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The Processing of an Orally Administered Protein Antigen in the Digestive Tract of Rainbow Trout, *Oncorhynchus mykiss*

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ABSTRACT. An assay was developed to assess the integrity of a protein antigen after its oral delivery to rainbow trout. Human gamma globulin (HGG) was detected by Western blotting in gut contents from all regions of the rainbow trout digestive tract and in the circulation for up to 48 hr after oral administration. The form in which HGG was detected differed considerably between gut regions suggesting the various roles which these play in antigen degradation. HGG was also found in small amounts in the bloodstream and the fragmentation pattern of the antigen partly reflected that seen in the gut lumen. In some cases HGG fragments were detected in the plasma which were not found in contents from any gut region. The use of gastric inhibitors to neutralise gastric acidity influenced the pattern of HGG fragmentation seen in gastric contents but did not abrogate gastric proteolysis. However, by altering the pH in the stomach it was possible to modulate the nature of the antigen subsequently detected in the circulation. COMP BIOCHEM PHYSIOL 117A;2:263–275, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Antigen degradation, digestive tract, gastric inhibitor, pH, rainbow trout, uptake, vaccine

INTRODUCTION

Gastrointestinal proteolysis and gastric acid secretion are recognised as obstacles to the oral delivery of vaccines in teleosts (32,58) and in higher vertebrates (41). A major role for digestive secretions in the destruction of orally administered protein antigens in fish was suggested 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 (27,28). In mammals, gastric acid has been demonstrated to have an adverse effect on the viability of live orally administered enteric organisms (21) and can alter the immunogenicity of inactivated oral vaccine preparations (5,46). Furthermore, proteolytic enzymes can lead to a rapid loss of vaccine antigenicity and convert antigens to low molecular weight fragments (47). 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 (9,31). The stomach of gastric teleosts secretes both hydrochloric acid and pepsinogen (51), a precursor from which active pepsin is produced by acidic hydrolysis and subsequently by autocatalysis in the optimal peptic environment of the stomach (53). The large number of proteases identified in the pyloric caeca and intestine of teleosts are pancreatic in origin 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. Pancreatic trypsinogen is cleaved by enterokinase (from the intestine) to form active trypsin which is consequently capable of autocatalysis (54) and activates the precursor forms of other pancreatic proteases such as chymotrypsin and elastase (6,7). Most enzymes tend to show a sharp decline in activity towards the posterior intestine and rectum (3) and there is some evidence for resorption of digestive enzymes in the posterior region of the teleost intestinal tract (24). Although there have been few investigations of the entire lumenal protease repertoire of individual species, comprehensive studies of catfish enzymes indicate that a number of pancreatic proteases of differing specificities are present in the intestine (59–64).

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*

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cholerae whole cell/B subunit vaccines in humans to prevent acid denaturation of the cholera toxin B subunit (5,46). The H_2 receptor antagonist, cimetidine, has also been used as a gastric inhibitor prior to the administration of irradiated *Escherichia coli* vaccine against enterotoxigenic *E. coli* infections (11). 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 model antigens (37) and protein hormones (39,49). Other studies have used protease inhibitors to enhance the uptake of antigens in intact form following their oral delivery. The use of soybean trypsin inhibitor led to an increased uptake of bovine serum albumin in mice (55) and of horseradish peroxidase in rainbow trout (37). Aprotinin has also been used as a protease inhibitor to inhibit gastric enzymes prior to the delivery of diphtheria and tetanus toxins in rabbits, leading to an increase in specific circulating antitoxin titres (40). A range of other delivery strategies exist to reduce the impact of gastric acid and digestive enzymes on labile vaccines including enteric coating (58) and microencapsulation (41).

The present study set out to develop a method to assess the extent of degradation of orally delivered human gamma globin (HGG) in the rainbow trout digestive tract *in vivo*. This could be used to determine the roles of different regions of the gastrointestinal tract in antigen breakdown and subsequently to assess whether oral delivery systems can protect an antigen from degradation in the gut and increase the amount of intact antigen reaching the circulation. An investigation into the susceptibility of test antigens to modification in the gut could provide a valuable initial step in the design of effective methods for the oral administration of vaccines to teleosts.

MATERIALS AND METHODS

Animals

Adult rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) 100–200 g of both sexes were maintained in a freshwater recirculating system at 14 ± 1°C and fed to satiation once daily on a commercial trout pellet preparation. Fish were deprived of food for 48 hr prior to commencing experiments.

Administration of Antigens and Gastric Inhibitors to Fish

Groups of fish ($n = 5$) were isolated in separate tanks for the duration of experiments. Fish were administered by oral gavage with a dose of 35 mg of HGG (Cohn fraction II, III (Sigma)) in 0.2 ml phosphate-buffered saline (PBS), pH 7.2. Solutions were delivered via 1 mm diameter polyvinyl chloride tubing attached to a 21-gauge needle and a 1 ml syringe. Various concentrations of the gastric inhibitors, sodium bicarbonate (Fisons) and cimetidine (Aldrich) were delivered to fish 1 hr before the antigen by the same means as for HGG.

Sample Collection

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 5800 × ***g*** for 5 min and plasma was stored in 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 further 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 and divided into four regions: stomach, pyloric caeca, and two equal segments of intestine arbitrarily termed anterior intestine and posterior intestine. The pH of each region was recorded by means of a glass pH microprobe (Aldrich). After making a longitudinal incision to expose the lumenal surface, mucosal contents were collected from each gut region and placed in plastic containers on ice. Contents from each group were made up to 6 ml with PBS, pH 7.2 and 50 μl of a 100 mM stock solution of phenylmethane sulphonate (PMSF (Sigma)) prepared in methanol was added. Samples were stored at −20°C until required.

