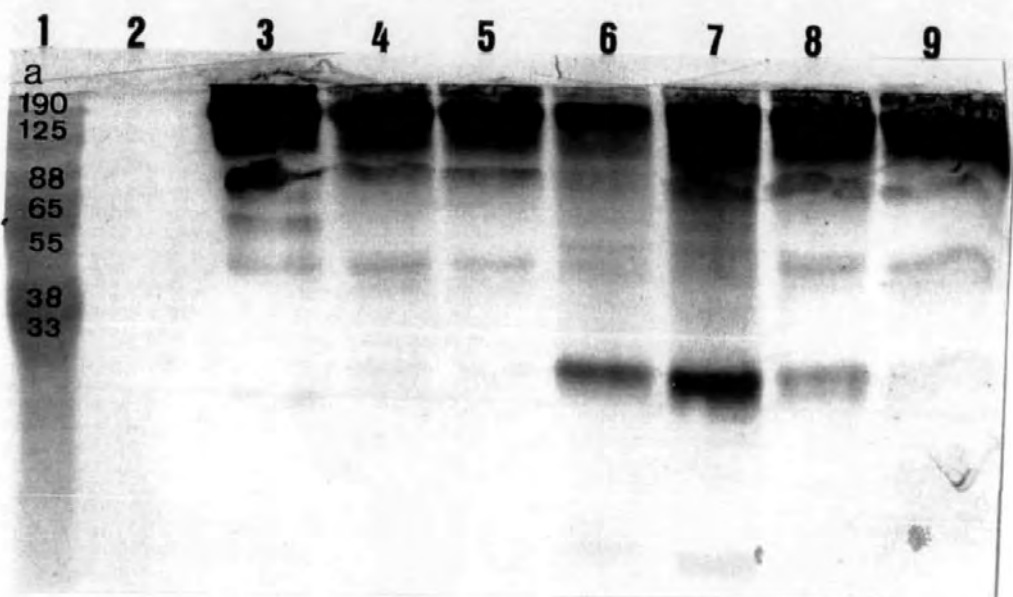


Fig. 4.14: Western blots showing processing of HGG and BSA by isolated intestinal cells. The molecular weights of the prestained markers are labelled in lane 1 of Fig 4.14(a) and the bars in lane 1 of Fig.4.14 (b&c) represent the same molecular weights.

Fig. 4.14(a): Time course of intracellular processing of HGG by isolated intestinal cells. Samples were electrophoresed under non-reducing conditions. Loading order: Lane 1, prestained molecular weight markers. Lane 2, negative control (cells only). Lane 3, positive control (HGG). Lanes 3 - 10, cell lysates from cells incubated with HGG for 1 min, 5 min, 15 min, 30 min, 60 min and 120 min respectively.

Fig. 4.14(b): Comparative analysis of intracellular processing of BSA by cells isolated from individual regions of the rainbow trout gut. Incubations were for 1hr in each case and samples were electrophoresed under non - reducing conditions. Loading order: Lane 1, prestained molecular weight markers. Lane 2, positive control (BSA). Lane 3, negative control (lysate from intestinal cells incubated in the absence of BSA). Lanes 4 - 8, cell lysates from cells isolated from gastric, pyloric caeca, anterior intestine, mid intestine and posterior intestinal regions respectively after a 1 hr incubation with BSA.

Fig. 4.14(c): Comparative analysis of supernatants after incubation of cells from individual gut regions with BSA. Incubations were for 1hr in each case and samples were electrophoresed under non - reducing conditions. Loading order: Lane 1, prestained molecular weight markers. Lane 2, positive control (BSA). Lane 3, negative control (lysate from intestinal cells incubated for 1 hr in the absence of BSA). Lanes 4 - 8, supernatants from cells isolated from gastric, pyloric caeca, anterior intestine, mid intestine and posterior intestinal regions respectively after incubation with BSA.



4.4 DISCUSSION

An *in vitro* system involving an isolated intestinal cell preparation was used to analyse the cellular processing of soluble proteins. The capacity of isolated intestinal cells to absorb BSA and HGG was clearly demonstrated and considerable differences were noted in the intracellular processing of the proteins. BSA was considerably degraded inside the cells while processing of HGG was less extensive. The rapid absorption of both BSA and HGG by trout gut cells in this study is in accordance with previous *in vivo* (Georgopoulou *et al.*, 1986; Jenkins *et al.*, 1992; Doggett *et al.*, 1993a) and *in vitro* studies (Georgopoulou *et al.*, 1986) which confirmed the uptake of these antigens from the gut into both the tissues and bloodstream. The rapidity of uptake of both antigens (< 1 min) also correlates with previous studies which demonstrated internalisation of HRP within as little as 5 seconds of exposure to tissue pieces (Iida and Yamamoto, 1985). It appeared in the present study that a greater quantity of BSA than HGG was absorbed by intestinal cells. *In vivo* studies on uptake of BSA and HGG enterically presented to tilapia, *Oreochromis mossambicus* and rainbow trout also suggest that higher levels of the former protein are absorbed by both species (Georgopoulou *et al.*, 1986; Doggett, 1989; Jenkins *et al.*, 1992). Levels of HGG absorption in rainbow trout appear very low in comparison to those observed in tilapia and carp (McLean and Ash, 1986; Jenkins *et al.*, 1992). It may also be significant that since HGG is a much larger molecule than BSA (150 kDa compared to 66 kDa) the incubation of cells with equal concentrations of both antigens would lead to exposure of cells to a much greater number of BSA molecules which may partly account for the greater uptake observed.

BSA was considerably fragmented by intracellular factors within one minute of exposure to trout gut cells but the degree of fragmentation did not increase greatly with time. This may have reflected overloading of the enzyme systems by the high concentration (10 mg ml⁻¹) of BSA used or alternatively may have been a result of the limited specificity of the cellular enzymes for cleaving certain regions of the BSA molecule. Intracellular processing of HGG appeared to be complex; only after 30 minutes incubation with cells was substantial fragmentation observed. At time points later than 30 min after incubation HGG detected intracellularly was largely intact.

Previous studies have shown that intracellular processing of HGG in intestinal enterocytes of both tilapia (Jenkins *et al.*, 1991) and rainbow trout (Georgopoulou *et al.*, [1986]) is a complex process possibly involving both degradative and non-degradative pathways for its intracellular processing after absorption. This hypothesis might account for the appearance of fragmentation only at certain time points after incubation since if HGG was channelled into a degradative lysosomal pathway the macromolecule would be progressively degraded leading to detection of immunogenic fragments only during an intermediate point between entry into the degradative *milieu* and digestion to non-antigenic components. Diversion of some HGG into an alternative non-degradative pathway could then account for the detection of apparently intact HGG at all time points. Previous studies have proposed the existence of alternative routes for antigen processing in teleosts. For example Rombout *et al.* (1985) proposed that HRP and ferritin were processed in different ways by the carp gut, only small amounts of HRP entering secondary lysosomes for transport to supranuclear vacuoles for degradation while ferritin was widely found in such structures. The latter authors proposed the existence of a 'protected' pathway for HRP involving a receptor-mediated pinocytotic mechanism resulting in the formation of clathrin coated vesicles which can protect macromolecules from fusion with lysosomes as described in mammals (Abrahamson and Rodewald, 1981; Rodewald and Abrahamson, 1982). In contrast, Rombout *et al.* (1985) proposed that the uptake of ferritin was via fluid phase pinocytosis involving accumulation of antigens in small vesicles or vacuoles which subsequently fuse with lysosome like bodies before reaching supranuclear vacuoles where the macromolecules are degraded (Rodewald, 1973; Walker, 1981). The pattern and extent of BSA fragmentation [] by trout gut cells reported in this study compares favourably with the results of Rombout *et al.* (1985). While exact absorption pathways cannot be defined from the present immunoblotting study, it is possible that the considerable differences in the patterns of intracellular processing of BSA and HGG reflect the existence of a selective pathway for HGG absorption in adult trout. Davidson (1991) found that the cell population resulting from collagenase digestion of gut tissue comprised 22% goblet and epithelial cells, 69.8% lymphocytes, 4.2% granulocytes and 3.6% macrophage-like cells. Since only a percentage of the isolated cells were of epithelial morphology a direct comparison with

studies which only investigated enterocyte uptake is only partly valid since it is likely that macrophages and other lymphoid cells also absorbed antigen and contributed to processing.

After the incubation of cells with antigens supernatants were analysed to determine if exocytosis of antigens occurs and/or factors present on the cell surface contribute to protein fragmentation. Rombout *et al.* (1985) described the exocytosis of absorbed HRP in cyprinids. Moreover a number of *in vivo* studies have previously demonstrated the presence of enterically administered antigens in the tissues and the circulation suggesting that in fish, as in mammals, antigens can be transported across the gut epithelium. Studies on teleosts and on mammalian species have indicated that peptidases present on the brush border membrane play a terminal role in protein hydrolysis prior to absorption by enterocytes (Ash, 1980; Ugolev and Kuzmina, 1994). In the present study BSA detected in supernatants was partially fragmented while HGG was modified to a lesser degree. This may be a consequence of the greater intracellular hydrolysis of BSA compared with HGG and may thus reflect exocytosis of intracellularly degraded antigen. The considerable intracellular fragmentation of HGG after 15 - 30 min incubation was not reflected in the antigen detected in supernatants which suggests that the fragments generated inside the cells were not exocytosed. This may be due to the presence of both degradative and non - degradative pathways as proposed by Rombout *et al.* (1985) which could account for the presence of intact antigen within the cells and also the subsequent appearance of antigen in the tissues. Alternatively, HGG may not be as readily degraded as BSA by gut cells. Although BSA detected intracellularly and in supernatants was extensively degraded the fragments present differed considerably. A greater amount of low molecular weight BSA fragments (< 20 kDa) were present in cell lysates than in supernatants suggesting that if these fragments were derived from within the cells there may have been an element of specificity which determined which fragments were exocytosed. The possibility also exists that some of the degradation of BSA apparent in supernatants was a result of proteolysis by factors on the cell surface. If the only membrane associated enzymes present on the cell surface of epithelial cells are di- and tri- peptidases then the fragmentation of native proteins would not be expected perhaps further suggesting that the fragmentation observed here

was a result of exocytosis. Additionally since the method which was used for the isolation of gut cells removes most of the surface epithelial layer (Davidson, 1991) it is perhaps unlikely that the brush border peptidases present on surface enterocytes were expressed at a high level.

A comparative study of intracellular processing of BSA and HGG by cells isolated from different gut regions indicated that cells from all areas of the gut possessed the capacity to absorb both BSA and HGG but that processing in different regions differed considerably. The intestinal tract of teleosts shows a regional differentiation with a proximal segment consisting of 60 - 75 % of gut length with enterocyte features characteristic of lipid absorption, a middle segment of 20 - 25 % gut length with epithelial cells capable of protein absorption by pinocytosis and a distal segment of 5 - 15 % of gut length with enterocytes characteristic of water and ion transport (Yamamoto, 1966; Iwai, 1969; Gauthier and Landis, 1972; Noaillac-Depeyre and Gas, 1976; Stroband and Debets, 1978; Stroband *et al.*, 1979). The intracellular digestion of macromolecules is reported to occur in the supranuclear vacuoles of enterocytes in the second gut segment in all larval teleosts and in adult cyprinids, this is supported by the observation of intense acid phosphatase activity in supranuclear vacuoles in this region (Noaillac-Depeyre and Gas, 1973, 1976; Stroband *et al.*, 1979; Ezeasor and Stokoe, 1981; Watanabe, 1981, 1982). The findings of this study showed that BSA was absorbed maximally by cells from the gastric region while HGG was maximally absorbed by cells from the anterior intestine. Interestingly, while BSA was absorbed by cells from the gastric region no fragmentation was apparent and it is possible that this absorption was by goblet cells which could not subsequently degrade the antigen. Uptake of soluble antigens by goblet cells in tilapia has been noted (Jenkins, pers. comm.) but may be non-productive for oral vaccination unless the antigen can be transported from these cells to the body tissues. In contrast, BSA detected within cells from the pyloric caeca was extensively degraded. BSA absorbed by the anterior and posterior intestinal regions appeared largely intact while the antigen was fragmented partially by cells from the mid intestine. This data suggests that the components necessary for the intracellular processing of BSA may be absent from or differ considerably between cells from different gut regions. Differences in intracellular processing of HGG between cells from different

gut regions were only evident when samples were electrophoresed under reducing conditions suggesting that the processing of HGG intracellularly may be less destructive in terms of gross digestion of the macromolecule than for BSA and that changes made to the antigen may be less acute. It is possible that the detection of apparently intact antigen under non-reducing conditions reflected the relatively low levels of HGG absorption rather than a lack of degradation. Differences in the form of HGG detected under reducing conditions suggests that as in the case of BSA the cellular handling of the antigen depended on the region of the gut from which cells were derived. Low molecular weight fragments of HGG were not detected in most cases and the 27 kDa band (presumably light chain) was present at a much lower level than in the control suggesting perhaps that binding of this fragment to cell components may have occurred accounting for the higher molecular weight forms of HGG detected. These results conflict with those of Georgopoulou *et al.* (1986) who found in an immunocytochemical study that the Fc portion of HGG was degraded intracellularly by rainbow trout intestinal enterocytes and that the Fab portion was not. However, if the light chain (Fab) has become associated with constituents inside the cell then though it might not migrate electrophoretically as "classical" light chain it would react normally with antisera against Fab in immunocytochemistry. An *in vivo* analysis of HGG uptake into the rainbow trout circulation after oral administration (section 5.3) found greater amounts of immunoreactive HGG in the plasma possessing determinants recognised by antisera to the Fc than the Fab portions of the molecule which correlates with the present findings and again conflicts with those of Georgopoulou *et al.* (1986). Numerous studies have demonstrated protein macromolecule absorption by the posterior intestinal enterocytes in teleost larvae (Iwai and Tanaka, 1968; Iwai, 1969) and in adult agastric teleosts such as goldfish (Gauthier and Landis, 1972), carp (Noaillac-Depeyre and Gas, 1973) and grasscarp (Yamamoto, 1966). The widely held supposition that macromolecular absorption by distally disposed enterocytes of agastric fish is an alternative mode of protein nutrition in fish devoid of a functional stomach (Iwai, 1969; Noaillac-Depeyre and Gas, 1973; Watanabe, 1982) has been challenged by the finding that the posterior intestine of gastric species such as channel catfish (Watanabe, 1984), perch (Noaillac-Depeyre and Gas, 1979), tilapia (Jenkins *et al.*, 1992; Doggett *et al.*, 1993a) and rainbow trout (Stroband and

Kroon, 1981; Georgopoulou *et al.*, 1985, 1986, 1988; McLean and Ash, 1986; Doggett *et al.*, 1989) has the capacity for macromolecular protein absorption. A number of studies have reported the slow rate of antigen degradation by enterocytes of the second gut segment of teleosts indicating that intracellular degradation is not the primary function of this region (Watanabe, 1982; Rombout *et al.*, 1985). Macromolecular uptake by anterior intestinal cells has been less frequently reported (Lamers, 1985). Scherbina *et al.* (1976) and Stroband and van der Veen (1981) however, found that in cyprinids 80% of protein absorption occurs in the first gut segment. Doggett (1989) also demonstrated significant uptake of HRP and ferritin by enterocytes in the anterior intestine of *Oreochromis mossambicus*. In addition similarities have been found between the distally disposed enterocytes of carp (Lamers, 1985) and tench (Noaillac-Depeyre and Gas, 1973) which are thought to be specifically adapted for macro-molecular absorption and those from the anterior intestine of *O. mossambicus* (Jenkins, 1992). In addition considerable absorption of HRP and ferritin by the proximal segment of carp albeit at a lower level than in the distal region, has been demonstrated (Rombout *et al.*, 1985). The present study is unique in analysing macromolecule absorption and processing by cells from all areas of the teleost gastrointestinal tract. The detection of antigen absorbed by cells from all gut regions may be partly artefactual since *in vivo* only the luminal aspect of surface epithelial cells is in contact with proteins whilst *in vitro* the macromolecules have access to all external cell surfaces. It is also likely that differences in the intracellular processing of soluble proteins from different regions of the gut partly reflects the presence of phagocytic lymphoid cells. Rombout and van den Berg (1989) found a greater number of and larger intraepithelial macrophages in the second gut segment of carp compared with the first segment and antigen was detected within these cells after oral intubation. This might account for the greater degradation of BSA by cells from the mid intestine rather than the anterior and posterior regions. However, since no characterisation of the cell populations present in the different gut regions was carried out such possibilities are open to conjecture. Many studies have revealed the presence of large numbers of immunologically responsive cells in the intestinal epithelium and lamina propria of various fish species (Davina *et al.*, 1980; Doggett, 1989; Rombout *et al.*, 1993a). In a study of the gastric species, tilapia, Doggett (1989) found roughly equal numbers of intraepithelial

leucocytes in all regions of the intestine, thus the recognition in this study of macromolecular absorption in all three intestinal zones may be significant if the intestinal cells involved in processing can present antigen to lymphocytes. Jenkins (1992) also used tilapia to demonstrate interactions between antigen and lymphocytes in the gut. The observation of antigen absorption by cells derived from the gastric region in this study may not be insignificant since studies have reported the presence of leucocytes in the stomach tissue of tilapia (Doggett and Harris, 1991) and elasmobranchs (Hart, 1987) but the numbers of lymphocytes described in this region is generally much less than in the intestinal regions. Mammalian studies indicate an important role for absorptive enterocytes in antigen processing and presentation and therefore in the induction of mucosal immune responses by the gut (section 2.5). Since teleosts do not possess the highly organised gut associated lymphoid tissues (GALT) present in higher vertebrates the absorptive enterocytes may assume the roles of antigen sampling and immunological regulation at the digestive mucosae.

To determine which enzymes were involved in cellular processing of BSA and HGG, inhibitory and other modulatory agents were utilised some of which have been previously applied *in vivo* to enhance antigen absorption by the teleost gut (Jenkins, 1992). In the present study leupeptin was found to completely abrogate intracellular processing of BSA. Leupeptin is a potent inhibitor of the lysosomal cysteine proteases cathepsins B, H and L (Seglen, 1983) and its ability to efficiently enter intact cells has been documented (Clarke and Williams, 1984). Pepstatin is an effective inhibitor of the lysosomal enzyme, cathepsin D (Barth and Afting, 1984) and partially inhibited the intracellular degradation of BSA in this study. However, it has been reported that pepstatin is taken up very slowly by mammalian cells (Gordon and Seglen, 1989) which may account for its partial effectiveness in this study. Cathepsin B is a cysteine proteinase whose action is similar to papain (Aronson and Barrett, 1978; Graf *et al.*, 1979, 1981) and Cathepsin D is an endopeptidase with a role similar to pepsin which functions in the degradation of endogenous and absorbed exogenous proteins (Barrett, 1972, 1977; Van der Westhuyzen, 1980; Okitani *et al.*, 1981; Barth and Afting, 1984). Cathepsins B and D are recognised as the most important

lysosomal enzymes and previous work has indicated that certain proteins have a specific vulnerability to either cathepsin B or D whilst other proteins may be attacked initially by either endopeptidase. Lysosomal proteolysis of haemoglobin in mammals for example is initiated by cathepsin D (Huang and Tappel, 1971) while cathepsin B or another thiol cathepsin is involved in the final stages of the degradation of albumin and other native proteins (Huisman *et al.*, 1974). The results of the present study suggest that a cysteine protease, possibly cathepsin B, is principally involved in intracellular processing of BSA by rainbow trout intestinal cells. The posterior intestine of trout was previously reported to be characterised by high catheptic activity which increased immediately after food ingestion (Georgopoulou *et al.*, 1985, 1986). Leupeptin is also an effective inhibitor of at least 1 cytoplasmic calcium activated neutral protease found in various tissues (Toyo-Oka *et al.*, 1978) so the complete abrogation of BSA degradation by leupeptin may also reflect an effect of the inhibitor on cytoplasmic enzymes. Georgopoulou *et al.* (1986) co-localised cathepsin D (by immunofluoresence) with absorbed HGG and hepatitis B surface antigen in the same vacuolar system and showed vacuolar localisation of cathepsin B in the same tissue suggesting a role for these enzymes in intracellular digestion in the rainbow trout intestine. In an earlier study, the activity of a lysosomal protease (cathepsin) and of acid phosphatase were shown to increase strongly at the rainbow trout posterior intestine after first feeding occurred also suggesting a role for cathepsins in the digestion of protein in juvenile fish (Georgopoulou *et al.*, 1985). Ammonium chloride did not appear to influence the intracellular processing of BSA in this study which is surprising since this agent can effectively inhibit pinocytosis in various cell types (Liversey *et al.*, 1980). A study on lysosomal degradation of radio-labelled albumin in yolk sacs (Clarke and Williams, 1979) indicated that tissues exposed to ammonium chloride rapidly regained most of their proteolytic activity after treatment which may explain the present results. Quil-A appeared to reduce intracellular fragmentation of BSA. Quil-A saponin has been reported to have inhibitory effects on certain proteolytic enzymes such as trypsin and pepsin (Ishaaya and Birk, 1965) and may have inhibited lysosomal cathepsins with similar properties. Cimetidine, a H₂ histamine receptor antagonist also appeared to partly inhibit BSA intracellular processing. Differences in the processing of HGG after treatment with potential

inhibitors were only apparent when lysates were electrophoresed under reducing conditions. While ammonium chloride, cimetidine and pepstatin appeared to have little effect on intracellular processing of HGG, leupeptin and Quil-A did alter its processing, both agents having similar effects. It is difficult to draw any firm conclusions from this data however since the differences may reflect subtle modifications in antigen processing due to the action of the various agents rather than the gross changes that were observed with BSA. Analysis of the effects of the inhibitors described previously on the nature of the antigens detected in supernatants indicated that leupeptin was most effective in reducing fragmentation of BSA and HGG. Pepstatin and Quil-A also appeared to have an inhibitory effect but in the case of both antigens the effects of inhibitors on degradation were minimal.

The evidence from this study supports the use of an *in vitro* methodology in the analysis of cellular processing of antigens in the teleost gut. Data indicates that considerable differences exist both in the modes of antigen handling in different gut regions and for different proteins by the same cells. Intracellular processing of BSA resulted in considerable degradation but processing of HGG appeared to be more complex. The observation of substantial antigen modification in cell supernatants indicated that fragmented and possibly intact antigen was exocytosed from cells after uptake. This study postulates that a great complexity exists in the mode of cellular antigen processing by the teleost gut involving, perhaps, a degree of specificity and that such fundamental work may be of value in devising strategies and formulations for oral vaccination of teleosts. Future work could apply this model to investigate the capacity for intestinal absorption of candidate antigens or of the value of modulatory agents in enhancing uptake prior to large scale *in vivo* experimentation.

CHAPTER 5

*AN INVESTIGATION INTO THE VALUE OF GASTRIC INHIBITORS IN THE ORAL DELIVERY
OF SOLUBLE PROTEIN ANTIGENS TO TELEOSTS*

5.1 INTRODUCTION

The gastric barrier has commonly been cited as an obstacle to the effective oral delivery of intact protein macromolecules to gastric teleosts (Lillehaug, 1989; Wong *et al.*, 1992). This contention was supported by the observation that the anal administration of soluble protein antigens and bacterins enhanced both their absorption and the resultant immune response when compared with oral administration (Johnson and Amend, 1983a, b; Jenkins *et al.*, 1992). In mammals, gastric acid has been demonstrated to have an adverse effect on the viability of live orally administered enteric organisms (Giannella *et al.*, 1973) and can alter the immunogenicity of inactivated oral vaccine preparations (Clemens *et al.*, 1986; Sanchez *et al.*, 1993a). Furthermore, trypsin, small intestinal contents or acid alone had little effect on *Escherichia coli* pilus protein colonization factor antigens (CFA's) whilst acid and pepsin or stomach contents led to a rapid loss of antigenicity and conversion of the antigens to low molecular weight fragments (Schmitt *et al.*, 1985).

The teleost stomach is lined by columnar epithelial cells with scattered goblet cells. Tubular glands are located in the cardiac and fundic regions of the stomach and open into foveolae (Fänge and Grove, 1979). In most sub-mammalian vertebrates gastric glands are formed of a single cell type, - oxynticopeptic cells, which secrete both HCl and pepsinogen; in mammals this cell differentiates into an acid secreting (oxyntic/parietal) cell and a pepsinogen secreting cell (Michelangeli *et al.*, 1988). Weinreb and Bilstad. (1955) indicated that the structure of gastric gland cells in rainbow trout resembled that of chief cells in other vertebrates. The piscine oxynticopeptic cell contains abundant secretory granules, presumed to be pepsinogen (Tan and Teh, 1974), and is also believed to produce HCl (Barrington, 1957; Iro, 1967; Mattisson and Holstein, 1980). A study which investigated the ultrastructure of gastric gland cells in teleosts described a basal region rich in rough endoplasmic reticulum and zymogen-like secretory granules which were released apically by exocytosis (Noaillac-Depeyre and Gas, 1978). These ultrastructural features are consistent with the hypothesis that gastric glands are active in both acid (HCl) production and pepsinogen synthesis and acidic gastric fluid has been frequently reported

in teleost species (Western and Jennings, 1970; Grabner and Hofer, 1985; Smith, 1989).

Gastric acid secretion in mammals is precisely regulated by neural (acetylcholine), hormonal (gastrin) and paracrine (histamine, somatostatin) mechanisms. The stimulatory effect of acetylcholine is mediated via an increase in cytosolic calcium and that of histamine by activation of adenylate cyclase and generation of cAMP (Shamburek and Schubert, 1993). Extensive research on the mechanisms involved in gastric acid secretion and the subsequent identification of specific receptor subtypes has led to the development of potent drugs capable of inhibiting acid secretion. These drugs include the histamine H₂ receptor antagonists such as cimetidine, ranitidine, famotidine, nizatidine and roxatidine acetate (Shamburek and Schubert, 1993). While most hormonal control of digestive tract physiology in higher vertebrates is extrinsic involving autonomic nerves and externally produced hormones, control in fish is mainly intrinsic with many of the hormone producing cells present in the intestinal epithelium (Smith, 1989). The mechanisms of action of some regulatory peptides found in the piscine gut have been described (Holmgren, 1985; Holmgren *et al.*, 1986). A role for histamine in stimulation of gastric acid secretion has been demonstrated in the European catfish, *Silurus glanis* (Gzgyzon and Kuzina, 1973) and in the Atlantic cod, *Gadus morhua* (Holstein, 1975). The observation that exogenous histamine leads to acid secretion indicates a physiological role for histamine in the regulation of acid secretion (Reite, 1969; Lorentz *et al.*, 1973). Holstein (1976) found that the injection of histamine or carbacholamine into cod led to considerable secretion of gastric acid, an effect which was blocked by the H₂ receptor antagonist metiamide. H₂ receptors have also been detected on a wide range of other cell types in mammals including lymphocytes (section 6.1).

A range of strategies have been used to reduce the impact of gastric secretions on orally administered macromolecules in both mammals and teleosts. Sodium bicarbonate has been administered prior to enteric vaccination with *Vibrio cholerae* whole cell/ B subunit vaccines in humans to prevent acid denaturation of the cholera toxin B subunit (Clemens *et al.*, 1986; Sanchez *et al.*, 1993). The H₂ receptor antagonist, cimetidine, has also been used as a gastric inhibitor prior to the administration of irradiated *Escherichia coli* vaccine against enterotoxigenic *E. coli* induced

diarrhoea (Dima *et al.*, 1992). The application of gastric inhibitors in teleost enteric delivery systems has been relatively limited, although sodium bicarbonate has been used to enhance the absorption of both standard antigens (McLean and Ash, 1989) and protein hormones (Thomas and Boyd, 1989; McLean *et al.*, 1990; Solar *et al.*, 1990). A range of strategies other than simply the neutralisation of gastric acid or inhibition of acid synthesis also exist for reducing the impact of gastric secretions on labile vaccines including enteric coating (Wong *et al.*, 1992) and microencapsulation (section 7.1).

The aims of the present study were to investigate the use of gastric inhibitors in reducing the impact of the gastric secretions of rainbow trout on the proteolysis of protein antigens and in enhancing antigen uptake. This involved determining the concentrations of sodium bicarbonate and cimetidine which were necessary to establish a desired gastric pH (in 100 - 150 g fish) and also devising an *in vivo* method for assessing the impact of gastric inhibitors (and other potential delivery systems) on luminal proteolysis. The criteria chosen for assessing the effects of the gastric inhibitors used in this study were gastrointestinal pH, stability of antigens in the gut lumen and the quantity and nature of antigen in the plasma. This work was also intended to test the predictive value of an *in vitro* model described in chapter 3 with regard to the pH dependence of the gastric proteolysis of HGG.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Rainbow trout were maintained as described in section 3.2.1.

5.2.2 Sample collection

Each group of fish was isolated in a separate tank for the duration of experiments. Animals were killed by administering a sharp blow to the head after which blood was collected from the caudal vein using a 23 gauge needle into heparinised syringes. Blood was stored overnight at 4°C before centrifugation at 5,800 x g for 5 min and plasma was stored in 200 µl aliquots at -20°C until required. After bleeding, a longitudinal incision was made in each fish from the anus to a point in line with the operculum, a transverse incision was made and one flank pulled aside to expose the gut. The gut was excised at the anterior end of the oesophagus and immediately anterior to the anus, divided into 5 regions (Fig 5.1) and the pH of each region was recorded by means of a glass pH microprobe (Aldrich, Poole, Dorset). After making a longitudinal incision to expose the luminal surface, mucosal scrapings were collected from each gut region with PBS, pH 7.2 and decanted into plastic containers on ice. Contents from each group (n=5) were made up to 6 ml with PBS and 50 µl of 100 mM PMSF was added. Samples were stored at -20°C until required.

Table.5.1: Overview of experimental protocols applied in the study of gastric inhibitors.

				Analysis performed			
Expt number	Gastric inhibitor used\ dose per fish	Antigen (HGG) dose given (Time 0)	Time before sample collection	Serum level of HGG (ELISA)	Serum presence of HGG (Blotting)	Mucosal presence of HGG (Blotting)	Gut pH values
1	-	0 - 75 mg	1 hr	+	-	-	-
2	NaHCO ₃ (0-35 mg)	35 mg	1 hr	+	+	-	+
3	Cimetidine (0-100 mg)	35 mg	1 hr	+	+	-	+
4	-	35 mg	15 min-48hr	+	+	-	-
5	NaHCO ₃	35 mg	15min-48hr	+	+	+	+
6	Cimetidine (1 mg)	35 mg	15min-48hr	+	+	+	+
7	Cimetidine (10 mg)	35 mg	15min-48hr	+	+	-	-
8	Cimetidine (50 mg)	35 mg	15min-48hr	+	+	+	+

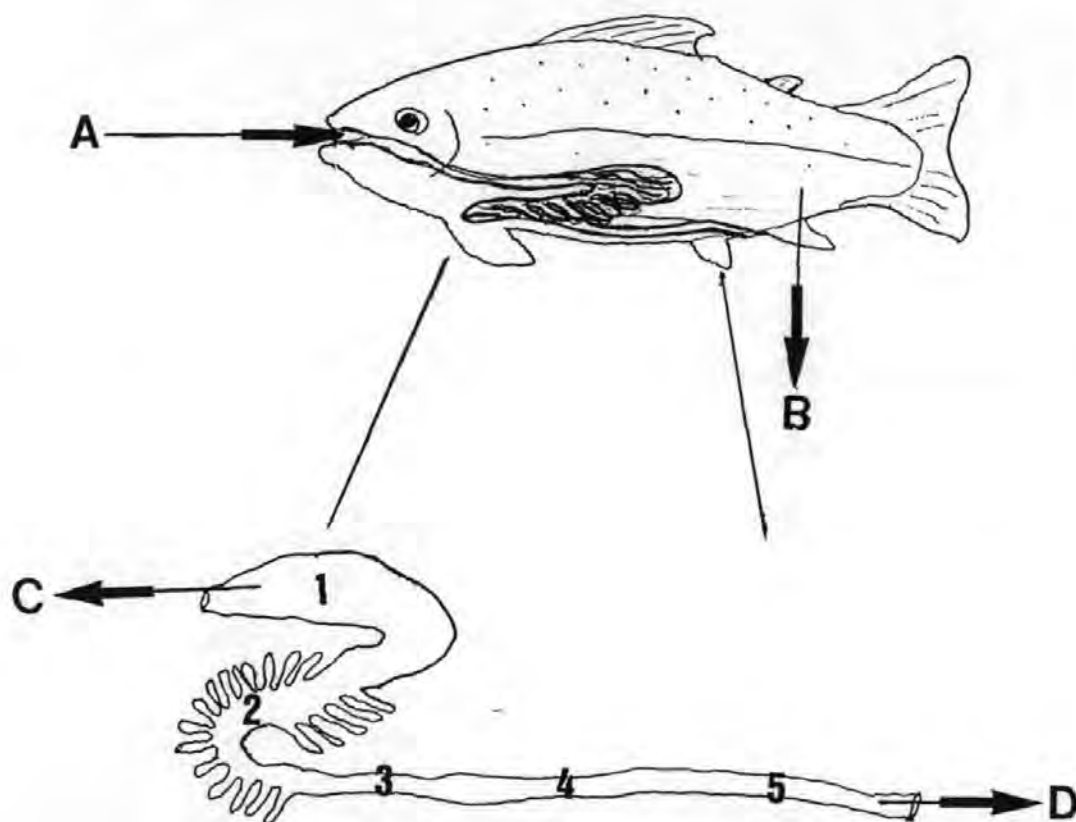


Fig. 5.1: *Experimental procedures used in the investigation of antigen proteolysis and absorption. A: oral intubation of antigen into pyloric curvature. B: Collection of plasma from the caudal sinus. C: Measurement of gut pH. D: Collection of scrapings from regions of the gut. Numbers 1 - 5 represent the regions of the digestive tract in which the pH was measured. 1, stomach. 2, pyloric caecae. 3, anterior intestine. 4, mid - intestine. 5, posterior intestine. For collection of mucosal scrapings, samples were taken and subsequently analysed from the stomach and pyloric caecae and the remainder of the intestine was bisected and referred to in the text as anterior and posterior intestine.*

5.2.3: Establishment of optimal doses of HGG and gastric inhibitors for oral administration

The experimental protocols used in this study are presented in condensed form in Table 5.1. To establish an optimal HGG dose, individual groups of fish ($n=5$) were intubated with a range of HGG doses from 1 to 75 mg per fish administered in 0.2 ml, PBS, pH 7.2. Solutions were delivered to fish through a 1 mm diameter polyvinyl chloride (PVC) tubing attached to a 21 gauge

needle and a 1 ml syringe. Blood samples were collected 1 hr after intubation. ELISA analysis of plasma samples (see section 5.2.5) indicated that a dose of 35 mg HGG per fish resulted in optimal plasma levels of the antigen and this dose was chosen as the basis for further studies. A range of doses of sodium bicarbonate or cimetidine were administered to fish 1 hr prior to HGG administration. Blood samples were collected 1 hr later and analysed by ELISA. Additionally, the pH of all regions of the trout gastrointestinal tract was measured by means of a glass microprobe (Aldrich). Following ELISA analysis, appropriate doses of cimetidine and bicarbonate were chosen and were subsequently used to investigate the effects of gastric inhibitors on the time course of HGG absorption.

5.2.4: Investigation of the effects of gastric inhibitors on the time course of HGG absorption

Time course experiments were performed with gastric inhibitors (Table 5.1). Doses of 1, 10 and 50mg cimetidine and 35 mg bicarbonate were administered to fish 1 hr prior to HGG administration. Samples were collected from fish at 15 min, 30 min, 45 min, 60 min, 2 hr, 6 hr, 12 hr, 24 hr and 48 hr after HGG intubation and subsequently analysed. To serve as a control for the gastric inhibitor study, 0.2 ml PBS was administered to fish 1 hr before HGG delivery and samples were collected and analysed over a 48 hr time course.

5.2.5: Enzyme linked immunosorbent assay (ELISA) for HGG detection.

The antigen capture ELISA used was a modification of that optimised previously (Jenkins *et al*, 1992) from the method of Ambler and Peters (1984). A representative example of the layout of a microtitre plate for HGG detection and a photograph of a developed ELISA plate are presented in Fig 5.2. Microtitre plates (Falcon) were coated with 100 μ l of a polyclonal goat anti-HGG antiserum (Sigma) at a 1:4000 dilution in carbonate-bicarbonate buffer, pH 9.6 (Appendix C), wrapped in aluminium foil and incubated in a humid box overnight at 4°C. Solutions were aspirated and wells washed x 4 by means of a Titertek Handiwash 110 plate washer (Labsystems, U.K) and dried thoroughly. Plasma samples (100 μ l) diluted 1:10 in PBS-Tween (Appendix C) and standard HGG solutions (100 μ l) at 10 μ g ml⁻¹ in 10% control trout serum were titrated down the

plate. Plates were wrapped in aluminium foil and incubated in a humid box at room temperature for 2 hr. Following this the plates were washed, dried and 100 μ l of goat anti-HGG peroxidase conjugate (Sigma) at a 1:2000 dilution in PBS-Tween was added to each well. In addition, control wells were included on each plate from which one reagent was omitted to determine if any cross reactions were present and to allow for the correction of sample absorbances. The plates were wrapped in foil and incubated at room temperature for 1 hr. After this final incubation, plates were washed, dried and 100 μ l chromagen (Appendix C) was added to each well. The reactions were terminated after 10 min by the addition of 50 μ l of 1 M sulphuric acid to each well. The absorbance values were read at 492nm by an automatic ELISA plate reader (Labsystems, U.K).

5.2.6: Modified Western blotting methods for detection of HGG.

A: Immunodetection with antisera to HGG (whole molecule)

The procedure described in section 3.2.12 was modified as follows to enable the detection of HGG in plasma and to abrogate cross reactions with control trout plasma.

The primary antiserum (goat anti-HGG peroxidase conjugate) was pre-adsorbed overnight with 5% control trout plasma to which 1% SDS had been added 24 hr previously and centrifuged at 11,600 x g for 10 min. The resultant supernatant was used as primary antiserum diluted 1:250 in Tris-saline + 4 % non-fat dried milk pH 7.5 (TSM). The secondary antiserum used was a rabbit anti-goat IgG peroxidase conjugate (Sigma) diluted 1:1000 in TSM.

Plasma samples, diluted 1:5 in electrophoresis sample buffer were electrophoresed under both reducing and non-reducing conditions. Lumenal scrapings were homogenised by vigorous shaking (WhirliMixer, Fisons) and centrifuged at 4,800 x g for 5 min to remove solid matter. The resultant supernatant was diluted 1:1 with non-reducing sample buffer, boiled for 3 min and incubated at room temperature for 2 hr prior to electrophoresis and immunoblotting.

B: Immunodetection with goat antisera to the Fc and Fab regions of HGG.

The protocol was identical to that described above except that the anti-Fc and anti-Fab antisera

(Sigma) were used as primary antisera at a dilution of 1:250 in TSM after pre-absorption as described in 5.2.6 (A)

5.2.7: Image analysis of immunoblots

Immunoblots were analysed using the UVP gel analysis suite program GelBase/ GelBlot (Ultra Violet Products Ltd, Science Park, Milton Road, Cambridge) and the results were subsequently expressed in tabular fashion as described in Fig.5.3.

5.2.8: Statistical analysis of results from ELISA analyses

ELISA data expressed as ng HGG ml⁻¹ was entered into Statgraphics software and an analysis of variance (ANOVA) test was performed to assess if significant differences existed between groups (at $p < 0.05$ and $p < 0.25$). If variances between groups were considerably different the data was log (log₁₀) transformed prior to analysis.

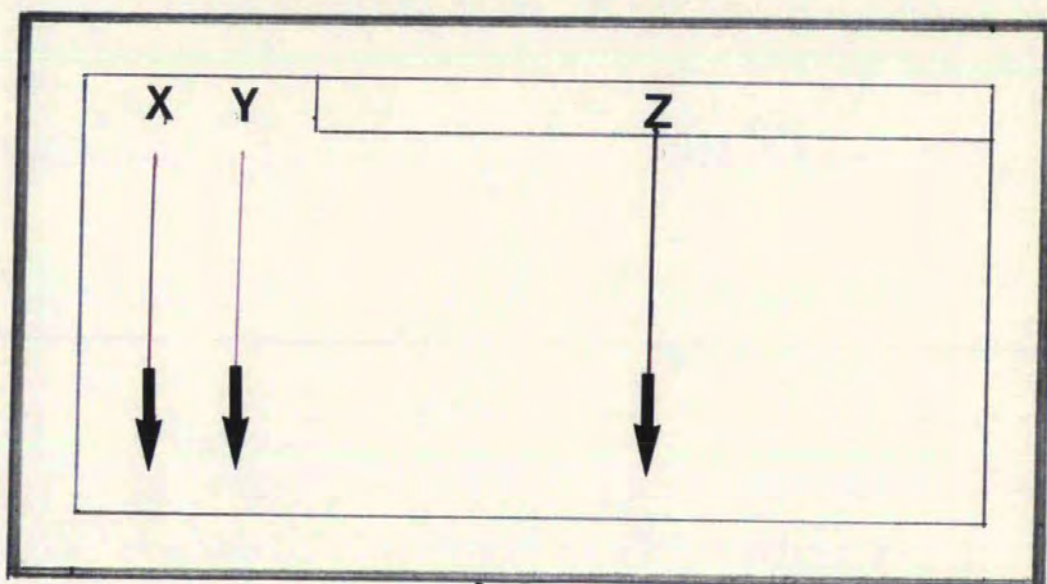


Fig. 5.2.(a): Example of the layout of an ELISA microtitre plate for assessment of plasma HGG levels. X - Doubling dilutions of HGG standard solution ($10\mu\text{g ml}^{-1}$) down column 2. Y- Doubling dilutions of control trout plasma down column 3. Z- doubling dilutions of test plasma down columns 4-11.

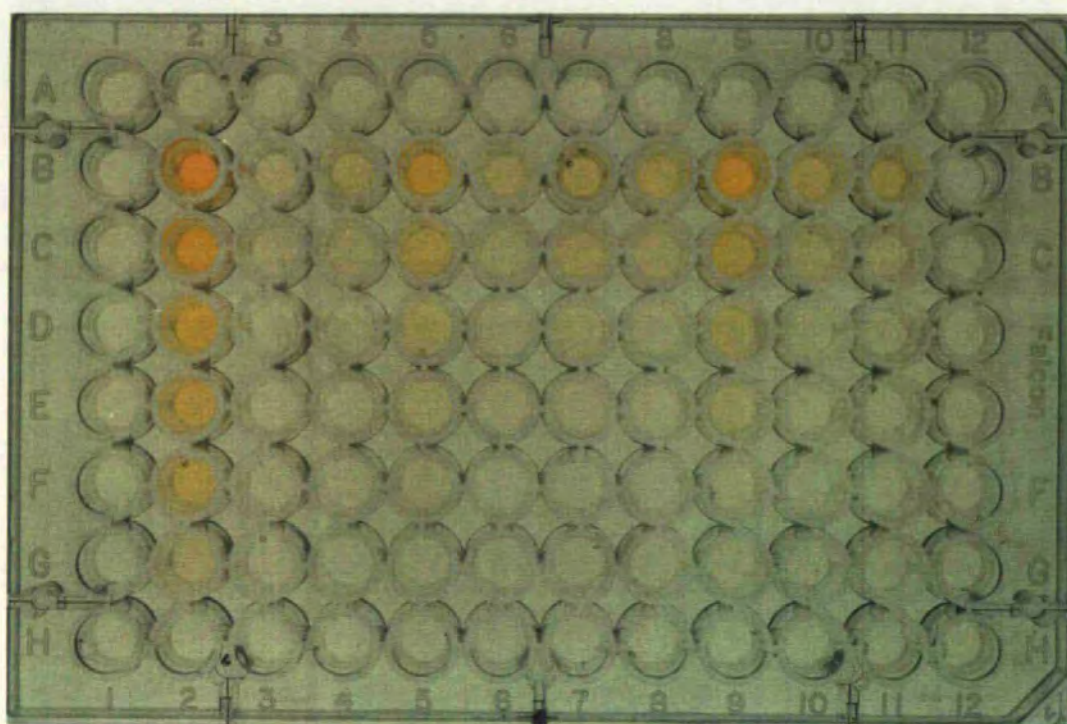


Fig. 5.2.(b): Photograph of a representative ELISA microtitre plate demonstrating the presence of HGG in rainbow trout plasma samples. The loading order on the plate was as outlined in Fig.5.2(a). The samples in columns 4-11 were of plasma, collected from fish orally intubated with 35 mg bicarbonate 1 hr prior to delivery of 35 mg HGG at 15 and 30 min and 1, 2, 6, 12, 24 and 48 hr after HGG delivery.

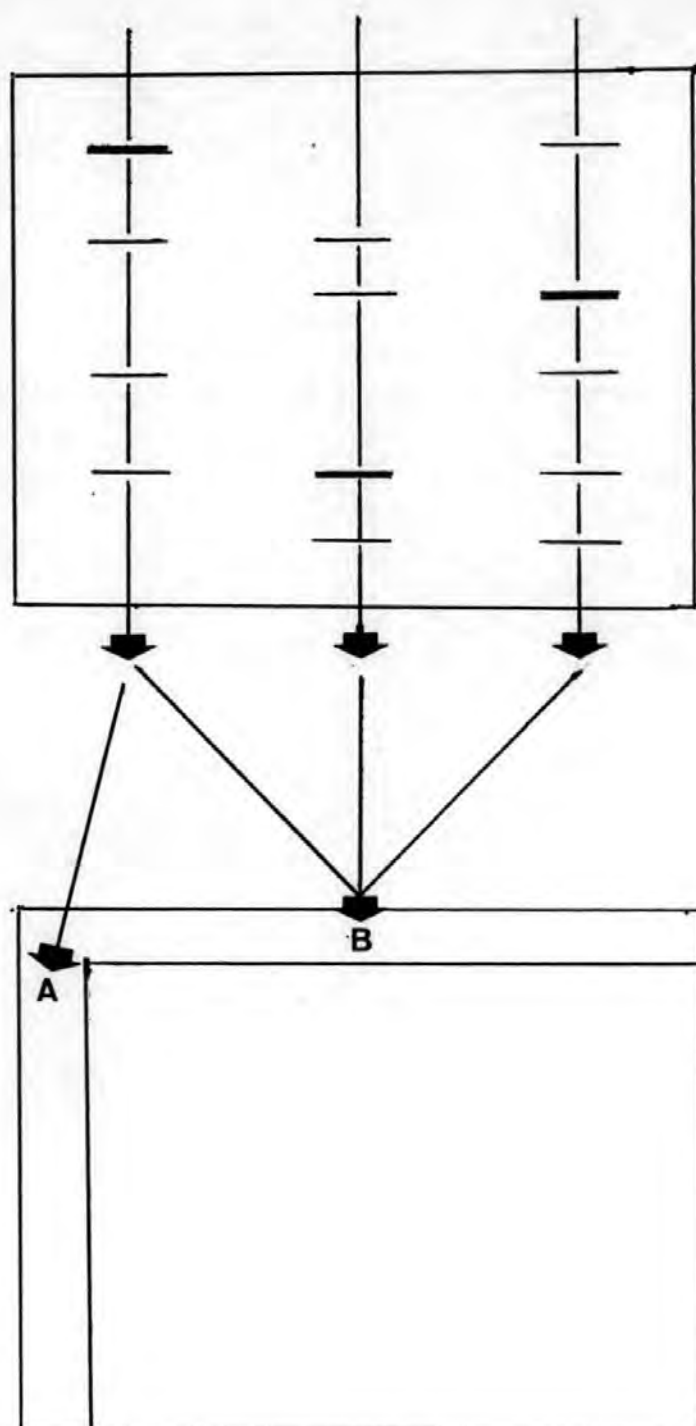


Fig.5.3. Derivation of semi - quantitative data from immunoblots by image analysis - origin of tabulated data in section 5.3. *A:* Represents the process used to derive data in columns of tables in section 5.3; single lanes were scanned by image analyser and relative percentages of each band calculated as a percentage of the total signal in that lane only. *B:* The process used to derive data in top row of tables in section 5.3; all lanes on immunoblot were scanned by image analyser and total signal for all antigen present calculated, the proportion of the total signal present in each single lane was then calculated.

5.3 RESULTS

5.3.1: *Dose response to HGG*

The relationship between the dose of HGG administered orally to rainbow trout and levels of HGG detected in the plasma of fish 1 hr after administration is presented in Fig 5.4. Doses of 35 and 50 mg HGG per fish resulted in plasma HGG levels (detected by ELISA) significantly greater ($p < 0.05$) than all other groups. The peak level was detected after administration of 35 mg HGG per fish resulting in plasma levels significantly ($p < 0.05$) greater than those detected after all other treatments. This was thus chosen as the standard dose for subsequent experiments.

5.3.2: *Dose effects of gastric inhibitors on gastrointestinal pH and HGG uptake*

A: Sodium bicarbonate

The effects of administering various doses of bicarbonate (1 hr before HGG delivery) on a range of parameters are presented in Fig 5.5 and Table 5.2. Regardless of the bicarbonate dose administered, the pH in all gut regions other than the stomach 1 hr after HGG delivery (i.e 2 hr after administration of bicarbonate) did not change significantly ($p < 0.05$). Gastric pH, in contrast increased almost linearly from pH 3.4 to 8.0 with increasing bicarbonate dose (Fig 5.5(a). All bicarbonate doses from 1.0 to 35 mg per fish resulted in significantly higher ($p < 0.05$) gastric pH compared with control PBS administration and differences in gastric pH induced by doses of 10, 20 and 35 mg bicarbonate per fish were not significant. ELISA analysis of HGG levels in plasma (Fig 5.5(b)) indicated that administration of 1, 20 or 35 mg bicarbonate per fish before HGG administration resulted in plasma HGG levels significantly ($p < 0.05$) greater than those detected after the delivery of PBS or all other doses of bicarbonate. Differences between the plasma levels of HGG detected after prior administration of 1, 20 or 35 mg bicarbonate were not significant. The semi-quantitative immunoblotting data presented in Table 5.2 shows that the highest total HGG signal was detected in plasma from fish which were administered with 20 or 35 mg bicarbonate prior to the delivery of HGG. This was similar to the results obtained by ELISA except that the relatively high signal detected by ELISA after prior delivery of 1 mg bicarbonate was not reflected in the immunoblotting data. The greatest number of HGG bands

were also detected in plasma from fish administered 20 (9 bands) or 35 mg (8 bands) bicarbonate. The qualitative nature of the HGG fragments detected after delivery of various doses of sodium bicarbonate also differed considerably. Plasma from fish orally intubated with control PBS or 0.1 mg bicarbonate contained greater amounts of fragments of low apparent molecular weight (10.6 and 7.4 kDa) than that from fish receiving 1 or 10 mg bicarbonate. At higher doses of bicarbonate similar sized fragments were again apparent although the 10.6 kDa fragment was present at a lower level. The high signal detected in plasma from fish given 20 or 35 mg bicarbonate was accounted for mainly by the 43.2 and 18.4 kDa fragments. It is important to note that the very high apparent molecular weight band (≈ 202 kDa) detected at the top of almost all immunoblots may be artefactual although it was not detected in control plasma. Intact HGG has a molecular weight of 150 kDa and the presence of a band at 200 kDa only in plasma of fish receiving HGG may have been a result of antigen precipitation prior to electrophoresis or to complexing with plasma factors. After consideration of the data a dose of 35 mg bicarbonate per fish was chosen for use in further experiments.

