THE ENTERIC DELIVERY OF MACROMOLECULES AND SUBSEQUENT IMMUNE RESPONSE IN THE CICHLID Oreochromis mossambicus.

BY

PAUL GEORGE JENKINS, BSc (Hons)

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Paul George Jenkins

Dr. J.E. Harris
(Supervisor)
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ABSTRACT.

Enhanced enteric delivery systems were examined in the cichlid *Oreochromis mossambicus* to evaluate the requirements for potential oral vaccination strategies in teleost fish. Human gamma globulin (HGG) was delivered enterically (orally or anally) to the intestine of *O. mossambicus* as a standard test antigen. The co-administration of the saponin *Quillaja saponaria* (Quil-A) was evaluated as a novel oral adjuvant, delivered with the antigen in a soluble form and also as a delivery vehicle in the form of micelles and immune-stimulatory complexes (ISCOMS). HGG absorption across the intestine was monitored utilising sensitive immunocytochemical techniques which showed that the enterically delivered antigen was transcytosed in a temporally complex manner, underwent extensive interaction with the enterocytes and gut-associated lymphoid tissue (GALT) cells and was eventually transported to the vicinity of the intestinal circulatory system. Co-administration of Quil-A resulted in an increase in the HGG absorbed, increased antigen localisation in the lamina propria and substantial interaction of the adjuvant with the enterocyte lumenal membranes.

The levels of HGG absorbed into the plasma were directly quantified by enzyme-linked immunosorbent assay (ELISA) and showed that administration of Quil-A concurrently with HGG greatly increased absorption above levels observed without adjuvant in the delivery system. Western blotting and laser densitometry demonstrated that HGG was absorbed as both an intact macromolecular antigen and as fragmented epitopes of distinct molecular weights. The fragmentation of the enterically delivered HGG was modified by the delivery of Quil-A suggesting that manipulation of conformational aspects of the delivered antigen may be possible. The systemic and mucosal immune responses to HGG administration were monitored and enteric immunisation of antigen with Quil-A was found to be effective in increasing specific antibody levels in the plasma, bile and cutaneous mucus of immunised fish.

Preliminary studies on the use of cholera toxin B-subunit, aluminium hydroxide and ammonium chloride showed that cholera toxin B-subunit acted to increase both level of absorbed antigen, after enteric delivery and the subsequent immune response to HGG whereas the other two adjuvants were unable to mediate any such responses.
List of publications and conference contributions:


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List of abbreviations.

A- angstroms

ABC- avidin-biotin complex

AlOH₃- aluminium hydroxide

APC- antigen presenting cell

B- bile

BCG- Bacille Calmette Guerin

BSA- bovine serum albumin

cAMP- cyclic adenosine monophosphate

C- degrees celsius

cm- centimetres

CM- cutaneous mucus

CMC- critical micellar concentration

CT- cholera toxin

CTB (or CtB)- beta subunit of cholera toxin

Da- daltons

DAB- 3’3 diamino benzidine tetrahydrachloride

DDA- dimethyldioadecylamide

DNP- dinitrophenol

DPX-

DRV- dehydration-rehydration vesicles

DTH- delayed-type hypersensitivity

ECP- extracellular products
EDTA- ethylene diamino tetracetic acid

ELISA- enzyme linked immunosorbent assay

EM- electron microscopy

F_{ab}^- antibody binding fragment of immunoglobulin

FAE- follicle associated epithelium

F_{c}^- crystallisable fragment of immunoglobulin

FCA- Freunds complete adjuvant

FIA- Freunds incomplete adjuvant

g- grammes

GALT- gut-associated lymphoid tissue

GCAT- glycerophospholipid cholesterol acyl transferase

GM1^- ganglioside of the enterocyte brush border

H&E-haematoxylin and eosin

HGG- human gamma globulin

HRP- horseradish peroxidase

HSF- heat sensitive factor

IEL- intraepithelial leucocyte

IFN- interferon

Ig- immunoglobulin

Ig^+^- immunoglobulin positive surface

IL- interleukin

ILL- intralaminal leucocyte

IP- intraperitoneally

IM- intramuscularly
pVC- polyvinyl chloride
QAC- quaternary ammonium complex
Quil-A- extract from *Quillaja saponaria* [Molina]
REV- reverse phase evaporation vesicles
sABC- streptavidin-biotin complex
sABC-HRP- streptavidin-biotin horseradish peroxidase complex
SBTI- soy-bean trypsin inhibitor
SC- subcutaneously
SDS-PAGE- sodium dodecyl sulphate polyacrylamide gel electrophoresis
SRBC- sheep red blood cells
SUV- small unilamellar vesicles
Tris- Tris (hydroxymethyl) aminomethane.
TBS- Tris buffered saline
TP- test plasma
V- volts
v/v- volume to volume ratio
w/w- weight to weight ratio
CHAPTER 1.
CHAPTER 1

INTRODUCTION

The epithelium of the gastrointestinal tract in vertebrates has an extensive surface area for the absorption of the digested food components needed for the normal nutritional requirements of the organism. Simultaneously it presents a barrier to large numbers of non-dietary antigens that continually pass along the alimentary tract and can potentially induce pathology. Most dietary components are broken down by digestive processes prior to absorption, thus most potential antigens in the gut never come into contact with the epithelial lining, as the luminal contents are physically directed along a central course away from the epithelium. However, it has been demonstrated that a small but immunologically relevant quantity of antigen does gain access to the body tissues, often in an antigenically intact form (Walker, 1981).

As a consequence the vertebrate gut has evolved a local immune system mediated by the cells of the gut-associated lymphoid tissue (GALT) which are distributed along its length. This system is diversified and extremely complex involving a wide range of cellular and soluble factors, coordinating to maintain a state that is able to sample some luminal antigens, exclude potential pathogens, carry out normal digestive and absorptive functions and allow a normal, beneficial, luminal bacterial ecosystem to co-exist with it.

GALT is more complex in evolutionarily recent vertebrates such as mammals than in lower vertebrates such as fish, where one of the major differences is an apparent lack of highly structured, organised lymphocyte populations. The nature of the underlying GALT is directly influenced by the
other aspects of the physiology of the alimentary tract such as the mode of nutrition of the species, which will have a major influence on gut passage times and hence on the eventual levels and qualitative status of any antigens that may be absorbed and come into contact with the GALT cells. Fish have widely divergent nutritional strategies and this is effected in species specific differences in gut structure and function.

Vertebrate intestines, including those of fish, have the capability to absorb a wide variety of antigens in a multitude of forms. This lends itself to the possibility of stimulating GALT cells via the oral delivery of antigenic substances and the production of oral vaccines.

Oral delivery of antigen remains potentially the most convenient route of vaccination against a wide variety of pathogenic organisms, particularly those which gain access to the host organisms body via the mucosal surfaces, whether in humans, other mammals or fish. Oral delivery maximises the speed of administration of vaccine and local action at the site of infection for many pathogens yet minimises the stress to the recipient organism, which may be an important contributing factor in disease e.g. in furunculosis in fish. In fish, oral vaccination regimes may also reduce labour costs, time of vaccination and reduce problems of accessibility of stock (Ellis, 1988).

Oral vaccination studies in fish to date have largely utilised standard protein molecules as test antigens or actual derivatives from enteric (often bacterial) pathogens. Recent increases in intensive aquaculture practices have resulted in a potential requirement for the delivery of a variety of biologically active macromolecules. Oral delivery systems in more sophisticated aquaculture
practices may also allow delivery of other macromolecules such as chemotherapeutics, hormones and colouring agents (McLean and Donaldson, 1990; McLean, Donaldson, Dye and Souza, 1990). Such systems largely rely on the ability of the gastrointestinal tract of vertebrates, especially the distal regions, to absorb macromolecules that are antigenically defined and possibly immunogenic or wholly intact. Such macromolecules are then thought to interact with the underlying GALT and also via mediators with other lymphoid tissue to provide a relevant and protective immune response (Mestecky, 1987).

Intact protein absorption, once thought to occur only in neonates, has been shown in adults of most classes of vertebrates and it appears that the capacity of the intestine to absorb macromolecules declines in adults but does not completely cease; i.e., gut "closure" is not necessarily complete. Intact protein macromolecules are also absorbed across the gut of embryonic, larval, juvenile and adult fish by a variety of mechanisms often analogous to those more thoroughly investigated in mammalian systems.

Macromolecules can be absorbed across the intestine by both transcellular and paracellular (intercellular) pathways. The precise mode of transport of an antigen across the intestine depends on several extraneous factors including, the nature of the antigen delivered, its dose and the presence of any modifying agents such as adjuvants.

Intact protein absorption may, under normal physiological conditions, have several roles including transfer of passive immunity in neonates, a nutritional role, provision of a selective barrier for dietary antigens and an enteropancreatic enzyme recirculation system. However "artificial" delivery of macromolecules
in a vaccination protocol requires the triggering of an immune response beyond all the roles outlined above. The fate of an orally delivered antigen is not as straight-forward as the direct, rapid, production of a suitable protective immune response, often generated by intraperitoneal administration of the same antigen. Oral delivery of antigen can lead to the induction of a mucosal immune response (mediated by secretory IgA, in mammals, and IgM in fish) at the site of administration. It may also allow systemic priming, resultant immunological memory and a secretory immune response at other mucosae. However, oral delivery of antigens can also result in low mucosal responses, no viable disease protection and it can also induce a state of systemic unresponsiveness to the antigen (oral tolerance). Oral tolerance does not totally preclude some cell-mediated immune responses per se but it does minimise the effectiveness of oral vaccination for some antigens. As such, oral vaccination regimens have been largely concerned with overcoming the problems associated with gastric destruction of antigens and the poor immune response sometimes elicited (Warren, Vogel and Chedid, 1986; Ellis, 1988).

Mammalian vaccine design techniques employ a variety of strategies such as genetically recombinant organisms as both antigen carriers or direct immunogens, live bacterial organisms (avirulent but immunogenic strains or aromatic-dependents), cloned epitopal vaccines, enhanced delivery systems (adjuvants, liposomes and microencapsulated antigens) and targeting agents for specific intestinal factors (Curtiss, 1990). Little is known of the true ability of orally delivered antigens to cross the intestine, interact with the local and systemic lymphoid tissue and elicit an immune response in fish. Fish vaccine...
technology is more basic, as yet, but all of these approaches are being considered as candidates for the production of useful vaccines against bacterial pathogens. Current fish bacterial vaccines are largely confined to the delivery of crude cellular products in en masse.

As part of a broad study on GALT and the absorption of enterically delivered antigens, carried out in this laboratory (Hart, 1987; Doggett, 1989) a number of aspects of macromolecular administration were examined. The specific aim of this study was:

1) To increase knowledge of the uptake of antigens in a species of teleost fish after oral delivery, in terms of their mode of absorption, temporal fluctuations, quantity of absorption and certain qualitative aspects of their uptake.

2) To gain some data on aspects of the interactions of orally delivered antigen with intestinal tissues and GALT.

3) To modify the delivery of the antigens by a variety of novel methods such as the use of adjuvants.

4) To monitor any subsequent immune response elicited.

5) To develop and use a wide variety of technologies for the study of oral antigen delivery in fish including classical microscopy, histochemistry, immunohisto- and cytochemistry, analytical protein techniques (electrophoresis and blotting) and serological immunoassays such as ELISA.

The species of teleost used in this study was the cichlid Oreochromis mossambicus, chosen for a variety of reasons, including the previous
characterisation of its GALT in our laboratory, its ability to absorb macromolecules across its intestine as an adult, its relative ease of use as an experimental animal and its potential importance as a commercially farmed fish, especially in the developing world.
CHAPTER 2.
CHAPTER 2. LITERATURE REVIEW.

This review is intended to discuss the variety of factors that can influence both the absorption of macromolecules delivered to the intestine and any subsequent immune responses generated, and the strategies currently being investigated as delivery systems for oral vaccination in mammals and fish. Previous studies have recently comprehensively reviewed mammalian and fish GALT (Hart, 1987; Kagnoff, 1987; Nicklin, 1987; Hart, Wrathmell, Harris and Grayson, 1988; Doggett, 1989) and this literature review is not intended as a comprehensive discussion of these areas.

2.1. THE STRUCTURE OF GALT.

The normal mammalian gastrointestinal tract encounters a massive antigenic load during the course of its normal function, including a wide variety and quantity of dietary antigens and microorganisms. The gut has evolved a complex immune system reflecting this: the associated lymphoid tissues, in mammals, are larger than all other lymphoid tissues combined (Nicklin, 1987). For example, the GALT in the human small intestine consists of $10^{10}$ IgA-producing cells per metre of its length as well as almost 70% of the body's antigen-presenting cells (Curtiss, 1990) and these account for only two aspects of GALT function (Brandzaeg, Thrane and Sollid, 1988; Schreiber and Walker, 1988).

The components of GALT can be divided into two major parts: organised lymphoid tissues such as the Peyer's patches, isolated lymphoid follicles and mesenteric lymph nodes and diffuse lymphoid tissue which is found scattered throughout the epithelium and the lamina propria.
(Kagnoff, 1987; Mowat, 1987). A third putative population of functional immunocytes has been suggested - a transient population of cells inhabiting the intestinal lumen itself, but having a positive protective role (Heatley and Bienenstock, 1982).

Peyer's patches are macroscopic aggregations of lymphoid tissue situated beneath a specialised membrane (Carlson and Owen, 1987) consisting of normal enterocytes interspersed by modified domed absorptive cells with few microvilli (M cells or follicle-associated epithelium (FAE), which allow GALT leucocytes to approach within 0.3 \mu m of the luminal source of antigen (Marsh and Swift, 1969; Owen and Jones, 1974; Kagnoff, 1987).

Large lymph nodes lie within the mesentery and are identical to peripheral lymph nodes with thymus-dependent areas, primary follicles and germinal centres. These develop in parallel with the Peyer's patches and the presence of intestinal antigen is required for their development. Mammalian mesenteric lymph nodes are probably a primary site of contact between the intestinal and systemic lymphoid compartments (Chanana, Schaedeli, Hess and Cottier, 1973; Barg and Draper, 1975). Isolated lymphoid follicles are similar to Peyer's patches in possessing an overlying cuboidal epithelium with M-cells (Owen and Nemancic, 1978).

The diffuse mucosal lymphoid tissue is composed of an enormous number of cells. The normal lamina propria is infiltrated by a wide variety of leucocytes including lymphocytes, plasma cells, macrophages, eosinophils, basophils and mast cells. Lymphocytes, in the lamina propria, consist of approximately equal numbers of T and B cells. However the most predominant cell is of the helper-
T phenotype (60-80% of laminal T-cells) (Selby, Janossy and Jewell, 1981; Selby, Janossy, Bofill and Jewell, 1983) whereas 80-90% of the B lymphocytes are terminally differentiated and committed to IgA synthesis (Tseng, 1984).

The epithelium is the layer in closest contact with luminal antigen. In mammals 60% of the intraepithelial leucocytes (IEL’s) are granulated and 15% have a "locomotor" aspect and are situated at the basement membrane or visualised passing across it (Toner, Carr and Wyburn, 1971). The majority of epithelial leucocytes are T-cells the majority of which have the T cytotoxic/suppressor phenotype (Cerf-Bensussan, Scheenburger and Bhan, 1984).

Mammalian GALT therefore consists of specialised absorptive structures (FAE and M-cells), normal absorptive enterocytes and both highly organised lymphoid structures and large numbers of diffuse lymphoid cells consisting of a wide variety of leucocyte-types often in unique numerical combinations (Bjerke, Brandtzaeg and Fausa, 1988).

Fish are the earliest vertebrates which have a well developed immune system characterised by both cellular and humoral branches, endowed with specificity (McCumber, Sigel, Trauger and Cuchens, 1982) and, in some cases memory (Cooper, 1976; McCumber et al., 1982; Rijkers, 1982). GALT is found in a number of evolutionarily diverse species of fish (Hart et al., 1988). Primitive fish, such as the cyclostomes have intraepithelial leucocytes with a few lymphoid accumulations (Tomonaga, Hirokane and Awaya, 1973) while the anterior gut of the sea lamprey has an invagination which is a primitive analogue of the spleen and similarly hagfish have haematopoietic foci in their lamina propria.
Elasmobranch intestines have lymphoid accumulations in the spiral valve as well as large numbers of diffuse leucocytes in the lamina propria. A few species of elasmobranchs also have encapsulated lymphoid accumulations underlying an epithelial layer that has some morphological similarities to the domed FAE in mammals (Tomonaga, Kobayashi, Hagiwara, Yamaguchi and Awaya, 1986; Hart et al., 1988).

In teleosts GALT consists mainly of diffuse leucocytes in the epithelium and lamina propria (Weinberg, 1975; Temkin and McMillan, 1986; Doggett, 1989), with some aggregations of small numbers of cells (Pontius and Ambrosius, 1972; Davina, Rijkers, Rombout, Timmermans and Van Muiswinkel, 1980). There are no direct analogues of M-cells reported in fish and the overlying epithelium in teleosts is not as specialised mammalian FAE. The only organised GALT in fish, exists as the elasmobranch spiral valve and a few small accumulations in the lower intestines of certain species. The percentages of leucocytes in the epithelium and lamina propria of teleosts and mammals appear similar.

There is less known about the phenotypic composition of the leucocytes in fish GALT. There is a predominance of surface-immunoglobulin negative (sIg-) lymphocytes in the epithelium of teleost species (Davina et al., 1980; Miller, Bly, van Ginkel, Elssaesser and Clem, 1987; Rombout, Bot and Taverne-Thiele, 1989) and as in mammals a greater number in the lamina propria. About 10% of the lymphocytes in the lamina are positive for surface Ig in carp (Rombout et al., 1989a), a smaller percentage than in other organs in the body eg kidney. Lymphocytes positive for cytoplasmic Ig were rare. It
appears that the lamina propria is conserved as a site of terminal differentiation of lymphocytes. Phenotypic analyses of the GALT lymphocytes using T-cell markers, anti-thymocyte antisera and conserved Thy\(^1\) cell markers derived from species specific brain tissue, have shown that positive cells are present in both the epithelium and the lamina propria (Davina et al., 1980; Elsaaesser, Bly and Clem, 1988). T-cell functions have also been indicated by rainbow trout, Oncorhynchus mykiss, cell mediated immune responses to Mycobacterium tuberculosis and M. salmoniphilum such as delayed type hypersensitivity (DTH) (Bartos and Somner, 1981). In vitro studies also suggested the possibility of similar lymphocyte subsetting as well as the existence of a range of soluble mediating factors analogous to the cytokines and monokines found in mammals (Miller, Deuter and Clem, 1986; Elsaaesser et al., 1988; Graham and Secombes, 1990).

Other leucocytes include a variable population of granulocytes of unclear lineage and function and macrophages that in some species appear to have morphological similarities with melanomacrophages and mammalian follicular dendritic cells (Doggett, 1989).

Fish GALT is less structurally organised than that of mammals but may still operate as its direct functional analogue (Temkin and Mcmillan, 1986) with respect to the ability of the intestine to absorb functionally intact macromolecules and to have a direct immunological interaction with them.
2.2. THE MECHANISMS OF UPTAKE OF MACROMOLECULES ACROSS THE GASTROINTESTINAL TRACT.

Before orally delivered antigens can have any interaction with the immune system the delivered macromolecules must cross the mucosal barrier from the lumen to the intestinal sites of the GALT, and also to the associated vascular supply for any systemic attention. The macromolecular absorptive processes are therefore important in determining the eventual fate of orally delivered antigens.

Several cell types throughout the vertebrate gut are involved with the uptake and transport of macromolecules. These include ubiquitous cell types such as the intestinal absorptive enterocytes (Schreiber and Walker, 1988), Paneth cells (Erlandson and Chase 1972a, 1972b), and the specialised mammalian M-cells (Owen and Jones, 1974; Wolf and Bye, 1984). Some of these cells are specific to mammals or to specific regions of the gut. However, there are some basic similarities in the way in which delivered macromolecules are absorbed in both fish and mammalian gastrointestinal tracts.

There are two principal modes of absorption, the first involving passage of macromolecules through the absorptive cell (transcellular pathway) and the second involving passage of material between the cells (paracellular pathway) and there are several controversial routes of absorption that are thought to be of less importance.

2.2.1. TRANSCELLULAR ABSORPTION.

Transcellular absorption involves endocytosis of macromolecules by one of three routes, depending on the nature and source of the macromolecules.
Fluid-phase macromolecular absorption, including soluble proteins is encompassed by the term "pinocytosis" and particulate macromolecules by the term "phagocytosis". Bacterial macromolecules (whole live or dead organisms) often pass across the intestinal mucosa, employing a transmural or resorptive process. Resorptive processes involve the passage across the intestine of immunologically or pathologically viable organisms which may have mechanisms influencing and/or increasing their own uptake (Nicklin, 1987).

Endocytosis has a defined sequence of events for the transport of material from the lumen. There are at least two endocytotic processes, one in which receptors specific for the delivered macromolecule are present on the luminal surface of the absorptive cell (receptor-mediated endocytosis) eg in the transfer of passive immunity via gamma globulins in neonatal mammals and in some larval fish. The second mechanism is termed fluid-phase endocytosis, where the luminal macromolecules are internalised in proportion to their exogenous concentration. The rate of uptake of macromolecules in both processes is largely determined by endocytotic vesicle size and the rate of vesicletic formation (McLean, 1987). However receptor-mediated endocytosis may allow the cell to concentrate and select specific macromolecules and exclude most coincident luminal material.

Receptor-mediated endocytosis is initiated by the binding of specific ligands to specific receptors in inward foldings of the apical surface of the plasma membrane (Udall and Walker, 1987). Binding of a ligand to a receptor (Goldstein, Anderson and Brown, 1979) causes clustering of receptors in the coated-pit of the cell and the conformational allowance of increased binding.
There may be more than one form of receptor for a certain ligand: one which allows its binding and another that allows its internalization (McLean, 1987). Fluid-phase endocytosis occurs by a generalised binding of macromolecules to the apical surface of the cell and although common to all cells may be of primary importance in M-cells (Nicklin, 1987; Udall and Walker, 1987). Bacteria may be endocytosed via either of these methods or in certain cases by highly specialised mechanisms. Many enterotoxigenic *E. coli* species (and others) have bacterial adhesins which allow specific binding to and translocation by enterocytes eg *E. coli* K88 (Udall and Walker, 1987), *E. coli* RDEC 101 which is specifically absorbed by rabbit M-cells (Mowat, 1987) and *Vibrio cholerae* which specifically adheres to the GM₁ ganglioside, a glycoprotein receptor on enterocytes (Boedeker and McQueen, 1988; Holmgren, Clemens, Sack and Svennerholm, 1989).

In teleosts and mammals it appears that adhesion of a macromolecule to the intestinal absorptive cell, by either method, is directly followed by internalisation. Fluidity of the cells plasma membrane changes and it becomes invaginated. This invagination has been described in many studies on the absorption of soluble protein antigens in teleosts (Georgopoulou, Sire and Vernier, 1985; Georgopoulou, Sire and Vernier, 1986; McLean, 1987; Georgopoulou, Dabrowski, Sire and Vernier, 1988; Doggett, 1989, Le Baii, Sire and Vernier, 1989). Invagination of material (receptor bound or otherwise) is followed by the formation of vesicles enclosing the absorbed material (Silverstein, Steinman and Cohn, 1977; Goldstein *et al.*, 1979).

Vesicular morphology is variable (Georgopoulou *et al.*, 1986, 1988) and the
time taken for these vesicles to migrate from the plasma membrane to the apical cytoplasm of the cell is also variable. Differential uptake dynamics occur for various delivered macromolecules (Fujino, Ono and Nagai, 1987; Georgopoulou et al., 1988; Rombout and van den Berg, 1989) though a defined sequence of events is maintained. The time course, however variable, results in vesicle fragmentation or multi-vesicular coalescence (Iida and Yamamoto, 1985; Georgopoulou et al., 1988) both in vitro and in vivo. Coalescing vesicles eventually form characteristic supranuclear vacuoles in teleosts within 5 hours for horseradish peroxidase (HRP) (Watanabe, 1982) and up to 10 hours for human gamma globulin (HGG, Georgopoulou et al., 1986) in rainbow trout. The rate of this phase of vesicular migration may also be dependent on the initial mode of absorption, mammalian studies indicating variability for receptor-mediated absorption of colostral IgG (Rodewald and Abrahamson, 1982).

The majority of supranuclear vacuoles coalesce with cellular lysosomes, effecting the formation of secondary lysosomes (Watanabe, 1981, 1982). It is at this juncture that the first level of post-lumenal processing of antigens occurs, with intracellular epithelial transcytosis exerting a major influence on the ultimate structure (and immunological fate) of a delivered macromolecule (Bland and Warren, 1986a, 1986b; Bland, 1989). Lysosomal degradation may move to completion, often in macrophages, for some delivered antigen. Receptor mediated endocytosis may provide some protection from this whereby "receptorsomes" may have a different fate from other lysosomes in their ultimate direction to the basal and lateral enterocyte membranes for exocytosis. Orally delivered macromolecules in fish may be biochemically deactivated by
fragmentation in lysosomes and still retain nascent immunologically recognisable epitopes (Rombout and van den Berg, 1989).

Two forms of transport systems have therefore been outlined (Abrahamson and Rodewald, 1981), one resulting in lysosomal merger and the other in intercellular exocytosis. Selective degradation or escape of proteins from such lysosomal activity may be due to distinct populations of vesicles (McLean, 1987). Vesicular transport of antigen across the cell may be augmented by and/or augment transport of macromolecules by cytoplasmic tubules; a system often noted in teleost absorption studies (Iida and Yamamoto, 1985) where cytoplasmic tubules can form complex, often fenestrated extensions linking pinocytotic vesicles with the basolateral membranes where deposited antigen can be detected.

Receptor delivered antigen requires a specific acidic intracellular compartment during its uncoupling and it is at this point that primary immunological interactions with orally delivered antigen can occur. Intestinal enterocytes in mammals, and possibly in fish, are known to have distinct major histocompatibility-complex antigens (MHC II) associated with their basolateral membranes and as an acidic environment is a prerequisite for antigen processing and presentation (Chain, Kaye and Shaw, 1988, Vallejo, Miller, Jorgensen and Clem, 1990) it can therefore be considered that under certain circumstances enterocytes may have an antigen presentation function (Bland and Warren 1986a, 1986b, Chain et al., 1988, Mayer, 1991). Enterocyte transcytosis may be the first stage in the immunological scrutiny of an antigen. Transcytotic-exocytotic mechanisms can therefore result in the degradation or the relatively intact
passage of macromolecular antigen together with the possibility of antigen presentation of certain epitopes.

2.2.2. THE PARACELLULAR PATHWAY AND OTHER MODES OF UPTAKE.

Classical transcellular absorption is not the only method of passage of macromolecules across the intestine. The other major route of uptake of macromolecules is via the paracellular pathway often termed persorption (Volkheimer, 1975, 1977; Nicklin, 1987). Persorption occurs when particulate antigens of varying sizes and forms (including starch, latex, Sephadex, pVC, solutions of colloidal metals, asbestos fibres and resin) are absorbed between the intestinal cells with the motive force being supplied by peristalsis. Persorption is restricted to single-cell layers of the intestine. The intestines of many species undergo a cycle of contraction and relaxation where old enterocytes on the villus tips are extruded and replaced by maturing cells. The integrity of the villus is partially compromised at such a stage and antigen can be directly absorbed into the lamina propria via this route. The membrane composition and architecture of the enterocytes is also intimately involved with absorption of macromolecules (Udall, Bloch, Vachine, Feldman and Walker, 1984) and may determine whether macromolecules or bacteria bind to the cell surface. Maturation of cells, paradoxically, may reduce transcellular absorption, as cellular protein content increases. Newborn vertebrates have enterocyte plasma membranes with reduced protein : phospholipid ratios. Such modifications can also be induced in adults, eg homeoviscous adaptation of teleost cells to temperature
(Bly, Cuchens and Clem, 1988) and in each case it results in the increased surface binding and internalisation of certain substances eg cholera toxin (Udall and Walker, 1987). Increased amounts of cholesterol (Egberts, Brinkhoff, Mouwen, van Dijk and Koninkx, 1985) are correlated with the ability of certain cells (M-cells) to absorb proteins such as HRP (Owen, 1977) and viral particles (Owen and Ermak, 1990). Orally intubated HRP has been detected in the goblet cells of mammals (Hugon and Borgers, 1968; McLean, 1987) though whether this mode of uptake is significant remains uncertain. Other controversial non-specific forms of uptake have also been postulated. These involve the potential uptake of antigen between the tight-junctions of enterocytes which can become "loosened" by the action of substances such as bacterial lipopolysaccharide (endotoxin), which in turn may be released by the gut bacterial populations and certain pathogens (Nicklin, 1987).

2.3. FACTORS INFLUENCING THE ORAL DELIVERY OF ANTIGENS.

Despite the variety of factors modulating the uptake of macromolecules, numerous studies have shown that intestinal absorption still occurs, in significant amounts, in mammals and teleosts (see McLean, 1987; Georgopolou et al., 1986, 1988; Doggett, 1989; Rombout and van den Berg, 1989).

Oral delivery of antigen can result in uptake of macromolecules that have been modified to a varying degree. Physiological functioning of the intestine while effecting qualitative aspects of delivered antigen also has a considerable influence on the overall efficacy of any vaccine by directly controlling the
A variety of more or less non-specific factors (Rijkers, 1982) influence the uptake of oral antigens. The overriding factor regulating oral delivery of antigen as vaccine in mammals and teleosts is presumed to be gastric degradation, which differs between species but is considerable in every case (Kraft, Rothberg and Kramer, 1967; McLean, 1987; Ellis, 1988). The importance of gastric secretions in limiting the amount of antigen in contact with the intestine, often beneficially, is exemplified by human patients with the hereditary syndrome, achlorhydria. This results in gastric HCl levels being reduced or even absent, hence causing substandard gastric secretions and as a consequence the formation of allergenic responses to dietary antigens (Kraft et al., 1967). Gastric secretions can also be influenced by a range of factors such as the nutritional status of the organism. During the acquisition of neonatal passive immunity, colostral inhibitors of enterokinase (the enzymatic convertor of trypsinogen to trypsin) mediate an increased lumenal content of intact immunoglobulins. Reduction of intestinal proteolysis by pancreatic enzymes also leads to an increased uptake of macromolecules, viruses, toxins and bacteria (Walker, 1981). Similarly in teleosts gastric and intestinal secretions have been found to greatly influence the level of uptake of bacterial antigens (Johnson and Amend, 1983a, 1983b).

Secretion of mucus provides an important physical barrier against macromolecular uptake, especially against bacterial attachment and the chemical action of toxins (Schreiber and Walker, 1988). An increase in mucus layer thickness slows down antigen absorption and increases the interaction time.
between a delivered antigen load and any lumenal digestive processes. Mucus is chemically complex and contains several substances that can influence absorption. Lysozyme (muramidase) is a bactereolytic enzyme, an obvious factor that will modulate the delivery of live or dead bacteria in a possible oral vaccine. Similarly lactoferrin (an iron sequestering agent) produces a bacteriostatic effect as a consequence of its competition for free iron. Iron is often required by surface receptors (siderophores) which are associated with virulence in some bacterial species eg *Aeromonas* spp (Dooley and Trust, 1988; Bayer, 1990, pers.comm.). Other less well characterised glycoproteins, such as transferrin, are produced which also specifically bind to bacterial toxins (Udall and Walker, 1987).

The indigenous intestinal flora deters overpopulation by pathogens. Both the quality and quantity of the flora is important for the competition of substrates with pathogens and the alteration of the microenvironment to sustain their own survival.

### 2.4. IMMUNOLOGICAL INTERACTIONS OF ABSORBED ANTIGEN WITH GALT.

Antigen delivered to the intestine can be seen to be absorbed and acted on, possibly in an immunologically relevant manner, by enterocytes. Antigen processing and presentation are among the central events involved in the induction of an immune response to certain (thymus-dependent) antigens. (Vallejo *et al.*, 1990). Absorbed orally delivered antigens interact with GALT tissue macrophages in teleosts in a manner analogous to classical antigen-
processing cells (APC) in mammals (Rombout and van den Berg, 1989). Antigen processing can be defined as the conversion of a molecule from a non-MHC binding to a MHC-binding form. The degree of processing required by an antigen is related to its structure (Chain et al., 1988). Antigen processing and presentation can occur prior to antigen interaction with GALT (see above). GALT cells capable of antigen presentation, characterised by MHC II expression, include dendritic cells, veiled cells (Mayer, 1991), macrophages and B-cells (Chesnut and Grey, 1981; Vallejo et al., 1990). Teleost cells, such as activated B-cells, are found to be efficient presenters of antigen in vitro. The initial interaction between antigen and APC is at the cell surface membrane although most antigens require passage through acidified endosomes intracellularly. Endosomes containing antigen rapidly interact with other compartments including the Golgi apparatus and plasma membranes (positive antigen processing at a neutral pH) or lysosomes (degradative processing). The final interaction of antigen with MHC complexes occurs in a trans-Golgi compartment and the complex is expressed at the cell surface. MHC complex expression has been defined in fish (Kaastrup, Stet, Tigchelgaar, Egberts and Van Muiswinkel, 1989) and it appears from such studies that MHC-restricted antigen processing may be necessary for some antigens delivered to GALT cells.

2.5. THE IMMUNE RESPONSE TO ORALLY DELIVERED ANTIGENS.

Immunological factors also have a selective role on the uptake of macromolecules and the potential for the induction of a local immune response is the primary reason for oral vaccination in both mammals and teleosts.
The existence of a protective local immune system functioning independently of systemic immunity was first proposed by Besredka in 1919. In this study it was shown that oral immunisation of rabbits with killed *Shigella flexneri* bacteria (Shiga bacilli) was protective against bacillic dysentery (Besredka, 1927). Similarly Davies (1919) successfully immunised soldiers against *Shigella* spp. and showed serum antibody responses against the bacteria. The local secretory immune system is largely mediated by a distinct subclass of immunoglobulins, in mammals - dimeric IgA (Tomasi, Tan, Solomon and Prenderport, 1965). IgA is also present in the serum but its structure differs as it is monomeric. Other immunoglobulin classes (primarily IgG and IgM) are also present in secretory immune responses but in much lower concentrations, than in the serum. Secretory IgA is heterogenous and usually occurs as a dimer of approximately 380 kDa molecular weight (consisting of a protective secretory component (SC) of 80 kDa and a joining (J) chain of approximately 15 kDa (Brandtzaeg, Baklien, Bjerke, Rognum, Scott and Valnes, 1987, Brandtzaeg et al., 1988, Brandtzaeg, 1990). Secretory IgA is locally-derived with only 2% of intestinal and 50% of biliary IgA being derived, in humans, from the serum (Delaacroix, Dive, Rambaud and Vaerman, 1982; Delacroix, Hodgson, McPherson, Dive and Vaerman, 1982). Other mammals may derive more IgA from the serum than humans (Orlans, Peppard, Payne, Fitzharris, Mullock, Hinton and Hall, 1984; Vaerman and Delacroix, 1984; Ahnen, Brown and Kloppel, 1985; Mestecky and McGhee, 1987). In these species secretory immunoglobulin is produced by plasma cells of the GALT, transported through the enterocytes and secreted into the mucus where it has the potential to cross-link
antigen, agglutinate bacteria, render bacteria mucophilic and neutralize viruses. The *modus operandi* of GALT is much more complex than just secretion of IgA into the mucus lining and lumen (Marsh, 1987). Laminal cells from guinea pigs, for example, orally immunised with a killed preparation of *Salmonella typhimurium* could passively transfer antigen specificity and sensitivity to naive guinea pigs (Owen and Ermak, 1990). Similar results were found with the oral delivery of other antigens such as bovine serum albumin (BSA) in rabbits and hamsters and also sheep red blood cells (SRBC) in mice (Brandtzaeg *et al.*, 1988). Such cells were characterised as antigen specific T-cells of the T-helper/inducer phenotype, which could have been capable of inducing an immunoglobulin or a cellular (cytotoxic T or macrophage) response. Interactions between T-cells and antigen presenting cells (macrophages, dendritic cells and veiled cells) and the secretory epithelium regulate the secretory-component dependent polymeric IgA (pIgA) response to oral antigens (Russel and Mestecky, 1989).

Oral immunisation routes lead to the induction of local and generalised immune responses manifested by the parallel appearance of sIgA to antigen in secretions of glands distant from the site of immunisation (Mestecky, 1987). This is the basis of the proposed common mucosal system, largely recognised in mammals. Table 2.1 shows some examples of specific antibodies secreted at mucosal sites after oral immunisation.
### Table 2.1. Examples of Secretory Antibody Production in Anatomically Remote Secretions After Oral Immunisation

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Delivery Form</th>
<th>Secretion</th>
<th>Comment</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>Live Bacteria</td>
<td>Milk</td>
<td>No serum response</td>
<td>Mestecky (1987)</td>
</tr>
<tr>
<td><em>H. influenzae</em></td>
<td>Killed bacteria plus enteric coat</td>
<td>Saliva</td>
<td>Increase in sIgA</td>
<td>Mestecky (1987)</td>
</tr>
<tr>
<td><em>S. mutans</em></td>
<td>Formalin killed bacteria in capsules</td>
<td>Saliva and tears</td>
<td>Specific antibodies in blood</td>
<td>Curtiss, Goldschmitt, Pastian, Lyons, Michaelke and Mestecky (1986)</td>
</tr>
<tr>
<td><em>S. mutans</em></td>
<td>Glucosyl transferase plus aluminium phosphate</td>
<td>Parotid saliva</td>
<td>Increase in sIgA</td>
<td>Curtiss et al., (1986)</td>
</tr>
</tbody>
</table>
Montgomery, Colin and Lally (1974) found antigen specific antibodies to orally administered dinitrophenol-Pneumococcus spp. conjugates in the milk of lactating mammals and through elegant ligation studies demonstrated that the antigen was not transported via the lymph or bloodstream (Montgomery, Lemaitre-Coehlo and Lally, 1976). Similar results were obtained with certain microbial and dietary antigens (Mestecky, McGhee, Russell, Michaelek, Kutteh, Gregory, Scholler-Guinad, Brown and Crago, 1985). The presence of sIgA in milk was found to be due to an active transport system (Sheldrake, Husband, Watson and Cripps, 1984) where immunocompetent cells seed the mucosal secretory site from the (distant) site of antigen contact.