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

Mucosal and plasma samples derived from experiments were subjected to discontinuous SDS-PAGE (30) to assess the extent of antigen proteolysis. Preparations were electrophoresed under non-reducing conditions using a mini Protean II system (Bio-Rad, Herts) with 11% gels as this enabled optimal visualisation and analysis of the wide range of peptides liberated by proteolysis. Lumenal contents were homogenised by vigorous shaking (WhirliMixer, Fisons) and then centrifuged at 5800 × ***g*** for 5 min to remove solid matter. The resultant supernatants were diluted 1:1 with non-reducing electrophoresis sample buffer, boiled for 3 min and incubated at room temperature for 2 hr prior to electrophoresis and immunoblotting. Plasma samples were diluted 1:5 in electrophoresis sample buffer and electrophoresed as with mucosal samples.

Gel Staining for Total Protein

For Coomassie Blue staining, Brilliant blue reagent (Sigma) was added to gels which were incubated overnight at room temperature. Gels were destained in fixative until bands were clearly visible. Molecular weight markers (Sigma) were

included on each PAGE gel to enable calculation of the apparent molecular weights of protein bands after electrophoresis and staining.

Western Blotting

For immunoblotting (Western blotting) and staining for specific antigen detection an adaptation of the procedure of Towbin *et al.* (52) was used. Subsequent to electrophoresis, proteins were electrotransferred to 0.45 μm nitrocellulose membranes (by a "wet" blotting procedure) at 30 mA constant current for 18 hr using a Trans-Blot™ cell (Bio-Rad). After transfer, blots were washed in 0.5 M Tris-saline buffer, pH 7.5 for 20 min. A blocking step was then performed for 1 hr in Tris-saline + 3% non-fat dried milk (Marvel) (TSM) (the blocking agent and diluent for all antisera for HGG immunoblotting). Blots were incubated in the primary antiserum (rabbit anti-HGG (Sigma) at a 1 in 500 dilution) for 6 hr. After washing three times in Tris-saline and twice in TSM, secondary antiserum (swine anti-rabbit IgG peroxidase conjugate) (Dako Ltd., High Wycombe, U.K.) at a dilution of 1 in 1000 was added for an additional hour. Blots were washed extensively in Tris-saline and developed in a 3,3′-diaminobenzidine (DAB) chromogen (Sigma) enhanced with nickel chloride (0.3% w/v) for 1–5 min. Reactions were terminated by thorough washing in Tris-saline.

Modified Western Blotting Method for the Detection of HGG in Plasma

The procedure described above was modified to enable the detection of HGG in plasma and to abrogate non-specific cross reactions. Primary antiserum (goat anti-HGG peroxidase conjugate) (Sigma) was pre-adsorbed overnight with 5% trout plasma (to which 1% SDS had been added 24 hr previously) and centrifuged at 11,600 × ***g*** for 10 min. The resultant supernatant was used as primary antiserum, diluted 1 in 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 in 1000 in TSM. The process was otherwise identical to the general method described above.

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). The data generated enabled quantification of the HGG present in samples on immunoblots allowing a comparison of the total amount of antigen present in different groups. Additionally the relative percentage of antigen present in each sample in intact form or as discrete fragments was calculated.

Statistics

The pH values measured in the gut regions of the various groups were compared by one way analysis of variance (ANOVA).

RESULTS

Dose Effects of Gastric Inhibitors on Gastrointestinal pH and HGG Uptake

The effects of prior delivery of bicarbonate and cimetidine on gastric pH and on the amount and nature of HGG detectable in the plasma were investigated 1 hr after antigen delivery.

I. Sodium Bicarbonate

After the delivery of sodium bicarbonate, gastric pH increased from pH 3.38 to 8.0 with increasing bicarbonate dose (Fig. 1). All bicarbonate doses from 1.0 to 35 mg per fish resulted in a significantly higher ($p < 0.05$) gastric pH compared with control PBS administration (Table 1) and differences in gastric pH induced by doses of 10, 20, and 35 mg bicarbonate per fish were not significant. The semi-quantitative immunoblotting data presented in Table 1 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. The greatest number of HGG bands were also detected in the plasma from fish administered 20 (9 bands) or 35 mg (8 bands)

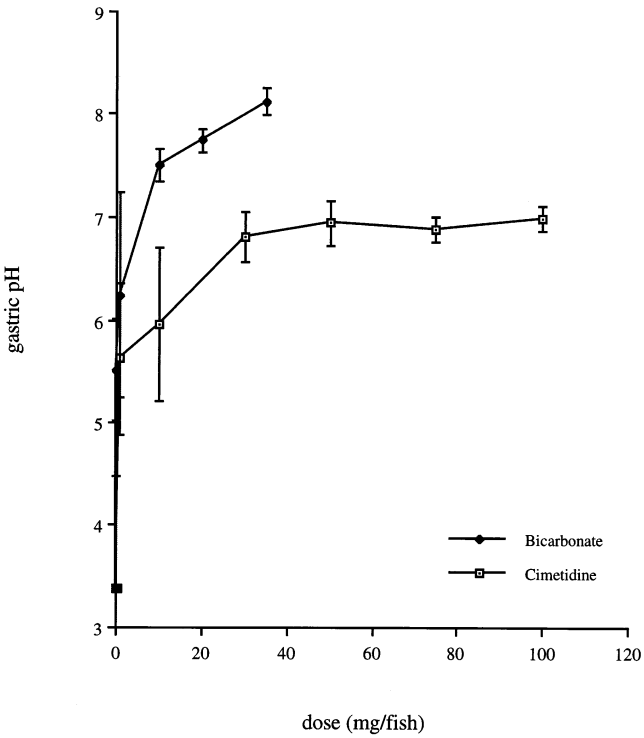

FIG. 1. Dose response of gastric pH to gastric inhibitors.