B: Cimetidine

The effects of increasing doses of the H_2 receptor antagonist cimetidine on antigen absorption and gut pH are presented in Fig. 5.6 and Table 5.3. Although the pH in the intestine only varied between 6.7 and 7.2 after different treatments the pH in the anterior and mid-intestinal regions of fish receiving control PBS prior to HGG was significantly greater than that recorded in these regions in all groups except untreated fish and fish receiving 100 mg cimetidine. Gastric pH increased with increasing dose from 1-30 mg cimetidine per fish to a $pH \approx 7.0$, pH values recorded after prior treatment with 30, 50, 75 or 100 mg cimetidine per fish were significantly greater than controls and fish receiving 1 mg cimetidine ($p < 0.05$) (Fig.5.6(a)). Differences between gastric pH values measured in fish receiving 30, 50, 75 or 100 mg cimetidine prior to HGG administration were not significant. The ELISA data on HGG absorption into the plasma (Fig 5.6 (b)) shows a remarkably large peak in the group given 1 mg cimetidine per fish, being significantly greater than all other groups ($P < .05$). In contrast, immunoblotting data indicated

that the greatest total HGG signal and greatest number of HGG fragments (10) were present in the plasma of fish given 75 mg cimetidine (Table 5.3). Plasma from fish receiving 1 mg cimetidine was only remarkable for the detection of high levels of the 10.2 kDa and 42.1 kDa fragments which were not present at a high level in other groups. The low apparent molecular weight fragments (10.2 and 8.2 kDa) were most noticeable in the group given 1 mg of cimetidine per fish. Between 38 and 55% of the signal detected in plasma from the fish intubated with 30, 50, 75 or 100 mg cimetidine was accounted for by the 20.4 and 18.9 kDa bands (similar to pattern obtained with high doses of bicarbonate). Due to the absence of an unambiguous dose response with cimetidine three separate doses (1, 10 and 50 mg per fish) were used to further investigate the use of this substance.

5.3.3 Effects of gastric inhibitors on a range of parameters over a time course after antigen administration

(A): Prior PBS administration

A number of time course experiments were pursued in order to examine in detail the nature and kinetics of the processing and absorption of HGG in the gut of rainbow trout. Firstly, a control time course was performed by delivering PBS prior to the administration of 35 mg HGG (Fig.5.7 and Table 5.4). A biphasic pattern of antigen uptake into the plasma was observed by ELISA. A small peak was apparent at 45 min (not significant at $p < 0.05$) and a larger peak at 12 hr which was significantly higher ($p < 0.05$) than at all other time points. Immunoblotting data showed that the very large peak at 12 hr found by ELISA was not reflected in terms of the total HGG signal detected (Table 5.4). A higher percentage (19.6%) of possibly intact HGG (152.3 kDa) was detected after 12 hr however than at any other time point. The highest signal in plasma was detected at 24 hr on Western blots at which point the greatest number (8) of HGG bands were also found. When the same plasma samples were probed with antiserum against the Fc portion of HGG 5 bands were detected and with antiserum against the Fab portion, 2 bands were recognised.

(B): Prior bicarbonate (35 mg) administration.

Fig 5.8(a) presents a PAGE gel showing the protein profile in plasma collected from fish over a 48 hr time course after the administration of 35 mg of sodium bicarbonate followed 1 hr later by the oral administration of HGG. The corresponding Western blot is presented in Fig. 5.8(b) demonstrating the presence of HGG in the plasma of these fish. The pH in all gastrointestinal regions except the stomach was relatively constant over time. Nonetheless, a significant increase in pH ($p < 0.05$) was noted 6 hr after HGG delivery in the pyloric caecae, anterior and mid-intestinal regions, greater than all times except 45 min and 12 hr after intubation (Fig.5.9(a)). Gastric pH remained at approximately pH 8.0 until 6 hr after intubation after which a significant decrease was found. Interestingly, the rise in intestinal pH found after 7 hr following bicarbonate administration occurred at the same time as the fall in gastric pH. A biphasic pattern of HGG absorption was observed by ELISA analysis with peak levels detected 30 min and 12 hr after HGG administration, the levels detected 15 min, 30 min, 6 hr and 48 hr after HGG delivery were also significantly greater than controls ($P < 0.05$). This biphasic pattern of uptake for can be seen in Fig.5.2(b). Peak values found after 30 min and 12 hr were not significantly different to each other. Immunoblotting data on these samples (Table 5.5) showed that the highest total signals were detected 2 hr and 12 hr after HGG delivery. Notable is the large amount of the 10.2 kDa band detected 45 min, 1 hr and 2 hr after delivery and that of the 18.4 kDa fragment 1, 2 and 12 hr after delivery. Using antisera to the Fc and Fab regions it was found over the 48 hr time course that anti-Fc antisera recognised 7 bands and anti-Fab antisera 5 bands, a number of bands (45.5, 18.4, 10.3 and 7.5 kDa) were recognised by all three antisera used.

Analysis of scrapings from the gastric mucus of these fish (Table 5.6) demonstrates the large number of HGG bands present (18) relative to those found in the plasma of these fish (10). HGG was only detected in scrapings up to 12 hr after HGG delivery. In scrapings collected 15 min - 6hr after intubation, only 20-40 % of HGG was detected in apparently intact form, much of the remainder being present as 107.5, 65.3, 52.7, 25.9 and 20.2 kDa fragments. Maximal levels were found in scrapings from the intestinal regions of these fish at 45 min and from 2 hr to 12 hr after antigen delivery (Table 5.7). Most fragments were found 2 hr (15) and 6 hr (14) after intubation.

The presence of the 18.5 and 10.4 kDa fragments in the intestine appears to reflect to some extent their presence in the plasma at 1 and 12 hr after HGG delivery (Tables 5.5 and 5.7).

(C): Prior cimetidine (1, 10 and 50 mg) administration

A photographic representation of the *in vivo* proteolysis of HGG in the trout gastrointestinal tract after administration of HGG subsequent to delivery of 1 and 50 mg cimetidine is presented in Fig. 5.10. In the cases of the time courses where 1 and 50 mg cimetidine were administered prior to HGG in addition to analysis of HGG levels in plasma and pH, data is presented on scrapings from 5 separate gut regions to demonstrate the effects of changing pH on *in vivo* luminal proteolysis. In the case of the time course where 10 mg cimetidine was delivered prior to HGG only data on the presence of the antigen in the plasma is presented.

1 mg cimetidine: pH data

A pH between 4.0 and 6.0 was measured in the gastric regions of fish for up to 12 hr after HGG delivery and differences between pH values at different time points up to 12 hr were not significant (Fig 5.11(a)). This may have been a result of the high degree of variability in gastric pH values between fish. The pH in the intestine ranged between 6.6 and 7.4 over the time course but was significantly higher at 15 min, 12 hr and 24 hr after intubation than at all other time points.

1 mg cimetidine: Analysis of HGG in plasma

ELISA data indicated that only at 30 min, 45 min, 1 hr, 2 hr and 6 hr after intubation were the HGG levels detected significantly greater than controls ($p < 0.25$). The level of HGG in the plasma 6 hr after intubation was significantly ($p < 0.05$) greater than that at all other time points. As before, the immunoblotting data (Table 5.8) did not reflect the ELISA data - the greatest total HGG signals being found 2 and 12 hr after delivery and the greatest number of HGG bands (12) were also found 12 hr after administration. A considerable amount of the HGG detected at 15 min, 1 hr, 2 hr and 24 hr after delivery was in the form of a 10.4 kDa fragment (57.2% of the total signal 2 hr after delivery which may reflect the ELISA peak at this time).

1 mg cimetidine: Analysis of mucosal scrapings - Immunoblotting data

In gastric scrapings from fish administered 1 mg cimetidine prior to HGG, 22 bands were detected. HGG was detectable at all time points analysed from 15 min to 6 hr after HGG administration after which time no HGG was found (Table 5.9). In scrapings taken from the pyloric caecae the highest HGG signals were found at 45 min and 6 hr - the latter peak possibly a result of gastric evacuation. HGG fragments of an apparent molecular weight less than 50 kDa were only present at a high level in the pyloric caecae of these fish at 2 hr (Table 5.10 and Fig 5.10). Most of the HGG detected at other time points was apparently intact or in the form of large fragments (> 100 kDa). In contrast, at the corresponding time points in the stomach a significant amount of the HGG present was in the form of fragments smaller than 50 kDa. In scrapings from the anterior intestine of these fish (Table 5.11) the highest HGG signal was detected at 2 and 6 hr, the greatest number of bands (15) were also present 6 hr after HGG intubation. In contrast to the pyloric caecae very little intact or high apparent molecular weight (> 100 kDa) antigen was detected in the anterior intestine; most fragments found being in the 30-65 kDa size range. This indicated that considerable breakdown of the intact antigen and large fragments by anterior intestinal enzymes had occurred. No antigen was detected in scrapings from the anterior intestine of these fish at time points later than 12 hr after delivery. The highest levels of HGG were found in scrapings from the posterior intestine at 30 min, 45 min and 24 hr after antigen delivery (Table 5.12). Between 64.7 and 94.3% of the HGG detected at 15 min, 30 min and 45 min after delivery was in the form of 30 - 52 kDa fragments. In contrast, fragments in this size range only represented 28.9% of the total signal at 12 hr and 6.6% at 24 hr and although present did not represent a significant percentage of the total at 2 and 6 hr. At 2 and 6 hr after delivery, 100% of the total signal detected in the posterior intestine was in the form of possibly intact HGG (149.8 kDa) but the intact antigen was not detectable at time points before 2 hr.

10 mg cimetidine: Analysis of HGG in plasma

The results of administering 10 mg cimetidine per fish prior to HGG are presented in Fig 5.12 and Table 5.13. The uptake of HGG as measured by ELISA was significantly higher ($p < 0.05$) at

6 hr than at any other time point. As found in some of the earlier time courses this peak was not reflected in terms of a greatly increased total HGG signal detected by immunoblotting (Table 5.13), rather the highest total signal was detected on Western blots 1 hr after antigen delivery. The only distinguishing feature in plasma collected from fish 6 hr after antigen delivery was the relatively high percentage of the 40.3 kDa fragment present which was also recognised by anti-Fc antisera.

50 mg cimetidine: pH data

After administration of this dose of cimetidine a pH $\approx$ 7.0 was measured in the stomach and in all intestinal regions until > 12 hr after HGG administration (Fig 5.13(a)). Gastric pH appeared to decline at 24 and 48 hr but this was not significant at the 5% level.

50 mg cimetidine: Analysis of HGG in plasma

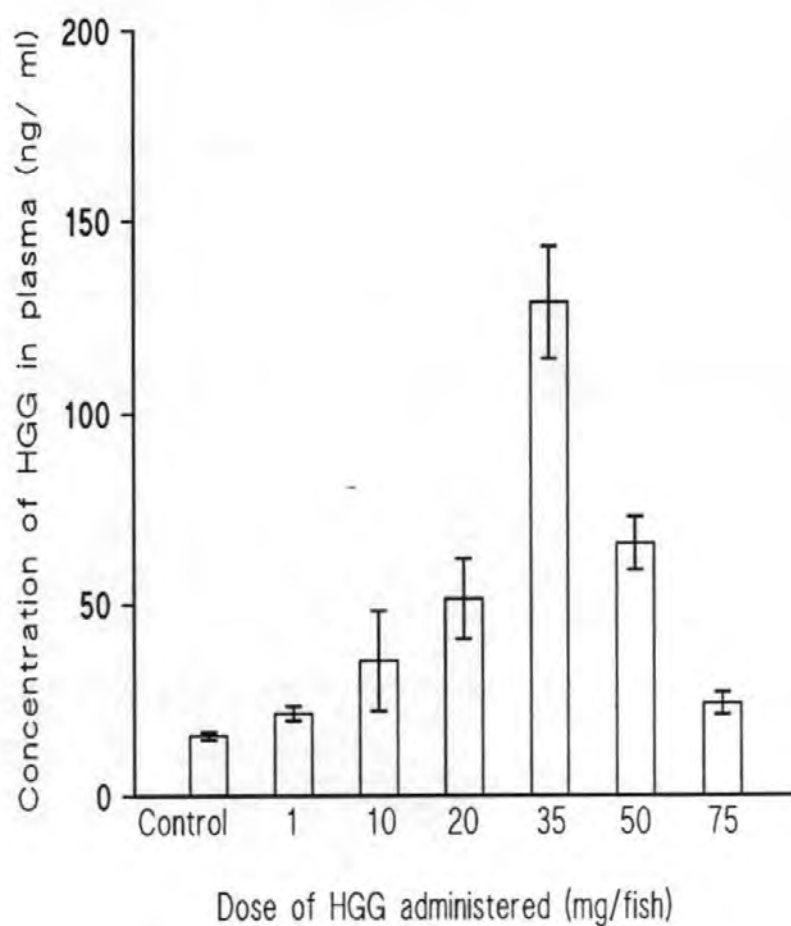
ELISA analysis of plasma HGG levels demonstrated a biphasic pattern with peaks at 45 min and 6 hr after antigen delivery which were significantly greater ($p < 0.05$) than values detected at all other times. Immunoblotting analysis of the plasma (Table 5.14) from these fish shows the highest total HGG signal was found at 6 hr and 24 hr after antigen delivery. Plasma collected from fish 6 hr after antigen administration was also notable for the high percentage of the 10.4, 82.4 and 98.9 kDa bands present.

50 mg cimetidine: Analysis of mucosal scrapings - Immunoblotting data

Immunoblotting analysis of scrapings from the gastric region of these fish (Table 5.15) shows that the total HGG signal detected was relatively constant until up to 2 hr post - administration after which a large decrease was found. Compared with fish given 1 mg cimetidine there was a greater amount of HGG fragments of 30-65 kDa (60.1% of the total HGG detected as compared with 30.6% in fish given 1 mg cimetidine) and an absence of a fragment of approximately 10 kDa. The highest levels of HGG in scrapings from the pyloric caecae of these fish were found at 2 hr and 12 hr after antigen delivery, the greatest number of bands (14) were also detected at 2 hr,

again possibly a consequence of gastric evacuation (Table 5.16). It is noteworthy as in Table 5.10 that very little HGG in any form < 50 kDa was detected in the pyloric caecae of these fish whereas up to 50% of the HGG detected in the gastric region of the same fish was in the form of fragments < 50 kDa. In scrapings from the anterior intestine of these fish the highest total HGG signal was detected at 6 hr and 12 hr after intubation. Much of the signal detected 45 min, 1 hr and 2 hr after delivery was in the form of the 29.2 and 23.9 kDa fragments while more intact HGG was found 6 hr, 12 hr and 24 hr after delivery (Table 5.17). Finally, in scrapings from the posterior intestinal region of these fish the highest total signal was found 1 hr after antigen delivery, this being mainly in the form of the 31.7 and 38.1 kDa fragments. The 38.1 kDa fragment constituted 35 - 63.3% of the total signal in scrapings taken from fish from 15 min to 2 hr after antigen delivery after which its presence was not detected. Very little antigen in this case was detectable in scrapings from the posterior intestinal regions of fish at time points later than 12 hr after HGG administration (Table 5.18).

Fig. 5.4: HGG dose Response measured 1 hr after antigen administration



Relationship between dose of HGG orally administered to trout and levels of HGG (mean $\pm$ SE) detected by ELISA in plasma 1 hr after antigen delivery. Control represents ELISA reading from fish receiving PBS only.

Fig. 5.5(a): Dose effect of sodium bicarbonate on gastrointestinal pH

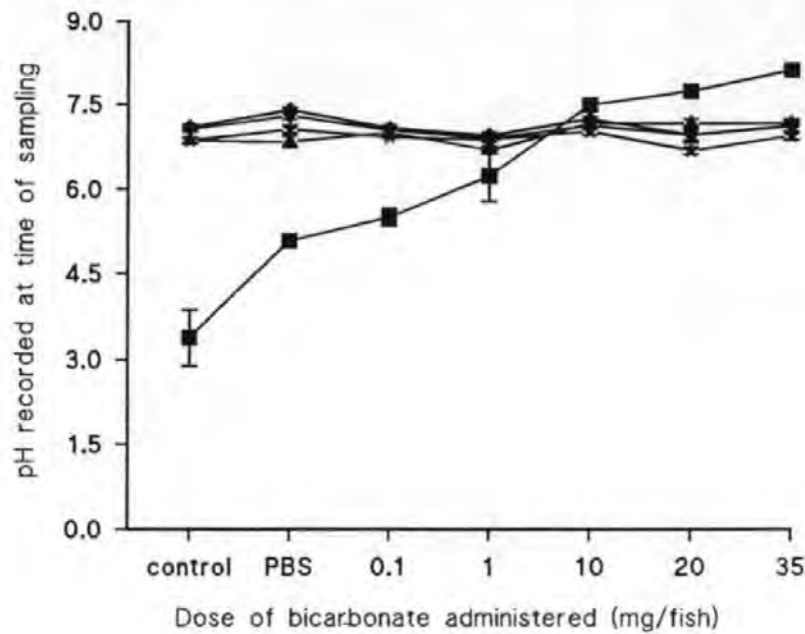


Fig.5.5(b): Bicarbonate Dose Response

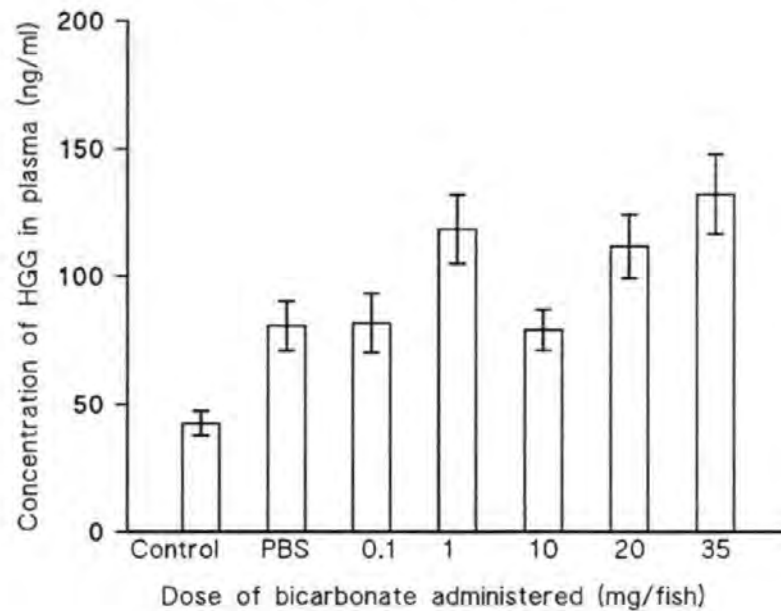


Fig. 5.5. (a): pH values (mean $\pm$ SE) recorded by pH microprobe in regions of the trout digestive tract 1 hr post HGG delivery after prior administration of sodium bicarbonate at a range of doses. Symbols used to represent gut regions in which pH was measured: ■ = Stomach. ▲ = Pyloric caecae. ▼ = Anterior intestine. ◆ = Mid intestine. ★ = Posterior intestine. **(b):** Effect of prior administration of bicarbonate at a range of doses on levels of antigen (mean $\pm$ SE) measured by ELISA in plasma of fish 1 hr after delivery of 35 mg HGG. Control represents measurements from untreated fish.

Table: 5.2: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in the plasma of fish orally intubated with 35 mg of HGG 1 hr after intubation with various doses of sodium bicarbonate.

0	0.1	1.0	10	20	35
HGG:Total (14.5%)*	HGG:Total (11.0%)*	HGG:Total (14.9%)*	HGG:Total (15.7%)*	HGG:Total (25.8%)*	HGG:Total (18.1%)*
202.1 (91.9%)	202.1 (61.9%)	202.1 (83.6%)	207.1 (42.8%)	202.1 (23.3%)	202.1 (40.9%)
	153 (23.8%)	153 (4.9%)	153 (21.8%)	153 (28.3%)	
80.5 (**)			80.5 (**)	80.5 (**)	80.5 (2.9%)
			68.8 (2.4%)	68.8 (2.2%)	68.8 (**)
48.1 (**)	48.1 (**)	48.1 (2.5%)	48.1 (**)	48.1 (1.4%)	48.1 (**)
43.2 (**)	43.2 (**)	43.2 (2.7%)	43.2 (**)	43.2 (29.6%)	43.2 (14.9%)
18.4 (**)	18.4 (**)	18.4 (3.3%)	18.4 (33%)	18.4 (2.3%)	18.4 (35.9%)
10.6 (8.1%)	10.6 (8.9%)	10.6 (3.1)		10.6 (5.6%)	10.6 (2.5%)
7.4 (**)	7.4 (5.1%)			7.4 (6.0%)	7.4 (2.9%)

* - Percentage of the entire amount of HGG detected on immunoblot present in lane.

** - Fragment present but yielding a value < 10 (arbitrary units) by image analysis.

Fig.5.6(a): Dose effect of cimetidine on gastrointestinal pH

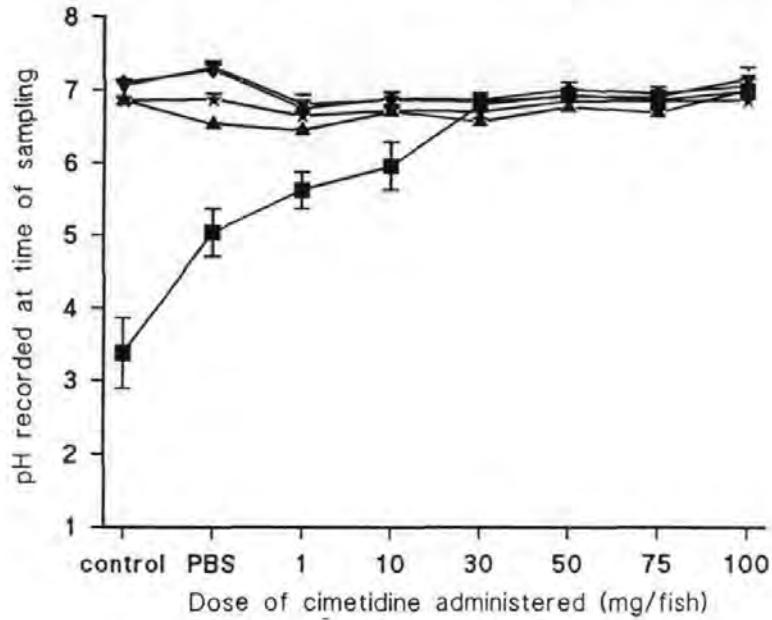


Fig.5.6 (b) : Cimetidine Dose Response

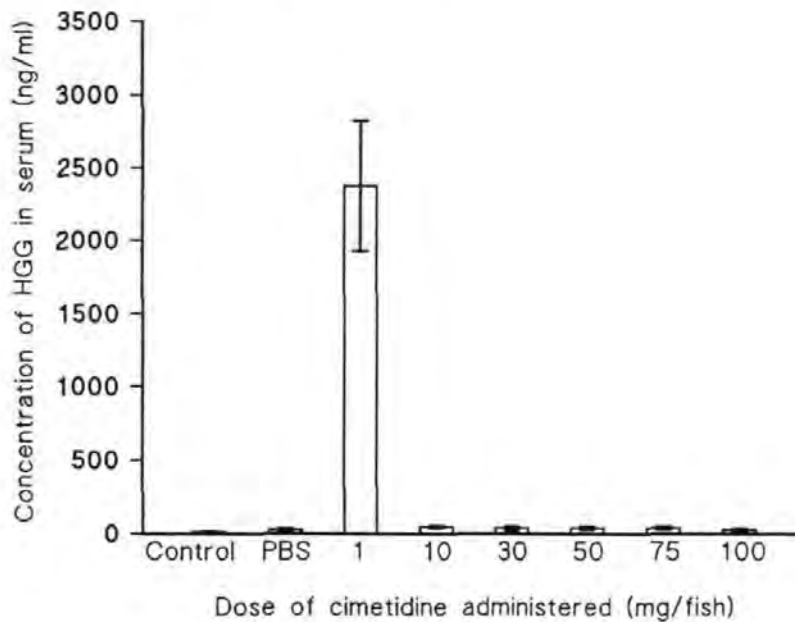


Fig. 5.6. (a): pH values (mean $\pm$ SE) recorded by pH microprobe in regions of the trout digestive tract 1 hr post HGG delivery after prior administration of cimetidine at a range of doses. Symbols used to represent gut regions in which pH was measured: ■ = Stomach. ▲ = Pyloric caecae. ▼ = Anterior intestine. ◆ = Mid intestine. ★ = Posterior intestine. **(b):** Effect of prior administration of cimetidine at a range of doses on levels of antigen (mean $\pm$ SE) measured by ELISA in plasma of fish 1 hr after delivery of 35 mg HGG. Control represents measurements from untreated fish.

Table 5.3: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in the plasma of fish orally intubated with 35 mg of HGG 1 hr after intubation with various doses of cimetidine.

0 mg	1.0 mg	10 mg	30 mg	50 mg	75 mg	100 mg
HGG:Total (6.0%) *	HGG:Total (13.2%) *	HGG:Total (5.7%) *	HGG:Total (8.8%) *	HGG:Total (13%) *	HGG:Total (39.3%) *	HGG:Total (14%) *
207.9 (100%)	207.9 (57.2%)	207.9 (76.8%)	207.9 (52.0%)	207.9 (48.7%)	207.9 (28.6%)	207.9 (54.7%)
187.0 (**)					187.0 (**)	
					151.8 (**)	
	73.5 (**)				73.5 (2.9%)	
					56.1 (4.7%)	
48.1 (**)	48.1 (**)	48.1 (**)	48.1 (**)	48.1 (**)	48.1 (7.0%)	
42.1 (**)	42.1 (15.5%)	42.1 (**)	42.1 (**)	42.1 (**)	42.1 (2.1%)	
			20.4 (31.6%)	20.4 (28.1%)	20.4 (33.4%)	20.4 (15.5%)
18.9 (**)	18.9 (14.3%)	18.9 (23.2%)	18.9 (16.4%)	18.9 (23.2%)	18.9 (21.3%)	18.9 (29.7%)
	10.2 (13%)				10.2 (**)	
	8.2 (**)					

* - Percentage of the entire amount of HGG detected on immunoblot present in lane.

** - Fragment present but yielding a value < 10 (arbitrary units) by image analysis.

Fig. 5.7: Time course of HGG absorption into plasma

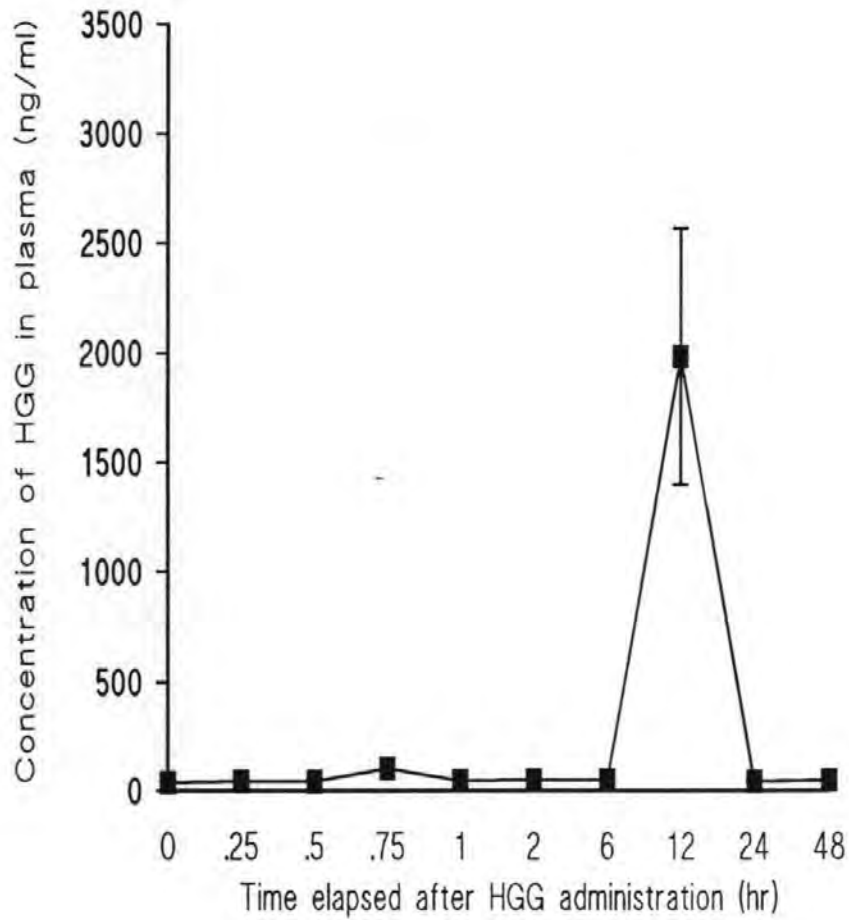


Fig. 5.7: Levels of HGG (mean $\pm$ SE) measured by ELISA over a 48 hr time course in plasma of fish orally intubated with 35 mg HGG 1 hr after administration of 0.2 ml PBS. The ELISA reading at time 0 was from fish which received PBS only.

Table. 5.4: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in the plasma of fish orally intubated with 35 mg of HGG 1 hr after intubation with control PBS.

15 min	45 min	1 hr	2 hr	6 hr	12 hr	24 hr	48 hr
HGG:Total (15.1%) *	HGG:Total (17.2%) *	HGG:Total (16.6%) *	HGG:Total (10.1%) *	HGG:Total (5.0%) *	HGG:Total (14.0%) *	HGG:Total (16.9%) *	HGG:Total (5.1%) *
198.6 (17.5%)	198.6 (29.5%)	198.6 (35.7%)	198.6 (50.5%)	198.6 (100%)	198.6 (80.4%)	198.6 (33.1%)	198.6 (51.0%)
	152.3 (**)				152.3 α Fc (19.6%)	152.3 (8.7%)	
108.1 (15.1%)	108.1 (**)	108.1 (9.1%)		108.1 α Fc (**)	108.1 α Fc (**)	108.1 α Fc (26.4%)	108.1 α Fc (7.7%)
		76.6 (14.2%)	76.6 α Fc (**)			76.6 (9.0%)	76.6 (10.0%)
48.2 α Fab (29.5%) α Fc	48.2 α Fab (31.2%) α Fc	48.2 α Fc (31.9%)	48.2 α Fab (39.5%) α Fc			48.2 α Fc (12.3%)	48.2 (24.5%)
40.7 (26.9%)	40.7 (24.6%)	40.7 (**)	40.7 α Fc (**)	40.7 (**)	40.7 (**)		
18.2 α Fab (11.0%)	18.2 α Fab (14.7%) α Fc	18.2 (9.1%)	18.2 α Fc (**)	18.2 (**)	18.2 α Fab (**)	18.2 α Fc (10.5%)	18.2 (6.7%)
10.2 (**)	10.2 (**)			10.2 (**)		10.2 (**)	
						8.4 (**)	

* - Percentage of total amount of HGG detected on immunoblot present in lane

** - Band present but yielding a value < 10 (arbitrary units) by image analysis

α Fc - Detected using antisera to the Fc region of HGG.

α Fab - Detected using antisera to the Fab region of HGG.

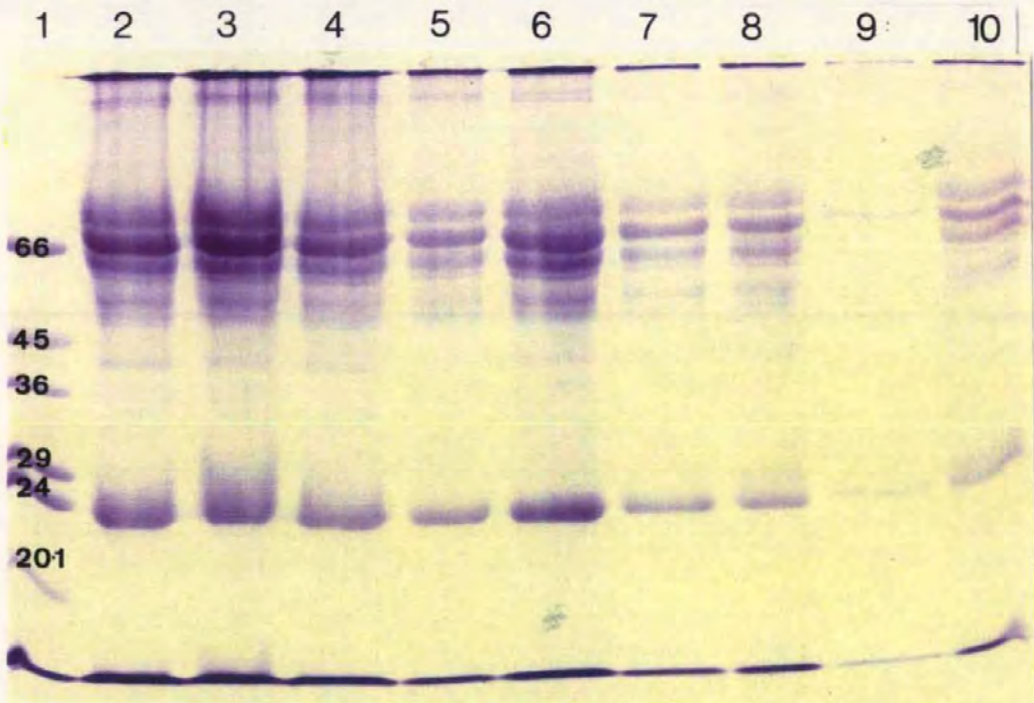


Fig. 5.8.(a): Coomassie Blue stained PAGE gel showing the presence of proteins in plasma samples collected from fish which were orally intubated with 35 mg of sodium bicarbonate 1 hr before administration of 35 mg HGG. Loading order. Lane 1, 7H molecular weight markers. Lane 2, control (plasma from fish intubated with control PBS). Lanes 3 - 10, samples collected from fish at 15 min, 45 min, 1 hr, 2 hr, 6 hr, 12 hr, 24 hr and 48 hr after HGG delivery.

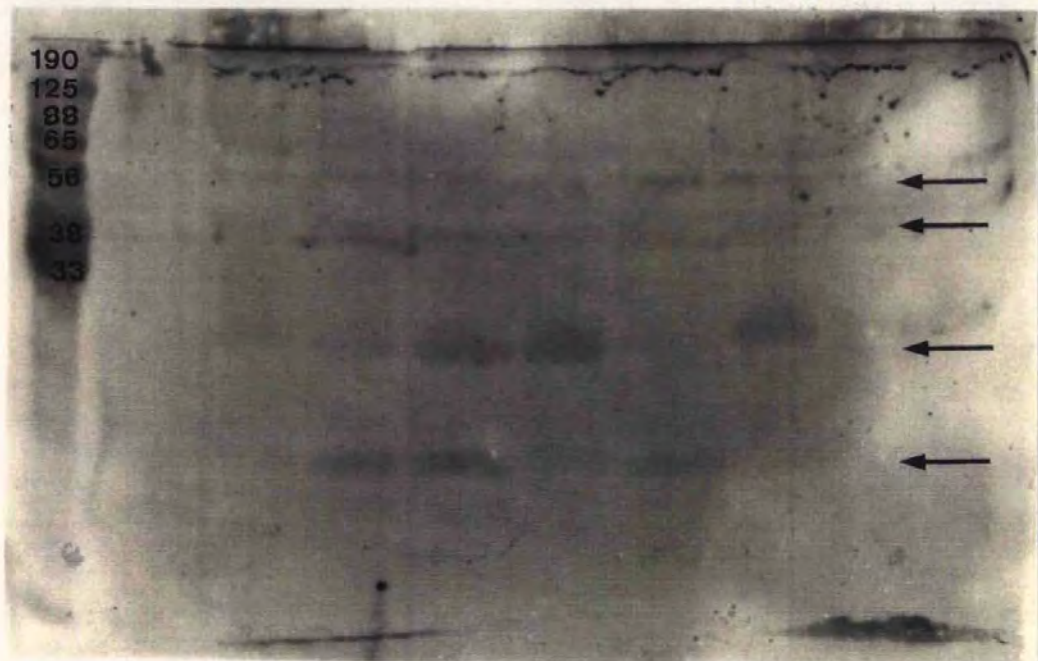


Fig. 5.8.(b): Corresponding immunoblot to the PAGE gel in (a) above showing the presence of HGG in the plasma. Loading order is identical to the above except lane 1 contains prestained molecular weight markers. The HGG bands present are arrowed.

Fig 5.9(a): Effect of bicarbonate (35 mg/fish) on gastrointestinal pH

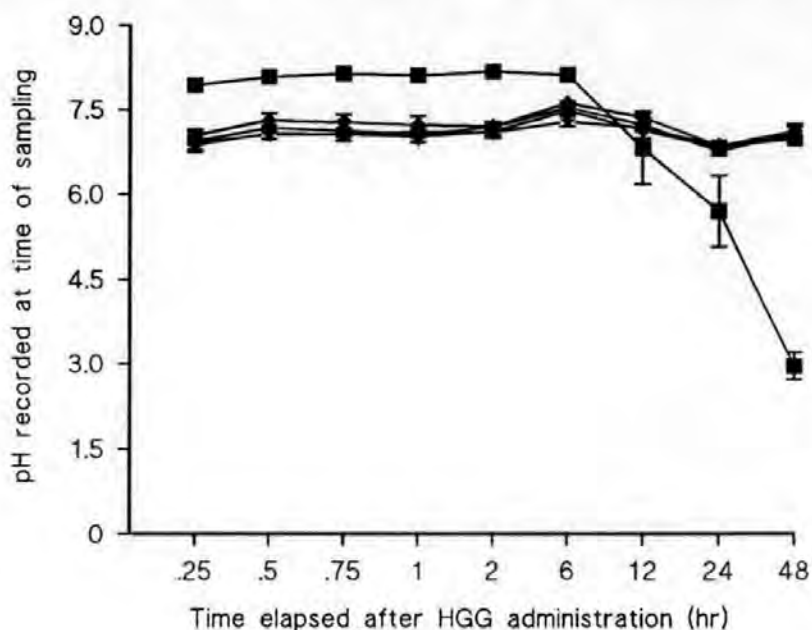


Fig. 5.9(b): Effect of bicarbonate (35 mg/fish) on HGG absorption into plasma

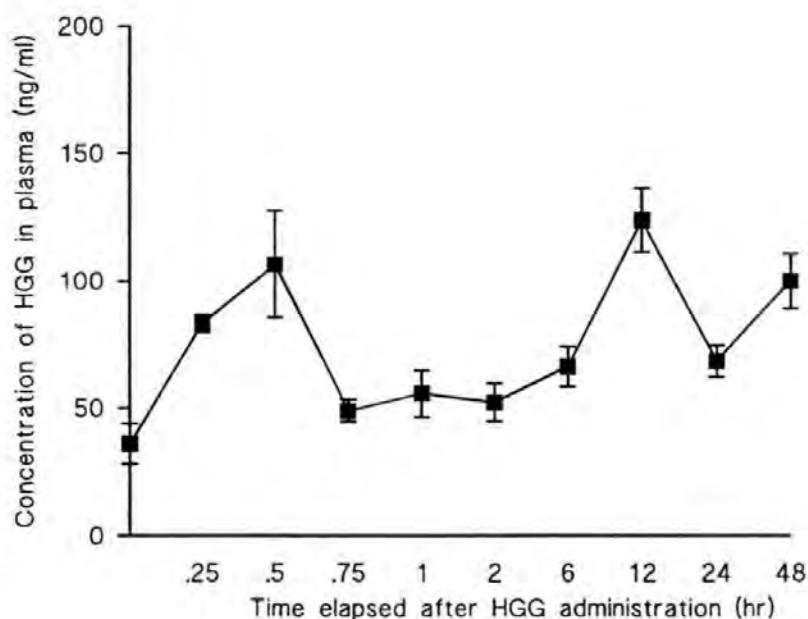


Fig. 5.9.(a): pH values (mean $\pm$ SE) recorded by pH microprobe in regions of the trout digestive tract over a 48 hr time course post HGG delivery after prior administration of 35 mg of sodium bicarbonate. Symbols used to represent gut regions in which pH was measured: ■ = Stomach. ▲ = Pyloric caecae. ▼ = Anterior intestine. ◆ = Mid intestine. ★ = Posterior intestine. **(b):** Effect of prior administration of 35 mg of sodium bicarbonate on levels of antigen (mean $\pm$ SE) measured by ELISA in plasma of fish over a 48 hr time course after delivery of 35 mg HGG. ELISA reading at time 0 was from fish receiving PBS only

Table. 5.5: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in the plasma of fish orally intubated with 35 mg HGG 1 hr after intubation with 35 mg sodium bicarbonate.

15 min	45 min	1 hr	2 hr	6 hr	12 hr	24 hr	48 hr
HGG:Total (5.8%) *	HGG:Total (11.8%) *	HGG:Total (13.4%) *	HGG:Total (22.6%) *	HGG:Total (16.5%) *	HGG:Total (22.3%) *	HGG:Total (4.1%) *	HGG:Total (3.5%) *
198.2 α Fc (100%)	198.2 α Fab (**) α Fc	198.2 α Fab (**) α Fc	198.2 α Fab (6.0%) α Fc	198.2 α Fab (12.6%) α Fc	198.2 α Fab (5.2%) α Fc	198.2 α Fab (100%) α Fc	198.2 α Fab (**) α Fc
		63.0 α Fc (**)	63.0 α Fc (**)	63.0 α Fc (**)	63.0 α Fc (**)		
				49.5 (20.6%)	49.5 (8.7%)		49.5 (100%)
47.0 α Fc (**)	47.0 (**)	47.0 α Fc (**)	47.0 α Fc (**) -	47.0 α Fc (**)	47.0 α Fc (**)		
45.5 α Fc (**)	45.5 α Fab (17%) α Fc	45.5 α Fab α Fc	45.5 α Fab (8.5%) α Fc	45.5 α Fab (16.5%) α Fc	45.5 α Fab (13.8%) α Fc		
	37.5 (11.8%)						
18.4 (**)	18.4 α Fc (**)	18.4 α Fab (42.5%) α Fc	18.4 α Fab (56.3%) α Fc	18.4 α Fab (15.3%) α Fc	18.4 α Fc (54.7%)		
				10.9 (23.7%)	10.9 (9.3%)		
	10.2 α Fab (48%) α Fc	10.2 α Fab (57.5%) α Fc	10.2 α Fc (12.9%)	10.2 (11.3%)	10.2 (8.3%)		
		7.5 α Fab α Fc	7.5 α Fc				

* - Percentage of total amount of HGG detected on immunoblot present in lane

** - Band present but yielding a value < 10 (arbitrary units) by image analysis

α Fc - Detected using antisera to the Fc region of HGG.

α Fab - Detected using antisera to the Fab region of HGG.

Table. 5.6: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in gastric scrapings of fish orally intubated with 35 mg HGG 1 hr after 35 mg bicarbonate.

15 min	30 min	45 min	1 hr	2 hr	6 hr	12 hr	24 hr	48 hr
HGG:Total (20.9%) *	HGG:Total (17.9%) *	HGG:Total (18.2%) *	HGG:Total (11.9%) *	HGG:Total (11.4%) *	HGG:Total (12.5%) *	HGG:Total (7.2%) *	HGG:Total (0.0%) *	HGG:Total (0.0%) *
157 (20.8%)	157 (31%)	157 (31.4%)	157 (34.2%)	157 (34.9%)	157 (27.0%)	157 (38.3%)		
133.1 (2.0%)	133.1 (**)	133.1 (**)	133.1 (3.2%)	133.1 (**)	133.1 (**)	133.1 (**)		
114 (**)	114 (**)	114 (**)	114 (2.0%)	114 (**)	114 (16.3%)	114 (17.8%)		
107.5 (10.6%)	107.5 (14.2%)	107.5 (13.3%)		107.5 (16.3%)				
77.3 (**)		77.3 (2.1%)		77.3 (1.1%)				
71.5 (3.0%)			71.5 (4.7%)	71.5 (0.8%)	71.5 (2.3%)			
65.3 (3.0%)	65.3 (12.2%)	65.3 (6.7%)		65.3 (1.7%)		65.3 (3.9%)		
		60.8 (2.6%)				60.8 (1.8%)		
52.7 (26.6%)	52.7 (24.1%)	52.7 (25.7%)	52.7 (33.3%)	52.7 (30.5%)	52.7 (25.4%)	52.7 (36.2%)		
		46.1 (**)	46.1 (**)	46.1 (6.0%)	46.1 (7.0%)	46.1 (**)		
39.7 (2.0%)	39.7 (**)	37.9 (**)	39.7 (**)	39.7 (**)				
37.8 (1.4%)	37.8 (**)	37.8 (**)	37.8 (**)	37.8 (**)				
33.2 (2.0%)	33.2 (2.9%)	33.2 (2.7%)	33.2 (**)	33.2 (1.5%)	33.2 (7.2%)	33.2 (**)		
30.5 (4.9%)								
25.9 (12.9%)	25.9 (7.9%)	25.9 (5.5%)	25.9 (6.8%)	25.9 (4.4%)	25.9 (8.0%)	25.9 (**)		
23.1 (4.6%)	23.1 (**)	23.1 (0.8%)	23.1 (**)	23.1 (**)	23.1 (**)			
20.2 (6.1%)	20.2 (7.7%)	20.2 (9.5%)	20.2 (3.6%)	20.2 (2.0%)	20.2 (5.1%)	20.2 (2.0%)		
		10.4 (**)	10.4 (**)	10.4 (1.1%)	10.4 (1.8%)			

* - Percentage of total amount of HGG on immunoblot present in lane

** - Band present but yielding a value < 10 (arbitrary units) by image analysis

Table. 5.7: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in scrapings from the intestine of fish orally intubated with 35 mg HGG 1 hr after intubation with 35 mg bicarbonate.