Peroral immunisation with Salmonella typhi led to the presence of predominantly IgA cells in the peripheral blood (Kantele, Avrilommi and Jokinen, 1986). Locally antigen-sensitized cells (plasma cells or T-cells) may migrate from one mucosal site eg the intestine after oral delivery of antigen to many others urogenital, respiratory mammary and salivary mucosae and may appear in any of the secretions.

Teleosts appear not to have multiple serum isotypes of immunoglobulin (Lobb and Clem, 1981a) and rely on a (usually) tetrameric IgM-like molecule (Hart et al., 1988) which has a species variable molecular weight (Lobb and Clem, 1981d; McCumber et al., 1982; Philips and Ourth, 1986; Hart, 1987; Glynn and Pulsford, 1990). Secretory IgM has been found in gut mucus (Diconza and Halliday, 1971; Doggett, 1989) cutaneous mucus (Lobb and Clem, 1981b; Rombout, Blok, Lamers and Egberts, 1986; Lobb, 1987; Peleteiro and Richards, 1988; Doggett, 1989; Rombout and van den Berg, 1989) and bile (Lobb
and Clem, 1981c; Lamers, 1985; Rombout et al., 1986; Hart, Wrathmell, Harris and Doggett, 1987; Doggett, 1989). It seems that teleosts, like humans and some other mammals, have immunoglobulins that are not derived from the serum (Lobb and Clem, 1981a-d) and that mucosa specific plasma cells are responsible for local IgM production with putative differences in the immunoglobulin heavy and light chains between those immunoglobulins and those in serum. A notoriously poor transport mechanism exists between teleost skin and the bloodstream (Harrell, Etlinger and Hodgins, 1976; Lobb and Clem, 1981b). Secretory component has not been recognised in fish (Hart et al., 1987, 1988) although the concentration of biliary Ig paralleled that of serum raising the possibility of active secretion, and fish Ig has been reported to be able to bind human SC (Underdown and Socken, 1978).

Particulate antigens can induce an immune response at teleost mucosal surfaces (Rombout et al., 1986). Peroral administration of antigen elicited antibody titres at distant mucosal surfaces that while low (Doggett, 1989) may be protective (Rombout and van den Berg, 1989) and indicate the possibility of a functional common mucosal system. Soluble protein antigens had less immunogenic effect in teleosts (see below) though the antigenic nature of the macromolecule may be the decisive factor. Oral delivery of antigen also caused direct cellular migration of granulocytes (Davina et al., 1980), and small macrophages (Rombout and van den Berg, 1989), into the intestine. It was postulated that such highly mobile populations of small macrophages and granulocytes could be a primary factor in the induction of systemic immunity. The efficacy of oral delivery of antigens to fish is also influenced by the state of
ontogenic development, especially in relation to the gut, as although antigen can be absorbed across the intestine of adults the level of absorption may be greater in larval and juvenile fish.

Initial delivery of a bacterial, viral, dietary protein, chemical or cellular antigen to the intestine by the oral route can lead to a marked decrease in the subsequent ability to initiate a systemic immune response to the same antigen even if it is delivered by another route (anally, parenterally, intravenously, intradermally or intramuscularly; MacDonald, 1982; Kagnoff, 1987). Table 2.2 shows some of the possible consequences of oral immunisation.

**TABLE 2.2. POTENTIAL IMMUNE RESPONSES TO ORAL IMMUNISATION.**

<table>
<thead>
<tr>
<th>INDUCTION.</th>
<th>INITIAL RESPONSE.</th>
<th>LEADS TO:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local injection or penetration of antigen.</td>
<td>Local.</td>
<td>Systemic and inflammatory.</td>
</tr>
<tr>
<td>Ingestion or inhalation.</td>
<td>General mucosal and/or secretory.</td>
<td>Systemic unreactivity.</td>
</tr>
</tbody>
</table>

This induction of systemic hyporesponsiveness to an intestinally delivered antigen is termed "oral tolerance" and is well documented in mammals (MacDonald, 1982; Strobel, Mowat, Drummond, Pickering and Ferguson, 1983). Immune tolerance to orally administered protein was first reported by Wells
The complete mechanism of oral tolerance induction has not yet been evaluated, however the presence of immune complexes has been linked to its induction (Kagnoff, 1987) but it is more likely that an antigen specific population of T-cells of the cytotoxic/suppressor phenotype from the intestinal epithelium (see above) are the mediating factors. This population would then migrate systemically lessening any antigen specific response (Bergmann and Waldman, 1988). Induction of oral tolerance in mammals depends on the nature of the antigen, its dose, the frequency and temporal kinetics of its administration and the recipient organisms current immune status (Peng, Turner and Strobel, 1990).

Orally delivered antigens were rendered tolerogenic by the normal physiological action of gastric enzymes (Michael, 1989). Untreated BSA was tolerogenic if orally but not ileally administered. Subsequent in vitro studies indicated that proteolysis degraded the BSA molecules, revealing molecular conformations suited for tolerance induction (Michael, 1989).

Teleost unresponsiveness has been directly linked to the form (particulate vs soluble) and nature (T-dependent vs T-independent) of the antigen. Particulate, T-independent antigens, such as some bacterial antigens (e.g. from Aeromonas salmonicida and Vibrio anguillarum) appear not to be tolerogenic. Route dependent immunosuppression was found with BSA (Serero and Avtalion, 1978) and HGG (Mughal, Farley-Ewens and Manning, 1986) but not with ferritin (Rombout and van den Berg, 1989). High doses of antigen at short intervals were found to be more efficient at tolerance induction but evidence suggests dose-dependent tolerance is not universal in teleosts (Whiskovsky and
Orally delivered antigen has several consequences as described above and a potential vaccine administered via this route must be designed in such a way as to maximise antigen delivery to effector GALT cells, induce a protective secretory immune response (possibly at mucosal sites divorced from that of immunisation) and to minimise systemic hyporesponsivness which often appears to be a consequence of intestinal antigen administration.

2.6. ORAL ANTIGEN DELIVERY SYSTEMS (VACCINES) POTENTIALLY USEFUL FOR THE INDUCTION OF A PROTECTIVE IMMUNE RESPONSE.

An efficient and long lasting topically induced secretory immune response generally requires repeated doses of antigen that may be several orders of magnitude greater than that administered parenterally (Mowat, 1987; Mestecky, 1987). As a consequence a variety of novel delivery systems are being investigated.

2.6.1. LIVE VACCINES.

Live microorganisms that are naturally or artificially non-pathogenic but are immunogenic and can multiply within the intestinal lumen, on enterocytes or within the GALT may provide a more effective stimulus for a secretory immune response than killed formulations (Waldman and Ganguly 1974; Mestecky 1987; Pierce, Kaper, Mekalanos, Cray and Richardson, 1987; Curtiss, 1990).

Inactivated whole cell vaccines of Salmonella spp orally administered are well tolerated but their efficacy could not be demonstrated (Germanier and...
Salmonella spp are organisms that preferentially colonise the Peyers patches. From this it was found that optimal protection was achieved by vaccination with live bacteria attenuated by general chemical mutagenesis or site-directed genetic lesions eg S.typhi strain Ty21a which lacks the enzyme UDP-4-galactose epimerase causing the lipopolysaccharide (LPS) virulence factor to be expressed only under conditions that cause autolysis of the bacteria (Levine,DuPont and Horlick, 1976; Germanier 1984; O’Callaghan, Maskell, Liew, Easmon and Dougan, 1988; Germanier and Cryz, 1989). Such live vaccines have been extremely protective - up to 96% protection in large scale field trials in humans (Wahdan, Serie, Cerisier, Sallam and Germanier, 1982). There remains sufficient scope for study of other genetic modifications as there are approximately 200-500 gene products involved in oral infection with S.typhi (Curtiss, 1990). Other attenuated S.typhi strains were derived via a series of genetic mutations : colE mutants (requiring exogenous galactose), purA gene mutants (effecting the synthesis of purines) and aroA gene mutants (effecting the synthesis of the shikimate pathway enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSP) which is required to synthesise aromatic amino acids (tyrosine, phenylalanine and tryptophan) and eventually folic acid and vitamin K. However derivatives of aroA and purA mutant strains failed to produce significant antibody titres though positive cell-mediated responses were elicited (Hackett, 1990). Similarly modifications in S.typhimurium have produced immunogenic mutants designated delta-crp (lacking the cAMP receptor protein) and delta-cya (lacking adenylate cyclase), respectively (Curtiss, 1990). Temperature-sensitive S.typhi mutants, with some of the above genetic
lesions, have been found to be especially useful in the induction of secretory immunity, especially for antigens that are only expressed *in vivo* (Hooke, Wang, Cerquetti and Bellanti, 1991).

Other bacteria have been studied for the production of live vaccines that are non-pathogenic and self-limiting via genetic (or chemical lesions). These include *Vibrio cholerae* where the production of a live vaccine could imitate a natural infection (which confers a high degree of protection against subsequent reinfection) and through a single dose protect susceptible age classes. Many pathogens such as *V. cholerae* are capable of (plasmid borne) genetic exchange, so putative vaccine strains must be rendered combination deficient so that reversions to wild-type through conjugation or transduction is less likely eg through lesions in toxin structural genes (*ctxA* or *ctxB*), *Zot*-gene the products of which alter the integrity of the enterocyte tight-junctions and regulatory genes (Kaper, Lockman, Baldini and Levine, 1984) including *tox R*, *tox S* and *tox T* genes (Curtiss, 1990). Similar strategies have been employed in other mammalian bacterial pathogens eg *Shigella* spp using non-invasive strains (Formal, Hale and Kaper, 1989; Linde, Dentschev, Bondarenko, Marinova, Randhagen and Bratoyeva, 1990) and *E. coli* strains that cause enterotoxigenic and enteroinvasive disease (ETEC and EIEC) where oral immunisation is found to be highly protective against intraintestinal challenge (Sack, Kline and Spira, 1988).

Studies on teleost pathogens have included the production of attenuated strains of *Vibrio anguillarum* made avirulent by transposon mutagenesis (Norquist, Hagstrom and Wolf-Watz, 1989). Such vaccines caused a 10 to 30 fold increase in the LD50 values in pathogenic challenge regimes. Protection was
elicited one week after immunisation and lasted up to 12 weeks with a limited associated antibody response (Norquist et al., 1989). AroA mutants of Aeromonas salmonicida have been constructed (Vaughan, Smith and Foster, 1990) and these appear to provide protection combined with avirulence when administered via the peritoneal route but have not been orally evaluated. Other live vaccines of Vibrio anguillarum, Aeromonas hydrophila, Cytophaga spp and Pseudomonas spp have also been recently patented but their efficacy remains unknown (Symbicom Corporation, 1989).

2.6.2. BACTERIA AS LIVE DELIVERY VEHICLES.

Organisms that colonise the intestinal tract or GALT such as Salmonella spp or Shigella spp make good candidates for genetic manipulation so that they can act as vehicles to deliver other antigens (Curtiss, 1990) from less-tractable bacteria, that are required for protective immunity.

Salmonella spp is often the chosen delivery organism and antigens can be introduced into it by cloning techniques (Hosieth and Stocker, 1981). The success of the hybrid ie containing cloned, heterologous, DNA depends on the stability of the introduced DNA, the level of expression of the product and its location in the carrier organism, the selection of the carrier strain eliciting the immune response (eg Ty21a has problems associated with its overattenuation so that it may only be moderately immunogenic in some instances) and the choice and inherent immunogenicity of the introduced antigen (Hackett, 1990).

Foreign antigenic sequences can often be inserted into a region that expresses a surface determinant eg into the lamB region in E.coli (Leclerc, Charbit, Molla, Cholla and Hofnung, 1989). Protein antigens from
Streptococcus mutans (glucosyl transferase and surface A protein) have been introduced into Salmonella typhimurium as recombinant plasmids (Katz, Michaelek, Curtiss, Harmon, Richardson and Mestecky, 1988). Orally delivered it induced a secretory IgA response in saliva (Mestecky, 1987). Rationally attenuated live vaccines can also be used to deliver recombinant bacterial toxins eg V.cholerae B-subunit or ETEC heat-labile toxin (LT). Triple hybrid (recombinant) vaccines have been proposed for Salmonella typhi, cholera and ETEC, using Ty21a as the carrier (Chatfield, Strugnell and Dougan, 1989; Clemens, Harris, Kay, Chakraborty, Sack, Ansaruzzaman, Rahman, Stanton, Khan, Khan, Yunus, Rao, Ciznar, Svennerholm and Holmgren, 1989). The choice of carrier organism throughout such studies is vital, to maximise immunogenicity and decrease the dose of vaccine eg the potential use of E.coli K12, Shigella flexneri, Listeria monocytogenes or Vaccinia virus (Linde, Abraham and Beer, 1991).

An important adjunct to the use of live expression vectors for the delivery of foreign antigens is the potential development of a foreign antigen secretion system in Mycobacterium kansasii (with a view to similar development in Mycobacterium bovis). Such a system would allow the delivery of antigen(s) in a vehicle that naturally resides intracellularly in macrophages and hence the possibility that the antigens could be processed and presented on the cell surface and continuously stimulate a relevant immune response (Matsuo, Yamaguchi, Yamazaki, Tasaka, Terasaka, Totsuka, Kobayashi, Yukitake and Yamada, 1990).

A novel antigen delivery method involves the use of "bacterial ghosts" which are produced by the genetically controlled release of the internal contents of a bacterium without the destruction of the bacterial sacculus. This system
allows delivery of intrinsic or cloned colonisation or virulence antigens that are present in the outer membrane (Curtiss, 1990).

Protective antigens, outside novel live regimes, are obvious candidates for oral vaccines and may be derived by traditional or cloning methods.

An oral vaccine to cholera is possible as its pathogenesis is more readily understood than any other organism (Holmgren et al., 1989). Cholera toxin consists of a binding region of five β-subunits into which is inserted the toxic active component, the α-subunit. The toxin binds to the GM₁ ganglioside on the brush borders of enterocytes. Pathogenesis occurs when the α-toxin subunit activates adenylate cyclase and intracellular cAMP levels rise.

β-subunit is widely used as a targeting agent for conjugated antigen and as a protective immunogen. The most advanced oral vaccine for cholera consists of whole formalin and heat-killed cells (Inaba, Ogawa and El Tor biovars) plus β-subunit. These antigens act synergistically to give a protective antibody response to toxin and structural protective antigens such as LPS, fimbrial adhesins and colonization factors (Holmgren et al., 1989; Ghose and Dasgupta, 1989; Srivastava, Srivastava and Jacob, 1989). Cholera β-subunit is also protective against organisms that have similar toxins and invasive strategies such as ETEC (Svennerholm, Holmgren and Sack, 1989; Svennerholm, Holmgren, Lopez-Vidal, Sanchez and Ahren, 1989) and Aeromonas spp (Ciznar, Hussain, Ahsan, Kay, Clemens and Sack, 1989). Vaccines using cholera β-subunit as an immunogen or a targeting agent have not, until now, been employed in teleosts but are likely to provide an efficient mechanism of antigen introduction and also possibly a protective immunogen against teleost Aeromonads.
The identification of the protective antigens for a teleost bacterial vaccine is a prerequisite. For *Aeromonas salmonicida*, the aetiological agent of furunculosis, a wide variety of antigens have been outlined as possibly protective and/or as being virulence factors. Such as A layer, LPS, crude extracellular products (ECP), toxoids, cell extracts, proteases and glycerophospholipid cholesterol acyl transferase ((GCAT); Ellis, 1988; Ellis, 1990) and salmolsin (Kawahara, Ueda and Nomura, 1991). Other bacterial species have their distinct antigens such as the aerolysin (siderophore) and ECP of *Aeromonas hydrophila* (Rodriguez, Fernandez and Nieto, 1990; Karunasagor, Rosalind and Karunasagor, 1990), O-antigen, LPS and heat-sensitive factor (HSF) of *Yersinia ruckeri* (Furones-Nozal, 1990). Fish bacterial vaccines are mostly restricted to heat or formalin killed products administered by routes other than perorally (Klontz and Anderson, 1970; Ellis, 1988) as no commercially successful oral administration regimes are used as yet (Campbell, Tatner and Adams, 1990). Such procedures are often unprotective for more intractable diseases such as *Renibacterium salmoninarum* (Sakai, Atsuta and Kobayashi, 1989a; Ellis, 1981, 1988) and β-haemolytic disease from *Streptococcus iniae* (Sakai, Atsuta and Kobayashi, 1989b). However, delivery of certain antigens orally to teleosts can elicit protection (Duff, 1942). Formalin killed *Vibrio anguillarum*, orally given in experimental field trials, was found to significantly reduce the mortality of ayu *Plecoglossus altivelis* with a 32% mortality in vaccinated groups as opposed to 52-61% in control groups (Kusuda, Kawai, Jo, Akizuki, Fukunaga and Kotake, 1978; Kawai and Kasuda, 1985). Oral delivery required a greater dose of bacterin but, unlike immersion methods with the same antigen
Kitao, 1990), elicited a protective, bacteriostatic, mucosal immune response. A similar result was found in carp, *Carassius auratus* with *V. anguillarum* (Rombout, van den Berg, van den Berg, Witte and Egberts, 1989). Similarly orally and anally administered *Yersinia ruckeri* elicited protection under challenge stimulus in rainbow trout *Oncorhynchus mykiss* (Johnson and Amend, 1983a; 1983b). Protection after orally delivered *A. salmonicida* ECP or ECP-toxoid has been reported (Rodgers and Austin, 1985). Chinook salmon *Oncorhynchus kisutch* can be orally immunised against *Vibrio* but have little specific serum antibody (Fryer, Rohovec, Tebbit, McMichael and Pilcher, 1976; Fryer, Amend, Harrell, Novotony, Plumb, Rohovec and Tebbit, 1977). Protection can be elicited by direct oral vaccination with derived bacterial products in teleosts and can be both rapid and long lasting (Kawai and Kasuda, 1985).

### 2.6.3. SYNTHETIC PEPTIDES.

The synthesis of peptides, corresponding in sequence to the primary structure of certain antigenic regions of a pathogen represents another way of constructing non-infectious surrogate vaccines (Lerner, 1982; Salk and Zanetti, 1989). Synthetic peptides can be constructed from primary amino acid sequences derived from nucleotide sequences. Synthetic peptides can be made that address different subpopulations of leucocytes via unique topographical conformations (Sercarz, 1989). There are usually minimal requirements for peptide antigens to elicit an immune response (Goodman-Smitkoff, Eisele, Heimer, Felix, Andersen, Fuerst and Mannino, 1990). Peptides can
be synthesized that are recognized by T-cells (Tₘ epitopes) or B-cells and trigger both protective immunity and immunological memory and also minimise conformations that are likely to induce T-suppressor cell activity ie factors that minimise oral tolerance. Synthetic peptide efficacy as an oral vaccine is especially dependent on the form of the presented antigen.

The immunomodulatory synthetic peptide K565 (Mine, Watanabe, Tawara, Yokata, Nishida and Goto, 1983; Kitao, Yoshida, Anderson, Dixon and Blanch, 1987) can specifically boost both B-cell responses (to antigen) and activate macrophages in mammals and fish. Levels of serum antibody and numbers of splenic antibody-forming cells were elevated against O-antigen preparations of *A. salmonicida* and *Y. ruckeri*, by increasing antigen processing and B-cell expression (Kitao et al., 1987).

Antigenic determinants of immunoglobulins can substitute for protein or carbohydrate antigens and elicit an immune response against these antigens (Lindeman 1973; Jerne, 1974, 1984; Salk and Zanetti, 1989). Such anti-idiotypic vaccines rely on the fact that antibody specificity is such that an immunoglobulin binding domain can act as a partial template for an antigen, as its mirror-image. A few potential vaccine studies using anti-idiotypes have been carried out in mammals but none in fish.

2.7. NOVEL DELIVERY SYSTEMS.

One method of increasing the amount of orally delivered antigen that reaches the mucosal lymphoid tissue is to provide it with direct physical protection from gastric degradation in a specialised capsule or coating.

Such a strategy involves the use of liposomes (Gregoriadis, 1990).
Liposomes are fluid-filled spheres formed spontaneously by the presence of amphipathic phospholipids, such as lecithin, in water (Bangham and Horne, 1962; Ostro, 1988). Liposomes represent an unbroken bimolecular sheet of lipids. Any antigens added to the formation solution will be enclosed in the aqueous spaces (if soluble) or in the phospholipid matrix. Liposomes range from 25 nm to 10 μm in diameter and may be multilayered (multilamellar vesicles; MLV's), single-layered (small or large unilamellar vesicles; SUV's or LUV's), dehydration-rehydration vesicles (DRV's) or reverse phase evaporation vesicles (REV's) (New, 1990). Uptake of liposomes is affected by their charge, size and degree of lipid unsaturation. Opsonization with IgG or IgM (Senior, 1987), greater negative charge and the presence of sterols can all increase liposome stability to gastric enzymes and uptake (Hsu and Juliano, 1982).

Liposomes can adsorb to almost all cell types (Ostro, 1988) and the phospholipid matrix may exchange lipid components with cell membranes and/or fuse with them (Gregoriadis, 1990). Delivered liposomes have a propensity for ingestion by macrophages (Alving, 1989) and from this an almost direct immunological interaction can be envisaged. Endocytosis can cause the direct delivery of antigen and lysosomal catabolism of liposomes can be circumvented by pH-sensitive vesicles that degrade in the endosomal acidic milieu and are released to the cytoplasm prior to lysosomal fusion (Gregoriadis, 1990). Liposomes have also been found to provide T-dependent help to weak antigens (such as some synthetic peptides) when rationally constructed complementary to the intrinsic nature of the delivered protein (Garcon and Six, 1991).
In mammals a wide variety of antigens have been included in liposomes for immunisation regimes (Alving, 1991) although there have been few studies in fish. Intraperitoneal and immersion delivery of antigens in liposomes in fish have largely concentrated on isolating the tissues involved in their sequestration (Rodgers and Austin, 1985; Rodgers, 1990). Table 2.3 shows some of the antigens studied in immunisation protocols using liposomes.
### TABLE 2.3. ANTIGENS DELIVERED IN LIPOSOMES TO INDUCE IMMUNITY.

<table>
<thead>
<tr>
<th>ANTIGEN SOURCE</th>
<th>SPECIFIC ANTIGEN</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Streptococcus pneumoniae</em> type 3</td>
<td>Wachsmann et al., 1989.</td>
</tr>
<tr>
<td></td>
<td><em>Streptococcus sobrinus</em> ribosomal protein</td>
<td>Wachsmann et al., 1989.</td>
</tr>
<tr>
<td></td>
<td>Gonococcal protein</td>
<td>Kersten et al., 1988a, 1988b.</td>
</tr>
<tr>
<td>BACTERIAL TOXIN</td>
<td>Cholera toxin</td>
<td>Pierce, Sacci, Alving and Richardson, 1984.</td>
</tr>
<tr>
<td>ANTIGEN SOURCE</td>
<td>SPECIFIC ANTIGEN</td>
<td>REFERENCE</td>
</tr>
<tr>
<td>---------------------</td>
<td>---------------------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>PROTEIN ANTIGENS</td>
<td>Horseradish peroxidase</td>
<td>Therian and Shahum, 1989.</td>
</tr>
<tr>
<td></td>
<td>Lysozyme</td>
<td>Gregoriadis, 1990.</td>
</tr>
</tbody>
</table>

Orally delivered liposomal antigens retain their biological activity eg both delivered glucose oxidase and insulin reduced blood sugar levels (Gregoriadis, 1976). Liposomes reduce the number and size of oral doses to elicit an immune response and direct interaction with enterocytes has been evaluated in vitro. Certain virulence factors introduced into liposomes, such as *V. cholerae* toxin, could both enhance the intestinal targeting of the vaccine and act as adjuvants (Pierce *et al.*, 1984; Alving, Richards and Moss, 1986; Chiacumpo and Pareiro, 1988).

The immune response to liposomal delivered antigen is increased with respect to free antigen and the physical association between the antigen and the phospholipid matrix is an integral cause of this (Therian and Shahum, 1988; Gregoriadis, 1990). The provision of a critical epitope density on the hydrophobic surface of the liposome is thought to be an important factor (Genco, Linzer and Evans, 1983). Drugs can be targeted to infected tissue and the release of vaccine antigen can be "pulsed" allowing a strong immune
response. Antibody levels can be increased up to 1000-fold (Ostro, 1988) because of the ability of liposomes to function as an antigen-depot for gradual, "pulsed", release of antigen to recipient lymphoid tissue. Liposomal ability to elicit a cell-mediated response lies in the degree of hydrophobicity of the matrix which influences antigen presentation to T-cells (Senior, 1987; New, 1990).

Liposomes, as yet, remain unused as oral delivery systems in fish. Parenteral administration of liposomes in rainbow trout showed that they targeted for similar cells and in a similar manner to mammals, with the spleen, anterior and posterior kidney being the primary sites of localisation (Power, Markham and Donald, 1990). A liposomal preparation of *A. salmonicida* antigen has also been studied by immersion (Rodgers, 1990) where a liposomally-encapsulated trivalent vaccine preparation of *Aeromonas salmonicida* was found to induce a greater degree of protective immunity (as evidenced by subsequent challenge) than the same, unencapsulated, preparation.

Other methods of protection against digestive degradation have been utilised. One is enteric coating which in its most common form involves the encapsulation of an antigen in a gelatin capsule and the subsequent spray-coating of the capsule with a substance such as hydroxyprophy-methyl-cellulose-phthalate or methyacrylic acid plus acrylic acid ethyl ester both of which are resistant to pH 1.5 for up to 2 hours but degrade at pH >6 in 10 minutes (Lehman and Dreher, 1981; Black, Levine, Ferreccio, Clements, Lanata, Rooney and Germanier, 1990; Lillehaug, 1989, 1990). This results in protection of antigen until its release in the intestine. This approach has been utilised in the oral delivery
of *Salmonella typhi* Ty21A in mammals where enteric coating of the live vaccine was found to increase protection to challenge with virulent strains (Levine, Ferrecio and Black, 1987; Black *et al.*, 1990; Forrest, Shearman and Labrooy, 1990). Other biodegradable microparticles have been investigated as controlled-release antigen delivery systems (O’Hagan, 1989; O’Hagan, Palin and Davis, 1989; O’Hagan, Rahman, McGee, Jeffery, Davies, Williams, Davis and Challacombe, 1991). These include the use of 6-7 µm PLGA (D,L,-lactide-co-glycolide) microparticles which can, reputedly, induce a humoral immune response of a similar magnitude to that obtained with the administration of antigen in Freund’s adjuvant (O’Hagan *et al.*, 1991) and the eventual production of nontoxic breakdown products (lactic and glycolic acids).

In fish, enteric coating has been used to test the oral administration of *V. anguillarum* bacterin to rainbow trout. Challenge regimes however resulted in less protection using this method than unprotected antigen (Lillehaug, 1989, 1990). The lack of effect of enteric coating may be due to the possible damage of protective antigens during encapsulation, the coating causing blockage of LPS-associated antigen or the requirement for gastric action on the antigen to reveal epitopes that are immunogenic (Lillehaug, 1989, 1990). In the same study another, related, protective delivery system was used. This was the incorporation of the same antigen preparation of *V. anguillarum* into a matrix of heavily degraded fatty acids - a "prill" (Baalsrud and Velle, 1974). "Prills" act by encompassing the delivered antigen in a matrix that is slowly broken down in the alimentary tract and appeared to allow greater uptake of antigen, as measured by greater protection, although were less protective than unprotected antigen.
Direct physical protection of vaccine can be seen to increase antigen uptake and immune response in some cases. Oral vaccine delivery, of all forms - live, dead, toxin, subunit and protected - is usually carried out in the presence of an immunomodulatory compound or adjuvant.

One method of antigen delivery, exclusively attempted in fish, is the use of bacterial antigens incorporated into a live zooplankton feed. Zooplankton *Branchionus plicatilis* were used to incorporate antigens from heat and formalin killed *V.anguillarum* and subsequently fed to ayu (Kawai, Yamamoto and Kusuda, 1989). These zooplankton would only incorporate certain antigens such as LPS but were found to elicit protection. On challenge, 92.4% and 64.2% of the vaccinated and unvaccinated ayu were protected, respectively. Such a method of vaccination may prove useful for certain antigens in certain fish.

2.8. THE USE OF ADJUVANTS IN VACCINATION.

The term adjuvant is used almost exclusively in current texts and its use is often ambiguous as it usually encompasses substances that can modify the immune response to an antigen when given with that antigen (the classical definition of an adjuvant) but can also apply to those substances that can modify the immune response independently of its action on the antigen (previously termed immunostimulators). The term immunostimulator or immunopotentiator is still applied to some substances that can increase non-specific defence mechanisms (Ellis, 1988). However due to the multiplicity of action of adjuvants (see below) "immunostimulator" is less fashionable, as most adjuvants, indirectly, act in this manner.

Adjuvants were first used in 1925 by Ramon who showed it was possible
to specifically increase the serum antibody response to diphtheria and tetanus toxins by the addition of unrelated substances, such as bread crumbs, tapioca, starch, lecithin and saponins, to the vaccine. Since then adjuvant development has largely been empirical (Warren et al., 1986) and an enormous number of very diverse compounds have been found to modulate the immune response in a wide range of species.

Adjuvants may be classified by a variety of means such as their mode of action on the delivered antigen (acting as an antigen repository, directly on antigen delivery or on recipient organism cells such as lymphocytes and macrophages) or their source (e.g., mineral, bacterial, plant, synthetic or host). The latter method of classification is useful as most antigens act by more than one mechanism but can usually be assigned by their origins (Warren et al., 1986). Table 2.4 shows one classification of the major adjuvants (Jolivet, 1989).
### TABLE 2.4. CLASSIFICATION OF THE PRINCIPAL ADJUVANTS.

<table>
<thead>
<tr>
<th>MINERALS</th>
<th>Aluminium hydroxide.</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Aluminum phosphate.</td>
</tr>
<tr>
<td></td>
<td>Calcium phosphate.</td>
</tr>
<tr>
<td></td>
<td>Fluoride.</td>
</tr>
<tr>
<td><strong>SURFACTANTS AND</strong></td>
<td><strong>Emulsions of oil in water.</strong></td>
</tr>
<tr>
<td><strong>HYDROPHOBIC SUBSTANCES</strong></td>
<td><strong>Emulsions of oil in water.</strong></td>
</tr>
<tr>
<td></td>
<td>RIBI formulation (monophosphoryl lipid A + trehalose dimycolate).</td>
</tr>
<tr>
<td></td>
<td>Adjuvant 65 (a mixture of Arlacel, oleic- mannide and aluminium monoesterate).</td>
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<tr>
<td></td>
<td>Squalene.</td>
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<tr>
<td></td>
<td>Liposomes.</td>
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<tr>
<td></td>
<td>Block polymers.</td>
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<tr>
<td></td>
<td>Saponins.</td>
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<tr>
<td></td>
<td>ISCOMS</td>
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<tr>
<td>SYNTHEtic Polynucleotides</td>
<td>Oligodeoxyribonucleotides.</td>
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<td>---------------------------</td>
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<tr>
<td></td>
<td>Polyribonucleotides.</td>
</tr>
<tr>
<td><strong>BACTERIA.</strong></td>
<td><strong>Mycobacteria</strong> ( (Mycobacterium) ) ( tuberculosis ) and ( M. b o v i s ).</td>
</tr>
<tr>
<td></td>
<td>Propionibacteriaceae.</td>
</tr>
<tr>
<td></td>
<td>Parvo and Enterobacteriaceae.</td>
</tr>
<tr>
<td><strong>BACTERIAL DERIVATIVES.</strong></td>
<td>Wax D.</td>
</tr>
<tr>
<td></td>
<td>WSA (soluble).</td>
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<tr>
<td></td>
<td>Cord factor (a mixture of trehalose and mycolin).</td>
</tr>
<tr>
<td></td>
<td>MDP and derivatives.</td>
</tr>
<tr>
<td></td>
<td>LPS.</td>
</tr>
<tr>
<td></td>
<td>Outer membrane proteins (such as TraT and Omp).</td>
</tr>
<tr>
<td>CLASSIFICATION OF PRINCIPAL ADJUVANTS.</td>
<td></td>
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<tr>
<td>-----------------------------------------</td>
<td></td>
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<tr>
<td>SYNTHETIC MOLECULES.</td>
<td></td>
</tr>
<tr>
<td>Imuthiol.</td>
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<tr>
<td>Oxaamisole.</td>
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<tr>
<td>Isoprinosine.</td>
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<tr>
<td>Levamisole.</td>
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<tr>
<td>Thiazalobenzimidazole.</td>
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<tr>
<td>Avridine.</td>
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<tr>
<td>DDA.</td>
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<tr>
<td>VITAMINS:</td>
<td></td>
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<tr>
<td>Vitamin A.</td>
<td></td>
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<tr>
<td>Retinol palmitate.</td>
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<tr>
<td>Retinoic acid.</td>
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<tr>
<td>CYTOKINES:</td>
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<tr>
<td>Interferons.</td>
<td></td>
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<tr>
<td>Interleukins.</td>
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</tbody>
</table>

2.8.1. NON-BACTERIAL ADJUVANTS:

The most widely used and the only currently commercially available adjuvant in humans is aluminium hydroxide (AHOH3,Klausner,1988). The first reported use of aluminium compounds as adjuvants was in 1926 (Glenny,Pope,Waddington and Wallace). In this study
administration of AlOH₃ plus diphtheria toxoid elicited significantly higher antibody titres than diphtheria toxoid on its own. Other substances used were cerium nitrate, colloidal iron hydroxide, zinc sulphate and calcium chloride. The main action of AlOH₃ is to act as an antigen repository ("depot") allowing a slow release of antigen which behaves as a secondary stimulus to previously released antigen (Warren and Chedid, 1988). AlOH₃ prolongs lymphocyte circulation in lymph nodes thus increasing the amount of contact between the antigen and lymphocytes (Edelman, 1980). These classes of compounds are relatively weak adjuvants, increasing the humoral immune response slightly and directly activating the complement pathway but having little effect on the cellular immune responses. Even these may be associated with local granuloma in tissues if injected or if sufficient laminal localisation occurs after oral delivery. Most of the above data is derived from mammalian studies (Warren and Chedid, 1988). In fish AlOH₃ has no adjuvant effect on injection in salmonids with keyhole-limpet haemocyanin-dinitrophenol (KLH-DNP) as an antigen, with the inducible serum antibody titres being decreased in some instances (Cossarini-Dunier, 1985). Similar compounds such as alum (potassium aluminium hydroxide) also offer no adjuvant effect in salmonid immunisation to *Vibrio* vaccine (Horne, Roberts, Tatner and Ward, 1984). Oral administration of *Vibrio* vaccine with alum caused increased protection to subsequent challenge but took longer to develop and was less effective than injected vaccine without adjuvant (Agius, Horne and Ward, 1983).

Administration of sodium fluoride (widely present in drinking water sources) is also reported to have a considerable immunopotentiating effect in
mammals, when delivered intragastrically (Butler, Satam and Ekstrand, 1990).

Synthetic chemical polymers have been produced in order to attempt to improve the immune response to antigens. Non-ionic polymers act as surfactants (surface-acting agents with or without an antigen repository function) and host responses to such compounds were dependent on both the local presentation of the antigen and its concentration. Oral administration of BSA was enhanced most efficiently by polyol compounds with a hydrophobic central region flanked by two hydrophilic regions (Warren et al., 1986). Copolymers of hydrophilic polyoxyethylene and hydrophobic polyoxy propylene in different proportions promoted an antibody response to BSA (Hunter, Strickland and Kedzy, 1981; Hunter and Bennett, 1984) possibly mimicking the effect of oil droplets (see below). Other synthetic substances such as aliphatic amines can be utilised as adjuvants (Dalsgaard, 1987) with C16 hydrocarbons such as dimethyldioadecylamine (DDA) and N,N-dioctadecyl-N,N-bis (2-hydroxyethyl) pronaediamine (Avridine) being the least toxic and most effective. Such compounds have only been tested in mammals and have been found to activate macrophages and cause interleukin-1 release, stimulating the cell-mediated and humoral immune mechanisms (Klausner, 1988). Similar substances are present in fish and therefore aliphatic amines may eventually be considered as practical adjuvants. Synthetic substances under consideration (in mammalian systems) include the polyanionic synthetic double-stranded polyribonucleotide complexes along with polyacrylic acid, dextran, bentonite and naturally occurring double-stranded RNA (Warren and Chedid 1988; Klausner, 1988) all of which act on receptor systems by antigen presentation and/or release mechanisms and act as
specific B-cell mitogens (Genco et al., 1983).