TABLE 1. Dose response to sodium bicarbonate, in terms of gastric pH values and sizes and relative percentages of HGG bands detected in plasma 1 hr after the delivery of HGG

Bicarbonate dose (mg/fish)	0	PBS (control)	0.1	1.0	10	20	35
HGG dose (mg/fish)	0	35	35	35	35	35	35
Gastric pH	3.38	5.1	5.51	6.24	7.5	7.74	8.12
Total signal (%) on Western blots	—	14.5	11.0	14.9	15.7	25.8	18.1
Band size (kDa) and relative %		202.1 (91.9%)	202.1 (61.9%)	202.1 (83.5%)	202.1 (42.8%)	202.1 (23.5%)	202.1 (40.9%)
			153 (23.9%)	153 (4.9%)	153 (21.8%)	153 (28.5%)	
		80.5 (*)			80.5 (*)	80.5 (*)	80.5 (2.9%)
					68.8 (2.4%)	68.8 (2.4%)	68.8 (*)
		48.1 (*)	48.1 (*)	48.1 (2.5%)	48.1 (*)	48.1 (1.6%)	48.1 (*)
		43.2 (*)	43.2 (*)	43.2 (2.7%)	43.2 (*)	43.2 (29.8%)	43.2 (14.9%)
		18.4 (*)	18.4 (*)	18.4 (3.3%)	18.4 (33.0%)	18.4 (2.4%)	18.4 (35.9%)
		10.6 (8.1%)	10.6 (9.0%)	10.6 (3.1%)		10.6 (5.7%)	10.6 (2.5%)
			7.4 (5.2%)			7.4 (6.1%)	7.4 (2.9%)

Sodium bicarbonate was administered to fish at a range of doses from 0–35 mg per fish 1 hr prior to the delivery of HGG and plasma was collected from fish 1 hr later. Gastric pH was measured at 3 points in the stomach of each animal and the average was taken, the result presented is the mean from five animals in each case. The total signal referred to in row 3 of the table represents the percentage of the total amount of HGG detected on Western blots comprised by summation of each individual experimental group. The data in the lower columns show the composition of the total signal in terms of the sizes and relative percentages comprised by particular bands on blots. Bands which were present but at a level below 10 absorbance units (chosen as the cut-off point to exclude background interference) are included followed by an asterisk.

of sodium 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 from fish receiving 1 or 10 mg bicarbonate. At higher doses of bicarbonate these HGG fragments were again apparent although the 10.6 kDa fragment was present at a lower relative level. A large amount of the HGG detected in plasma from fish given 10, 20, or 35 mg bicarbonate was accounted for by the 43.2 and 18.4 kDa fragments which were not present at a high level in the gastric regions of fish administered with lower doses of bicarbonate (Table 1). The very high apparent molecular weight band ($\approx$ 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 the plasma of fish receiving HGG may have been a result of antigen aggregation or to complexing with plasma factors.

II. Cimetidine

Gastric pH increased with increasing dose of cimetidine from 1–30 mg 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. 1). Differences between gastric pH values measured in fish receiving 30, 50, 75, or 100 mg cimetidine prior to HGG administration were not significant. Immunoblotting analysis demonstrated 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 2). Plasma from fish receiving 1 mg cimetidine was 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 molecular weight fragments (10.2 and 8.2 kDa) were most noticeable in the group given a low dose (1 mg) of cimetidine per fish, a pattern similar to that observed with low doses of sodium bicarbonate. There was a considerable similarity in the presence and relative percentages of the 18.9 and 20.4 kDa fragments in fish administered with a dose of 30–100 mg cimetidine, the very similar pH values recorded in these different groups may explain the similar results. This is in contrast to the case of bicarbonate where the fragmentation pattern differed considerably between groups of fish receiving high doses of the antacid, this probably reflects the wide variation in the gastric pH values recorded in these groups. As with bicarbonate which at high doses led to the detection of the 10.6 kDa fragment in plasma, a 10.2 kDa fragment was detected in the plasma of fish admin-

TABLE 2. Dose response to cimetidine in terms of gastric pH values and sizes and relative percentages of HGG bands detected in plasma 1 hr after the delivery of HGG

Cimetidine dose (mg/fish)	0	PBS (control)	1	10	30	50	75	100
HGG dose (mg/fish)	0	35	35	35	35	35	35	35
Gastric pH	3.38	5.04	5.62	5.96	6.81	6.94	6.87	6.98
Total signal (%) on Western blots	N/A	6.0	13.2	5.7	8.8	13.0	39.3	14.0
Band size (kDa) and relative %		207.9 (100%)	207.9 (57.2%)	207.9 (76.8%)	207.9 (52%)	207.9 (48.7%)	207.9 (28.6%)	207.9 (54.7%)
		187 (*)					187 (*)	
							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 (*)					

Cimetidine was administered to fish at a range of doses from 0–100 mg per fish 1 hr prior to the delivery of HGG and plasma was collected from fish 1 hr later. Gastric pH was measured at 3 points in the stomach of each animal and the average was taken, the result presented is the mean from five animals in each case. The total signal referred to in row 3 of the table represents the percentage of the total amount of HGG detected on Western blots comprised by summation of each individual experimental group. The data in the lower columns show the composition of the total signal in terms of the sizes and relative percentages comprised by particular bands on blots. Bands which were present but at a level below 10 absorbance units (chosen as the cut-off point to exclude background interference) are included followed by an asterisk.

istered with high doses of cimetidine. However, the amount of this fragment detected in the case of cimetidine was considerably less. This may reflect the higher gastric pH in fish administered with high doses of bicarbonate. The pattern of results obtained using high doses of bicarbonate and high doses of cimetidine were relatively similar, as a result bicarbonate was omitted from further studies which compared the effects of a low dose and a high dose cimetidine treatment.

Measurement of pH Over a Time Course After the Delivery of Cimetidine

No significant difference was found between the gastric pH of fish 1 hr after the delivery of HGG in PBS or 1 mg cimetidine/fish. A pH between 4.0 and 6.0 was measured in the gastric regions of fish for up to 12 hr after HGG delivery in animals previously administered with 1 mg cimetidine and differences between pH values at different time points up to 12 hr after delivery were not significant (Fig. 2). This may have been a result of the high degree of variability in gastric pH values between fish. After administration of 50 mg cimetidine a pH $\approx$ 7.0 was measured in the stomach until $>$ 12 hr after HGG administration (Fig. 2). A pH greater than 6 was measured up to 48 hr after HGG delivery, the latest time point sampled.