15 min	30 min	45 min	1 hr	2 hr	6 hr	12 hr	24 hr	48 hr
HGG:Total (2.4%) *	HGG:Total (3.3%) *	HGG:Total (18.5%) *	HGG:Total (6.0%) *	HGG:Total (19.5%) *	HGG:Total (27.8%) *	HGG:Total (18.7%) *	HGG:Total (3.5%) *	HGG:Total (0.3%) *
152.2 (34.1%)	152.2 (44.2%)	152.2 (11.8%)	152.2 (14.2%)	152.2 (12.8%)	152.2 (11.8%)	152.2 (7.9%)	152.2 (8.4%)	152.2 (40.1%)
139.0 (**)	139.0 (**)	139.0 (2.8%)	139.0 (**)	139.0 (**)	139.0 (**)	139.0 (**)	139.0 (**)	139.0 (**)
121.7 (20.2%)	121.7 (24.8%)	121.7 (10.7%)	121.7 (**)	121.7 (15.6%)	121.7 (18.9%)	121.7 (5.0%)	121.7 (3.3%)	
			91.3 (1.4%)	91.3 (**)	91.3 (13.1%)	91.3 (6.7%)	91.3 (10.0%)	
79.2 (4.2%)	79.2 (1.6%)		79.2 (1.1%)	79.2 (**)		79.2 (2.3%)		
							72.0 (3.8%)	
	69.5 (2.0%)	69.5 (4.5%)	69.5 (2.2%)	69.5 (**)	69.5 (2.8%)	69.5 (6.4%)		
55.5 (36.5%)	55.5 (1.5%)	55.5 (18.7%)	55.5 (21.6%)	55.5 (18.0%)	55.5 (15.3%)	55.5 (7.8%)	55.5 (8.6%)	
47.6 (5.0%)	47.6 (26%)	47.6 (8.0%)	47.6 (14.6%)	47.6 (12.9%)	47.6 (8.0%)	47.6 (14.2%)	47.6 (32.0%)	47.6 (16.7%)
		41.9 (2.8%)		41.9 (**)	41.9 (**)	41.9 (7.8%)	41.9 (13.5%)	41.9 (43.1%)
		34.7 (9.1%)	34.7 (10.6%)	34.7 (12.4%)	34.7 (10.9%)	34.7 (15.7%)	34.7 (14.2%)	34.7 (**)
		27.3 (19.3%)	27.3 (9.8%)	27.3 (18.9%)	27.3 (17.0%)	27.3 (8.5%)		
		18.5 (4.4%)	18.5 (5.5%)	18.5 (7.9%)	18.5 (10.2%)	18.5 (9.5%)		
		16.5 (1.7%)		16.5 (1.2%)	16.5 (**)			
				14.2 (0.3%)	14.2 (0.3%)			
		10.4 (6.2%)	10.4 (5.2%)	10.4 (**)	10.4 (11.8%)	10.4 (8.2%)	10.4 (6.5%)	

* - Percentage of total amount of HGG on immunoblot present in lane

** - Band present but yielding a value < 10 (arbitrary units) by image analysis

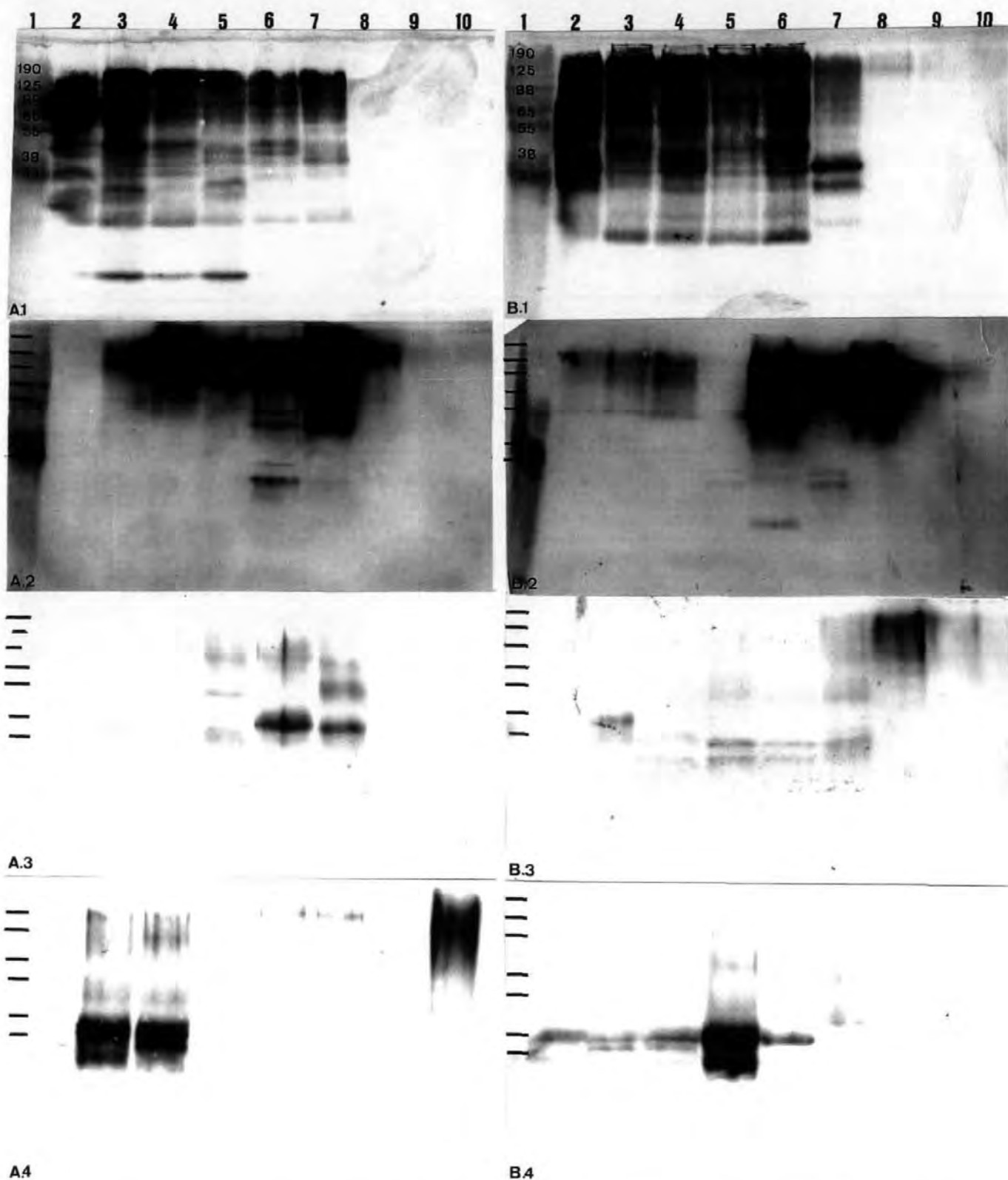


Fig.5.10: Photographic representations of immunoblots demonstrating the presence of HGG in regions of the rainbow trout gut at various times after administration of HGG and prior administration of cimetidine. A (1-4), scrapings from the stomach, pyloric caecae, anterior intestine and posterior intestine respectively of fish orally intubated with 1 mg cimetidine 1 hr before delivery of 35 mg HGG. B(1-4), scrapings from the stomach, pyloric caecae, anterior intestine and posterior intestine respectively of fish orally intubated with 50 mg cimetidine 1 hr before delivery of 35 mg HGG. Loading order. Lane 1, prestained molecular weight markers (molecular weights in kDa). Lanes 2-10, scrapings collected from fish at 15 min, 30 min, 45 min, 1 hr, 2 hr, 6 hr, 12 hr, 24 hr and 48 hr after HGG administration. Bars on prestained marker

Fig 5.11(a): Effect of cimetidine (1 mg/fish) on gastrointestinal pH

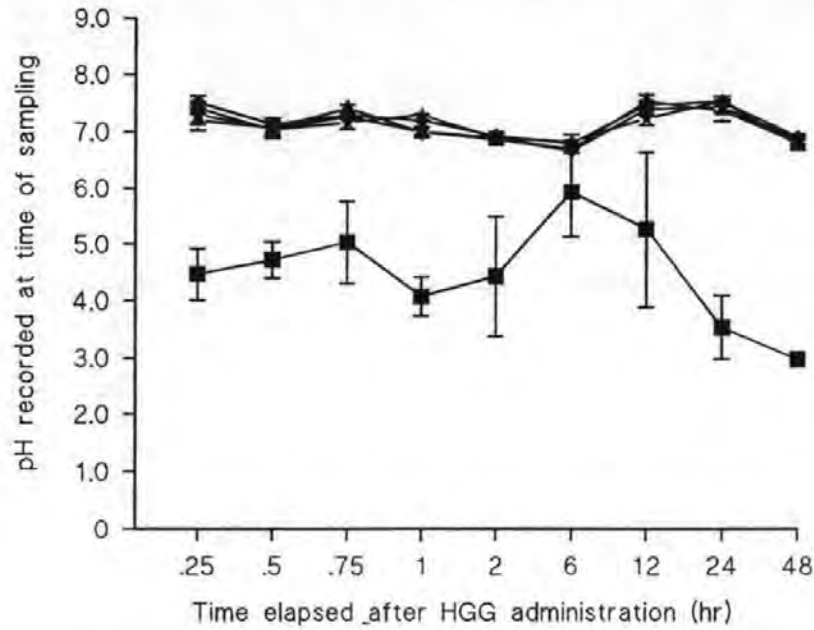


Fig.5.11(b): Effect of cimetidine (1 mg/fish) on absorption of HGG into plasma

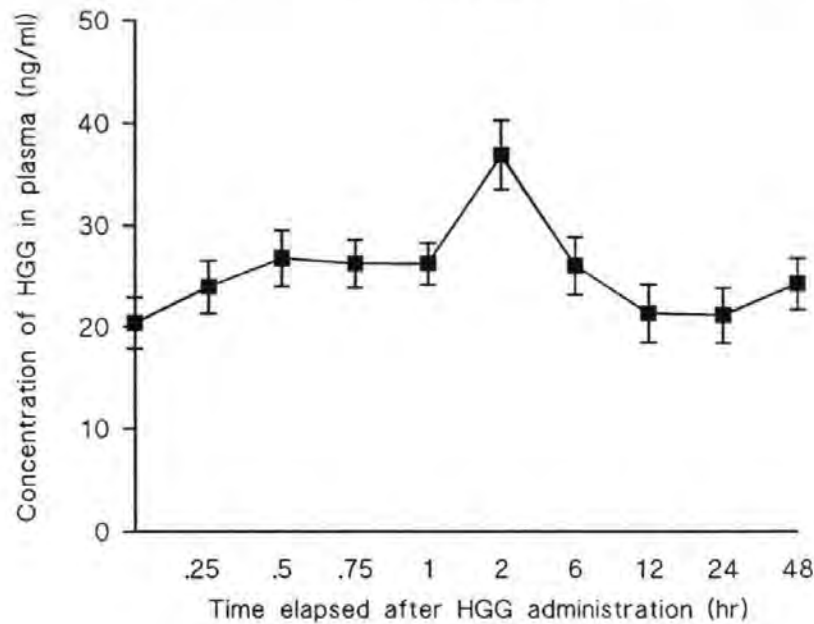


Fig. 5.11.(a): pH values (mean $\pm$ SE) recorded by pH microprobe in regions of the trout digestive tract over a 48 hr time course post HGG delivery after prior administration of 1 mg cimetidine. Symbols used to represent gut regions in which pH was measured: ■ = Stomach, ▲ = Pyloric caecae, ▼ = Anterior intestine, ◆ = Mid intestine, ★ = Posterior intestine. **(b):** Effect of prior administration of 1 mg cimetidine on levels of antigen (mean $\pm$ SE) measured by ELISA in plasma of fish over a 48 hr time course after delivery of 35 mg HGG. ELISA reading at time 0 was from fish which received control PBS only.

Table. 5.8: Apparent molecular (kDa) weights and relative percentages of HGG bands detected in the plasma of fish orally intubated with 35 mg HGG 1 hr after intubation with 1 mg cimetidine.

15 min	45 min	1 hr	2 hr	6 hr	12 hr	24 hr	48 hr
HGG:Total (4.4%) *	HGG:Total (7.3%) *	HGG:Total (14.8%) *	HGG:Total (19.2%) *	HGG:Total (12.3%) *	HGG:Total (20.5%) *	HGG:Total (8.6%) *	HGG:Total (12.9%) *
201.5 (26.9%)	201.5 (12.6%)	201.5 (7.4%)	201.5 (7.9%)	201.5 (23.2%)	201.5 (13.4%)	201.5 (12.0%)	201.5 (55.2%)
	151.2 (9.9%)	151.2 (7.4%)	151.2 (1.8%)	151.2 (13.8%)	151.2 (16.7%)	151.2 (19.1%)	151.2 (29%)
109.1 (**)	109.1 (**)	109.1 (**)	109.1 (**)	109.1 (**)	109.1 (2.8%)	109.1 (9.8%)	
	76.4 (**)	76.4 (**)	76.4 (**)	76.4 (**)	76.4 (2.9%)		
			63.2 (**)		63.2 (2.8%)		63.2 (9.6%)
45.6 (**)	45.6 (**)	45.6 (1.2%)	45.6 (1.9%)	45.6 (**)	45.6 (**)	45.6 (**)	45.6 (**)
37.9 (**)	37.9 (7.2%)	37.9 (10.3%)	37.9 (12.5%)	37.9 (29%)	37.9 (**)		37.9 (8.2%)
30.6 (**)	30.6 (20.9%)	30.6 (7.3%)	30.6 (1.9%)		30.6 (**)		
26.9 (**)	26.9 (16.1%)	26.9 (21.7%)	26.9 (4.4%)		26.9 (15.4%)	26.9 (**)	
	27.3 (25.8%)						
	25.6 (10.5%)	25.6 (3.8%)	25.6 (11.1%)	25.6 (12%)	25.6 (38.4%)		
			18.8 (**)				
10.4 (56.7%)		10.4 (39.9%)	10.4 (57.2%)	10.4 (14.9%)	10.4 (6.0%)	10.4 (67.0%)	
7.5 (4.6%)	7.5 (**)	7.5 (**)	7.5 (1.3%)	7.5 (7.1%)	7.5 (**)		
7.1 (11.7%)							

* - Percentage of the total amount of HGG on immunoblot present in lane

** - Band present but yielding a value < 10 (arbitrary units) by image analysis

Table. 5.9: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in gastric scrapings of fish orally intubated with 35 mg HGG 1 hr after intubation with 1 mg cimetidine.

15 min	30 min	45 min	1 hr	2 hr	6 hr	12 hr	24 hr	48 hr
HGG:Total (22.8%) *	HGG:Total (26.7%) *	HGG:Total (15.3%) *	HGG:Total (12.4%) *	HGG:Total (11.9%) *	HGG:Total (10.9%) *	HGG:Total (0.0%) *	HGG:Total (0.0%) *	HGG:Total (0.0%) *
152.4 (12.2%)	152.4 (16.8%)	152.4 (24.4%)	152.4 (27.2%)	152.4 (25.6%)	152.4 (42.8%)			
	97.6 (1.6%)		97.6 (3.7%)		97.6 (9.5%)			
	87.1 (3.8%)	87.1 (7.0%)		87.1 (11.1%)	87.1 (10.5%)			
75.6 (18%)	75.6 (6.1%)	75.6 (13.7%)	75.6 (12.9%)	75.6 (19.6%)	75.6 (5.3%)			
	69.3 (1.7%)							
65.9 (2.7%)	65.9 (3.2%)	65.9 (3.0%)	65.9 (14.1%)	65.9 (16.7%)	65.9 (**)			
63.4 (8.8%)	63.4 (10.8%)	63.4 (9.1%)						
60.1 (12.9%)	60.1 (**)	60.1 (**)	60.1 (**)	60.1 (**)	60.1 (**)			
			55.4 (3.1%)		55.4 (6.5%)			
	52.7 (5.8%)	52.7 (2.9%)		52.7 (1.3%)				
47.0 (8.9%)	47.0 (15.4%)	47.0 (17.1%)	47.0 (4.9%)	47.0 (10.3%)	47.0 (6.3%)			
	43.6 (**)	43.6 (**)	43.6 (4.9%)	43.6 (8.4%)	43.6 (3.6%)			
39.5 (4.8%)	39.5 (2.6%)		39.5 (4.4%)	39.5 (3.1%)	39.5 (12.2%)			
	36.0 (2.3%)	36.1 (1.4%)	36.0 (0.6%)					
33.6 (9.9%)	33.6 (5.6%)	33.6 (5.7%)	33.6 (2.0%)	33.6 (1.7%)	33.6 (2.9%)			
29.0 (6.1%)	29.0 (10.4%)	29.0 (1.9%)	29.0 (6.3%)		29.0 (0.3%)			
25.6 (3.2%)	25.6 (**)	25.6 (1.5%)	25.6 (3.8%)	25.6 (0.7%)				
21.6 (3.0%)	21.6 (2.0%)	21.6 (2.5%)	21.6 (0.7%)					
19.5 (6.6%)	19.5 (4.7%)	19.5 (6.5%)	19.5 (2.7%)	19.5 (1.4%)	19.5 (2.2%)			
	14.5 (0.9%)	14.5 (**)						
	12.2 (0.5%)	12.2 (**)						
10.2 (2.9%)	10.2 (5.7%)	10.2 (3.3%)	10.2 (7.8%)					

* - Percentage of total amount of HGG detected on immunoblot present in lane

** - Band present but yielding a value < 10 (arbitrary units) by image analysis

Table. 5.10: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in scrapings from the pyloric caecae of fish orally intubated with 35 mg HGG 1 hr after intubation with 1 mg cimetidine.

15 min	30 min	45 min	1 hr	2 hr	6 hr	12 hr	24 hr	48 hr
HGG:Total 0.0% *	HGG:Total 10.2%*	HGG:Total (24.9%)*	HGG:Total (10.6%)*	HGG:Total (18.7%)*	HGG:Total (27.7%)*	HGG:Total (7.9%)*	HGG:Total (0.0%) *	HGG:Total (0.0%) *
	148.3 (32.2%)	148.3 (87.4%)	148.3 (76.7%)	148.3 (61.7%)	148.3 (33.7%)	148.3 (86.4%)		
					113.8 (12.2%)			
	110.0 (60.5%)				110.0 (**)			
					90.0 (14.3%)			
					75.1 (15.4%)			
			66.8 (**)	66.8 (**)	66.8 (**)	66.8 (5.0%)		
					65 (17.3%)			
	62.3 (2.3%)		62.3 (**)	62.3 (**)	62.3 (**)	62.3 (1.8%)		
	58.6 (2.2%)	58.6 (12.6%)	58.6 (4.0%)	58.6 (**)	58.6 (7.1%)			
		56.7 (**)	56.7 (19.2%)	56.7 (**)	56.7 (**)			
	52.0 (2.8%)	52.0 (**)		52.0 (14.3%)	52.0 (**)			
			45.3 (**)	45.3 (13.7%)	45.3 (**)			
	42.1 (**)	42.1 (**)		42.1 (**)	42.1 (**)			
				30.7 (**)	30.7 (**)			
				26.1 (8.9%)	26.1 (**)			
				22.2 (1.6%)	22.2 (**)			

* - Percentage of total amount of HGG detected on immunoblot present in lane

** - Band present but yielding a value < 10 (arbitrary units) by image analysis

Table. 5.11: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in the anterior portion of the intestine of fish orally intubated with 35 mg HGG 1 hr after intubation with 1 mg cimetidine.

15 min	30 min	45 min	1 hr	2 hr	6 hr	12 hr	24 hr	48 hr
HGG:Total (0.0%) *	HGG:Total (5.9%)*	HGG:Total (4.0%)*	HGG:Total (21.6%)*	HGG:Total (30.9%)*	HGG:Total (35.3%)*	HGG:Total (2.3%)*	HGG:Total (0.0%) *	HGG:Total (0.0%) *
		154.1 (**)	154.1 (**)		154.1 (3.5%)			
					135 (3.2%)	135 (**)		
			123.9 (3.6%)					
					109.5 (2.1%)			
			104 (7.0%)					
					99.8 (1.5%)			
				92.6 (9.5%)				
		85.5 (**)						
			80.3 (21.6%)	80.3 (6.3%)	80.3 (5.9%)			
				75.5 (18.2%)	75.5 (4.4%)			
			63 (5.0%)					
					60.4 (14.1%)			
					58.3 (12.6%)			
			52.2 (17.4%)		52.2 (**)			
	36.4 (91%)			36.4 (66%)	36.4 (46.6%)	36.4 (69%)		
					35.7 (**)			
			33.9 (18.4%)			33.9 (31%)		
	32.7 (9.0%)		32.7 (12.9%)	32.7 (**)	32.7 (**)			
		31.6 (38.3%)			31.6 (1.2%)			
		29.6 (61.7%)	29.6 (14.2%)		29.6 (3.0%)			
					28.2 (2.0%)			

* - Percentage of total amount of HGG detected on immunoblot present in lane

** - Band present but yielding a value < 10 (arbitrary units) by image analysis

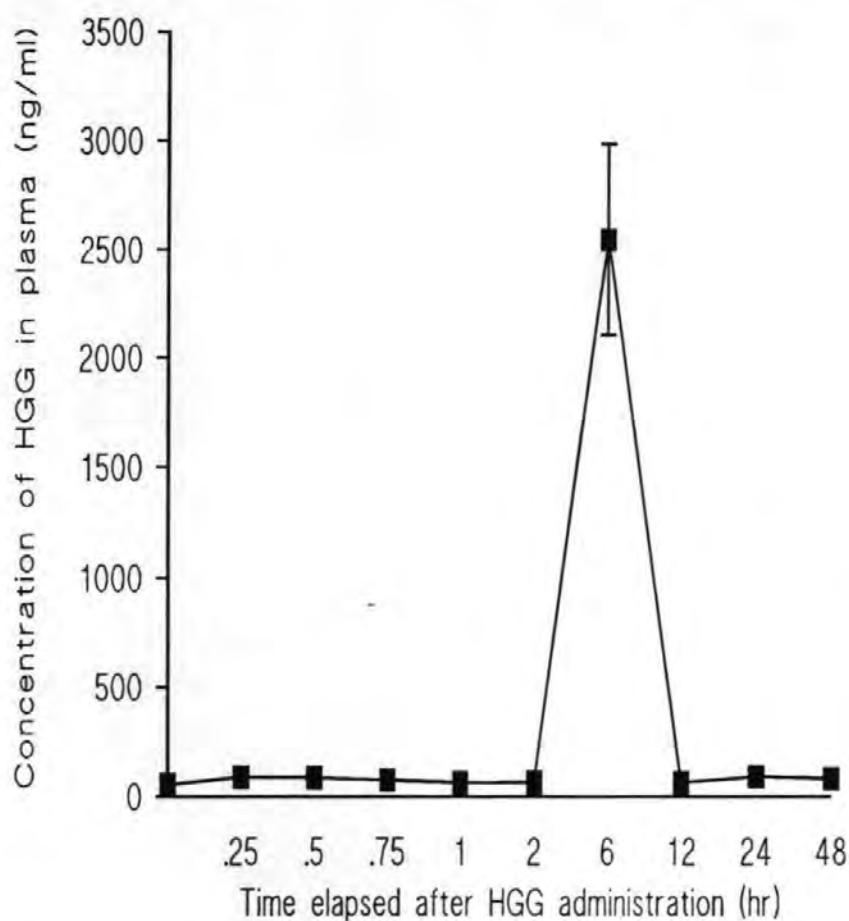
Table. 5.12: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in scrapings from the posterior portion of the intestine of fish orally intubated with 35 mg HGG 1 hr after intubation with 1 mg cimetidine.

15 min	30 min	45 min	1 hr	2 hr	6 hr	12 hr	24 hr	48 hr
HGG:Total (7.6%) *	HGG:Total (36.6%) *	HGG:Total (30.8%) *	HGG:Total (0.0%) *	HGG:Total (0.3%) *	HGG:Total (1.5%) *	HGG:Total (2.3%) *	HGG:Total (20.9%) *	HGG:Total (0.0%) *
				149.8 (100%)	149.8 (100%)		149.8 (38.4%)	
	134.7 (0.7%)							
	98.4 (9.6%)	98.4 (6.3%)						
				90.8 (**)	90.8 (**)		90.8 (15.9%)	
83.8 (5.7%)	83.8 (3.8%)	83.8 (18.9%)						
							77.6 (33.1%)	
	73.3 (10.2%)							
				71.2 (**)	71.2 (**)			
						66.2 (50.5%)		
	64.8 (1.4%)			64.8 (**)				
		59.6 (4.4%)		59.6 (**)				
	56.5 (9.7%)				56.5 (**)	56.5 (11.5%)	56.5 (5.8%)	
						53.8 (7.1%)		
51.4 (11.9%)	51.4 (7.9%)	51.4 (12.9%)				51.4 (23.7%)	51.4 (2.0%)	
							48.4 (4.6%)	
				46.8 (**)	46.8 (**)	46.8 (8.2%)		
				42.7 (**)				
39.7 (18.2%)								
					37.9 (**)			
37.5 (17.7%)	37.5 (37.5%)	37.5 (34.6%)						
34.9 (27%)		34.9 (23%)		34.9 (**)	34.9 (**)			
	33.1 (19.3%)			33.1 (**)				
31.3 (19.4%)		31.3 (**)						
				29.3 (**)				

- Percentage of total amount of HGG detected on immunoblot present in lane

** - Band present but yielding a value < 10 (arbitrary units) by image analysis

Fig.5.12: Effect of cimetidine (10 mg/fish) on absorption of HGG into plasma.



Effect of prior administration of 10 mg cimetidine on levels of HGG (mean $\pm$ SE) measured by ELISA in plasma of fish over a 48 hr time course after administration of 35 mg HGG. ELISA reading at time 0 was from fish which received PBS only.

Table. 5.13: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in the plasma of fish orally intubated with 35 mg HGG 1 hr after intubation with 10 mg cimetidine.

15 min	45 min	1 hr	2 hr	6 hr	12 hr	24 hr	48 hr
HGG:Total (6.6%) *	HGG:Total (10.7%) *	HGG:Total (27.6%) *	HGG:Total (16.7%) *	HGG:Total (16.2%) *	HGG:Total (16.3%) *	HGG:Total (4.6%) *	HGG:Total (1.3%) *
201.4 α Fc (1.9%)	201.4 α Fc (10.0%)	201.4 α Fab (10.2%) α Fc	201.4 α Fab (14.5%) α Fc	201.4 α Fab (0.0%) α Fc	201.4 α Fab (4.2%) α Fc	201.4 α Fab (41.3%) α Fc	201.4 α Fab (100%) α Fc
152.2 (46.3%)	152.2 (27.0%)	152.2 (**)	152.2 (13.3%)	152.2 α Fc (19.7%)			
	107.4 (14.1%)	107.4 (31.4%)	107.4 (13.1%)	107.4 α Fc (16.4%)			
76.2 (17.3%)		76.2 (10%)	76.2 (20.3%)	76.2 (10.8%)			
47.6 (5.1%)	47.6 α Fc (**)	47.6 α Fc (16.4%)	47.6 (20.1%)		47.6 (19.8%)		
40.3 α Fc (29.5%)	40.3 α Fc (14.2%)		40.3 (**)	40.3 α Fc (38.9%)	40.3 (12.5%)		
	37.8 (8.0%)		37.8 α Fc (**)	37.8 (14.2%)	37.8 α Fc (24.2%)		
			18.5 (17.8%)		18.5 (29.8%)	18.5 (25.6%)	
	10.8 (27.7%)	10.8 α Fab (31.9%) α Fc			10.8 α Fab (9.5%) α Fc	10.8 (33.2%)	
		7.6 α Fab (**) α Fc	7.6 α Fc (**)		7.6 α Fab (**) α Fc		

* - Percentage of total amount of HGG on immunoblot present in lane.

** - Band present but yielding value < 10 (arbitrary units) by image analysis.

Fig 5.13(a): Effect of cimetidine (50 mg/fish) on gastrointestinal pH

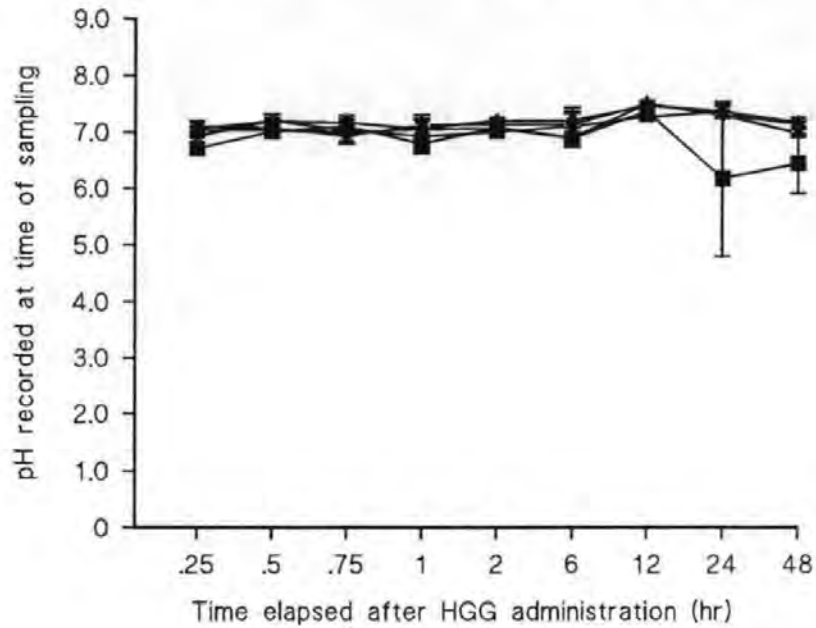


Fig.5.13(b): Effect of cimetidine (50 mg/fish) on absorption of HGG into plasma

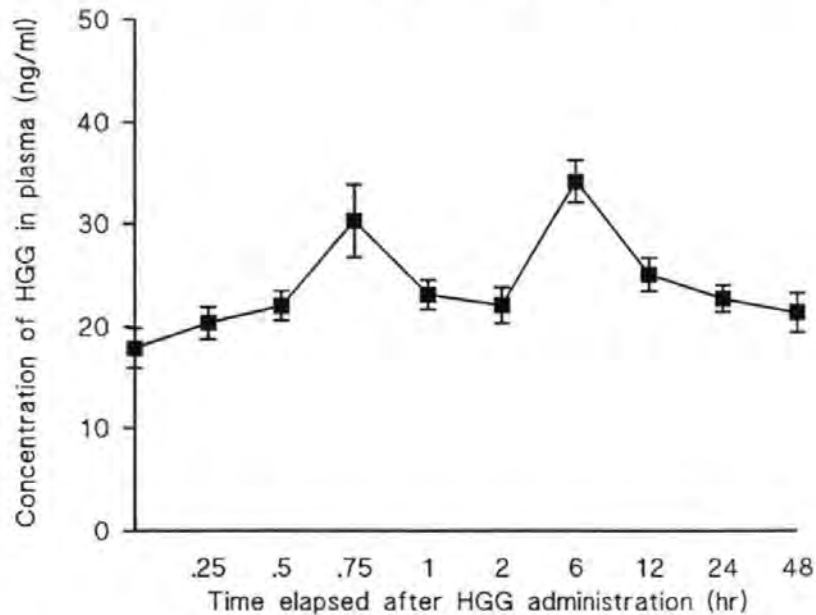


Fig.5.13.(a): pH values (mean $\pm$ SE) recorded by pH microprobe in regions of the trout digestive tract over a 48 hr time course post HGG delivery after prior administration of 50 mg cimetidine. Symbols used to represent gut regions in which pH was measured: ■ = Stomach, ▲ = Pyloric caecae, ▼ = Anterior intestine, ◆ = Mid intestine, ★ = Posterior intestine. **(b):** Effect of prior administration of 50 mg cimetidine on levels of antigen (mean $\pm$ SE) measured by ELISA in plasma of fish over a 48 hr time course after delivery of 35 mg HGG. ELISA reading at time 0 was from fish which received PBS only.

Table. 5.14: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in plasma of fish orally intubated with 35 mg HGG 1 hr after intubation with 50 mg cimetidine.

15 min	45 min	1 hr	2 hr	6 hr	12 hr	24 hr	48 hr
HGG:Total (0.0%)*	HGG:Total (0.0%)*	HGG:Total (9.4%)*	HGG:Total (0.0%)*	HGG:Total (26.3%)*	HGG:Total (21.8%)*	HGG:Total (37.6%)*	HGG:Total (4.9%)*
		197.4 (**)		197.4 (19.5%)	197.4 (35%)	197.4 (14.5%)	197.4 (20.4%)
		144.1 (**)	144.1 (**)	144.1 (**)	144.1 (30.5%)	144.1 (19.5%)	144.1 (29.2%)
	98.9 (**)	98.9 (**)	98.9 (**)	98.9 (23.7%)	98.9 (35.4%)	98.9 (**)	
	82.4 (**)	82.4 (**)	82.4 (**)	82.4 (34.3%)	82.4 (**)	82.4 (**)	
		56.1 (**)			56.1 (**)	56.1 (**)	
47.2 (**)	47.2 (**)	47.2 (**)	47.2 (**)	47.2 (**)	47.2 (**)	47.2 (**)	47.2 (**)
38.1 (**)	38.1 (**)	38.1 (**)	38.1 (**)	38.1 (**)	38.1 (**)	38.1 (11.1%)	38.1 (**)
		29.2 (**)	29.2 (**)		29.2 (**)	29.2 (**)	
		25.8 (**)					
						18.5 (46.3%)	18.5 (50.4%)
		10.4 (100%)		10.4 (22.5%)		10.4 (8.6%)	
		8.3 (**)	8.3 (**)				

* - Percentage of total amount of HGG detected on immunoblot present in lane

** - Band present but yielding value < 10 (arbitrary units) by image analysis

Table. 5.15: *Apparent molecular weights (kDa) and relative percentages of HGG bands detected in gastric scrapings of fish orally intubated with 35 mg HGG 1 hr after intubation with 50 mg cimetidine.*

15 min	30 min	45 min	1 hr	2 hr	6 hr	12 hr	24 hr	48 hr
HGG:Total (22.4%)*	HGG:Total (18.5%)*	HGG:Total (18.7%)*	HGG:Total (14.2%)*	HGG:Total (18.1%)*	HGG:Total (7.8%)*	HGG:Total (0.2%)*	HGG:Total (0.1%).	HGG:Total (0.0%)*
149.3 (17.6%)	149.3 (18.2%)	149.3 (16.1%)	149.3 (26.5%)	149.3 (18.9%)	149.3 (26.1%)	149.3 (100%)	149.3 (100%)	
	103.2 (3.4%)	103.2 (3.2%)			103.2 (7.3%)			
91.6 (5.2%)	91.6 (3.5%)	91.6 (2.7%)	91.6 (5.7%)					
		79.5 (4%)			79.5 (13%)			
72.9 (7.9%)	72.9 (12.1%)	72.9 (4%)	72.9 (14.9%)	72.9 (16.6%)				
		68.2 (4.5%)						
65.1 (5.1%)	65.1 (7.6%)	65.1 (5.9%)	65.1 (12.3%)	65.1 (8%)	65.1 (6.5%)			
60.0 (11.8%)	60.0 (3.4%)	60.0 (8.9%)		60.0 (2.8%)	60.0 (4.6%)			
	57.3 (4.7%)		57.3 (4.8%)	57.3 (5.3%)				
50.9 (7.5%)		50.9 (6.6%)	50.9 (5.9%)					
	45.3 (16.8%)		45.3 (9.6%)	45.3 (16%)				
	43 (**)							
40.4 (9.4%)	40.4 (5.3%)	40.4 (12.4%)	40.4 (7.1%)	40.4 (7.3%)				
37.6 (3.2%)	37.6 (1.8%)	37.6 (13.4%)			37.6 (29.1%)			
35.5 (8.6%)	35.5 (5.5%)		35.5 (4.4%)	35.5 (7.6%)				
31.1 (10.9%)	31.1 (4.4%)	31.1 (6.4%)	31.1 (3.4%)	31.1 (3.4%)	31.1 (13.4%)			
	28.6 (2.5%)	28.6 (6.4%)		28.6 (**)				
23.2 (3.5%)	23.2 (2.0%)	23.2 (1.2%)	23.2 (**)	23.2 (5.7%)				
21.1 (2.7%)		21.1 (3.8%)			21.1 (**)			
18.2 (4%)	18.2 (8.6%)	18.2 (7.2%)	18.2 (5.6%)	18.2 (8.4%)				

* - Percentage of total amount of HGG detected on immunoblot present in lane.

** - Band present but yielding value < 10 (arbitrary units) by image analysis.

Table. 5.16: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in scrapings from pyloric caecae of fish orally intubated with 35 mg HGG 1 hr after 50 mg cimetidine.

15 min	30 min	45 min	1 hr	2 hr	6 hr	12 hr	24 hr	48 hr
HGG:Total (5.4%)*	HGG:Total (5.1%)*	HGG:Total (8.0%)*	HGG:Total (0.0%)*	HGG:Total (30.7%)*	HGG:Total (19.1%)*	HGG:Total (27%)*	HGG:Total (4.7%)*	HGG:Total (0.0%)*
151.2 (34.7%)	151.2 (36.5%)	151.2 (50.7%)		151.2 (34.4%)	151.2 (82.6%)	151.2 (37.6%)	151.2 (100%)	
103.9 (10.7%)	103.9 (14.5%)			103.9 (**)				
91.3 (19.3%)	91.3 (22.5%)	91.3 (28.9%)		91.3 (**)				
80.7 (**)				80.7 (**)		80.7 (34.5%)		
				74.3 (19.7%)				
	68.7 (9.2%)							
61.8 (7.8%)	61.8 (5.1%)			61.8 (**)				
	58.1 (1.1%)			58.1 (11.9%)				
53.1 (12.5%)	53.1 (7.9%)	53.1 (9.2%)		53.1 (28.5%)		53.1 (26.8%)		
45.3 (8.6%)				45.3 (**)	45.3 (5.2%)			
41.0 (6.4%)	41.0 (3.4%)				41.0 (2.1%)	41.0 (**)		
	35.4 (**)			35.4 (2.2%)	35.4 (1.5%)	35.4 (1.1%)		
			31.9 (**)	31.9 (**)	31.9 (1.2%)			
				30.4 (1.7%)	30.4 (2.5%)			
		27.4 (1.6%)	27.4 (**)	27.4 (**)	27.4 (4.9%)			
				19.3 (1.6%)				

* - Percentage of total amount of HGG detected on immunoblot present in lane

** - Band present but yielding value < 10 (arbitrary units) by image analysis

Table. 5.17: Apparent molecular weights (kDa) and relative percentages of HGG bands detected in scrapings from the anterior portion of intestine of fish orally intubated with 35 mg HGG 1 hr after intubation with 50 mg cimetidine.

15 min	30 min	45 min	1 hr	2 hr	6 hr	12 hr	24 hr	48 hr
HGG:Total (0.0%)*	HGG:Total (9.1%)*	HGG:Total (3.7%)*	HGG:Total (7.5%)*	HGG:Total (7.3%)*	HGG:Total (24.8%)*	HGG:Total (36.9%)*	HGG:Total (9.3%)*	HGG:Total (1.4%)*
					150.3 (12%)	150.3 (61%)	150.3 (63.2%)	
		124.9 (10.2%)	124.9 (10.5%)		124.9 (9.3%)			
					111.5 (7.2%)		111.5 (9.3%)	
			91.7 (5.7%)		91.7 (10.6%)	91.7 (22.6%)		
				80.2 (4.6%)	80.2 (8.6%)		80.2 (12.7%)	
	70.9 (4.0%)							
				68.5 (5.9%)	68.5 (4.6%)	68.5 (3.3%)		
	65.1 (4.8%)	65.1 (8.6%)						
	61.4 (3.3%)	61.4 (7.3%)			61.4 (5.1%)		61.4 (13.8%)	
		55.1 (12.1%)	55.1 (7.8%)	55.1 (13%)	55.1 (9.8%)	55.1 (6.9%)		
		47.2 (13.4%)	47.2 (17.5%)	47.2 (31.7%)	47.2 (18.9%)	47.2 (2.2%)		
				39.6 (7.0%)		39.6 (2.0%)		
	35.9 (83.5%)							
	34.0 (**)	34.0 (9.0%)			34.0 (3.7%)	34.0 (2.0%)		
			29.2 (18.7%)	29.2 (6.5%)	29.2 (6.2%)			
		23.9 (25.3%)	23.9 (33.8%)	23.9 (16.6%)	23.9 (4.1%)			
	22.7 (4.4%)							
		20.2 (9.1%)	20.2 (6.0%)	20.2 (7.4%)				
				18.1 (7.1%)				

* - Percentage of total amount of HGG detected on immunoblot present in lane

** - Band present but yielding value < 10 (arbitrary units) by image analysis

Table. 5.18: Apparent molecular weights (kDa) and relative percentages of HGG scrapings from the posterior portion of the intestines of fish orally intubated with 35 mg HGG 1 hr after intubation with 50 mg cimetidine.

15 min	30 min	45 min	1 hr	2 hr	6 hr	12 hr	24 hr	48 hr
HGG:Total (18.7%)*	HGG:Total (9.9%)*	HGG:Total (17.7%)*	HGG:Total (36.3%)*	HGG:Total (7.7%)*	HGG:Total (6.5%)*	HGG:Total (1.4%)*	HGG:Total (1.8%)*	HGG:Total (0.0%)*
150 (12.8%)	150 (10.1%)	150 (6.7%)	150 (1.4%)	150 (16%)	150 (**)	150 (**)		
97.1 (1.0%)								
		76.0 (11.4%)						
73.3 (12.6%)			73.3 (4.8%)		73.3 (6.3%)	73.3 (**)	73.3 (**)	
		68.2 (7.0%)	68.2 (5.9%)	68.2 (**)				
66.8 (3.1%)		66.8 (**)						
		61.2 (**)		61.2 (**)				
55.3 (6.8%)								
50.9 (7.3%)		50.9 (8.0%)	50.9 (11.4%)					
					48.1 (15.7%)			
		44.4 (**)						
		42.4 (**)	42.4 (**)		42.4 (34.1%)	42.4 (100%)	42.4 (100%)	
38.1 (35%)	38.1 (39.5%)	38.1 (43.9%)	38.1 (41.7%)	38.1 (63.3%)				
	35.2 (50.3%)	35.2 (**)						
33.0 (21.4%)					33.0 (16.7%)			
		31.7 (23.1%)	31.7 (32.3%)	31.7 (20.7%)				
		27.9 (**)			27.9 (23.2%)			
			22.9 (2.6%)					

* - Percentage of total amount of HGG detected on immunoblot present in lane

** - Band present but yielding value < 10 (arbitrary units) by image analysis

5.4 DISCUSSION

The approach adopted in this study represents the first attempt to delineate the exact nature of the luminal processing of a protein antigen in the teleost gut *in vivo*. The results indicate that luminal processing is a complex process with different gut regions apparently playing distinct but complementary roles in protein antigen handling. Additionally, it was demonstrated that the form of orally delivered antigen ultimately reaching the circulation may be predicted to some degree by understanding the mechanics of *in vivo* luminal proteolysis. HGG delivered into the trout gut either alone or after the administration of gastric inhibitors was absorbed into the bloodstream. Gamma globulins have previously been used as marker proteins to assess the intestinal uptake of protein macromolecules in fish (Fujino *et al.*, 1987; Hart, 1987; Georgopoulou *et al.*, 1986; Jenkins *et al.*, 1992) but their processing in the lumen has not been described. The present study has investigated the condition of orally delivered HGG in the gut and quantified the levels of HGG reaching the rainbow trout circulation using ELISA and Western blotting methods. This was in contrast to previous studies of the absorption of HGG by the rainbow trout which had centred on cytochemical and immunohistochemical techniques.

A relationship was noted in the present study between the quantity of HGG administered to fish and the levels of antigen subsequently detected in the plasma. This result is similar to previous studies on the uptake of soluble proteins in *Oncorhynchus mykiss*. McLean (1987) reported maximal absorption of HRP after administering an oral dose of 50 mg and Georgopoulou *et al.* (1988) found a clear correlation between dose of HRP delivered (within the range from 10 to 30 mg per fish) and subsequent plasma levels detected. Studies on *Oreochromis mossambicus* found the optimal absorption of HRP and HGG respectively after an oral dose of 2 mg per fish (Doggett, 1989; Jenkins, 1992). The present study has found that the optimal uptake of HGG by rainbow trout as determined by ELISA occurred after an oral dose of 35 mg per fish but higher doses resulted in significantly lower levels than this peak. McLean (1987) suggested that these dose responses may reflect saturation - type kinetics on coated pits at the microvillous surface. If this is correct one might expect the levels of antigen reaching the bloodstream to increase with

increasing dose until a saturation state was reached and that doses greater than this saturation level would result in similar levels of antigen uptake. Jenkins (1992) reported that optimal levels of HGG absorption occurred in tilapia after the oral delivery of 2 mg per fish but found significantly lower levels after the delivery of 4 or 10 mg HGG per fish. In the same study, when HGG was administered via the anal route maximal absorption was found after intubation with a dose of 4 mg per fish but less absorption was found after delivery of 10 mg. Similarly, Doggett (1989) found maximal absorption of BSA in tilapia (by rocket immunoelectrophoresis) after the administration of 12.5 mg per fish but lower uptake after delivering 15 mg. These results may reflect inadequacies in the techniques used. Alternatively, in the case of orally delivered proteins the greater viscosity of the more concentrated solutions may have slowed their gut passage and since the dose responses described above were only assessed 1 hr after intubation then less of the more concentrated solutions may have reached the absorptive sites of the intestine. Differences in the intracellular processing of particular orally administered antigens in teleosts have been described (chapter 4) so the observed dose responses may partly reflect the mode of intracellular processing in the intestine prior to entering the circulation. The capacity for uptake is therefore likely to depend both on the physiology of a particular fish species and the nature of the protein antigen administered. Nonetheless such approaches did serve as a rational basis for the determination of appropriate antigen doses for subsequent experimentation.

The overall levels of HGG absorption measured in this study were in accordance with those reported for uptake of other soluble proteins by rainbow trout (McLean, 1987; Dorin *et al.*, 1993). These uptake levels (generally in range 1 - 50ng ml⁻¹) were considerably less than those reported for the same antigens in tilapia where peak values as high as 300 µg HRP ml⁻¹ (Doggett, 1989) and 700 µg HGG ml⁻¹ (Jenkins, 1992) have been reported. Such wide differences in the levels of soluble protein uptake were also reported between rainbow trout and carp (McLean and Ash, 1986, 1987b) where the tissue levels of orally delivered HRP were up to 1000-fold greater in the "agastic" carp than in the "gastric" trout. Differences in the degree of macromolecular protein uptake into the bloodstream may reflect differences in gastrointestinal physiology (Ash, 1985) such

as the absence of an initial peptic gastric phase or in the extent and rates of intracellular digestion between species (Watanabe, 1982). The gastric phase of digestion may be of less importance to the destruction of intact protein than previously thought since high levels of macromolecular protein antigen were found to be absorbed by the "gastric" species, tilapia (Jenkins *et al.*, 1992; Doggett *et al.*, 1993a). A comparative study of the absorption of soluble proteins in carp, tilapia and rainbow trout found that the uptake of protein was greatest in tilapia and least in rainbow trout with carp occupying an intermediate position (Doggett, 1989). In the present study a biphasic temporal pattern of HGG uptake was found (by both ELISA and immunoblotting analyses) with an increase in absorption from 45 min-2hr after antigen delivery and a second (usually larger) peak 6-12 hr after antigen administration. A similar pattern was reported for the uptake of HGG in tilapia (Jenkins *et al.*, 1992) where levels of HGG detected in plasma were maximal 6 hr after oral administration but an initial smaller peak was found 30 min after delivery. Studies of the oral administration of HGG to rainbow trout reported its absorption after 1 hr *in vitro* (using an everted gut sac technique) and its intracellular detection 5 hr after oral administration *in vivo*; in the same species the absorption of rabbit IgG into intestinal epithelial cells was detected 3 hr after oral administration (Fujino *et al.*, 1987). HRP which had been orally administered to rainbow trout was first detected in the bloodstream after 7 to 8 hr and reached a maximum after 16 hours (Georgopoulou *et al.*, 1988). However the first sample was taken 3 hr after delivery so an earlier peak may have existed. This possibility is supported by the results of McLean and Ash (1987b) which showed that maximal absorption of HRP into the plasma of rainbow trout occurred 30 min after delivery although uptake was only monitored for a total time of 75 min. The present work and the findings of Jenkins *et al.* (1992) and McLean and Ash (1987b) indicate that regardless of the quantitative differences the absorption of soluble antigens into the bloodstream of teleosts occurs very rapidly, within 15 min of delivery. The biphasic patterns of uptake reported in these studies may reflect gastric evacuation rates which have been reported to be biphasic in tilapia fry (Hofer and Newrkla, 1983) and this may also occur in rainbow trout. Obviously the dynamics of such processes would be influenced by physiological differences between species.

A number of techniques have been used in the past to investigate the uptake of protein antigens into the bloodstream of teleosts. These include rocket immunoelectrophoresis (Doggett, 1989), ELISA (McLean, 1987; Doggett, 1989; Davidson, 1991; Jenkins, 1992), ELISA combined with luminescence (Georgopoulou *et al.*, 1988) and Western blotting (Jenkins, 1992). The present study used ELISA and Western blotting to analyse plasma levels of absorbed HGG and found that in some cases large peaks in absorption which were detected by ELISA were not found when the same samples were analysed by Western blotting. The large peaks of absorption detected by ELISA may have been a consequence of the presence of particular antigenic fragments identified on Western blots. The presence of such highly antigenic fragments may have influenced the ELISA results leading to apparent uptake levels of greater than 1 $\mu\text{g HGG ml}^{-1}$ in some groups while in general, values in the range 0-50 ng ml^{-1} were found. Jenkins (1992) also used these techniques to investigate HGG absorption in tilapia and in some cases found a correlation between peaks of absorption detected by ELISA and the recognition of particular fragments by Western blotting. In the latter study the very high absorption values reported ($> 100\mu\text{g ml}^{-1}$) may have been less prone to alteration by a highly immunogenic fragment than was the case in the present study where low levels of absorption were found. There is a wide discrepancy between the reported levels of absorption of HRP into the circulation of rainbow trout which may reflect differences in the techniques used. McLean and Ash (1987b) found that uptake amounted to only 0.5% of the intubated dose while Georgopoulou *et al.* (1986) found that 6% of the intubated dose was absorbed. The detection of HRP was dependent upon the retention of functional enzymic peroxidase activity (McLean and Ash, 1986) while detection of HGG in the present study relied on retention of antigenicity. HGG was detected in the present study in intact and fragmented forms so a higher apparent level of uptake (compared with HRP) might have been expected. The results suggest that in rainbow trout or in other species exhibiting low levels of macromolecular protein absorption the use of ELISA alone to quantify absorption may result in misleading findings but by the application of Western blotting or another technique to assess the qualitative nature of the antigen present such an interpretation may be avoided. Considerable differences in the sizes and relative abundances of HGG fragments detected at various times after antigen administration

were noted. Previous investigations involving a variety of fish species have reported the generation of antigenic fragments after the oral delivery of BSA (Doggett *et al.*, 1991), ferritin (Rombout and van den Berg, 1989), HGG (Jenkins *et al.*, 1992) and antigens from *Vibrio anguillarum* (Rombout and van den Berg, 1989). Hemmings (1979) in a study on rats similarly found that 57.7-68.8 % of the BSA which was absorbed after oral administration was in the form of 20 - 50 kDa breakdown products. It has previously been found in teleosts that the processes of absorption of macromolecules and their retention in enterocytes may differ from that of antigenic fragments (Georgopoulou *et al.*, 1988; Doggett, 1989; Rombout and van den Berg, 1989; Jenkins, 1992). Intact antigen may be selectively retained intracellularly while smaller antigenic fragments may gain rapid entry to the bloodstream (Rombout and van den Berg, 1989). Jenkins (1992) found increased plasma levels of intact HGG from 24-48 hr after antigen delivery when the total quantitative levels of the antigen were in decline. Likewise in the present study, albeit at a much reduced level, lower molecular weight fragments appeared in the plasma more rapidly than either high molecular weight fragments or the apparently intact antigen which, although present rarely and in very small amounts, appeared predominantly more than 12 hours post intubation. This suggests that intestinal processing of HGG may be similar in trout and tilapia despite the considerably greater uptake of the protein in the latter species. In the present study the levels of HGG in the plasma which were detected by ELISA and Western blotting decreased considerably after 12-24 hr. This reduction was probably a result of clearance mechanisms involving antigen trapping in the liver, kidney and spleen (McLean and Ash, 1987; Doggett, 1989; Smedsrud *et al.*, 1984; Dannevig *et al.*, 1990, 1994). The kinetics of rainbow trout plasma clearance has been described as monophasic and exponential at a rate of 3 % per minute by Georgopoulou *et al.* (1988) and as biphasic by McLean (1987). The exact nature of this process will determine the quantity and form of orally administered antigen present in the plasma at various times after delivery.