Liposomes have been examined as delivery systems in their own right, but it should be mentioned that part of their phospholipid structure, if absorbed intact can act as an adjuvant and can decrease the thymic dependency of an antigen where applicable (Yasuda, Dancey and Kinsky, 1977; Yasuda, Kanegasaki, Tsumita, Tadakuma, Homma, Inage, Kusumoto and Shiba, 1982; Taubman, Ebersole, Smith and Stack, 1984). Liposomes can act as efficient adjuvants for carbohydrate antigens and therefore may be useful in vaccines utilising bacterial capsules such as Meningococcal vaccine. The essential hydrophobicity of the liposome phospholipid may be a controlling influence on its adjuvanticity to a particular antigen. Liposomes have adjuvant action by either direct action on the immune system or by presenting the relevant epitopes more efficiently to the immunocompetent cells (Garcon and Six, 1991).

One obvious method of potentially increasing immunological responses to orally delivered antigen is by the use of substances that neutralize gastric acidity or peptic activity such as sodium bicarbonate, pepstatin or carbonic anhydrase (McLean, 1987). Sodium bicarbonate is used as both a solution and tablet prior to, or concurrently with, oral cholera vaccination in large scale field trials as cholera toxin is known to be strictly pH sensitive (Clemens, Jetborn, Sack, Stanton, Holmgren, Khan and Huda, 1986). Similarly, the addition of proteolytic inhibitors such as aprotinin (affects chymotrypsin) and soy-bean trypsin inhibitor (SBTI; affecting trypsin) can increase uptake of BSA in new-born rabbits (Udall et al., 1984) and other mammals. Administration of SBTI to rainbow trout was found to enhance the mean tissue accumulation of
HRP by 55-220% in the liver and kidney but did not influence its plasma levels (McLean, 1987) in contrast to mammals where aprotinin caused a 60% increase in plasma-borne antigen (Seifert, Sass and Dreyer, 1984). Such adjuvants seen to be effective solely due to their power to reduce the gastric degradation of antigen and some studies have suggested that increased protection to bacterial challenge after anal administration of antigen (thus bypassing the major degradative routes) is a direct result of greater antigen dose across the intestine (Johnson and Amend, 1983a; 1983b) rather than any modifications on the antigen or recipient tissue.

Adjuvants that affect the pinocytotic, endocytotic or lysosomal-degradative processes are also theoretically capable of acting as adjuvants to certain antigens. However the antibiotic monensin was found to be ineffective as an adjuvant for the increase of HRP in rainbow trout after oral or anal delivery (McLean, 1987). Chloroquine and ammonium chloride are also both lysomotrophic agents and deserve further study as adjuvants (Bland and Whiting, 1990).

One of the most studied classes of adjuvants are the detergents (Edelmann, 1980; Dalsgaard, 1987). There are an enormous variety that have been tested eg the polyoxyethylene sorbitan monolaurate (Tween) class of substances (Warren and Chedid, 1988). One of the major groups recognised as potentially powerful adjuvants are the saponins and they are already in use as adjuvants for veterinary vaccines against equine influenza and feline leukaemia virus. Saponins are a naturally occurring and widespread group and, though not fully defined chemically, consist of glycosides mainly a triterpenoid aglycan in
glycosidic linkage with several naturally occurring steroids (Dalsgaard, 1978, 1987; Campbell and Bede, 1989). The most studied of these is a purified saponin extract of *Quillaja saponaria* [Molina] or Quil-A the separation and characterization of the active components of which is complex, resulting in the delineation of several immunologically active components (Kensil, Patel, Lennick and Marciani, 1991). Quil-A acts as a surfactant, directly binding to surface cholesterol and causing several membrane effects including pore formation (Dalsgaard, 1987; Bomford, 1980, 1982, 1988). It also has several other effects, acting as an antigen repository, a B-cell mitogen and a non-specific stimulator of macrophage activity and interleukin release (Campbell and Bede, 1989). In mammals Quil-A increased the cell-mediated and plaque-forming cell (PFC) responses to T-dependent and T-independent antigens (Bomford, 1980; Flebbe and Braley-Mullen, 1986). Quil-A, whilst being a B-cell mitogen, cannot induce an antibody response in conditions where one would not already occur and as such differs in its mode of action from bacterial LPS. Potentiation of a mammalian oral vaccine to rabies virus, by Quil-A, resulted in strong antibody response and protection from intercerebral challenge from live virus (Maharaj, Froh and Campbell, 1986). Injection studies with saponins in fish showed no adjuvanticity (Horne et al., 1984; Cossarini-Dunier, 1985) and immersion studies in rainbow trout also showed no increase in protection but an increase in the non-specific mechanisms involved in the bacterial clearance of *Yersinia ruckeri* (Grayson, Williams, Wrathmell, Munn and Harris, 1987). Saponins can also be induced to form three-dimensional structures due to their physical nature (micelles and immune-stimulatory
complexes, ISCOMS) that may give a novel aspect to their adjuvanticity (Morein, Sundquist, Hoglund, Dalsgaard and Osterhaus, 1984; Morein and Simon, 1985; Morein, Lovgren, Hoglund and Sundquist, 1987) and act to induce an immune response that would otherwise be unobtainable often by overcoming specific antigen tolerance (Mowat, Donachie, Reid and Jarrett, 1991). ISCOMS have been used in several studies in mammals for the delivery of viral proteins but can also be produced with the incorporation of standard proteins (Mowat et al., 1991) and proteins derived from bacterial pathogens (Kersten et al., 1988a, 1988b). Other detergents have been used as adjuvants for orally delivered antigens in fish such as Mega 9 (McLean, 1987). This detergent enhanced the uptake of orally and anally administered antigen, but had a 2-4 times greater effect anally. Mega 9 had its effect by gelatinising the mucus layer of the intestine, thinning or removing it altogether, and allowing a greater, direct, interaction of the antigen with the enterocytes surface as well as possibly other surfactant attributes.

Recent attention has been focused on the use of vitamins as potential adjuvants (Friedman, 1991). Water-miscible vitamin A, and several of its bioactive components, were found to act as extrinsic adjuvants ie. they induced an immune response to ovalbumin without being immunogenic itself, of a similar level to that obtained with the delivery of ovalbumin with MDP (Friedman, 1991).

These adjuvants are derived from many different sources and many are in current use as experimental vaccine adjuvants.
2.8.2. BACTERIAL ADJUVANTS.

The most widely used adjuvants in test systems are bacterial in nature - Freunds Complete Adjuvant (FCA). This adjuvant is largely restricted to use with injected antigens but is mentioned due to its importance and because its use has motivated research into the active components which can be used orally and oil-droplet based adjuvants which still remains the most potent method of enhancing administration (Freund, 1956; Taubman et al., 1984). The active components of FCA are a mineral oil and a *Mycobacterium tuberculosis* strain which in combination are very potent stimulating both the cellular and humoral immune systems but are, however, very toxic (Freunds Incomplete Adjuvant, FIA lacks the bacterium and is often used as a booster adjuvant but remains potent; Warren et al., 1986; Warren and Chedid, 1988). It acts on antigen by its presentation in an organised manner, exposed on the surface of microdroplets of the mineral oil thus facilitating its interaction with, already stimulated, immunocompetent cells (Dalsgaard, 1987), but has also been reported to confer protection against subsequent challenge non-specifically in fish (Olivier, Evelyn and Lallier, 1985). The active component of such mixtures is one of the smallest bioactive peptides known; consisting of two peptides flanked by one sugar residue, \( \text{-N-acetylmuramyl-L-alanyl-D-isoglutamine} \) (muramyl dipeptide, MDP; Ellouz, Adam, Ciorbaru and Lederer, 1974). MDP retains its adjuvanticity when delivered by the oral route, especially with T-dependent antigens (Mestecky, McGhee, Arnold, Michael, Prince and Babb, 1978; Smith, Taubman and Ebersole, 1979). Oral delivery of MDP with antigens enhanced the secretory immune responses from increased antibody
titres to the induction of isotype specific T-suppressor cells (Langbeheim, Arnon and Sela, 1978) and can elicit an antibody response when given orally even if the antigen is given by another route (Warren et al., 1986). MDP can stimulate macrophages to produce interleukin 1, decrease their migration, increase their adherence and spreading and cause them to release prostaglandins or act as T-cell replacing factors for B-cells (Watson and Whitlock, 1978; Byers, 1982; Taubman et al., 1984). MDP has been used in numerous studies on oral delivery of antigens in mammals (Warren and Chedid, 1988). In fish MDP had no effect on the immune response of rainbow trout when injected peritoneally (Cossarini-Dunier, 1985) on its own, in terms of elevated serum antibody titres. It has not been used in the oral delivery of antigens in fish. Natural water in oil emulsions have been produced to mimic the effect of this form of adjuvant with a mixture of lecithin + glycerin + peanut oil (LIPOVANT) being used as an effective adjuvant in equine encephalitis vaccine (Dalsgaard, 1987). In fish Bacille Calmette-Guerin (BCG) mycobacterial preparation was found not to act as an effective adjuvant for Y. ruckeri in immersion vaccines (Grayson et al., 1987).

The other main bacterial adjuvants are LPS, Bordetella pertussis and a variety of detoxified bacterial products (Klausner, 1988).

The use of LPS as an adjuvant was first described in 1956 (Johnson, Gaines and Landy). LPS (endotoxin) can affect the tight junctions of enterocytes, facilitating greater levels of uptake of orally delivered antigen across the intestine. The classical effect of LPS, however, is its T-cell independent mitogenic effect on B-cells where it can stimulate production of non-specific antibody (-antigen) and specific antigen in the presence of antigen (Warren et
al., 1986). LPS can also directly affect macrophages by stimulating monokine production and by altering their level of Ia antigens thereby influencing antigen presentation. As an adjuvant LPS is very toxic due to the Lipid-A portion of the molecule. Most studies in fish (Ellis, 1988) have utilised LPS as a candidate antigen for vaccines rather than an adjuvant although it has been shown in mammals that non-specific LPS (eg from Kleibsella spp) can afford protection to mice against pathogens such as *Listeria monocytogenes* (Fujiki, Kutsukake, Imai and Tanaka, 1988).

*Bordetella pertussis* LPS and pertussis toxin are also used as adjuvants in mammalian test systems (Waksman, 1979). Pertussis-based adjuvants enhances cell-mediated immunity by its modulation of the recirculation of lymphocytes and the subsequent alteration of the balance of T-cells in the secondary lymphoid tissue (Tamura, Tanaka, Takayama, Sato and Uchida, 1985). Such adjuvants can cause an elevated immune response (antibody titre) against SRBC.

Other microbial products being commercially evaluated as potential adjuvants are monophosphoryl Lipid A, *Propionibacterium aranes*-pyridine extract, trehalose dimycolate, mycobacterial cell wall skeleton and *Corynebacterium parvum/rubrum* DNA (Klausner, 1988). The co-administration of antigen with non-pyrogenic integral membrane proteins (Imps) derived from *E.coli* has also been evaluated recently (Croft, Walsh, Lloyd and Russell-Jones, 1991). The extraction and use of the TraT outer membrane protein and its conjugation to standard antigens (its use as a carrier-molecule adjuvant) was found to elicit a significantly increased antibody titre to a protein derived from *Plasmodium falciparum* (Croft et al., 1991).
The range of adjuvants available for use with oral vaccines is immense and it is almost certain that optimisation of vaccination regimes to produce an efficient and protective immune response in fish and mammals will require their use.

Oral vaccination to achieve a viable, long-lasting and commercially feasible protective response is likely to require an enhanced form of specific, purified antigen (or antigens in multivalent vaccines) in a precise delivery system, with the presence of an adjuvant.
CHAPTER 3.
CHAPTER 3. IMMUNOCYTOCHEMISTRY OF THE UPTAKE OF PROTEIN ANTIGENS AND THE USE OF ENHANCED DELIVERY SYSTEMS.

3.1 INTRODUCTION.

The ability to absorb intact protein macromolecules has been demonstrated in several studies on mammals (Walker, Isselbacher and Bloch, 1972; Walker and Bloch, 1983a, 1983b) and in larval and adult fish including elasmobranchs (Hart, 1987), rainbow trout *Oncorhynchus mykiss* (McLean and Ash, 1987), goldfish *Carassius auratus* (Iwai, 1969; Watanabe, 1982), carp, *Cyprinus carpio* (Iwai, 1969; Rombout, Lamers, Helfrich, Dekker and Taverne-Thiele, 1985; McLean and Ash, 1987) and others (Watanabe, 1984) including tilapia *Oreochromis mossambicus* (Doggett, 1989). Some studies have dealt with the accumulation of antigen in the bloodstream and other organs of the body (McLean, 1987; McLean and Ash, 1987, 1990; McLean and Donaldson, 1990) while others have addressed the mechanisms of the temporal passage of antigens across the intestine and its associated cellular interactions *in vivo* including those with cells of the gut-associated lymphoid tissue (GALT) (Georgopoulou *et al*., 1986; Georgopoulou *et al*., 1988; Rombout and Van Den Berg, 1989).

Most of the studies on intestinal absorption of protein antigens in teleosts have relied on the direct physical visualisation of the antigen (under light or electron microscopy) by the immediate colorimetric development of a delivered enzymatic antigen e.g. the direct physical development of horseradish peroxidase (HRP); Graham and Karnovsky, 1966); or by a radioimmunoassay detection
Both of the above techniques have shortcomings with radioimmunoassays being less effective for monitoring protein antigen uptake for a variety of reasons including the effect of the isotope on the detecting antibody and the possible splitting of the isotope from its associated antibody and its subsequent spurious detection (Larssen, 1988). Direct visualisation of antigens that can be degraded after delivery is problematic, especially for the cytochemical detection of macromolecules such as HRP and ferritin, as the signal-generating reaction is largely dependent on the presence of relatively intact macromolecules (Larssen, 1988; Rombout and van den Berg, 1989). Immunocytochemical detection has several distinct advantages over these more conventional methods, for analysis of absorption of delivered protein antigens (Bullock and Petrusz, 1982; Polak and Van Noorden, 1986; Beesley, 1988; Larssen, 1988) as they can potentially visualise both intact macromolecules and derived antigenic fragments that may be important as nascent immunogens (Rombout and van den Berg, 1989).

Immunocytochemical detection systems appear to allow greater sensitivity of antigen detection along with a less artifactual visualisation of antigen distribution. To date, the technique has been used to monitor macromolecular absorption after oral delivery in relatively few studies in fish (Hart, 1987; Georgopoulou et al., 1986; Rombout and van den Berg, 1989). Proteins have been shown being absorbed across the intestinal enterocytes of various species of gastric (Georgopoulou et al., 1985, 1986, 1988) and agastric fish (Davina, et al., 1980, Rombout, et al., 1985; Rombout, et al., 1986). Proteins, once thought to
be absorbed predominantly across the enterocytes of the posterior intestines of fish (Noaillac-Depeyre and Gas, 1973; Watanabe, 1984), have been reported being absorbed across the anterior regions of the intestine (Lamers, 1985) as is the case for HRP and ferritin in tilapia (Doggett, 1989).

Orally delivered protein macromolecules have been reported being absorbed very rapidly in an intact form across the posterior and anterior regions of the intestine of tilapia (Doggett, 1989). However, protein antigens delivered to *O. mossambicus* are also recognised as being heavily degraded by a variety of digestive processes, which could lead to the production of definable and potentially immunogenic fragments which could have value in the oral induction of a relevant immune response.

Orally delivered protein macromolecules have been observed in association with the various cells of the GALT underlying the intestinal enterocytes of fish (Davina et al., 1980; Georgopoulou et al., 1986; Rombout and van den Berg, 1989). The levels of antibodies generated by oral delivery of proteins, such as ferritin, are relatively low but may be substantially protective for certain bacterial antigens (Rombout et al., 1989). One method of increasing both the levels of absorbed protein macromolecules after oral delivery and the resultant immune responses to these proteins is by the use of adjuvants (Ellis, 1988). The saponin adjuvant, Quil-A, has been shown to enhance the uptake and immune response to orally delivered antigens in mammals for antigens from a wide variety of sources (Dalsgaard, 1978; 1987; Maharaj et al., 1986; Campbell and Bede, 1989) and by a variety of mechanisms. Quil-A, and saponin adjuvants in general, have been largely neglected for oral delivery.
systems in teleost fish and only rarely used via the intraperitoneal and immersion routes (Cossarini-Dunier, 1985; Tatner and Horne, 1985; Grayson et al., 1987).

The current study attempts to analyse the tissue localisation and mechanics of absorption of orally and anally delivered protein macromolecules utilising a variety of sensitive immunocytochemical techniques and the effects on absorption of the saponin adjuvant Quil-A.

3.2. MATERIALS AND METHODS.

3.2.1. ANIMALS.

Adult tilapia, *Oreochromis mossambicus*, of both sexes weighing between 30-50g were retained in a recirculating freshwater system at 24-26°C.

3.2.2. INTUBATION PROCEDURES.

Groups of tilapia (n=3) were intubated with test antigen, human gamma globulin (HGG, molecular weight 150kDa, Cohn fraction II, Sigma, Poole Dorset). Each fish received 0.2ml of a 10mg/ml HGG solution using phosphate buffered saline as diluent (PBS, pH 7.2; Appendix I). Peroral and peranal intubation was carried out using thin bore pVC tubing (1.00mm and 0.75mm external diameter for oral and anal administration, respectively) attached to a 1ml syringe via a 23g needle. Control groups of tilapia (n=3) were intubated with PBS pH 7.2. Oral and anal administration of HGG was carried out over a structured time course and tissue sampled as follows: after 5, 15, 30, 60 minutes and 2, 6, 12, 24 and 48 hours.

HGG was administered orally and anally with the purified saponin
adjuvant Quil-A, derived from *Quillaja saponaria* [Molina] (Superfos, Denmark).

Quil-A was administered with antigen in several forms:

1) At various concentrations (100μg/ml; 10μg/ml and 1μg/ml), 1 hour prior to the intubation of HGG, by a complementary route (*i.e.* orally or anally).

2) Concurrently with the antigen, at various concentrations (as above), by a complementary route.

3) As a micellar package (see 3.2.3.).

4) As an immune stimulatory complex (ISCOM)(see 3.2.4.).

5) At 100μg/ml (in PBS pH 7.6; 0.2ml dose) on its own for the determination of any direct effects on the intestine.

### 3.2.3. THE FORMATION OF MICELLES WITH QUIL-A.

All saponins, including Quil-A, have the ability to spontaneously form micelles when in an aqueous solution above a defined critical concentration. For this purified preparation of Quil-A this critical micellar concentration (CMC) was approximately 0.03% (Dalsgaard, 1978).

Micelle formation is characterised by a drop in the overall conductivity of the solution as levels of free ions lessen, with the hydrophilic regions of the saponin molecules being bound up in the micelle structure (Dalsgaard, 1978). The formation of Quil-A micelles with HGG was monitored by detection of conductivity of the solution with a Electrical Instruments conductivity meter (Kent Meters, LtD, Luton, U.K.). Micelles were prepared by prolonged vortexing of 10mg/ml HGG with Quil-A above the CMC and 0.2ml of this solution was subsequently administered to groups of fish (n=3).
3.2.4. THE PREPARATION OF IMMUNE STIMULATORY COMPLEXES (ISCOMS)

ISCOMS were produced utilising concentrations of Quil-A above the CMC but were structurally distinct from micelles. ISCOMS were prepared according to the method of Morein et al.,(1984). Briefly, 6ml of HGG (10mg/ml) in 0.05M Tris-HCl - 0.1ml NaCl (Tris-saline, pH 7.4) buffer plus 60μl (1%) E.coli strain L:026:B6 lipopolysaccharide (LPS; Sigma) and 0.03% Quil-A was layered over approximately 30ml of a 10-40% sucrose density gradient also containing 0.03% Quil-A, and was ultracentrifuged at 44000rpm (150 000g) for 4 hours at 20°C using a 70 T.I rotor head in a Beckman L7 ultracentrifuge (Beckman, High Wycombe).

A tight white band of ISCOMS were formed and fractions of approximately 1ml were eluted and dialysed against 0.05M ammonium acetate for 48 hours at 4°C. All fractions were tested for protein content using a commercial, Bradford (Bradford, 1976), total protein assay system (Biorad, Watford) and also for antigen content using a HGG specific enzyme-linked immunosorbent assay (ELISA) as described in Chapter 4. The concentration of ISCOMS was subsequently adjusted in Tris-saline to approximately 10mg/ml HGG and 0.2ml administered to fish.

Control groups of tilapia (n=3) were immunised with an uncentrifuged HGG/Quil-A/E.coli LPS mixture, a HGG/LPS mixture or HGG alone by a complementary route.

3.2.5. TISSUE COLLECTION.

Fish were sacrificed by a sharp blow to the head, and the intestine, from
the pyloric sphincter to the anus, immediately excised. The intestine was divided into the anterior, mid and posterior regions of approximately equal length (Doggett, 1989).

3.2.6. IMMUNOHISTOCHEMISTRY.

Tissue was fixed for immunohistochemistry in 2% paraformaldehyde, buffered in PBS pH 7.6, for 2 hours at 4°C and subsequently washed for 2x10 minutes in PBS. Tissue was prepared for paraaffin wax immunohistochemistry by automatic dehydration through graded alcohols, cleared in xylene and was embedded in molten Fibrowax (BDH, Bristol) at 56-58°C. Sections were cut at 5-6μm on a Reichert-Jung rotary microtome (Reichert-Jung, London).

3.2.6.1. IMMUNOPEROXIDASE STAINING.

An antigen-specific streptavidin-biotin-HRP complex staining procedure was used to localise antigen in tissue sections as described by Hsu, Reine and Farger (1981). All reagents are as outlined in Appendix I. The antibody concentrations were optimised, on section, using a checkerboard titration method of primary antisera from 1/100 to 1/1000 and secondary antisera from 1/300 to 1/1000.

Sections were deparaffinised and rehydrated, equilibrated in Tris-saline (TBS) pH 7.5 for 10 minutes and endogenous chemical activity suppressed using 3% v/v H₂O₂ for 5 minutes (for endogenous peroxidase activity) and 0.01% v/v streptavidin followed by 0.001% v/v biotin (for endogenous avidin activity). Sections were washed for 10 minutes in Tris-saline and incubated in normal goat serum (1/5 in TBS) for 20 minutes to eliminate cross-reactivity with the
secondary antiserum (Bourne,1983).

All antisera (Dako Ltd, High Wycombe, Bucks) were diluted in TBS plus 0.1% bovine serum albumin (BSA) as a blocking agent and the sections were washed for 3x 5 minutes in TBS after each incubation. Sections were incubated with rabbit anti-HGG (Sigma) antiserum optimised at 1/500 for 1 hour at ambient temperature (primary antiserum) followed by incubation in biotinylated goat anti-rabbit IgG antiserum (Dako, Ltd) optimised at 1/1000 (secondary antibody) for 1 hour at ambient temperature. After washing as previously, the sections were incubated in streptavidin-biotin-HP (sABC-HP) complex for 30 minutes at ambient temperature and subsequently developed in peroxidase substrate solution consisting of 6mg of 3'3' diaminobenzidine tetrahydrochloride (DAB) in 10ml TBS plus 100μl of 3% v/v H2O2 as the reaction initiator, for 3-5 minutes. Positive, specific, peroxidase activity is defined by a deep-brown colour reaction product that is insoluble in alcohol. The reaction was stopped by extensive washing in distilled water and the sections counterstained, dehydrated and mounted in DPX.

3.2.6.2. TWO-STAGE IMMUNOPEROXIDASE STAINING.

Two-stage immunoperoxidase staining was carried out by sequential antisera incubations and washings as previously, using TBS as the washing medium and TBS-BSA as the incubation solution. Primary antisera incubation was as in 3.2.6.1. and secondary antisera incubation was with goat anti-rabbit peroxidase conjugate (Sigma) at 1/1000 for 1 hour at ambient temperature. The development of the peroxidase-labelled conjugate was carried out using the DAB substrate as previously described.
3.2.6.3. **IMMUNOGOLD-SILVER STAINING.**

A silver-enhanced immunogold staining method was also used to detect absorbed HGG based on the method of Hacker, Springall, Van Noorden, Bishop, Grimelius and Polak (1985). Some sections were pre-treated in Lugol’s iodine (a 1% w/v iodine in 2% v/v aqueous potassium iodide solution) for 5 minutes followed by bleaching in sodium thiosulphate until colourless, in an attempt to accentuate staining.

Sections were rehydrated and rinsed in 10mM PBS pH 7.2 and incubated in 5% heat-inactivated (52°C for 20 minutes) normal goat serum diluted in PBS for 20 minutes. All incubations were carried out in antisera diluted in PBS plus 0.1% BSA and were followed by sequential washing in PBS for 3x 5 minutes. Sections were incubated in rabbit anti-HGG (primary antibody) at 1/500 for 2 hours at ambient temperature followed by goat anti-rabbit IgG 5nm gold conjugate (1/100 for 1 hour) diluted in PBS-BSA plus 5% porcine gelatin. The sections were washed in PBS and distilled water (3x3 minutes) prior to silver enhancement.

The sections were silver enhanced according to the manufacturers instructions (Amersham Ltd, Amersham, Herts.) for approximately 12 minutes and the reaction was stopped by rinsing in distilled water.

A brief diagrammatical representation of the immunostaining methods used is shown in Figure 1.
FIGURE 3.1. METHODS OF IMMUNODETECTION OF HGG ANTIGENS.

3.2.7. COUNTERSTAINING AND CONTROLS.

Comprehensive technical controls were carried out using blocking steps for endogenous peroxidase (Lewis, Johnson and Cruse, 1983) and avidin (Wood...
and Warnke, 1981), a serum blocking stage which reduces any potential cross-reactions with the secondary antisera; control sections without primary or secondary antisera or ABC-HRP bridging-complex and pre-absorption of the antisera with appropriate antigen (HGG) and its subsequent use on sections.

Counterstaining was carried out with Mayer's haematoxylin (Drury and Wallington, 1976), Wright's stain (Russell, pers. comm.), Giemsa's stain (McWann, pers. comm.) and haematoxylin and eosin (Drury and Wallington, 1976).

**3.2.8. IMMUNO-ELECTRONMICROSCOPY.**

Intestinal tissue was fixed in a solution of 2% paraformaldehyde plus 0.5% glutaraldehyde for 2 hours at 4°C. Tissue was then sequentially treated in 70%, 90% and 100% alcohol, equilibrated and embedded in LR White medium-grade hydrophilic resin at 48°C for 48 hours. Other tissue blocks were fixed, as previously, and prepared for embedding in Nanoplast FB101 water soluble melamine resin. For this, tissue was pre-stained in 1% v/v aqueous uranyl acetate for 4 hours in the dark, embedded in Nanoplast and dried in a desiccator for 48 hours at 40°C. Post-curing was carried out at 60°C for 48 hours.

Silver sections were cut at 500-800Å on a Reichert-Jung Ultracut microtome and mounted on inert (gold or nickel) 200-400 mesh grids.

The sections were stained using the two-stage immunoperoxidase and immunogold methods with the antisera being diluted as previously. All staining was carried out on droplets of the appropriate solutions at ambient temperature. The sections were washed by transferring the grids, step-wise, into droplets of
PBS buffer and blotting them carefully. The peroxidase-conjugated secondary antisera for the immunoperoxidase method was developed with a solution of DAB (as previously described) for 3-5 minutes. Immunogold stained grids were unenhanced. The sections were counterstained with lead citrate and examined under a Philips 300 transmission electron microscope.

3.2.9. THE CYTOCHEMICAL DETECTION OF QUIL-A.

Intestinal tissue blocks were fixed for cytochemistry in 2% glutaraldehyde for 2 hours and then post-fixed in 1% osmium tetroxide (OsO₄) for 60 minutes. Osmium tetroxide can act as a general stain for unsaturated lipids (Pearse, 1968; Bayliss-High, 1984) and was used to probe for Quil-A in this study. Tissue was dehydrated through a series of graded alcohols, embedded in Spurrs' resin (TAAB, Reading) and sectioned and examined as previously. Control tissue (without Quil-A) was processed and examined as above.

3.3. RESULTS.

3.3.1. THE ABSORPTION OF HGG.

HGG was detected free in the gut lumen and associated with the mucus layer of the intestine 5 minutes after both oral and anal administration but was not observed in the intestinal epithelium itself (not shown).

HGG was detected immunohistochemically in all regions of the intestine 15 minutes after oral and anal intubation in the terminal webs of the enterocytes and, rarely, in the lamina propria (Figure 3.2e). Oral intubation generally resulted in less observable antigen throughout the intestine up to 1 hour and in less accumulated antigen (Figure 3.2a) although antigen was also observed in apparent contact with the intraepithelial leucocytes (IEL's) with this route of
delivery (Figures 3.2a and 3.2b). Maximum levels of HGG were detected in all
regions of the intestine approximately 6 hour after oral delivery (Figures 3.3a
and 3.3e) and antigen was observed in accumulations in the supra-nuclear
cytoplasm of the enterocytes and throughout the lamina propria. Thirty minutes
after anal intubation (Figure 3.2c) HGG was observed more often in the apical
cytoplasm of the enterocytes and in the lamina propria free or apparently in
contact with the IEL's situated near the basement membrane. Maximum levels
of antigen were detectable in the posterior intestine 1 hour after anal intubation
of antigen and was widely distributed after 2 (Figure 3.2d) to 6 hours (Figure
3.3c). The amount of antigen after both oral and anal delivery decreased after
6 hours. After 12 hours (Figures 3.3b and 3.3.d) there is less observable antigen
in all the intestinal regions. Anally delivered antigen is still visualised in
accumulations in the enterocytes but in reduced levels. Little trace of HGG
could be detected 24 to 48 hours after administration.

Experimental (PBS intubations) and technical controls were negative for
endogenous, cross-reactive and specific staining (not shown).

3.3.2. THE MECHANICS OF HGG ABSORPTION

Orally delivered antigen was visualised in contact with the enterocyte
microvilli (Figure 3.4e) of the posterior intestine, with associated invaginations
being formed in the lumenal plasma membranes of the cells (Figure 3.4a). HGG
appeared to be absorbed by such invaginations being pinched off from the
plasma membrane and compartmentalised in large spherical vesicles. HGG was
also observed near cytoplasmic tubules (Figure 3.4a) that were associated with
the plasma membrane. Vesicular formation appeared to be a composite of
antigen deposition from both pinocytotic invaginations of the enterocyte membrane and cytoplasmic tubule coalescence (Figure 3.4b) forming both amorphous (less-structured) deposits of antigen and structured pinocytotic vesicles. Anally delivered antigen was pinocytosed in a similar manner, with an apparent migration pattern towards the supranuclear cytoplasm (Figure 3.4c). Some anally delivered antigen appeared to be associated with phagolysosomes after approximately 1 hour. Small amounts of HGG were also observed in association with the goblet cells of the posterior intestine (Figure 3.4d). Antigen appeared to be situated in the basal regions of the mucus cell and in the phagolysosomes of the adjacent cellular cytoplasm.

HGG was absorbed through the enterocytes by vesicular transport to the lateral intercellular regions of the cells (Figure 3.5e) or to the basal membrane and was then exocytosed from the enterocytes to the lamina propria and the underlying vasculature (Figure 3.5a). One hour after delivery, exocytosed antigen could be visualised in intimate contact with the capillaries of the intestine (Figure 3.5a; closed black triangle Figure 3.5b). There was an observable morphological difference between antigen vesicles on the basis of their size and apparent lysosomal activity (Figures 3.5b and 3.5c). Some exocytosed vesicles had been broken down leaving amorphous, degraded, antigen (open black triangle, Figure 3.5c) in the lamina propria.

After absorption, exocytosed antigen came into contact with a variety of GALT leucocytes. HGG was found being degraded in the phagolysosomes of macrophages in the lamina propria and epithelium after 2 hours and up to 12 hours (not shown). Leucocytes with both macrophage and lymphocyte-like
morphologies were also found to bind HGG at their plasma membranes (Figure 3.5d). Antigen could be observed being directed to the intercellular spaces between these leucocytes and the surrounding enterocytes or laminal cells.

Antigen was largely absent after 12 hours and none was detected after 48 hours.

3.3.3. THE EFFECT OF VARIOUS MODES OF DELIVERY OF QUIL-A ON THE ABSORPTION AND PROCESSING OF HGG.

Orally administered Quil-A (100μg/ml;0.2ml dose) on its own was cytochemically distinguished in various areas of the enterocytes of the intestine in large amounts 1 hour after delivery (Figures 3.6a and 3.6b).

Quil-A caused extensive pinocytosis of the luminal contents (Figure 3.6c). Quil-A appeared to be absorbed directly across intercellular junctions and by a cytoplasmic tubule system. Conversely, micellar Quil-A could be observed coalescing with the enterocyte plasma membranes (Figure 3.6c). Quil-A also affected the cellular microvilli of the enterocytes causing them to become shortened and damaged (Figure 3.6d).

Quil-A administration orally and anally conspicuously affected the extent of uptake of HGG when given prior to (Figure 3.7) and concurrently with the antigen (Figures 3.8 and 3.9). The concentrations of Quil-A administered appeared to have similar effects on the increase of observable HGG absorbed.

The prior administration of Quil-A caused a more extensive uptake of orally delivered HGG throughout the intestine (Figures 3.7a and 3.7b) when compared with the delivery of the antigen on its own, with more being present
in the epithelium and lamina propria 1 hour after the administration of Quil-A. Transcellular protein uptake mechanisms appeared to be largely similar to that described earlier *ie* by pinocytotic vesicles and prior administration of Quil-A caused anally delivered HGG to be rapidly absorbed and increasingly associated with the capillaries (Figures 3.7c and 3.7d).

Concurrent Quil-A administration also caused increased uptake of HGG (Figure 3.8). Both oral (Figures 3.8a and 3.8b) and anal (Figures 3.8c, 3.8d and 3.8e) concurrent delivery of adjuvant caused extensive localisation and retention of antigen in the lamina propria. Large antigen-containing micelles could be observed coalescing with the intestinal epithelium after anal delivery (Figure 3.8d).

Delivery of ISCOMS and micelles had a similar effect on HGG absorption as the concurrent administration of Quil-A but with greater localisation in the lamina propria (Figure 3.9). Predominantly with micelles and ISCOMS, HGG was localised in large spherical vesicles in the lamina propria often near the vascular system. This was the major observable modification due to the delivery of ISCOMS and micelles. Some antigen was observed intracellularly (Figure 3.9a) and tubule-like connections were visualised projecting from the surrounding laminal cells and contacting the intercellular antigen (Figure 3.9b).

Control administration of HGG largely gave results similar to those described above.
3.4. DISCUSSION.

The present study indicates that orally and anally delivered proteins are rapidly absorbed by the intestinal enterocytes of *Oreochromis mossambicus*, by a complex mechanism of absorption, and that the administration of the saponin adjuvant Quil-A in several forms of delivery system has a significant effect on the absorption of protein. The study also shows that the intestine of *O. mossambicus*, can have complex interactions with intact, or epitopes of, orally and anally delivered protein throughout its length. This contrasts with the "classical" view of protein absorption which was thought to occur solely in the form of amino acids and small oligopeptides, which has also been challenged in mammals (Walker *et al.*, 1972; Walker 1981; Gardner, 1983). Recent studies in other species of fish have also indicated that the intestines of teleosts have the potential to absorb intact proteins or potentially immunogenic fragments that can interact with GALT cells (Georgopoulou *et al.*, 1986; Rombout *et al.*, 1989).

Protein absorption was also once thought to predominantly occur in the absorptive cells of the posterior intestine of fish (Noaillac-Depeyre and Gas, 1973; Stroband and Kroon, 1981; Stroband and van der Veen, 1981; Georgopoulou *et al.*, 1985; Rombout *et al.*, 1986) but has recently been demonstrated in the anterior intestinal regions in gastric species such as *O. mossambicus* (Doggett, 1989) and agastric species such as carp, *Cyprinus carpio* (Rombout *et al.*, 1985).