Analysis of Mucosal Contents Over the Time Course

In fish administered with 1 mg of cimetidine prior to HGG delivery there was a progressive decline in the amount of HGG detected in the gastric region from 15 min to 6 hr after HGG administration (Fig. 3). Subsequently there was a rapid decrease in the amount of HGG detected and little antigen was found from 12–48 hr after administration. The HGG was detected in intact form and as 21 additional fragments (Table 3). In gastric contents from fish which were administered with 50 mg cimetidine prior to HGG, antigen was detectable at all time points analysed from 15 min to 6 hr after HGG administration after which time no HGG was detected (Fig. 3). The total HGG signal detected was relatively constant until up to 2 hr post-administration after which a large decrease was found. The passage of antigen from the stomach of fish administered with 50 mg cimetidine appeared to be delayed compared with those receiving the 1 mg dose. The number of fragments smaller than 20 kDa in size present in the gastric region of fish administered with the higher dose of cimetidine was less (1 fragment)

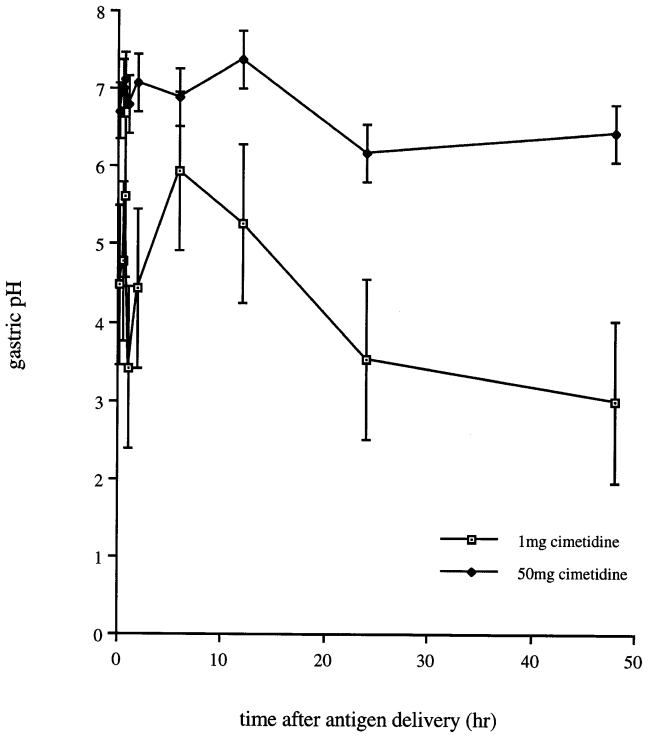

FIG. 2. Gastric pH over a time course after the delivery of cimetidine.

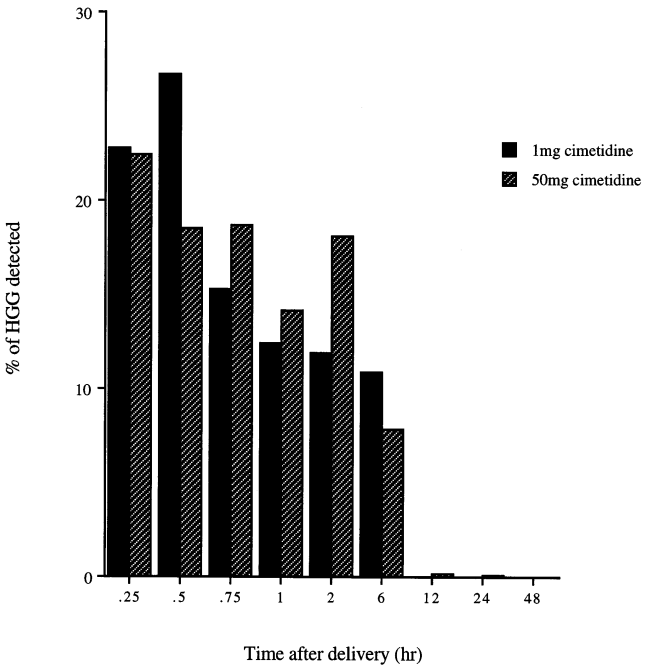

FIG. 3. Detection of HGG in gastric region of fish after oral gavage.

than when a dose of 1 mg of cimetidine was delivered (4 fragments) (Table 4). However, the relative amount of intact antigen present was considerably greater in the case of fish receiving the higher dose of cimetidine (40.40% compared with 24.84%).

In contents taken from the pyloric caeca of fish administered with 1 mg of cimetidine the highest HGG signals were found at 45 min and 6 hr after HGG administration (Fig. 4). HGG fragments of an apparent molecular weight of less than 50 kDa were only present at a high level in the pyloric caeca of these fish at 2 hr after HGG delivery. Most of the HGG detected at other time points was apparently intact or in the form of large fragments (> 90 kDa) (Table 3). In contrast, at the corresponding time points in the gastric regions of these fish a significant amount of the HGG present was in the form of fragments smaller than 50 kDa. The highest levels of HGG in contents from the pyloric caeca of fish administered with 50 mg cimetidine were found at 2 hr and 12 hr after antigen delivery (Fig. 4). It is noteworthy as in the case of fish administered with 1 mg cimetidine that very little HGG in any form < 50 kDa was detected in the pyloric caeca of these fish whereas over 30% of the HGG detected in the gastric region of the same fish was in the form of fragments < 50 kDa in size (Table 4). There appeared to be two phases to the transit of antigen through the pyloric caeca, an initial minor phase from 15–45 min after delivery and a second greater phase from 2–24 hr after delivery. The greatest number of bands (14) were also detected at 2 hr after delivery. As was the case with the results from gastric contents, the transit of antigen into the pyloric caeca appeared to be delayed in the group administered with the higher dose of cimetidine.