Western blot analysis in this investigation showed that antigenic fragments containing determinants recognised by antisera to the Fab and Fc regions of HGG were absorbed into the bloodstream of

rainbow trout after oral delivery. A greater number of fragments were recognised by antisera to the Fc portion in this study which is in agreement with the findings of Jenkins (1992) in tilapia. In contrast, Georgopoulou *et al.* (1986) found that 30 min after the incubation of everted trout gut sacs with HGG, antisera to both Fc and Fab regions located epitopes in the apical vacuoles of lining epithelial cells but 60 or 90 minutes after incubation no material reactive with antisera to the Fc component was detected. The authors proposed that the Fc region underwent intracellular digestion. However, an *in vivo* analysis in the same study showed that HGG reactive with antisera to both the Fc and Fab components was present within intestinal cells. The results in section 4.3 of the present thesis indicated that the heavy chain of HGG was found intact more often intracellularly than the light chain in cells isolated from the trout intestine. Since the present study provides unequivocal evidence for absorption of HGG fragments possessing determinants recognised by antisera to the Fab and particularly the Fc components these must have passed through the gut epithelium intact. If some of the absorbed antigen was selectively exocytosed from the cells used by Georgopoulou *et al.* (1986) this may explain the apparent loss of Fc-related antigenicity.

Two gastric inhibitors were used in this study which altered gastric pH in different ways. Sodium bicarbonate resulted in acid neutralisation leading to a rise in gastric pH to a level approximating that of the intubated solution itself (> pH 8.0). Cimetidine administered at doses greater than 10 mg per fish resulted in a pH of approximately 7 which was consistent with its role as a competitive histamine H₂ receptor antagonist. The measurement of a gastric pH of 7 at the first sampling point, 75 minutes after cimetidine administration, indicated that a constant and considerable secretion of HCl occurred in the rainbow trout stomach in order to maintain an acidic environment. This study determined that the intubation of control PBS alone, because of its inherent buffering capacity resulted in a significant rise in gastric pH. This outcome has not been considered previously in studies of antigen absorption in teleosts but may be of importance. The oral administration of antigen to rainbow trout in volumes of up to 2 ml of buffered diluent has been reported (Georgopoulou *et al.*, 1988) and this would be expected to result in neutralisation

of gastric pH for some time. This may be important for future studies which should take appropriate measures in order to avoid the generation of possibly misleading results. In the present study, changing the gastric pH altered the qualitative nature of HGG fragments detected in the gut lumen and in the plasma. Fragments of low apparent molecular weight were more prominent in the plasma of fish with a low gastric pH (4 - 5) while a greater quantity of somewhat larger fragments was found in the plasma of fish with a gastric pH $\approx$ 7.0. These findings were in accordance with the predictions of the *in vitro* model described in section 3.3. However, the use of gastric inhibitors did not appear to greatly increase the quantitative levels of HGG reaching the plasma. Gastric inhibitors may be of considerable value in cases where orally administered antigens are highly acid-labile. In such cases the incorporation of bicarbonate or other inhibitor may significantly increase the quantity of intact antigen available for absorption, as found for example in the case of cholera toxin B subunit in mammalian studies (Clemens *et al.*, 1986; Sanchez *et al.*, 1993b). Previous studies on both teleosts and mammals have assumed that if the gastrointestinal destruction of protein molecules could be reduced then the enhanced absorption of such molecules would result. The efficacy of PLG microparticles in mammals has for example been partly attributed to the protection of antigens from acidic degradation and intestinal proteolysis (section 7.1). Studies on teleosts have also suggested that by modifying the conditions in the digestive tract, the extent of protein macromolecular absorption may be enhanced. The use of soybean trypsin inhibitor (McLean, 1987; McLean and Ash, 1989) increased the quantity of orally delivered HRP in the liver and spleen of rainbow trout. Moreover Jenkins (1992) found that the prior or concurrent treatment of HGG - intubated tilapia with Quil-A saponin increased plasma levels of HGG and altered the fragmentation patterns of the HGG detected, an effect which may have involved Quil - A mediated inhibition of luminal proteases. Additional evidence is provided by Wong *et al.* (1992) who found that the enteric coating of orally delivered *Vibrio* bacterins resulted in enhanced protection to challenge as compared with unprotected vaccine. It may be concluded that by modifying the gut environment or protecting labile protein antigens the efficacy of oral vaccines may be enhanced.

To date this study is the first to attempt to analyse in depth the exact nature of *in vivo* proteolysis of a protein antigen in the teleost gastrointestinal tract. The methods of analysis which were used have allowed a detailed assessment of the roles of various regions of the gut in antigen degradation. This is a critical step in assessing the likely efficacy or otherwise of oral delivery systems for protein or peptide molecules. If detailed information on the factors involved in the degradation of proteins *in vivo* can be determined then specifically tailored delivery protocols may be developed. Using this method a relatively large proportion of the antigen detected in the stomach was found in intact form indicating that the intubated proteins were only partly digested and would have left the stomach in this form. This is in accordance with the predictions of the *in vitro* model described in section 3.3 of this thesis and with a previous study on protein digestion in the Atlantic cod (Lied and Solbakken, 1984). The finding that a similar amount of degradation of the intact HGG occurred in the trout stomach at pH 7 as at pH 4 - 5, although the fragmentation patterns of the processed antigen differed, indicated that the enzymes in the trout stomach can operate over a wide pH range (see also section 3.4). This is in contrast to the suggestion that abrogation of gastric acidity would lead to reduced proteolysis and possibly in enhanced macromolecular uptake (McLean and Donaldson, 1990).

The role of the pyloric caecae in salmonids has been a matter of some debate. Lipid absorption, enzyme synthesis and vitamin production have all been proposed as functions but little evidence exists to support this (Smith, 1989). The surface area of the rainbow trout pyloric caecae is 3 times that of the remainder of the midgut and twice the area of the entire intestine and this has led to suggestions that it is merely an expansion of the surface area of the midgut (Ezeasor and Stokoe, 1980). Studies on the inner epithelium of salmonid pyloric caecae indicated that cells secreting digestive enzymes were absent (Fänge and Grove, 1979). Utilising isolated everted pyloric caecae it was found that this tissue can absorb amino acids and sugars across its epithelial cells (Diamond and Buddington, 1985) and it has been determined that intestinal contents move into and out of the pyloric caecae by contraction of the caecal musculature. Pyloric caeca have been used as a source of intestinal proteases in salmonids. The pancreas in these species has been

described as a diffuse tissue dispersed over the mass of cells comprising the mucous epithelium of the pyloric caecae (Kolodzeiskaya *et al.*, 1988) thus making isolation of enzymes from the pancreas difficult. Digestive proteases have been isolated from the pyloric caecae of a number of teleost species (section 3.1). Previously there has been little information concerning the role of the pyloric caecae *per se* in the digestion of proteins. The present study indicates an important role for the pyloric caecae in the degradation of soluble proteins. A number of HGG fragments present in the stomach were not detected in the pyloric caecae of these fish which suggested that either the fragments were degraded in the pyloric caecae to peptides or amino acids which were not detectable by the methodologies used or absorbed. Most of the antigen which was detected was apparently intact or in the form of large (> 100 kDa) fragments. It thus appears that enzymes residing in the pyloric caecae of rainbow trout are highly effective in digesting peptides released by prior gastric treatment but not in fragmenting large proteins. A study which used the same division of the digestive tract as the present work analysed protein and free amino acid levels in regions of the rainbow trout digestive tract after feeding (Dabrowski and Dabrowska, 1981) and found a 20-200 fold increase in the amount of free amino acids released from dietary protein in the pyloric caecae as compared to the stomach. Overall the evidence indicates that the proteolytic activity in the lumen of the rainbow trout pyloric caecae is considerable. In agreement with this are the results of Lied and Solbakken (1984) which found mainly proteins and polypeptides in the stomach but polypeptides, short peptides and amino acids in the pyloric caecae and anterior intestine. Ulla and Gjedrom (1985) found a significant correlation between the length of the rainbow trout intestine and protein digestibility but found no such correlation between digestibility and either the number or the length of pyloric caecae leading the authors to propose that pyloric caecae were not involved in protein digestion. It is conceivable that in the present study that HGG fragments produced in the stomach were subsequently absorbed in the pyloric caecae (see section 4.3) but since such large scale antigen absorption has not been previously reported to occur *in vivo* previously this is perhaps unlikely. This region is more probably implicated in the proteolysis of peptides.

In contrast to the results on HGG degradation in the pyloric caecae, the anterior half of the post - pyloric caecae gut appeared to possess enzymes which were highly effective in degrading intact proteins and large protein fragments. Very little intact antigen was found in this region. This is in agreement with reports of the presence of highly active proteases and peptidases in the teleost intestine (section 3.1). The results collected in this study on scrapings from the posterior portion of the intestine are more difficult to assess. Antigen was detected in this posterior intestinal region soon after intragastric intubation and this is difficult to envisage in the context of normal gut physiology. Intact HGG was detected in scrapings from the posterior intestine up to 24 hr after HGG delivery, at which time no HGG fragments were detected. This suggested that antigen may have reached the posterior intestine under the force of injection and remained intact in this region. If this is indeed the case then the results are highly artefactual since if antigen was incorporated into food and administered to fish the normal processes of bulk food handling and gastric evacuation would presumably dictate that antigen would not reach such posterior gut regions for some time. A number of previous studies have used similar intubation procedures (McLean and Ash, 1986, 1987b; Georgopoulou *et al.*, 1988; Jenkins *et al.*, 1992; Doggett *et al.*, 1993) and the biphasic patterns of antigen uptake into plasma reported in the present and earlier studies (Jenkins *et al.*, 1992) may be a result of this procedure and not a *de facto* physiological phenomenon. Whatever the reason it appeared that little antigen proteolysis occurred in this posterior intestinal region; antigen being found up to 24 hr after delivery in apparently intact and high molecular weight forms. A decline in the activity of intestinal proteases in the posterior intestine has been reported previously (Hofer and Schiemer, 1981) as has an important role for this region in intact protein absorption (McLean and Ash, 1987a; Georgopoulou *et al.*, 1988; Dorin *et al.*, 1993). The rainbow trout hindgut is deeply folded, possibly resulting in a functional separation between the central lumen and the spaces between the folds (Ezeasor and Stokoe, 1981). Nonvacuolated cells line the central lumen while the sides of the folds are lined by vacuolated cells indicative of macromolecular uptake by pinocytosis, the latter authors postulated that this folding pattern increases food retention time in the hindgut and therefore presumably increases the capacity for absorption.

It was not possible in this study to predict the exact nature of plasma-borne HGG fragments by examining the nature of the antigen which was present in the gastrointestinal tract. In some cases fragments were detected in the plasma which were not detectable in any gut region. It is thus likely that such fragments were the result of post-luminal processing possibly involving membrane-associated factors (Ash, 1980; Ugolev and Kuzmina, 1994), intracellular processing (Georgopoulou *et al.*, 1986; Dorin *et al.*, 1993) or possibly even peptidases in the bloodstream (McLean, 1987). It is perhaps noteworthy that a HGG fragment of approximately 10 kDa, similar in size to that detected in the plasma of fish with a mildly acidic gastric pH (pH 4 - 5), and not detectable in any region of the gut was found to be produced within cells isolated from the intestine of rainbow trout (section 4.3). It is conceivable that intracellular processing followed by exocytosis generated fragments which subsequently reached the circulation. In conclusion, the results of this *in vivo* investigation support the results derived from the *in vitro* model described in chapter 3, particularly with regard to the occurrence of gastric proteolysis of HGG when the stomach pH is neutralised. In addition, the finding of HGG fragments in the plasma of orally intubated fish which were not detectable in any region of the digestive tracts of these fish indicates as suggested in chapter 4, that intracellular proteolysis may contribute to the degradation of orally administered proteins.

CHAPTER 6

THE NATURE AND KINETICS OF THE SYSTEMIC AND MUCOSAL ANTIBODY PRODUCED TO HGG FOLLOWING ORAL AND PARENTERAL ADMINISTRATION.

6.1 INTRODUCTION

The gut mucosa of a number of fish species has been shown to be capable of responding immunologically to orally administered antigen. Gut associated lymphoid tissue (GALT) in fish comprises a repertoire of lymphoid cells capable of such functions as antigen binding, antigen presentation, antibody secretion and cell-mediated immunological reactivity (section 2.4.1). In mammals the existence of a common mucosal immune system possessing a number of features distinct from those of the systemic system is well documented (McGhee and Kiyono, 1993). In a number of cases a lack of correlation between serum antibody titre and resistance to infection by enteric pathogens has been demonstrated; the induction of a mucosal immune response being the most effective means of protection. A vital component of this defence is the production of secretory IgA (S-IgA), an antibody isotype uniquely adapted to its role in defence at the gut mucosa and lumen. The importance of the role now attributed to this facet of mucosal immunity is indicated by the use of intestinal mucus S-IgA as the 'gold standard' for assessing the efficacy of enteric vaccines (Forrest, 1992).

The existence of a common mucosal immune system in teleosts has similarly been proposed (see section 2.4.2). Antibody has been detected in the bile (Lobb and Clem, 1981a; Lamers, 1985; Rombout *et al.*, 1986; Davidson, 1991; Jenkins, 1992), cutaneous mucus (Harrell *et al.*, 1976; Lobb and Clem, 1981b; Ourth, 1980; St. Louis-Cormier *et al.*, 1984; Rombout *et al.*, 1986, 1989a; Lobb, 1987; Burgess, 1988; Davidson, 1991; Jenkins, 1992) and in the intestinal mucus (Fletcher and Grant, 1969; Bradshaw *et al.*, 1971; Di Conza and Halliday, 1971; Harris, 1972; Fletcher and White, 1973; Rombout *et al.*, 1986). Fletcher and Grant, 1969; Fletcher and White, 1973). A protective role for such antibody has rarely been demonstrated although Horne and Baxendale (1983) demonstrated a reduced adherence of *Vibrio anguillarum* to excised sections of rainbow trout gut in vaccinated fish and a reduction in bacterial establishment in the skin mucus of ayu, *Plecoglossus altevelis* has been described after oral immunisation (Kawai *et al.*, 1981). Some authors have described differences between mucosal and systemic derived antibody (Lobb and Clem, 1981c) leading to suggestions that secretory immunoglobulins are not derived from the

general circulation. Secretory component (SC) and J chain, pivotal features of mammalian S-IgA, have not been unequivocally identified in the secretory immunoglobulin of teleosts to date. A 'J' chain has been described as a component of IgM in the sheepshead, *Archosargus probatocephalus*, (Lobb and Clem, 1981d), and rainbow trout (Sanchez *et al.*, 1989) but could not be detected in chum salmon, *Oncorhynchus keta* (Kobayshi *et al.*, 1982) or in flounder, *Platichthys flesus* (Glynn and Pulsford, 1990). Lobb and Clem (1981b) suggested that a peptide associated with the cutaneous mucus IgM in the sheepshead, *Archosargus probatocephalus* may represent a secretory component type molecule and Underdown and Socken (1978) demonstrated that the secretory component - binding site is present on high molecular weight immunoglobulin in vertebrate species as primitive as the nurse shark, *Ginglymostoma cirratum*.

There is some evidence to suggest that in teleosts as in mammals enterically presented soluble protein antigens are poor mucosal immunogens compared with particulate antigens (Rombout *et al.*, 1989; Davidson, 1991). The former study demonstrated the presence of specific antibody to *Vibrio anguillarum* in cutaneous mucus and bile but not in serum after enteric administration. In contrast, most reports of enteric immunization with soluble proteins have found serum titres which were significantly greater than those in secretions (Burgess, 1988; Doggett, 1989; Rombout *et al.*, 1989a; Davidson, 1991; Jenkins, 1992). In the elasmobranch, *Scyliorhinus canicula* the oral administration of sheep red blood cells and *Vibrio anguillarum* bacterins resulted in detectable biliary antibody but no systemic response (Hart *et al.*, 1987). Specific antibody secreting cells have been identified in the gut mucosa of rainbow trout after oral delivery of both soluble (Georgopoulou and Vernier, 1986) and particulate antigen (Davidson *et al.*, 1993). Lobb (1987) immunised catfish, *Ictalurus punctatus* by immersion in dinitrophenylated-horse serum albumin and found that a greater number of fish possessed specific antibody in the cutaneous mucus than in the serum. The importance of antibody levels in determining protection of teleosts against certain infections has frequently been questioned and many studies have reported little correlation between protection from bacterial challenge and serum antibody levels (Kawai *et al.*, 1981; Kawai and Kusuda, 1983; Ellis, 1988). Support for the belief that other factors are of greater importance

may be derived from a recent study on Atlantic salmon, *Salmo salar*, (Barratt and Leadbeater, pers comm) which showed that following oral administration of a commercial furunculosis vaccine (Furovac) in a water in oil emulsion serum antibody was barely detectable but protection against challenge was comparable to that resulting from parenteral administration of the vaccine. Smith *et al.* (1980) found that oral vaccination against furunculosis did not result in an enhanced antibody response but did enhance the cell - mediated immune response. In many cases only systemic antibody titres have been investigated and the possibility exists that a specific and perhaps protective humoral response may have been induced at the mucosae has not been examined. In addition, a non-specific response which may have been induced either systemically or locally by antigen administration must also be considered in instances where oral immunisation leads to protection from subsequent challenge.

HGG has previously been demonstrated to induce antibody production in rainbow trout after both parenteral (Tatner *et al.*, 1987; Burgess, 1988; Davidson, 1991) and oral (Burgess, 1988; Davidson, 1991) administration. Jenkins (1992) found that the plasma and mucosal antibody titres to HGG enterically administered to tilapia could be increased by the addition of Quil-A saponin in various formulations. This indicated the potential for the design of oral delivery systems which increase the extent of antigen uptake across the gut and the resultant immune response. In mammals oral feeding of soluble antigen may induce a state of specific unresponsiveness and the oral delivery of fragments of protein antigens generated by digestive protease action has been found to result in the induction of oral tolerance (section 2.8). Therefore the protection of antigens from proteolysis in the gut may reduce the likelihood of oral tolerance resulting from the oral administration of antigens.

The use of gastric inhibitors to alter gastric pH was found to alter the nature of the HGG fragments absorbed (section 5. 3). As a result an investigation to determine if these changes in the nature of HGG absorbed influenced the subsequent antibody response was carried out. In addition to their presence on parietal cells, H_2 receptors have also been found on a range of other

cell types in mammals and cimetidine has been shown to exert a number of immunological effects (Markiewicz *et al.*, 1985; Bury *et al.*, 1992). Cimetidine has been found to increase synthesis of immunoglobulins both *in vitro* and *in vivo* (Friedman *et al.*, 1982; Badger *et al.*, 1983). In this study it was hoped to determine if prior administration of cimetidine could increase the antibody response to HGG by a method not related to its role as a gastric inhibitor. This study also sought to determine if fragments of HGG generated by enzymes in the rainbow trout gut retain antigenicity by probing antigen fragments with antisera from immunised fish. Additionally, using this system attempts were made to determine if differences existed in the recognition of such fragments by antibody present in the plasma and secretions from fish immunised parenterally or orally.

6.2: MATERIALS AND METHODS

6.2.1 *Animals*

Rainbow trout, *Oncorhynchus mykiss* of 100 - 150 g in weight were maintained as described in section 3.2.1.

6.2.2 *Immunisation protocol*

Four groups of fish (n = 30) were immunised either orally or intra-peritoneally (i.p.) with HGG. For i.p. injection, each fish received 35 mg HGG dissolved in 0.1 ml 0.15 M PBS, pH 7.2 emulsified in 0.1 ml Freund's complete adjuvant (FCA). Orally immunised fish were divided into 3 groups; those which

- a) received 0.2 ml PBS 1 hr before receiving 35 mg HGG (in 0.2 ml PBS).
- b) received 35 mg Na₂CO₃ in 0.2 ml PBS 1 hr before receiving 35 mg HGG.
- c) received 10 mg Cimetidine in 0.2 ml PBS 1 hr before receiving 35 mg HGG.

Five weeks after primary immunisation an identical booster immunisation (including prior administration of gastric inhibitor) was administered to the oral groups but for the i.p. immunised groups Freund's incomplete adjuvant (FIA) was used to emulsify the antigen in place of FCA. Each week for 10 weeks following primary immunisation 3 fish were sacrificed from each group. Samples of plasma, bile, cutaneous, intestinal and gastric mucus were collected from each fish and antibody levels to HGG were assessed by ELISA and Western blotting. To serve as controls, a group of fish were orally intubated with two concurrent administrations (1 hr apart) of 0.2 ml PBS and samples of plasma and secretions were collected 3 weeks after administration. The antibody titres detected for these fish by ELISA were treated as baseline levels against which titres for the immunised fish were determined.

6.2.3 *Procedure for the collection of blood and secretions from fish.*

Fish were starved for 24 hr prior to sampling to ensure the presence of bile in the gall bladder and

were sacrificed by a sharp blow to the head before the collection of samples.

Cutaneous mucus

Immediately after sacrifice that portion of the fish anterior to the operculum was clothed in tissue paper to prevent any potential contamination of skin mucus with blood. Fish were placed in dry polythene bags and a small volume of PBS was added. Mucus was collected from the dorsal body surface by gentle stroking with cotton buds (Boots Ltd) from the operculum to the tail to minimise scale detachment. Two cotton buds were used to collect mucus from each fish after which the cotton was stripped off, placed in plastic tubes with 2 ml of PBS and mixed for 1 min (Whirlimix). Samples were centrifuged at 4, 800 x g for 10 min and the supernatant was removed and stored at -20°C until required.

Plasma

Blood samples were drawn from the caudal sinus of fish via 23 gauge needles into heparinised 2.5 ml syringes. Blood was left overnight at 4°C and subsequently centrifuged at 4,800 g for 10 min. Supernatants were taken off and stored at -20°C until required.

Bile

After collection of cutaneous mucus and plasma, fish were dissected ventrally to expose the digestive tract (section 5.2). Bile was collected from the gall bladder via 23 gauge needles into 1 ml syringes and stored undiluted at -20°C until required.

Intestinal mucus

The digestive tract from immediately posterior to the pyloric caecae to the anus was excised and opened to expose the lumen (section 5.2). Mucus was collected by gently scraping the gut wall with a spatula. The mucus was washed with PBS into plastic tubes on ice. This volume was made up to 2 ml with PBS and 50 µl of 100mM PMSF was added. The mixture was vortexed for 1 min, centrifuged at 4,800 x g and the supernatant stored at -20°C until required.

Gastric Mucus

After dissecting out the gastric region of each fish (see section 5.2) gastric mucus was collected and processed as described for intestinal mucus.

6.2.4 Production of rabbit antiserum to rainbow trout IgM

Rainbow trout IgM was purified from the serum of fish immunised i.p. with 5 mg HGG emulsified in FIA. Serum was separated by gel filtration chromatography on an ACA-22 column (LKB, Bromma, Sweden). Fractions were tested by ELISA (see section 6.2.6) for anti-HGG activity, protein concentration was measured and reactive fractions were pooled and dialysed against 200 mM Tris-saline, pH 7.5 to remove azide. The second separation step was performed by anion exchange chromatography using a 0.5 M sodium acetate stepped gradient at room temperature (Burgess, 1988; Grayson *et al.*, 1991). A mono-Q HR5/5 column was connected to a Fast Protein Liquid Chromatography system (Pharmacia, Milton Keynes, U.K). Fractions were monitored at 280 nm (UV-1, Pharmacia) and peaks collected (Frac-100, Pharmacia). Fractions were again assessed for anti-HGG activity by indirect ELISA, reactive fractions were pooled and 200 µg of the resultant protein administered sub-cutaneously (sc) to Dutch rabbits. After 24 days the rabbits were given a booster injection of 150 µg of the protein and 3 weeks later blood samples were taken and tested by immunoelectrophoresis (Hudson and Hay, 1988) against rainbow trout IgM (anti-HGG reactive fractions from column; see above). The precipitin band after being excised and homogenised in 1.5 ml saline, pH 7.2 was used to immunise the rabbits 5 weeks after the first booster immunisation. The animal was re-immunised 6 months later as described above and bled 8 months after the final injection.

6.2.5 Determination of the specificity of the rabbit anti-trout IgM antiserum and of the susceptibility of trout IgM to degradation by rainbow trout intestinal proteases.

Samples of plasma, bile, cutaneous mucus, intestinal mucus and gastric mucus collected from fish as described above were diluted in reducing electrophoresis sample buffer, boiled for 3 min, subjected to SDS-PAGE and electroblotted (sections 3.2.9 & 3.2.12). Blots were washed in Tris-

saline, pH 7.5 and blocked in Tris-saline + 4% non fat dried milk (TSM) (section 3.2). Blots were incubated for 4 hr in rabbit anti-trout IgM antiserum at a dilution of 1: 500 (in TSM), and for 1 hr in swine anti-rabbit peroxidase conjugate (Sigma) at a dilution of 1:1000 in TSM. Blots were developed as described in section 3.2.12 To determine the susceptibility of any IgM which may have been present in these secretions to rainbow trout digestive enzymes, samples of each were taken and added to an equal volume of a solution containing enzymes from the rainbow trout intestine (section 3.2) for 2 hr. The resultant mixture was diluted with reducing electrophoresis sample buffer and boiled for 3 min. SDS-PAGE and Western blotting was then performed on these samples.

6.2.6 Indirect ELISA for detection of antibody to HGG.

This assay was performed essentially as described previously (Burgess, 1988; Jenkins, 1992). Ninety six well microtitre plates (Falcon) were coated with 100 μ l per well of 100 μ g ml⁻¹ HGG in 0.5 M carbonate-bicarbonate buffer, pH 9.6 (Appendix C) and incubated in a humid box overnight at 4°C. Some wells were left uncoated to serve as controls. Plates were washed 4 times with 0.5 % PBST (Titertek Handiwash 110, Labsystems, U.K) and patted dry on paper towels. Samples (200 μ l) of plasma (starting dilution 1:10), bile (starting dilution 1:2), cutaneous mucus (starting dilution 1:2), intestinal mucus (starting dilution 1:2) or gastric mucus (starting dilution 1:2) in PBST were added to plates and doubly diluted down a single column (intestinal and gastric mucus samples) or 2 columns (plasma, bile and cutaneous mucus) in PBST. Control samples from fish administered PBS only were included on each plate and titrated according to the same pattern. After incubation at room temperature for 2 hr the samples were aspirated and the plates were washed 4 times with PBST and patted dry. To each well, except controls, was added 100 μ l of rabbit anti-rainbow trout IgM antiserum at a dilution of 1:500 in PBST and the plates were incubated at room temperature for 1 hr. Samples were again aspirated, washed 4 times with PBST and patted dry. Subsequently, 100 μ l of swine anti-rabbit IgG peroxidase conjugate (Sigma) at a dilution of 1:1000 in PBST was added to each well and the plates were incubated at room temperature for 1 hr. Samples were aspirated and the plates were washed 4 times with PBST and

patted dry. Finally 100 μ l of developing solution (Appendix C) was added to each well, reactions were terminated by addition of 50 μ l of 1M H₂SO₄ and the absorbance at 492 nm was read spectrophotometrically (Titertek Multiscan, Labsystems, U.K)

6.2.7 Determination of the specificity of rainbow trout antisera in plasma and secretions.

HGG (4 mg ml⁻¹ in Tris-HCl buffer, pH 7.75) was added to an equal volume of a solution of rainbow trout intestinal enzymes (see section 3.2) and incubated at 15°C for 30 min. The reaction was terminated by adding non-reducing electrophoresis sample buffer and then boiling for 5 min. SDS-PAGE and immunoblotting were carried out as described previously (section 3.2) except in this case the 'comb' for creating wells in the stacking gel possessed only a single 'tooth', the remainder creating a large well occupying most of the gel length (Fig 6.1). Prestained marker proteins (Sigma) were inserted into the first well while 100 μ l of digested HGG sample was added to the large well. After electrophoresis, gels were electroblotted (section 3.2) for 18 hr. Blots were washed and placed in blocking solution (see section 3.2) after which the blots were inserted into a multiscreen apparatus (Miniprotean II Multiscreen, Biorad) which enabled screening of a single antigen preparation by a number of different antisera (Fig 6.1). Samples (600 μ l) of plasma or secretions made up in TSM at the same dilutions as the starting dilutions outlined for ELISA above were added to 'slots' and incubated at room temperature for 4 hr. Samples were carefully removed from slots and each slot was washed 3 times with Tris-saline, pH 7.5 and twice with TSM. Rabbit anti-rainbow trout IgM (600 μ l diluted 1:500 in TSM) was added to each slot and the blots were incubated at room temperature for 2 hr. Blots were washed as described above and 600 μ l of a 1:1000 dilution of swine anti-rabbit IgG peroxidase conjugate (Sigma) added to each slot. Blots were incubated for 1 hr at room temperature, washed 3 times in Tris-saline, pH 7.5, removed from the apparatus and developed as described in section 3.2. Image analysis of immunoblots was performed as outlined in section 5.2.

6.2.8 Expression of antibody titres and statistical comparison of groups of immunised fish

End point titres for each sample were determined by comparing absorbance values at each sample

dilution with that of a control sample using Chi square (Wardlaw, 1987), at a 5% level of significance. Mean titres from groups ($n = 3$) were compared by analysis of variance (ANOVA) on Statgraphics software.

6.3 RESULTS

6.3.1 *Specificity of the polyclonal rabbit anti-rainbow trout IgM antiserum and susceptibility of trout IgM to rainbow trout intestinal proteases.*

Western blots showed that the rabbit anti-trout IgM antiserum recognised two bands in the plasma of molecular weight 72 and 26 kDa which may correspond to the heavy and light chains of trout IgM. In addition, bands of apparent molecular weights 63, 59, 54 and 51 kDa were recognised by the antiserum as well as bands > 100 kDa albeit very weakly (Fig 6.2(a)). After 30 min incubation of plasma with intestinal enzymes the 72 kDa band was considerably reduced as a proportion of the total positive signal (from 25.2 % to 9.1 %) and a number of additional bands of between 10 and 70 kDa were detected (Fig 6.2(b and c)).

Similarly, in the cutaneous mucus a band of approximately 72 kDa was recognised by the polyclonal anti-trout IgM antiserum in addition to bands of 252, 167, 111 and 62 kDa. After incubation with intestinal enzymes the 72 kDa band appeared to be almost completely absent. Interestingly, in the case of biliary and mucus samples a greater number of bands of molecular weight > 72 kDa were detected on blots after digestion with intestinal enzymes. These may have represented breakdown products of high MW proteins in the secretions. In the bile, 3 strongly staining bands of 211, 196 and 143 kDa were found in addition to bands of 69, 54, 72 and 26 kDa (the latter two possibly represented IgM heavy and light chains respectively). In the intestinal mucus, strongly staining bands of 201 and 183 kDa were detected in addition to a number of less intense bands including one of 72 kDa. Unlike plasma, bile and cutaneous mucus the undigested intestinal mucus sample possessed positively staining low molecular weight (< 10 kDa) bands. After incubation with intestinal enzymes the band of 72 kDa was absent but the strongly staining band at 183 kDa did not appear to be susceptible to digestion. In gastric mucus only weakly staining bands were apparent after blotting with rabbit anti-trout IgM primary antiserum. However, a band of 72 kDa was found which was not detectable in samples after incubation with intestinal enzymes (result not shown), extra bands of 118 and 90 kDa were detected after digestion with enzymes.

6.3.2 Detection of antibody to HGG in plasma, bile and mucus

A: Plasma

An example of the dilution profiles of peak titre plasma samples from all 4 groups is presented in Fig 6.3. The levels of antibody to HGG determined by ELISA were highest in the i.p. immunised group which over the 10 week period were significantly greater ($p < 0.05$) than all three oral groups. Titres in the i.p. immunised group increased to > 12 ($\log_2$) by week 4 after a primary injection but decreased significantly after boosting at week 5, thereafter titres remained low (< 8) until 4 and 5 weeks after boosting at which time titres were not significantly different from those immediately prior to boosting (Fig 6.5(a)). By Western blotting (Table 6.1) it was determined that antibodies in plasma from trout immunised i.p. recognised a number of HGG fragments more strongly than controls. It was remarkable that the intensity of staining of the 52 kDa band appeared to correlate very closely with the peak titres determined by ELISA (Fig 6.4 & Fig 6.5(b)). Although other bands were also recognised, their intensity did not appear to reflect the ELISA titres or to follow a clearly discernable pattern.

Although antibody was detectable in the plasma of all three groups of orally immunised fish, patterns in the titres were difficult to define. Boosting did not result in a significant increase or decrease in titres in any of the 3 groups. Antibody titres in the group administered bicarbonate prior to HGG were significantly higher than in the other oral groups 5 weeks after the primary immunisation but appeared to decrease after boosting (Fig 6.5(a)). In the group administered PBS prior to HGG the differences between titres measured from 3-10 weeks post-immunisation were not significant ($p < 0.05$). Administration of cimetidine prior to HGG did not significantly alter plasma antibody titres but by lowering the level of significance to 25% the titres from samples collected 3 weeks after primary immunisation and 5 weeks after boosting were significantly greater than those 1 and 5 weeks after primary and 2 weeks after secondary immunisation. The signal detected on Western blots with plasma from groups of fish immunised orally was very weak compared with i.p. immunised fish. Nevertheless, greater numbers of fragments were recognised by plasma from orally immunised fish after boosting (Table 6.1). In contrast with the i.p. group

the fragment of approximately 52 kDa was rarely detected by plasma antibodies from orally intubated groups although a fragment of 70 kDa was recognised by plasma from all 3 oral groups.

B: Cutaneous mucus

The kinetics of antibody production in cutaneous mucus collected from the i.p. immunised fish was very similar to that found in plasma from these fish (Fig. 6.6). The titres initially increased rapidly but differences between titres at weeks 3-5 after primary injection were not significant ($p < .05$). Titres decreased significantly after boosting but recovered relatively rapidly and 4 and 5 weeks after boosting the titres were not significantly different from those 5 weeks after primary injection. The number of fragments recognised by antisera in Western blotting appeared to reflect the kinetics of antibody production found by ELISA (Table 6.2) and as was the case with plasma from these fish the intensity of staining of a fragment of approximately 52 kDa appeared to match the peaks of absorbance found by ELISA. Titres in cutaneous mucus collected from fish orally intubated with HGG were significantly less ($p < 0.05$) than those from fish immunised i.p. and differences in titres between the 3 orally immunised groups were not significant. The titres in orally intubated fish were relatively erratic and no clear pattern was evident. Boosting did not result in a significantly increased titre in any of the 3 oral groups. The signals produced using these samples in Western blotting were very weak but as for plasma samples a greater number of fragments were recognised by samples collected from fish after boosting (Table 6.2).

C: Bile

Antibody titres of bile samples collected from fish which were injected i.p. with HGG were highest 4 and 5 weeks after boosting. Unfortunately the groups of fish 4 and 5 weeks after primary immunisation were inadvertently fed prior to sample collection so no bile was collected from these fish (Fig 6.7). Western blots probed with samples from these fish showed that a 52 kDa fragment was relatively weakly recognised by biliary antibody in samples collected 4 and 5 weeks after boosting. This recognition appeared to reflect the peaks of absorbance measured by

ELISA (Table 6.3). Antibody titres in bile collected from fish orally intubated with HGG were erratic and titres in the range 0-4 were recorded. Boosting did not result in a significant rise in titre (Fig 6.7). Western blots were only very weakly stained using these samples and in contrast with plasma and cutaneous mucus samples, boosting did not appear to alter this (Table. 6.3).

D: Intestinal mucus

Antibody titres >4 were measured in intestinal mucus from i.p. immunised fish 4 and 5 weeks after boosting (Fig.6.8). A high background staining was found on Western blots when intestinal mucus was used as a primary antiserum but a 52 kDa fragment was present which appeared to reflect the ELISA peak absorbance (Table.6.4). Specific antibody was only detected by ELISA in the intestinal mucus of a small number of orally immunised fish and the titres were rarely significantly greater than controls (Fig 6.8). No bands were detected on immunoblots probed with mucus from fish administered PBS prior to HGG and for the 2 other oral groups weak and inconsistent results were recorded.

E: Gastric mucus

Following the primary immunisation, antibody titres in gastric mucus of i.p. immunised fish were not significantly different from controls. However, after boosting titres of between 1-3 were found (Fig.6.9). This finding was supported by Western blotting (Table.6.5), although a fragment of 52 kDa was not detected. Antibodies to HGG were barely detectable in gastric mucus from fish orally intubated with HGG until after boosting. In the case of the group administered prior cimetidine the titres at weeks 2 and 5 after boosting were not significantly different from those in the i.p. injected group. Using Western blotting a greater number of fragments were detected by samples taken from orally immunised groups after boosting (Table 6.5). It should be noted that the signal was extremely weak and barely detectable by the techniques used.

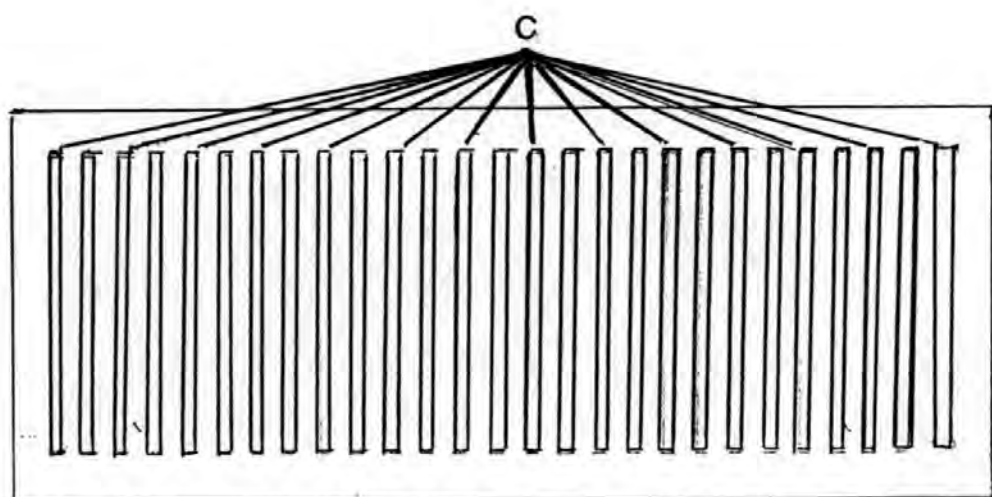
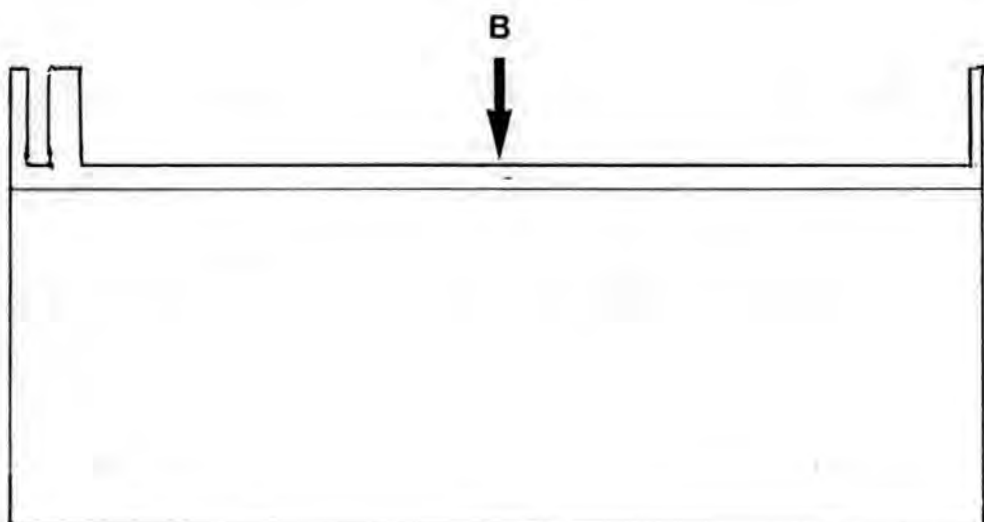
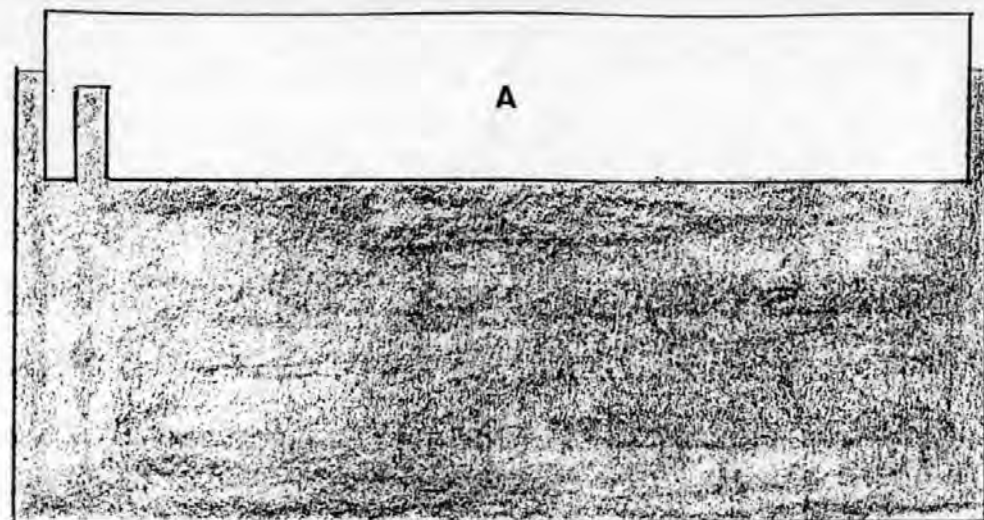


Fig. 6.1: Procedure used to screen partially digested HGG with a range of antisera from fish on Western blots. A: Use of a comb with a single large 'tooth' to produce a large well for loading of antigen samples. B: Application of antigen to well after polymerisation of acrylamide and removal of comb, after electrophoresis the gel was electroblotted and transferred to the 'Multiscreen' apparatus. C: 'Multiscreen' apparatus showing the application of a number of antiserum samples to a single nitrocellulose blot.

Fig. 6.2: *Western blots demonstrating the specificity of the polyclonal rabbit-anti-trout IgM antiserum and the susceptibility of rainbow trout IgM to digestion by intestinal proteases.*

Fig.6.2(a): *Recognition of heavy (H) and light (L) chains of trout IgM present in plasma by the polyclonal rabbit anti-trout IgM antiserum. Loading order: Lane 1, prestained molecular weight markers. Lanes 2 & 3, Plasma samples from fish at a dilution of 1:100 and 1:400 respectively.*

Fig.6.2(b&c): *Bands recognised in plasma and secretions of trout and the effects of intestinal proteases. Fig 6.2(b) is an 11% PAGE gel which shows the low molecular weight bands clearly and Fig 6.2(c) is a 4-15% gradient gel showing the high molecular weight bands more clearly. Loading order; Lane 1, prestained molecular weight markers. Lane 2, Plasma at a dilution of 1:20. Lane 3, plasma at a dilution of 1:20 after digestion with enzymes. Lane 4, cutaneous mucus at a dilution of 1:5. Lane 5, cutaneous mucus at a dilution of 1:5 after digestion with enzymes. Lane 6, bile at a dilution of 1:5. Lane 7, bile at a dilution of 1:5 after digestion with enzymes. Lane 8, intestinal mucus at a dilution of 1:2. Lane 9, intestinal mucus at a dilution of 1:2 after digestion with enzymes. Lane 10, gastric mucus at a dilution of 1:2. Lane 11 (Fig 6.2(c) only, gastric mucus at a dilution of 1:2 after digestion with enzymes.*

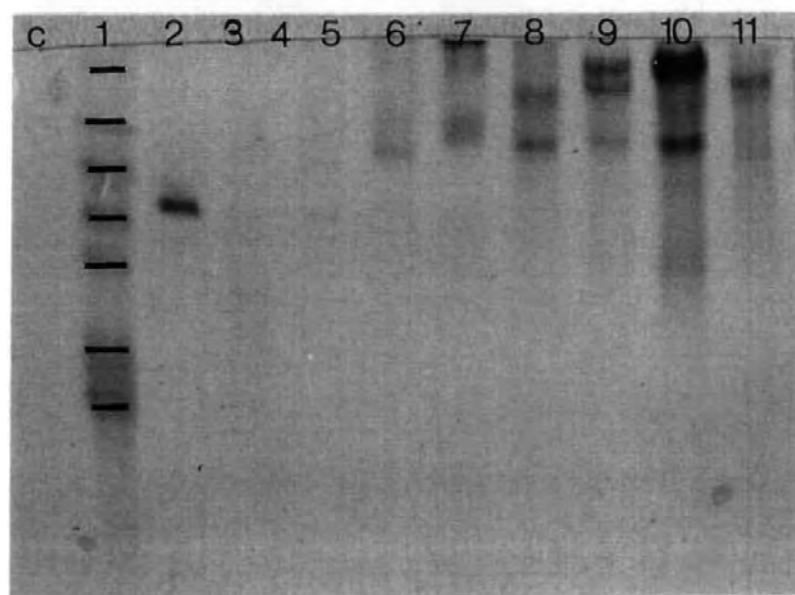
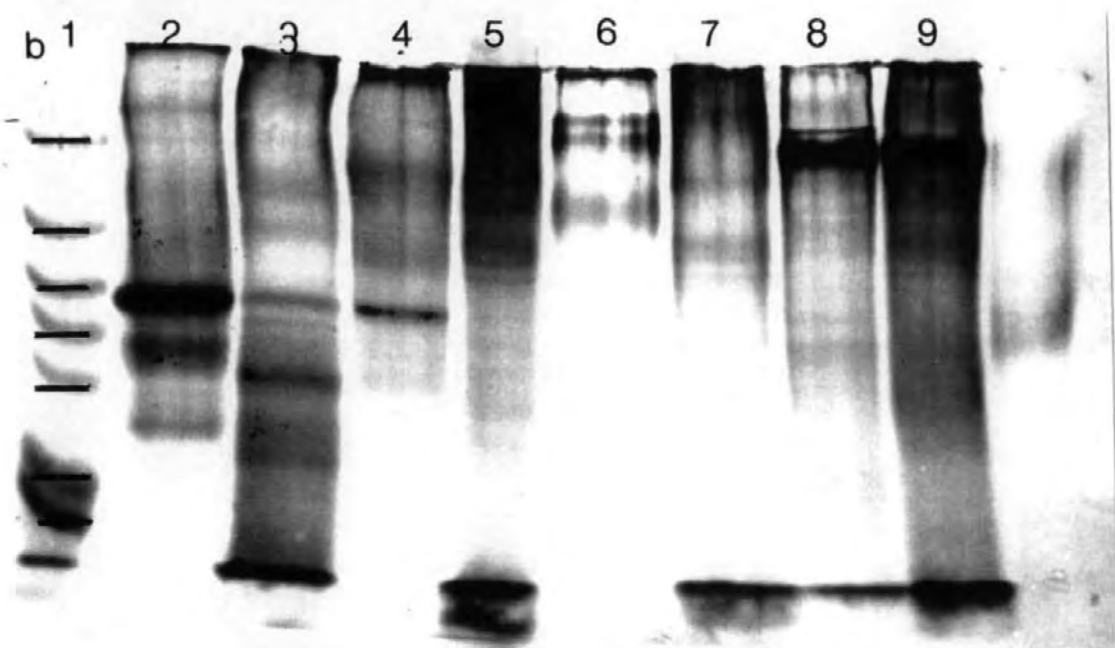
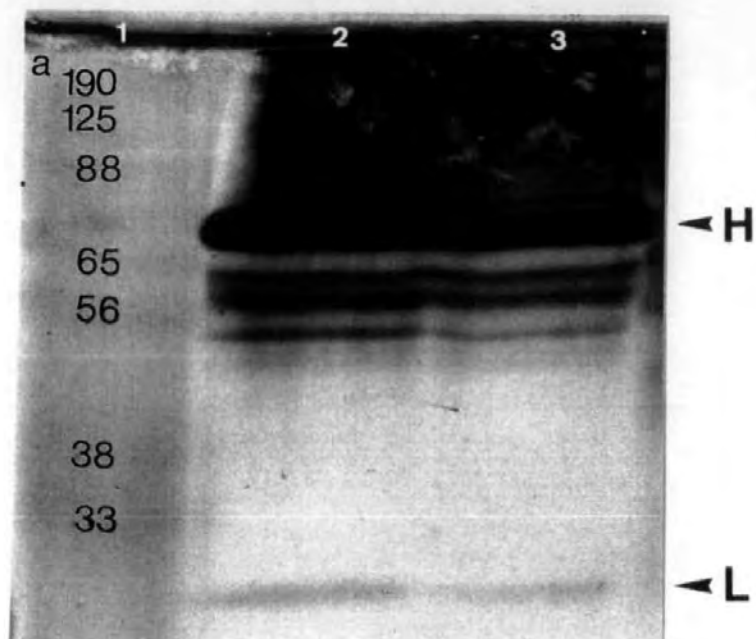
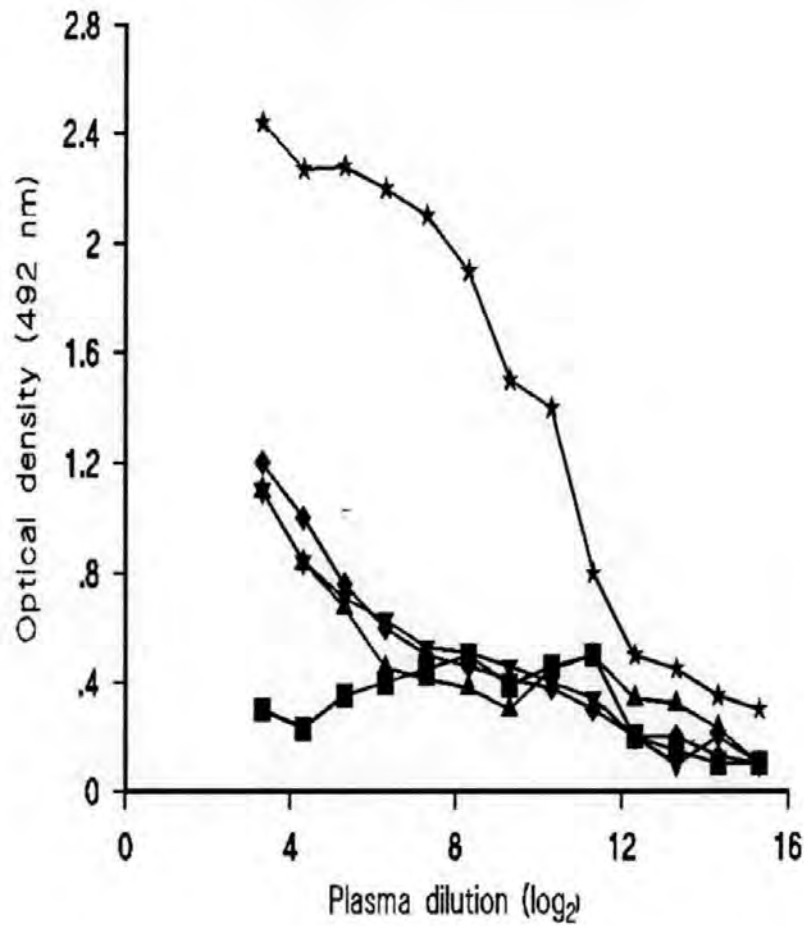


Fig.6.3: Dilution profiles of plasma samples from fish orally or intra - peritoneally immunised with HGG.