HGG is absorbed across the intestinal enterocytes of *O. mossambicus* 15 minutes after oral and anal delivery and localised accumulations of antigen are apparent in the apical cytoplasm and the supra-nuclear cytoplasm after 30-60
minutes and near the intestinal vasculature after an equivalent time. Levels of antigen increase intracellularly after delivery reaching a maximum after 6 hours and 1 hour for oral and anal administration, respectively. Gamma globulins have only been rarely investigated as candidate antigens for absorption across the intestine of fish (Georgopoulou et al., 1986; Hart, 1987; Fujino et al., 1987). In rainbow trout, Oncorhynchus mykiss, HGG absorbed after 60 minutes in vitro but specific intracellular localisation only occurred 5 hours after oral administration in vivo (Georgopoulou et al., 1986), whereas direct administration of rabbit IgG perorally caused absorption after 3 hours (Fujino et al., 1987). In O. mossambicus orally and anally HRP was absorbed after 5 minutes and was present throughout the enterocytes and in the phagolysosomes of macrophages 2 hours after oral delivery (Doggett, 1989). Ferritin uptake took longer in tilapia and was retained for longer in the enterocyte supranuclear vacuoles and GALT macrophages (Doggett, 1989). Ferritin is absorbed across the intestine in other species such as carp (Rombout and van den Berg, 1989) and it was observed that the distribution of biologically intact macromolecules and immunoreactive fragments is different (Rombout and van den Berg, 1989). Proteins appear to be retained in the macrophages of several species from 24 hours to 4 days, such as ferritin in carp (Rombout and van den Berg, 1989), rainbow trout (Georgopoulou et al., 1986) and perch, Perca fluviatilis (Noaillac-Depeyre and Gas, 1979). The observed temporal variability in the absorption of antigens across the gut in O. mossambicus may depend on the molecular weights of the antigens as has been suggested, for example, in rainbow trout (Georgopoulou et al., 1986) and possibly carp (Rombout and van den Berg, 1989). Macromolecules such as bovine growth
hormone (molecular weight 22kD) are absorbed after 1 hour, HRP (molecular weight of 44kD) absorbed after 2 hours, ferritin (molecular weight of 460kD) after 18 hours and hepatitis B surface antigen (molecular weight of 2x10⁶kD) taking some 20 hours (Georgopoulou et al., 1985, 1988; Le Bail et al., 1989). However there is contradictory evidence which indicates that antigenically discernable protein fragments are detectable in the bloodstream 15 minutes after oral delivery (McLean and Ash, 1987). There is also a wide discrepancy in the temporal kinetics of protein absorption across the intestines of teleosts, some of which may be due to the evolutionary divergence between the species studied, their (often widely) different modes of nutrition and the detection systems employed (McLean, 1987; Georgopoulou et al., 1988; Doggett, 1989; Rombout and van den Berg, 1989). Mammalian studies also demonstrate variability with both the species and antigen studied but most orally delivered proteins are detectable in the intestine after 1 hour and in the bloodstream (or lymphatic vessels) after 2 hours (Walker, 1981; Walker and Bloch, 1983a; 1983b).

HGG orally and anally delivered to O. mossambicus appears to be absorbed across the intestinal enterocytes by a pinocytotic mechanism similar to that described for HRP and ferritin in tilapia (Doggett, 1989) and HRP in rainbow trout (Georgopoulou et al., 1985, 1988) and in vitro in isolated hindgut enterocytes from goldfish, C. auratus (Iida and Yamamoto, 1985). HGG absorption also seems to be mediated by a system of intracellular cytoplasmic tubules as well as pinocytotic vesicularisation. A similar system is implicated in the intracellular transcytosis of HRP in goldfish (Iida and Yamamoto, 1985) and
in the active mechanisms of absorption and transport in other species (Noaillac-Depeyre and Gas, 1979; Ezeasor and Stokoe, 1981). The status of such tubular systems is unclear, however, with their role alternately being implicated as sites of antigen deposit, after pinocytosis (Iida and Yamamoto, 1985) and also as mechanisms of cellular access which ultimately form or combine with vacuoles and vesicles (Georgopoulou et al., 1988). In this study it appears that the absorption of HGG in tilapia occurs by a dual mechanism of uptake implicating both tubules and pinosomes.

A multiplicity of fates of HGG absorbed after oral and anal delivery were observed with exocytosis to the lamina propria; direct absorption into the vascular system; lysosomal degradation or contact with the GALT cells all being possible. Orally and anally delivered HGG was observed being degraded in phagolysosomes (as evidenced by the amorphous nature of some of the antigen) possibly in a similar manner to the degradation of orally delivered HGG in rainbow trout where degradation was initiated at the Fc portion of the molecule after 1 hour to 90 minutes (Georgopoulou et al., 1986). In carp, degraded (though antigenic) and intact ferritin follow different routes after absorption with degraded ferritin reaching the lamina propria and eventually the bloodstream whereas the intact protein remained in the enterocyte vacuoles (Rombout and van den Berg, 1989). The degradation of absorbed proteins, after oral administration, has been positively correlated with the activities of the enzymes acid phosphatase and cathepsin B and cathepsin D (Georgopoulou et al., 1986).

There was a greater level of visualisable antigen in the intestinal tissues of tilapia after the anal administration of HGG by comparison with oral
administration. A similar pattern was noted by Johnson and Amend (1983a, 1983b) after the oral and anal delivery of bacterial antigens from *Vibrio anguillarum* and *Yersinia ruckeri* and in this study the circumvention of the gastric conditions in the stomach of rainbow trout was considered to be the major cause for the increased level of antigen absorbed and the increased antibody response achieved with anal delivery. Similarly, the amounts of antigen absorbed into the plasma and the resultant antibody responses of *O. mossambicus* altered with the various modes and routes of delivery of HGG (this study).

Absorbed HGG was also observed interacting with the plasma membranes of lymphocyte-like cells and macrophages in a manner similar to that described for ferritin and *V. anguillarum* antigens orally delivered to carp (Rombout and van den Berg, 1989) and which was considered to be antigen presentation activity. It is well established that cells of the macrophage lineage and B-cells can effectively present antigen in lymphoid tissue in mammals (Chain et al., 1988; Pierce, Morris, Grusby, Kaumaya, van Buskirk, Srinvasan, Crump and Smolenski, 1988) and a similar function is likely for comparable cells in fish (Vallejo et al., 1990). Such activity, if truly indicative of antigen presentation, is thought to be one of the central events required for the generation of a local and/or systemic immune response and could indicate the potential of oral delivery for the establishment of an immune response. Oral and anal delivery of HGG can also result in the rapid transfer of antigen to the vicinity of capillaries and its subsequent passage to the systemic circulation and major body organs.

Administration of the saponin adjuvant Quil-A caused an appreciable
increase in the uptake of orally and anally delivered HGG, which may result in, eventually, an enhanced specific immune response (Maharaj et al., 1986). However, intraperitoneal injection and immersion administration (Cossarini-Dunier, 1985; Grayson et al., 1987) of Quil-A failed to potentiate an immune response to antigens from Aeromonas salmonicida and Y. ruckeri respectively, and indeed caused a decreased response in some instances. Saponins have not been previously used in oral or anal delivery systems in fish but in mammals the immune responses to orally delivered antigens such as rabies G antigen, can be increased by the prior or concurrent administration of Quil-A (Maharaj et al., 1986). Quil-A was observed having a number of physical effects on the intestinal enterocytes of tilapia such as "loosening" intercellular junctions, increasing the pinocytosis of coincident luminal contents, fusion with the plasma membrane and direct effects on the microvilli all of which probably serve to increase the permeability of the intestine to delivered HGG. These effects are similar to those reported for the oral delivery of saponins in mammalian intestines (Campbell, Maharaj and Roith, 1985; Campbell and Bede, 1989). Quil-A binds to cholesterol in the plasma membranes of the enterocytes and causes the formation of "pores" (Bangham and Horne, 1962), again increasing intestinal permeability. Such direct effects may explain the increase in observed antigen absorption in this study along with other possible modes of action such as the inhibition of the gastric and intestinal proteolytic enzymes such as trypsin, chymotrypsin and pepsin, observed for other saponins, which increase the levels of antigen reaching the intestine in mammals (Ishaaya and Birk, 1965). Quil-A may also have a direct stimulatory effect on the macrophages of the
underlying GALT in the intestine by causing them to produce interleukins and potentiate any resultant immune response. Quil-A has also been reported to have an adjuvant effect by acting as an antigen "depot" for the effective concentration and presentation of antigens, the structure of which it may or may not modify by its presence (Maharaj et al., 1986).

The administration of HGG in ISCOMS and micelles directly increased the amounts of antigen accumulated in the lamina propria along with its retention time. The interaction observed between the intercellular antigen after this mode of delivery and the surrounding laminal cells may be a result of the increased retention time. ISCOMS may give HGG an almost "particulate" nature, a factor which may induce different cellular processing. Micelles have a less well-defined tertiary structure but act, like ISCOMS, as both concentrating agents and delivery vehicles (Dalsgaard, 1987; Morein, 1987; Morein, et al., 1987).

The current study indicates that the oral and anal delivery of protein macromolecules to the intestine of *O. mossambicus* results in complex patterns of absorption and cellular interactions (see Chapters 4 and 5). The levels of absorbed protein detected in the intestinal tissue are increased by several modes of delivery of Quil-A which has a direct effect on the enterocytes of the intestine and may contribute to an enhanced immune response (see Chapter 6).
FIGURE 3.2. THE ABSORPTION OF HGG

The absorption of HGG in the intestine of *Oreochromis mossambicus* between 30 minutes and 6 hours after oral (3.2a, 3.2b and 3.2e) and anal (3.2c and 3.2d) delivery. Posterior intestine.

3.2a, HGG 1 hour after oral intubation in the epithelium (E, black arrows). The lamina propria (LP) and goblet cells (G) are not positive for HGG after this time. Intraepithelial leucocytes (IEL) are observed in intimate association with antigen (open black triangle).

3.2b, HGG 2 hours after oral intubation. The gut lumen (GL) is largely free from staining for antigen which can now be detected (black arrows) throughout the epithelium (E) and lamina propria (LP).

3.2c, anally intubated HGG after 30 minutes. The antigen is increasingly found in accumulations (AC) in the epithelium but also was detected penetrating the lamina (black arrow) and in contact with IEL's (open black triangle).

3.2d, anally intubated HGG after 2 hours. Pink, unenhanced, colloidal gold can be seen (black arrows) in the epithelium and lamina propria. Antigen is widely distributed throughout the terminal webs of the enterocytes (t).

Figure 3.2(a-d) is immunogold/silver stained with Wrights (3.2a, 3.2c and 3.2d) and Giemsa's (3.2b) counterstaining.

3.2e, orally intubated antigen (insert) after 15 minutes showing staining of antigen largely confined to the epithelium (E) with a clear lamina propria (LP).
Figure 3:2e is two-stage immunoperoxidase stained with H&E counterstaining.
FIGURE 3.3. THE ABSORPTION OF HGG

The absorption of HGG between 6 and 12 hours after oral (3.3a and 3.3b) and anal (3.3c and 3.3d) delivery of antigen. Posterior intestine.

3.3a, orally delivered HGG after 6 hours. HGG is beginning to be found in accumulations (white arrows) in the epithelium (E) while being cleared from the lamina propria (LP).

3.3b, HGG 12 hours after oral intubation. There is less antigen (black arrows) present in both the epithelium (E) and lamina propria (LP).

3.3c, 6 hours after the anal intubation of HGG. Very similar to oral delivery at the same time, with accumulations (AC) in many parts of the epithelial (E) enterocytes, including the supranuclear regions.

3.3d, anally intubated HGG after 12 hours. There appears to still be considerable staining in the terminal web region (T) and large conglomerates of antigen (C) in the epithelium (E) but little in the lamina propria (LP).

3.3e, orally delivered HGG after 6 hours (insert) showing typical accumulations of antigen in supranuclear vacuoles (SNV) in the enterocytes.

Figure 3.3 is immunogold/silver stained with Wrights counterstaining.
Figure 3.3
FIGURE 3.4. THE MECHANICS OF THE ABSORPTION OF HGG.

Figure 3.4 (a-c) mid intestine, 3.4d posterior intestine.

3.4a, orally intubated HGG after 1 hour. Antigen is detected between the microvilli (MV) of the enterocytes, binding to the apical membrane often in intermicrovillous invaginations (small arrows). Antigen is also detected around the intercellular junctions (D, large arrows). Pinocytotic vesicles (PV) containing HGG are found in the apical cytoplasm (AC) in tubules (open triangles) that can be observed running from the enterocyte membrane into the apical cytoplasm.

3.4b, similar to 3.4a, with amorphous antigen (large arrows) present along with pinocytosed antigen (PV).

3.4c, anally intubated HGG in the apical cytoplasm (AC) of a mid intestinal enterocyte, after 2 hours. Pinocytosed antigen (PV) is directed towards the supranuclear cytoplasm (SNC). Antigen is also localised inside structures resembling phagolysosomes (PL).

3.4d, amorphous antigen (black arrow) 1 hour after the anal delivery of HGG inside a goblet cell (G). The cytoplasm surrounding the mucus body of the cell also contained phagolysosomes (PL).

Figure 3.4 (a-d) is two-stage immunoperoxidase stained.

3.4e (insert), antigen is intimately associated with the microvilli (MV) as well as the enterocyte lumenal membrane during uptake of orally delivered antigen.

Figure 3.4e is immunogold stained (5nm particles).
FIGURE 3.5. THE MECHANISM OF ABSORPTION OF HGG: CELLULAR INTERACTIONS.

Figure 3.5 (a-c) mid intestine, 3.5d posterior intestine.

3.5a, absorption of orally intubated HGG after 2 hours. HGG was detectable in the lamina propria, near the capillaries (CP) containing erythrocytes (RBC) in several forms including intact vesicles (IV, large solid arrow), active vesicles (AV), as amorphous antigen (open triangle) and as very small vesicles (small solid arrows).

3.5b, similar to 3.5a showing the channelling of some HGG towards the capillary lumen (solid black triangle).

3.5c, vesicles, with varying morphologies, containing HGG 2 hours after oral delivery. Legend as in 3.5b.

3.5d, the interaction of orally delivered (12 hours) antigen (A) with an intralaminal leucocyte (N) with a lymphocyte-like morphology. The antigen is incorporated (solid black triangles) into the cells plasma membrane (PM).

3.5e (insert), absorbed antigen is often associated with intercellular passage at the desmosomes (D).

Figure 3.5 is two-stage immunoperoxidase stained.
FIGURE 3.6. THE CYTOCHEMICAL DETECTION OF QUIL-A IN THE INTESTINE.

Figure 3.6, the tissue localisation of orally delivered Quil-A (100μg/ml) after 1 hour. Figure 3.6 is anterior intestine.

3.6a, heavily stained Quil-A, spherically arranged (arrows, QA) intracellularly, in the epithelium (E) of the posterior intestine.

3.6b, accumulated Quil-A (arrows, QA) similar to 3.6a but deeper in the epithelium (E).

3.6c, Quil-A from the gut lumen (GL), associated with the microvilli (MV) as small micelles (M). Quil-A causes copious cytoplasmic tubule formation (CT) and vesicularisation of both Quil-A (VAQ) and coincident lumenal material (P).

3.6d, the direct physical effects of Quil-A on the epithelium (E) its damage to the microvilli (DMV) and effects at tight-junctions (TJ) and enhanced pinocytosis (P) as in 3.6c.
**FIGURE 3.7. THE UPTAKE OF HGG WITH PRIOR ADMINISTRATION OF QUIL-A.**

Figure 3.7, the distribution of HGG 1 hour after the oral delivery of 100 μg/ml Quil-A, after oral (3.7a and 3.7b) and anal (3.7c and 3.7d) antigen intubation. Figure 3.7 (a-c) mid intestine, 3.7d posterior intestine.

3.7a, the terminal web (T) of the epithelium (E) is heavily stained (dark arrows; ST) for HGG. The lamina propria (LP) shows some staining for antigen. There is some excess staining in the gut lumen (GL) that is likely to be due to antigen adhering to the lumenal mucus.

3.7b, similar to 3.7a with the absorbed antigen in the terminal web (T) and positive inclusions (PI) of antigen extending into the apical cytoplasm. The goblet cells (G) are negative.

3.7c, anally intubated HGG after 1 hour showing staining in the epithelium (E) and in the lamina propria (LP, white arrow) near the basement membrane (BM). Staining for antigen is also detectable near the capillaries (CP).

3.7d, similar to 3.7c but from a region nearer the anus. There appears to be extensive staining of the epithelial membrane (E; black arrows) and in the lamina propria (LP; white arrows). There is also heavy staining in and around the underlying vascular supply (CP).

Figure 3.7 is streptavidin-biotin-HRP (sABC-HRP) stained, with Mayer's haematoxylin counterstaining.
Figure 3.7
FIGURE 3.8. THE ABSORPTION OF HGG WITH CONCURRENT ADMINISTRATION OF QUIL-A IN VARIOUS FORMS.

Figure 3.8, the distribution of HGG after oral (3.8a and 3.8b) and anal (3.8c, 3.8d and 3.8e) intubation of antigen concurrently with 1μg/ml Quil-A. Posterior intestine.

3.8a, 1 hour after oral intubation, antigen can be detected in the epithelium (Large arrow; E) and lamina propria (LP; small arrow; st). Antigen is accumulated in the lamina.

3.8b, similar to 3.8a showing the strong association of some antigen with the intralaminal leucocytes (ILL’s, white arrow) and the retention of Quil-A in the lamina rather than in the basal areas of the enterocytes. Legend as above.

3.8c, anal intubated HGG 1 hour after concurrent administration of HGG similar to 3.8a. Staining is intensely associated around the small capillaries in the intestine (CP).

3.8d (insert), Quil-A administered as a micelle (M-arrows) apparently in the process of fusing with the epithelium (E).

3.8e, HGG 2 hours after anal intubation with antigen localised in the lamina propria (LP) and the epithelium (E) largely negative.
Figure 3.8
FIGURE 3.9. THE ACCUMULATION OF HGG AFTER DELIVERY OF QUIL-A.

Figure 3.9 are mid and posterior intestine.

3.9a, accumulated intercellular antigen (heavy black arrows, IAG) in the lamina propria (LP) 1 hour after the oral delivery of antigen as micelles or ISCOMS. In other areas of the lamina propria antigen is found within the cells (CAG; open black triangle) possibly being degraded.

3.9b, similar to 3.9a, showing intercellular antigen (broad black arrows, IAG) in the lamina propria. Cytoplasmic tubules (CT; thinner black arrows) originating from the laminal cells may be seen in contact with the HGG.

Figure 3.9 is two-stage immunoperoxidase stained.
CHAPTER 4.
CHAPTER 4 QUANTITATIVE SEROLOGICAL ASPECTS OF THE UPTAKE OF PROTEINS WITH ENHANCED ENTERIC DELIVERY SYSTEMS.

4.1 INTRODUCTION.

The intestinal epithelium acts as a selective barrier between the intestinal lumen and the systemic circulation. In mammals it has been shown that antigen delivered to the intestinal lumen by the oral or anal route is, under normal circumstances, transported across the enterocytes largely by a transcellular mechanism (Colony and Neutra, 1985) and is capable of causing an immune response (Forrest et al., 1990) that can be substantially protective against pathogens such as Salmonella typhi. Adult mammals have specialised absorptive cells, M cells situated in the Peyer's patches, that allow the passage of intact proteins (Telemo, Westrom and Karlson, 1982), or antigenic fragments of proteins that arise from the processes of luminal and intracellular degradation, to the blood circulation (Morris and Morris, 1978). Transport of antigens in mammals has been demonstrated for a wide variety of antigens, including standard proteins such as BSA (Rothberg, Kraft, Farr, Kriebel and Goldberg, 1970), human serum albumin (HSA), HRP (Owen, 1977), and a wide variety of bacterial and viral antigens (Mestecky 1987). Such transport occurs across the specialised M-cells when in low concentrations (Bye, Allan and Trier, 1984) and through the absorptive enterocytes when present in higher concentrations (Owen and Ermak, 1990). Antigens are therefore absorbed by mammalian intestines in levels which are considered to be capable of eliciting an immune response...
(Walker, 1981). Such antigens may interact with local, underlying, gut-associated lymphoid tissue (GALT) cells to give a mucosal IgA response or reach the blood and lymphatic circulation and eventually generate a systemic IgG and IgM response (Owen and Ermak, 1990). Orally delivered proteins can also cause cells to respond antigen-specifically in tissues such as the spleen, indicating that a broad-range of immune responses can be induced by oral administration of antigen (Clarke, Wilson, Williams and Stokes, 1991).

Absorption of intact proteins and antigenic fragments occurs through the intestines of larval and adult teleosts, to varying degrees, in both gastric (Georgopoulou et al., 1986; McLean, 1987; Georgopoulou et al., 1988; Doggett, 1989) and agastric species (Davina et al., 1980; Rombout et al., 1986; Rombout and van den Berg, 1989). This absorption of protein antigens may be directly quantified on their reaching the systemic circulation (McLean, 1987; Georgopoulou et al., 1988; Doggett, 1989; Doggett, Wrathmell and Harris, 1991). Despite the relative simplicity of teleost GALT (Hart et al., 1988) and its general lack of (morphologically) specialised absorptive cells and organized lymphoid tissue, oral and anal delivery of antigen can result in intestinal accumulation (Davina et al., 1980; Watanabe, 1982; Georgopoulou and Vernier, 1985), transport to plasma and secondarily to major organs such as liver, spleen and kidney (McLean, 1987; Doggett, 1989), observable interactions with lymphoid tissue (Davina et al., 1980; Rombout and van den Berg, 1989) and eventually to relevant immune responses (Johnson and Amend, 1983a, 1983b) that can be protective (Rombout et al., 1989b). Gamma globulins are transcytosed across the intestine, after oral delivery, in large quantities in neonatal mammals (such as suckling rats), by a
receptor-mediated process that effectively confers protective immunity (Breuton, 1980). Absorption of gamma globulins across the intestine of fish appears to be less efficient and slower (Georgopoulou et al., 1986) than in mammals and the relatively-lower immune responses to soluble antigens delivered orally (by comparison with injection) is thought to be due to decreased amounts of antigen being absorbed across the intestine (Johnson and Amend, 1983a, 1983b) or possibly the induction of a tolerogenic response. One strategy to increase the levels of absorbed antigen after oral delivery is to employ the use of an adjuvant or immunopotentiating agent in the delivery system. The use of saponin adjuvants such as Quil-A has been shown to increase the levels of antigen absorbed in mammals after oral administration (Mahraj et al., 1986; Campbell and Bede, 1989) and potentiate the immune responses to these antigens (Campbell et al., 1985; Crick, Brown, King, Williams, Thompson and Fearne, 1985). This involves the use of a variety of preparations that can impart unique physical properties to an antigen (Morein et al., 1984; Morein and Simon, 1985; Morein, 1987). Such delivery systems may allow the use of protein antigens, orally, that would otherwise be non-immunogenic or tolerogenic (Mowat et al., 1991).

Saponins have been largely neglected in studies on the oral administration of antigens in fish and this study aimed to quantify the levels of HGG absorbed into the plasma of Oreochromis mossambicus, along with the temporal kinetics of absorption and any modifying effect that various modes of delivery of the saponin adjuvant Quil-A had on these systems.
4.2. MATERIALS AND METHODS.

4.2.1. ANIMALS.

Tilapia, *Oreochromis mossambicus*, of both sexes weighing 30-50 g were maintained in a recirculating freshwater system at 24-26 °C until use.

4.2.2. INTUBATION PROCEDURES.

Tilapia were intubated perorally and peranally (as described in 3.2.2.) using human gamma globulin (HGG, Sigma) as test antigen.

The effect of dose in groups of tilapia (n = 5) to orally and anally delivered HGG was investigated, at 1 hour, using a range of solutions from 1 mg/ml to 50 mg/ml (intubated volumes of 0.2 ml). Control groups (n = 5) were sham intubated with 0.2 ml PBS pH 7.2.

0.2 ml of an optimised, 10 mg/ml solution of HGG was administered orally or anally to groups of tilapia (n = 5) and the plasma tested over a structured time course viz: after 5, 15, 30 minutes and 1, 2, 6, 12, 24 and 48 hours. Control groups (n = 5) were intubated with 0.2 ml PBS pH 7.2. All intubations were carried out in fixed volumes (5 L) of freshwater in individual, non-circulating, (12 L) tank systems, kept at approximately 24 °C.

4.2.3. ADJUVANT IMMUNISATION.

Quil-A (Superfos, Denmark) was administered, orally and anally to groups of tilapia (n = 5), prior to (1 hour before) and concurrently with HGG, at doses of 0.2 ml from 1 mg/ml to 100 pg/ml viz: 1 mg, 100 μg, 10 μg, 1 μg, 100 ng, 10 ng, 1 ng and 100 pg, by a complementary route, and also over the same time course as above. Quil-A plus HGG was administered as micelles (for preparation see 3.2.3.) and ISCOMS (for preparation see 3.2.4.). Controls were either PBS pH 7.2, HGG
(10mg/ml;0.2ml,anally) on its own or 10mg/ml HGG plus 10μg/ml E.coli lipopolysaccharide (Sigma;0.2ml intubated) for the ISCOM and micelle study, as described previously (3.2.4.).

4.2.4. SAMPLE COLLECTION.

Fish were sacrificed by a sharp blow to the head and blood was collected in heparinised (10 units heparin (Sigma)/ml saline) capillary tubes (25μl volumes) and microhaematocrit tubes (100μl volumes). Blood was pooled for each test group, centrifuged at 4000rpm for 4 minutes and the plasma removed and stored at -20°C until required.

Volumes of water (5ml) were randomly removed from different areas of each test group tank and retained for analysis of levels of voided HGG (regurgitated or excreted) from the test system. Water samples were retained at -20°C until use.

4.2.5. DETECTION OF ABSORBED HGG USING AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA).

The general procedure described by Voller,Bidewell and Bartlett (1979) and Ambler and Peters (1984) was employed as the basis for the optimisation of the assay. All ELISA buffers are listed in Appendix I. All samples and reagents were added at 100μl per well to 96 well flat-bottomed polystyrene microtitre plates (Falcon,Ltd,Becton Dickinson,New Jersey,U.S.A.), unless stated (all immunoreagents were obtained from Sigma, unless stated).

Extensive studies were undertaken to optimise the system for the detection of HGG in tilapia plasma. Two prospective ELISA systems were evaluated, each employing a sandwich antigen-capture technique, one utilising a
homologous antisera system and the other heterologous antisera (Figure 4.1).

**FIGURE 4.1. ELISA SYSTEMS EMPLOYED FOR THE DETECTION OF HGG.**

**HETEROLOGOUS ANTISERA.**

1) Adsorption of goat anti-HGG to plate.
2) Wash with PBST.
3) Addition of HGG in plasma or as standards in PBST.
4) Wash with PBST.
5) Addition of rabbit anti-HGG in PBST (secondary antibody).
6) Wash with PBST.
7) Addition of swine anti-rabbit peroxidase conjugate (Dako, Ltd).
8) Develop.
9) Read at 492nm.

**HOMOLOGOUS ANTISERA.**

1) Adsorption of goat anti-HGG to plate (coating antisera).
2) Wash with PBST.
3) Addition of HGG in plasma or as standards in PBST.
4) Wash with PBST.
5) Addition of goat anti-HGG peroxidase conjugate.
6) Wash with PBST.
7) Develop.
8) Read at 492nm.

All antisera concentrations were optimised using a checkerboard titration method. Plates were coated with primary antiserum diluted in 0.05M carbonate/bicarbonate buffer at pH 9.6 (see Appendix I) overnight at 4°C. The plates were covered in aluminium foil and placed in a humid chamber to prevent evaporation. Plates were sequentially washed (4x3 minutes) after each incubation with approximately 150µl PBST (PBS plus 0.5% Tween 20, Polyoxyethylene sorbitan monolaurate), by aspiration using a Titertek
Handiwash 110 washer (Labsystems, Basingstoke, U.K) and patted dry on paper towels. Antigen containing solution (test plasma, 100% tank water, plasma + known concentrations of HGG or PBST + known concentrations of HGG) was incubated for 2 hours at ambient temperature. This was followed by the addition of either secondary antibody followed by antibody conjugate (heterologous system) or directly by peroxidase conjugate (homologous system) for 1 hour at ambient temperature. Standards of a known amount of HGG were analysed on each plate (from 40μg/ml to 300pg/ml with sequential 1:5 dilutions) to generate a standard curve. All reactions were developed using 2mM orthophenyldiamine plus 3mM hydrogen peroxide in 0.02M citrate-phosphate buffer at pH 5.0 at ambient temperature for up to 10 minutes. Reactions were stopped by the addition of 50μl 1M H₂SO₄ and the plates were read at 492nm on a Titertek Multiscan Plus II (Labsystems, Basingstoke, U.K.) utilising data storage software.

Optimisation of the ELISA system also involved the use of blocking agents such as 1% w/v BSA or 5% w/v non-fat dried milk and the use of test plasma at 2% concentration in PBST, the concentration at which there was the least endogenous cross-reaction caused by the presence of plasma, which was occasionally observed with plasma concentrations of over 10%.

These studies lead to the subsequent use of the homologous ELISA system throughout the study to test the pooled, experimental, tilapia plasma using primary (coating) antisera at 1/4000 dilution, secondary (conjugate) antisera at 1/2000, no protein blocking steps and a development time of approximately 6 minutes.
4.2.7. STATISTICAL ANALYSES.

All ELISA-derived data was analysed using one and two-way analyses of variance using the ANOVA programs on MINITAB software. Briefly, analyses of variance are used to identify sources of variation between data groups e.g. if differences between data are truly due to experimental results or due to natural variability in the uptake of macromolecules by the test groups.

Analyses of variance usually derive a final value of variance ratio (F-statistic) that can be tabulated and significance derived from a follow up test (Wardlaw, 1987). The follow-up test involves the use of the pooled standard deviations (PSD) derived from the ANOVA program in the following equation. Least significant difference (LSD) =

\[
\frac{\sqrt{PSD^2 \times 2}}{N} \times t_{a, \nu} \times t_{a, \nu}
\]

where \( t_{a, \nu} = \) the degrees of freedom. The t-distribution of the LSD is then checked for significance at \( p = 0.025 \). This is the system for the determination of LSD's with groups of data of equal numbers.

Unequally sized groups have LSD's determined by:

\[
[\sqrt{(PSD)^2 \times \left(\frac{1}{n_1} + \frac{1}{n_2}\right)}] \times t_{a, \nu}
\]

Standard error of means (SEM's) were derived by:

\[
\frac{\sum N-1}{\sqrt{N}}
\]
which equivilates to a 75% confidence limit. 95% confidence limits were derived by: 95% limit = SEM x t-statistic.

The derived absorbance readings were statistically treated and the 75% confidence limits added to the representative Figures

4.3. RESULTS.

4.3.1. ABSORPTION OF HGG.

The standard curves typically obtained with the homologous antisera ELISA are shown in Figure 4.2.a. The titration of an antibody response over such a wide range of antigen concentrations is shown, with a typical optimal range of sensitivity from approximately 1.6μg/ml to 2.5ng/ml and an end point of an absorbance value of approximately 1.5 units generally being chosen. Figure 4.3 (a-d) shows typical ELISA systems on microtitre plates with the titration of test plasma in several columns.

The dose response to orally and anally delivered HGG was investigated after 1 hour in order to optimise both the amounts of HGG absorbed and minimise the levels of HGG that are voided by regurgitation and excretion. Figure 4.2.b shows the dose responses to HGG. There is a statistically significant increase (p<0.025) in the amount of detectable HGG absorbed into the plasma 1 hour after anal delivery on comparison with levels detected after oral delivery, for all concentrations of HGG tested. Lower concentrations of anally delivered HGG (1mg/ml and 2mg/ml) resulted in lower absolute levels of uptake of antigen into the plasma and maximal absorption of HGG occurred with 20mg/ml concentrations of antigen though these levels were not significantly higher than those detected after 10mg/ml HGG intubation.
10mg/ml HGG orally delivered was absorbed in significantly (p < 0.05) greater quantities than other concentrations but anally delivered antigen at 20mg/ml was only statistically significantly greater than 1mg/ml. Control delivery of PBS resulted in background levels of approximately 0.2μg/ml (not shown).

HGG was also voided 1 hour after oral and anal delivery (Figure 4.2.c). Orally delivered HGG was voided in greater quantities than anally delivered antigen with the greatest levels of antigen voiding occurring with the intubation of the higher HGG concentrations (20mg/ml and 50mg/ml). The maximal absolute values (in Figure 4.2.c) of voided antigen, however, did not correlate with the values as percentages of the delivered antigen dose (Table 4.1) eg the maximum percentage of voided antigen was approximately 30% of the orally delivered dose (0.2ml of the 1mg/ml preparation) and only between 1.6% and 3.75% of the delivered doses of the higher concentration preparations (10mg/ml, 20mg/ml and 50mg/ml).

As a result a dose of 10mg/ml HGG was chosen for the study in order to maximise levels of absorption after oral and anal delivery, to retain dose standardisation and to keep voided antigen to a minimum.
### TABLE 4.1. VOIDED HGG AS A PERCENTAGE OF DELIVERED ANTIGEN DOSE.

<table>
<thead>
<tr>
<th>CONCENTRATION OF ANTIGEN PREPARATION</th>
<th>DELIVERED DOSE</th>
<th>PERCENTAGE OF THE DOSE VOIDED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mg/ml</td>
<td>200μg</td>
<td>Oral delivery = 30%. Anal delivery = 25%.</td>
</tr>
<tr>
<td>2 mg/ml</td>
<td>400μg</td>
<td>Oral delivery = 25.8%. Anal delivery = 7.5%</td>
</tr>
<tr>
<td>10 mg/ml</td>
<td>2mg</td>
<td>Oral delivery = 3.75%. Anal delivery = 2.5%</td>
</tr>
<tr>
<td>20 mg/ml</td>
<td>4mg</td>
<td>Oral delivery = 1.6%. Anal delivery = 2.4%</td>
</tr>
<tr>
<td>50 mg/ml</td>
<td>12.5mg</td>
<td>Oral delivery = 2.2%. Anal delivery = 1.1%</td>
</tr>
</tbody>
</table>

#### 4.3.2. TEMPORAL ASPECTS OF HGG ABSORPTION.

The absorption of HGG was observed to be temporally variable and significant differences in the levels of absorption at various times due to the route of antigen delivery were observed. Bars on Figures 4.4.a and 4.4.b represent the 95% confidence limits for these data points, all other bars on figures represent the SEM's.
Orally delivered antigen (Figure 4.4.a) was absorbed into the bloodstream after 15 minutes and two peaks of absorption were observed; after 30 minutes and, maximally, after 6 hours. The second absorptive event indicated levels of plasma HGG that were significantly greater than at other times and greater than background values obtained after PBS-intubated controls that resulted in approximate values of 0.1µg/ml (not shown).

Anally intubated antigen (Figure 4.4.b) was absorbed in a temporally different manner (compared to oral delivery) with the uptake being more rapid as shown by peaks of absorption 30 minutes after intubation and maximal absorption after 60 minutes. Both routes of antigen delivery show that the levels of HGG reaching the plasma rapidly decline after 6 to 12 hours resulting in very-low amounts of HGG from 24 to 48 hours. Anal delivery of HGG resulted in both a more rapid absorption and an earlier peak response than oral intubation, with significantly greater amounts detected after 1 hour and significantly less after 6 hours.

4.3.1. ABSORPTION OF HGG 1 HOUR AFTER PRIOR INTUBATION OF QUIL-A.

Quil-A was administered 1 hour prior to delivery of antigen by a complementary route (ie orally or anally) at various concentrations in order to evaluate the dose effect of Quil-A on the absorption of optimised HGG. The optimal dose for the increase of absorption of antigen by both routes was 100µg/ml (Figure 4.4.c) with detectable levels in the plasma being significantly (p<0.05) greater than control administration of HGG. However, outside this concentration, Quil-A appeared to have a negative effect on the absorption of
HGG with levels in the plasma being below control values, albeit not statistically significantly (for anally delivered antigen).

Temporally, prior administration of Quil-A (Figure 4.4.d) altered the uptake kinetics of HGG absorption and the relative levels absorbed. The maximal uptake of anally delivered HGG (after 30 minutes) was significantly higher ($p < 0.05$, almost two-fold greater) than oral delivery (maximal absorption $218 \mu g/ml$, 2 hours after delivery). Increased absorption due to prior Quil-A administration was observed up to 6 hours after delivery.

4.3.3. ABSORPTION OF HGG WITH CONCURRENT ADMINISTRATION OF QUIL-A.

Quil-A administered concurrently with HGG also caused a dose-dependent increase in the amounts of antigen absorbed into the plasma (Figure 4.5.a), 1 hour after intubation. The adjuvant effect of Quil-A administered in this system was observed with concentrations of Quil-A from $1 mg/ml$ to $1 \mu g/ml$ with the peak modification, occurring at $10 \mu g/ml$ for both orally and anally delivered antigen. Oral antigen delivery with concurrent administration of adjuvant was increased above control values (HGG only) but was lower than the values detected for anally delivered antigen (approximately $700 \mu g/ml$). Both routes of delivery showed no increase in the levels of antigen absorbed with concentrations of adjuvant below $100 ng/ml$.

The administration of an optimised dose of Quil-A with HGG, orally and anally, yielded a temporal pattern of absorption that was similar to the temporal kinetics of HGG on its own, but had significantly elevated levels (Figure 4.5.b). Anally administered HGG plus Quil-A was detected maximally after 1 hour; it
was increased 10-fold over control levels of anally intubated antigen and this peak of absorption was significantly ($p < 0.025$) greater than oral administration of antigen plus adjuvant. Oral delivery resulted in biphasic absorption peaks at 1 and 6 hours of 220 and 250 $\mu$g/ml antigen, respectively, which, while being significantly higher than the control values, were not different from each other.

### 4.3.4. ADMINISTRATION OF HGG IN DERIVED TERTIARY FORMATIONS WITH QUIL-A.

The addition of Quil-A in tertiary conformations with HGG *ie* as ISCOMS caused an increase in the levels of antigen absorbed, 1 hour after administration, when compared to the control administration of HGG on its own or with LPS plus HGG (Figure 4.5.c) by 3-fold. Anal administration caused slightly greater absorption of HGG than oral delivery but these differences were not significant for each mode of absorption. Micelle delivery resulted in levels of absorbed HGG that were less significantly higher than that observed with controls. Delivery of HGG plus LPS was not significantly greater than HGG on its own, but the differences between the two routes of absorption were more pronounced as the overall levels were lower.

### 4.3.5. THE VOIDING OF HGG INTO THE TANK WATER.

The pattern of voiding of HGG into the tank water was temporally variable with both anal and oral delivery (Figure 4.5.d). Delivered antigen appeared to be regurgitated into the water in greater amounts after oral administration. The peaks of voiding roughly correlated with the peaks of absorption into the plasma (*ie* 1 hour after anal delivery and 6 hours after oral
delivery). The levels of voided antigen decreased with time and were largely absent after 48 hours.