In contents from the anterior intestine of fish administered with 1 mg cimetidine prior to HGG delivery the highest HGG signal was detected at 2 and 6 hr after HGG delivery (Fig. 5), the greatest number of HGG bands (15) were also present 6 hr after HGG intubation. No antigen was detected in contents from the anterior intestine of these fish at time points later than 12 hr after delivery (Fig. 5). In contrast to the pyloric caeca very little intact or high molecular weight (> 90 kDa) antigen was detected in the anterior intestine of fish administered with 1 mg cimetidine; most fragments found being in the 30–65 kDa size range (Table 3). This indicated that considerable breakdown of the intact antigen and large fragments by anterior intestinal enzymes had occurred. In contents from the anterior intestine of fish administered with 50 mg cimetidine the highest total HGG signal was detected at 6 hr and 12 hr after intubation, as before the transit was delayed relative to fish which received 1 mg cimetidine prior to HGG. Much of the signal detected 45 min, 1 hr, and 2 hr after delivery was in the form of 29.2 and 23.9 kDa fragments while more intact HGG was found 6 hr, 12 hr, and 24 hr after delivery. There were considerable differences between the relative percentages of individual fragments present in the two groups, for example,

TABLE 3. Apparent molecular weights (kDa) and relative percentages of HGG bands detected on Western blots from contents of various regions of the digestive tract over a 48-hr time course after the delivery of 1 mg cimetidine prior to HGG

Site from which sample was taken				
Stomach	**Pyloric caeca**	**Anterior intestine**	**Posterior intestine**	**Plasma**
152.4 (24.84)	148.3 (63.1)	154.1 (1.76)	149.8 (34.17)	201.5 (19.74)
97.6 (2.47)	113.8 (2.11)	135.0 (0.47)	134.7 (0.21)	151.2 (12.12)
87.1 (5.4)	110.0 (10.16)	123.9 (0.05)	98.4 (2.32)	109.1 (1.49)
75.6 (12.6)	90.0 (2.46)	109.5 (0.03)	83.8 (4.17)	76.4 (0.27)
69.3 (0.29)	75.1 (2.65)	104.0 (1.21)	77.6 (4.84)	63.2 (1.46)
65.9 (6.62)	66.8 (0.91)	99.8 (0.19)	73.3 (1.57)	45.6 (0.3)
63.4 (4.79)	65.0 (2.96)	92.6 (1.62)	71.2 (*)	37.9 (8.31)
60.1 (2.15)	62.3 (0.76)	85.5 (*)	66.2 (7.32)	30.6 (3.67)
55.4 (1.61)	58.6 (4.40)	80.3 (5.57)	64.8 (0.31)	27.3 (3.14)
52.7 (1.66)	56.7 (3.28)	75.5 (3.81)	59.6 (0.71)	26.9 (7.11)
47 (10.49)	52.0 (2.93)	63.0 (0.77)	56.5 (3.97)	25.6 (9.39)
43.6 (2.82)	45.3 (2.36)	60.4 (2.29)	53.8 (1.24)	18.8 (*)
39.5 (4.52)	42.1 (*)	58.3 (2.15)	51.4 (8.43)	10.4 (30.12)
36.0 (0.72)	30.7 (*)	52.2 (2.84)	48.4 (0.71)	7.5 (1.54)
33.6 (4.64)	26.1 (1.56)	36.4 (45.37)	46.8 (1.28)	7.1 (1.34)
29.0 (4.17)	22.2 (0.36)	35.7 (*)	42.7 (*)	
25.6 (1.53)		33.9 (8.27)	39.7 (2.81)	
21.6 (1.37)		32.7 (3.69)	37.9 (*)	
19.5 (4.02)		31.6 (6.55)	37.5 (12.94)	
14.5 (0.15)		29.6 (13.09)	34.9 (7.25)	
12.2 (0.09)		28.2 (0.27)	33.1 (2.87)	
10.2 (3.05)			31.3 (2.88)	
			29.3 (*)	

The data presented shows the entire range of HGG bands detected on Western blots of samples collected at all time points over the time course for each gut region or for plasma. The figures in parentheses are the amounts of each band present as a percentage of the total amount of HGG detected at that site over the time course.

TABLE 4. Apparent molecular weights (kDa) and relative percentages of HGG bands detected on Western blots from contents of various regions of the digestive tract over a 48-hr time course after the delivery of 50 mg cimetidine prior to HGG

Site from which sample was taken				
Gastric	**Pyloric caeca**	**Anterior intestine**	**Posterior intestine**	**Plasma**
149.3 (40.40)	151.2 (53.87)	150.3 (19.52)	150.0 (5.91)	197.4 (17.86)
103.2 (1.71)	103.9 (3.69)	124.9 (4.34)	97.1 (0.16)	144.1 (15.81)
91.6 (2.11)	91.3 (10.18)	111.5 (2.41)	76.0 (1.46)	98.9 (11.8)
79.5 (2.10)	80.7 (5.02)	91.7 (5.61)	73.3 (2.99)	82.4 (6.82)
72.9 (6.91)	74.3 (2.89)	80.2 (3.75)	68.2 (1.64)	56.1 (*)
68.2 (0.53)	68.7 (1.39)	70.9 (0.62)	66.8 (0.42)	47.2 (*)
65.1 (5.65)	61.8 (1.93)	68.5 (1.92)	61.2 (*)	38.1 (2.20)
60.0 (3.91)	58.1 (1.94)	65.1 (1.96)	55.3 (0.88)	29.2 (*)
57.3 (1.82)	53.1 (12.21)	61.4 (4.26)	50.9 (3.37)	25.8 (*)
50.9 (2.47)	45.3 (2.06)	55.1 (7.14)	48.1 (1.99)	18.5 (19.31)
45.3 (5.27)	41.0 (1.78)	47.2 (12.01)	44.4 (*)	10.4 (26.20)
43.0 (*)	35.4 (0.77)	39.6 (1.34)	42.4 (29.29)	8.3 (*)
40.4 (5.16)	31.9 (0.26)	35.9 (11.98)	38.1 (27.96)	
37.6 (5.91)	30.4 (0.68)	34.0 (2.15)	35.2 (6.33)	
35.5 (3.32)	27.4 (1.01)	29.2 (4.54)	33.0 (4.79)	
31.1 (5.21)	19.3 (0.32)	23.9 (11.45)	31.7 (9.54)	
28.6 (1.08)		22.4 (0.68)	27.9 (2.91)	
23.2 (1.52)		20.2 (3.26)	22.9 (0.36)	
21.1 (0.78)		18.1 (1.06)		
18.2 (4.23)				

The data presented shows the entire range of HGG bands detected on Western blots of samples collected at all time points over the time course for each gut region or for plasma. The figures in parentheses are the amounts of each band present as a percentage of the total amount of HGG detected at that site over the time course.