- - Control (plasma from fish 5 weeks after oral intubation with PBS)
- ▲ - Plasma collected from fish 8 weeks after oral immunisation (3 weeks after boosting) with HGG after prior administration of PBS.
- ▼ - Plasma collected from fish 3 weeks after oral immunisation with HGG after prior administration of 35 mg sodium bicarbonate.
- ◆ - Plasma collected from fish 10 weeks after immunisation with HGG after prior administration of 10 mg cimetidine
- ★ - Plasma collected from fish 5 weeks after i.p. immunisation with 35 mg HGG in FCA.

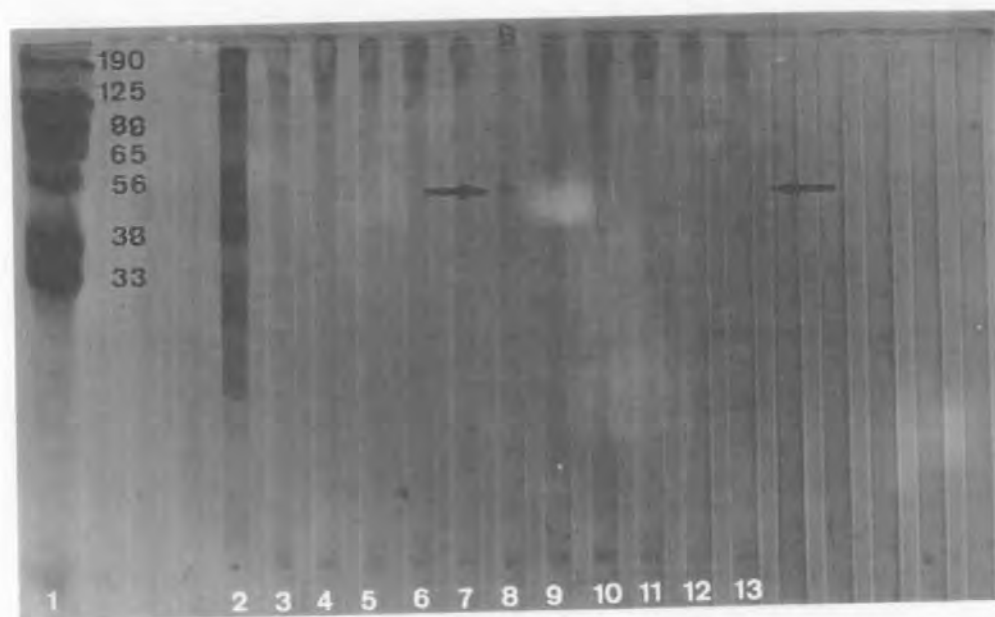


Fig. 6.4: Western blot showing the recognition of HGG fragments produced by intestinal enzyme degradation with antisera from i.p. immunised trout. Lane 1, prestained molecular weight markers. Lane 2, partially digested HGG screened with a rabbit-anti HGG polyclonal antiserum. Lanes 3, partially digested HGG screened with control trout plasma. Lanes 4-13, partially digested HGG screened with plasma from rainbow trout collected 1-10 weeks after immunisation with 35mg HGG in FCA. B - Booster immunisation with 35mg HGG in FIA. Arrow points to the 52kDa HGG fragment.

Fig.6.5(a): Anti-HGG antibody titres in plasma from fish immunised orally or i.p with 35 mg HGG

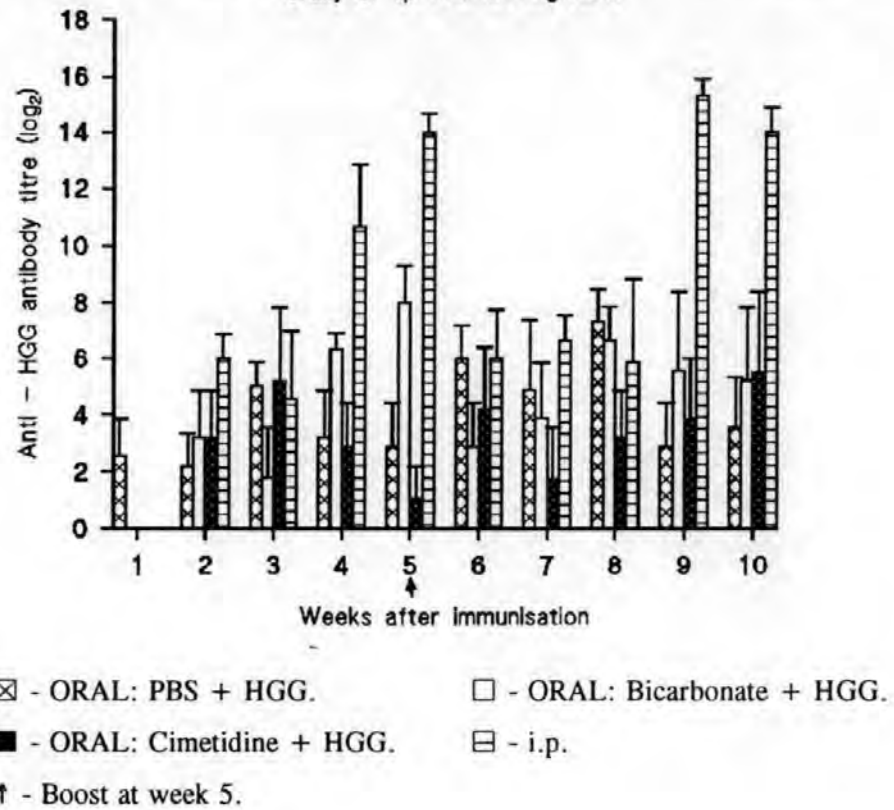


Fig.6.5(b): Comparison of anti-HGG antibody titres in plasma with the recognition of a 52 kDa HGG fragment

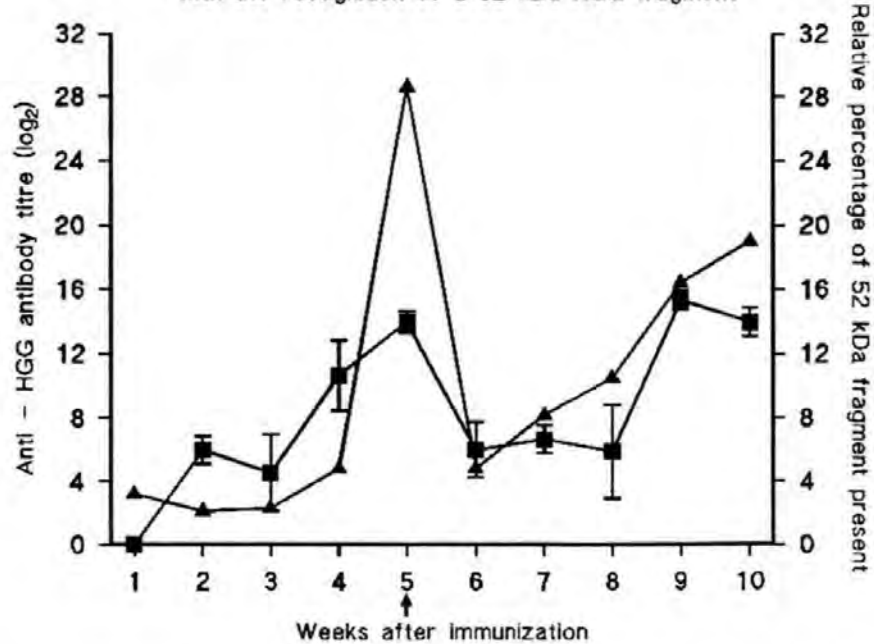


Table. 6.1: HGG fragments produced by partial digestion of HGG by rainbow trout intestinal enzymes which were recognised by antibody in plasma from fish immunised orally or i.p. with 35 mg HGG.

Immunisation regime	Fragment size (kDa)	Week No									
		1	2	3	4	5(Boost)	6	7	8	9	10
ORAL:PBS + HGG	146				+		+	+	+	+	
	70.6				+		+	+	+		
	51				+				+		
	13							+	+		
ORAL: Bicarbonate + HGG	151.2	+	+	+			+	+	+		
	70.2			-				+			
	49.7		+	+				+			
	39.2									+	
	7.5							+	+	+	+
ORAL: Cimetidine + HGG	150.6			+	+				+	+	+
	94		+							+	
	70.6								+	+	+
	39.2							+	+		+
	13		+	+							
	7.5			+	+	+					
i.p. injected	153			+		+				+	+
	126.5	+	+		+		+	+	+		
	80	+					+	+	+	+	+
	51.8	+		+	+	+		+	+	+	+
	7.5	+		+	+	+	+	+	+	+	+

Fig.6.6: Anti-HGG antibody titres in cutaneous mucus of fish immunised orally or i.p. with 35 mg HGG

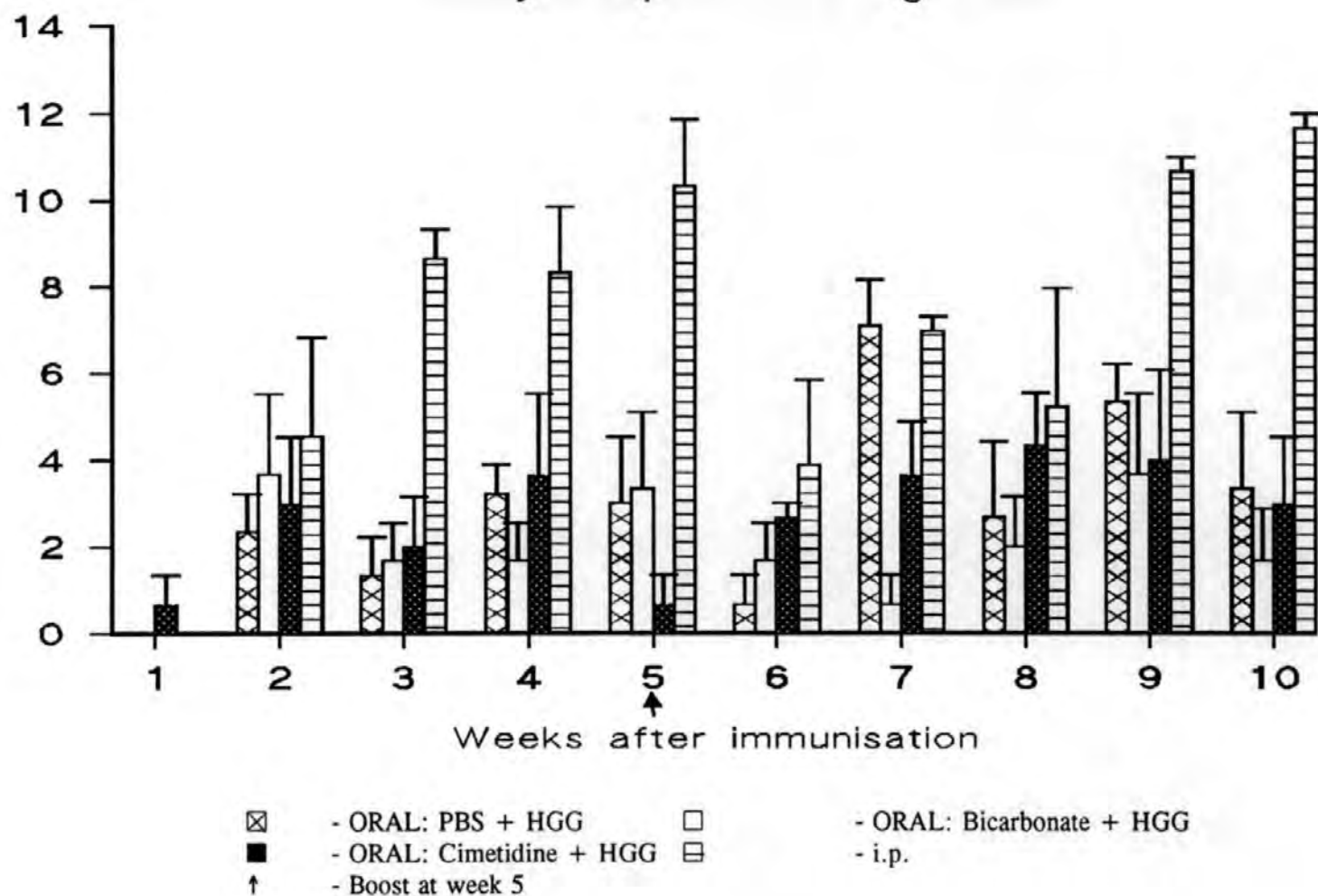


Table. 6.2: *HGG fragments produced by partial digestion of HGG by rainbow trout intestinal enzymes which were recognised by antibody in cutaneous mucus from fish immunised orally or i.p. with 35 mg HGG.*

Immunisation regime	Fragment size (kDa)	Week No									
		1	2	3	4	5 (boost)	6	7	8	9	10
ORAL: PBS + HGG	148.5			+				+	+		
ORAL: Bicarbonate + HGG	148.5									+	+
	90									+	+
	50.9									+	+
ORAL: Cimetidine + HGG	148.5							+	+	+	
	101.3							+		+	+
	50.9								+	+	+
	38.6							+	+	+	
injected	153		+	+					+	+	+
	121.4		+		+				+	+	+
	52.7					+				+	+
	7.5		+		+	+			+	+	+

Fig.6.7: Anti-HGG antibody titres in bile of fish immunised orally or i.p with 35 mg HGG

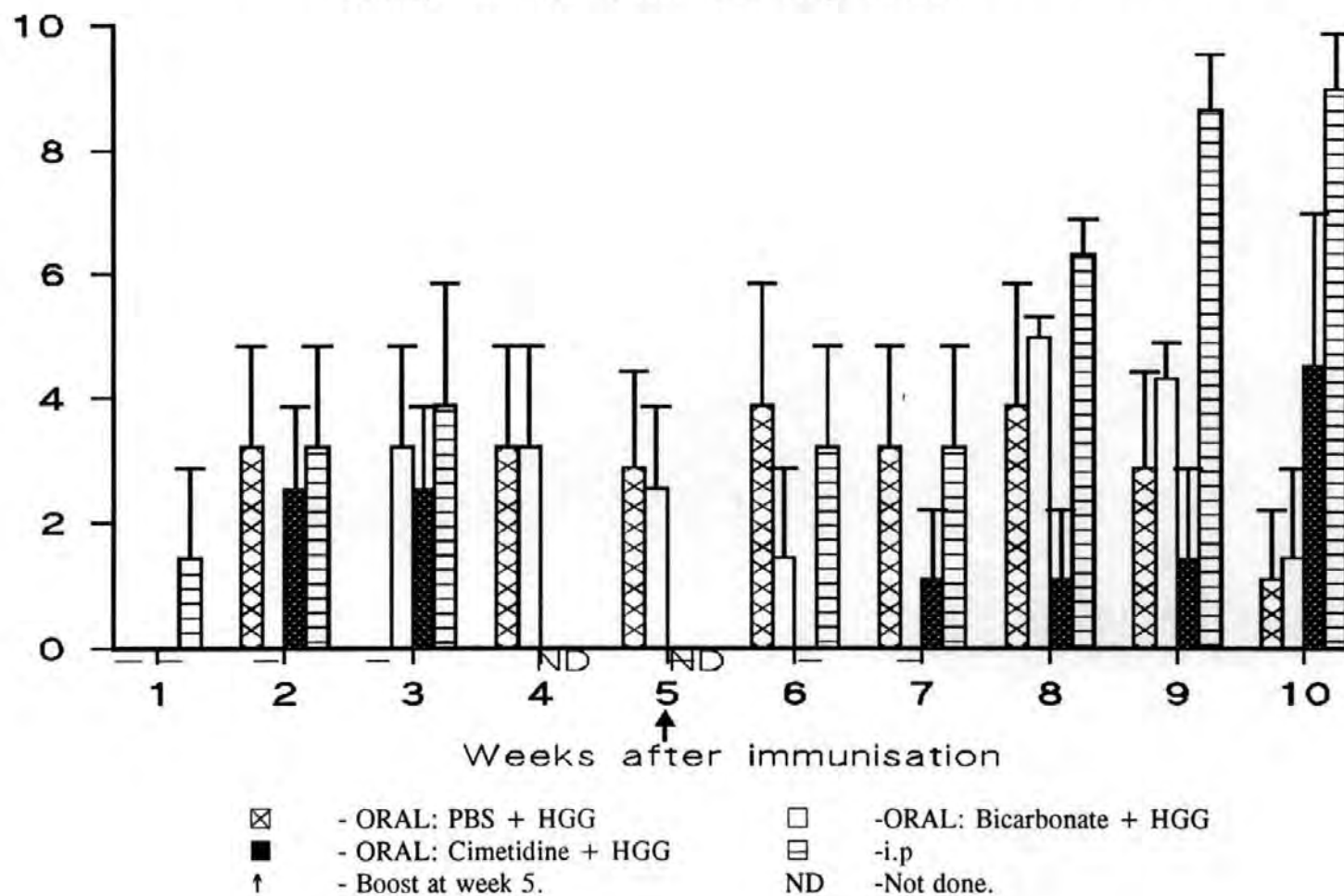


Table. 6.3: *HGG fragments produced by partial digestion of HGG by rainbow trout intestinal enzymes which were recognised by antibody in the bile of fish immunised orally or i.p. with 35 mg HGG.*

Immunisation regime	Fragment size (kDa)	Week No									
		1	2	3	4	5(boost)	6	7	8	9	10
ORAL:PBS + HGG	153		+	ND	+				+	+	
	100.6		+	ND	+				+		
ORAL: Bicarbonate + HGG	155.3		ND		+	+					
	100.6		ND	+		+					
	61.8		ND		+	+					
ORAL: Cimetidine + HGG	151.6			+	+	ND		+	+	+	
	46.7					ND		+			
injected	151.6				ND	ND	+		+	+	+
	73.1				ND	ND	+			+	+
	52.2				ND	ND				+	+

Fig.6.8: Anti-HGG antibody titres in intestinal mucus from fish immunised orally or i.p with 35 mg HGG

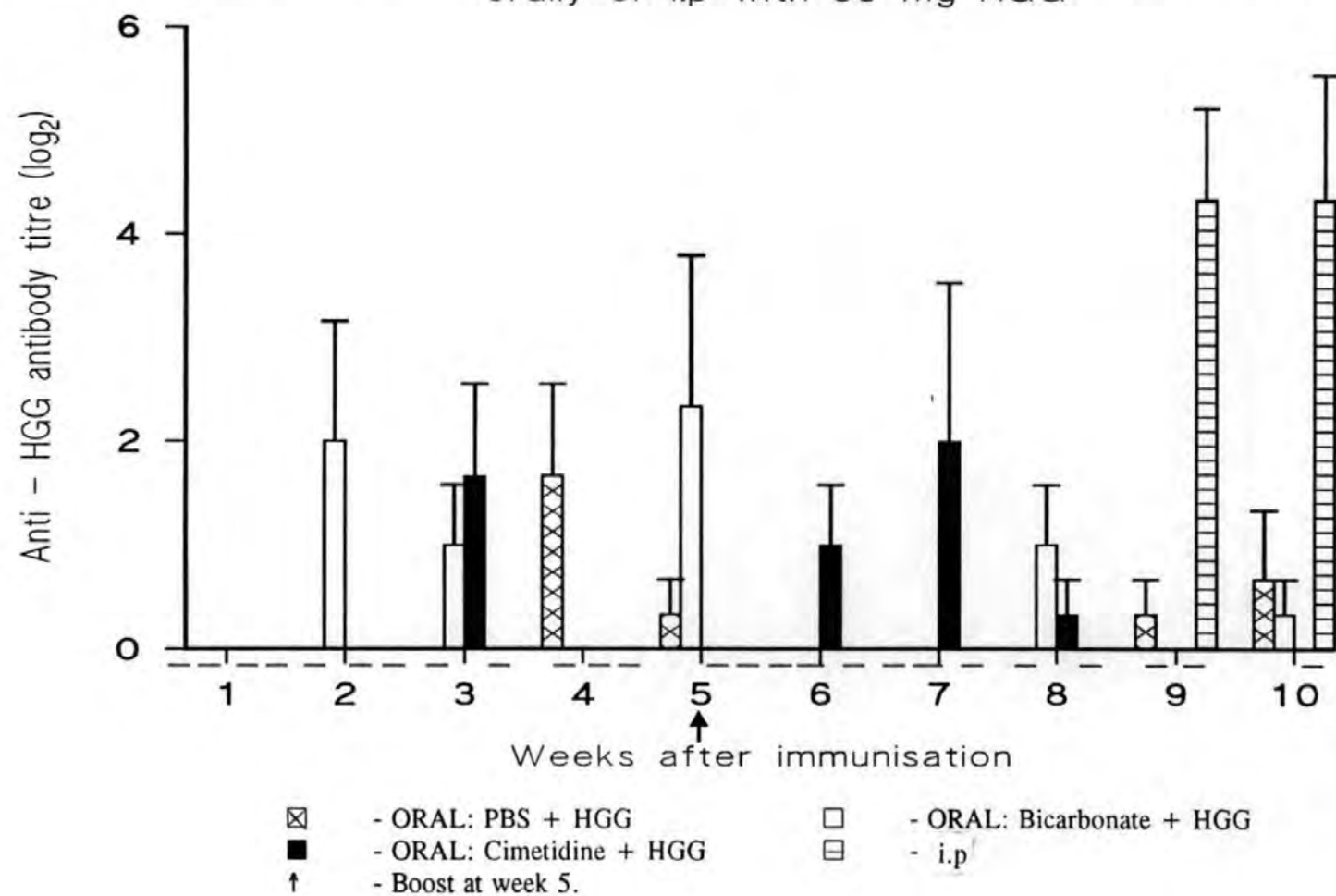


Table. 6.4: *HGG fragments produced by partial digestion of HGG by rainbow trout intestinal enzymes which were recognised by antibody in the intestinal mucus of fish immunised orally or i.p. with 35 mg HGG.*

Immunisation regime	Fragment size (kDa)	Week No									
		1	2	3	4	5(boost)	6	7	8	9	10
ORAL: PBS + HGG											
ORAL: Bicarbonate + HGG	152					+		+		+	
	103.4									+	
ORAL: Cimetidine + HGG	152		+	+		+					
	103.5			+						+	
injected	152.5		+						+	+	+
	76.6									+	+
	51.4				+	+		+		+	+

Fig.6.9: Anti-HGG antibody titres in gastric mucus from fish immunised orally or i.p with 35 mg HGG

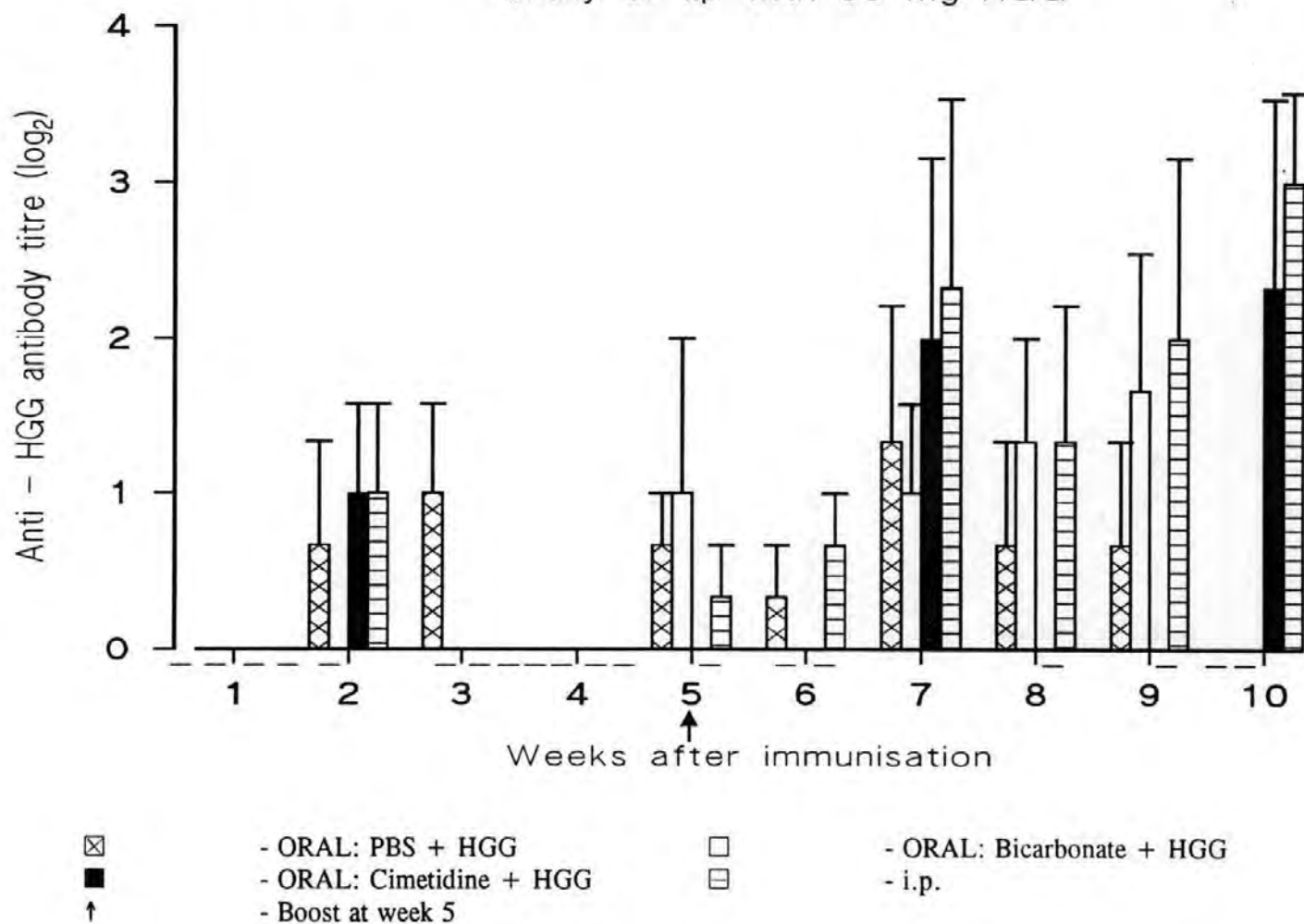


Table. 6.5: HGG fragments produced by partial digestion of HGG by rainbow trout intestinal enzymes which were recognised by antibody in the gastric mucus of fish immunised orally or i.p. with 35 mg HGG.

Immunisation regime	Fragment size (kDa)	Week No									
		1	2	3	4	5 (boost)	6	7	8	9	10
ORAL: PBS + HGG	150.4		+				+		+	+	+
	103.2									+	+
ORAL: Bicarbonate + HGG	150.4					+		+	+	+	
	99.6							+		+	
ORAL: Cimetidine + HGG	150.4		+	+			+		+	+	+
	102.8		+				+		+	+	
	46.8										+
	7.7					+		+	+	+	+
injected	151.4			+			+		+	+	+
	101.3			+			+		+	+	+
	62.4										+
	46.8								+		

6.4 DISCUSSION

This study has demonstrated the induction of a humoral immune response to HGG with specific antibody detectable both in plasma and in secretions after oral and parenteral delivery. At all sites the magnitude of the antibody response was greater after injection of antigen than after oral delivery. Prior administration of the gastric inhibitors cimetidine and bicarbonate did not appear to significantly influence the resultant antibody response to HGG. The most important findings of this work were a recognition that the kinetics of antibody production to parenterally administered HGG were similar in both the plasma and secretions and that the antibody produced reacted strongly on Western blots with a fragment of HGG which was released by incubation of the antigen with rainbow trout intestinal enzymes. Indeed, in the case of the antibody response in the plasma and cutaneous mucus the peak titres determined by ELISA appeared to parallel with the recognition of this 52 kDa fragment by immunoblotting.

Relatively few studies have examined the nature and kinetics of antibody production to soluble antigens in teleosts, particularly in secretions but interest is likely to grow with the increased application of modern vaccine technology and the generation of protein or peptide vaccines (Leong, 1993). There have been suggestions that soluble antigens and antigens which are known to be T-dependent in mammals are poorly immunogenic in fish (Etlinger *et al.*, 1979; Ellis, 1982). While definitive evidence for a distinct T cell subset is presently lacking in fish, processes indicative of T cell - B cell interactions, particularly in the catfish have been well documented (Ellis, 1982; Miller and Clem, 1984; Miller *et al.*, 1985; Vallejo *et al.*, 1991, 1993). However, antibody responses to soluble proteins have been found to depend on the particular antigens and fish species used (Trump and Hildeman, 1970; Burgess, 1988). The latter study found that BSA was a very poor immunogen in rainbow trout while Trump and Hildeman (1970) detected high titres of anti-BSA antibody in immunised goldfish. Variation in the nature of antigens administered, the route of delivery, stock genotype and the fish species used make it difficult to generalise about such findings.

The indirect ELISA assay used in this study for detection of antibody to HGG in the plasma, bile and mucus of rainbow trout was a method optimised by Burgess (1988). The author found that the use of this method enabled the detection of specific antibody to HGG without interference from non-specific 'background antibody'. Using this system in the present study, antibody against HGG was detected in plasma, cutaneous mucus, bile and more rarely in the intestinal and gastric mucus. Western blotting demonstrated that the polyclonal rabbit anti-rainbow trout IgM antiserum appeared to be raised primarily against the heavy (H) chain of rainbow trout IgM (72 kDa band) but also reacted with a band of 26 kDa which may represent light (L) chain. A number of additional bands of 51, 54, 59 and 63 kDa were also recognised. Heavy and light chains of similar sizes have been described in a number of other fish species (Acton *et al*, 1971; Lobb and Clem, 1981c; Lobb *et al*, 1984; Ghaffari and Lobb, 1989; Glynn and Pulsford, 1990). Sanchez *et al*. (1989) reported the detection of two H chains of 70 and 60 kDa in rainbow trout IgM which may offer some explanation for the detection of bands of 51-63 kDa in rainbow trout plasma in the present study. A faint immunoreactive band of 72 kDa in addition to a number of higher molecular bands were detected in the bile and mucus but the significance of this result is at present unknown. The expression of antibody titres from mucus samples is a somewhat contentious field due to variability in the quantity of mucus collected from individual fish and the variable dilution factor resultant from the amount of water collected with the mucus. The use of protein content as a means to standardise the quantity of mucus present has been reported (Davidson, 1991), although to the authors knowledge an assessment of the degree of variability in the protein content of teleost cutaneous mucus has not been carried out. It may be possible that handling stress, anaesthesia, and differences in stock genotype and feeding regimes affect both the volume and protein content of the mucus. If this were the case then protein may not be a good standard against which to gauge antibody level. Additionally, in cases where antibody constitutes a higher or lower than average proportion of the total protein then correction for total protein content would result in a distortion of the antibody titres. Obviously a similar difficulty may pertain to the use of protein content as a standard for correcting intestinal mucus titres since there may be a residual food content which varies according to gut evacuation rates (section 5.3). There may also be

differences in the levels of luminal enzymes present in the gut. In the absence of a definitive standard the present study adopted a relatively arbitrary approach. Fish of similar sizes were used in each group and mucus samples from each fish were made up to an identical volume, titres were then expressed with reference to this original volume. Further study is required to determine a feature which reliably indicates mucus content such as viscosity or a mucus constituent such as a polysaccharide or glycoprotein. In extensive mammalian studies investigating intestinal antibody responses, no correction for the protein content of the mucus has been made (Forrest, 1992).

The finding in this work that the heavy chain of rainbow trout IgM was rapidly degraded by rainbow trout intestinal proteases is perhaps significant since if there is only a single isotype of IgM present in rainbow trout as suggested by Harrell *et al.* (1976), for this to be functionally effective it would require some form of protection akin to the secretory component of mammalian S-IgA to prevent it from degradation in the gut. Using antiserum against human secretory component, a positive reaction with bile or mucus samples was not found (results not presented), a similar finding has recently been reported for carp (Rombout *et al.*, 1993b). The polyclonal antiserum used, although appearing to be directed principally to the heavy chain in plasma, detected or cross reacted with a number of high molecular weight bands in the bile and mucus. The identity of these proteins was not investigated but it is possible that they represent forms of IgM complexed with other constituents in these secretions. The finding of low molecular weight protein bands reactive with our polyclonal antiserum only in the intestinal mucus of fish indicated that some digestion of this mucosal IgM may occur *in vivo*, alternatively processing of the sample may have made the antibody susceptible to protease action.

The highest antibody titres detected at all sites were found after injection of HGG with Freund's complete adjuvant (FCA). FCA has been found to have a significant immunopotentiating effect on the humoral immune response of rainbow trout to the parenteral but not the oral administration of HGG (Burgess, 1988). A number of studies have found that the levels of antibody detected in plasma and secretions were greater after i.p. than oral immunisation with soluble antigens

(Burgess, 1988; Rombout *et al.*, 1989a; Davidson, 1991; Jenkins, 1992). The induction of a systemic antibody response to HGG after parenteral delivery has previously been reported in rainbow trout (Tatner *et al.*, 1987; Burgess, 1988; Davidson, 1991) and in tilapia, *Oreochromis mossambicus* (Jenkins, 1992). The present finding that parenteral boosting led to a significant reduction in the plasma antibody titre may have been a result of antibody-antigen complexing but the rapid increase in titre which was found after this temporary decline did not indicate the occurrence of any immune suppression. Tatner *et al.* (1987) found a very similar pattern after i.p. immunization of rainbow trout with 2.5 mg HGG emulsified in FCA.

Specific antibody to HGG was also detected at a higher level in bile and mucus of fish after i.p. injection than in any of the orally immunised groups. Interestingly, the kinetics of antibody appearance in the cutaneous mucus of the parenterally immunised fish appeared to parallel those seen in the plasma and boosting resulted in a similar decline and subsequent rise in titre. This suggested either that the injected antigen reached the skin mucosa to complex locally produced antibody or formed complexes with some of the circulating antibody pool which was destined for the cutaneous mucus. Burgess (1988) could only detect antibody to HGG in the skin mucus and bile of parenterally immunized rainbow trout which had detectable levels of plasma anti-HGG antibody, a result which may indicate that the secretory antibody was derived from the general circulation. The finding that the kinetics of antibody appearance in plasma and secretions were similar after i.p. injection was in accordance with previous studies on rainbow trout (Davidson, 1991) and the tilapia, *Oreochromis mossambicus* Jenkins (1992). Detection of specific antibody in the bile of teleosts has been reported less often than in the cutaneous mucus. In mammals the importance of biliary immunoglobulin is species-dependent, in certain species such as man intestinal polymeric IgA is mainly derived from a trans-epithelial transport mechanism while in rabbits, rats and in birds for example transport of intestinal immunoglobulin is principally by the hepatobiliary route (Brown and Klappel, 1989; Rombout *et al.*, 1992). It was additionally found in some species that disruption of the flow of bile led to a depletion in intestinal mucus antibody (Andrew and Hall, 1982). A recent study (Rombout *et al.*, 1993b) using an antiserum raised in

rabbits to cutaneous mucus immunoglobulin from carp found positive staining by immunohistochemistry with skin epithelia and with bile ducts and capillaries but not with the intestinal epithelium and postulated that the hepatobiliary route may be the principal transport route for intestinal immunoglobulin in carp. The antibody responses of teleosts to immunisation with soluble preparations appear to contrast with responses to particulate antigens. Agius *et al.* (1983) found that soluble extracts of *Vibrio anguillarum* resulted in better protection than whole cells via the intra-peritoneal route while the reverse was true via the oral route. Rombout *et al.* (1986) detected antibody in intestinal and cutaneous mucus and in bile of carp after anal and oral administration of *V. anguillarum* but in serum only after anal administration.

In the present study antibody was detected in a greater number of fish in the bile than in the intestinal mucus. It is possible as suggested by Davidson (1991) that the levels of intestinal Ig may depend on biliary evacuation and if fish are starved prior to collection of samples less intestinal antibody will obviously be detected, this hypothesis awaits investigation however. Antibody titres in the bile were highest after i.p. injection in the present study, the highest titres being found 4 and 5 weeks after booster injection. Interestingly, no antibody was detectable in fish from this group 4 and 5 weeks after the primary injection although the plasma and skin mucus titres were elevated, additionally these fish were accidentally fed prior to sampling the bile contents were liberated into the gut. Since no antibody was detectable in the intestine of these fish one may suggest either that biliary antibody was not present or that the antibody was degraded in the intestine. Burgess (1988) also found low levels of specific anti-HGG antibody in the intestinal mucus of i.p immunised trout but in contrast Davidson (1991) could not detect specific antibody in the intestinal mucus of rainbow trout immunised i.p. with KLH. The mucus samples in the latter study were dialysed extensively before analysis which may have resulted in degradation of any IgM present since in addition to the finding that what appeared to be the H chain of rainbow trout IgM was highly susceptible to intestinal protease action, dialysis was found (see section 3.3) to be a highly effective means of degrading luminal gut contents and isolating intestinal proteases. However a number of other authors have also reported an inability to detect antibody in the

intestinal mucus (Smith *et al.*, 1980; Kawai *et al.*, 1981). An important factor to consider in the case of the present results at least, since little processing of bile and mucus samples was carried out, is the finding by Burgess (1988) that a depression of apparent titres in an anti-BSA antibody reference sample was caused by addition of secretions in the following order; reference sample > PBS > skin mucus > bile > gut mucus. Therefore it is possible that titres reported in such samples in the present work were artificially low. This study also attempted to detect antibody in the gastric mucus since it was felt that if intestinal mucus antibody is bile-derived then antibody detection in the stomach might indicate local secretion of antibody as lymphoid cells have occasionally been described in the gastric region of both elasmobranchs (Hart *et al.*, 1986) and teleosts (Doggett, 1989). Very little antibody was detected in the gastric mucus however, only in a few cases were titres significantly greater than controls; antibody was detectable 2, 4 and 5 weeks after booster injection in the i.p. immunised fish antibody but only in a single fish (of 3) in each case.

Specific antibody was rapidly synthesised in the plasma of fish which had been orally immunised with HGG after oral delivery of antigen. The titre increased relatively rapidly but in most cases there was no clear peak or pattern in the response and boosting did not result in a significant increase in titre. The low levels of antigen absorbed after the oral delivery of HGG (section 5.3) were presumably not great enough to cause the same complex forming effect as seen following i.p. booster administration. Since the titres in the plasma of orally immunized fish did not decline after boosting it is possible that this second immunisation actually sustained antibody production. Burgess (1988) could not detect specific antibody to HGG in the plasma of fish given 2 mg of HGG by the oral or anal route. This may not be surprising due to the small dose administered, since administration of doses of 5 mg per fish resulted in very little antigen in any form reaching the intestine (section 7.3). Results from a number of studies on both mammals (Rothberg *et al.*, 1970; Nicklin, 1987) and fish (Rombout *et al.*, 1989a) have indicated that repeated enteric exposure to antigen may be required to induce a strong immune response. Evidence exists to suggest that 'trickle' vaccination of fish with vaccine incorporated into food over a protracted

period is a more effective regime in inducing a protective response in fish to bacterial challenge indicating that if a sufficient dose of vaccine is administered, oral vaccination may indeed be a feasible alternative to other modes of vaccine delivery (Smith, pers comm). Such findings cannot be compared with the present work since the immunological basis of protection has frequently not been established. The finding that prior administration of gastric inhibitors did not appear to influence the antibody response to orally delivered HGG indicated that differences in the HGG fragments absorbed after delivery of cimetidine and bicarbonate (section 5.3) had no effect on the subsequent immune response.

The use of immunoblotting in addition to serving as a second method to assess the specificity of the antibody under investigation enabled the determination of whether HGG fragments released by exposure to intestinal proteases retained antigenicity. The finding of an apparent correlation between the detection of a 52 kDa HGG fragment by the same antisera responsible for the peak antibody titres determined by ELISA suggests that antigen fragments generated by intestinal protease action may indeed retain antigenicity in this species and possibly indicates the existence of an immunodominant epitope (at least for rainbow trout) on the molecule. That immunodominance may be an important phenomenon in lower vertebrates has been postulated by Vallejo *et al.* (1993) who found that catfish lymphocytes responded to a single highly immunogenic peptide of the cytochrome c antigen. The method used in the present study did not set out to determine the importance of individual HGG epitopes in the induction of a humoral immune response and was limited in that SDS treatment and electrophoresis result in linearisation of proteins and thus the only antibodies detectable by this process are those which recognise sequential determinants. It is possible and perhaps likely that conformational epitopes on the HGG molecule are also important in the immune response to the antigen in rainbow trout. Nevertheless the finding of a correlation between the recognition of this fragment and the ELISA titre suggests that it is of some importance. Additionally, the finding that this fragment is recognised almost exclusively by plasma and secretions from i.p. immunised fish in addition to the similarities in the kinetics of antibody detection in the plasma and secretions after i.p. immunisation indicates a

possible homogeneity in this response. Unfortunately, little definitive information on the nature of antibody induced by oral immunisation was gained due to the poor sensitivity of this technique and the low titre of antibody detected. However, although the 52 kDa fragment detected by antibodies from immunised fish was not detected by plasma antibodies from orally intubated fish plasma from all 3 groups of orally immunised fish appeared to detect a 70 kDa fragment whose detection appeared to coincide to some extent with the peak ELISA titres. This, although being far from definitive, indicates that differences exist in the specificity of antibody generated by oral or parenteral antigen delivery. The majority of HGG after oral delivery (see section 5.3) was absorbed in the form of fragments of less than 50 kDa in size, and although a fragment of approximately 52 kDa was detected in many cases in the gut lumen it was not detectable in the plasma. A small amount of possibly intact and high apparent molecular weight HGG fragments was also absorbed which may have possessed the epitope present on the 52 kDa fragment. It would be interesting to determine the minimum size of this fragment retaining antigenicity and if it possesses any characteristics which are particularly effective in inducing an antibody response in this species. This finding may simply reflect the recognition of fewer epitopes on protein antigens by teleosts than by mammals. A number of previous studies found that salmonids responded to relatively fewer vaccine components after immunisation than mammals (Hastings and Ellis, 1990; Grayson *et al.*, 1991). If this is a general feature of teleost antibody responses then it would appear necessary to determine the specificity of antibody produced after vaccination in addition to its titre.

CHAPTER 7

A PRELIMINARY INVESTIGATION INTO THE USE OF BIODEGRADABLE (POLY-LACTIDE-CO-GLYCOLIDE) MICROPARTICLES FOR THE ORAL IMMUNISATION OF TELEOST FISH.

7.1 INTRODUCTION

Exploitation of a new generation of vaccine antigens and the delivery of peptides and proteins has been hampered by a lack of appropriate delivery systems (Almeida *et al.*, 1993); a situation which is particularly acute in the case of the oral delivery route. Antigens capable of inducing protective immunity may be attached to a suitable carrier which possesses adjuvant or sustained release properties in order to induce effective and long - lasting local immunity by interacting with MALT and/ or eliciting protective systemic immune responses on reaching immune competent organs such as spleen (Gregoriadis, 1990). The administration of bioactive molecules in microparticles is an expanding area of mammalian oral vaccine research (McGhee *et al.*, 1992; Morris *et al.*, 1994) and accumulated evidence indicates that biodegradable micro - and nanoparticles may act as efficient antigen delivery vehicles (Damgé *et al.*, 1988; Eldridge *et al.*, 1990, 1991; Michel *et al.*, 1991; O'Hagan *et al.*, 1991; Aguado, 1993; Moldoveanu *et al.*, 1993). The ability of the mammalian gut to absorb microparticulate material is now firmly established (section 2.2.3), although its extent and nature remain highly contentious issues

The rationale behind the use of controlled release systems for vaccine delivery is to reduce the number of repeated administrations required to establish long - term protection since the number of doses required for a vaccine to be effective against an infectious agent is pivotal in achieving the appropriate level of immunity (Aguado and Lambert, 1992). Spherical sustained release polymer particles may be classified into the 'reservoir type' in which the agent is in solution in cavities formed by the polymeric material and which are normally referred to as microcapsules and the 'monolithic type' where material is evenly dispersed throughout the polymeric matrix which are referred to as microspheres (Tice and Cowsar, 1984; Kissel *et al.*, 1990; reviewed by Morris *et al.*, 1994). Using such controlled release systems two types of delivery may be possible:- continuous antigen release where antigen is progressively released over a period of time and pulsed antigen release where a mixture of particles of different sizes and compositions are used to effect pulses of antigen release somewhat akin to conventional booster immunisation with vaccines (Aguado and Lambert, 1992). A wide range of antigens including influenza virus (Moldoveanu

et al., 1993), *Bacillus pertussis* filamentous haemagglutinin (Cahill *et al.*, 1993), *Escherichia coli* colonization factor antigen 1 (Edelman *et al.*, 1993), tetanus toxoid (Almeida *et al.*, 1993) and cholera toxin B subunit (O'Hagan *et al.*, 1993) have been incorporated into biodegradable microspheres for use in parenteral and oral immunization. Microsphere technology has the potential advantages of reducing the number of inoculations, enhancing the immune response after both parenteral and oral immunisation and reducing the total antigen dose needed to achieve protection (Morris *et al.*, 1994). The favoured co - polymer for oral (Peyer's patch) vaccination studies is presently poly (DL-lactide-co-glycolide) (DL-PLG) (Beck *et al.*, 1980, 1981, 1983). Their preferential status results from their biocompatibility (Vischer *et al.*, 1987), biodegradability, composition of non - toxic pharmaceutically approved components, adequate rates of absorption and a history of safe use in humans (Eldridge *et al.*, 1990). *In vivo* the polymer undergoes random non - enzymatic hydrolysis of its backbone ester linkages into the endogenous metabolites lactic and glycolic acids at a rate influenced by the molecular weight of its components, surface area, monomer stereoregularity, and lactide : glycolide ratio (Morris *et al.*, 1994). Polymers with different characteristics can be produced by altering a number of parameters (Lewis, 1990) including the ratio of the monomers lactide and glycolide and the incorporation of surfactants (Alonso *et al.*, 1993). By changing the ratio of polylactide to polyglycolide the delivery system may be 'programmed' to degrade and thus liberate antigen at predetermined intervals (Aguado and Lambert, 1992). Antigen is released from microparticles both by diffusion through matrix pores and by matrix degradation (Morris *et al.*, 1994). A possible problem with the use of PLG microparticles as a delivery system for delivery of labile macromolecules is the instability of the molecules in the acid environment created *in vivo* as some of the polymer degrades (Pitt, 1990).

Particle size appears to be a critical determinant of the fate of microparticles after absorption and possibly of the elicited immune response to the antigen (Aguado and Lambert, 1992; O'Hagan *et al.*, 1993; Jenkins *et al.*, 1994). Particles of less than 10 μm are absorbed by M cells and translocated to the Peyer's patch T and B cell zones (Eldridge *et al.*, 1989, 1990, 1991; Jenkins *et al.*, 1994). Studies with PLG microparticles found that those of less than 5 μm diameter were

endocytosed and transported by MAC-1 (a cell surface marker for macrophages) positive cells through efferent lymphatics to systemic lymphoid tissues to stimulate a serum antibody response. Particles greater than 5 μm diameter remain in the Peyer's patch leading to the sustained release of antigen in this SIgA inductive area (Eldridge *et al.*, 1990). These particles may protect antigen from breakdown in the acidic stomach and from proteolysis in the gut (Eldridge *et al.*, 1990; Challacombe *et al.*, 1992; Edelman *et al.*, 1993; Morris *et al.*, 1994). The reported absence of exposed antigen on vaccine-containing microparticles is in contrast with live recombinant vectors (chapter 8) whose adherence to and uptake by Peyer's patches may be inhibited by pre-existing antibody (McGhee *et al.*, 1992). Accurate quantification of microparticle uptake in the rat gut by Jenkins *et al.*, (1994) indicates that the levels of microparticle absorption may be insufficient for effective drug delivery but may be sufficient to deliver an immunogenic dose of antigen to the immune system. Oral immunisation with antigen incorporated in microparticles has been demonstrated to induce systemic (Eldridge *et al.*, 1989; Challacombe *et al.*, 1992; Edelman *et al.*, 1993; Moldoveanu *et al.*, 1993) and secretory (Eldridge *et al.*, 1989, 1990; Challacombe *et al.*, 1992; Moldoveanu *et al.*, 1993) antibody responses. Additionally, proliferative and cytotoxic T cell responses have been found after intraperitoneal injection of encapsulated antigen (O'Hagan *et al.*, 1993). It has been suggested that the efficacy of PLG microparticles in enhancing immune responses to antigens may reflect the need for high local antigen concentrations in the Peyer's patches and associated GALT in order to induce immune responses. Encapsulation may fulfil this requirement (Reid *et al.*, 1992) and reduce the dose necessary for oral immunisation. The exact means by which PLG microparticles exert their immunopotentiating effects by sustained antigen release are uncertain but may include a depot effect as with aluminium salt adjuvants, the delivery of antigen directly to antigen presenting cells or continuous exposure to antigen as with chronic infections (Morris *et al.*, 1994).