Voiding also exhibited a dose response with the oral and anal delivery of the adjuvant Quil-A when given prior to (Figure 4.6.a) and concurrently with (Figure 4.6.c) HGG, at various concentrations, after 1 hour. Voiding of antigen was only significantly increased by the higher concentrations of Quil-A (1mg/ml and 100μg/ml), when given 1 hour prior to the antigen (Figure 4.6.a). Concentrations of Quil-A below 1μg/ml did not cause an increase in the levels of HGG evacuated, above control anal administration of HGG. Concurrent administration of HGG plus antigen caused an increase in the levels of expelled antigen over a similarly narrow concentration range, below which levels were the same as controls (Figure 4.6.c). Absolute levels voided at 1mg/ml and 10μg/ml administered orally with both systems were similar (approximately 73-75μg) but were disparate for anal administration (15μg prior and 50μg concurrent).

Voiding of antigen after the intubation of Quil-A (Figure 4.6.b) decreased over time with the greatest levels being voided rapidly after intubation. Levels of voiding were similar, except after 15 minutes where anal delivery resulted in approximately 140μg of antigen being voided. The voiding of HGG with the concurrent administration of Quil-A was much more temporally complex (Figure 4.6.d) with the expulsion of antigen paralleling its absorption into the plasma. However, peak levels in both systems appeared to be consistent at approximately 50μg, for most time periods.

4.4. DISCUSSION.

Complex patterns of absorption after oral and anal delivery are evident
in tilapia, for HGG, which is absorbed into the plasma by mechanisms that can be altered by the action of the adjuvant Quil-A causing a direct increase in the levels of antigen taken into the plasma after its administration as a direct, soluble adjuvant and as a three-dimensional vehicle.

The appearance of detectable HGG in the plasma is closely correlated with its appearance in the intestinal enterocytes as determined by immunocytochemistry. There was a clear dose-response of absorption of HGG into the plasma of tilapia 1 hour after the oral administration of antigen, with a maximum being detected after a single dose of 0.2ml of a 10mg/ml solution. A similar dose response was observed after the oral delivery of a single dose of HRP, in tilapia, with maximal absorption also occurring with a 10mg/ml antigen concentration (Doggett, 1989). Dose responses to orally delivered antigen have also been noted in other species such as Oncorhynchus mykiss where peak intestinal absorption was observed after a single oral dose of 50mg/ml HRP (McLean, 1987) and there appeared to be a direct correlation between the quantity of antigen ingested and the quantity transferred to the blood in O. mykiss in another study employing HRP (Georgopoulos et al., 1988). Such similarity in responses may indicate that the processes of absorption for these antigens are similar between these species and are indicative of saturation-type kinetics on coated-pits at the microvillous surface (McLean, 1987). Multiple doses of antigen applied orally and anally to O. mossambicus did not increase the overall levels of antigens absorbed across the intestine (Doggett, 1989) and were deemed an ineffective strategy for the oral delivery of soluble proteins in tilapia (Doggett et al., 1991).
HGG has been visualised being absorbed rapidly after oral and anal delivery across the intestinal enterocytes of *Oreochromis mossambicus* in antigenically recognisable forms, similar to the absorption of ferritin and HRP, in this species (Doggett, 1989). Absorption occurs by a process involving both transcellular pinocytosis by vesicles and cytoplasmic tubules in the intracellular transportation as described in other species (Iida and Yamamoto, 1985; Rombout et al., 1986; Rombout and van den Berg, 1989; Georgopoulou et al., 1988).

The pattern of absorption of HGG across the intestine is temporally complex and route dependent. Initial absorption of HGG into the plasma was very rapid, being detectable 15 minutes after administration. Maximal levels of HGG were detected in the plasma 6 hours after oral delivery and 60 minutes after anal delivery. Such absorptive kinetics differ from those observed in other studies on the uptake of protein macromolecules by the intestine of tilapia. Oral delivery of ferritin resulted in maximal levels of approximately 300μg/ml in the plasma after 60 minutes (Doggett, 1989), whereas the time course of absorption of HRP was remarkably similar to HGG i.e it was detectable after 15 minutes. However maximal peaks of HRP absorption occurred after 60 minutes and were 7-fold greater than the peak levels of HGG absorption at 6 hours (700μg/ml HRP compared to 100μg/ml HGG; Doggett, 1989). Anally administered HGG was absorbed in greater quantities, more rapidly, in tilapia, than orally delivered antigen, as was observed for ferritin absorption (Doggett, 1989) but in contrast to the results of anal delivery of HRP, the levels of which were almost 2-fold lower anally than orally (Doggett, 1989). Increased absorption of anally delivered antigen, through the circumvention of the gastric conditions of the stomach (in
gastric species), is generally regarded as, possibly, the major reason for the increased efficacy of this route for immunisation (Johnson and Amend, 1983a, 1983b; Ellis, 1988). The stomach also has a complex series of physiological interactions controlling its rate of activity, including its emptying (Jobling, 1988, Dabrowski, 1985), the mechanics of which are generally regarded to be biphasic in nature (Hofer and Newrkla, 1983). Such physiological functions will have a large effect on the temporal patterns of absorption of orally delivered antigens and are also, theoretically, influenced by factors such as the nutritional state of the organism (Soothill, 1976), which may be more important in some species than others (McLean, 1987; Doggett, 1989). Absorption of orally delivered macromolecules is temporally variable in other species. HGG absorption in *O. mykiss* first occurs 10 hours after administration *in vivo* and 90 minutes *in vitro* in isolated enterocytes, and is first observed absorbed 5 hours after anal delivery (Georgopoulou et al., 1986). Reports of studies on absorption of other antigens have been conflicting, with HRP being reported as being detectable in the plasma of *O. mykiss* after 15 minutes and maximally after 1 hour (McLean, 1987; Doggett, 1989) and 7-8 hours after oral intubation in a third study (Georgopoulou et al., 1988). Such differences may possibly be due to variations in the experimental protocol or assay techniques used with some studies (McLean, 1987; Doggett, 1989), relying on either an antigen-capture ELISA (McLean, 1987) and yet others on a combination of ELISA and chemiluminescence (Arrio, Lecuyer, Dupaix, Volfin, Jousset and Carrette, 1980; Georgopoulou et al., 1988). In agastric species the absorption of antigen appears to be equally as rapid, with ferritin being detectable in the
intestinal tissues (Rombout et al., 1986; Rombout and van den Berg, 1989) and plasma (Doggett, 1989) after 60 minutes. There are also disparities in the detectable levels of various antigens after oral and anal delivery in several species with absorbed values from ng (HRP, *O. mykiss*) to µg levels (HRP and ferritin, *O. mykiss*, carp *Cyprinus carpio* and tilapia). Quantitatively, almost 1000 times the amount of antigen was absorbed across the intestine of tilapia than in other species such as rainbow trout and carp (McLean, 1987; Doggett, 1989; Georgopoulou et al., 1986, 1988). In mammals, oral delivery of protein macromolecules results in detectable levels of intact proteins and antigenic fragments of those proteins in the blood (Walker, 1981). 0.1% BSA solution administered to rabbits in drinking water resulted in approximately 60 ng of detectable protein being observed in the circulating plasma, approximately 0.01% of the administered dose. In vitro studies in mammals indicated that 0.1% of human serum albumin, used as antigen, was recoverable from the plasma in definable form 2 hours after oral delivery (Hemmings, 1978). It appears that the levels of protein absorption are approximately 0.02% of the ingested dose in humans and 0.01%-0.1% in other mammals whereas fish show a much greater percentage absorption of proteins, with levels of approximately 4% absorbed in this study. Direct comparisons of antigenic levels must be made with caution as different antigens are likely to be processed in different manners (intra and interspecifically) and fragmentation of antigen can be envisaged where certain macromolecules are split into a variety of potentially antigenic (and immunogenic) portions e.g. a possibility in the processing of ferritin which is made up of 24 identical sub-units
HGG, like other antigens delivered to the intestines of fish, is likely to be primarily absorbed to the plasma via a transcapillary route rather than through any lymphatic system (McLean, 1987) and this is further suggested by the fact that it is visualised near the intestinal capillary beds rapidly after absorption (Chapter 3) in tilapia. The intestinal capillaries of both mammals and fish are thought to be permeable to macromolecules as up to 60% of their surface area is fenestrated which may facilitate the rapid transport of small macromolecules through open fenestrae and incomplete intracellular junctions (Hemmings, 1978). Indeed in mammals studies indicated that of the total of 2% of plasma absorbed BSA 1.1% was transported through the capillary systems underlying the epithelium and 0.8% through the lymphatics.

HGG was detected in the plasma 48 hours after oral and anal delivery but the direct quantitative level decreased after 6-12 hours. This is likely to be an indirect measure of the ongoing antigenic clearance systems which include the transfer of delivered antigen to major organs such as the kidney, liver and spleen (Georgopoulou et al., 1988) which appear to act as repositories for orally and anally delivered HRP and ferritin in tilapia (Doggett, 1989), HRP in rainbow trout and carp (McLean, 1987) and immune-complexes of BSA and antibody in plaice, Pleuronectes platessa (Ellis, 1981). Direct tissue clearance patterns were not measured in this study due to the levels of cross-reactivity obtained with whole body organs in ELISA microtitre systems. Temporally the decrease in the plasma levels of HGG in this study correlate with other studies where most antigen was absent at 24 hours post-delivery (Georgopoulou et al., 1988).
exact mechanics of plasma clearance of antigen are unclear but have been surmised as involving macrophage activity in the venous sinusoids, especially in the pronephros (Smedsrud, Dannevig, Tolleshaug and Berg, 1984), melanomacrophage centres in the spleen, kidney and liver (Ellis, 1981). Indirect stimulation of macrophages, by bacterial challenge, increases plasma clearance mechanisms (Grayson et al., 1987) thus highlighting the probable importance of these cells. The clearance kinetics of antigen from plasma are not fully determined and are described as both monophasic and biphasic for HRP orally delivered to rainbow trout (McLean, 1987; Georgopoulou et al., 1988), but are probably antigen dependent. There was a considerable visual increase in the amounts of HGG absorbed across the intestine on administration after, or concurrently with, the saponin adjuvant, Quil-A (Chapter 3). This increase in the intestinal accumulation of HGG was paralleled by a substantial increase in the amount of antigen in the plasma. There was a distinct dose response to the oral and anal administration of Quil-A 1 hour prior to HGG by a complementary route. Maximum increase in absorbance was observed with the prior delivery of 100 μg/ml Quil-A with the amounts of HGG being absorbed being increased by 66%, orally, and 33%, anally over control levels. Outside this concentration, Quil-A had a negative effect on absorption. Quil-A at this optimal concentration caused a more rapid absorption of anally and orally delivered HGG, in significantly greater levels (10-fold, anally and 5-fold, orally) up to 6 hours post-delivery. A similar pattern of modification was observed on rabies antigen administered to mice after oral treatment with Quil-A (Maharaj et al., 1986), the level of which increased with time up to 16 hours.
In mammals, oral and anal delivery of saponins can increase the absorption of macromolecules such as BSA (Udall et al., 1984) by up to 60% in rats treated with aprotinin (Seifert, Sass and Dreyer, 1984; Telemo et al., 1982).

Concurrent administration of Quil-A with HGG also potentiated the absorption of antigen to the plasma over a narrow concentration range (10μg/ml), after 1 hour. Peak oral and anal levels were increased by 6-7-times and 15-times respectively. The temporal patterns of HGG appeared to be unaffected by this mode of delivery but the overall levels were increased for up to 12 hours after intubation, in contrast to the production of a peak of absorption 2 hours after delivery in mice. Optimal doses of orally delivered Quil-A with rabies antigen, in mice was much higher, 10mg, though potentiation of the immune response was achieved with 125μg of saponin (Maharaj et al., 1986; Campbell et al., 1985).

Administration of antigen as ISCOMS or micelles allows unique properties and adjuvant effects to be imparted to orally delivered antigens. Both ISCOM and micellar delivery of antigens increased the level of absorbed antigen in the plasma but not greater than that observed with other forms of Quil-A administration. However, ISCOMS impart a well-defined (cage-like) three-dimensional structure on the normally soluble HGG which would give it aspects of a particulate antigen as well as inherent adjuvant effects from Quil-A (Dalsgaard, 1987; Morein et al., 1984; Morein, 1987). Particulate antigens appear to be more suited to initiating a mucosal immune response in both fish (Mughal et al., 1986; Rombout et al., 1989b) and mammals (Mestecky, 1987) and HGG administered in these forms was observed accumulated and possibly retained in
the lamina propria of the intestine. Micelles are less-well defined structures that probably act by partially enclosing the antigen and acting as a protective vesicle as well as an adjuvant. The presence of LPS, a known B-cell mitogen did not appear to increase HGG absorption appreciably possibly due to its low concentration or inappropriate mode of delivery.

The absorptive and adjuvant effects of Quil-A are likely to occur by a combination of direct effects on the permeability of the intestine itself (Bangham and Horne, 1962), effects on GALT cells such as macrophages (Campbell and Bede, 1989), direct modification of the antigen ("depot effect") and inhibitory effects on digestive enzymes such as trypsin, pepsin and chymotrypsin that are considered to be the major abrogators of useful oral delivery of antigen. The various modes of delivery of antigen with Quil-A are probably enhanced by most of these systems acting synergistically although the primary relevance of each form of action may alter eg prior delivery may have a greater effect by reducing digestion on antigens by enzymes as it contacts them prior to the delivery of antigen.

Orally and anally administered HGG was found to be voided into the water by regurgitation (observable after oral delivery) or excretion (after anal delivery), one of the few instances in which this observation has been recognised and quantified. HGG was voided in a dose dependent and temporally complex manner with larger absolute amounts being expelled with greater concentrations of HGG delivered and over shorter time periods (15 minutes to 1 hour). More antigen was voided after oral delivery and this contrasted with the expulsion of HRP in tilapia with levels of up to 30% of the intubated dose of HGG.
compared to 3.32% HRP being voided (Doggett, 1989). It is likely that it is the absolute levels of voided antigen that is relevant in the possibility of initiation of an immune reaction via introduction into the organism through another route. Generally, the presence of Quil-A in the delivery system increased the levels of voided antigen. Such demonstrations of expelled antigen through oral and anal delivery into the surrounding tank water lends itself to the possibility of the antigen being absorbed via an immersion form of delivery, being subsequently re-swallowed or having a direct effect on the gills or integument. Immersion vaccines have been found to be substantially protective in certain cases (Ellis, 1988) although their major mode of stimulation has been recently postulated as being through swallowing of the vaccine (Tytler, Tatner and Findlay, 1990) and subsequent stimulation of the GALT. Therefore substantial evacuation of intubated antigen into the immediate surroundings may have unique repercussions on the overall uptake of antigen, its route of introduction and hence on overall antigen delivery strategies.
FIGURE 4.2.

Figure 4.2. 4.2.a shows two standard curves, after HGG titration, from the homologous-antiserum ELISA. 4.2.b is the dose response levels of HGG in the plasma after oral and anal delivery (●●●; oral delivery;大方块; anal delivery). 4.2.c is the dose response of levels of HGG in the tank water after oral and anal delivery (●●●; oral delivery;大方块; anal delivery).
Figure 4.2

Concentration of HGG standards (μg/ml)

Concentration of HGG administered (mg/ml)

Dose of HGG administered (mg/ml)

Level of HGG (μg/ml)
FIGURE 4.3

ELISA MICRO TitRE PLATES SHOWING TYPICAL TITRATION PATTERNS FROM THE HGG ANTIGEN-CAPTURE ELISA.

4.3a, 4.3b and 4.3c show the absorption of HGG (0.2 ml of a 10 mg/ml solution) into the plasma after oral delivery of a range of concentrations of Quil-A from 1 mg/ml to 100 pg/ml, by a complementary route.

4.3a, shows the titration of plasma-borne HGG after intubation of 1 mg/ml (A), 100 μg/ml (B) and 10 μg/ml (C) Quil-A prior to HGG, with controls of negative plasma (NP, no HGG) and no plasma (-P). Standards (STDS) are HGG titrated 1:5 in PBST from 40 ng/ml to 500 ng/ml. Antibody controls are with the absence of primary antibody (1°c) and secondary antibody (2°c) which are also negative in the assay.

4.3b, similar plate layout to 4.3a with the concentrations of Quil-A 1 μg/ml (A), 100 ng/ml (B) and 10 ng/ml (C). Other columns are the same as A). Asterisk (*) shows an anomalous result in the negative plasma column.

4.3c, similar to 4.3a with concentrations of Quil-A at 2 ng/ml (A), 1 ng/ml (B) and 100 pg/ml (C) with other columns being the equivalents of A).

4.3d, shows the titration of voided water samples (plus voided HGG) from the same ELISA as 4.3a, with orally administered Quil-A followed by HGG. Quil-A concentrations are 1 mg/ml (A), 100 μg/ml (B) and 10 μg/ml (C). Plate protocol is as previously with normal water (NW) from controls and columns lacking water (-W). Antisera controls are as previously (1°c and 2°c).
FIGURE 4.4.

Figure 4.4. Figure 4.4.a is the temporal absorption of HGG into the plasma after oral delivery. 4.4.b is the temporal absorption of HGG after anal delivery. Controls are delivery of PBS by a complimentary route (not shown). 4.4.c is the dose-response absorption of HGG 1 hour after the prior intubation of Quil-A from 1mg/ml to 1pg/ml (oral delivery; anal delivery and control anal delivery of HGG). 4.4.d is the temporal absorption of HGG 1 hour after the prior intubation of 100μg/ml Quil-A (Legend as in 4.4.c).
FIGURE 4.4.

Figure 4.4. Figure 4.4.a is the temporal absorption of HGG into the plasma after oral delivery. 4.4.b is the temporal absorption of HGG after anal delivery. Controls are delivery of PBS by a complimentary route (not shown). 4.4.c is the dose-response absorption of HGG 1 hour after the prior intubation of Quil-A from 1mg/ml to 1pg/ml (O oral delivery; △ anal delivery and □ control anal delivery of HGG). 4.4.d is the temporal absorption of HGG 1 hour after the prior intubation of 100μg/ml Quil-A (Legend as in 4.4.c).
Figure 4.4

a)...

b)...

c)...

d)...

Figure 4.4

Figure 4.4

Figure 4.4

Figure 4.4
Figure 4.5. Figure 4.5.a is the dose-response absorption of HGG with the concurrent administration of Quil-A, from 1 mg/ml to 100 pg/ml (O oral delivery; △ anal delivery; □ control anal delivery of HGG). 4.5.b is the temporal absorption of HGG with the concurrent administration of 10 μg/ml Quil-A (Legend as in 4.5.a). 4.5.c is the absorption of HGG into the plasma 1 hour after the oral and anal delivery of Quil-A as ISCOMS and micelles and the control delivery of HGG + LPS and HGG on its own (◼ oral delivery; □ anal delivery). 4.5.d is the temporal voiding of HGG into the tank water in μg/tank (O oral delivery; △ anal delivery).
**Figure 4.5**

- **a)** Concentration of Quilotoa (Log_10 g/ml) over time.
- **b)** Level of HGG in plasma (µg/ml) over time.
- **c)** Mode of distribution of micelles, LPS + HGG, and HGG.
- **d)** Level of HGG in water (µg/l) over time.
**FIGURE 4.6.**

Figure 4.6. Figure 4.6 (a-d) shows the level of voiding of HGG into the tank water (μg/tank). 4.6.a is the dose-response voiding of HGG into the tank water, 1 hour after the prior intubation of Quil-A from 1mg/ml to 100pg/ml ( ○ oral delivery; △ anal delivery and □ control anal delivery of HGG). 4.6.b is the temporal voiding of HGG after the prior intubation of 100μg/ml Quil-A (Legend as in 4.6.a). 4.6.c is the dose-response voiding of HGG with the concurrent administration of Quil-A ( ○ oral delivery; △ anal delivery; □ control anal delivery of HGG). 4.6.d is the temporal voiding of HGG with the concurrent administration of 10μg/ml Quil-A (Legend as in 4.6.c).
CHAPTER 5.
CHAPTER 5. THE QUALITATIVE PROCESSING AND ABSORPTION OF PROTEIN MACROMOLECULES AND THE EFFECTS OF ENHANCED ENTERIC DELIVERY SYSTEMS.

5.1. INTRODUCTION.

The normal physiological function of the alimentary tract in mammals and fish acts to rapidly degrade the majority of antigen after the bulk oral administration of protein (Jobling, 1988; McLean, 1987; Georgopoulou et al., 1988). Such degradative activity is (qualitatively) species-specific and is dependent on a variety of factors including the nature of the antigen delivered and both the mode and state of nutrition of the organism (Hofer, 1982). The oral delivery of protein macromolecules, such as BSA, to mammals results in the uptake of a small percentage (approximately 0.01%-0.1%) of serologically intact proteins and a larger percentage of antigenic breakdown products. These are generated by proteolytic conditions in the stomach and intestine and also by post-absorptive intracellular enzymatic degradation (Warshaw, Walker, Cornell and Isselbacher, 1971; Walker et al., 1972). Such breakdown products may contain degraded fractions that may be non-reactive with antisera to the native whole molecule but still be capable of eliciting an immune response (Hemmings, 1978; Morris and Morris, 1978). Similar fragmentation of proteins has been observed after the oral administration of protein antigens to fish (Georgopoulou et al., 1986, 1988) together with subsequent intracellular release and contact with GALT cells. This may have immunological implications with the eventual induction of a local immune response (Rombout et
However, the production of fragments after the oral delivery of protein antigens may result in the revelation of protein conformations that can act as tolerogenic epitopes (Michael, 1989). Thus, the relatively lower immune responses to orally delivered soluble protein antigens (by comparison with injection) may be due to the decreased amounts of antigen available for absorption across the intestine or the revelation and subsequent presence of tolerogenic epitopes of the antigen. The incorporation of the saponin adjuvant Quil-A may allow the use of protein antigens, orally, that would otherwise be non-immunogenic and possibly induce tolerance (Mowat et al., 1991). Quil-A has been found to substantially increase both the levels of absorbed orally delivered antigens (Maharaj et al., 1986) and the resultant cellular and humoral immune responses (Mowat et al., 1991).

The present study was carried out in order to examine the antigenic fragmentation of orally and anally delivered HGG, the quantitative and temporal aspects of the passage of these fragments to the plasma and any modifying effects that various modes of delivery of the saponin adjuvant Quil-A on these systems.

5.2. MATERIALS AND METHODS.

5.2.1. ANIMALS.

Adult tilapia, Oreochromis mossambicus of both sexes, weighing 30-50g were held, as previously (Chapter 3), in a freshwater recirculating system at 24-26°C, until use.
5.2.2. THE ADMINISTRATION OF HGG AND QUil-A.

Optimised doses of human gamma globulin (HGG MW 150kDa, Crohn fraction II, Sigma; 10mg/ml, 0.2ml dose) were administered to groups of fish (n=5) orally and anally over a structured time course, as previously (as in 3.2.2.). Control groups (n=5) were sham intubated with PBS pH 7.2.

The effect of administration of the purified saponin adjuvant, Quil-A on the qualitative absorption of HGG was also investigated at previously optimised doses (Chapter 4) prior to and concurrently with the antigen, over a structured time course. Quil-A was also administered with the antigen as ISCOMS (as in 3.2.3.) and micelles (as in 3.2.5.).

Control groups of fish were immunised with HGG orally and anally or with a mixture of HGG plus E.coli L:026:B6 LPS (Sigma), for the ISCOM and micelle study.

Fish were sacrificed by a sharp blow to the head and blood obtained in heparinised (10 units heparin (Sigma)/ml saline) microcapillary (25μl volumes) and microhaematocrit tubes (100μl volumes). Blood was centrifuged at 4000rpm for 4 minutes and the plasma removed, pooled for each test group, and stored at -20°C until required.

5.2.3. SDS-PAGE ELECTROPHORESIS.

All reagents, including the 6H, 7H and pre-stained molecular weight markers were composed as in Appendix I (Sigma, Poole, Dorset, U.K.).

A discontinuous sodium dodecyl-sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) system was used throughout the study to analyse plasma
samples from the test and control groups of fish. Discontinuous polyacrylamide gels were used, based on the method of Laemmli (1970) utilising a Mini Protean II electrophoresis system (Bio-Rad, Watford, U.K.) or a LKB 2001 system (LKB, Bromma, Sweden).

SDS-PAGE was carried out on plasma under reducing (ie. sample buffer plus 2-β mercaptoethanol) and non-reducing (ie. without 2-β mercaptoethanol) conditions. Various percentage gels were prepared according to Table 5.1 and polymerisation was carried out using an ammonium persulphate/TEMED system (Hames and Rickwood, 1990).

**TABLE 5.1. THE COMPOSITION OF SOME STACKING AND RESOLVING GELS FOR SDS-PAGE.**

<table>
<thead>
<tr>
<th>11% SEPARATING GEL</th>
<th>7% SEPARATING GEL</th>
<th>3% STACKING GEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>3ml Separating gel buffer.</td>
<td>3ml Separating gel buffer.</td>
<td>1ml Stacking gel buffer.</td>
</tr>
<tr>
<td>9.5ml Separating gel solution.</td>
<td>6ml Separating gel solution.</td>
<td>2ml Stacking gel solution.</td>
</tr>
<tr>
<td>11.5ml SDS solution containing 17mg ammonium persulphate.</td>
<td>11.5ml SDS solution containing 17mg ammonium persulphate.</td>
<td>4ml SDS.</td>
</tr>
<tr>
<td></td>
<td>3.5ml water.</td>
<td>1ml water containing 8mg ammonium persulphate.</td>
</tr>
</tbody>
</table>
Other percentage gels may be made by alteration of the above compositions by manipulation of the following formula:

\[ \% gel(\%) = \frac{g. acrylamide + g. bis}{total. acrylamide} \times 100 \]

Gel sandwiches were assembled on a levelling table. The desired concentration of separating gel was prepared and de-gassed for 15 minutes without the ammonium persulphate. The de-gassed separating gel had the ammonium persulphate added to it and was poured into the gel former and overlaid with water-saturated n-butanol, to exclude air, and left to polymerise for 45 minutes.

The overlay solution was rinsed off completely with distilled water and the stacking gel prepared by degassing and poured onto the separating gel in the presence of a 10-well comb. The stacking gel was left for 45 minutes to polymerise.

Plasma samples were added to the wells, at various concentrations, each optimized for the particular staining or blotting technique employed (see below), in reducing or non-reducing sample buffer. Various preparations of molecular weight markers were used, 6H for high molecular weight protein determination, 7H for low molecular weight determination or pre-stained standards for determinations on immunoblots. Varying quantities of sample were loaded onto the gels - 10μl per well for the Mini-Protean II system and 50μl per well for the LKB 2001 system. Electrophoresis was carried out at a
constant voltage of 200V for 45 minutes (Mini-Protean II) or 300V for 3-4 hours (LKB 2001). Gels were removed and were directly stained or immunoblotted.

5.2.3.1. COOMASSIE BLUE STAINING.

Gels were fixed in Coomassie brilliant blue-R stain overnight at ambient temperature on an orbital shaker and subsequently destained for 4-5 hours with several changes of destain. The sensitivity of detection of this staining system was 0.1-0.5µg of protein per lane (Harlow and Lane, 1988). The optimal concentration of plasma for this form of detection system was found to be 5%.

Coomassie brilliant blue-G staining was carried by fixation of the gel in a trichloroacetic acid-salicylic acid fixative followed by staining in the Coomassie working solution (Appendix I) for 1-2 hours. Destaining was carried out in 10% acetic acid plus 25% methanol for 60 seconds followed by immersion in a 25% methanol solution for 24 hours. Maximal detection sensitivity was 0.05-0.1µg of protein per lane.

5.2.3.2. NEUTRAL SILVER STAINING.

Plasma concentrations were optimised at 2% for this method of detection. Gels were fixed for 30 seconds, washed and equilibrated in an appropriate volume of silver solution for 30 minutes. The silver stain was developed, stopped and a reduction of the stain carried out for 10-30 seconds if necessary. Detection limits were 1-10ng of protein per lane.

5.2.4. IMMUNOBLOTTING (WESTERN BLOTTING).

Electrophoresed plasma proteins were transferred, in Trans-Blot apparatus (Bio-Rad), onto 0.45µm or 0.2µm pore nitrocellulose membranes (Sigma; Schleicher and Schull, Anderman and Co., Kingston-Upon-Thames, U.K.)
according to the method of Towbin, Staehelin and Gordon (1979) in 0.025M Tris, 0.192M glycine plus 20% v/v methanol at pH 8.3 for 18-20 hours at a constant current of 30mA.

5.2.4.1. STAINING OF IMMUNOBLOTS FOR TOTAL PROTEIN.

Initial imunoblots were stained for total protein content to ascertain whether the general pattern of the transferred protein resembled that of equivalent gels. Total protein staining was carried out by 2 methods: the first involving the use of amido black and the second the use of India ink staining.

Amido black staining was carried out by sequentially washing of the immunoblot with PBST (Appendix I) and immersion in Amido black solution for 5 minutes followed by destaining. India ink staining was similarly carried out on a washed blot and was compatible with further immunostaining (Harlow and Lane, 1988).

5.2.4.2. IMMUNOSTAINING.

Western blotting was optimised with respect to blocking protocol, antisera concentrations and incubation times for the detection of absorbed fragments of HGG. Blots were washed in Tris-saline pH 7.5 for 20 minutes and subsequently blocked, for 2 hours, in Tris-saline plus 5% low fat skimmed milk (Tris-saline-milk, TSM), on an orbital shaker at ambient temperature. All antisera were diluted in TSM for incubations and each incubation was preceded by thorough washing of the membranes for 3x3 minutes in Tris-saline and 2x3 minutes in TSM. Blots were incubated in primary antiserum (rabbit anti-HGG) at 1/1500 for 6 hours at ambient temperature. For peroxidase conjugate-labelling, the blots
were incubated with secondary antiserum (swine anti-rabbit IgG peroxidase conjugate) at 1/2000 for 2 hours at ambient temperature. Blots were developed in Tris-saline plus 0.5% w/v 3'3' diaminobenzidine-tetrahydrachloride (DAB) substrate plus 0.03% v/v H₂O₂ for 4-5 minutes and the reaction was terminated by thorough washing in Tris-saline.

For immunogold-silver staining, the blots were treated as above until incubation in secondary antiserum. The secondary antiserum was goat anti-rabbit IgG 5nm gold conjugate in PBS pH 7.2 plus 1:20 porcine gelatin (Sigma), for 2 hours at ambient temperature. The blots were washed for 2x5 minutes in PBS and subsequently for 2x1 minute in distilled water and developed with silver enhancing solution as per manufacturers instructions (Amersham Ltd, Amersham, Herts., U.K.) for 10-15 minutes and the reaction terminated by washing with PBS pH 7.2.

Blots were also analysed for the molecular origin of the absorbed HGG fragments. Blots were incubated with either goat anti-human IgG Fₐb or goat anti-human Fₖ region antiserum at 1/250 in TSM for 4 hours followed by rabbit anti-goat peroxidase conjugate at 1/2500 in TSM for 1 hour and developed as above.

5.2.5. QUANTIFICATION OF GELS AND BLOTS.

All gels and blots were run in the presence of appropriate molecular weight markers. This allowed the accurate estimation of the molecular weights of the HGG fragments by interpolation from log weight versus relative mobility curves. For fragments that appeared to be differentially mobile with the %T of the gels (often apparent for glycoproteins) an asymptotic minimum was
calculated from several gels. Gels and blots were analysed by laser densitometry with a LKB Ultroscan XL densitometer equipped with a helium-neon laser, at 633nm. Densitometry scans were compared to blots directly and a relative quantification of protein bands was carefully made.

5.2.6. STATISTICAL ANALYSES.

All densitometry derived data were analysed using one-way analyses of variance with the incorporated follow-up test that allows derivation of 75% and 95% confidence limits (see Chapter 4).

5.3. RESULTS.

Tilapia plasma is, de facto, very complex and contains a large number of proteins of variable size (molecular weight) and structure (Figure 5.1.a). Figure 5.1.a shows the optimisation of plasma at 5% for Coomassie-blue detection (Lanes c and d) with overloaded plasma in two lanes (at 25%(a) and 15%(b), respectively).

Administration of HGG altered the observable plasma protein profile, in gels, in a temporal and route dependent manner. Reducing gels of plasma after the oral and anal administration of HGG, had noticeable alterations in the overall band patterns (Figures 5.1.b and 5.1.c). Immunoblotting of similar gels and subsequent probing for the antigenic determinants of HGG with specific rabbit antisera revealed a wide range of antigenic fragments of HGG in the plasma (Figures 5.4.a and 5.4.b). These bands were first detectable after 15 minutes and remained, at some level, throughout the study. Table 5.2 shows the apparent molecular weights (Mw's) of the antigenic fragments of HGG detected in the plasma as derived after curve interpolation.
TABLE 5.2. THE APPARENT MOLECULAR WEIGHTS (kDa) OF THE ANTIGENIC FRAGMENTS OF HGG.

<table>
<thead>
<tr>
<th>THE MODE OF ADMINISTRATION OF HGG.</th>
<th>HGG</th>
<th>PRIOR QUIL-A</th>
<th>CONCURRENT QUIL-A</th>
<th>ISCOMS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ORAL</td>
<td>ANAL</td>
<td>ORAL</td>
<td>ANAL</td>
</tr>
<tr>
<td>15.1*</td>
<td>15.1*</td>
<td>27.0*</td>
<td>27.0*</td>
<td></td>
</tr>
<tr>
<td>37.7</td>
<td>37.7</td>
<td>30.2</td>
<td>30.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>42.9*</td>
<td>36.1</td>
<td></td>
</tr>
<tr>
<td>48.8</td>
<td>48.8</td>
<td>41.0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>59.9</td>
<td>59.9</td>
<td>47.2</td>
<td>47.2</td>
<td></td>
</tr>
<tr>
<td>70.3</td>
<td>70.3</td>
<td>-</td>
<td>52.8</td>
<td></td>
</tr>
<tr>
<td>79.8</td>
<td>79.8</td>
<td>-</td>
<td>59.0*</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>92.5</td>
<td>64.5</td>
<td>64.5</td>
<td>48.1</td>
</tr>
<tr>
<td>93.8</td>
<td>93.8</td>
<td>73.8*</td>
<td>73.8*</td>
<td>91.4</td>
</tr>
<tr>
<td>99.7</td>
<td>99.7</td>
<td>-</td>
<td>73.4</td>
<td>109.2</td>
</tr>
<tr>
<td>119.9</td>
<td>119.9</td>
<td>-</td>
<td>75.9*</td>
<td>127.5</td>
</tr>
<tr>
<td>130.9*</td>
<td>130.9*</td>
<td>-</td>
<td>81.2</td>
<td>139.3</td>
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<td></td>
<td></td>
<td></td>
<td>83.0</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>90.8</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>111.1*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>124.2</td>
<td></td>
</tr>
</tbody>
</table>
The mode of administration of HGG

<table>
<thead>
<tr>
<th>HGG</th>
<th>PRIOR QUIL-A</th>
<th>CONCURRENT QUIL-A</th>
<th>ISCOMS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ORAL</strong></td>
<td><strong>ANAL</strong></td>
<td><strong>ORAL</strong></td>
<td><strong>ANAL</strong></td>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>141.2</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>145.3</td>
<td>145.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>149.9*K</td>
<td>--</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* denotes the fragments that are identified by Western blotting as having determinants identified with anti-F<sub>ab</sub> antisera.

§ denotes the fragments that are identified with anti-F<sub>c</sub> antisera.

**Table 5.3. Correlation of relative levels of fragments of HGG with ELISA maxima.**

<table>
<thead>
<tr>
<th>DELIVERY SYSTEM</th>
<th>FRAGMENT M&lt;sub&gt;w&lt;/sub&gt; (kDa)</th>
<th>RELATIVE LEVEL (%)</th>
<th>MEAN PLASMA LEVEL OF HGG (by ELISA; µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGG (ORAL)</td>
<td>59.9</td>
<td>46.4</td>
<td>96.1</td>
</tr>
<tr>
<td></td>
<td>70.3</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>79.8</td>
<td>28.7</td>
<td></td>
</tr>
<tr>
<td>HGG (ANAL)</td>
<td>59.9</td>
<td>31.3</td>
<td>74.1</td>
</tr>
<tr>
<td></td>
<td>119.9</td>
<td>31.3</td>
<td></td>
</tr>
<tr>
<td>PRIOR Q.A. (ORAL)</td>
<td>64.5</td>
<td>31</td>
<td>218.6</td>
</tr>
<tr>
<td>PRIOR Q.A. (ANAL)</td>
<td>64.5</td>
<td>38.3</td>
<td>407.6</td>
</tr>
<tr>
<td>CONC. Q.A. (ORAL)</td>
<td>51.9</td>
<td>41.5</td>
<td>251.8</td>
</tr>
<tr>
<td>CONC. Q.A. (ANAL)</td>
<td>51.9</td>
<td>24.2</td>
<td>687.3</td>
</tr>
</tbody>
</table>
Several of the fragments are positive for determinants associated with the Fc and/or Fab regions of the molecule (Table 5.2). There appears to be less fragments with complete Fab determinants present on comparison with fragments with Fc epitopes and the fragments that are Fab positive are in the Mw size-range that is suggestive of the presence of the whole molecule or the intact Fab portion itself. The data for the Fc is more complex and suggests the presence of determinants on intact immunoglobulin heavy chains.