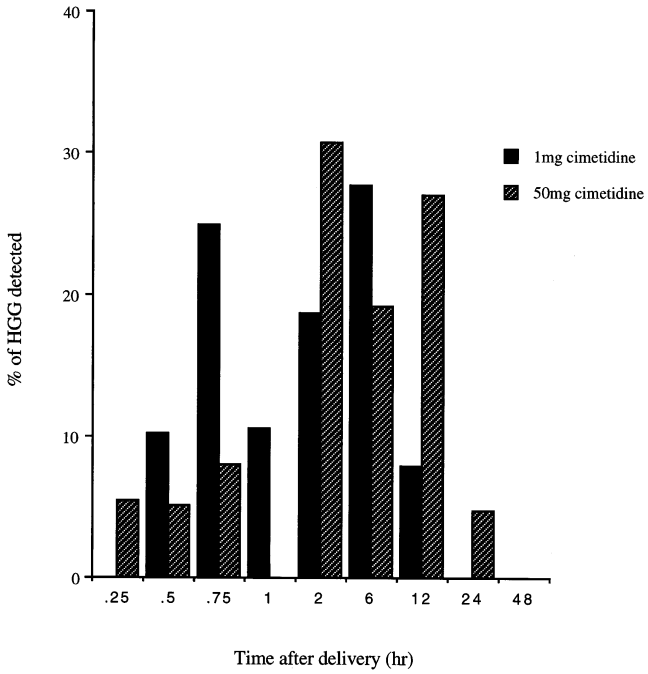

FIG. 4. Detection of HGG in pyloric caeca after oral gavage.

the high percentage of the 36.4 kDa fragment in fish administered with 1 mg of cimetidine (Table 3). However, the relative amount of intact antigen present in the anterior intestine was considerably greater after the administration of 50 mg cimetidine (Tables 3 and 4).

The highest levels of HGG found in contents from the

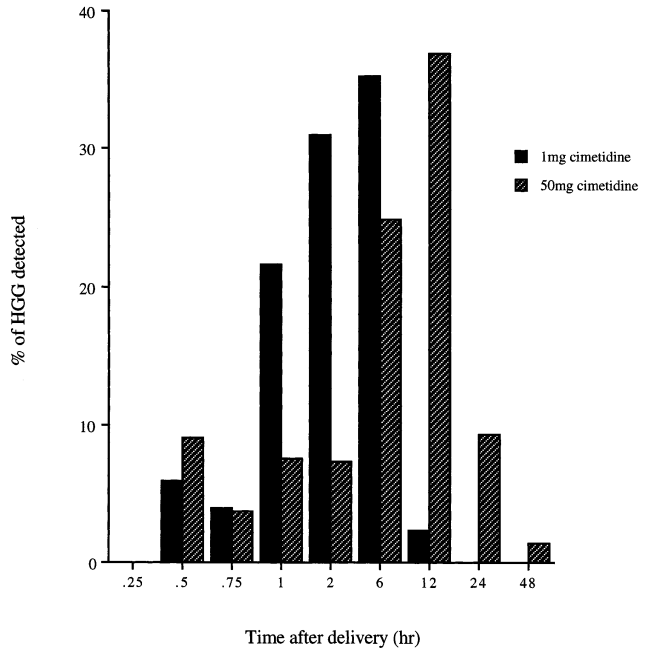

FIG. 5. Detection of HGG in anterior intestine after oral gavage.

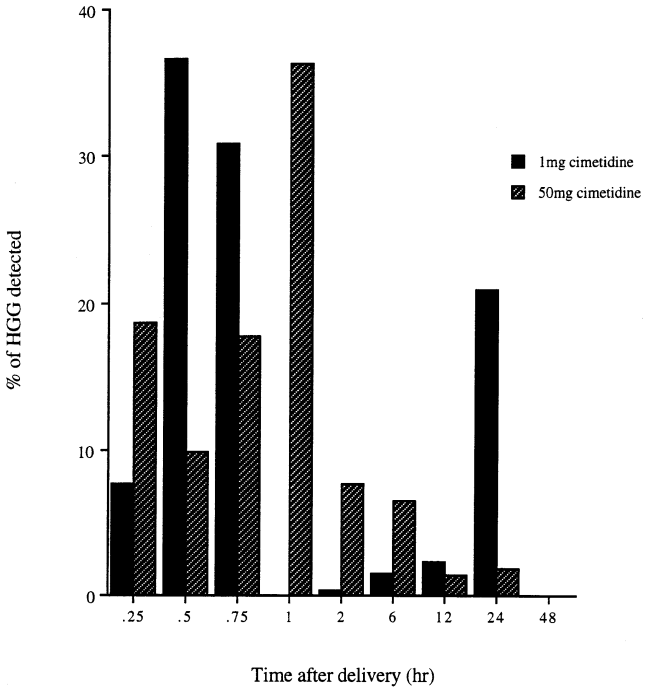

FIG. 6. Detection of HGG in posterior intestine after oral gavage.

posterior intestine of fish administered with 1 mg cimetidine were at 30 min, 45 min, and at 24 hr after antigen delivery (Fig. 6). 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 fragments between 30–52 kDa in size. 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. Over the entire time course this possibly intact band represented more than 34% of the total HGG detected while intact HGG represented only 1.76% of the total in the anterior intestine of these animals (Table 3). In contents from the posterior intestinal region of fish administered with 50 mg cimetidine the highest total signal was found 1 hr after antigen delivery (Fig. 6). Very little antigen in this case was detectable at time points later than 6 hr after HGG administration. Much of the HGG detected 1 hr after antigen delivery was in the form of the 31.7 and 38.1 kDa fragments. The 38.1 kDa fragment constituted 35.0–63.3% of the total signal in contents taken from fish from 15 min to 2 hr after antigen delivery after which its presence was not detected. In contrast to the results with contents from the anterior intestine, a higher percentage of intact HGG was found in the posterior intestine of fish administered with 1 mg cimetidine compared with 50 mg cimetidine (Tables 3 and 4).