Very little work investigating microparticulate absorption by the teleost gut has been performed. Teleosts do not appear to possess M cell analogues but an antigen sampling function has been attributed to the second gut region/ hindgut (Davina *et al.*, 1982; Rombout and van den Berg,

1989) although whether this function extends to uptake of inert particulate matter is uncertain. A number of studies have demonstrated the absorption of bacterins by the teleost intestine (Davina *et al.*, 1982; Rombout and van den Berg, 1985) and some have reported that absorbed bacterial cells were not transported further but remained in the mucosa after uptake (Nelson *et al.*, 1985). Evensen *et al.* (1993) detected the uptake of *Aeromonas salmonicida* bacterins in the hindgut epithelial cells of Atlantic salmon up to 7 days after oral administration. In a study of particulate absorption by the gut of larval teleosts, Olafsen and Hansen (1992) found that the hindgut epithelial cells which resembled those of adult teleosts endocytosed a range of bacterial strains but did not absorb fixed erythrocytes or bacterial sized latex particles. Studies in our laboratory and at the University of Nottingham using a sensitive flow cytometric technique to measure microparticle absorption (Jamieson *et al.*, unpublished observations) have indicated that all areas of the rainbow trout gut can absorb polystyrene microspheres of 1.0 μm and 0.1 μm in quantities similar to those observed in mammals. If secretory immune responses may be induced by the administration of particulate antigen then it could be expected that microparticles, if absorbed, may be effective in inducing immune responses to encapsulated antigen. The use of biodegradable microparticles as a delivery system in fish has not been extensively studied but initial results using parenterally delivered PLG microparticles appear encouraging (Davidson, pers comm). The present study sought to apply the strategies devised in earlier parts of this study (chapters 5&6) to assess whether encapsulation of HGG in PLG microparticles protects the antigen from degradation in the gastric and intestinal gut regions, increases its presence in the circulation and results in an enhanced antibody response to the antigen.

7.2 MATERIALS AND METHODS

7.2.1 Animals

Groups of rainbow trout (150-200g in weight) were maintained as described in section 3.2.1.

7.2.2 Preparation and characterisation of PLG (Poly-lactide-co-glycolide) microparticles incorporating HGG.

Microparticles were formulated in Nottingham University by Dr P.G Jenkins using a water - in - oil - in - water (w/o/w) emulsion evaporation method adapted from the processes of Beck *et al.* (1979) and Jeffery *et al.* (1991). A 6% solution of Poly (DL-lactide-co-glycolide; PLG) copolymer, composition 50:50 (Resomer RG 503, Boehringer-Ingelheim, Germany) in dichloromethane (HPLC grade, May and Baker, Essex) was emulsified with 5ml of distilled water containing 250 mg of HGG (Sigma) at 13,000 x g for 7 min with a Silverson homogeniser (Silverson Machines, Chesham, Bucks). The resultant oil-in-water (o/w) emulsion was again emulsified with 60 ml poly-vinyl alcohol (20% w/v in water) at 13,000 x g for 10 min to produce a water-in-oil-in-water (w/o/w) emulsion. This was stirred for 12-18 hr under ambient conditions while microparticles formed. Microparticles were collected and washed by centrifugation (3 times at 15,000 x g for 15 min), (Beckman), freeze dried overnight and stored at 4°C.

Microparticles were characterised by scanning electron microscopy to check surface characteristics such as shape and lack of porosity and to serve as a primary evaluation of the poly-dispersity of the batch of particles. Particles were measured by laser photon correlation spectroscopy (PCS) and sizes expressed as 'volume mean diameter'. The content of HGG in microparticles was established by a BCA protein assay (Pierce). Aliquots (15 mg) of each batch of encapsulated antigen was degraded in 1 ml of 5% SDS in 0.1 M NaOH overnight with shaking under ambient conditions. 100 µl of each sample and of standards (ovalbumin) was mixed with BCA reagent (49 ml BCA + 1 ml copper sulphate (Sigma)) at 65°C for 30 min. The protein content of each sample was assessed spectrophotometrically at 562 nm and interpolated into an ovalbumin standard curve.

7.2.3 Antigen delivery and collection of samples.

For the determination of whether encapsulation protected HGG from gastrointestinal proteolysis and increased its uptake into the bloodstream, fish were intra-gastrically intubated with either 5 mg HGG in 0.2 ml PBS or 58.4 mg PLG-encapsulated HGG (equivalent to 5 mg HGG in the first batch of microparticles) in 0.2 ml PBS after being starved for 48 hr. Three fish were sacrificed from each of the 2 groups 15, 30 and 45 min and 1, 2, 6, 12, 24 and 48 hr after antigen delivery and samples of gastric mucus, intestinal mucus and plasma collected as described in section 5.2.2. For the antibody response study groups of 18 fish were intra - gastrically immunised with either 2 mg HGG in 0.6 ml PBS or 100 mg PLG-encapsulated HGG (equivalent to 2 mg HGG in the second microparticle batch) in 0.6 ml PBS or with control PBS. Cutaneous mucus, intestinal mucus, bile and plasma samples were collected (section 6.2.3) over a period of 9 weeks after immunisation when a second immunisation of 2 mg HGG in 0.6 ml PBS was administered to the remaining fish in both groups. Samples were again collected bi - weekly for a further 6 weeks.

7.2.4 Analysis of antigen proteolysis, antigen uptake and antibody responses to HGG.

Mucus samples from gastric and intestinal regions and plasma samples were diluted 1:5 with non - reducing sample buffer, boiled for 15 min and subjected to SDS- PAGE and Western blotting as described in section 5.2.6(A). The system for detection and evaluation of antibody in secretions and in plasma was as described in section 6.2.6.

7.3 RESULTS

7.3.1 Influence of encapsulation on the retention time and fate of HGG in the gastric region of the trout digestive tract after oral delivery.

A scanning electron micrograph showing the spherical shape and relatively uniform size of the microparticles containing HGG is presented in Fig 7.1. The effect of encapsulation on the intra-gastric fate of HGG is presented in the form of immunoblots in Fig 7.2. In addition to the detection of intact HGG a large number of HGG fragments were detectable in the gastric region of fish administered soluble HGG alone 15 min after antigen delivery. The number of bands detected did not subsequently increase greatly but at times later than 45 min post-delivery little or no intact antigen was detected. After 2 hr post-intubation no antigen in any form was detectable suggesting that it had been evacuated from the stomach or degraded completely. The pattern of fragmentation detected in the gastric regions of fish administered encapsulated HGG was similar although the overall staining was less intense (Fig 7.2 (B)). However the intensity of staining for this group fluctuated considerably between time points. By comparing immunoblots for each of the immunised groups it was found that the total antigen detected in the gastric region by image analysis for the PLG-encapsulated HGG group was 44 % of that present in the soluble HGG group suggesting that most of the antigen in the PLG group was within the microparticles. Most of the antigen detected in these fish was in a fragmented form and in contrast with fish administered soluble HGG, antigen was still detectable in the gastric region of these fish up to 12 hr after delivery (Fig 7.3).

7.3.2 Effect of encapsulation on the form of HGG detected in the intestines of fish after oral delivery.

Semi - quantitative data demonstrating the presence of HGG in the intestinal regions of fish at various times after antigen administration is presented in Table 7.1. It is apparent that no intact or high molecular weight HGG was detectable in the intestines of fish administered HGG in soluble form. Antigen was only detected at a low level at 15 min and, in particular, at 30 min after administration in the form of a 43.6 kDa fragment. No HGG in any form was detected

between 45 min and 48 hr. In contrast, when scrapings from the intestines of fish administered encapsulated HGG were analysed no antigen in any form was detected until after 2hr post-delivery. Subsequently, antigen was detectable up to 24 hr post-delivery. At 2 hr and 12 hr most of the antigen detected was apparently in an intact form, although a considerable amount of fragmented HGG was detected after 6 hr and 24 hr.

7.3.3 Effect of encapsulation on the form of HGG detected in the plasma of fish after oral delivery.

As was the case with the intestinal scrapings from fish intubated with soluble antigen little HGG was detected in the plasma and none in high molecular weight or intact form. Fragmented HGG was detectable 15 min, 1 hr and 6 hr after delivery. In the case of fish administered encapsulated HGG a number of fragments and also intact antigen were detectable although at an extremely low level (Table 7.2).

7.3.4 Antibody responses to soluble and encapsulated HGG in plasma and secretions.

Fig 7.4 presents the antibody titres recorded in the plasma of fish orally intubated with soluble or PLG-encapsulated HGG over a period of 15 weeks. The primary response in the group immunised with encapsulated antigen appeared to be delayed compared to the group immunised with soluble antigen, although by week 5 titres were very similar in both groups. The response declined rapidly to a level not significantly different from controls by 9 weeks post immunisation. After boosting with soluble HGG at week 9 the responses in both groups increased, the highest titres recorded in the entire study were at week 11 (2 weeks after boosting) in 2 of 3 fish which were initially primed with PLG-encapsulated HGG. The mean titre of this group was reduced since a third fish did not respond so the overall mean was not significantly different from the titre in the soluble HGG group. As with the response after priming the titres declined rapidly after boosting to a low level by week 13.

Antibody was not detected in bile or cutaneous mucus of fish from either group (results not presented). Similarly, titres in the gut mucus from both groups were not significantly different

from controls at any week other than in fish primed with PLG-encapsulated HGG at week 11 (week 2 after boosting) when titres greater than controls were detected in all 3 fish and relatively high titres were recorded in 2 of the 3 fish (Table 7.3).

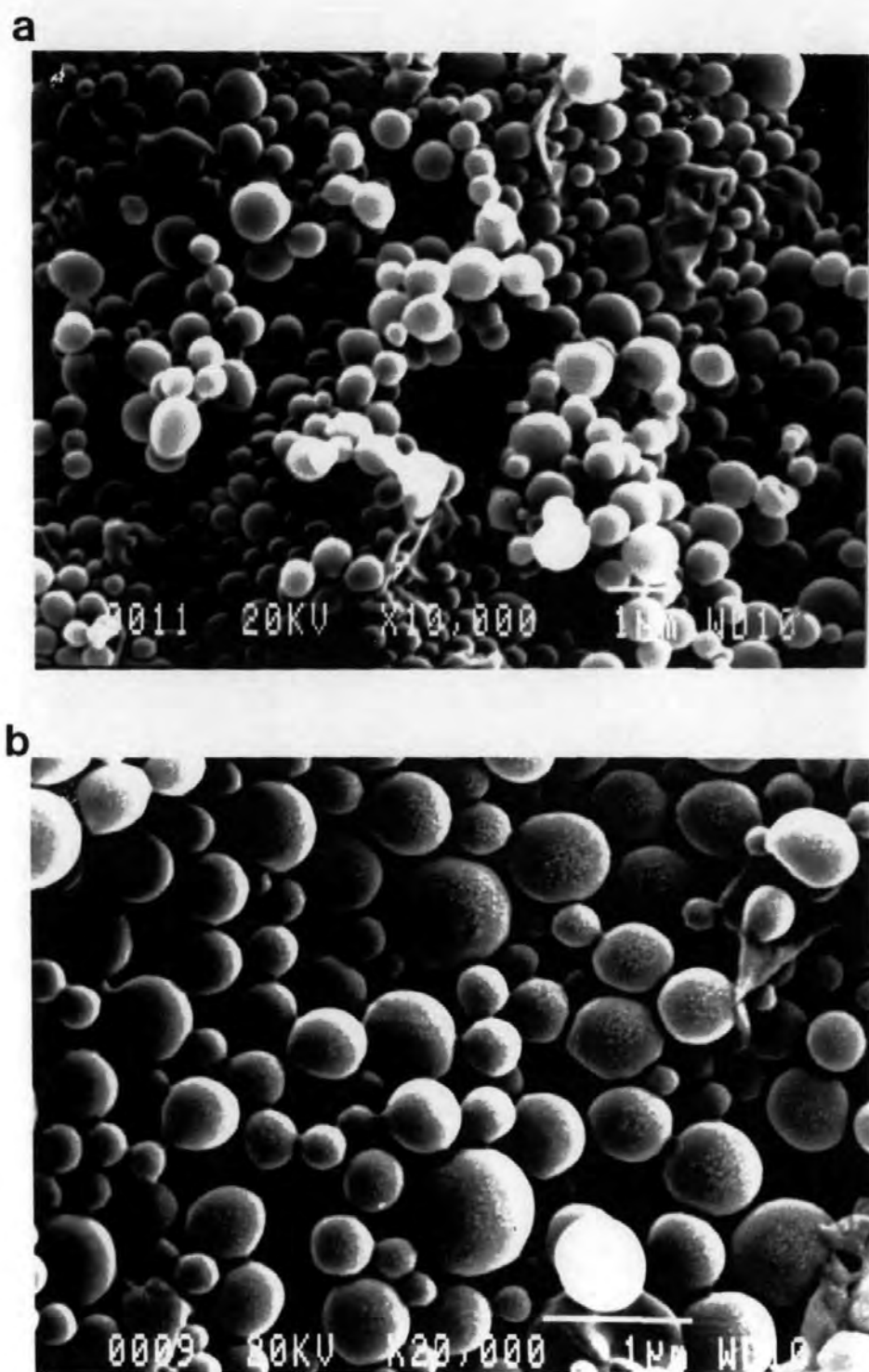


Fig. 7.1: Scanning electron micrographs of PLG microparticles incorporating HGG. *a:* Magnification x 10, 000. *b:* Magnification x 20, 000.

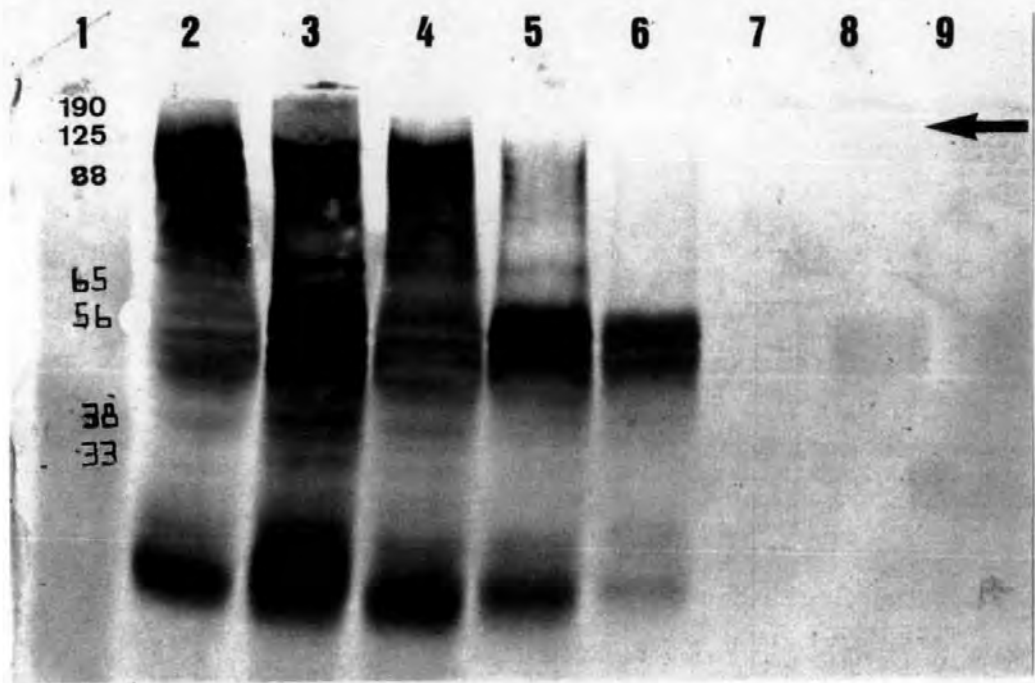


Fig.7.2. (a): Western blot demonstrating the presence of HGG in gastric scrapings of rainbow trout over a time course after administration of 5 mg HGG in 0.2 ml PBS. Loading order: Lane 1, prestained molecular weight markers. Lanes 2-9, gastric scrapings collected from fish 15 min, 45 min, 1 hr, 2 hr, 6 hr, 12 hr, 24 hr and 48 hr after antigen administration. ← - Intact HGG.

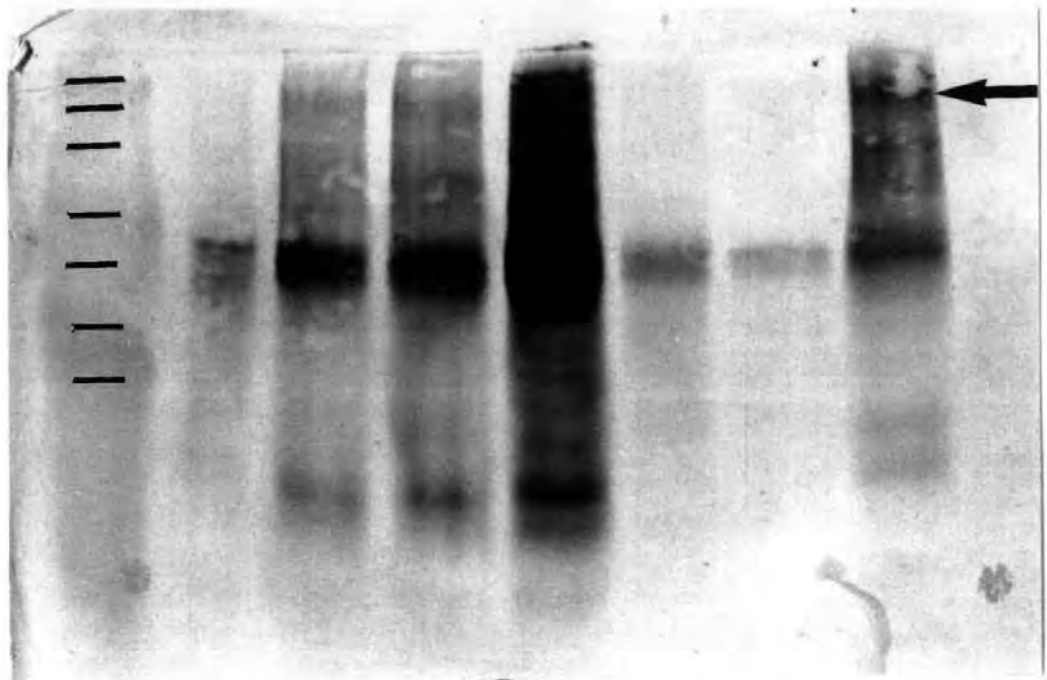
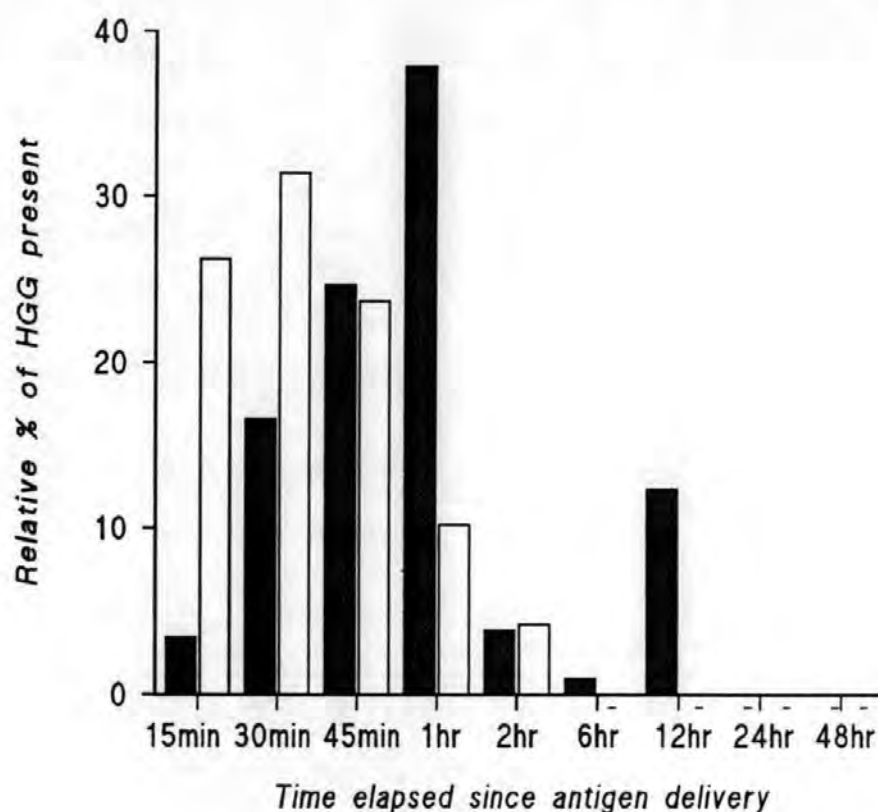


Fig.7.2. (b): Western blot demonstrating the presence of HGG in gastric scrapings of rainbow trout over a time course after administration of 58.4 mg of PLG-encapsulated HGG (containing 5mg antigen) in 0.2 ml PBS. Loading order: Lane 1, prestained molecular weight markers (molecular weights in kDa). Lanes 2-9, gastric scrapings collected from fish 15 min, 45 min, 1 hr, 2 hr, 6 hr, 12 hr, 24 hr and 48 hr after antigen administration. ← - Intact HGG. Bars in lane 1 represent molecular weight markers as in (a) above

Fig.7.3: Effect of microencapsulation on retention time of HGG in stomach



Data generated by image analysis of immunoblots. Relative percentages represent the proportion of the signal detected in gastric scrapings at a particular time point as a proportion of the total signal detected in samples collected over the entire time course after a particular treatment. The percentages are therefore not directly comparable between treatments ($n = 3$).

□ = Group administered soluble HGG.

■ = Group administered encapsulated HGG.

- = No signal detected.

Table. 7.1: *Apparent molecular weights and relative percentages of HGG bands detected in intestinal scrapings from fish administered either soluble HGG or HGG encapsulated in PLG microparticles.*

		Time elapsed after antigen delivery								
	Fragment size(kDa)	15 min	30 min	45 min	1 hr	2 hr	6 hr	12 hr	24 hr	48 hr
Soluble HGG	50.2	100%								
	43.6		69.2%							
	32.3		13.3%							
	31.1		17.6%							
PLG encapsulated HGG	151.4					100%		87.3%	7.7%	
	77.5						13%			
	63.7						46.7%			
	51.2						9.7%	12.7%		
	45.5						31.5%		92.3%	

Data was generated by image analysis of individual lanes on immunoblots and percentages represent the percentage of a particular band as a proportion of the total HGG signal detected in that lane.

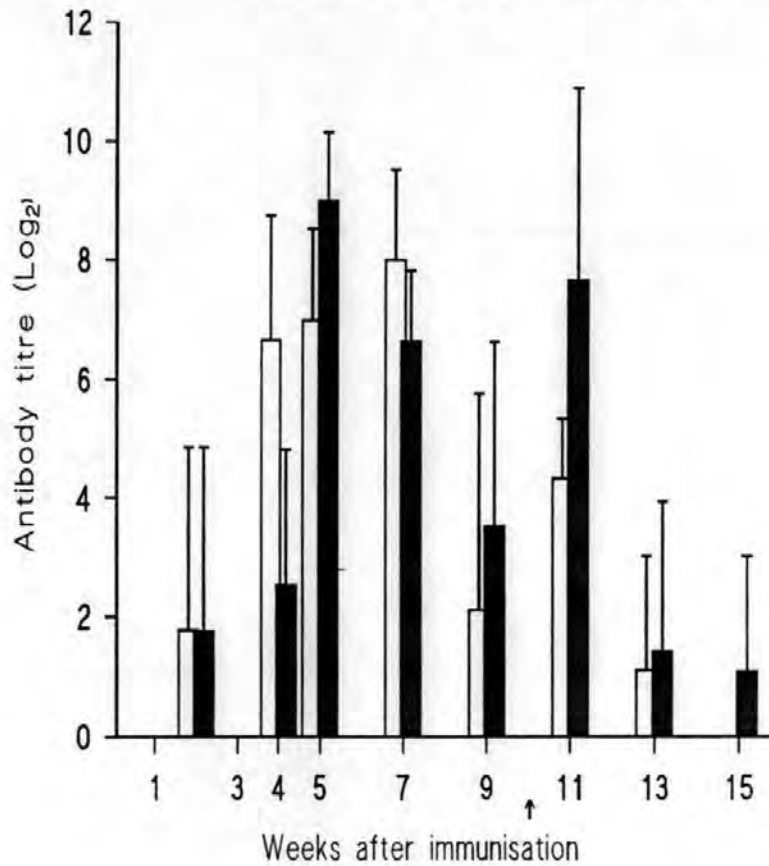
Table. 7.2: Apparent molecular weights and relative percentages of HGG bands detected in plasma from fish administered either soluble HGG or HGG encapsulated in PLG microparticles.

		Time elapsed after antigen delivery							
	Fragment size(kDa)	15 min	45 min	1 hr	2 hr	6 hr	12 hr	24 hr	48 hr
Soluble HGG	80.1	(**)							
	52.4	100%		(**)					
	42.5			100%		36.6%			
	36.5					53.4%			
PLG encapsulated HGG	149.4	28.1%	-	47.5%	44.1%	62.2%	56.7%	60.4%	
	61.4	15.5%							
	51.7	41.4%	22.1%	18.2%	28.1%			19.8%	
	44.5		44.8%	19.4%	27.8%	14.9%	23.3%	19.8%	
	33.8	15%		14.9%		22.9%	20%		

Data was generated by image analysis of individual lanes on immunoblots and percentages represent the percentage of a particular band as a proportion of the total HGG signal detected in that lane.

(**) - Band present but yielding value < 10 (arbitrary units) by image analysis.

Fig.7.4: Antibody levels in plasma after oral immunisation of fish with soluble or encapsulated HGG



Antibody titres determined by ELISA in plasma of fish immunised with soluble (□) or PLG - encapsulated (■) HGG at week 0 and boosted with soluble HGG at week 9 (↑).

Table 7.3 Antibody titres measured in intestinal mucus of fish after boosting with soluble HGG.

	Mean Titre (Log ₂) (n=3)	Standard deviation
Soluble HGG	NS	-
PLG - encapsulated HGG	7.44	2.26

Antibody titres determined by ELISA in mucus of fish immunised with soluble or PLG - encapsulated HGG 2 weeks after boosting with soluble HGG.

NS - not significantly different from controls ($p < 0.05$)

7.4 DISCUSSION

The evidence from the present study suggests that the encapsulation of antigens in PLG microparticles may be a useful oral delivery system for teleost fish. Encapsulation was found to protect a percentage of the antigen from proteolysis in the digestive tract and to increase the level of intact antigen subsequently reaching the bloodstream. The detection of fragmented HGG in the gastrointestinal tract of fish orally intubated with PLG-encapsulated antigen indicated that a considerable amount of HGG was present on the microparticle surface. This suggests that caution must be exercised in the interpretation of results after oral compared to parenteral delivery of PLG encapsulated antigen since in addition to the uptake of encapsulated (intact) antigen fragmented and/or intact soluble antigen may also be absorbed. Microencapsulation of HGG did not result in an enhanced plasma antibody response compared with the response produced to soluble HGG. However, detection of antibody in the gut mucus of fish primed with PLG-encapsulated HGG and boosted with soluble HGG may provide encouragement for further research.

The uptake of particulate matter by the gut is a controversial field in mammalian research. Indeed, O'Mullane *et al.* (1987) concluded that "the transport of intact carriers across the gastrointestinal tract is restricted to exceptional and unusual circumstances". Although this is in conflict with a number of reports of microparticulate uptake across the mammalian gut (see section 2.2.3), it serves to demonstrate the extent of the debate regarding the extent and role of such processes. Little information is available on the uptake of inert particulate matter from the teleost gut. Previous work in our laboratory (Jamieson *et al.*, unpublished observations) indicated that 0.1 μm and 1.0 μm inert fluorescent latex microspheres were absorbed by gastric and intestinal regions of the trout gastrointestinal tract. Although the uptake of HGG was not quantified by ELISA in the present work the increased plasma levels of intact HGG detected on Western blots after delivery of microencapsulated antigen suggested that the particles were absorbed across the trout gut.

A number of *in vitro* studies on mammals have found that there are two stages in the release of antigen from PLG microparticles. An initial period when 15-35 % of antigen is released, possibly

by diffusion through water filled channels and a later prolonged period over which antigen is slowly released (Eldridge *et al.*, 1990; Alonso *et al.*, 1993). The details of this second phase of release depends on the particular characteristics of the microparticles used. The release kinetics of encapsulated antigen have been found to depend on the ratio of lactic/ glycolic acid, polymer molecular weight, surface characteristics, vaccine composition, vaccine loading and microparticle size (Aguado and Lambert, 1992; Alonso *et al.*, 1993). The results of the present investigation indicate that this classic pattern of a 'spike' release of surface associated antigen and subsequent slow release of antigen may occur if microparticles are parenterally administered when given orally it is likely that surface associated antigen will be degraded in the gut.

The strategy devised and described earlier in this thesis (section 5.2) for analysing the extent of antigen degradation in the trout gastrointestinal *in vivo* was successfully applied in this investigation to determine whether the encapsulation of HGG in PLG microparticles protected the antigen from proteolysis in the gut. The results obtained with gastric scrapings from fish orally intubated with 5 mg soluble HGG were similar to those described previously (section 5.3) with substantial degradation of the protein to peptides as quickly as 15 min post-administration. The absence of detectable intact antigen in the gastric scrapings of these fish at time points later than 1 hour post intubation may reflect either gastric evacuation and/or the complete degradation of the intact antigen in the stomach. If the intact protein was fully degraded in the stomach then the earlier findings that a proportion of HGG remained intact for up to 15 hr in gastric secretions (sections 3.4, 5.4) may have been a result of the high levels of antigen used. These high doses may have exceeded the enzymic capacity of the gastric proteases. Analysis of gastric scrapings from fish administered encapsulated HGG were difficult to interpret because of the variation in the amount of antigen detected at different time points by immunoblotting. This may reflect the inadequacy of boiling samples in electrophoresis sample buffer to release antigen from the microparticles. The method was not optimised and it is likely that there was variability between samples in the amount of antigen released since organic solvents are required for complete dissolution of particles (Jenkins, pers comm). Nonetheless, it was evident that the greater amount of the surface

associated and/ or released antigen in the stomach was in the form of fragmented HGG. The pattern of fragmentation of the released antigen was very similar to that observed after delivery of the soluble protein. The microencapsulation of HGG resulted in an increase in gastric retention time from 2 to 12 hr. Furthermore, encapsulation delayed the time before HGG was first detectable in the intestine from 15 min to 2 hr. It was suggested earlier (section 5.4) that reports of a biphasic pattern of soluble antigen uptake in teleosts (Jenkins, 1992) may have been a result of the intubation procedure used and of the use of soluble HGG in fluid form. The finding of HGG in the intestinal region of fish administered soluble antigen at 15 and 30 minutes after intubation may add further support to this contention. The results with the encapsulated antigen on the dynamics of gut passage may therefore be closer to that of antigen delivered in feed *in vivo*. Antigen was only detectable up to 30 minutes after delivery and in the form of small peptides in the intestines of fish administered 5 mg soluble HGG which is in contrast to earlier findings in this thesis (section 5.3). This again may have reflected the higher dose of HGG administered (35 mg per fish) and indicates that high doses of protein antigens may be necessary to effect transport of soluble proteins in detectable quantities to the intestinal regions.

A considerable percentage of the HGG which was detected in scrapings from the intestinal regions of fish was apparently intact which indicated that encapsulation protected the antigen from proteolysis in the gut. The detection of HGG mainly as intact antigen and high molecular weight fragments indicated that the fragmented HGG found in the gastric region of these fish was either fully degraded or absorbed from the lumen. A greater amount of both intact and fragmented antigen was found to be present in the plasma of fish receiving encapsulated HGG as compared with fish receiving the soluble antigen. After administration of soluble HGG, antigen was detectable in the plasma after 15 minutes and at a higher level after 1 hour and 6 hours post delivery, a pattern similar to that described previously (section 5.3). No intact antigen was detected at any time in the plasma of these fish however. Since only 8.6 percent (w/w) of the microparticles used in this study consisted of HGG and since a considerable amount of the associated antigen was degraded it was encouraging to detect any antigen in the plasma. Indeed,

if the loading of antigen and the proportion of entrapped antigen could be increased one may assume that a considerably greater amount of antigen would be absorbed since the determining factor is the extent of particle absorption.

A number of studies in mammals have reported increased levels of systemic antibody after oral administration of antigen in PLG microparticles (Challacombe *et al.*, 1992; Edelman *et al.*, 1993; O'Hagan *et al.*, 1993). These have partly attributed the efficacy of PLG microparticles in enhancing the immune response against soluble protein antigens to the protection of the antigen from gastric and intestinal destruction by the polymer wall (Challacombe *et al.*, 1992; Edelman *et al.*, 1993). Although, in the present work encapsulation protected HGG from degradation in the gut and increased its plasma presence it did not significantly increase the specific antibody titres in the plasma. For the purposes of immunisation the absorption of fragmented antigen derived from the microparticle surface into the bloodstream may raise some complications. If oral tolerance is the normal outcome of antigen feeding (Challacombe and Tomasi, 1980; Strober *et al.*, 1983; Enders *et al.*, 1986; O'Hagan, 1990) then the absorption of degraded soluble protein might be expected not to result in an immune response; the observation of considerably enhanced immune responses after oral immunisation with PLG-encapsulated antigen (Challacombe *et al.*, 1992; Edelman *et al.*, 1993; Moldoveanu *et al.*, 1993) however suggests that this is not the case. It would be interesting to produce microparticles with no surface antigen and to compare uptake and immune responses with those possessing surface associated and entrapped antigen since protein antigen fragments have been found to have a role in the induction of oral tolerance (Michael, 1989, 1992; Hachimura *et al.*, 1993). The adsorption of antigen onto biodegradable microparticles has however been shown to enhance secretory immune responses in comparison with soluble antigen in gastrically immunised rats (O'Hagan *et al.*, 1989). This may indicate that the PLG particles exert an adjuvant effect unrelated to antigen encapsulation but this was not supported by the present findings. The plasma antibody titres in fish immunised with microparticles in this study initially appeared lower than in fish administered soluble antigen but five weeks after primary immunisation the responses in both groups were similar and both declined to control

levels by week 9. The slower kinetics of antibody production in the fish intubated with PLG-encapsulated antigen may have reflected the degradation rates of the particles used which were formulated to release antigen in 4-6 weeks (Jenkins, pers comm). Two weeks after boosting with soluble HGG high levels of antibody were detected in 2 of 3 fish primed with PLG-encapsulated HGG but as with the response after priming the titres declined rapidly. Therefore, further studies with larger numbers of fish are necessary to further establish the significance of these results.

Antibody was undetectable in most cases in the secretions of immunised fish in these experiments. This was in contrast to the results described earlier (section 6.3) when antibody in the cutaneous mucus was regularly detected albeit at a low level in fish orally immunised with 35 mg HGG and probably reflects the lower antigen dose used (2.2 mg). A previous study on rainbow trout (Burgess, 1988) also found very little antibody in the cutaneous mucus, intestinal mucus or bile of fish orally immunised with 1 mg HGG. Perhaps a higher antigen dose is needed to generate local rather than systemic responses. Similarly, biliary antibody was not detectable in any of the fish receiving either soluble or encapsulated HGG. In contrast while antibody was not detectable in the intestinal mucus of any of the fish immunised with soluble HGG it was detected in the gut mucus of all 3 PLG immunised fish 2 weeks after boosting and was present at a high level in 2 of these fish. Oral immunisation with 35 mg HGG did not result in gut antibody levels of this magnitude (section 6.3). In fact, in the latter investigation high titres were only found in the intestinal mucus of parenterally immunised fish. Due to the small number of fish tested and the absence of gut antibody titres in this group at all other times it is difficult to assess the significance of this finding but if it is a general feature this may be highly promising for the prospects of oral vaccination. Only a single priming immunisation with microparticles was administered in the present work which is in contrast to mammalian studies where microparticles are administered on three or more occasions. However, a number of these studies found that the secretory antibody responses to encapsulated antigens may be short lived (Eldridge *et al.*, 1989; Challacombe *et al.*, 1992; Edelman *et al.*, 1993). Therefore, the transient detection of antibody in the gut mucus of fish immunised with PLG - encapsulated HGG here may not be anomalous but may be a feature

of this delivery system. Davidson *et al.* (1993) detected considerable numbers of antibody secreting cells in the head kidney and intestinal mucosa of rainbow trout 3 weeks after peroral intubation with *Aeromonas salmonicida* bacterins and this response declined rapidly in both sites to undetectable levels by week 4. This may help to explain the transient presence of antibody to encapsulated HGG in the gut mucus. Georgopoulou and Vernier (1986) induced an antibody-secreting cell response in the intestine of rainbow trout after oral administration of HGG but it was not determined if the antibody produced was destined for the gut lumen. In contrast, Davidson (1991) was unable to detect an antibody secreting cell response to orally intubated soluble keyhole limpet haemocyanin (KLH) and suggested that particulate antigens may more effectively stimulate local immunity in the teleost gastrointestinal tract. Previous studies on teleosts have reported the detection of secretory antibody after the repeated enteric administration of particulate but not soluble antigen (chapter 6) but it is difficult to determine at present if a similar outcome is possible using PLG microparticles as a delivery system.

While the present results are relatively encouraging a considerable amount of work is required to establish whether the encapsulation of antigen in PLG is a viable oral delivery system. Unfortunately the microparticles used for the antibody response study had an antigen loading of only 2.2%. Typical loading amounts are in the range 1 - 10% but may be considerably increased (Aguado and Lambert, 1992) and further studies should investigate the effects of increased antigen loading. Detection of antigenically intact HGG in the gut and plasma and the detection of specific antibody in the plasma and gut mucus indicated that the encapsulation process did not damage the antigen. A previous study found that the molecular weight and antigenicity of ovalbumin remained unaltered by the same encapsulation process as used in the present study (Jeffrey *et al.*, 1993). The use of particles of different sizes and release rates should be applied to determine if responses can be enhanced and sustained. There is some evidence to suggest that in mammals that fast release particles may be more effective in inducing primary immune responses while delayed release microspheres are more effective in boosting the response (Staas *et al.*, 1991; Aguado and Lambert, 1992). Similarly, it will be interesting to determine if larger beads are retained in the

mucosa to generate a local response as occurs in mammals (Eldridge *et al.*, 1989; Jani *et al.*, 1992). Much more fundamental work on microparticulate uptake and the capacity for inducing local immunity in teleost species is required. It will be of particular importance to determine which cells are involved in absorption of microparticulate material in teleosts since the efficacy of PLG microparticles in inducing local immunity in mammals may be a result of the accumulation of large antigen doses inside Peyer's patches. If the uptake in the teleost gut is diffuse this localised concentration of antigen may not occur and the potential advantages of PLG microparticles as an oral delivery system for inducing secretory immune responses for such species might not materialise. However, if the adjuvant effect of PLG particles which has been noted in mammals also applies in fish then encapsulation may be of value in increasing the effectiveness of oral vaccines. Additionally, the finding in the present work that encapsulated antigen was absorbed into the bloodstream indicates that orally delivered microparticles may reach the primary lymphoid organs to induce a systemic immune response.

CHAPTER 8

*THE ADHERENCE TO AND INVASION OF SALMONID EPITHELIAL CELLS BY WILD TYPE AND ATTENUATED (Δ aroA) STRAINS OF *Aeromonas salmonicida*.*

8.1 INTRODUCTION

Amongst the greatest advances in the quest for effective vaccines in recent years has been the development of a potential new generation of live attenuated organisms resulting from the application of modern molecular biology and improved understanding of microbial pathogenicity at the molecular level (Chatfield *et al.*, 1993). The use of such organisms as vaccines and as carrier vehicles for delivery of heterologous antigens is an approach being increasingly applied in mammals and more recently in piscine species (Leong, 1993). These vaccines can induce better protection than conventional killed vaccines, probably as a result of their ability to establish limited infections in the host thus mimicking the early stages of natural infection to stimulate a potent immune response. Live replicating antigens have been described as more effective mucosal immunogens than their dead, non-replicating counterparts which generally induce weak mucosal responses (Lycke and Svennerholm, 1986; van der Heijden *et al.*, 1991; McGhee *et al.*, 1992). Additionally, mass production, distribution and administration are easier and less costly for such live attenuated organisms than for purified component vaccines (Cárdenas and Clements, 1992).

A considerable amount of work has been performed investigating the use of recombinant live viruses as potential vaccine vectors, including vaccinia (Andrew *et al.*, 1992; Tartaglia *et al.*, 1992), adenovirus (Gallichan *et al.*, 1993; Zheng *et al.*, 1993) and varicella zoster virus (Lowe *et al.*, 1987). The present study, however, focuses only on the application of attenuated bacterial strains as mucosal vaccines and as enteric delivery systems incorporating foreign antigens.

The use of chemical or UV mutagenesis to attenuate bacteria was the original approach to the generation of a number of effective vaccines (Chatfield *et al.*, 1993). The *Salmonella typhi* Ty21a oral vaccine generated by chemical mutagenesis, for example, has resulted in some degree of success in field trials (Germanier and Furer, 1975; Wahdan *et al.*, 1982) and has been used experimentally as a carrier vehicle for foreign antigen (Baron *et al.*, 1987; Tacket *et al.*, 1990).

Similarly, the live oral cholera vaccine CVD103-HgR has shown better efficacy than the killed whole cell B subunit vaccine (wc\bs) in field trials (Dragonsky *et al.*, 1992). Unfortunately such methods result in poorly defined genetic lesions, batch - to - batch variation in vaccine formulations, are hampered by the potential for reversion to virulence and have been largely superseded by the application of modern molecular biological techniques. The application of such strategies has resulted in the identification of genes vital for *in vivo* growth and survival and it is now possible to introduce defined mutations into individual genes thus attenuating strains in a rational manner and producing safe, non - reverting vaccines that can be reliably quality controlled. Research into the use of attenuated bacterial species including *Yersinia enterocolitica* (Van Damme *et al.*, 1992), *Escherichia coli* (Newland *et al.*, 1992), *Bordetella pertussis* (Roberts *et al.*, 1990), *Bacillus anthracis* (Ivins *et al.*, 1990) and *Shigella flexneri* (Verma and Lindberg, 1991; Kärnell *et al.*, 1992) as live vaccines and as carriers is ongoing but the organism of choice for much of this work has been *Salmonella*. This is because apart from *Escherichia coli*, *Salmonella* is the most genetically defined organism in existence and the transfer of genetic material between the latter two species is feasible. Manipulation and characterisation work can therefore be performed in *E. coli* and DNA subsequently introduced into the *Salmonella* strain (Chatfield *et al.*, 1993). The ability to adhere to and invade the gut epithelium is obviously a prerequisite for gaining access to the underlying GALT and the subsequent presentation of foreign antigen at this site. *Salmonella* is an invasive organism and can replicate in the immunologically responsive tissues of the gut. Therefore the organism may be used to deliver foreign antigens to the appropriate site for processing and presentation to immunologically responsive cells (Cárdenas and Clements, 1992). A wide range of attenuated *Salmonella* strains have been produced which are deficient in such characteristics as purine biosynthesis (O'Callaghan *et al.*, 1988), LPS biosynthesis, galactose metabolism (Hone *et al.*, 1987), adenylate cyclase regulation (Curtiss III and Kelly, 1987), phosphate metabolism (Miller *et al.*, 1989a), regulation of porin genes (Dorman *et al.*, 1989), porins (Chatfield *et al.*, 1991), stress response (Chatfield *et al.*, 1992a), PABA

synthesis (Stocker, 1990), haem biosynthesis (Benjamin *et al.*, 1991) and aromatic compound biosynthesis (Miller *et al.*, 1989b; Jones *et al.*, 1991; Chatfield *et al.*, 1992b). Studies have mainly focused on genetically defined attenuated auxotrophic *Salmonella* strains, especially on genes encoding enzymes of the prechorismate biosynthetic pathway (*aro* genes). *Salmonella* strains with defined lesions in several *aro* genes including *aro* A, *aro* C and *aro* D either alone or in combination are effective single dose vaccines in several animal models (Dougan *et al.*, 1988; Miller *et al.*, 1989; Jones *et al.*, 1991). Genetically defined candidate oral typhoid vaccines based on double *aro* mutants of *Salmonella typhi* have been produced (Hone *et al.*, 1991; Chatfield *et al.*, 1992), one of which, CVD908 after a single dose resulted in a high rate of seroconversion to O-antigen and a specific IgA-antibody secreting cell response in the gut of 100% of volunteers. Using such strains as vaccine vectors, antigens from eukaryotic and prokaryotic species as diverse as malaria (Sadoff *et al.*, 1988), hepatitis B virus (Wu *et al.*, 1989), human immunodeficiency virus (Charbit *et al.*, 1993), *Clostridium tetani* (Fairweather *et al.*, 1990), *Streptococcus pneumoniae* (Paton *et al.*, 1993) and *Streptococcus sobrinus* (Doggett *et al.*, 1993b) have been cloned into and expressed in *Salmonella* strains in attempts to produce multivalent vaccines. Induction of both a specific mucosal (Doggett *et al.*, 1993b) and systemic antibody response to a variety of antigens expressed by orally delivered recombinant *S. typhimurium* (Stabel *et al.*, 1991; Chatfield *et al.*, 1992c; Sjöstedt *et al.*, 1992) has been demonstrated and in some cases these responses accompanied protection against a subsequent challenge with the pathogen (Chatfield *et al.*, 1992c; Poirier *et al.*, 1988; Sjöstedt *et al.*, 1992). Attenuated *Salmonellae* have additionally been shown to induce cytotoxic T - cell responses to carried antigens (Tite *et al.*, 1990) so the capacity exists to elicit a range of immune responses to antigens incorporated in live bacterial vaccines.

An aromatic deficient strain of *Aeromonas salmonicida*, the aetiological agent of the disease furunculosis in salmonids, has been constructed and shown after parenteral immunisation to induce

a protective immune response in salmon and trout (Vaughan *et al.*, 1993). The *aro A* gene codes for the synthesis of 3-enolpyruvylshikimate-5 phosphate synthetase which catalyses the conversion of phosphoenolpyruvate and shikimate-3-phosphate into 5-enolpyruvoylshikimate-3-phosphate in a pathway eventually leading to the synthesis of chorismate, a common intermediate compound in the synthesis of aromatic amino acids, *p* - aminobenzoic acid (essential for folate synthesis), 2,3 -dihydroxybenzoic acid (needed for synthesis of enterochelin-an iron chelator), *p*-hydroxybenzoic acid (precursor of ubiquinone) and o-succinylbenzoic acid (a precursor of vitamin K). The latter two compounds are not essential for bacterial growth and survival; tyrosine, tryptophan and phenylalanine are essential but are available in mammalian cells so their *de novo* synthesis is unnecessary. 2, 3-dihydroxybenzoic acid and *p*-aminobenzoic acid, however, are essential nutrients which are not available in mammalian tissues and mutants unable to synthesize these metabolites are avirulent since they cannot replicate in host cells (Clements, 1987; Pitard, 1987). Studies are ongoing to incorporate the gene encoding β galactosidase into the *A. salmonicida* Δ *aro A* strain and to examine the feasibility of using this strain to stimulate an immune response against heterologous antigens whose genes are incorporated in its genetic material (DeVoy and Foster, 1993). The present study sought to determine if this *aro A* strain possessed the capacity to adhere to and invade primary intestinal epithelial cells of rainbow trout. The molecular basis of the pathogenicity of *A. salmonicida* is relatively poorly understood at present, nonetheless important roles for extracellular factors including proteases (Price *et al.*, 1989; Gudmundsdottir *et al.*, 1990), haemolysins/ cytolytins (Nomura *et al.*, 1988; Lee and Ellis., 1990) and enzymes involved in lipid metabolism (Campbell *et al.*, 1990; Lee and Ellis, 1990) have been suggested as virulence determinants. Indeed the glycerophospholipid: cholesterol acyltransferase (GCAT) - LPS complex and a 70 kDa protease have been correlated with cytotoxicity, production of furuncles and mortality in salmonids (Kawahara *et al.*, 1990; Ellis., 1991; Huntly *et al.*, 1992). A regular paracrystalline surface array (S - layer) composed of a 50 kDa A - protein attached to the cell surface by the O - polysaccharide chains of LPS with which it interacts to form the A

layer (Belland and Trust., 1985) is a macromolecular structure which has been implicated in the pathogenesis of furunculosis (Ishiguro *et al.*, 1981; Munn *et al.*, 1982; Chu *et al.*, 1991; Karczewski *et al.*, 1991). The A-layer has been demonstrated to mediate binding to and penetration of macrophages (Trust *et al.*, 1983; Garduño and Kay, 1992; Garduño *et al.*, 1992) and binding to the extracellular matrix proteins fibronectin, laminin (Doig *et al.*, 1992) and collagen (Trust *et al.*, 1993). A study using a novel method of growing *A. salmonicida* inside intraperitoneal diffusion chambers *in vivo* found that recovered bacteria had acquired a capsular layer, apparently covering the A-layer and associated with complete resistance to host lytic factors and phagocytosis (Garduño *et al.*, 1993). Questions still remain therefore as to the precise role of the A-layer in the overall pathogenesis of furunculosis. Despite a large body of accumulated knowledge regarding epidemiology, pathology, virulence mechanisms and vaccination against *Aeromonas salmonicida* infections, the route of entry of the organism into the fish is still uncertain. Studies have indicated that the A layer increases the capacity of *A. salmonicida* to adhere to fish tissue culture cells and to excised rainbow trout intestinal tissue (Parker, 1985; Parker and Munn, 1985). To date, however, an invasive potential for *Aeromonas salmonicida* has not been definitively established. An invasive capacity has been suggested for a number of mesophilic aeromonad species implicated as enteropathogens in humans including *A. hydrophila*, *A. sobria* and *A. veronii* (Pitarangsi *et al.*, 1982; Lawson *et al.*, 1985; Watson *et al.*, 1985; Krovacek *et al.*, 1989; Gray *et al.*, 1990; Grey and Kirov, 1993) although little is known about colonization factors, receptors or adhesins (Atkinson *et al.*, 1987; Nandopalan and Chang, 1989; Hokama and Iwanaga, 1991). The ability to invade cells is an important virulence attribute of a number of mammalian enteropathogens and endows these organisms with the opportunity to gain access to the internal milieu and body tissues (Finlay and Falkow, 1989; Falkow *et al.*, 1992; Rosenshine and Finlay, 1993). In view of the paucity of information pertaining to the invasiveness of *A. salmonicida*, this study sought to investigate the interactions between rainbow trout intestinal cells and the wild type and live-attenuated *A. salmonicida* strains. If the attenuated strain of

Aeromonas salmonicida does possess the capacity to invade the salmonid gut then this may provide a putative oral vaccine as well as a means to deliver foreign antigens to the salmonid GALT.