There was an overall difference in the numbers of fragments derived after oral (10 fragments) and anal (12 fragments) administration of HGG. The apparent Mw’s of the fragments derived from unreduced immunoblots were remarkably similar for both routes of administration. Extensive laser densitometry was carried out on immunoblots of plasma after delivery of HGG and the differences highlighted using this technique correlated with the observable differences on the immunoblots. Figures 5.7 to 5.12 show the quantified levels of each antigenic fragment as a relative percentage of the total detectable HGG level. Such data was derived from scans as exemplified in

<table>
<thead>
<tr>
<th>DELIVERY SYSTEM</th>
<th>FRAGMENT Mw (kDa)</th>
<th>RELATIVE LEVEL (%)</th>
<th>MEAN PLASMA LEVEL OF HGG (by ELISA; µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISCOMS (ORAL)</td>
<td>36.8</td>
<td>46</td>
<td>145.5</td>
</tr>
<tr>
<td>ISCOMS (ANAL)</td>
<td>36.8</td>
<td>46</td>
<td>155.5</td>
</tr>
<tr>
<td>MICELLES (ORAL)</td>
<td>27</td>
<td>51</td>
<td>65.6</td>
</tr>
<tr>
<td>MICELLES (ANAL)</td>
<td>27</td>
<td>49.7</td>
<td>64.3</td>
</tr>
</tbody>
</table>
Figures 5.5 and 5.6. Figure 5.5 shows an immunoblot (5.5.c) of an orally delivered ISCOM preparation and the corresponding laser scan (5.5.a) and quantification (5.5.b) of detected bands and Plate 5.6 shows similar data for a scan of a 7H molecular weight standard protein sample after Coomassie staining. Typical differences may be noticed between the two scans (5.5.a and 5.6.a), showing the complexity of antigenic fragments in the plasma in contrast to the relative simplicity and homogeneity of a predetermined protein sample.

From such laser scans of immunoblotted and probed HGG-containing plasma it was observed that there were temporally variable patterns of absorption or production of antigenic fragments from HGG, from small portions of the molecule to its almost entirely intact structure (Figures 5.7-5.12). There appeared to be several fragments that had a similar pattern of appearance in the plasma, with time, after oral and anal delivery.

Antigenic fragments of 15.1kDa, 37.7kDa, 99.7kDa and the largely intact 130.9kDa were processed in a route-independent manner after delivery. The levels of the two low Mw fragments, 15.1kDa and 37.7kDa, decreased rapidly with time and were at 5% levels 6 hours after intubation. In contrast, the higher Mw fragments, 99.7kDa and 130.9kDa, increased with time after delivery making up 70-80% of the detectable antigen after 12-48 hours. The other fragments of HGG, while being present at the same weights after both routes of delivery, were quantitatively different in their patterns of appearance in the plasma. Anal delivery of HGG resulted in the production of two more fragments than oral delivery, one of 42.9kDa and another of 92.5kDa.

Prior administration of Quil-A, in various concentrations ranging from
1mg/ml to 100pg/ml, 1 hour before HGG delivery resulted in detectable differences in the numbers and apparent quantities of the plasma protein profiles after electrophoresis and neutral silver staining (Figure 5.2). Prior administration of Quil-A reduced the number of bands produced, and their apparent Mw's (Table 5.2, Figures 5.3.c and 5.3.d) with no fragments of over 73.8kDa being detectable. The quantitative processing of the fragments was route-dependent (Figure 5.13) with the apparently preferential production (approximately 30-40% of the total detectable protein) of a fragment of 64.5kDa after both oral and anal delivery (Table 5.3). Anal administration resulted in the production of more bands than oral delivery but there was a significant similarity between the relative levels of the common fragments produced.

Concurrent administration of Quil-A with HGG resulted in an observable alteration in the plasma protein profiles derived after oral and anal delivery (Figure 5.3.a and 5.3.b). Delivery of HGG with Quil-A in this manner appeared to cause a greater degree of division of the antigen into fragments than the other modes of delivery. Orally and anally delivered antigen was processed into a number of fragments that were unique to each route, with only 10 of the 18 bands sharing a common approximate apparent Mw (Figure 5.14). Comparison of immunoblots (Figure 5.3.c lanes 4 and 5) shows that the production of fragments after this form of delivery differed from prior administration of Quil-A (Figure 5.3.c lanes 1, 2 and 3). The lower molecular weight (below 75kDa) bands appeared to be produced in similar levels after both oral and anal administration, whereas route differences were most apparent in the levels of higher molecular weight fragments (90.8kDa, 111.1kDa and 124.2kDa) and the
intact molecule. Greater relative levels of intact proteins were produced by this mode of delivery than any other.

There appeared to be an antigenic fragment of similar Mw 59kDa produced with the oral and anal administration of HGG on its own, with or after the administration of Quil-A.

Oral and anal delivery of ISCOMS and micelles resulted in the production of fragments that were similar to each other but unlike those from other modes of delivery (Figure 5.4.c). There was little route variation in the levels of production of each fragment (Figures 5.15 and 5.16) and maximal levels of antigen represented as a 36.8kDa antigen (Table 5.3).

The maximal relative levels of certain fragments of HGG appeared to correlate with the overall peak levels of HGG as detected by ELISA (Table 5.3).

5.4. DISCUSSION.

The oral and anal administration of HGG to *O. mossambicus* resulted in the absorption of a number of antigenic fragments, of various sizes, being absorbed in various quantities across the intestine to the plasma as well as the largely intact protein. The antigenic fragments ranged from small (18kDa in molecular weight) to almost intact protein (130.9kDa in molecular weight). Analysis revealed that the absorption and qualitative processing of orally and anally delivered HGG varied with time with respect to the relative levels of the fragments detectable in the plasma. More antigenic fragments were derived after anal intubation of HGG than oral delivery, possibly due to the anally administered protein bypassing the stomach and its degradative milieu and hence having an increased retention of detectable fragments. Fragmentation of
orally delivered HGG in *O. mossambicus* resulted in the production of several fragments that were associated with the Fc and/or Fab regions of the molecule. The levels of the Fab positive fragments generally decreased with time possibly indicating that this region of the molecule was more susceptible to degradation. The oral delivery of HGG in other species such as rainbow trout also resulted in the rapid production of antigenic fragments that could be specifically identified as belonging to the Fc and Fab portions of human IgG (Georgopoulou *et al.*, 1986). However this study revealed contrasting results where the Fc region of the molecule was primarily degraded, utilising an everted gut-sac model.

Some fragments, of similar molecular weight, were present after oral and anal delivery of HGG and two fragments, one of 59.9kDa and the other of 70.3kDa, had peaks of relative abundance that correlated with the maximal levels of HGG, as quantified by ELISA. The production of highly antigenic epitopes from orally delivered proteins has been documented for other protein antigens in teleosts, such as BSA (Doggett, 1989; Doggett *et al.*, 1991) and ferritin and antigens from *Vibrio anguillarum* (Rombout and van den Berg, 1989) and is correlated with increased overall levels of plasma-borne antigen. Partially degraded antigenic fragments from BSA, of approximately 40kDa, were detected after oral administration in tilapia (Doggett, 1989). In mammals small fragments and complexes of higher molecular weight, thought to be indicative of BSA in immune-complexes, contributed to the overall plasma levels of the antigen (Schreiber and Walker, 1988). Up to 5% of the total delivered dose of BSA, and between 57.7% and 68.8% of the total absorbed antigen appeared as breakdown products, of between 20-50kDa in mammals (Hemmings, 1978).
Qualitative and quantitative fragmentation of antigen is also dependent on its inherent structure eg macromolecules such as ferritin are likely to be fragmentable into immunologically recognisable epitopes as it is composed of 24 identical subunits (Rombout and van den Berg,1989) whereas BSA is composed of three non-identical domains. Fragmentation of orally delivered antigen is mostly caused by the normal physiological functioning of the stomach and other areas of the alimentary tract where primary degradative events occur. The complex temporal patterns of antigen appearance in the plasma may be due to the mechanisms of fluid retention and gastric emptying that operate in the alimentary tract of teleosts as well as species specific nutritional functions (Jobling,1988). The direct intubation of relatively large volumes of oral antigens, may alter gut retention times, the absolute level of degradation on the antigen and the size and peptide composition of the fragments obtained, as compared to intra-ileal delivery in fish and mammals (Michael,1989).

Intact HGG and fragments of higher molecular weights appeared in increasing amounts 24 to 48 hours after delivery both with and without Quil-A adjuvant, when the overall detectable level of plasma antigen was decreasing. Such an absorptive pattern could be due to the retention of intact antigens in the intestinal tissue. The differential absorption and the retention of intact antigen, in association with GALT cells and the supranuclear vacuoles of the enterocytes, was observed for HGG (Chapter 3), HRP and ferritin in tilapia (Doggett,1989) for ferritin and *V.anguillarum* in carp, *C.carpio* (Rombout and van den Berg,1989) and HRP in Rainbow trout (Georgopoulou et al.,1988) for up to 48 hours whereas antigenic fragments appeared to be more rapidly directed.
to the plasma (Rombout and van den Berg, 1989). Another possibility is that low Mw breakdown products are more rapidly cleared from the vasculature resulting in an apparent accumulation of intact HGG in the plasma, with time.

Administration of Quil-A prior to and concurrently with HGG altered the numbers of fragments, their molecular weights and the temporal pattern of their appearance in the plasma on comparison with the absence of adjuvant. Prior Quil-A delivery resulted in the preferential production of detectable fragments of HGG of low molecular weight, after both oral and anal delivery, (the largest fragment weighing 73.8kDa). Such a process has also been observed for the oral administration of rabies antigen preparations after the delivery of Quil-A (Campbell et al., 1985; Maharaj et al., 1986) where a rapid absorption of fragments of low molecular weight occurred after 2 hours. Concurrent administration of Quil-A with HGG resulted in the widest size range of fragments in the study and the highest levels of absorbed antigen, quantitatively. The level of intact proteins absorbed after 1 hour into the plasma increased. Such differences in the antigen fragment profiles in the plasma can be directly attributed to the adjuvant effects of the saponin Quil-A on the degradative enzymes of the gut, the absorptive capacity of the enterocytes and also on the antigen itself which may include direct structural changes, as noted for the complex rabies G antigen when orally delivered to mice in the presence of Quil-A (Maharaj et al., 1986). Administration of ISCOMS and micelles resulted in altered HGG antigen patterns in the plasma probably via the mechanisms outlined above and also due to the addition of a defined three-dimensional structure to the antigen. (Dalsgaard, 1987; Morein, 1987). Particulate antigens are processed and retained
in a different manner by teleost alimentary tracts and can expect to arrive in the plasma altered in comparison to the administration of the same antigen by other mechanisms. This appeared to be the case in tilapia where the fragmentation profile was different to all others obtained.

Protein fragments derived after the oral and anal delivery of antigen in fish and mammals retain the potential to act as both antigens (Hemmings, 1978; Rombout et al., 1989b) and immunogens (Morris and Morris, 1978). Orally delivered ferritin and *V.anguillarum*, after fragmentation in the enterocytes of the hindgut of carp *C. carpio* produced detectable serum and mucosal antibody titres that appeared to be protective (for *V.anguillarum*) after subsequent challenge (Rombout et al., 1989b). However, oral delivery of antigens such as BSA to mice and the subsequent action of the stomach caused the revelation of epitopal conformations that preferentially induced a tolerogenic response to the subsequent administration of the antigen (Michael, 1989).

The production of breakdown products from delivered proteins and the resultant facilitation of an immune response is enhanced by the use of the saponin adjuvant Quil-A, as observed in mammals on the administration of rabies G antigen in mice (Campbell et al., 1985; Maharaj et al., 1986). It appears that the immune responses to orally delivered antigens in mammals and fish may be directly influenced by the epitopes of the breakdown products that are formed by both intra and extracellular degradation.

This study did not address itself to the immunological consequences of the fragmentation of HGG *ie.* whether the direct production of specific fragments could specifically act as tolerogens or immunogens. The current study
did, however, show that the enteric delivery of HGG results in the production of fragments of the antigen and that the relative levels of the fragments could be differentially altered by various modes of delivery of Quil-A in a complex and specific manner.
FIGURE 5.1.
SDS-PAGE GELS OF TILAPIA PLASMA AFTER THE ADMINISTRATION OF HGG ORALLY AND ANALY.

5.1.a) A 7% reducing SDS-PAGE gel showing various dilutions of normal tilapia plasma from 25% (a), 15% (b) and 5% (c and d) showing the overloading of bands (a&b) and an optimal protein concentration (c&d). Molecular weight markers (6H) show protein distributions from 205kDa to 29kDa. Coomassie blue stain.

5.1.b) A 6% reducing gel of 5% tilapia plasma after the oral administration of HGG (0.2ml of 10mg/ml) at various times: 15 minutes (1), 30 minutes (2), 60 minutes (3), 2 hours (4), 6 hours (5), 12 hours (6), 24 hours (7) and 48 hours (9). Control HGG is shown in lane 8. Molecular weight markers (6H) are as in 1. Coomassie blue staining.

5.1.c) A 6% reducing gel of plasma after the oral and anal administration of HGG after 15 minutes (2), 30 minutes (3), 60 minutes (4), 2 hours (5), 6 hours (6), 12 hours (7), 24 hours (8) and 48 hours (9). Molecular weight markers (6H, lane 1) are as in 1. Coomassie blue staining.
Figure 5.1
FIGURE 5.2.  

SDS-PAGE GELS SHOWING THE PLASMA PROTEINS AFTER PRIOR ORAL AND ANAL ADMINISTRATION OF QUIL-A FOLLOWED BY HGG.

5.2.a) A 11% reducing SDS-PAGE gel showing plasma, optimised at 2%, after the oral administration of Quil-A at various concentrations, 1 hour prior to the delivery of HGG (0.2ml of 10mg/ml). The concentrations of administered Quil-A were 1mg/ml (1), 100µg/ml (2), 10µg/ml (3), 1µg/ml (4), 100ng/ml (5), 10ng/ml (6), 1ng/ml (7) and 100pg/ml (8). Low molecular weight markers (7H) are as in 2. Neutral silver staining.

5.2.b) A 11% reducing SDS-PAGE gel of 2% plasma after the anal administration of Quil-A, at various concentrations, 1 hour prior to the administration of HGG. The Quil-A concentrations were the same as 1) except lane 7 being a 2mg/ml solution. Low molecular weight markers (7H). Neutral silver staining.
FIGURE 5.3.

SDS-PAGE GELS AND IMMUNOBLOTS SHOWING THE PLASMA PROTEINS AND ANTIGENIC FRAGMENTS OF HGG WITH THE ADMINISTRATION OF QUIL-A.

5.3.a) A 8% SDS-PAGE reducing gel showing plasma after the delivery of 100μg/ml Quil-A concurrently with HGG, with oral intubation (O) and anal intubation (A) after 15 minutes (1), 30 minutes (2) and 60 minutes (3). Molecular weight markers (6H) are shown with the same weights as in Plate 10.1 and as shown in Appendix 1. Coomassie blue stain.

5.3.b) A 12% reducing SDS-PAGE gel of plasma showing the distribution of proteins after the administration of 10μg/ml Quil-A concurrently with HGG orally (O) and anally (A) after 15 minutes (1) and 30 minutes (2). Neutral silver staining.

5.3.c) An immunoblot of a non-reducing 11% SDS-PAGE gel showing the distribution of the absorbed HGG fragments 1 hour after the administration of 100μg/ml Quil-A orally (1&3) and anally (2) and with the concurrent administration of 10μg/ml Quil-A orally (4) and anally (5). Pre-stained molecular weight markers (PS) have weights as shown. Immunogold-silver staining.

5.3.d) An immunoblot of a non-reducing 7% SDS-PAGE gel showing the fragmentation of absorbed HGG 1 hour after the administration of 100μg/ml delivered orally (1&3) and anally (2&4) after 60 minutes (1&2) and 2 hours (3&4). Pre-stained molecular weight markers (ps) are as in 3. DAB staining of peroxidase conjugate antisera.
Figure 5.3
FIGURE 5.4.

IMMUNOBLOTS SHOWING THE PRESENCE OF ANTIGENIC FRAGMENTS OF HGG AFTER ORAL AND ANAL DELIVERY.

5.4.a) An immunoblot of a non-reducing 7% SDS-PAGE gel with 2% tilapia plasma after the oral (lanes 1,3&5) and anal (lanes 2&4) delivery of HGG after 15 minutes (1&2), 30 minutes (3&4) and 60 minutes (5) after oral delivery. Pre-stained molecular weight markers (PS) are as in 2). Immunogold-silver staining.

5.4.b) A higher magnification of 1) with the same lane protocol showing observable differences in the band patterns of HGG fragments after oral (1) and anal (2) delivery, with respect to both their numbers and intensity of staining. Molecular weight markers (PS) have weights (kD) as shown. Immunogold-silver staining.

5.4.c) An immunoblot of a 7% reducing SDS-PAGE gel of plasma after the oral and anal administration of ISCOMS and micelles containing HGG. ISCOMS are shown in 3 (anally administered) and 4 (orally administered) and micelles in 1 (anally administered) and 2 (orally administered). Pre-stained molecular weight markers (PS) are as previously. DAB staining of peroxidase-conjugate antisera.
Figure 5.4
FIGURE 5.5.

A TYPICAL LASER DENSITOMETRY SCAN FOR THE PRESENCE OF ANTIGENIC FRAGMENTS OF ABSORBED HGG.

Figure 5.5 shows a typical laser scan of an immunoblot of orally and anally delivered ISCOMS (5.5.a and 5.5.b respectively) stained with DAB. The laser scan (5.5.a) is carried out from the bottom of the blot upwards (directional arrow of c and a) and the relative absorbances and derived percentages shown in 5.5.b.
FIGURE 5.6.

A TYPICAL LASER DENSITOMETRY SCAN FOR THE MOLECULAR WEIGHT MARKERS (7H) OF A SDS-PAGE GEL.

Figure 5.6 shows a typical laser scan of the 7H molecular weight markers on a 11% reducing SDS-PAGE gel. The composition of the markers is shown in Appendix I. The laser scan (5.6.a) is from the bottom of the gel (5.6.c) in the direction shown (arrows). The subsequent quantitative output is shown in 5.6.b. The scan of such a standard protein mixture is much more easily relatable to its appearance compared to the fragments of HGG derived on an immunoblot (Figure 5.5). Coomassie blue staining of a gel. Standards at 10%.
FIGURE 5.7.

THE RELATIVE PROCESSING OF A 15.1kDa AND 37.7kDa ANTIGEN FRAGMENT AFTER ORAL AND ANAL ADMINISTRATION OF HGG.
The processing of orally intubated HGG:
The appearance of a 15.1kDa antigen in the plasma.

The processing of anally intubated HGG:
The appearance of a 37.7kDa antigen in the plasma.
FIGURE 5.8.

THE RELATIVE PROCESSING OF A 48.8kDa ANTIGEN FRAGMENTS AFTER ORAL AND ANAL ADMINISTRATION AND A 42.9kDa ANTIGEN FRAGMENT AFTER ANAL ADMINISTRATION OF HCG.
The processing of orally intubated HGG:
the appearance of a 48.8kDa antigen in the plasma.

The processing of anally intubated HGG:
the appearance of a 48.8kDa antigen in the plasma.

The processing of anally intubated HGG:
the appearance of a 42.9kDa antigen in the plasma.
FIGURE 5.9.

THE RELATIVE PROCESSING OF 59.9kDa AND 70.3kDa ANTIGEN FRAGMENTS AFTER ORAL AND ANAL ADMINISTRATION OF HGG.
The processing of orally intubated HGG:
the appearance of a 59.9kDa antigen in the plasma.

The processing of anally intubated HGG:
the appearance of a 59.9kDa antigen in the plasma.

The processing of orally intubated HGG:
the appearance of a 70.3kDa antigen in the plasma.

The processing of anally intubated HGG:
the appearance of a 70.3kDa antigen in the plasma.
FIGURE 5.10.

THE RELATIVE PROCESSING OF A 79.8kDa ANTIGEN FRAGMENT AFTER ORAL AND ANAL ADMINISTRATION AND OF A 92.6kDa ANTIGEN FRAGMENT AFTER ANAL ADMINISTRATION OF HGG.
The processing of orally intubated HGG:
the appearance of a 79.8kDa antigen in the plasma.

The processing of anally intubated HGG:
the appearance of a 79.8kDa antigen in the plasma.

The processing of anally intubated HGG:
the appearance of a 92.6kDa antigen in the plasma.
FIGURE 5.11.

THE RELATIVE PROCESSING OF 93.8kDa AND 99.7kDa ANTIGEN FRAGMENTS AFTER ORAL AND ANAL ADMINISTRATION OF HGG.
The processing of orally intubated HGG:
the appearance of a 93.8kDa antigen in the plasma.

The processing of anally intubated HGG:
the appearance of a 93.8kDa antigen in the plasma.
FIGURE 5.12.

THE RELATIVE PROCESSING OF 119.9kDa AND 130.9kDa ANTIGEN FRAGMENTS AFTER ORAL AND ANAL ADMINISTRATION OF HGG.
The processing of orally intubated HGG:
the appearance of a 119.9kDa antigen in the plasma.

The processing of anally intubated HGG:
the appearance of a 119.9kDa antigen in the plasma.
FIGURE 5.13.

THE RELATIVE PROCESSING OF ANTIGEN FRAGMENTS AFTER ORAL AND ANAL ADMINISTRATION OF QUIL-A PRIOR TO THE DELIVERY OF HGG.
The processing of HCG after the admin. of Galt-A prior to oral delivery of HCG.

Level of antigen in plasma (% HCG).

Molecular weight of antigen (kDa).

The processing of HCG after the admin. of Galt-A prior to oral delivery of HCG.

Level of antigen in plasma (% HCG).

Molecular weight of antigen (kDa).
FIGURE 5.14.

THE RELATIVE PROCESSING OF ANTIGEN FRAGMENTS AFTER ORAL AND ANAL ADMINISTRATION OF QUIL-A CONCURRENTLY WITH HGG.
The processing of HGG after oral admin. concurrently with Quil-A.

Level of antigen in plasma (% HGG).

Molecular weight of antigen (kDa).

The processing of HGG after the oral admin. concurrently with Quil-A.

Level of antigen in plasma (% HGG).

Molecular weight of antigen (kDa).
FIGURE 5.15.

THE RELATIVE PROCESSING OF ANTIGEN FRAGMENTS AFTER ORAL AND ANAL ADMINISTRATION OF HGG AS ISCOMS.
The processing of HGG after the oral administration of ISCOMs.

Level of antigen in plasma (% HGG).

Molecular weight of antigen (kDa).

The processing of HGG after the oral administration of ISCOMs.

Level of antigen in plasma (% HGG).

Molecular weight of antigen (kDa).
FIGURE 5.16.

THE RELATIVE PROCESSING OF ANTIGEN FRAGMENTS AFTER ORAL AND ANAL ADMINISTRATION OF HGG AS MICELLES.
The processing of HGG after the oral administration with Oul-A as micelles.

Level of antigen in plasma (E HGG).

Molecular weight of antigen (kDa).

The processing of HGG after the oral administration with Oul-A as micelles.

Level of antigen in plasma (E HGG).

Molecular weight of antigen (kDa).
CHAPTER 6.
CHAPTER 6. THE ANTIBODY RESPONSES TO ORAL AND ANAL DELIVERY OF PROTEIN ANTIGEN IN ENHANCED ENTERIC DELIVERY SYSTEMS.

6.1 INTRODUCTION.

The absorption of antigens from the gastrointestinal tract, in mammals, can lead to the stimulation of specific antibodies locally, systemically and also in secretions at mucosal sites "remote", anatomically, from the site of antigen delivery (Mestecky, 1987; Nicklin, 1987). The oral delivery of a wide range of antigens to mammals can result in their subsequent absorption and a specific immune response that can be protective against infectious agents such as Salmonella typhi (Black et al., 1990; Forrest et al., 1990) that gain access to the host organism via the gastrointestinal tract. The repeated oral delivery of standard antigens such as bovine serum albumin (BSA) has been reported in mammals, to result in a serum immunoglobulin response that is comparable to parenteral immunisation with respect to titre and antibody isotype production (Rothberg et al., 1970; Nicklin, 1987) and to the generation of antibody-secretory cells in the plasma (Kantele, 1991). The antibody response generated at mucosal sites, especially the intestine, largely consists of dimeric secretory IgA (sIgA), an isotype that is uniquely adapted to secretion across epithelial surfaces and to resistance against lumenal proteolysis (Brandtzaeg et al., 1988; Mestecky, McGhee and Elson, 1988). Fish have been reported as only having one major isotype of immunoglobulin, a tetrameric IgM-like molecule, although the systemic and mucosal forms may have some structural differences and indeed recent evidence...
lends itself to the possibility of more widespread differences in immunoglobulin structure than was previously presumed (Lobb and Clem, 1981a-d; Kobayashi and Tomonaga, 1988; Davidson, 1991). Similar studies have resulted in the observation that the oral delivery of some proteins, especially dietary and standard, T-dependent, antigens cause a specific systemic hyporesponsiveness (tolerogenesis) that is possibly mediated by the induction of specific T-suppressor cells (Elson, 1985; Nicklin, 1987; Michael, 1989). Mammalian studies therefore indicate that the immune response to orally delivered antigen is variable and dependent on a series of interrelated factors including the nature of the antigen delivered, the dose, timing and frequency of its administration and the nutritive status and genotype of the recipient organism (Ada, 1990; Peng et al., 1990).

The oral and anal delivery of a variety of protein antigens has been reported to elicit systemic and/or mucosal immune responses, both of a humoral and cellular type in fish (Davina et al., 1980; Johnson and Amend, 1983a, 1983b; Ellis, 1988; Rombout et al., 1989b; Doggett, 1989; McLean and Donaldson, 1990). The oral delivery of protein antigens to fish has shown that it is possible to immunise enterically with both soluble and particulate antigens (Georgopoulou et al., 1986; Doggett, 1989; Rombout et al., 1989b), and for such immunisation to be effective in generating a protective immune response in secretions anatomically separated from the alimentary tract. This may indicate the existence of a common mucosal immune system in fish (Kawai, Kusuda and Itami, 1981; Kawai and Kusuda, 1983; Rombout et al., 1985; Rombout et al., 1989b; Davidson, 1991), similar to that in mammals (Arya, 1990). However, studies also show similar variation to oral stimulation in mammals, with the
induction of an appropriate immune response being variable, at best. Oral immunisation of teleosts has been found to induce protective immunity against subsequent bacterial challenge (Kawai et al., 1981; Rombout et al., 1989b), but to also possibly result in a route-dependent tolerogenic response to soluble protein antigens (Whiskovsky and Avtalion, 1982; Mughal et al., 1986; Davidson, 1991) and especially to those characterised as thymus-dependent (Rombout et al., 1989b).

The abrogation of potential oral tolerance, and the possible enhancement of any resultant immune responses, may be facilitated by the use of specific adjuvants in oral delivery systems. The saponin adjuvant Quil-A has been reported in several studies in mammals, as being highly effective in enhancing the immune response to both the oral and parenteral delivery of protein antigens (Dalsgaard, 1987; Kensil et al., 1991; Mowat et al., 1991). The delivery of Quil-A has been reported to increase the absorption of standard protein antigens and to increase the systemic antibody titre to molecules such as BSA, by 100-fold (Kensil et al., 1991). Quil-A can also be manipulated to form specialised immune-stimulatory complexes (ISCOMS) that can be used to impart a degree of immunogenicity (by their three-dimensional nature) on proteins that are otherwise tolerogenic when administered orally, in their native state (Mowat et al., 1991). The delivery of soluble protein macromolecules to Oreochromis mossambicus perorally or peranally has been shown to be enterically absorbed in amounts that are potentially immunologically significant (see Chapter 4) and that the quantitative levels of the proteins, their qualitative nature and their absorption kinetics may all be significantly altered by the incorporation of the saponin adjuvant Quil-A in the delivery system (see Chapter 5).
The aims of the current study were to monitor one aspect of the immune response notably antibody production to an enterically delivered standard protein antigen (HGG). The current study also investigated the results of the use of the saponin adjuvant Quil-A in the enhancement of any specific immune responses obtained following its oral and anal delivery in a variety of forms with the antigen.

The test of the efficacy of the delivery methods was the initiation of an antigen-specific antibody response, both systemically and mucosally. However, this imposes a certain degree of limitation to the investigation with respect that this is only one indicator of an efficient, specific, immune response (albeit that it is the parameter most often measured in investigations of this type and that the formation of an antibody response, at a minimum, requires cellular interactions in the form of antigen presentation and appropriate T-cell involvement) (Sanderson and Walker, 1991). There are other assay systems that can potentially be employed to monitor the efficacy of oral immunisation, especially with respect to the direct induction of an observable cellular immune response. This study tests the efficacy of the delivery systems employed, coupled with the analysis of mucosal secretions which may give an indication of the possibility of the induction of a specific adaptive immune response at mucosae distanced from the delivery site, through the operation of a common mucosal immune system. Immunisation studies employing different antigens may require emphasis on the enhancement of a different aspect of the immune response.
6.2. MATERIALS AND METHODS.

6.2.1. ANIMALS.

Adult tilapia, *Oreochromis mossambicus*, of both sexes, weighing 50-100g, were held in a recirculating freshwater system at 24-26 °C throughout the study.

6.2.2. IMMUNISATION PROTOCOLS.

Groups of fish (n=5) were immunised once (on day 0) with HGG with or without optimised concentrations of Quil-A (see Chapter 4), as follows:

1) **HGG immunisation:** orally, anally or intraperitoneally with 0.2ml HGG solution (10mg/ml in 0.85% saline).

2) **Prior administration of Quil-A with HGG:** orally or anally with 0.2ml Quil-A (10μg/ml in 0.85% saline) followed after 1 hour by 0.2ml HGG solution (10mg/ml in 0.85% saline).

3) **Concurrent administration of Quil-A with HGG:** orally or anally with 0.2ml HGG solution (10mg/ml in 0.85% saline) plus 1μg/ml Quil-A.

4) **Quil-A used as vehicles for HGG:** orally or anally with ISCOMS or micelles prepared as in 3.2.2.

5) **Control immunisation:** orally or anally with 0.2ml 0.85% saline *in lieu* of antigen and/or adjuvant, to act as an experimental control.

6.2.3. COLLECTION OF SERUM, BILE AND MUCUS.

Fish were bled from the caudal sinus 0, 7, 14, 21, 28 and 35 days after immunisation using heparinised (10 units heparin (Sigma)/ml saline) syringes (Luckham, Watford, U.K.) with 19g needles. The blood was pooled, centrifuged at 4000rpm for 5-6 minutes and the plasma removed and stored at -20 °C prior to their assay for specific antibody via ELISA.
Bile was removed, by syringe, from the gall bladder and dialysed against 0.05M Tris 0.15M NaCl pH 7.5 (Tris-saline) overnight at 4°C, with stirring, to remove the bile salts. The resultant solution was further dialysed against flake polyethylene glycol (Aquacide III, Calbiochem, Behring Diagnostics Ltd, U.K.) until volumetrically similar to the original sample.

Cutaneous mucus was removed using a method based on the protocol of Burgess (1988). Briefly, mucus (in aliquots of approximately 120 μl) was collected by the use of absorbent cotton wool buds (Boots, Ltd) and subsequently eluted by washing and squeezing with approximately 680 μl PBS (pH 7.2). The resultant solution was dialysed against 0.05M Tris 0.15M NaCl pH 7.5 (as above) and subsequently dialysed against flake polyethylene glycol until volumetrically similar to the original sample. The cutaneous mucus was stored at -20°C until use.

6.2.4. ELISA FOR THE DETECTION OF ANTI-HGG ANTIBODIES.
6.2.4.1. PRODUCTION OF RABBIT ANTI-TILAPIA IgM ANTISERUM.

Large adult tilapia, 150-200 g were initially immunised intraperitoneally with a 0.2 ml solution of sheep red blood cells (SRBC) emulsified in an equal volume of Freund's Complete Adjuvant (FCA). The fish were boosted by repeated immunisation (as above) three times at two-weekly intervals with antigen (SRBC) emulsified in Freund's Incomplete Adjuvant (FIA).

Fish were bled, using non-heparinised syringes and needles, via the caudal sinus and the blood centrifuged at 4000 rpm for 5-6 minutes. The serum was removed and subjected to haemagglutination against a 2% solution of SRBC.
The tilapia IgM-SRBC agglutinate was harvested and emulsified in an equal volume of FCA and 1ml injected into Dutch rabbits at two sites subcutaneously (SC). The rabbits were boosted by two SC injections of antigen, with FIA, at 6-weekly intervals. Rabbits were bled via the marginal ear vein, the blood stored at 4°C overnight and serum collected following centrifugation for 5-6 minutes at 4000rpm. The serum was then tested for its specificity for tilapia IgM by immunoelectrophoresis.

Immunelectrophoresis was carried out using the method outlined by Hudson and Hay (1989). Briefly, microscope slides (75 x 21 x 1 mm) were cleaned in methanol and coated with a thin gel of 1% (w/v) agar in 0.08M barbitone buffer pH 8.2. Electrophoresis templates were cut in the agar and 10μl of fish serum placed in the side wells with specific rabbit antiserum in the central trough. Serum was electrophoresed at 30mA constant current for approximately 2 hours with bromophenol blue dye as a front marker. Electrophoresed gels were left overnight for development of precipitin arcs, washed in PBS, thoroughly dried and stained with Coomassie blue.

A single precipitin arc was produced corresponding to the specific reaction of the rabbit antiserum with the tilapia IgM.

This rabbit anti-tilapia IgM antiserum was aliquoted and stored at -70°C until use.

6.2.4.2. SPECIFIC ELISA FOR THE DETECTION OF ANTI-HGG ANTIBODIES.

Tilapia plasma, bile and cutaneous mucus were assayed for the presence of anti-HGG antibodies using a specific, indirect, enzyme-linked immunosorbent
assay (ELISA) employing heterologous antisera, based on the method of Voller et al., (1979).

Samples and reagents were added at 100μl per well to 96-well flat bottomed polystyrene microtitre plates (Falcon, Ltd, Becton Dickinson, New Jersey, U.S.A.) unless stated. Extensive studies were carried out to optimise the coating dilution of HGG on the plates, the initial dilution of the plasma, bile and cutaneous mucus added to the plate and the dilution of the rabbit anti-tilapia IgM (by checkerboard titration).

Plates were coated with 20μg/well HGG in carbonate/bicarbonate buffer pH 9.6 overnight at 4°C. Plates were subsequently washed (4x3 minutes) after each incubation with approximately 150μl phosphate-buffered saline plus 0.5% Tween (PBST) by aspiration, using a Titertek Handiwash 110 (Labsystems, Basingstoke, U.K.) and then patted dry on paper towels.

Test tilapia plasma was optimised at an initial dilution of 10% (in PBST and also in non-immune tilapia serum), bile at 10% (in PBST) and cutaneous mucus at 2.5% (in PBST). Plasma, bile and cutaneous mucus were doubly diluted, from these initial dilutions, down the plate and incubated for 2 hours at ambient temperature in a humid environment.

The system employed for the detection of the captured anti-HGG antiserum involved the use of the rabbit anti-tilapia IgM (optimised at 1/500, in PBST) for 1 hour at ambient temperature followed by swine anti-rabbit immunoglobulin peroxidase conjugated antiserum (optimised at 1/1000, in PBST) for 1 hour at ambient temperature.

All reactions were developed using 2mM ortho-phenyldiamine plus 3mM
hydrogen peroxide (OPD-H$_2$O$_2$) substrate solution in 0.02M citrate-phosphate buffer at pH 5.0 at ambient temperature for up to 10 minutes.

The reactions were terminated by the addition of 50μl 1M H$_2$SO$_4$ to each well of the plate. Absorbances were read at 492nm on a Multiscan Mk II microplate reader (Labsystems,Basingstoke,U.K.) utilising data handling and storage software.

6.2.3.3. CONTROLS.

The experimental controls were as outlined in 6.2.2. Technical controls involved the omission of the test solutions (plasma,bile and cutaneous mucus),the primary antiserum and the secondary antiserum, independently, to test for residual binding and background activity in the test assay.

6.2.4. STATISTICAL ANALYSES.

Statistical analyses were carried out on the derived antibody titres. The degree of significance of difference between specific antibody titres was determined using the chi-squared test (Wardlaw,1987).

6.3. RESULTS.

A typical standard curve derived from the ELISA assay for the presence of anti-HGG antibodies in tilapia plasma, bile and cutaneous mucus is shown in Figure 6.1.

Figure 6.1. specifically shows the antiserum titration from the ELISA assay of plasma, 14 days after the oral and anal immunisation of HGG with the concurrent delivery of 10μg/ml Quil-A. Control immunisation of saline resulted in background absorbances of approximately 0.1 absorbance units (Figure 6.1) throughout the study. The control levels were mathematically removed from the
derivation of the antibody titres with the use of the ELISA-hardware data storage software, and are not shown on the remaining figures.