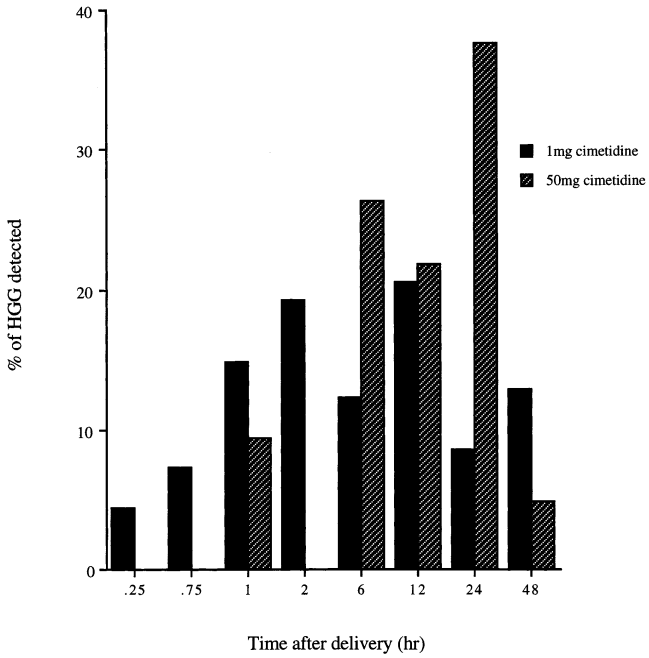

FIG. 7. Detection of HGG in plasma after oral gavage.

After prior treatment with 1 mg cimetidine the greatest total HGG signal found in plasma was at 2 and 12 hr after HGG delivery (Fig. 7). 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, similar to the results found 1 hr after antigen delivery with prior administration of 1 mg cimetidine in the dose response study (Table 2). Immunoblotting analysis of the plasma (Fig. 7) from fish administered with 50 mg cimetidine 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 notable for the high percentages of the 10.4, 82.4, and 98.9 kDa bands present. Considering the wide variation in the relative percentage of intact HGG present in different regions of the digestive tract, the similarity in the amount of intact antigen (including aggregates) in the plasma of fish administered with 1 mg (31.86%) and 50 mg (33.67%) of cimetidine was remarkable (Tables 3 and 4). Another similarity between the groups was the large amount of a fragment of approximately 10.4 kDa present, this was particularly striking in the case of fish administered with the higher dose since fragments of less than 18 kDa in size were not detected in the digestive tract. The principal difference between the HGG detected in the plasma of the two groups was the presence of a large amount of the 18.5 kDa fragment in fish administered with 50 mg of cimetidine, this fragment was present in the plasma of fish receiving the lower dose but not at a significant level. A similar pattern was noted in the dose response study but the trend was less marked than in this case. As was the case with contents from the various gut regions, the detection of HGG in the plasma of fish administered with the higher dose of cimetidine was delayed compared with those receiving the lower dose suggesting a correlation between gut passage and uptake into the bloodstream.

DISCUSSION

This study describes the processing of a protein antigen in the rainbow trout gut *in vivo*. Proteolysis of HGG in the gastrointestinal tract is a complex process with different gut regions playing distinct but complementary roles. It was demonstrated that the nature and quantity of orally delivered antigen ultimately reaching the circulation may be predicted to some degree by understanding the mechanics of *in vivo* lumenal 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 (16,17,27) but their processing in the lumen was not described. Studies have revealed the capacity for macromolecular uptake in fish and proposed a role for luminal enzymes in antigen degradation in the gut (27,37). The present work investigated the condition of orally delivered HGG in the gut and circulation using Western blotting methods. The methods of analysis which were used have allowed a detailed assessment of the roles of various regions of the gut in antigen degradation.

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 of greater than 10 mg per fish resulted in a pH of approximately 7 which was consistent with its role as a competitive histamine H_2 receptor antagonist (48). The measurement of a gastric pH of 7 at the first sampling point, 75 min after administration of 50 mg cimetidine indicated that a constant and considerable secretion of HCl occurred in the rainbow trout stomach in order to maintain an acidic environment (pH approximately 3.4 in control fish). A role for histamine in the stimulation of gastric acid secretion has been demonstrated in the European catfish, *Silurus glanis* (23) and in the Atlantic cod, *Gadus morhua* (25). The observation that exogenous histamine led to acid secretion indicated a physiological role for histamine in the regulation of acid secretion (34,43). It was also found that the injection of histamine or carbocholamine into cod led to considerable secretion of gastric acid, an effect which was blocked by the H_2 receptor antagonist metiamide (26).

Intubation of control PBS alone, because of its 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 (18) which would increase gastric pH for some time. The delivery of test antigens in a small volume of water may be a more rational approach in the future. 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 larger fragments was found in the plasma of fish with a gastric pH ≈ 7.0. In a study of the gastric proteases of cod three enzymes were detected which were optimally active at pH 3.0–5.0 (50). 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. Similarly, in the present study activity was demonstrated in the gastric region at a pH of 4–5 and at pH 7 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 dependent. For example it was found that the optimal pH for pepsin from Arctic capelin, *Mallotus villosus* varied according to the substrate used (19) and marked effects of pH on rainbow trout gastric chitinase activity which were also substrate dependent have been reported (33). A study on Dover sole, *Solea solea* gastric proteases found three separate peaks of protease activity with a casein substrate at pH 1.7, pH 6.0, and pH 10.0 (4). Similarly, a pepsin from capelin 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 (19). 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 (42). Some of the antigen detected in the gastric region of fish in the present study was found intact indicating that the antigen was only partly digested and would have left the stomach in this form. This is in accordance with a study on protein digestion in the Atlantic cod, *Gadus morhua* (31). It is also possible that the delivery of a relatively large amount (35 mg) of purified protein overloaded the proteolytic capacity of the gastric enzymes. The finding that considerable degradation of the intact HGG occurred in the trout stomach at pH 7 and 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. This supports previous findings on rainbow trout (9), cod (31), and other species (45) 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 (20). Only very small amounts of free amino acids were found to be released from proteins in the stomach (9,31). 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 caeca. 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 (2).