8.2 MATERIALS AND METHODS

8.2.1 Bacterial strains

Aeromonas salmonicida 644 Rb Nal^R, virulent A layer positive strain isolated from clinical disease outbreak (*in vivo* passaged) was obtained from the fish disease group, University College Galway, Ireland.

Aeromonas salmonicida 644 Rb wild type and 644 Rb / M. Aro A⁻ aro A:: Kan^R mutant were kindly provided by L. Vaughan. Trinity College Dublin.

Renibacterium salmoninarum MT 444, a natural isolate from infected Atlantic salmon was supplied by the Marine Laboratory SOAFD fish cultivation unit. Strains of *Yersinia ruckeri*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Escherichia coli* K-12 strain DH1 were obtained from the culture collection of the Department of Biological Sciences. University of Plymouth.

8.2.2 Bacteriological media and growth conditions

A. salmonicida strains were grown in brain heart infusion (BHI, Difco) broth or trypticase soy agar or broth (TSA\ TSB, Difco). Kanamycin was included for growth of *A. salmonicida* Δ aro A on TSA at a concentration of 40 μ g/ ml. Stock cultures were maintained in glycerol at - 20 °C.

R. salmoninarum was grown in Mueller - Hinton broth including 0.1 % cysteine hydrochloride, pH 6.5 at 15° C for 6 weeks in static conditions. All other bacterial strains were taken from liquid nitrogen, grown once in TSB, plated on TSA and inoculated into fresh TSB to produce cultures for invasion studies.

A. salmonicida and *Y. ruckeri* strains were incubated with shaking in TSB at 20 °C for 24 hr before use. *E. coli*, *S. faecalis* and *S. aureus* strains were grown overnight with shaking at 37°C in TSB.

8.2.3 Isolation and culture of primary intestinal cells and tissue culture cells

A) Primary rainbow trout intestinal cells

Cells from the intestinal epithelial layer and lamina propria were isolated and cultured as outlined in section 4.2.

B) Atlantic salmon (AS) epithelial -like tissue culture cells

Atlantic salmon cells (Flow) were grown at 20° C in Eagles modified minimal essential medium with Earle's salts containing 10% foetal calf serum (FCS) in a 5 % CO₂ atmosphere. Cells (grown on coverslips) reached confluency in 7 - 10 days and were sub - cultured at a split ratio of 1 : 4.

8.2.4 Infection of cells with bacteria

Cells were counted using a haemocytometer and their viability established by trypan blue exclusion. *Aeromonas salmonicida* were added to cells at multiplicities of infection (m.o.i) of 10:1, 100:1 or 1000:1 and allowed to incubate in polystyrene tubes in L -15 medium with 5% FCS with shaking over a time course from 1 min to 2hr. *A. salmonicida* were washed 3 times in PBS + 0.1% SDS and enumerated with reference to a standard curve of bacterial number v optical density at 490 nm. After incubation with bacteria cells were washed three times (500 x g for 5 min in each case) in L -15 + 5% FCS and pellets were processed for transmission electron microscopy. A m.o.i of 100:1 was chosen as optimal (based on TEM analysis) for visualisation of bacteria-cell interactions and was used subsequently as the bacteria:cell ratio for SEM, light and fluorescence microscopic investigations. For all other bacterial species enumeration of a washed bacterial culture was achieved by staining for 10 min in crystal violet and placing in a bacterial counting chamber. For investigations of bacterial uptake into tissue culture cells an arbitrary number (10⁶) of bacterial cells were added to each coverslip. To examine the effect of cytochalasin D (Sigma) on bacterial invasion this agent was added to host cells at a concentration of 40 µg/ ml for 15 min prior to addition of bacteria and for the entire duration of the subsequent incubation.

8.2.5 Transmission electron microscopy (TEM).

Cell pellets were resuspended and fixed in a solution of 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 1 hr at 4°C. Samples were centrifuged and washed three times (500 x g for 5 min) with cacodylate buffer, resuspended and post - fixed in 1% osmium tetroxide in sodium cacodylate buffer for 1 hr at 4° C and washed twice with buffer as above. Pellets were dehydrated through an ascending graded series of ethanols of 30, 50, 70, 90 % and finally two changes of 100 % ethanol (for 5 min in each case). Samples were then infiltrated in Spurr's resin (Spurr, 1969) by adding to mixtures in ratios of absolute alcohol : Spurr's of 3:1, 1: 1, 1:3 (for 2 hr in each) and completed by incubating in pure resin first for 2 hr and then overnight. Specimens were placed in pure resin in BEEM capsules and allowed to polymerise at 60°C for 12 hr. Ultrathin ('gold' or 'silver') sections were cut (Reichert OmU3 Ultracut microtome) and mounted on inert (nickel) mesh grids. Sections were routinely stained with aqueous uranyl acetate for 15 min, washed in distilled water, counterstained with Reynolds lead citrate for 15 min, washed with distilled water, dried and observed under a Jeol JEM 1200 - EXII transmission electron microscope operated at 80KV and 100 kv.

8.2.6 Scanning electron microscopy (SEM)

A) Conventional SEM

Specimens for conventional SEM were fixed in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 at 4°C for 1 hr. Subsequently samples were rinsed in cacodylate buffer, dehydrated in a graded series of ethanols and critically point dried using CO₂ as the transitional fluid. Dried material was mounted on brass specimen stubs, sputter coated with gold or palladium (6nm thickness) and observed under a Jeol JSM 6100 SEM operated at 10 - 15 KV.

B) Cryo - SEM (Droplet method -using rivets)

Specimens were lightly fixed in 1% glutaraldehyde for 1 min. Material was collected on

millipore/ nucleopore filters by connecting a 10 μ m filter to a 2ml syringe containing the resuspended cellular material. Tissue was then subjected to a rapid plunge fix in reduced liquid nitrogen (nitrogen 'slush'/ supercritical nitrogen) at -212 °C and transformed under vacuum to the cryoprep chamber of the oxford CT1500 cryotrans unit. The rivet preparation was manipulated to reveal a fractured droplet surface. Specimens were transferred to the SEM cryo - stage (operated at -170°C) and observed at 2-3 KV. Surface ice was carefully etched away (sublimation) by gradually raising the cryo-stage temperature to - 80°C. When sufficient sublimation (etching) had occurred to reveal all the required detail, samples were transferred back to cryo - prep and copper coated prior to observation on the SEM cryo-stage (-170°C) at 15 KV.

8.2.7 Light microscopy- Giemsa staining

Glass slides were smeared with a small volume of foetal calf serum and dried prior to use to enhance adherence of cells to slides. Smears produced from control epithelial cells and after incubation with bacteria were stained with giemsa for 10 min (Appendix D) after prior fixing with methanol for 10 min. Cells were viewed under a Canon Vannox research microscope.

8.2.8 Acridine orange/ crystal violet fluorescence microscopy

The procedure used was adapted from those of Goldner *et al.* (1983) and Miliotis (1991). Samples derived from incubations of bacteria and cells were washed (3 times at 500 x g) with L-15 +5% FCS . Pellets were resuspended in 0.5 ml of 0.05 % acridine orange (Sigma) and allowed to stain for 1 min, made up to 20 ml with PBS and washed twice (500 g). The pellets were then resuspended in 0.5 ml of 0.05 % crystal violet (Sigma) and allowed to stain for 1 min, made up to 20 ml with PBS and finally washed (3 times at 500 x g). Resultant preparations were smeared on glass slides and mounted with DPX. Preparations were viewed  by fluorescence microscopy incorporating a narrow pass excitation filter for blue fluorescence at 450-495 nm and a long pass barrier filter at 405 nm. Photographs were taken onto Fujicolour super G 400 ASA

film.

8.2.9 Specific immunostaining for actin.

This process was adapted from that described by Teyssie *et al.* (1992). Samples from incubations of bacteria with cells were centrifuged at 500 x g and washed three times (500 x g) in L-15 + 5% FCS. Pellets were fixed for 30 min in 3% paraformaldehyde in cacodylate buffer and subsequently washed twice in L - 15 medium. Cells were permeabilized by resuspending for 1 hr in 0.1% Triton X-100. A blocking step in Tris-Saline pH 7.5 + 3% non fat dried milk (TSM) for 1 hr was then carried out. Samples were washed X 3 (500 x g) in Tris-saline, pH 7.5 and incubated for 2 hr in a 1:50 dilution of rabbit anti-actin (Sigma) in TSM. Samples were washed X 3 in Tris saline and twice in TSM. Goat anti rabbit fluorescein isothiocyanate (FITC) conjugate at 1: 100 in TSM was added for 1 hr, cells were washed X 3 in Tris-saline, pH 7.5, mounted, viewed and photographed as outlined in 8.2.6 above.

8.3 RESULTS

8.3.1 *Interaction of Aeromonas salmonicida strains with rainbow trout enterocytes*

Wild type and attenuated *Aeromonas salmonicida* strains were visualised interacting with primary trout enterocytes by SEM (Fig. 8.1) but little information on the nature of the invasion process was gained. By transmission electron microscopy both wild type and attenuated ($\Delta aro A$) strains were observed to adhere to and invade rainbow trout enterocytes within 5 min of incubation. Bacteria were seen to adhere very closely to cells (Fig. 8.2), an interaction which appeared to result in the formation of a 'pedestal' type structure on the cell surface immediately beneath the bacteria. The close interactions between host cells and bacteria appeared to involve an extracellular bacterial layer since close contact between the bacterial outer membrane and the host cell surface was not observed (Fig 8.2). Cell organelles appeared to be excluded from the region around this pedestal structure and fibrillar material possibly indicative of cytoskeletal components was also apparent in this area. In Fig 8.3 (A) there appears to exist a zone separating the cytoplasmic material surrounding the bacterium from the general cellular cytoplasm. Bacteria were internalised into cells within 5 min of exposure and could subsequently be observed inside endosomes in the cell cytoplasm (Fig 8.4). In general, the number of bacteria seen inside individual cells was in the range 0 - 5. These interactions were observed over a time course from 5 min to 3 hr but at time points later than 30 min of incubation of bacteria with cells few adherent or internalised bacteria were apparent and a considerable amount of cell debris was visible. When cells incubated with *A. salmonicida* were stained with giemsa large numbers of bacteria were visible interacting with cells although it was not possible to determine whether these cells were adherent or intracellular (Fig 8.5).

8.3.2 *Determination of the viability of intracellular bacteria.*

Staining of the bacteria alone with acridine orange resulted in their fluorescing an intense green/yellow colour. After counterstaining with crystal violet this fluorescence disappeared leaving only a faint background around the bacteria. Similarly, when samples were observed after staining with

acridine orange a number of bacteria were visible on the surface of cells although counterstaining reduced this fluorescence and only internalised bacteria continued to exhibit a green fluorescence. Applying this procedure to cells after 5 min, 30 min, 2 min and 6 hr incubation with bacteria led to visualisation of green (viable) intracellular bacteria at all time points (Fig 8.6). Most intracellular bacteria even after 6 hr incubation appeared viable although at this time a number of the host cells appeared dead (red fluorescence). No differences were apparent between the wild type and attenuated *Aeromonas* strains with respect to the extent of bacterial invasion and bacterial cell viability.

Using the same technique to examine whether a number of other bacterial strains were internalised by rainbow trout enterocytes indicated that *Renibacterium salmoninarum*, *Yersinia ruckeri*, *Enterococcus faecalis* and *Staphylococcus aureus* did not invade these cells. A strain of *Escherichia coli* (K12 - DH1) did appear to invade to some degree. Using the Atlantic salmon epithelial cell line very similar results to the above were obtained (Table 8.1). Both *A. salmonicida* strains were visualised intracellularly in a viable condition 1 hr after incubation with tissue culture cells. Of the other bacterial species tested only *E. coli* appeared to invade and this bacterium was only observed inside cells very occasionally.

8.3.3 The role of actin in the internalisation of bacteria.

Using specific indirect immunofluorescence, control cells which had been stained for actin possessed a filamentous web-like pattern of fluorescence (Fig 8.7). In contrast, cells stained after incubation with *A. salmonicida* displayed intense foci of fluorescence. This effect was visible within as little as 1 min after incubation of cells with bacteria. Additionally, incubation of enterocytes with cytochalasin D, an inhibitor of actin polymerisation, at a concentration of 40 $\mu\text{g/ml}$ for 15 min prior to addition of bacteria appeared to inhibit bacterial entry as assessed by the acridine orange\ crystal violet fluorescence staining method and no intracellular bacteria were visible inside cells 5 min, 15 min, 30 min or 1 hr after incubation (Table 8.1).

Table 8.1: Overview of methodologies used and results obtained in the investigation of the invasiveness of bacterial species.

STRAIN	TEM (5 min - 3 hr)		SEM(5min,1hr only)		LIGHT MICROSCOPY (Giemsa)		FLUORESCENCE ((Acridine orange/crystal violet)		ACTIN STAINING (Indirect Immunofluorescence)	
	PRIMARY TROUT ENTEROCYTES	ATLANTIC SALMON CELLS	PRIMARY TROUT ENTEROCYTES	ATLANTIC SALMON CELLS	PRIMARY TROUT ENTEROCYTES	ATLANTIC SALMON CELLS	PRIMARY TROUT ENTEROCYTES	ATLANTIC SALMON CELLS	PRIMARY TROUT ENTEROCYTES	ATLANTIC SALMON CELLS
<i>A. salmonicida</i> (wild type)	✓	-	✓	-	✓	-	✓ (+++)	✓ (+++)	✓	-
<i>A. salmonicida</i> (Δ aroA)	✓	-	✓	-	✓	-	✓ (+++)	✓ (+++)	✓	-
<i>R. salmoninarum</i>	-	-	-	-	-	-	✓ (-)	✓ (-)	-	-
<i>Y. ruckeri</i>	-	-	-	-	-	-	✓ (-)	✓ (-)	-	-
<i>E. faecalis</i>	-	-	-	-	-	-	✓ (-)	✓ (-)	-	-
<i>S. aureus</i>	-	-	-	-	-	-	✓ (-)	✓ (-)	-	-
<i>E. coli</i>	-	-	-	-	-	-	✓ (++)	✓ (+)	-	-

✓ -Test carried out
- -Test not performed
(-)-Non - invasive
(+)-Few bacteria within cells
(++)-invasive
(+++)-Highly invasive

Fig. 8.1: Scanning electron micrographs showing the interactions between *A. salmonicida* (Δ aro A) and isolated rainbow trout enterocytes. Samples were from a 5 min incubation of bacteria with cells.

A - C - Micrographs showing bacteria interacting with enterocytes.

scale bar = 5 μ m

b - bacterium

e - enterocyte

n - nucleus

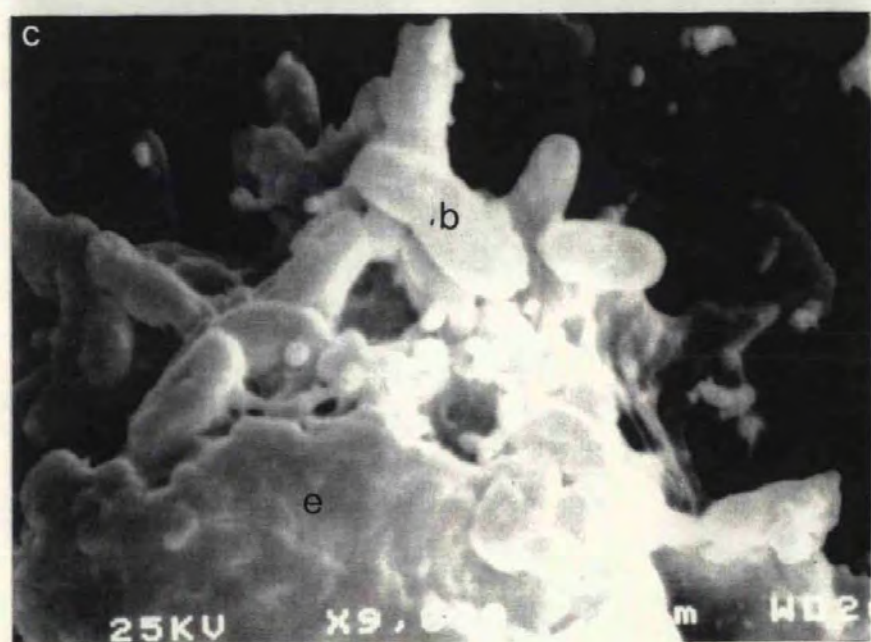
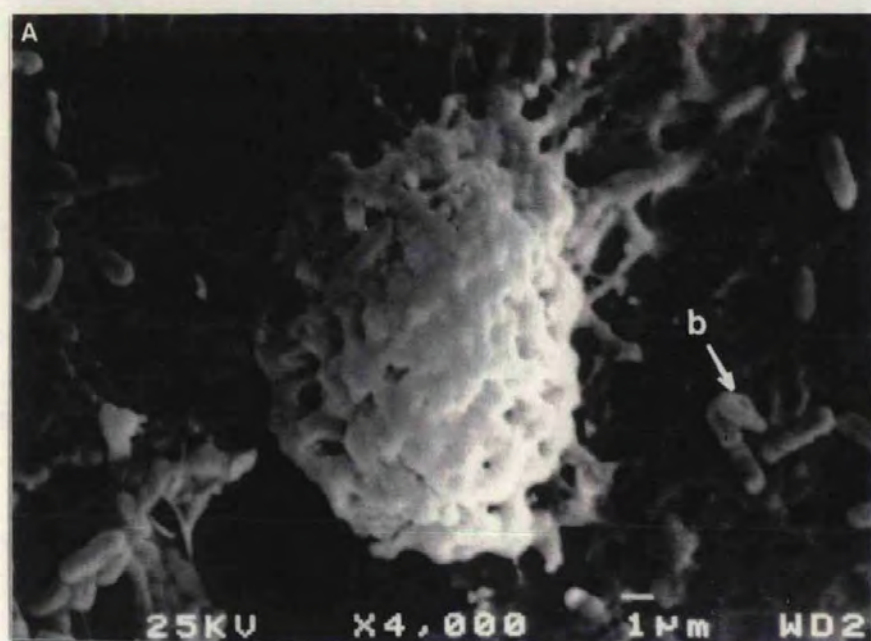


Fig. 8.2: *Transmission electron micrographs demonstrating the adherence of A. salmonicida (Δaro A) to isolated rainbow trout enterocytes. Samples were from a 1 min incubation of bacteria with cells.*

A: *Bacteria surrounded by cell debris.*

scale bar = 500 nm

B - Bacterium

PM - Plasma membrane

OM - Outer membrane.

B - D: *Pedestal formation on cells beneath adherent bacteria.*

The cup-like pedestal structure is most apparent in D opposite.

P - Pedestal

F - Fibrillar material beneath the pedestal.

note the exclusion of cytoplasmic organelles from the region around the pedestal

B: *scale bar = 200 nm*

C: *scale bar = 100 nm*

D: *scale bar = 200 nm*

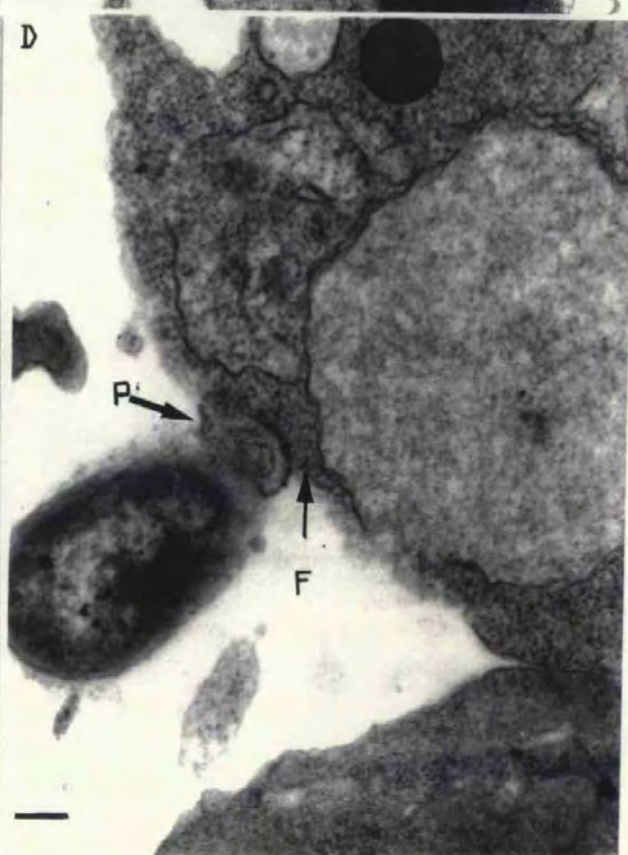
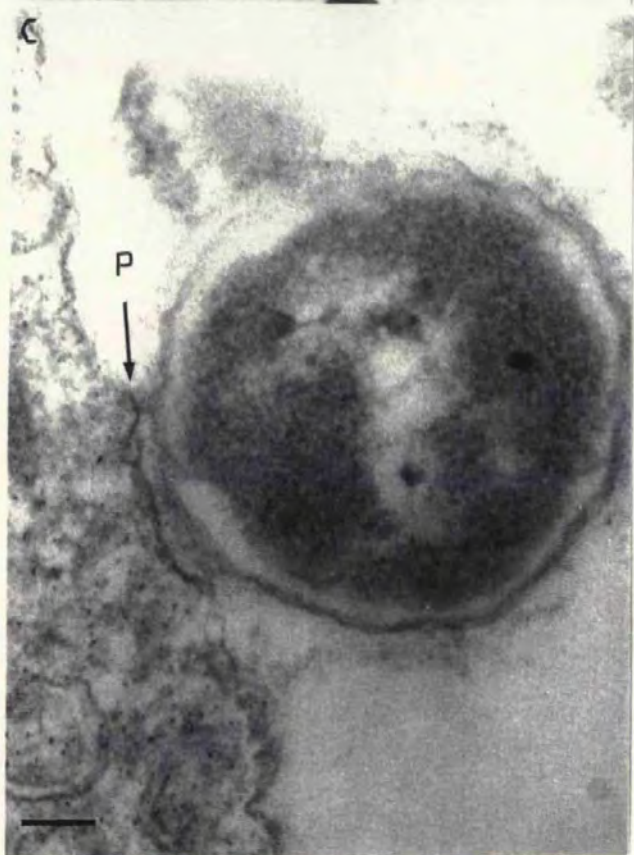
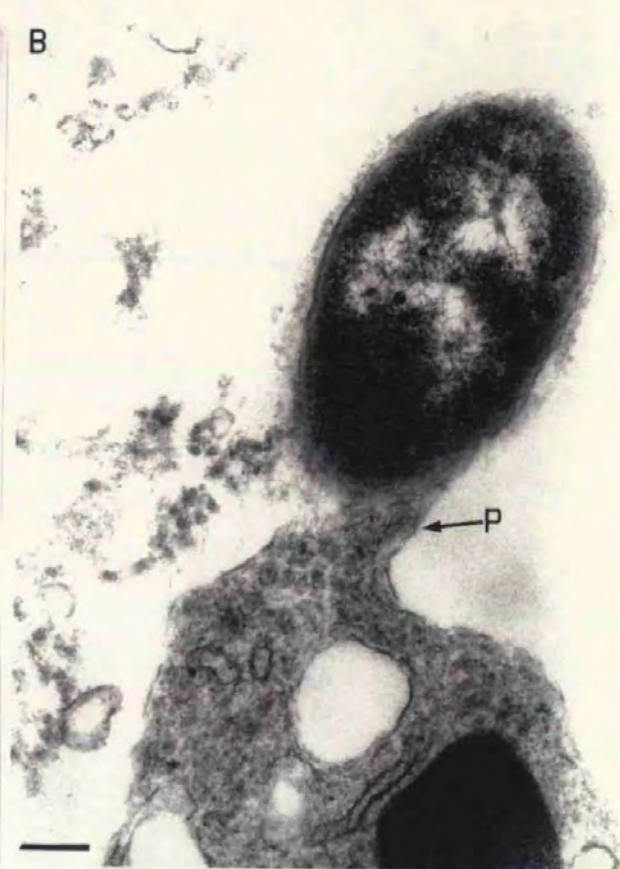
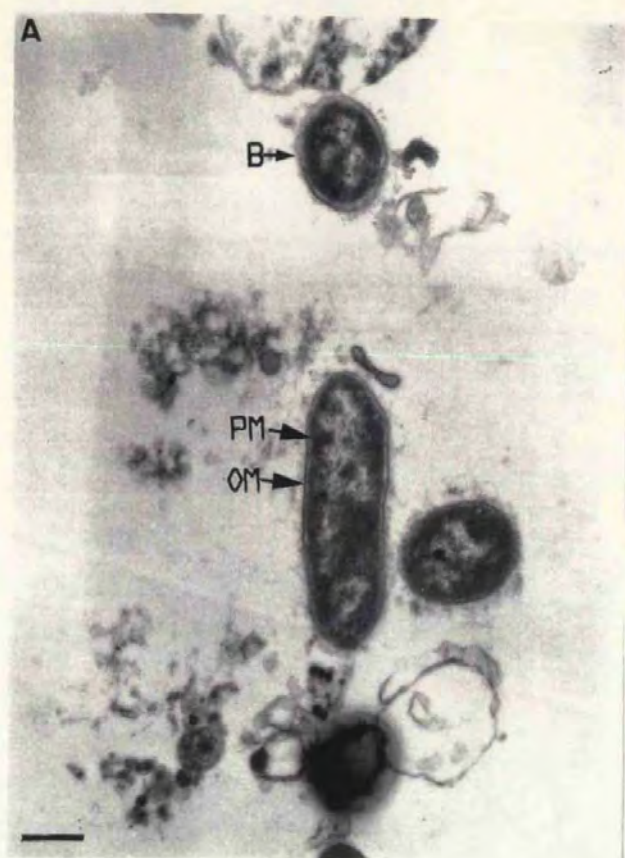


Fig. 8.3: Transmission electron micrographs demonstrating stages in the invasion of isolated rainbow trout enterocytes by *Aeromonas salmonicida* (Δ aro A). Samples were from a 5 min incubation of bacteria with cells.

Note the presence of fibrillar material (F) in the region around the bacterium and the exclusion of organelles.

In addition to an invading organism an internalised bacterium (I) is visible in (C).

A: scale bar = 200 nm.

B: scale bar = 200 nm.

C: scale bar = 200 nm.

D: scale bar = 1 μ m

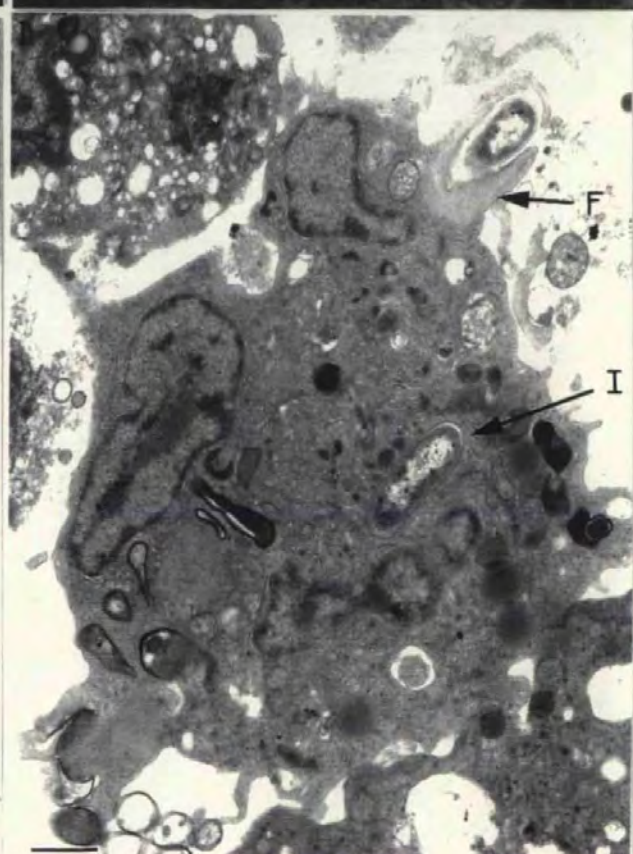
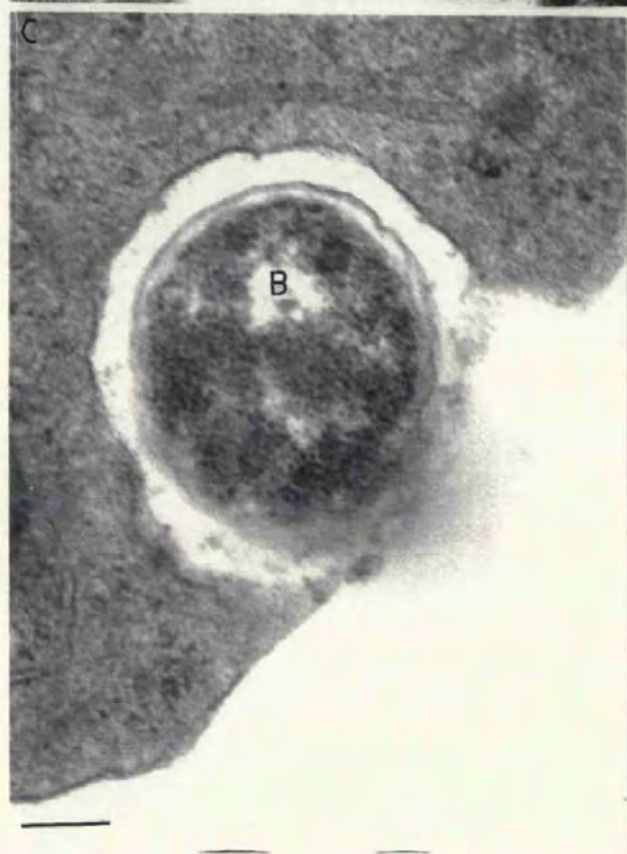


Fig. 8.4: *Transmission electron micrographs showing the presence of internalised A. salmonicida (Δ aro A) bacteria. Samples were from a 15 min incubation of bacteria with cells.*

In each case bacteria (B) are present in the cytoplasm within vacuoles (V).

A: scale bar = 500 nm.

B: scale bar = 500 nm.

C: scale bar = 500 nm.

D: scale bar = 1 μ m.

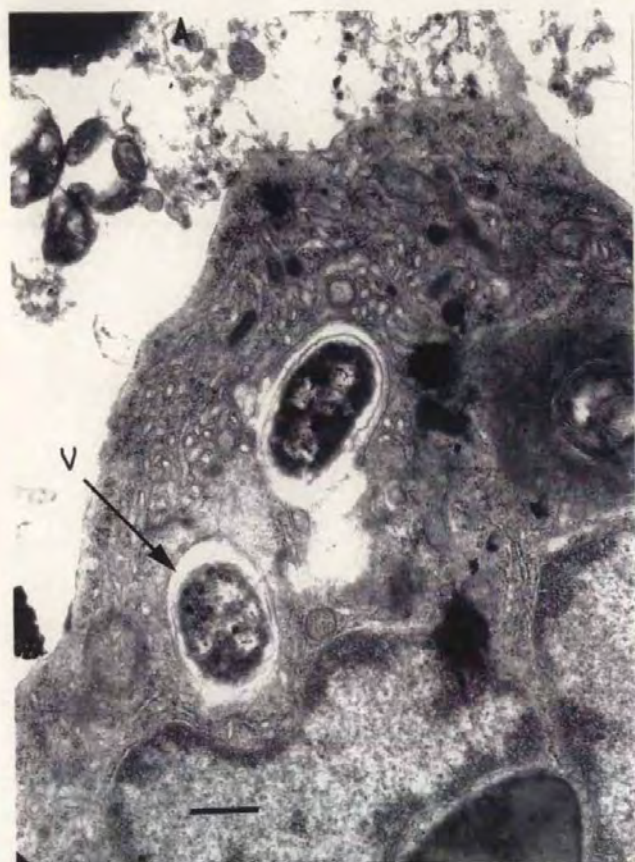


Fig. 8.5: *Giemsa stained smears of cells after 30 min incubation with bacteria*

b - bacterium

scale bars represent 5 μ m in each case.

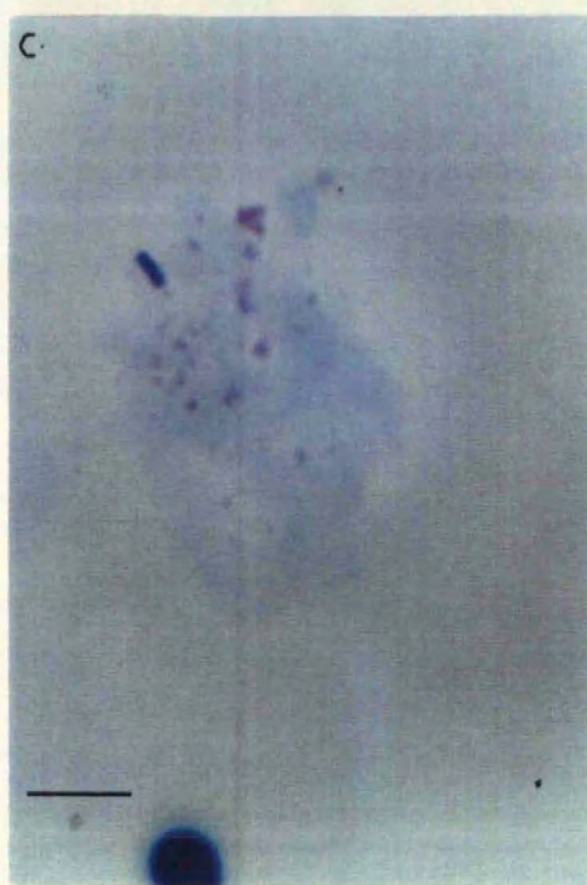
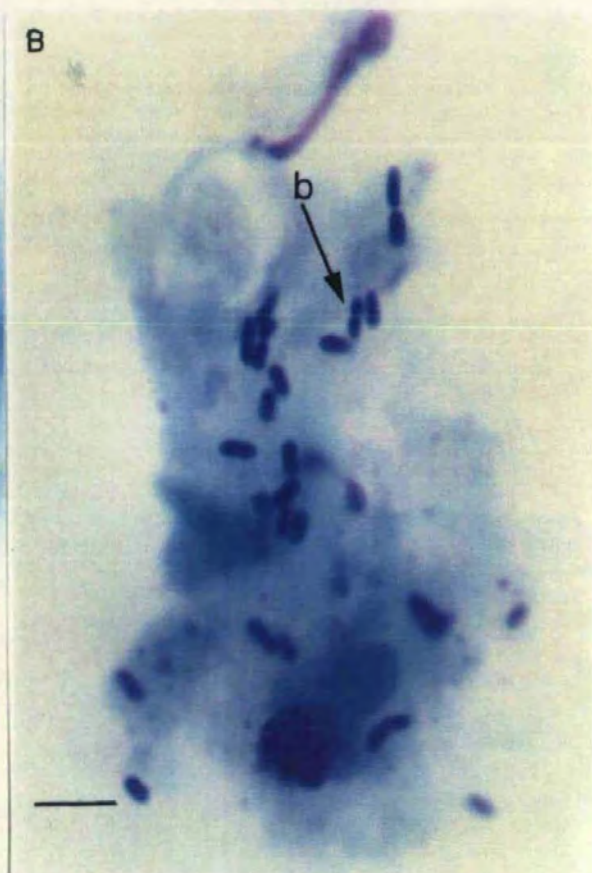
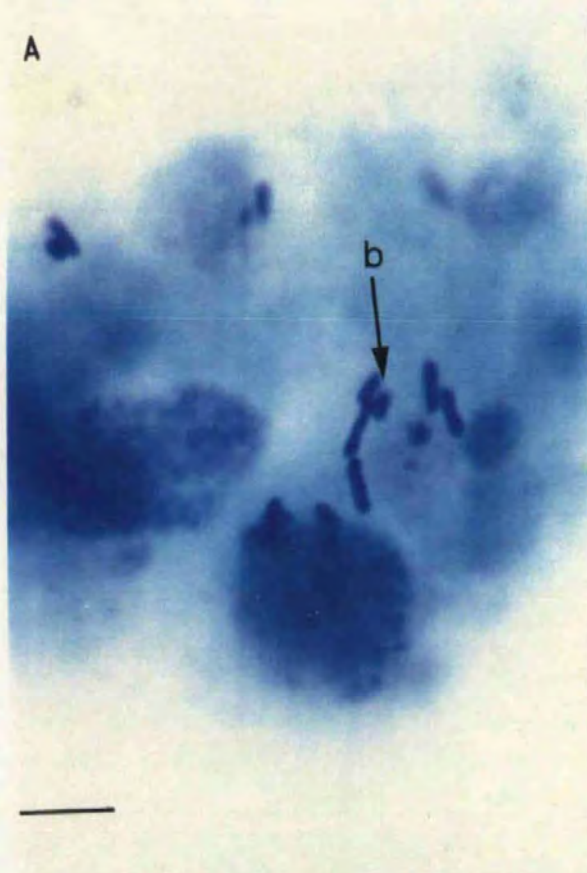


Fig. 8.6: *Smears of enterocytes stained with acridine orange and counterstained with crystal violet after a 15 min incubation with A. salmonicida (Δ aro A).*

scale bar = 5 μ m

e - enterocyte

n - nucleus

b - bacteria

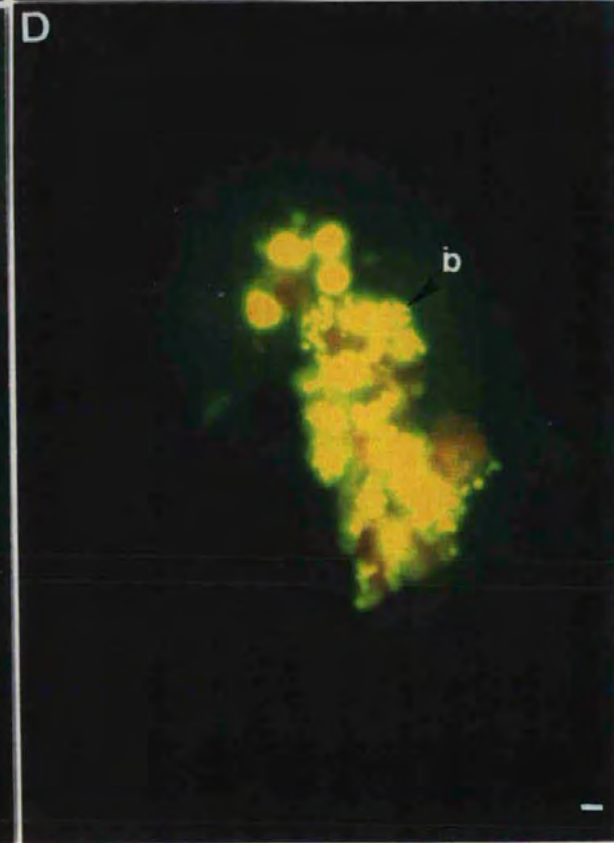
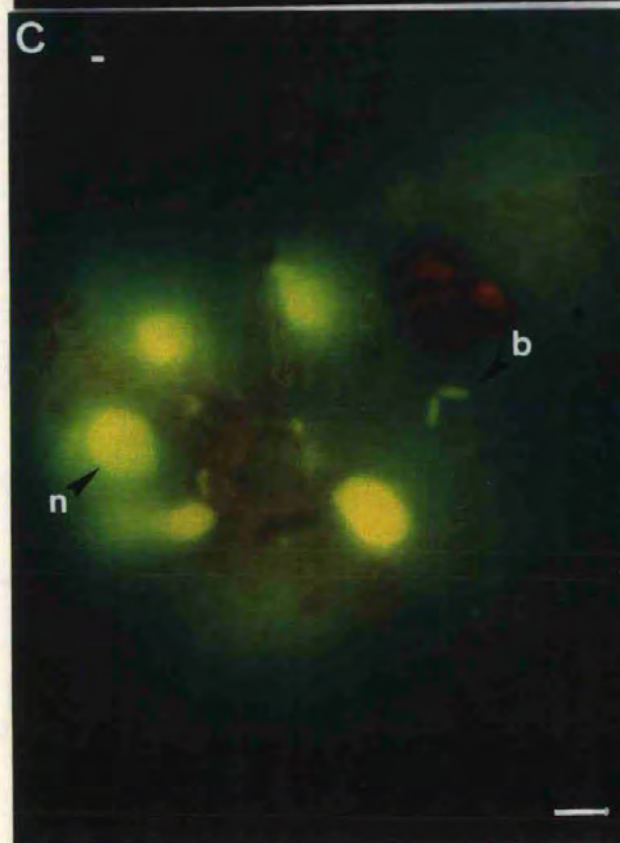
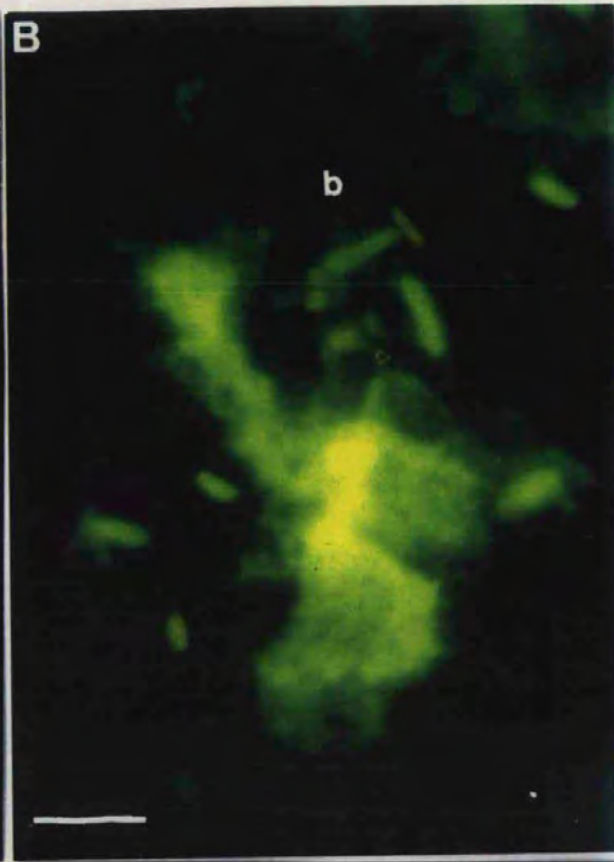
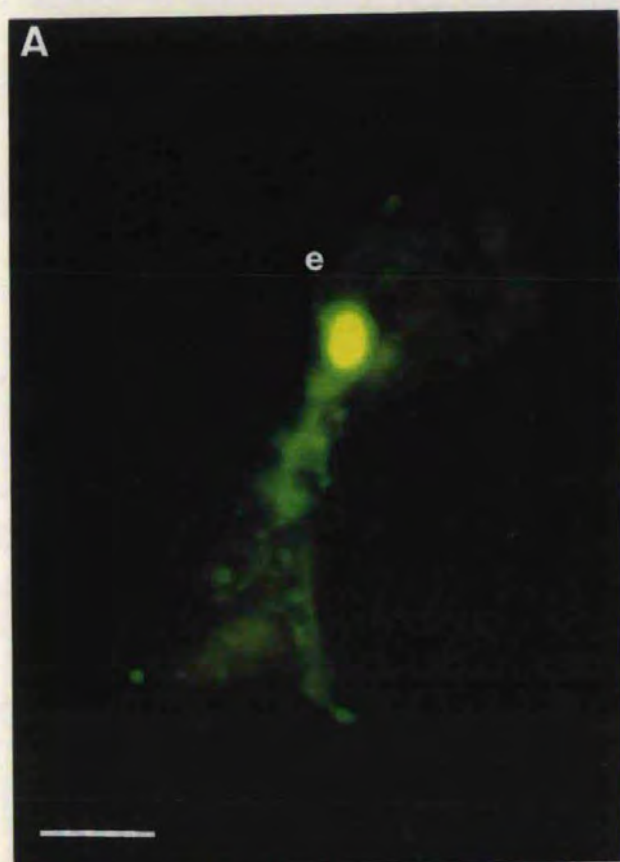
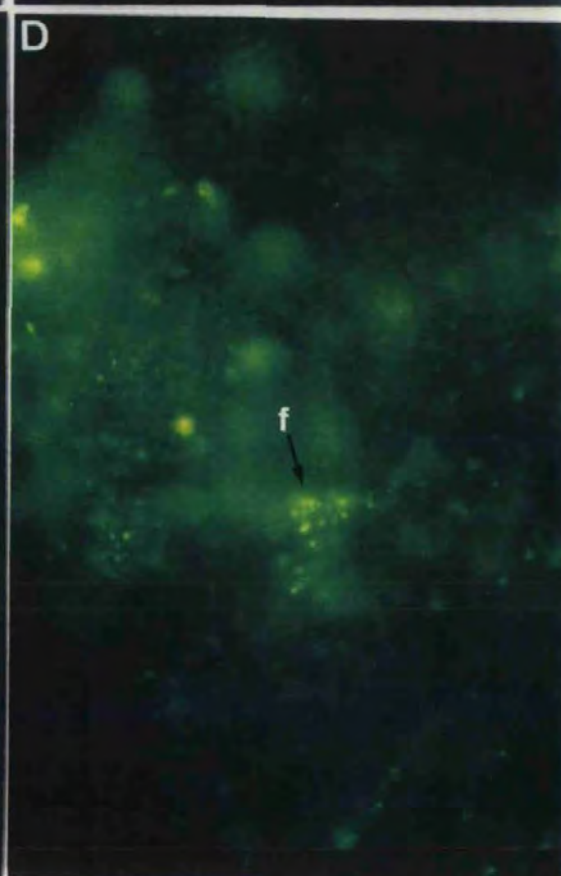
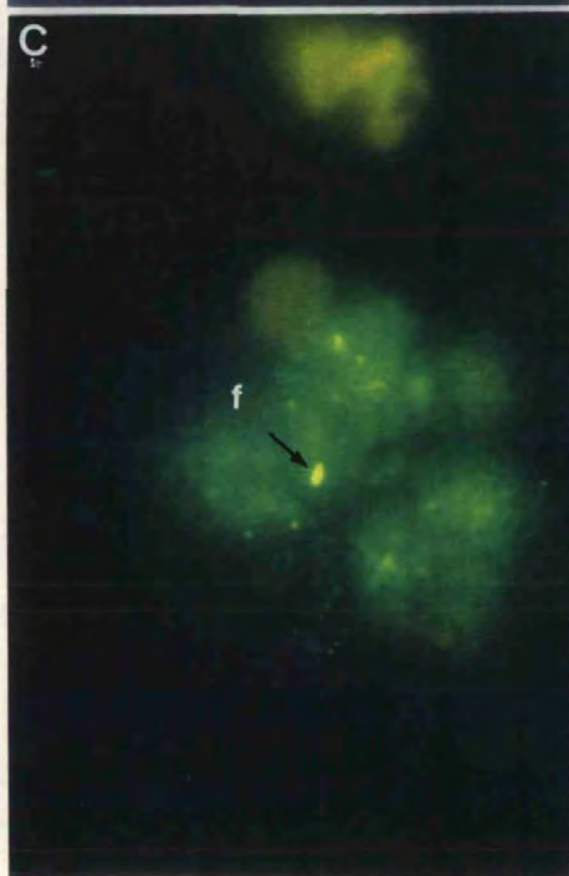
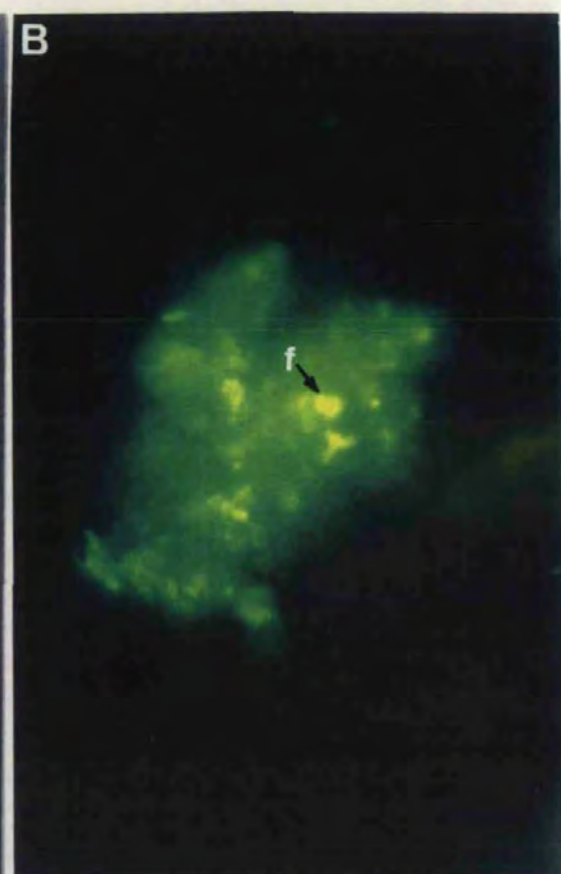


Fig. 8.7: *Indirect immunofluorescent staining of enterocytes before and after infection with A. salmonicida (Δ aro A).*

Note the web-like (W) pattern of staining on the control cells in A opposite

In B-D the sharp foci (F) of fluorescence in cells exposed to bacteria for 5 min.



8.4 DISCUSSION

This study has demonstrated the ability of an attenuated live aromatic dependent strain of *Aeromonas salmonicida* and its parental wild type to adhere to, invade and survive within primary rainbow trout intestinal enterocytes and epithelial - like Atlantic salmon cells. No differences were apparent in the extent and nature of these processes between the wild type strain and its attenuated derivative. The invasion process appeared to be dependent on actin polymerization and was morphologically similar to the processes observed with some mammalian enteropathogens. The process was very rapid and resulted in substantial modification to the host cell membrane in the vicinity of the adherent bacterium. In contrast to the uptake of inert macromolecules or microparticles (chapters 4, 5 and 7) invasive bacteria have been shown to encode factors promoting their own adherence and invasion mechanisms (Falkow *et al.*, 1992). The present study was a preliminary work which yielded limited but valuable information on the invasive nature of *Aeromonas salmonicida*. Because few similar studies have previously been undertaken in relation to fish pathogens much of this discussion concentrates on similarities between the processes observed in this study and those of invasive mammalian enteric pathogens.