6.3.1. HGG IMMUNISATION WITHOUT QUIL-A.

The results of oral, anal and intraperitoneal (IP) HGG immunisation are shown in Figures 6.2 (a-c), respectively. IP immunisation of HGG resulted in peak titres of specific plasma anti-HGG antibodies that were significantly greater ($p < 0.05$) than the maximal titres obtained after oral and anal delivery (Figure 6.2). Plasma antibody responses to IP immunisation were significantly greater ($p < 0.05$) than mucosal antibody titres, in the bile and cutaneous mucus. IP delivery of HGG resulted in rapid initial antibody responses in the plasma (after 7 days), which peaked after 30-35 days. IP mucosal titres were also significantly greater than those obtained after oral and anal immunisation (Figures 6.2.b and 6.2.c). The antibody response in the cutaneous mucus was, after IP immunisation, lower than the response in the plasma and bile.

Oral and anal delivery of HGG resulted in lower antibody titres in the plasma, bile and mucus than after IP immunisation. Anal immunisation resulted in higher antibody titres, in the plasma and bile (Figures 6.2.a and 6.2.c) than oral immunisation, albeit not statistically significantly. Anal immunisation resulted in a more rapid peak in plasma and biliary antibody responses than after IP or oral delivery. Oral immunisation consistently resulted in longer peak antibody generation times (Figures 6.2.b and 6.2.c). There were very low antibody responses generated in the cutaneous mucus after either oral or anal immunisation of HGG.
6.3.2. THE ADMINISTRATION OF QUIL-A PRIOR TO HGG IMMUNISATION.

The administration of 100μg/ml Quil-A 1 hour prior to the delivery of HGG (by a complementary route) resulted in a significant alteration in the temporal kinetics and levels of the antibody response (Figures 6.3 (a-c)) by comparison with HGG administered alone. Quil-A was not administered via the IP route due to the toxicity of saponins delivered to fish in this manner (Lindbland, 1985)

Oral immunisation of HGG, with the prior delivery of Quil-A (Figure 6.3.a), resulted in a rapid peak plasma antibody response (after 14 days) which was approximately 15-fold greater than that after immunisation with HGG alone. Similarly, mucosal responses after oral immunisation were initiated more rapidly (Figures 6.3.b and 6.3.c) and were of greater peak levels than their non-adjuvanated counterparts and in the case of the biliary response, by almost 2-fold. Significant levels of antibodies were detectable in the cutaneous mucus after this form of delivery (Figure 6.3.c).

Anal HGG administration, with the prior delivery of Quil-A, resulted in a very rapid antibody response in the plasma (often after < 7 days), which declined more rapidly than that observed after oral delivery but which was of an equal magnitude (Figure 6.3.a). However anal immunisation resulted in a more rapidly initiated response, and greater peak levels of antibodies in bile and cutaneous mucus than after oral delivery (Figures 6.3.b and 6.3.c).
6.3.3. THE IMMUNISATION OF QUIL-A CONCURRENTLY WITH HGG.

The concurrent administration of 10μg/ml Quil-A with HGG orally and anally resulted in generation of antibody responses in the plasma (Figure 6.4.a) that were lower by comparison with those obtained after immunisation of HGG following prior administration of Quil-A.

Oral immunisation resulted in peak antibody levels in the plasma (after 28 days) (Figure 6.4.a) that were approximately 4-fold less than those obtained after oral immunization with prior Quil-A delivery but significantly greater than those obtained with the oral delivery of HGG alone. Biliary and cutaneous mucus antibody levels after oral immunisation (Figures 6.4.b and 6.4.c) were similar to those achieved with antigen delivered after Quil-A treatment. Peak plasma and mucus antibody levels were similar after oral and anal delivery, after 14-28 days (Figures 6.4.a and 6.4.c) but peak biliary levels were greater after anal delivery (Figure 6.4.b). Mucosal antibody titres (in the bile and cutaneous mucus) were considerably higher than obtained after HGG immunisation on its own.

6.3.4. THE IMMUNISATION OF QUIL-A WITH HGG AS ISCOMS.

The delivery of HGG as ISCOMS resulted in antibody responses in the plasma, bile and cutaneous mucus that were quantitatively very similar (Figures 6.5. (a-c)). Plasma antibody levels paralleled each other after oral and anal immunisation with ISCOMS but resulted in lower titres (Figure 6.5.a) than with either delivery of Quil-A prior to or concurrently with HGG. However, plasma antibody levels after the oral and anal delivery of ISCOMS were greater than
with HGG, alone.

Mucosal antibody responses were higher in the bile than the cutaneous mucus after both oral and anal HGG delivery as ISCOMS (Figures 6.5.b and 6.5.c). However the mucosal responses after the oral and anal delivery of ISCOMS (Figures 6.5.b and 6.5.c) were not significantly different than after other methods of immunisation. Peak responses in the bile and cutaneous mucus occurred 14 days after anal delivery whereas they occurred 21 and 28 days after oral delivery, respectively. Plasma, bile and mucus antibody titres were considerably elevated above those observed after the oral and anal delivery of HGG alone.

6.3.5. THE IMMUNISATION OF QUIL-A WITH HGG AS MICELLES.

The antibody responses to the oral and anal immunisation with micellar HGG in the plasma and cutaneous mucus (Figures 6.6.a and 6.6.c) were temporally and quantitatively similar to those observed after the corresponding immunisation with ISCOMS. The biliary antibody response with micellar HGG immunisation resulted in marginally higher peak titres after oral immunisation by comparison with anal immunisation (Figure 6.6.b); however the overall levels of both were lower than the corresponding biliary antibody responses after immunisation with ISCOMS.

Table 6.1 is a table showing the peak levels of antibody production in the plasma, bile and cutaneous mucus after the various modes of delivery of HGG and the time taken for the generation of this response, post-immunisation.
TABLE 6.1. ILLUSTRATING THE PEAK LEVELS OF ANTIBODY PRODUCTION (AND THEIR TIMES OF GENERATION POST-IMMUNISATION).

<table>
<thead>
<tr>
<th>ROUTE OF ADMINISTRATION.</th>
<th>Intraperitoneal</th>
<th>Oral</th>
<th>Anal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyte</td>
<td>P.</td>
<td>B.</td>
<td>CM.</td>
</tr>
<tr>
<td>HGG</td>
<td>5.42</td>
<td>2.11</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>(28)</td>
<td>(35)</td>
<td>(28)</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>(28)</td>
<td>(35)</td>
<td>(28)</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>1.7</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>(21)</td>
<td>(21)</td>
<td>(28)</td>
</tr>
<tr>
<td>QA&gt; HGG</td>
<td>3.31</td>
<td>1.81</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>(14)</td>
<td>(28)</td>
<td>(28)</td>
</tr>
<tr>
<td></td>
<td>3.31</td>
<td>2.11</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>(7)</td>
<td>(21)</td>
<td>(14)</td>
</tr>
<tr>
<td>HGG+QA</td>
<td>2.71</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>(28)</td>
<td>(28)</td>
<td>(28)</td>
</tr>
<tr>
<td></td>
<td>2.71</td>
<td>2.11</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>(21)</td>
<td>(21)</td>
<td>(14)</td>
</tr>
<tr>
<td>ISCOMS</td>
<td>2.41</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>(14)</td>
<td>(21)</td>
<td>(28)</td>
</tr>
<tr>
<td></td>
<td>2.71</td>
<td>1.81</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>(14)</td>
<td>(14)</td>
<td>(14)</td>
</tr>
<tr>
<td>MICELLES</td>
<td>2.41</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>(21)</td>
<td>(21)</td>
<td>(21)</td>
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<tr>
<td></td>
<td>2.41</td>
<td>1.2</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>(14)</td>
<td>(14)</td>
<td>(21)</td>
</tr>
</tbody>
</table>

Key: Unparenthesised numbers refer to the maximal HGG-specific titres observed (1/ - Log_{10} antibody titre) and the numbers in parenthesis refer to the time of generation of the titres, post immunisation (Days).

Analyte refers to the fluid analysed, where P is plasma, B is bile and CM is cutaneous mucus.

Columns marked - indicate that this particular test was not carried out.
The nomenclature in the left hand column refers to the mode of delivery of HGG where: HGG equals delivery without adjuvant; Q.A. < HGG refers to the delivery of Quil-A prior to HGG; HGG + Q.A refers to the delivery of HGG plus Quil-A; ISCOMS refers to the delivery of HGG as immune stimulating complexes; MICELLES refers to the delivery of HGG as a micellar package with HGG.

6.4. DISCUSSION.

The present study shows that the oral, anal and intraperitoneal delivery of a single, standard dose of HGG to *O. mossambicus* results in a primary immune response evidenced by considerable levels of antigen-specific antibodies in the plasma, bile and cutaneous mucus. The production of specific antibodies, was monitored by the utilisation of a specific ELISA system which quantified levels of specific antibodies rather than overall immunoglobulin (Ig) levels. Detection of specific antibodies rather than overall Ig levels is extremely valuable in comparing the relative immunogenicity of antigens (Ellis, 1985; Burgess, 1988) and in the determination of any possible induction of specific immunity in secretions anatomically remote from the intestine, and hence in determining the potential existence of a common mucosal immune system. Intraperitoneal injection of HGG resulted in significantly higher titres of antibodies in the plasma than by oral or anal immunisation. A similar route-dependent increase in the plasma levels of antibodies was observed after intraperitoneal injection of BSA in *O. mossambicus* by comparison with oral and anal immunisation (Doggett, 1989). In this study intraperitoneal immunisation
of BSA resulted in specific titres of 1/209600 and oral and anal immunisation in titres of only 1/32 and 1/16, respectively. Administration of HGG intraperitoneally to carp, *Cyprinus carpio*, resulted in similar levels of antibody production by comparison with those seen in the current study, when a specific indirect haemagglutination assay was used; with peak antibody titres of $\log_2 14-20$ and $\log_2 7-8$ being produced after the administration of HGG and antigens from *Aeromonas salmonicida*, respectively (Mughal et al., 1986) although the ELISA system is generally regarded to be more sensitive than haemagglutination.

Anal administration of HGG generated greater levels of plasma antibodies than oral delivery in tilapia but less than those achieved by intraperitoneal injection. The efficacy of anal immunisation has also been observed in carp where the repeated anal delivery of ferritin resulted in antibody responses that approximated to the levels achieved with intramuscular injection (Rombout et al., 1989b). The delivery of ferritin (associated with food) *per diem*, for a period of 8 days, resulted in antibody titres that were at least 2-fold greater than those achieved against HGG in this study (Rombout et al., 1989b).

The delivery of HGG orally (*per diem*, for 5 or 10 days) in rainbow trout, *Oncorhyncus mykiss*, resulted in detectable antibody responses in the plasma which were approximately 6-fold greater than the plasma antibody titre generated after a single intraperitoneal injection of the antigen (Davidson, 1991). However, in contrast, multiple doses of antigen did not result in an increase in the levels of antigen absorbed from the intestine or in the resultant antibody response in *O. mossambicus* (Doggett, 1989).

The oral and anal delivery of HGG also resulted in lower titres of
antibodies in the bile and cutaneous mucus by comparison with intraperitoneal injection, although the disparity was not as great as that evidenced between the respective plasma responses. Several other studies have identified antibodies in the secretions of fish including the bile of channel catfish Ictalurus punctatus (Lobb and Clem, 1981d), sheepshead, Archosargus probatocephalus (Lobb and Clem, 1981c), dogfish, Scyliorhinus canicula (Hart, 1987), carp, Cyprinus carpio (Rombout et al., 1986) and rainbow trout Oncorhyncus mykiss (Davidson, 1991). BSA administered orally and anally also resulted in low, but specific, titres of antibodies in the cutaneous mucus and bile of O. mossambicus (Doggett, 1989). Soluble antigens such HGG and ferritin have been reported as being poor immunogens, at least for the stimulation of specific mucosal antibodies in fish. No specific cutaneous mucus antibodies were found after the oral administration of ferritin in carp (Rombout et al., 1989b), and very low levels of antibodies were found in the bile and cutaneous mucus of rainbow trout after immunisation with HGG (Burgess, 1988). However antibodies have been generated in the cutaneous mucus of channel catfish, Ictalurus punctatus, after bath immunisation (Lobb, 1987) and antibodies have been detected in the cutaneous mucus of both rainbow trout Oncorhyncus mykiss (Harrell et al., 1976; St Louis Cormier et al., 1984; Davidson, 1991) and the sheepshead A. probatocephalus (Lobb and Clem, 1981b). The oral delivery of particulate, T-independent bacterial antigens such as those from Vibrio anguillarum resulted in relatively high levels of specific, protective, antibodies in the cutaneous mucus with no detectable systemic titres (Rombout et al., 1989b). Such results may lend some explanation to the observation that protection against bacterial challenge, after vaccination, is very
often correlated with a definite lack of specific, systemic antibodies (Bienenstock and Befus, 1980; Kawai et al., 1981; Kawai and Kusuda, 1983; Ellis, 1988; Davidson, 1991) and can also be efficiently initiated by the enteric delivery of suitable antigens (Johnson and Amend, 1983a, 1983b).

The kinetics of the primary immune response to HGG generated after oral, anal and intraperitoneal immunisation of tilapia, was monitored for up to 35 days post-immunisation. The antibody generation curves show that the immune responses to HGG in the plasma, bile and cutaneous mucus all paralleled each other, with the antibody response after oral and anal immunisation being largely short-lived (of approximately 21 days duration) after a single HGG dose. Anal immunisation appeared to elicit slightly greater levels of antibodies more rapidly than oral immunisation. Intraperitoneal immunisation generated a plasma response with a short latent period, that appeared to be tenable past 35 days. A transient antibody response was also observed after single oral, anal and intraperitoneal immunising doses of BSA in tilapia (Doggett, 1989), resulting in plasma antibody kinetics that were very similar to those observed here after immunisation with HGG. Anti-HGG antibody titres generated in the serum and bile peaked after 28-35 days and after 21-28 days in the mucus and the persistence of these antibody titres were shorter than those generated in carp, after boosted, oral and anal administration of ferritin and V.anguillarum (Rombout et al., 1989b). In this study the antibody responses after oral immunisation appeared to be tenable for approximately 28 days, after which time a boosting immunisation was required to maintain the levels of antibody (Rombout et al., 1989b). This study also confirmed the observation that
protection against bacterial infection is often not correlated with the levels of plasma antibodies but may be associated with mucosal titres or other factors. Other studies have also shown that oral and anal delivery of antigens can generate an antibody response that can be remarkably long-lived (Ourth, 1980) and maintained so that it can be almost totally protective against subsequent bacterial challenge for up to 1 year post-immunisation (Fryer, Rohovec and Garrison, 1978). Mucosal antibody titres have also been shown to be specifically elevated for extended periods of time, after oral and anal immunisation; for example up to 63 days in carp (Rombout et al., 1986) and for up to 96 days after bath immunisation, in channel catfish (Lobb, 1987).

The current study therefore provides further evidence for the existence of a common mucosal immune system in fish as the enteric delivery of antigens is clearly able to generate substantial levels of specific antibodies in mucosal tissues divergent from the intestine. Specific antibodies have also been reported as being variably inducible in the intestinal mucus, after oral stimulation (Fletcher and White, 1973; Davidson, 1991). Oral immunisation in the plaice Pleuronectes platessa resulted in antibody levels that were maintained for up to 1 year after delivery of the antigen possibly indicating that there was a significant memory component in the lymphoid tissue responsible for the generation of this population of antibodies (Fletcher and White, 1973). Specific antibodies have also been induced in the intestinal mucus after immunisation in carp (Rombout et al., 1986). However, conflicting evidence has been shown where chub experimentally infected with the Acanthocephalan, Pomphorhynchus laevis, failed to produce any discernable antibodies in the intestinal mucus.
(Harris, 1972) and more recently no intestinal antibody could be induced, after the delivery of HGG and αKLH, in the rainbow trout *O. mykiss* despite considerable levels of specific antibodies generated in other secretions, such as the bile and cutaneous mucus (Davidson, 1991). Parenteral immunisation has also been reported to be able to induce specific antibodies in mucosae such as the female genital tract of the dogfish, *Scyliorhinus canicula* (Hart, 1987). Mammalian studies have also indicated that local and generalized immune responses, manifested by the parallel appearance of sIgA, in mucosal secretions distant from the site of immunisation, can be induced after the enteric delivery of antigens (Mestecky *et al.*, 1985; Mestecky, 1987). Oral immunisation in mammals has led to the observation that antibodies can be induced in milk after natural infections, or immunisation, with enteric pathogens such as *Salmonella typhimurium* (Montgomery, Lemaitre-Coelho and Vaerman, 1980; Sheldrake *et al.*, 1984; Mestecky, 1987) and that enteric stimulation of GALT triggered antibody responses in saliva, bronchial, biliary and intestinal secretions often with only minimal serum antibody titres (Montgomery *et al.*, 1980; Gyure, Hall, Hobbs and Jackson, 1991).

There appears to be a general consensus in both fish and mammalian studies that the enteric, and especially the oral, delivery of antigens is less efficacious in producing systemic antibody titres than parenteral immunisation (Mestecky, 1987; Nicklin, 1987; Rombout *et al.*, 1989b; Davidson, 1991) but are still capable of eliciting considerable levels of mucosal antibodies. One of the major factors that may cause such observed disparities in the levels of antibodies generated after enteric and parenteral immunisation is the decreased levels of
antigen encountering target lymphoid tissue, after enteric immunisation and the resultant requirement for a greater antigen dose to generate equivalent immune responses to those obtained after parenteral immunisation (Ellis, 1988). However the present study shows that the oral and anal delivery of human gamma globulins (HGG), which consists, mostly, of the active IgG component, results in the absorption of detectable amounts of antigen across the intestinal enterocytes of \emph{O. mossambicus} over a wide range of time. HGG is rapidly absorbed and transcytosed, in a complex manner, across the enterocytes and is exocytosed into the lamina propria where it comes into contact with a variety of laminal cells including GALT cells. HGG was observed in contact with GALT cells apparently of the macrophage lineage and also with non-granular lymphocyte-like cells. Immunoreactivity of these cells was thought to parallel similar results in carp (Rombout and van den Berg, 1989) where it was considered to be indicative of active antigen presentation processes and it has also been established in mammals that such cells are an \textit{a priori} requirement for the initiation of local immune responses (Chain \textit{et al.}, 1988).

Other studies have also indicated that the oral and anal immunisation with soluble protein antigens can result in measurable levels of absorbed antigens and subsequent interactions of such antigen with the local immune cells of the GALT (Davina \textit{et al.}, 1980; Georgopoulou \textit{et al.}, 1986, 1988; Rombout \textit{et al.}, 1986; Davidson, 1991). Such interactions of absorbed antigen with immunocompetent cells at the site of absorption may be a major factor in the observation that enterically delivered antigens can elicit relatively high local immune responses and the eventual generation of immune responses at other
mucosae. Absorbed antigen has been observed interacting with GALT cells in *O.mossambicus* (Doggett, 1989; see Chapter 3), rosy barb, *Barbus conchonius* (Davina *et al.*, 1980) and carp (Rombout *et al.*, 1986; Rombout and van den Berg, 1989) and antigen-specific antibody secreting cells have been detected and enumerated in the disaggregated intestine of rainbow trout, *O.mykiss*, after oral immunisation with HGG (Davidson, 1991) but the eventual fate of the released antibody in such cells could not be apportioned, with any degree of certainty, to the luminal or serosal compartments of the intestine (Davidson, 1991).

Surface-immunoglobulin positive cells (Ig+) cells have also been observed in the intraepithelium of the intestine of the carp (Rombout *et al.*, 1989a), where they comprise approximately 10% of the cells; in the lamina propria of the rosy barb (Davina *et al.*, 1980) and histochemically in the intestinal lymphoid tissue of the dogfish (Hart, 1987; Hart *et al.*, 1988). The production of a mucosal immune response in secretions such as the cutaneous mucus is not thought to be due to the sequestration of serum antibodies (Lobb and Clem, 1981d) as a poor transport system is believed to exist, in fish, for the transport of immunoglobulins from the serum to mucosae such as the skin epidermis (Harrell *et al.*, 1976; Lobb and Clem, 1981c; Rombout *et al.*, 1989b).

The generation of mucosal antibodies, such as those observed in this study, are thought to be initiated, after oral immunisation, at a local level by either direct antigen stimulation of resident lymphoid tissue at each respective mucosal surface or that antigen specific lymphocytes (both of B and T cell lineage) are generated in the GALT and eventually migrate and seed the relevant mucosal surfaces (Rombout *et al.*, 1989b; Davidson, 1991).
The results of mammalian studies vary as to the generation of a mucosal immune response. Some mammals, such as humans, directly derive approximately 50% of their biliary sIgA and 2% of their intestinal sIgA from the serum (Delacroix et al., 1982a, 1982b) whereas in other mammals these values are considerably elevated (Vaerman and Delacroix, 1984; Mestecky and McGhee, 1987). Other studies have indicated that the GALT can be stimulated to preferentially produce antigen-specific antibody secreting cells after the oral immunisation of ovalbumin (van der Heijden, Bianchi, Bokhout, Dol, Scholten and Stok, 1989) and that populations of such antibody-secreting cells in the intestinal mucosa can seed other mucosal surfaces (Kagnoff, 1987; Mowat, 1987; Nicklin, 1987). The relatively low levels of antibodies generated after oral and anal immunisation of HGG in *O. mossambicus* may be due to the initiation of systemic hyporesponsiveness (tolerance) that appears to be observed after the oral delivery of soluble protein antigens in fish (Whiskovsky and Avtalion, 1982; Mughal et al., 1986; Rombout et al., 1989b; Davidson, 1991) and mammals (Andrew and Hall, 1982; Mowat et al., 1991). Tolerogenesis is usually manifested as the reduced efficacy of the generation of a secondary immune response, and although secondary immune responses were not investigated in this study, intestinally mediated immunosuppression of circulating antibody titres has been reported in carp (Mughal et al., 1986; Rombout et al., 1989b) to a variety of antigens such as ferritin and soluble and particulate HGG and also in rainbow trout, where the simultaneous administration of HGG orally and intraperitoneally caused a dose-dependent tolerance (Davidson, 1991). Such systemic vulnerability to a decrease in antibody titre may be dependent on the form of the antigen and
its degree of T-dependency (Davidson, 1991).

The enteric delivery of HGG to *O. mossambicus* results in its detectable fragmentation (see Chapter 5), and the serum appearance of such fragments may also be partly responsible for any systemic hyporesponsiveness after oral antigen delivery. The absorption of soluble protein antigens across the mammalian gastrointestinal tract results in the revelation of epitopes of the molecule that are stimulatory for T-suppressor cells and also in the corresponding loss of determinants specific for T-helper cells (Kay and Ferguson, 1989a, 1989b) both of which result in immune tolerance to the antigen and subsequently to minimal antibody responses (Bruce and Ferguson, 1986a, 1986b; Michael, 1989).

The delivery of the saponin adjuvant Quil-A has been reported, in mammals, to be an efficient adjuvant and to also allow generation of specific immune responses to orally delivered antigens that otherwise induce tolerance (Maharaj *et al.*, 1986; Mowat *et al.*, 1991). The oral and anal administration of Quil-A in combination with HGG to *O. mossambicus*, in a variety of delivery systems, resulted in a substantial increase in the primary immune response (as monitored by an increase in HGG-specific antibody titres in the plasma, bile and cutaneous mucus) compared to the levels achieved after immunisation without adjuvant. The current study records the first demonstration of Quil-A as an enteric adjuvant and its effectiveness in increasing both mucosal and systemic antibody responses in these animals.

The oral and anal administration of Quil-A, prior to the delivery of HGG by a complementary route, resulted in the generation of similar antibody titres in the plasma and bile. However anal immunisation resulted in greater antibody
titres in the cutaneous mucus. Immunisation with HGG, orally and anally, after Quil-A resulted in an approximately 100-fold increase in the plasma and a 2-3-fold increase in the biliary antibody levels over those achieved after HGG immunisation on its own, but were still not of a comparable level to those following the IP administration of the antigen (1/2048 titres generated after oral delivery of Quil-A followed by antigen compared to 1/64 and 1/300000 titres raised after oral and IP administration of HGG on its own). Antibody titres in the cutaneous mucus after delivery of HGG, preceded by Quil-A, were elevated to approximately the same levels as those derived after the intraperitoneal injection of HGG on its own. These adjuvant-modified antibody titres were initiated more rapidly than the direct delivery of HGG but their persistence was not increased. The antibody titres achieved after the enteric delivery of HGG, in combination with Quil-A in this manner, were comparatively higher than those obtained after the oral, anal and intramuscular immunisation of carp with ferritin or V.anguillarum (Rombout et al., 1986, 1989b) without adjuvant.

The oral and anal delivery of HGG concurrently with Quil-A elicited raised antibody titres above those observed with the comparable non-adjuvanated immunisations but were slightly lower than those with HGG delivered after Quil-A. Similarly, the mucosal antibody titres were raised 4-6 fold over their non-adjuvanated counterparts.

The oral and anal immunisation of O.mossambicus with HGG plus Quil-A in the form of ISCOMS and micelles resulted in elevated antibody titres compared to oral and anal HGG immunisation. ISCOMS yielded slightly greater antibody responses than micelles in all the secretions studied and the
temporal disparity between the antibody generation curves was greatest in the mucosal immune responses after oral and anal immunisation with ISCOMS.

The addition of Quil-A, prior to or concurrently with HGG significantly raised the levels of antigen that were absorbed after oral and anal delivery (Chapter 4). The amounts of HGG absorbed and retained in the intestine of *O. mossambicus* were greatly increased by the oral and anal administration of the antigen with the saponin adjuvant Quil-A. Quil-A also increased the levels of antigen absorbed into the plasma. Any potential immune response to orally or anally delivered antigen is influenced by both the level of antigen present in the intestine and its qualitative nature *ie* if it is intact or fragmented, the nature of the fragments produced, whether it is soluble or particulate and its degree of T-dependency (Whiskovsky and Avtalion, 1982; Mughal *et al*., 1986). The delivery of Quil-A, enterically, also has a direct effect on the intestinal enterocytes of *O. mossambicus* (Chapter 3), such as increasing the intestinal permeability, shortening and damaging the villi and causing pinocytosis of coincident luminal material. The oral delivery of Quil-A, in combination with both soluble protein antigens and more complex glycoproteins derived from micro-organisms, was reported to elicit and increase both the levels of antigen absorbed from the mammalian gastrointestinal tract and to cause a resultant increase in the immune response to the antigen (Maharaj *et al*., 1986; Kersten *et al*., 1988a, 1988b; Mowat *et al*., 1991) in some studies but to be ineffective via the oral route in others (Trudel, Nadon, Seguin, Boulay and Lussier, 1987). The use of Quil-A, in fish, to date is limited to its incorporation in an immersion vaccine against *Yersinia ruckeri* (Grayson *et al*., 1987) and to its administration with
Antigen preparations (Cossarini-Dunier, 1985), both of which were ineffective and indeed the latter appeared to have a negative effect on the generation of antibody titres (Cossarini-Dunier, 1985).

The increased antibody responses with prior and concurrent Quil-A delivery with HGG may reflect the increase in antigen absorption observed with these delivery systems, but Quil-A administration also greatly alters the patterns of antigen fragmentation observed and also the temporal appearance of these fragments in the plasma (Chapter 4). The immune response to oral delivery of antigen may possibly be tolerogenic for some soluble antigens, as outlined previously, with gastric degradation playing an important role in the production of the epitopes responsible. Thus, as Quil-A alters the profile of degraded antigens appreciably in both a qualitative and quantitative manner, the reduction of tolerogenic protein conformations and/or the increase in the revelation of epitopes specific for T-helper cells may be as important as the direct increase in antigen absorption in the production of the observed increased antibody response to orally and anally delivered antigen. The delivery of HGG as ISCOMS and micelles also increase the antibody titres generated to levels approximating those observed with prior and concurrent Quil-A administration with HGG, which would not be expected if the sole adjuvant effect of Quil-A was its direct ability to increase antigen absorption, as the delivery of ISCOMS and micelles caused significantly lower peak levels of antigen absorption. The implications of this are that the alterations in the plasma antigen fragmentation patterns observed are indeed immunologically relevant and that Quil-A has a direct effect on the lymphoid tissue it encounters (primarily the GALT) in some
manner that causes an increased immune response or that, specifically, the delivery of HGG as ISCOMS and micelles imparts a degree of "particularity" to the antigen due to their intrinsic three-dimensional natures. All these factors have received a degree of support in recent investigations where the derivation of specific fragments of soluble protein are highly linked to immunosuppression and oral tolerance (Kay and Ferguson, 1989a, 1989b). Quil-A has been observed as being able to increase the production and release of immunomodulators such as cytokines and monokines (Campbell and Bede, 1989) and that particulate antigens may be more immunogenic at mucosal surfaces than, unmodified, soluble ones (Rombout et al., 1989b) and that ISCOM delivery in particular can preferentially cause a greater increase in mucosal immune responses (Mowat et al., 1991). The production of a delivery system that allows the generation of a relevant and protective immune response with a single immunising dose is likely to be a requirement for any oral vaccination regime, considering the various problems encountered with oral tolerance and immune response variability observed in both mammals and fish.

In conclusion, the present study shows that the oral, anal and intraperitoneal delivery of a single dose of HGG results in the generation of considerable levels of antibodies in the plasma, bile and cutaneous mucus of O. mossambicus and that the enteric delivery of the antigen in combination with the saponin adjuvant Quil-A in a variety of delivery systems can cause a significant increase in the specific antibody responses elicited, both systemically and mucosally.
FIGURE 6.1.

Figure 6.1 shows the plasma antiserum titration 14 days after the oral and anal delivery of 10mg/ml (0.2ml intubated) HGG concurrently with or after Quil-A. HGG + Q.A. (O) is the oral delivery of HGG concurrently with Quil-A; Q.A. > HGG (O) is the oral delivery of HGG after the delivery of Quil-A; HGG + Q.A (A) is the anal delivery of HGG concurrently with Quil-A; Q.A. > HGG (A) is the anal delivery of HGG after the delivery of Quil-A; the control is the anal delivery of saline.
Fig. 6.1 Antibody response in the plasma after the oral and anal delivery of HGG after or concurrently with Quil-A.

Absorbance (492nm).

14 day response.

- □ HGG+Q.A (0)
- ▲ Q.A>HGG (0)
- × HGG+Q.A (A)
- ◊ Q.A>HGG (A)
- Control
FIGURE 6.2.

Figure 6.2.a shows the temporal antibody response in the plasma after the oral, anal and intraperitoneal (IP) immunisation with 10mg/ml (0.2ml dose) without adjuvant. Figure 6.2.b shows the temporal antibody response in the bile after the oral, anal and intraperitoneal (IP) immunisation with 10mg/ml (0.2ml dose) without adjuvant. Figure 6.2.c shows the temporal antibody response in the cutaneous mucus after the oral, anal and intraperitoneal (IP) immunisation with 10mg/ml (0.2ml dose) without adjuvant.
Fig. 6.2a  Antibody response in the plasma after the delivery of HGG.

Fig. 6.2b  Antibody response in the plasma after the delivery of HGG.

Fig. 6.2c  Antibody response in the cutaneous mucous after the delivery of HGG.

Antibody response (−1/log 10).

Plasma response.

- - - Oral immunisation.
- - - Anal immunisation.
- - - IP. Immunisation.
FIGURE 6.3.

Figure 6.3.a shows the temporal antibody response in the plasma after the oral and anal immunisation with 10mg/ml HGG (0.2ml dose) after the prior administration of 100μg/ml Quil-A. Figure 6.3.b shows the temporal antibody response in the bile after the oral and anal immunisation with 10mg/ml HGG (0.2ml dose) after the prior administration of 100μg/ml Quil-A. Figure 6.3.c shows the temporal antibody response in the cutaneous mucus after the oral and anal immunisation with 10mg/ml HGG (0.2ml dose) after the prior administration of 100μg/ml Quil-A.
Figure 6.3

**Figure 6.3.a** Antibody response in the plasma after the delivery of Quilt-A prior to HGG.

**Figure 6.3.b** Antibody response in the bile after the delivery of Quilt-A prior to HGG.

**Figure 6.3.c** Antibody response after the delivery of Quilt-A prior to HGG.
FIGURE 6.4.

Figure 6.4.a shows the temporal antibody response in the plasma after the oral and anal immunisation with 10mg/ml HGG (0.2ml dose) with the concurrent administration of 10μg/ml Quil-A, by a complementary route. Figure 6.4.b shows the temporal antibody response in the bile after the oral and anal immunisation with 10mg/ml HGG (0.2ml dose) with the concurrent administration of 10μg/ml Quil-A, by a complementary route. Figure 6.4.c shows the temporal antibody response in the cutaneous mucus after the oral and anal immunisation with 10mg/ml HGG (0.2ml dose) with the concurrent administration of 10μg/ml Quil-A, by a complementary route.
Figure 6.4. Antibody response in the plasma after the concurrent delivery of HGG and Qui-A.

Antibody response ($-1/\log 10$).

Time after immunisation (days).

Fig. 6.4.a Antibody response in the plasma after the concurrent delivery of HGG with Qui-A.

Antibody response ($-1/\log 10$).

Time after immunisation (days).

Plasma response.

Oral immunisation.

Anal immunisation.
FIGURE 6.5.

Figure 6.5.a shows the temporal antibody response in the plasma after the oral and anal immunisation with 10mg/ml HGG (0.2ml dose) with Quil-A as immune-stimulatory complexes (ISCOMS). Figure 6.5.b shows the temporal antibody response in the bile after the oral and anal immunisation with 10mg/ml HGG (0.2ml dose) with Quil-A as immune-stimulatory complexes (ISCOMS). Figure 6.5.c shows the temporal antibody response in the cutaneous mucus after the oral and anal immunisation with 10mg/ml HGG (0.2ml dose) with Quil-A as immune-stimulatory complexes (ISCOMS).
Figure 6.5

Figure 6.5.a Antibody response after the delivery of HGG as ISCOMS.

Figure 6.5.b Antibody response after the delivery of HGG as ISCOMS.

Figure 6.5.c Antibody response after the delivery of HGG as ISCOMS.

Antibody response $(-1/\log 10)$.

- Oral immunisation.
- Anal immunisation.

Plasma response

- Oral immunisation.
- Anal immunisation.
FIGURE 6.6.

Figure 6.6.a shows the temporal antibody response in the plasma after the oral and anal immunisation with 10mg/ml HGG (0.2ml dose) with Quil-A as micelles. Figure 6.6.b shows the temporal antibody response in the bile after the oral and anal immunisation with 10mg/ml HGG (0.2ml dose) with Quil-A as micelles. Figure 6.6.c shows the temporal antibody response in the cutaneous mucus after the oral and anal immunisation with 10mg/ml HGG (0.2ml dose) with Quil-A as micelles.
Figure 6.6

Antibody response after the delivery of HGG as micelles.

**Figure 6.6.b**

Antibody response $(-1/\log 10)$

<table>
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<tr>
<th>Time after Immunisation (days)</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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</tr>
<tr>
<td>5</td>
<td>0.4</td>
</tr>
<tr>
<td>10</td>
<td>0.6</td>
</tr>
<tr>
<td>15</td>
<td>0.8</td>
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<tr>
<td>20</td>
<td>1.0</td>
</tr>
<tr>
<td>25</td>
<td>1.2</td>
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</tr>
<tr>
<td>35</td>
<td>1.6</td>
</tr>
<tr>
<td>40</td>
<td>1.8</td>
</tr>
</tbody>
</table>

- Biliary response
- Oral immunisation

**Figure 6.6.c**

Antibody response $(-1/\log 10)$

<table>
<thead>
<tr>
<th>Time after Immunisation (days)</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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</tr>
<tr>
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<td>0.4</td>
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<tr>
<td>25</td>
<td>1.2</td>
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<td>30</td>
<td>1.4</td>
</tr>
<tr>
<td>35</td>
<td>1.6</td>
</tr>
<tr>
<td>40</td>
<td>1.8</td>
</tr>
</tbody>
</table>

- Cutaneous mucous
- Oral immunisation

**Figure 6.6.a**

Antibody response after the delivery of HGG as micelles.

**Antibody response ($-1/\log 10$)**

<table>
<thead>
<tr>
<th>Time after Immunisation (days)</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>5</td>
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<td>1.6</td>
</tr>
<tr>
<td>40</td>
<td>1.8</td>
</tr>
</tbody>
</table>

- Plasma response
- Oral immunisation
- Anal immunisation
CHAPTER 7.
CHAPTER 7. PRELIMINARY STUDIES ON THE USE OF TARGETING AGENTS AND ADJUVANTS IN POTENTIAL DELIVERY SYSTEMS TO *O. mossambicus*.

7.1. INTRODUCTION.

The oral administration of soluble, T-dependent antigens has produced variable results in attempts to generate a specific, protective, immune response. The delivery of protein antigens, orally, often results in the conversion of the protein, by the normal physiological processes of the gastrointestinal tract, into a form that results in immunological hyporesponsiveness and eventually specific immunosuppression to the protein (Bruce and Ferguson, 1986a, 1986b). This mode of administration often requires a larger immunising dose than that required on systemic injection of the same protein, to elicit an immune response, and may reflect both the degradative processing of the gastrointestinal tract and/or the trans-lumenal processing effects of the intestinal enterocytes (Mayer, 1991; Sanderson and Walker, 1991). The enteric delivery of protein antigens, in mammalian systems, can also result in the generation of an antigen specific immune response locally in the intestine that can be recognised in mucosal sites distanced from the intestine which comprises the common mucosal immune system (Befus and Bienenstock, 1980; Mestecky, 1987; van der Heijden et al., 1989; Arya, 1990; van der Heijden, Bianchii, Dol, Pals, Stok and Bokhout, 1991). Lymphocytes of the gastrointestinal tract, stimulated with antigen, are induced to express immunoglobulin that is evolutionarily designed for mucosal translocation.
to local secretions (sIgA, in mammals). Antigens delivered orally to the lumen are absorbed and interact with resident T and B cells and accessory cells. Antigen sensitized B-cells enter the general blood and lymphatic circulations until they lodge in mucosae-associated tissue where terminal differentiation into secretory plasma cells occurs (Bockman, Boydston and Beezhold, 1983). From this it can be observed that the oral immunisation of non-replicating, protein antigens may have a variable effect on the potential generation of an immune response with a spectrum of possible responses from hyporesponsiveness to a specific immune response (van der Heijden et al., 1989; Davidson, 1991).