Gastric inhibitors may be of considerable value in cases where orally administered antigens are highly acid-labile. 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 (5,46). 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 be facilitated (38). The efficacy of PLG microparticles as a delivery system for oral vaccines in mammals has for example been partly attributed to the protection of antigens from acidic degradation and intestinal proteolysis (41). 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 (37) increased the quantity of orally delivered horseradish peroxidase in the liver and spleen of rainbow trout. Additional evidence is provided by a study which found that enteric coating of orally delivered *Vibrio* bacterins resulted in enhanced protection to challenge as compared with unprotected vaccine (58). It may be concluded that by modifying the gut environment or protecting labile protein antigens the efficacy of oral vaccines may be enhanced. However, the results of the present study indicate that neutralisation of gastric acidity does not completely abrogate proteolysis of HGG in the stomach or greatly increase the quantity of intact antigen reaching the circulation.

The role of the pyloric caeca in salmonids has been a matter of some debate. Lipid absorption, enzyme synthesis and vitamin production have all been proposed as functions. The surface area of the rainbow trout pyloric caeca is three times that of the remainder of the midgut and twice the area of the entire intestine indicating that it represents an expansion of the surface area of the midgut (14). Studies on the inner epithelium of salmonid pyloric caeca indicated that cells secreting digestive enzymes were absent (15). 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 caeca (29) thus making isolation of enzymes from the pancreas difficult. A significant correlation was found between the length of the rainbow trout intestine and extent of protein digestion but no such correlation between digestion and either the number or the length of pyloric caecae was found (57) suggesting that the

pyloric caeca were not involved in protein digestion. Utilising isolated everted pyloric caeca it was found that this tissue can absorb amino acids and sugars across its epithelial cells (10) and it has been determined that intestinal contents move into and out of the pyloric caeca by contraction of the caecal musculature. Previously there has been little information concerning the role of the pyloric caeca *per se* in the digestion of proteins. The present study suggests an important role for the pyloric caeca in the degradation of soluble proteins. A number of HGG fragments present in the stomach were not detected in the pyloric caeca of these fish which suggested that either the fragments were degraded in the pyloric caeca to peptides or amino acids which were not detectable by the methodologies used or were absorbed. Most of the antigen which was detected was apparently intact or in the form of large fragments which was in contrast to the results from gastric contents where a higher proportion of the antigen detected was in the form of smaller molecular weight fragments. It thus appears that enzymes residing in the pyloric caeca 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 divisions 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 (8) and found a 20–200-fold increase in the amount of free amino acids released from dietary protein in the pyloric caeca as compared to the stomach. In agreement with this are the results of a later study (31) which found mainly proteins and polypeptides in the stomach but polypeptides, short peptides and amino acids in the pyloric caeca and anterior intestine. The evidence indicates that the proteolytic activity in the lumen of the rainbow trout pyloric caeca is considerable. It is conceivable that HGG fragments produced in the stomach were subsequently absorbed in the pyloric caeca but since such large scale antigen absorption has not been previously reported this is perhaps unlikely.

In contrast to the pyloric caeca, the anterior half of the post-pyloric caeca gut appeared to possess enzymes which were highly effective in degrading intact proteins and large protein fragments. Very little intact antigen or high molecular weight fragments ($>$ 100 kDa) were found in this region. This rapid and extensive proteolysis is in agreement with reports of the presence of highly active proteases and peptidases in the intestinal region of rainbow trout (9,22) and in cod (31). The results from the present study on contents from the posterior portion of the intestine are more difficult to assess. Antigen was detected in this region soon after intra-gastric intubation which is difficult to envisage in the context of normal gut physiology. Intact HGG was detected in 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 (12,18,27) and the biphasic patterns of antigen uptake into plasma reported in the present and earlier studies (27) may be a result of this procedure and not a *de facto* physiological phenomenon. Whatever the reason, it appeared that little proteolysis of HGG occurred in this posterior intestinal region; antigen being found up to 24 hr after delivery in intact form and as high molecular weight fragments. A decline in the activity of intestinal proteases in the posterior intestine has been reported previously (24) as has an important role for this region in protein absorption (13,18,36). The rainbow trout hindgut is deeply folded, possibly resulting in a functional separation between the central lumen and the spaces between the folds (14). Nonvacuolated cells line the central lumen while the sides of the folds are lined by vacuolated cells indicative of macromolecular uptake by pinocytosis, which may increase food retention time in the hindgut and therefore presumably increases the capacity for absorption.

Many studies have suggested 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 after oral administration (18,44). If this is the case then some degree of antigen protection from luminal secretions appears necessary. The present study indicates that there is a greater correlation between the levels of HGG detected in the anterior intestine and in the plasma than between the posterior intestine and plasma which suggests that the majority of protein uptake may occur in the anterior intestinal region. However, if uptake from the posterior intestinal region is rapid the antigen detected in anterior intestinal contents may have been absorbed upon entry into the posterior intestine and thus was not detectable at this site. It is therefore possible that the small amounts of antigen detected in the posterior intestinal region reflect large-scale protein uptake. In some cases antigen fragments were detected in the plasma which were not detectable in any gut region. It is likely that such fragments were the result of post-lumenal processing possibly involving membrane-associated factors (1,56), intracellular processing (13,17), or possibly peptidases in the bloodstream (35).

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