The pathogenesis of many bacterial diseases involves the colonization of body sites effected by the sequential engagement of their surface-bound adhesins with cognate receptors on host cells or components of the extracellular matrix. This recognition process is believed to be necessary to establish infection and is thought to determine the tissue and host type targeted by an organism (Hopelman and Tuomanen, 1992). Adhesin proteins occur in fimbrial and non-fimbrial forms and generally recognise carbohydrates on eukaryotic cells (Ofek and Sharon, 1990; Krogfelt, 1991) although strict protein-protein interactions may also occur. Many bacteria can display a number of adhesins; more than 9 in the case of *E. coli*, for example, and 7 or more in the case of *Bordetella pertussis* (Hopelman and Tuomanen, 1992). As a consequence of bacterial adherence, adhesins can fulfil roles both in toxin delivery and in facilitating subsequent invasion of cells. Fimbriae are widely distributed among gram negative bacteria and are implicated in attachment to many types of receptor (Willems *et al.*, 1993). Enterotoxigenic bacterial pathogens such as *Vibrio cholerae* and *Escherichia coli* for example carry specific appendages to allow colonization of the host ileum (Krogfelt., 1991;

Smyth *et al.*, 1991). Special classes of protein fimbriae which promote mucosal adhesion of enterotoxigenic *E. coli* (ETEC) have been identified and termed colonization factor antigens (Knutton *et al.*, 1989). The adhesion of enteropathogenic (EPEC) and enterotoxigenic (ETEC) *E. coli* adhesion to enterocytes is mediated by fimbriae (Milon *et al.*, 1990). In many cases adhesin receptors on the cell surface have been shown to be sugar molecules with binding specificities which have been elucidated by using a range of defined sugar molecules to inhibit the adherence process (Ofek and Sharon, 1990). Glycolipids have been demonstrated to serve as receptor molecules for a number of adhesins (Payne *et al.*, 1993) and in most cases the oligosaccharide portion of the glycolipid appears to predominantly determine the specificity of the ligand - receptor interaction. The K88 fimbrial adhesin of ETEC binds β 1-linked galactosyl residues which may form the molecular basis of glycoprotein and glycolipid receptors for K88 fimbrial adhesins in the porcine small intestine (Payne *et al.*, 1993). The nature of the factors mediating adherence of *A. salmonicida* is poorly understood but may involve mainly non-specific hydrophobic interactions. Hydrophobic interactions have long been recognised as important in the adhesion of bacteria to host cells (Macura, 1987; Doyle and Rosenberg, 1990; Wibawan *et al.*, 1992). Hydrophobic bonding can overcome short distance electrostatic repulsive forces between bacteria and host cells due to their mutual negative charges (Jones, 1977) and may also stabilize receptor-ligand complexes (Doyle *et al.*, 1982). Aggregating strains of *A. salmonicida* were found to adhere to human, rabbit and fish leukocytes and to fish intestinal mucosal cells (Udey and Fryer, 1978). Parker (1985) found that among a variety of cell types *A. salmonicida* (A layer⁺ and A layer⁻ strains) adhered best to a fish epithelial cell line and suggested this specificity might indicate the presence of specific receptors on fish cells. The A layer appeared to be important in this adhesion since the adherence of A⁺ cells was greater than A⁻ to all cell types and to excised fish tissue (Parker, 1985; Parker and Munn, 1985). A comparison of the amino acid sequence of *A. salmonicida* A protein revealed similarities with *E. coli* K88 antigen, fimbriae of enterotoxigenic strains and with the outer membrane pore protein of *E. coli* K12 (Evenberg and Lugtenberg, 1982) so it is conceivable that this serves as an adhesin in *A. salmonicida*. Pili have been demonstrated on some strains of the bacterium (Parker, 1985) but a role in adhesion has not been established. The presence of surface adhesins on *A. salmonicida* has been demonstrated by haemagglutination with erythrocytes (Brooks and Trust, 1983). Ishiguro and Trust (1981) found

that specific adhesins on A-layer negative strains could be inhibited by D-mannose although the importance of this is difficult to assess since these would be masked by the A layer in virulent A⁺ strains. Parker (1985) demonstrated specific yeast agglutinins and haemagglutinins on A layer - negative cells and suggested that *A. salmonicida* adhesins were non - specific since attachment was not affected by either pH or the addition of simple sugars. Such a non - specific process might enable the organism to interact with a wide array of cell types. The observation in the present study that *A. salmonicida* strains adhere to and invade Atlantic salmon epithelial- like tissue culture cells suggests the bacteria may indeed have the capacity to adhere to a range of different cell types. All the surfaces of fish exposed to the environment are mucosal and this may allow a greater degree of latitude for pathogens to invade than is the case with mammals. In addition to the obvious advantage of possessing strategies to adhere to and invade cell, it is accepted that bacterial adherence endows pathogens with a capacity to withstand cleansing mechanisms operating at mucosal surfaces and may thus be an important early stage in the colonisation of host tissue (Beachey, 1981; Beachey *et al.*, 1982).

The process of cellular invasion in the present study appeared to involve substantial perturbation of cell surface structure. This was manifest firstly in intimate attachment, followed by the formation of a cup-like pedestal structure and finally in the engulfment of bacteria in large structures extending from the cell surface. The formation of a pedestal structure is reminiscent of the lesions resultant upon enteropathogenic *E. coli* (EPEC) adhesion to tissue culture cells (Knutton *et al.*, 1989; Dytoc *et al.*, 1993). This process involves peripheral attachment to the intestinal brush border followed by close adhesion which results in the disappearance of microvilli on the cell surface in the vicinity of the invading organism. An 'attaching and effacing' (ae) lesion now regarded as characteristic of EPEC forms and the microvillar architecture is lost leading to a cup-like association between the enterocyte and bacteria (Milon *et al.*, 1990). The *eae A* gene of EPEC is part of a chromosomal gene cluster necessary for this phenotype of intimate attachment (Donnenberg *et al.*, 1993). In the present study microvilli were never observed in the vicinity of adherent or invading *A. salmonicida* but were frequently observed on the surface of control cells. Whether this reflects an equivalent of 'effacement' is uncertain but could be investigated by *in vivo* studies of these interactions using intubated bacteria.

Fibrillar material possibly indicative of cytoskeletal components was visible beneath invading *A. salmonicida* by TEM, similar to descriptions for other invasive bacteria (Mathan *et al.*, 1993). The perturbations of cell surface structure during the process of *Salmonella* invasion into epithelial cells is a particularly disruptive process involving prominent cytoplasmic structures 5 - 10 μm in diameter which expand outward to surround and internalise the adherent bacterium (Finlay and Falkow, 1990). This contrasts with the uptake of some other invasive enteropathogens such as *Yersinia* which cause little cell disruption.

Internalization of *A. salmonicida* occurred very rapidly, within 5 min of incubation with cells, which is similar to the speed of uptake reported for *Salmonella* (Francis *et al.*, 1992). The field of bacterial invasion and its molecular basis has undergone remarkable expansion in the last few years resulting in the recognition of a considerably larger number of invasive organisms than was formerly believed (Falkow *et al.*, 1992). Indeed it is increasingly appreciated that many microorganisms have evolved the capacity to interact with host cell receptor molecules in order to induce their own internalization (Moulder, 1985; Falkow, 1991; Isberg, 1991). Only in a limited number of cases is the cellular and molecular basis of these processes even partly understood. A well studied example of an invasive microorganism is the enteric pathogen *Yersinia* in which a bacterial surface protein 'invasin' can mediate bacterial internalization into non - phagocytic cells (Isberg *et al.*, 1987; Leong *et al.*, 1990; Tran Van Nhieu and Isberg, 1991). Invasin promotes bacterial adherence and entry into eukaryotic cells by binding to multiple β_1 integrins (Isberg and Leong, 1990; Bliska *et al.*, 1993). The very high affinity binding of invasin with integrin results in integrin clustering, triggering host tyrosine protein kinase leading to cytoskeletal rearrangements facilitating bacterial uptake. Integrins are a class of dimeric transmembrane protein cell adhesion molecules (Hemler, 1990) which bind to cytoskeleton-associated proteins (Otey *et al.*, 1990). Other microbial invasion determinants including the *Yersinia enterocolitica* Ail protein (Miller and Falkow, 1988), *Listeria monocytogenes* internalin (Gaillard *et al.*, 1991) and Ipa proteins of *Shigella* species (Sansonetti, 1991) have been characterised but the host molecules with which most of these proteins interact have not yet been identified.

Large rearrangements of the host cell surface were observed in the present work but without any

information on the cellular and molecular basis of the interaction there seems little point in speculating on the exact nature of *A. salmonicida* invasion. Elements of the process appear similar to those of a number of mammalian enteropathogens and it will be interesting to determine if these are reflected at the molecular level. Considerable variation exists in the location and numbers of genes encoding proteins which have been shown to be associated with invasion. The *Shigella ipa* invasion genes and the enteroinvasive *E. coli* (EIEC) invasion genes are plasmid borne (Sansonetti *et al.*, 1981, 1982) while the invasion loci on *Salmonella* species are located on the chromosome (Elsinghorst *et al.*, 1989). Interestingly, *Yersinia pseudotuberculosis* has been demonstrated to possess two invasion systems - a highly efficient chromosomally-based *inv* mediated process (Isberg and Falkow, 1985; Isberg *et al.*, 1991), and a less efficient virulence plasmid-based *yad A* mediated process (Yang and Isberg, 1993). Additionally, while single genes appear capable of enabling invasion of *Yersinia* species and *Listeria monocytogenes* (Isberg and Falkow, 1985; Rosenshine and Finlay, 1993), invasion by other bacteria such as enteropathogenic *E. coli*, *Salmonella* and *Shigella* appears to be encoded by multiple gene systems (Galán *et al.*, 1992a; Ginocchio *et al.*, 1992; Stone *et al.*, 1992; Rosenshine and Finlay, 1993). Despite large phenotypic differences in the nature of the invasion strategies and the previously held belief that little homology existed between invasion genes a recent study has found that an assemblage of chromosomal genes involved in the invasion processes of *Salmonella* are very similar in order, arrangement and sequence to gene clusters on the *Shigella* virulence plasmid which control the presentation of surface antigens (Groisman and Ochman, 1993). Considerable similarities have also been noted between the derived amino acid sequences of gene products implicated in attachment of ETEC and EPEC to epithelial cells and the invasion proteins of *Y. enterocolitica* and *Y. pseudotuberculosis* (Donnenberg *et al.*, 1993). It has been appreciated for some time that in addition to the presence of specific gene(s), environmental factors also play a role in the regulation of bacterial cell entry (Di Rita and Mekalanos, 1989; Mekalanos, 1992). Enteric pathogens experience severe environmental changes when they enter the host via the oral route such as low pH, increased temperature (in the case of endothermic animals), low oxygen tension and nutrient deprivation. It has been found that bacteria respond to these environmental changes by modulating expression of different pools of genes and that many of these genes are coordinately regulated (Gross *et al.*, 1989; Miller *et al.*, 1989b). *Salmonella* entry appears to be regulated by growth phase (Finlay *et al.*, 1989) and

oxygen tension (Ernst *et al.*, 1990; Shierman and Shope, 1991) possibly mediated by the state of DNA supercoiling in the bacterium (Higgins *et al.*, 1990). A recent study found using Int - 407 monolayers that there was no difference in adhesion or invasion by *S. typhimurium* from log or stationary phase cultures (Kusters *et al.*, 1993). This study found that adherence did not require either host cells or bacteria to be viable but the process was saturable suggesting the existence of only a limited number of receptors. In contrast, invasion was found to be dependent on live host cells and bacterial protein synthesis. Recent evidence shows that there is a distinct overlap between the stimuli growth phase and osmolarity in the regulation of adherence to and invasion of human intestinal cells by *Salmonella typhimurium* (Tartera and Metcalf, 1993). High osmolarity conditions were found to be necessary in late-log phase for the optimal induction of the adherent and invasive phenotype. The *S. flexneri* *ipa* invasion genes and *Yersinia* *inv*, *ail*, and *yad A* loci are temperature regulated (Maurelli *et al.*, 1985; Small *et al.*, 1987). Invasion of epithelial cells by *Salmonella* and *Shigella* species requires active RNA and protein synthesis but does not appear to require DNA synthesis (Finlay and Falkow, 1988; Finlay *et al.*, 1989). In contrast, organisms such as *Yersinia* continue to invade despite treatment with formalin, UV light inactivation or inhibition with RNA and protein synthesis inhibitors (Vesikari *et al.*, 1983; Finlay and Falkow, 1989). Host cell energy production and viable bacterial cells are also required for the invasion of human epithelial cells by *Campylobacter jejuni* (Kashel and Jones, 1989). Great caution must be exercised in the interpretation of these studies since it has been demonstrated that the invasion property in *Shigella* is unstable and readily lost upon subcultivation (Sansone *et al.*, 1991). Similarly, the production of a D-mannose resistant adhesin by *E. coli* was shown to be dependent on culture medium and temperature (Milon *et al.*, 1990). Furthermore, the composition of culture media has been demonstrated to have a considerable effect on the adherence of *A. salmonicida* to fish cells (Parker, 1985).

The finding in the present study that *A. salmonicida* appeared to induce actin polymerisation at the site of bacterial attachment is in accordance with a number of previous studies (Falkow *et al.*, 1992). Furthermore, although no attempt was made to quantify the process, cytochalasin D (a fungal inhibitor of actin polymerization) appeared to completely prevent cell invasion. The uptake of several bacterial

pathogens by nonprofessional phagocytes involves cytoskeletal actin microfilaments (Ewanowich and Peppler, 1990). Indeed, cytochalasin D appears to be antagonistic to the internalization of a large number of invasive bacterial pathogens (Finlay and Falkow, 1988). It has frequently been shown with a number of mammalian enteropathogens that short filaments of polymerized actin accumulate beneath the host cell cytoplasmic membrane at the site of bacterial entry (Goldberg and Sansonetti, 1993). The cellular processes commensurate with bacterial invasion are perhaps best understood for the invasive pathogen *Salmonella typhimurium*. Upon coming into close proximity to epithelial cell brush border *Salmonella* induces profound but transient changes in microvillar architecture (Finlay and Falkow, 1992; Ginocchio *et al.*, 1992) which are limited to the point of bacterial contact. Its subsequent internalization is accompanied by considerable changes in the host cell cytoskeleton, with the accumulation of a number of cytoskeletal proteins including actin, α - actinin, talin, tubulin and ezrin around the entering bacterium (Finlay *et al.*, 1991; Ginocchio *et al.*, 1992). These events suggest that the bacterium sends signals to the cell to induce its own uptake. The exact nature of this signal is unknown but it was initially found that stimulation of the epidermal growth factor receptor (EGFR) appeared necessary for *S. typhimurium* invasion into cultured Henle - 407 cells (Galán *et al.*, 1992b). It has also been demonstrated that free intracellular calcium mobilization, phospholipase A₂ activity and leukotriene D₄ synthesis are needed for *Salmonella* entry into Henle 407 cells (Pace *et al.*, 1993). Stimulation of the EGFR (possibly through tyrosine phosphorylation) results in a variety of immediate (Ca²⁺ mobilization and membrane ruffling) and long term cell responses (DNA replication) (Schlessinger., 1988; Carpenter and Cohen, 1990). Since *Salmonella* invasion is a very rapid process the immediate responses to EGFR activation were presumed to be of greatest importance. Increased levels of intracellular Ca²⁺ which may be induced by the bacterium from outside the cell and reorganisation of the host cytoskeleton appear to be necessary for *Salmonella* invasion (Pace *et al.*, 1993). Attachment of the bacterium and stimulation of the EGFR are genetically separate phenomena and probably are mediated by different molecules. A signalling cascade results in depolymerization of actin microfilaments leading to the formation of membrane blebs and possibly to the release of profilin from the membrane which could participate in the reorganization of the cytoskeleton seen during *Salmonella* invasion (Pace *et al.*, 1993). A recent study has found that *Salmonella typhimurium* induces membrane ruffling by a growth factor receptor - independent mechanism (Jones

et al., 1993). It has additionally been shown that invasive *Salmonellae* can enter a line that does not express EGFR (Francis *et al.*, 1993) at a similar rate to a cell line expressing the receptor so it appears that EGFR is not essential for invasion. Ruffles are specialised plasma membrane ultrastructures of mammalian cells which are thought to be integral to growth, development and locomotion. Induced by growth factors, mitogens, or oncogene expression ruffles are sites of filamentous actin rearrangement (Mellström *et al.*, 1988) and are temporally associated with enhanced pinocytosis (Bar-Sagi and Feramisco, 1986; Haigler *et al.*, 1979). Structures resembling ruffles have been observed to be associated with the site of entry of invasive *Salmonella typhimurium* (Francis *et al.*, 1993) and it has been shown that ruffles elicited by invasive *Salmonellae* and other factors mediate internalisation of non-invasive bacteria or inert polystyrene beads in a macropinocytotic fashion - a phenomenon termed 'passive entry'. *Salmonella*-induced ruffling unlike that resulting from other factors is localised to the site of bacterium-host cell interaction. Ruffle formation and subsequent *Salmonella* entry are sensitive to inhibitors of actin polymerisation but not to microtubule inhibitors (Finlay and Falkow, 1988). Stimulation of EGFR and Fc and of *Salmonella* invasion receptor in cells lacking EGFR results in ruffle formation which facilitates macropinocytosis of bacteria (Francis *et al.*, 1993), and it appears that a common pathway for ruffle induction may exist which allows for parallel entry pathways (Galán *et al.*, 1992). The cascade pathways outlined above may therefore be the eventual outcome. In contrast to the above, polystyrene beads were not internalised when the same HEP - 2 cells were infected with non - invasive bacteria or with invasive *Yersinia enterocolitica* indicating that passive entry correlated with ruffling activity (Francis *et al.*, 1993). The evidence appears to suggest that separate genetic loci are involved in adherence and in transmembrane signalling in the case of *Salmonellae* whereas the *Yersinia* invasin protein alone appears sufficient for the adherence and entry of this organism. Similarly, a dichotomy between the genetic loci involved in intimate attachment and those genes necessary for signalling to the cytoskeleton also appears to exist for EPEC (Donnenberg *et al.*, 1993). Obviously it is impossible to understand the mechanism for the entry of *Aeromonas salmonicida* into epithelial cells at present but the similarities in membrane perturbation and the requirement for actin polymerization found in the present study indicate that similarities may exist in the molecular bases of these processes.

The use of primary intestinal cells in the present study enabled the invasion process to be investigated in some detail. In general, the analysis and the elucidation of the molecular basis of such phenomena relies on the use of tissue culture cells. A number of experimental cell tissue culture models have been developed to study steps in invasion and to identify both the genes involved and the regulatory factors involved in invasion (Small *et al.*, 1987; Miller and Falkow, 1988; Elsinghorst *et al.*, 1989). Such systems have a number of advantages including their well characterised nature, ease of culture and attachment to a solid substratum. In some cases, such as Caco 2 and MDCK cells (Finlay *et al.*, 1988; Finlay and Falkow, 1990) the cells are highly differentiated producing microvilli and tight junctions as with epithelial cells *in vivo* (Mounier *et al.*, 1990) although it is possible that transformed cell lines possess altered receptors which are absent from host epithelial cells (Babakhani and Joens, 1993). For example, it was found that *Treponema denticola* attached more to primary cultured epithelial cells than to continuous cell lines (Keulers *et al.*, 1993). A comparative study found considerable differences in the extent of cellular invasion by *Salmonella choleraesuis*, *Shigella flexneri* and *Yersinia enterocolitica* depending on the cell line used (Finlay and Falkow, 1988). Additionally, the *Y. enterocolitica* Ail invasion protein has been shown to exhibit remarkable cell line specificity in its ability to promote bacterial uptake, being capable of mediating bacterial binding to the surfaces of a wide variety of mammalian cell lines but only in a few cases leading to efficient internalization (Miller and Falkow, 1988). The extent of invasiveness by *L. monocytogenes* has also been demonstrated to be dependent on the cell line used (Meyer *et al.*, 1992). Despite such limitations some studies have established a degree of correlation between bacterial invasion of epithelial cells *in vitro* and bacterial virulence *in vivo* (Moulder, 1985; Finlay and Falkow, 1988). Gálan and Curtiss (1989) for example found that a *Salmonella* mutant strain which was unable to invade cells *in vitro* was reduced in virulence after oral but not intraperitoneal infection of mice indicating that efficient penetration of the intestinal epithelial layer requires uptake via the pathways analysed in cultured cells. Primary intestinal epithelial cells were used in the present study to approximate the cell type found in the trout intestine and because no well differentiated trout gut epithelial cell line was available. The present study was not without precedent since primary small intestinal enterocytes from the intestines of piglet and guinea pig gut have been previously used to assay the invasiveness of *Campylobacter jejuni* (Babakhani and Joens, 1993) and *L. monocytogenes* (Meyer *et al.*, 1992). The present

investigation used cells that were in suspension and consequently all aspects of the cell surface were exposed to bacteria whereas *in vivo* or with a well defined cell line only the luminal\ apical aspect is available for interaction with bacterial cells. Therefore no information was gained in this study on the specificity of the uptake process with respect to the area of the cell involved. This may be of considerable importance since it has been demonstrated that *Shigella flexneri* enters polarized mammalian cells through the exposed basolateral surfaces rather than the apical cell surface and additionally that the paracellular junctions of confluent monolayers are opened allowing bacterial access to the basolateral surfaces for invasion (Mounier *et al.*, 1992). It has also been suggested that endocytosis by the lateral epithelial cell membrane is the main invasion route of the enteropathogen *Providencia alcalifaciens* (Mathan *et al.*, 1993). This is in striking contrast to *Salmonella* which invade directly through the brush border of polarized cells after inducing cytoskeletal rearrangements (Finlay and Falkow, 1990). Another disadvantage of the cell suspension system used in the present study is that tight junctions as a route of mucosal invasion cannot be investigated as although this is quite rare for enteric pathogens such an invasion strategy has been demonstrated for *S. typhimurium* (Takeuchi, 1967) and *Providencia alcalifaciens* (Mathan *et al.*, 1993).

It was determined in this study that bacteria appeared to survive for some time within the gut epithelial cells. Using TEM, bacteria were only observed intracellularly within endosomes and not free in the cytoplasm and since these cells were in suspension no information pertaining to the subsequent fate of the bacteria could be gathered. Using the fluorescence microscopy method with extracellular quenching proved a valuable means to assess the viability of intracellular microbes and indicated that *A. salmonicida* remained viable for a number of hours after internalization. Among invasive bacteria two intracellular strategies have been observed in epithelial cell lines. The intracytoplasmic site of multiplication distinguishes such organisms as *Rickettsiae* (Teyssie *et al.*, 1992), *Shigella flexneri* (Goldberg and Sansonetti, 1993) and *Listeria monocytogenes* (Kocks *et al.*, 1993) from other intracellular organisms that grow either in phagolysosomes or in phagocytic vacuoles by inhibition of lysosomal fusion (Ishibashi and Arai, 1990; Falkow *et al.*, 1992). The latter species utilise actin polymerization to move within the cell and *S. flexneri* and *L. monocytogenes* have been shown to have the extraordinary property of lateral cell-cell spread. Once inside the host cell cytoplasm these

bacteria can accumulate short filaments of polymerized cytoplasmic actin and other actin-associated proteins including plastin, filamin and vinculin are also located in the tail at one extremity of the bacterium (Goldberg and Sansonetti., 1993). Actin is bundled to form an actin-containing tail behind the microbe as it moves in the cytoplasm which enables rapid bacterial movement (Bernaddini *et al.*, 1989; Kocks *et al.*, 1993). Continuous addition of polymerized actin filaments to the tail behind the bacterium propels the bacterium forward (Sanger *et al.*, 1992; Theriot, 1992; Theriot *et al.*, 1992; Tilney and Tilney, 1993). *S. flexneri* and *L. monocytogenes* are extruded from infected cells in macrovilli or pseudopod-like structures which can be internalised by neighbouring cells in a process involving the formation of finger-like protrusions from the infected cell surface. These protrusions contain the bacterium and its actin tail and the tip of the protrusion penetrates the surface membrane of the adjacent cell resulting in phagocytosis (Goldberg and Sansonetti, 1993; Niebhuhr *et al.*, 1993). The bacteria lyse the double membrane to become free in the cytoplasm and begin a new cycle of growth and infection (Teyssie *et al.*, 1992; Tilney and Tilney, 1993; Goldberg and Sansonetti, 1993). In the case of *L. monocytogenes* an Act A bacterial surface protein has been implicated in actin accumulation. Mutants defective in this gene are incapable of actin accumulation, intracellular and intercellular cell - cell spread and are highly attenuated *in vivo* (Niebuhr *et al.*, 1993). Similarly, the *S. flexneri* plasmid encoded Ics A protein is vital for intracellular movement and intercellular spread (Vasselon *et al.*, 1992). *Salmonella typhimurium* and *Yersinia* on the other hand remain within a membrane bound inclusion throughout the intracellular phase (Falkow *et al.*, 1992). In the present study bacteria were only observed within the cytoplasm inside endosomes and no evidence of intracellular replication was found. The production of membrane damaging agents by *A. salmonicida* is well documented (see below) therefore the possibility of internalized bacteria escaping from the phagosome/ phagolysosome is not inconceivable.

The demonstration that a range of other bacterial species in this study did not invade rainbow trout epithelial cells indicate that the internalisation of the *A. salmonicida* does not simply represent an 'antigen sampling' function which has been suggested for posterior intestinal enterocytes of teleosts (chapter 4). It is more likely to reflect a selective process requiring specific bacterial factors somewhat analogous to findings in mammals. However, since the expression of an invasive phenotype

depends on environmental conditions and growth phase it is conceivable that the negative results reported here merely reflect the experimental conditions. Obviously if this work substantiates an invasive capacity for *A. salmonicida* this may have implications with respect to the pathogenesis of furunculosis. Indeed, the demonstration that the strains used can invade both skin and gut cells suggests that the pathogen may infect by a number of routes. Parker (1985) demonstrated that *A. salmonicida* can adhere to trout gills and also that A layer positive strains of *A. salmonicida* could adhere to the outer skin surface of rainbow trout but could not traverse the integument and suggested this might not thus represent a portal of entry. Similarly, Bowers and Alexander (1982) failed to demonstrate passage of bacteria across trout skin. Discussion of the possible implications are outside the scope of the present work whose sole object was to evaluate the potential use of the attenuated strain as an oral vaccine vehicle for delivery of antigens to salmonids. It appears that assuming the bacterium can survive the biophysical, chemical and enzymic barriers in the proximal gastrointestinal *A. salmonicida* may be capable of invading the gut epithelium and potentially of delivering antigen to the underlying GALT and/ or the primary lymphoid organs of the fish. Obviously before such a strain can gain access to the intestinal epithelium it must be capable of surviving or be protected from the effects of gastric acidity, pancreatic enzymes and bile salts. Indeed, if a live oral vaccine survives the gastric barrier antigens expressed by such a strain could be synthesized *de novo* in their natural environment and thus be delivered intact to the teleost GALT. Parker (1985) found that *A. salmonicida* was relatively resistant to the effects of bile salts and a range of proteases but that the bacterium did not grow to a detectable extent at a pH less than 5. The pH pertaining in the unfed trout stomach of 2 - 4 (see section 5.3) may therefore be inhibitory to the strain. Preliminary observations (Jones, unpublished observations) indicate that a small percentage of these bacteria can survive a pH as low as 2.75 for up to 6 hr. The use of gastric inhibitors to protect the strain from acid-mediated killing may thus be of some advantage.

The demonstration of high affinity binding of *A. salmonicida* to type IV collagen found in basement membranes (Trust *et al.*, 1993) and to fibronectin and laminin (Doig *et al.*, 1985) may be of considerable importance. If the organism can invade the intestinal epithelium and subsequently associate with the basement membrane this might facilitate its passage to deeper tissues. Several

microbial pathogens can bind extracellular matrix components including collagen and fibronectin and these bridging molecules generally mediate cellular adhesion but not entry (Isberg, 1991). Entry of *Trypanosoma cruzi* and *Chlamydia trachomatis* into cultured mammalian cells can occur via a trimolecular mechanism, heparin bridging the interaction between the host cell and receptor (Ortega and Pereira, 1991; Zhang and Stevens, 1992). The β_1 integrin receptors for *Y. pseudotuberculosis* Inv A protein are involved in cellular adhesion to fibronectin, collagen and laminin (Aargraves *et al.*, 1987; Hemler, 1990).

It has been suggested that lectin-mediated adherence may confer growth advantage and enhanced toxicity to pathogens due to the restricted diffusion of products secreted by both target cells and bacteria (Zafiri *et al.*, 1987). There is some evidence that when compared with nonadherent bacteria, the toxins produced by adherent bacteria are targeted more efficiently and become relatively inaccessible to neutralization by toxin inhibitors. Toxicity to mammalian cells due to heat labile enterotoxin secreted by a K - 12 *E. coli* strain was greatly enhanced in bacteria adhering to cells compared to monolayers exposed to organisms whose adherence was inhibited by mannoside (Ofek *et al.*, 1990). In this case, the growth advantage and enhanced toxin efficacy was shown to result from the accumulation of products secreted both by the tissue cells and the bacteria in crypts formed by the ruffle structure of the tissue cells and 'lids' formed by the adherent bacteria. Similarly, the cytolytic activity towards tissue culture cells and mouse peritoneal macrophages caused by *Streptococcus pyogenes* streptolysin S was greatly enhanced in mixtures containing organisms capable of adhering to the target cells compared to nonadherent bacteria (Ofek *et al.*, 1990). In the present study, although no quantification was attempted it appeared that cell lysis was occurring when cells were incubated with *A. salmonicida* at a multiplicity of infection greater than 100:1, so after more than 30 min incubation a large amount of cell debris was visible and by TEM, relatively few cells containing intracellular bacteria were apparent. It was not possible to determine if this resulted from cytotoxin secretion by extracellular bacteria or lysis due to internalized cells. Cipriano *et al.* (1981) found a correlation between virulence and toxicity of *A. salmonicida* extracellular products to rainbow trout gonad cells. Similarly Munro *et al.* (1980) and Anderson (1972) described toxicity of *A. salmonicida* to rainbow trout gonad cells and lysis of fathead minnow tissue culture cells by *A.*

salmonicida ECP respectively. A cytopathic effect of A layer positive strains of *A. salmonicida* on murine (Garduño *et al.*, 1992) and trout (Garduño and Kay, 1992) macrophages has also been observed and this resulted in cell detachment from the substratum only in the case of trout macrophages. Obviously if a similar effect occurs with trout gut cells in-depth analysis of the longer term interactions of such cells with the pathogen will be difficult.

In conclusion, the present preliminary study indicates that the attenuated *aro* A strain of *A. salmonicida* and its wild type can adhere to and invade intestinal epithelial cells from rainbow trout. Obviously without a further assessment of the subsequent fate of the organism in the fish tissues it is impossible to speculate as to the *in vivo* potential of the attenuated strain as an oral vaccine vehicle. However, if this apparent invasive capacity is reflected *in vivo* and the strain gains access to the immunologically responsive tissues it may well be of value as an oral delivery vehicle for salmonids and provide a template for the immunization of a number of economically important teleost species.

CHAPTER 9

GENERAL DISCUSSION

This study has demonstrated the complexity of antigen processing in the rainbow trout gastrointestinal tract. The rapidity and extent of protein antigen degradation in the gut indicated that proteolytic enzymes in the digestive tract pose a considerable barrier to the oral delivery of protein antigens and suggested that a means to protect labile antigens in the gut may enhance the efficacy of oral vaccination. The *in vivo* and *in vitro* methods used in this investigation for the study of antigen degradation by gut cells and secretions may be of use in future as a preliminary step in the rational design of enteric delivery systems for teleosts.

Chapter 3 described the isolation of luminal enzymes from the trout gut and their effects on HGG and BSA. The findings indicated that both gastric and intestinal enzymes can degrade protein antigens *in vitro* but degradation by intestinal enzymes was most extensive leading to complete digestion of the intact antigens. In order to determine if antigen degradation could be reduced by altering the conditions in the digestive tract, the effects of temperature, pH and inhibitors on proteolysis were investigated. Degradation of both antigens by intestinal enzymes increased with increasing temperature from 2°C to 35°C and was greatly reduced at temperatures less than 10°C. It has been shown that certain teleosts exhibit a high degree of adaptability in regulating digestion, particularly with regard to temperature (Lee and Cossins, 1986) so the use of an *in vitro* system to investigate luminal processing of proteins may not necessarily directly reflect processing of the same antigens *in vivo*.

The activity of intestinal enzymes was found to increase with increasing pH from pH 2-9 but little activity was noted at pH values less than 5. Results of the investigation of the pH dependence of gastric enzyme activity were unexpected, gastric enzymes appeared to degrade both HGG and BSA maximally at two distinct pH values, pH 4-5 and at pH 7, little degradation being apparent at the intermediate pH 6. It was not determined if these two pH optima were a result of the presence of distinct proteases with different pH optima or of a single enzyme(s) with different activities depending on pH. To further investigate this surprising result the *in vivo* proteolysis of HGG after oral administration alone or after delivery of gastric inhibitors was studied (chapter 5). The use

of Western blotting to investigate *in vivo* luminal proteolysis proved very useful and demonstrated, as found *in vitro*, that proteolysis occurred in the trout stomach at pH 7. Additionally the pattern of HGG fragmentation detected in the trout stomach at pH 4-5 and pH 7 was similar to that observed *in vitro*. By altering the gastric pH with gastric inhibitors it was found that the nature of HGG fragments absorbed into the plasma could also be affected. The analysis of HGG in various regions of the gut after oral intubation indicated that different regions played distinct but complementary roles in proteolysis but the detection of HGG fragments in the plasma of fish which were not detectable in any region of the gut indicated that these were a result of processing at a stage following luminal degradation.

There have been encouraging indications that the application of enteric delivery protocols developed in mammals may enhance antigen uptake and immune responses in fish (Jenkins, 1992; Wong *et al.*, 1992). A preliminary investigation into the use of PLG microparticles as an oral delivery vehicle for soluble protein antigen in teleosts found that considerable fragmentation of the microparticle - associated HGG occurred in the trout stomach (chapter 7). This indicated that a proportion of the antigen was on the particle surface. However, analysis of HGG in the intestinal region of fish after oral intubation demonstrated that encapsulation did protect a percentage of the antigen from proteolysis. Furthermore, encapsulation of HGG resulted in greater amounts of the antigen reaching the bloodstream. Relatively little is known about the uptake of particulate materials from the teleost gut. Despite the absence of Peyer's patches, which appear primarily responsible for particulate uptake in the mammalian gut (Jenkins *et al.*, 1994) unpublished work from our laboratory has demonstrated the uptake of 0.1 μ m and 1.0 μ m fluorescent microspheres by the trout gut. Studies of the gut regions and cells involved in this uptake and of the subsequent fate of the particles is necessary to determine the value of particulate delivery systems. Our findings that these microspheres were detectable in the blood of fish after oral administration may be of importance since, in mammals, particulate materials are believed to localise in the lymphatic system. The second particulate delivery system investigated in this study was a live attenuated aromatic amino acid dependent (Δ aroA) strain of *A. salmonicida*. The finding that this strain which has proved effective as a parenterally administered vaccine in salmonids can invade

enterocytes suggests that it may be useful as an oral carrier vehicle for heterologous antigens. Further investigations on the invasiveness of the bacterium *in vivo* and of its capacity to withstand the harsh luminal environment are necessary before the value of this mutant strain in oral vaccination can be fully ascertained. In comparing the advantages of live recombinant bacteria and biodegradable microparticles as enteric delivery systems it has been suggested that in contrast with bacteria, the absence of exposed antigen on microparticles prevents exclusion of antigen as a result of pre-existing antibody (McGhee *et al.*, 1992). The finding in this study that antigen appears to be present on the microparticle surface is in conflict with this contention. However, if the degree of internalisation of antigen in the microparticle could be increased and the surface exposed antigen removed, oral immunisation may be more effective.

The possibility of antigen degradation occurring within intestinal cells was addressed in chapter 4. Using a previously described method (Davidson, 1991) gut cells were isolated and incubated with antigens. The results indicated that BSA was extensively degraded within gut cells while HGG was only modified to a limited extent. The intracellular processing of HGG appeared to be complex since HGG fragments were only detected within cells from 30-45 min after incubation, within cells, at later time points the HGG detected appeared to be intact. This indicated either that these fragments were fully degraded within the cells or exocytosed from the cell and therefore no longer detectable. If exocytosis did occur this may explain the presence of HGG fragments in the plasma which were not detectable in the gut after oral administration of HGG. Indeed, a 10kDa HGG fragment was identified within gut cells which was very similar in size to a fragment detected in the plasma of fish with a gastric pH of 4-5 and which was not detectable in the lumen. Investigation of the intracellular processing of HGG and BSA by cells isolated from different regions of the gut indicated, particularly in the case of BSA that degradation was much more extensive in some regions than others. Unfortunately, the identity of the cells isolated from different regions of the gut was not established so the roles of enterocytes, macrophages, granulocytes and other cell types in processing is unknown. Dorin *et al.* (1993) found that after anal administration of recombinant trout somatotropin or native bovine somatotropin that both

proteins were detectable within intestinal macrophages while only the heterologous bovine somatotropin was found within eosinophilic granular cells in the gut. This suggests that a study of the cell types responsible for the processing of proteins observed in the present study may provide useful information on the differential roles of gut cells. The identification of genes encoding MHC class I and II genes in fish (Okamura *et al.*, 1993; Bartl and Weissman, 1994) indicates that in future by *in situ* hybridisation or by immunocytochemical or flow cytometric techniques, if antibodies to the proteins are produced, the identity of cells potentially capable of antigen presentation in the gut mucosa may be identified. From a comparative viewpoint it would be interesting to determine the nature of cells involved in antigen processing and presentation in the teleost gut since this may cast light on the role of the diffuse GALT of higher vertebrates.

A study of the nature and kinetics of antibody produced against HGG after parenteral and oral administration found that IgM detected using a polyclonal anti - rainbow trout IgM antiserum was degraded by trout intestinal enzymes which may indicate that unless the immunoglobulin is somehow protected from proteolysis *in vivo* it is unlikely to function as a secretory immunoglobulin in the gut. However, specific antibody to HGG was detected in the intestinal mucus after i.p injection and after boosting with soluble HGG in fish which were orally administered with PLG - encapsulated HGG (chapter 7). Further study is necessary to determine the origin of intestinal antibody and to determine if it is resistant to proteolysis *in vivo*. The highest antibody titres to HGG were detected in the plasma and secretions of i.p immunised fish. This result was similar to earlier studies on the antibody responses of teleosts to soluble protein antigens (Davidson, 1991; Jenkins, 1992) and contrasts with earlier reports that i.p injection was not an efficient route for the induction of mucosal immune responses (Fletcher and White, 1973; Rombout *et al.*, 1989a). The latter studies investigated the response to particulate antigens which may explain the contrasting results. These studies may indicate that in teleosts as in mammals particulate antigens are more effective mucosal immunogens than soluble proteins.

Although the existence of a local immune system and even a common mucosal immune system in teleosts has been proposed (chapter 2) there is relatively little evidence in the literature for a

functional immune compartment in the gut independent of the systemic system. To clarify the role of the gut in immune protection it will be necessary in future to determine the aspects of the immune response responsible for protection after immunisation since in many cases the limited protection observed may have been the result of non-specific factors. It has been suggested that the lymphatic system in the teleost gut may not be as independent of the circulatory system as in mammals (Nakanishi, pers comm). If the lymphatic system in the teleost gut can act as a 'secondary circulatory system' allowing exchange of components with capillaries in the gut, then it would be difficult to envisage a local immune system acting completely independently of its systemic counterpart. It would therefore appear necessary to investigate the degree of interconnection between these two pivotal transport systems in the piscine gut since it may elucidate the degree of separation between the gut and systemic lymphoid compartments. Further study on the uptake of particulate materials from the gut, particularly with regard to the roles of lymphatics and capillaries in transport may cast some light on this area. If there is a greater degree of exchange between the lymphatic and blood circulatory systems this may explain the detection of antibody in the cutaneous mucus after enteric delivery of antigen (chapter 6). However, it would not explain the finding of antibody in the cutaneous mucus but not in the plasma after enteric immunisation (Rombout *et al.*, 1989a). It is possible that plasma cells if induced in the gut might retain a predilection for the mucosae and thus preferentially localise in the skin. The wide degree of variability between teleosts (Ellis, 1986) indicates that it is likely that the function of local immunity will differ considerably between species. Therefore until the capacity for local immunological responsiveness and characterisation of the lymphoid populations in the gut of a range of teleosts is performed, any general statements on the role of local immunity in teleosts should be made with caution.

The peak titres determined by ELISA in plasma of secretions of i.p immunised fish appeared to parallel the recognition of a 52 kDa HGG fragment on Western blots. It would be interesting to purify this fragment and to determine if it is immunogenic in trout, it is possible that this region of the HGG molecule possesses motifs which are immunogenic in this species. Indeed studies into

the fine specificity of teleost antibody to protein antigens to determine if epitope immunodominance is a feature of such species would be very useful as an aid to vaccine design. The recent finding by Vinitnantharat and Plumb (1993) that administration of feed impregnated with *Edwardsiella ictaluri* cell extract maintained antibody levels in fish which were vaccinated by injection while levels in fish which did not receive oral vaccine continued to decline suggests that until effective oral vaccines are developed the oral administration of vaccines as a booster may be effective. If this is a general feature, then an investigation into the means by which oral immunisation stimulates antibody production in parenterally immunised fish may be valuable. However, the possibility of inducing tolerance by administering antigen orally after parenteral delivery exists however (Davidson *et al.*, 1994).

In conclusion, the trout digestive tract has been shown to possess a number of potent enzymic barriers to the oral delivery of protein antigens indicating that a means of protecting labile antigens through this environment is a prerequisite to the development of oral vaccines incorporating labile protein antigens for teleosts. The strategies developed in this study may provide a rational starting point in assessing the potential of oral delivery systems and may reduce the numbers of fish required in the preliminary stages of future investigations of this kind.

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APPENDICES

APPENDIX A: REAGENTS FOR SODIUM DODECYL SULPHATE - POLYACRYLAMIDE GEL ELECTROPHORESIS.

All reagents and formulations were prepared according to instructions provided by and with materials from Sigma (Poole, Dorset, U.K).

1: SEPARATING GEL BUFFER (Reagent A)

Tris. 36.3g

N,N,N',N'- Tetramethylethylenediamine 0.3 ml

Dissolved and diluted to 90 ml with water, adjusted to pH 8.9 at 25°C with concentrated HCl and diluted with water to a final volume of 100 ml.

2: SEPARATING GEL SOLUTION (Reagent C)

Acrylamide 28.0g

N,N' - Tetramethylethylenediamine 0.46 ml

Dissolved and diluted with water to a final volume of 100ml and filtered to remove insoluble material.

3: STACKING GEL BUFFER (Reagent B)

Tris 5.98g

N,N,N',N' - Tetramethylethylenediamine 0.46 ml

Dissolved and diluted to 80ml with water, adjusted to pH 6.7 at 25°C with concentrated HCl and diluted with water to a final volume of 100ml.

4: STACKING GEL SOLUTION (Reagent D)

Acrylamide 10.0g

N,N' - Methylenebisacrylamide 2.5g

Dissolved and diluted with water to a final volume of 100 ml and filtered to remove insoluble material

5: SDS SOLUTION

Sodium Dodecyl sulphate(SDS)0.21g

Dissolved and diluted with water to a final volume of 100 ml.

6: PREPARATION OF 11% SEPARATING GELS:

3.0 ml Reagent A

9.5 ml Reagent C

11.5 ml Reagent E containing 17 mg of Ammonium Persulphate (freshly prepared)

7: PREPARATION OF 7% SEPARATING GELS

3.0 ml Reagent A

6.95 ml Reagent C

11.5 ml Reagent E containing 17 mg of Ammonium Persulphate
(freshly prepared)

3.5 ml Water

8: PREPARATION OF STACKING GELS

1.0 ml Reagent B

2.0 ml Reagent D

4.0 ml Reagent E

1.0 ml Water containing 8 mg Ammonium Persulphate (freshly prepared)

9: PREPARATION OF 2 X SAMPLE BUFFER (Reducing Sample Buffer)

Tris 1.51 g

Glycerol 20.0 ml

Dissolved in 35 ml water and adjusted to pH 6.75 with concentrated HCl. To this the following reagents were added.

SDS 4.0g

2- mercaptoethanol 10 ml

Bromophenol Blue 2mg

This final solution was diluted with water to a final volume of 100ml.

For non - reducing Sample Buffer 2 - mercaptoethanol was replaced with water.

10: ELECTRODE BUFFER

Tris 6.05g

Glycine 28.8g

SDS 2.0g

Dissolved and diluted with water to a final volume of 2.0 litres

11: FIXATIVE SOLUTION (FOR DESTAINING GELS)

Methanol 400 ml

Glacial acetic acid 70 ml

Water 530 ml

12: COOMASSIE BLUE STAINING SOLUTION

Brilliant Blue R (Coomassie Brilliant Blue R) 1.25 g

dissolved in 500ml Fixative solution

13: REAGENTS FOR NEUTRAL SILVER STAINING OF PROTEINS

A: FIXATIVE

30 % (Vol/Vol) Ethanol	300 ml
10 % (Vol/Vol) Acetic acid	100 ml
Distilled water	600 ml

B: SILVER EQUILIBRATION SOLUTION

Silver nitrate concentrate	7.5 ml
Distilled water	292.5 ml

C: DEVELOPING SOLUTION

Sodium carbonate	30 ml
Distilled water	120 ml
Formaldehyde	0.17 ml

D: STOPPING SOLUTION

1% Acetic acid.

E: REDUCING SOLUTION

Potassium ferricyanide	2.0 ml
Sodium thiosulphate	4.0 ml
Sodium carbonate	0.7 ml
Distilled water	393.3 ml

14: MOLECULAR WEIGHT MARKERS

A: 6h HIGH MOLECULAR WEIGHT MARKERS

	Molecular weight
Carbonic Anhydrase (Bovine Erythrocytes)	29.4 kDa
Ovalbumin (egg)	45.0 kDa
Albumin (bovine plasma)	66.0 kDa
Phosphorylase B (rabbit muscle)	97.4 kDa
β Galactosidase (<i>Escherichia coli</i>)	116 kDa
Myosin (rabbit muscle)	205 kDa

B: 7h LOW MOLECULAR WEIGHT MARKERS

α Lactoglobulin (Bovine milk)	14.2 kDa
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Trypsin inhibitor (soybean)	20.1 kDa
Trypsinogen (Bovine pancreas)	24.0 kDa
Carbonic Anhydrase (Bovine erythrocytes)	29.0 kDa
Glyceraldehyde-3-Phosphate Dehydrogenase (rabbit muscle)	36.0 kDa
Albumin (egg)	45.0 kDa
Albumin (Bovine plasma)	66.0 kDa

APPENDIX B: REAGENTS FOR WESTERN BLOTTING.

1: TRANSFER BUFFER

Tris	6.1 g
Glycine	28.8 g
Methanol	400 ml
Distilled water	1600 ml

2: STAINING OF NITROCELLULOSE FOR TOTAL PROTEIN

PONSEAU S CONCENTRATE

Ponseau S	2.0 %
Trichloroacetic acid	30 %
Sulfosalicylic acid	30 %
-	

PONSEAU S WORKING SOLUTION

Ponseau S Concentrate	2 ml
Deionised water	180 ml

DESTAINING SOLUTION

5% Acetic acid

3: PREPARATION OF BLOCKING SOLUTIONS

A: TRIS - SALINE

Sodium Chloride	11.68 g
Tris	6.05 g

Made up to 1 litre with water and adjusted to pH 7.5.

B: To prepare blocking solution for HGG immunoblotting Tris - Saline was adjusted to pH 7.7 and 3 % (w/v) "Marvel" very low fat dried milk was added.

C: To prepare blocking solution for BSA immunoblotting 0.8 % (w/v) Glycine (porcine skin) and 1 % (v/v) Tween 20 was added to Tris - Saline pH 7.5.

3: DEVELOPING SOLUTION (For peroxidase activity)

3'3' Diaminobenzidine	50 mg
Hydrogen peroxide	100 μ l
Tris - Saline pH 7.5	100 ml
Nickel chloride (enhancer)	0.3 % (w/v)

4: PRESTAINED MOLECULAR WEIGHT MARKERS.

Triosephosphate isomerase (Rabbit muscle)	33.0 kDa
Lactic dehydrogenase (Rabbit muscle)	38.0 kDa
Fumarase (Porcine heart)	56.0 kDa
Pyruvate kinase (Chicken muscle)	65.0 kDa
Fructose - 6 - Phosphate kinase (Rabbit muscle)	88.0 kDa
β Galactosidase (<i>Escherichia coli</i>)	125.0 kDa
α 2 Macroglobulin (Human plasma)	190.0 kDa

APPENDIX C: REAGENTS FOR ENZYME LINKED IMMUNOSORBENT ASSAY

1: PHOSPHATE BUFFERED SALINE + TWEEN 20 (PBST)

Disodium hydrogen orthophosphate	1.15 g
Potassium dihydrogen orthophosphate	0.2 g
Potassium chloride	0.2 g
Sodium chloride	0.2 g

Adjusted to pH 7.4 and 0.5 % Tween 20 was added.

2: CARBONATE-BICARBONATE BUFFER (COATING BUFFER)

Sodium carbonate	1.59 g
Sodium hydrogen carbonate	2.93 g
Made up to 1 litre with distilled water and adjusted to pH 9.6	

3: CITRATE-PHOSPHATE BUFFER

Citric acid (anhydrous)	4.23 g
Disodium hydrogen orthophosphate (anhydrous)	5.80 g
Made up to 1 litre with distilled water and adjusted to pH 5.0	

4: DEVELOPING SOLUTION

Orthophenylenediamine	20 mg
Hydrogen peroxide	20 μ l
Citrate-Phosphate buffer	50 ml

APPENDIX D: FORMULATION OF GIEMSA STAIN

Giemsa buffer (BDH pH 6.5)	2 parts
methanol	1 part
distilled water	8 parts

1 volume of giemsa stain (BDH, Gurr traditional formula) was diluted with 3 volumes of the above mixture (Wrathmell, pers comm) to prepare a working solution.