It has also been reported that the prolonged feeding of protein antigens, in mammals, can similarly result in either specific local and inducible secretory immune responses (Mestecky, 1987; Kantele, 1991) or in systemic hyporesponsiveness (Bruce and Ferguson, 1986a, 1986b). The oral administration of protein antigens over several days, in repeated doses, in teleosts, can also result in a measurable antibody response in both the mucosal and systemic immune compartments (Rombout et al., 1986, 1989b; Davidson, 1991). However, as in mammalian systems, the oral administration of soluble protein antigens, to fish, has also been reported to result in a form of systemic immunosuppression that may be similar to tolerance (Harrell et al., 1976; Whiskovsky and Avtalion, 1982; Rombout et al., 1989b; Davidson, 1991). Analysis of the resident gut-associated lymphoid tissue (GALT) in the intestine of teleosts appears to show that the mechanisms responsible for the induction of a common network of immunologically-interlinked mucosae, in the form of an inducible antibody-secreting cell population exists (Davidson, 1991), and in a manner analogous to
that observed in mammals (Czerkinsky, Prince, Michaelek, Jackson, Russell, Moldoveanu, McGhee and Mestecky, 1987; Lycke, Bromander and Holmgren, 1989; Lycke, Bromander, Ekman, Karlsson and Holmgren, 1989; Czerkinsky, Svennerholm, Quiding, Jonsson and Holmgren, 1991; van der Heijden et al., 1991). However it also appears that the cellular phenotypes required for the generation of a mammalian-type tolerogenic response that is antigen-specific (intraepithelial T-cells of the suppressor phenotype) is possibly also present in the teleost gastrointestinal tract (Miller et al., 1986, 1987).

Recent studies have evaluated the requirement for adjuvants, targeting agents and carrier molecules that will allow the oral delivery of protein macromolecules in a manner that will avoid tolerogenesis (Mestecky, 1987; Mowat et al., 1991; van der Heijden et al., 1991). One potential delivery vehicle, that has received considerable attention in mammalian studies, is the use of Vibrio cholerae toxin or its purified B-subunit (McKenzie and Halsey, 1984; Lycke and Holmgren, 1986; Kay and Ferguson, 1989a, 1989b; Lycke et al., 1989a, 1989b; van der Heijden et al., 1991). Cholera holotoxin is composed of α and β sub-units with the α subunit (approximately 30 kDa in molecular weight) being the primary causative agent for the clinical symptoms of cholera in mammals via its activation of adenylate cyclase resulting in raised intracellular levels of cAMP in the intestinal enterocytes and in the active efflux of electrolytes and fluid (Liang, Lamm and Nedrud, 1988). The β-subunit is a pentamer (each monomer being approximately 10.6 kDa in molecular weight) which adheres to the GM1 gangliosides on the intestinal enterocytes and allows the active translocation of the holotoxin in vivo (Liang et al., 1988). Both the holotoxin (CT)(Lycke and
Holmgren, 1986, 1989; Lycke et al., 1989a, 1989b) and its β-subunit (McKenzie and Halsey, 1984; Liang et al., 1988) have been reported as being able to increase the local (intestinal) and systemic immune responses and to alter the isotype profile of the antibody response (Jetborn, 1988) to antigens when delivered mixed together or conjugated with them (by a variety of mechanisms) (Liang et al., 1988). Cholera toxin and its β-subunit have not, as yet, been used as adjuvants or carriers for the oral delivery of protein macromolecules in fish although the potential usefulness of this procedure has been noted (Ellis, 1988).

Aluminium hydroxide (Al(OH)₃) is one of the most commonly used clinical adjuvants and is currently the only adjuvant licensed for widespread commercial and veterinary use (Warren et al., 1986; Jolivet, 1989; Lise and Audibert, 1989). Al(OH)₃ has been reported to be an efficient inducer of humoral immune responses through its ability to specifically induce the T-cell subset Th₂ in mammals, and the resulting B-cell stimulation (Lise and Audibert, 1989). Al(OH)₃ has been variously reported as a slow-release ("depot") type adjuvant (Warren and Chedid, 1988) that allows a continuous trickle of antigen at the site of delivery, and as an immunopotentiating agent (Lise and Audibert, 1989) by causing an inflammatory focus that attracts immunocompetent cells to the vicinity of the antigen.

Al(OH)₃ has been used in injection, immersion and oral vaccination regimes in fish, with a variety of antigens, to little beneficial effect (Agius et al., 1983; Tatner and Horne, 1983; Cossarini-Dunier, 1985).

Ammonium chloride (NH₄Cl) has been reported as acting as a lysosomotrophic agent when delivered to fish cells in vivo and in vitro.
Immersion of rainbow trout in quaternary ammonium compound (QAC), a presumptive analogue, prior to *Aeromonas salmonicida* bacterin caused an increase in the resultant immune response observed, although apparently largely by acting as a non-specific immunostimulant (Jeney and Anderson, 1991, in press).

The aims of the current study were to carry out preliminary investigations into the applicability of the use of cholera toxin B-subunit as a targeting agent and adjuvant, AlOH₃ and NH₄Cl as adjuvants and immunostimulants in enteric delivery systems in *O. mossambicus*. The study represents the first use of cholera toxin B-subunit in oral delivery systems in fish and represents one of the few reports to use AlOH₃ and NH₄Cl as enteric adjuvants. Furthermore, it is designed to make preliminary observations of the effects of these substances on the absorption of HGG and on a single measurable parameter of any resultant immune response - the systemic antibody levels specifically raised against the antigen. Thus this investigation is not designed as an exhaustive study on the mechanisms of action of any of the substances studied or on the affects they may have on all the parameters of an immune response *in vivo*.

### 7.2. MATERIALS AND METHODS

#### 7.2.1. ANIMALS

Tilapia, *Oreochromis mossambicus*, of both sexes, weighing between 30-50g were maintained in a recirculating freshwater system at 24-26°C until required.

#### 7.2.2. PREPARATION OF ADJUVANT-ANTIGEN COMBINATIONS

a) **Cholera toxin B-subunit**: (containing approximately 20ng cholera
holotoxin, Sigma, Poole, Dorset, U.K.) was prepared in 0.05M Tris, 0.2M NaCl buffer plus 3mM NaN₃ and 1mM Na₂EDTA (ethylene diamino tetracetic acid) at pH 7.5. 50μg of cholera toxin β-subunit solution was mixed, by thorough vortexing, with 2mg human gamma globulin (HGG) in 0.2ml 50mM Tris, 0.2M NaCl buffer and stored at 4°C until required.

b) Aluminium hydroxide: (AlOH₃; Aldrich Chemical Company, Gillingham, Dorset, U.K.) was added at a 1:1 w/w ratio with 10mg/ml HGG and mixed by thorough vortexing, in saline. The mixed antigen and adjuvant was adsorbed for 8 hours at ambient temperature and subsequently stored at 4°C, until required.

c) Ammonium chloride: (NH₄Cl; BDH, Poole, Dorset, U.K.) was added at a 1:1 w/w ratio with 10mg/ml HGG and mixed by thorough vortexing, in saline. The antigen-adjuvant mixture was stored at 4°C until required.

7.2.3. INTUBATION AND IMMUNISATION PROCEDURES.

7.2.3.1. INTUBATION PROCEDURES.

For the antigen absorption study groups of fish were intubated perorally and peranally with 0.2ml of a 10mg/ml solution of HGG, as a positive control, the fish sacrificed, and the plasma tested over a structured time course as follows: after 15 minutes, 1, 2, 6, 12 and 24 hours.

Test groups of fish (n=3) were intubated perorally and peranally with 0.2ml of each of the adjuvant-antigen mixtures, prepared as outlined above (cholera β-subunit/AlOH₃/NH₄Cl plus HGG) and sampled over the same structured time course as the positive control. Control fish (n=3) were intubated with 0.2ml PBS pH 7.2, by a complementary route under the same conditions.
7.2.3.2. IMMUNISATION PROCEDURES.

Groups of fish (n=3) were also immunised (for the antibody response study) perorally, peranally and intraperitoneally with 0.2ml of each antigen-adjuvant mixture, solely with HGG (as a positive control) and with PBS pH 7.2 as a negative control. The fish were marked subcutaneously, by panjetting, with an Alcian blue (Gurr stains, BDH, Bristol, U.K.) solution in ethanol to aid subsequent identification.

7.2.4. SAMPLE COLLECTION.

7.2.4.1. FOR THE ANTIGEN ABSORPTION STUDY.

Fish were sacrificed by a sharp blow to the head and blood was collected in heparinised (10 units of heparin (Sigma)/ml saline) microcapillary tubes (25μl volumes) and microhaematocrit tubes (100μl volumes). Blood was pooled for each test group, centrifuged at 4000rpm for 5 minutes and the plasma removed and stored at -20°C until required.

7.2.4.2. FOR THE ANTIBODY RESPONSE STUDY.

The test groups of fish were bled, at days 0, 7, 14, 21, 28, and 35 days post-immunisation using 25g needles and heparinised (as previously) syringes from the caudal sinus. The plasma was removed, by centrifugation, as previously described and stored at -20°C until required.
7.2.5. THE DETECTION OF HGG AND FISH ANTI-HGG ANTIBODIES USING AN ENZYME LINKED IMMUNOSORBENT ASSAY SYSTEM (ELISA).

The procedures employed for both the detection of absorbed HGG and the production of HGG-specific antibodies in the plasma are identical to those developed and described in Chapters 4.2.5. and 6.2.5., respectively. All ELISA assay results were subjected to statistical analyses.

7.2.6. STATISTICAL ANALYSES.

The ELISA derived data was analysed using one and two-way analyses of variance for the antigen absorption study (as in Chapter 4.2.7.) and the data form the antibody generation study was analysed using chi-squared tests (as in Chapter 6.2.7.)

7.3. RESULTS.

7.3.1. THE ABSORPTION OF HGG AFTER ORAL AND ANAL DELIVERY AND THE EFFECT OF ADJUVANTS ADMINISTERED IN COMBINATION WITH THE ANTIGEN.

The ELISA systems utilised in this investigation were largely based on those optimised in the earlier studies on HGG absorption after enteric delivery (see Chapter 4.2.5) and antigen-specific antibody responses to immunisation (see Chapter 6.2.5). The kinetics of the titration curves obtained with these systems were extremely similar to those obtained in the earlier assays and the control values were not significantly altered (approximately 0.1-0.15 absorbance units). The background absorbances were mathematically subtracted from the peak
absorbances in the ELISA systems using data storage software.

7.3.1.1. **ANTIGEN ABSORPTION AFTER HGG DELIVERY WITHOUT ADJUVANT.**

The absorption patterns obtained after the oral and anal delivery of HGG (Figure 7.1) were similar to those obtained previously and were used in this study as both a positive control and as a baseline against which to make comparisons of any effect of the adjuvants on the levels of antigen absorption. Differences in the levels of absorption of HGG from the intestine were temporally variable and route-dependent. HGG absorption appeared to be biphasic with peaks of absorption 1 hour and maximally, 6 hours after oral delivery (Figure 7.1). These maxima were significantly greater (p < 0.1) than the other levels of HGG detected in the plasma and was also significantly greater than background levels obtained (not shown).

The temporal kinetics of absorption of anal delivered HGG were different from those seen after oral delivery with maximal absorption occurring after 1 hour. The levels of HGG reaching the plasma declined after 6-12 hours following either oral or anal delivery.

7.3.1.2. **ANTIGEN ABSORPTION AFTER THE DELIVERY OF HGG WITH CHOLERA TOXIN β-SUBUNIT.**

The oral and anal delivery of HGG with cholera toxin β-subunit (CTB) altered the levels and the pattern of absorption of HGG from the intestine. Oral delivery of HGG plus CTB resulted in peak levels of antigen absorption
(Figure 7.2) that were over 2-fold greater than those observed after the oral delivery of HGG alone (Figure 7.1). Similarly, anal delivery of HGG plus CTB caused an increase in the levels of antigen absorbed (Figure 7.2). The levels of HGG absorbed after enteric delivery with CTB substantially increased above those achieved with HGG delivery on its own from 6-12 hours after delivery. The temporal levels of absorption were also different with peak levels occurring 12 hours after anal delivery, although the maximum uptake still occurred 6 hours after oral delivery.

7.3.1.3. ANTIGEN ABSORPTION AFTER THE DELIVERY OF HGG WITH ALUMINIUM HYDROXIDE (AlOH₃).

The oral and anal delivery of HGG plus aluminium hydroxide (AlOH₃) resulted in the absorption of HGG in a similar manner (temporally and quantitatively) to that of HGG delivered without adjuvant. The oral delivery of HGG plus AlOH₃ resulted in peak levels of antigen absorption 6 hours after delivery that were approximately 1/3-fold greater (Figure 7.3) than those observed after HGG delivery on its own. The kinetics of antigen absorption were similar to that of HGG delivered on its own although the plasma levels remained significantly greater (almost 2-fold) between 12 and 24 hours after administration.

Anal delivery of HGG with AlOH₃ (Figure 7.3) resulted in levels of antigen absorption that were statistically similar to those observed after oral delivery. Anal administration of HGG plus AlOH₃ resulted in a biphasic pattern of absorption with peaks 15 minutes and 6 hours after administration.
The levels of plasma HGG declined more swiftly between 12-24 hours after anal delivery than those observed after the oral delivery of HGG plus AlOH$_3$.

7.3.1.4. ANTIGEN ABSORPTION AFTER THE DELIVERY OF HGG WITH AMMONIUM CHLORIDE (NH$_4$Cl).

The oral and anal delivery of HGG plus NH$_4$Cl resulted in significantly lower (p<0.1) peak levels of antigen absorption (Figure 7.4) by comparison with the peak levels observed after the delivery of HGG alone. The oral delivery of HGG plus NH$_4$Cl resulted in peak levels of absorption (Figure 7.4) after 6 hours that were significantly greater than those observed after the anal delivery of HGG with NH$_4$Cl. The temporal kinetics of HGG absorption after its oral delivery with NH$_4$Cl were similar to those observed after the delivery of the antigen plus AlOH$_3$. Peak antigen levels were observed 6 hours after oral delivery and 15 minutes after anal delivery. Levels of antigen decreased between 6 and 24 hours after both oral and anal delivery.

The anal delivery of HGG with NH$_4$Cl resulted in lower levels of antigen absorption, over the entire time course studied, by comparison with levels observed after oral delivery. Peak levels of antigen were detected 15 minutes after anal delivery and the levels of plasma antigen decreased after this time, in marked contrast to the trend observed after oral antigen delivery. The levels of antigen absorbed 1 to 24 hours after anal delivery of HGG with NH$_4$Cl were significantly lower than those observed after oral delivery over the same time. The levels of HGG absorbed after the enteric delivery of antigen with NH$_4$Cl were lower than those observed after the administration of HGG with
7.3.2. THE ANTIBODY RESPONSE IN THE PLASMA AFTER PARENTERAL AND ENTERIC IMMUNISATION WITH HGG.

The presence of anti-HGG antibodies in the plasma was investigated following the immunisation of the antigen in combination with several adjuvants.

The standard curves derived from the ELISA assay (developed in Chapter 6) are exemplified by data derived from immunisation of HGG with CTB in Figure 7.5 and were similar to those obtained previously. Control immunisation of saline regularly resulted in background absorbances of 0.1-0.15 absorbance units throughout the study. The control values are not illustrated on the figures as they were mathematically removed from the ELISA data utilising data storage software.

Intraperitoneal (IP) immunisation with HGG resulted in an antigen-specific antibody response (Figure 7.6). IP immunisation resulted in peak antibody titres that were significantly greater \((p<0.05)\) than those obtained after oral and anal delivery (Figure 7.6). IP immunisation of HGG resulted in the generation of detectable antibody titres 7 days post-immunisation that peaked 28 days post-immunisation.

The oral and anal delivery of HGG resulted in significantly lower antibody titres in the plasma than after IP immunisation. Oral and anal immunisation of HGG resulted in peak antibody titres that were quantitatively and temporally similar, peaking 21-28 days post-immunisation. The oral and anal delivery of HGG resulted in a longer latent period, prior to the generation of a
plasma antibody response, than after IP delivery.

7.3.3. THE ANTIBODY RESPONSE IN THE PLASMA AFTER PARENTERAL AND ENTERIC IMMUNISATION WITH HGG AND CHOLERA TOXIN β-SUBUNIT.

IP immunisation with HGG with CTB did not result in a statistically significant increase in the levels of antibodies (Figure 7.7) generated against HGG by comparison with HGG delivered without adjuvant. However, the delivery of HGG orally and anally in combination with CTB caused a moderate increase in the levels of antibodies generated in the plasma (Figure 7.7). The peak levels of antibodies generated were similar after both the oral and anal delivery of HGG plus CTB.

Temporally, the delivery of HGG in combination with CTB stimulated different antibody generation curves by comparison with delivery of HGG alone. Peak level of antibody was observed 35 days after immunisation via the enteric route compared with 28 days after the IP delivery of HGG with CTB. The inclusion of CTB resulted in a decrease in the latent period between immunisation and the appearance of antibodies in the plasma after oral and anal administration, with peak responses 21-28 days after delivery (for both routes of immunisation) that appeared to be tenable beyond 35 days, in the case of the orally delivered immunogen.
7.3.4. THE ANTIBODY RESPONSE IN THE PLASMA AFTER PARENTERAL AND ENTERIC IMMUNISATION WITH HGG PLUS ALUMINIUM HYDROXIDE (AlOH₃).

The IP immunisation of HGG plus AlOH₃ resulted in the generation of a plasma antibody response with a peak titre (Figure 7.8) approximately 8-10 fold less than the maximum titre observed after the IP immunisation with HGG alone. IP immunisation of HGG adsorbed to AlOH₃ resulted in plasma antibody titres that were significantly \((p < 0.05)\) greater than those detected after oral and anal delivery.

The enteric (oral and anal) delivery of HGG adsorbed to AlOH₃ resulted in peak titres that were slightly (but not statistically) greater than those observed after the enteric delivery of HGG alone.

IP immunisation of antigen with AlOH₃ caused a peak antibody response 35 days post-immunisation and a more rapid generation of the antibody response (as evidenced by a steeper curve over the early points in the time course) than the antibody generation curve stimulated by HGG alone (Figure 7.6).

The oral and anal delivery of HGG adsorbed to AlOH₃ resulted in similar antibody generation curves with oral delivery causing a peak after 28 days and anal delivery a peak after 35 days. The oral delivery of HGG plus AlOH₃ resulted in plasma antibody titres that were of a comparable magnitude to those obtained after the equivalent immunisation with HGG and CTB, whereas for immunisation via the anal and intraperitoneal routes the antibody response was considerably reduced.
7.3.5. THE ANTIBODY RESPONSE IN THE PLASMA AFTER PARENTERAL AND ENTERIC IMMUNISATION WITH HGG AND NH₄Cl.

The immunisation with HGG and NH₄Cl, by both the parenteral and enteric routes, resulted in both quantitative and temporal differences in the antibody generation curves by comparison with immunisation of HGG alone.

IP immunisation resulted in peak specific antibody titres that were lower (Figure 7.9) than those observed after the IP immunisation with HGG on its own or in combination with CTB (by approximately 10-fold) but were approximately equal to the levels observed after the IP delivery of HGG adsorbed to Al(OH)₃. Oral and anal delivery of HGG with NH₄Cl resulted in lower antibody titres by comparison with IP immunisation (Figure 7.9). Oral delivery resulted in detectable higher antibody titres than anal delivery. The enteric delivery of HGG with NH₄Cl resulted in lower levels of specific antibodies than any of the other immunisation protocols (Figures 7.6, 7.7 and 7.8). Maximal antibody titres occurred 35 days after IP immunisation and 28 days after the enteric delivery of HGG plus NH₄Cl.

7.3.6. A TABLE SUMMARISING THE PEAK ANTIBODY RESPONSES GENERATED IN THE STUDY.

Table 7.1 is a tabulated summary of the peak antibody titres achieved with the various modes of antigen delivery with or without adjuvant and the times these levels were detected post-immunisation.
TABLE 7.1. ILLUSTRATING THE PEAK LEVELS OF ANTIBODY PRODUCTION (AND THEIR TIMES OF GENERATION POST-IMMUNISATION).

<table>
<thead>
<tr>
<th>ROUTE OF ADMINISTRATION.</th>
<th>Intraperitoneal</th>
<th>Oral</th>
<th>Anal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyte</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HGG</td>
<td>4.82 (28)</td>
<td>1.81 (28)</td>
<td>1.81 (21-28)</td>
</tr>
<tr>
<td>HGG + Cholera tox B</td>
<td>5.12 (35)</td>
<td>2.71 (21-28)</td>
<td>2.71 (21-28)</td>
</tr>
<tr>
<td>HGG + Al(OH)₃</td>
<td>4.21 (35)</td>
<td>2.71 (28)</td>
<td>2.41 (35)</td>
</tr>
<tr>
<td>HGG + NH₄Cl</td>
<td>4.21 (21)</td>
<td>2.11 (28)</td>
<td>1.5 (28)</td>
</tr>
</tbody>
</table>

Key: Unparenthesised numbers refer to the maximal HGG-specific titres observed (1/ - Log₁₀ antibody titre) and the numbers in parenthesis refer to the time of generation of those titres, post immunisation (Days).

Analyte refers to the immunogen analysed.

The nomenclature in the left hand column refers to the mode of delivery of HGG where: HGG equals delivery without adjuvant; Cholera tox B refers to the delivery of the B-subunit of cholera toxin in combination with HGG; Al(OH)₃ refers to the delivery of HGG adsorbed to aluminium hydroxide; NH₄Cl refers to the delivery of HGG with ammonium chloride.
7.4. DISCUSSION.

The present study is one of the first demonstrations of the viability of the use, in fish, of a variety of adjuvants on the absorption of enterically delivered antigen. It is also the first study to demonstrate a potential use of the B-subunit from cholera toxin (CT) as an adjuvant and targeting agent in the delivery of antigen to the gastro-intestinal tract of fish.

HGG delivered enterically without adjuvant resulted in detectable levels of absorbed antigen in the plasma 15 minutes after oral or anal delivery. The appearance of HGG in the plasma correlated with similar temporal patterns of absorption as observed in earlier studies (Chapters 4 and 5) using the same optimised dose of 10mg/ml (0.2ml intubated dose). This rapid absorption, as outlined earlier, is also observed for optimised doses of other orally and anally delivered soluble protein antigens such as bovine serum albumin (BSA) in *O. mossambicus* (Doggett, 1989), horseradish peroxidase (HRP) and HGG in rainbow trout *Oncorhynchus mykiss* (McLean, 1987; Lavelle, unpbl. obs).

The absorption of HGG in *O. mossambicus* delivered enterically without adjuvant was rapid, temporally complex and also dependent on route of administration. The initial absorption of anally delivered HGG was rapid with anal delivery resulting in maximal levels of absorption into the plasma after 1 hour. The levels of plasma HGG observed at this time were greater than those observed after oral delivery at this time. Oral delivery resulted in peak levels of plasma antigen absorption after 6 hours that were approximately equal to the peak levels of plasma antigen after anal delivery. Levels of plasma antigen appeared to decrease from 6-12 hours after both oral and anal delivery. The
temporal patterns and quantitative levels of HGG absorption into the plasma, after oral and anal delivery, were largely similar to those observed earlier (Chapter 4). The oral and anal delivery of other protein macromolecules, such as ferritin and HRP, to *O. mossambicus* (Doggett, 1989) resulted in similar times of appearance of these antigens in the plasma but at considerably elevated levels by comparison with the levels of absorbed HGG in this study. The maximal levels of absorbed ferritin were 4-5 fold greater and maximal levels of HRP 7-fold greater (Doggett, 1989) than the peak levels of absorbed HGG 6 hours after oral delivery. The anal delivery of these antigens resulted in variable patterns of absorption, ferritin being absorbed in a similar manner to HGG and HRP in a distinctly different mode. Studies of the absorption of enterically delivered antigens in other species such as *O. mykiss* have shown that the temporal patterns of antigen absorption may be related both to the nature of the antigen administered and to the detection method used (Georgopoulou *et al.*, 1986; McLean, 1987; Doggett, 1989).

The enteric delivery of HGG alone, in this study, is assumed to result in absorption across the intestinal epithelium in a similar manner to that observed earlier (Chapter 3) with the bulk of antigen being absorbed across the enterocytes transcellularly. HGG enterically delivered to *O. mossambicus* was also observed interacting with cells of the gut-associated lymphoid tissue (GALT). Such interactions, with both the enterocytes and GALT cells, will profoundly affect the temporal patterns of antigen absorption from the intestine as well as the quantitative levels of antigen observed in the plasma. The oral delivery of growth hormone was shown to have considerable interactions with
granular cells in the intestine of *O. mykiss* that affected overall absorption into the plasma (Le Bail *et al.*, 1989). Levels of antigen reaching the intestinal enterocytes are further influenced by a variety of other factors associated with both the nutritive functions of the gastrointestinal tract and overall gut physiology (Jobling, 1988) and in the case of orally delivered proteins, the gastric pH may also be especially important (Smith, 1989).

The enteric delivery of HGG in combination with a standard dose of cholera toxin B-subunit resulted in an increase in the overall levels of antigen absorbed into the plasma, over the time course studied. Oral delivery of cholera toxin B-subunit mixed with HGG resulted in more rapid absorption of HGG into the plasma; the levels of orally administered antigen reaching the plasma after 15 minutes being significantly greater (by almost 2-fold) than those observed 15 minutes after the delivery of HGG without adjuvant. Oral administration of HGG plus cholera toxin B-subunit altered the temporal patterns of antigen absorption such that peak absorption was still observed 6 hours after antigen delivery but the biphasicity of the absorption curve was absent. Peak levels of HGG absorption were also 2-fold greater than the peak levels of antigen absorbed when delivered alone.

Anally delivered HGG plus cholera toxin B-subunit also caused an increase in the levels of HGG absorbed into the plasma. Temporally, the anal administration of HGG plus cholera toxin B-subunit caused an alteration in the pattern of absorption of antigen into the plasma, with the levels of absorption up to 1 hour being similar to that of HGG alone and less than that observed after oral delivery without adjuvant. Between 1 and 6 hours the levels of HGG
absorbed into the plasma was similar to that observed after oral delivery. Peak levels of anally administered antigen were reached 12 hours after delivery. By comparison with HGG delivery without adjuvant the duration of antigen absorption was increased, with levels only declining 12 hours after either oral or anal delivery. The levels of antigen absorbed after the enteric delivery of HGG in combination with cholera toxin β-subunit were generally lower (by up to 5-fold) than those observed after the delivery of Quil-A prior to or concurrently with HGG. Peak levels of antigen absorbed were similar to the peak levels observed after the delivery of HGG with Quil-A as ISCOMS or micelles (Chapter 4).

The current study represents the first recorded practical use of cholera toxin β-subunit as an adjuvant in oral delivery systems in fish although the potential use of cholera toxin β-subunit for enhanced delivery of enteric antigens (in vaccination regimes) has recently been theoretically discussed (Ellis, 1988; Davidson, 1991) as it is known that the primary mechanism of cellular recognition for the β-subunit - the GM1 gangliosides - are probably present on most, if not all, eukaryotic cells (Kay and Ferguson, 1989a). Most of the information concerning the applicability of the use of cholera toxin (CT) or the β-subunit (CTB) as an adjuvant for enteric delivery studies and resultant immune responses has been observed from investigations on mammals, and frequently, humans (McKenzie and Halsey, 1984; Lycke and Holmgren, 1986; Liang et al., 1988; Kay and Ferguson, 1989a, 1989b; van der Heijden et al., 1989; Clarke et al., 1991; Lycke, Karlsson and Magnusson, 1991; van der Heijden et al., 1991). The enteric delivery of antigens mixed with cholera toxin was found to be efficacious
in increasing antigen absorption (and specific resultant immune responses) in mammals (McKenzie and Halsey, 1984). The delivery of HRP in combination with CTB was found to cause increased binding of the adjuvant/antigen to the GM1 gangliosides, and hence an increased uptake of the antigen by M-cells and enterocytes. This was coupled with an increased active processing of the antigen by the intestinal lymphocytes and macrophages, possibly caused by increased cytokine release, including raised levels of interleukin-1 and gamma-interferon (Quiding, Nordstrom, Kilander, Andersson, Hanson, Holmgren and Czerkinsky, 1991) that were not mediated by a rise in the intracellular levels of cAMP (Lycke and Strober, 1989).

Delivery of cholera toxin B-subunit mixed with Sendai virus also resulted in an increase in antigen absorption by direct conjugation of the B-subunit to the virus (Liang et al., 1988), which was thought to cause increased binding of the antigen-CTB complex to the GM1 gangliosides and further increase the resultant immune response (van der Heijden et al., 1991).

In contrast, other studies have suggested that delivery of antigen plus free CTB does not increase the immunogenicity of the administered antigen ie. does not act as an adjuvant (Kay and Ferguson, 1989a, 1989b; Lycke et al., 1991; van der Heijden et al., 1991). These studies have suggested that the overall permeability of the gut is one of the vital factors in determining the levels of orally administered macromolecules absorbed and their relative immunogenicity (Lycke et al., 1991) and that both cholera toxin and its B-subunit are required for increasing the intestinal permeability and that the cooperation between the two components to achieve this is synergistic (Clarke et al., 1991). The delivery of
CTB in this study may, to some extent, be influenced by such factors as those outlined above as commercial preparations of cholera toxin B-subunit (such as the one used in this study) generally have cholera toxin contaminants (equivalent to approximately 20ng per dose in the preparation used in this study) and as little as 100ng of CT is required to generate a strong immune response to an antigen (Lycke and Holmgren, 1986). It is therefore likely that the increase in the absorption of HGG enterically delivered with cholera toxin B-subunit in *O. mossambicus* is due to a multiplicity of factors including an increase in the binding of the antigen to the intestinal enterocyte surface (via the GM1 ganglioside), a possible increase in the permeability of the intestinal epithelium and also an alteration in the pattern of antigen passage and processing in the enterocytes via increases in soluble immunological mediators (IL's and IFN's) which may influence functions of the enterocytes (Bland and Warren, 1986a, 1986b; Mayer, 1991).

The oral and anal absorption of HGG adsorbed to aluminium hydroxide resulted in increased levels of antigen absorption into the plasma over a limited period which occurred towards the end of the time course studied in this investigation.

Oral administration of HGG adsorbed with aluminium hydroxide did not result in increased levels of absorption of antigen to the plasma until at least 6 hours after delivery, where peak levels were approximately 1/3 greater than the peak levels detected after the delivery of HGG on its own. Levels of plasma HGG decreased rapidly 12 hours after oral delivery of antigen. Anal delivery of HGG adsorbed to aluminium hydroxide also resulted in an increase in the
levels of HGG absorbed into the plasma. However, this increase was only observed between 2 to 6 hours after administration. Levels of adsorbed HGG were not increased significantly outside this time period, by comparison with delivery of HGG alone. The levels of HGG absorbed after the enteric delivery of the antigen adsorbed with aluminium hydroxide were lower than those observed after the delivery of the antigen with cholera toxin β-subunit at any time.

Aluminium hydroxide has been widely used as an adjuvant in clinical situations (Warren et al., 1986) and has been found to act as a "depot" for antigen, where the delivery of antigen in situ is delayed (Warren and Chedid, 1988) resulting in prolonged interaction between the absorbed antigen and immunocompetent cells at the area of introduction (Warren et al., 1986). Mammalian studies also show that precise conditions of antigen-adjuvant adsorption are required for the complete efficacy of aluminium compounds as depots for antigen (Edelman, 1980) and deviations from these conditions may result in the subsequent loss of adjuvant activity. The current study represents the first use of aluminium hydroxide as an enteric adjuvant for the delivery of protein antigens in fish. Related compounds, such as potassium aluminium sulphate, have been shown to significantly increase the uptake of *Vibrio anguillarum* bacterin in rainbow trout *O. mykiss*, in an immersion vaccination regime (Tatner and Horne, 1983). Such an increase in antigen absorption may also be partially attributed to the action of the antigen-adjuvant complex in the gastrointestinal tract, through the drinking of the bath vaccine and the subsequent passage of the antigen across the intestine (Tytler et al., 1990). The
increase in the levels of antigen absorbed were specifically observed between 6 to 12 hours after enteric administration and may correlate with the protraction of antigen release ("depot" effect) as evidenced in mammalian studies (Edelman, 1980; Warren et al., 1986).

The oral and anal delivery of HGG in combination with ammonium chloride generally resulted in significantly lower peak levels of antigen absorption by comparison with either enterically delivered HGG alone or HGG delivered with cholera toxin β-subunit or adsorbed to aluminium hydroxide.

Orally administered HGG in combination with ammonium chloride resulted in peak levels of antigen absorption into the plasma 6 hours after delivery, at levels that were approximately 2-fold greater than those observed after the anal delivery of HGG plus simultaneous ammonium chloride. Administration of HGG plus ammonium chloride, orally, resulted in a rapid, biphasic, pattern of absorption that, although quantitatively lower than that observed after the delivery of HGG on its own, was temporally similar.

The anal administration of HGG in combination with ammonium chloride resulted in peak levels of absorption after 15 minutes and levels of antigen absorbed into the plasma rapidly declined after this time. Levels of enterically administered HGG plus ammonium chloride declined to very low levels after 12 hours.

This study again represents the first attempt to enhance the levels of antigen absorption with the use of ammonium chloride, enterically in an in vivo situation in fish. The levels of absorbed antigen, however, indicate that ammonium chloride acts in vivo in O. mossambicus in such a manner as to
actively reduce the levels of antigen absorption. In contrast to this, ammonium chloride has been reported to function as an efficient agent for the prevention of proteolysis of antigen in vitro in fish (Smedsrud et al., 1984). The use of ammonium chloride in vitro in whole kidney homogenates of arctic char, *Salvelinus alpinus*, resulted in the reduction of proteolysis of delivered $^{125}$-HSA (human serum albumin), mediated by intracellular cathepsins, by its direct lysosomotrophic action (Smedsrud et al., 1984). As a consequence the potential use of ammonium chloride to increase the absorption of and/or the resultant immunogenicity of enterically delivered antigen, in fish, has recently been postulated by McLean (1987).

Recent mammalian studies have indicated that enterocytes (and possibly the majority of cell types) can, under certain circumstances, express MHC II molecules on their surfaces and in the case of the intestinal enterocytes may have considerable antigen processing capabilities and an antigen presenting role (Bland and Warren, 1986a; 1986b; Bland and Whiting, 1990; Mayer, 1991). The administration of ammonium chloride to isolated mammalian enterocytes, in combination with ovalbumin, significantly reduced their ability to process the antigen and effectively blocked antigen presentation to T-cells (Bland and Whiting, 1990).

It is possible that ammonium chloride has similar functions on the lysosomal/endosomal pathways in vivo, in fish, and hence on the overall levels and rates of transcytosis of antigen as well as on antigen processing/presentation to which such factors are inextricably linked (Chain et al., 1988; Bland and Whiting, 1990). Such mechanisms of action could partially explain the lowering
of the overall levels of antigen absorption of HGG into the plasma after its enteric delivery with ammonium chloride, as HGG appears to be absorbed across the intestine largely by a transcellular route that is partially dependent on pinocytotic vesicles. The prevention of endocytosis, the intracellular processing, presentation and exocytosis of enterically delivered antigens, by ammonium chloride, may also account for the decrease in the resultant levels of antibodies after immunisation involving this adjuvant. If ammonium chloride acts in such a manner then the greater decrease in the absorption of anally delivered antigen, by comparison with orally delivered antigen, is explainable. The anal route, by bypassing the denaturing conditions of the stomach, theoretically allows more active substances (in this case adjuvant) to reach and interact with the intestine.

The current study represents the first in vivo use of ammonium chloride as an enteric adjuvant, in fish. Related ammonium compounds, termed quaternary ammonium compounds (QAC) (of indeterminate nature, and which may or may not contain ammonium chloride) have been used in bath immersion regimes (Jeney and Anderson, 1991, in press). QAC's have been reported to increase neutrophil activity and phagocytosis, by non-specific mechanisms but have also been reported to be largely inefficient in increasing protection to challenge with *Vibrio anguillarum* in this regime (Jeney and Anderson, 1991, in press).

The immune responses to the various parenteral and enteric delivery systems was measured by the direct quantitation of the plasma antibody levels, at selected times post-immunisation, after the administration of HGG with and
without adjuvant. The direct quantification of plasma antibody titres is the parameter most often studied in determining the efficacy of antigen delivery systems enterically and parenterally, in mammals and fish (Warren et al., 1986; Mestecky, 1987; Ellis, 1988) and presupposes specific cellular interactions for its generation (Sanderson and Walker, 1991).

The immunisation of *O. mossambicus* with HGG, in the absence of adjuvant, by the enteric (oral and anal) or parenteral routes (IP) resulted in plasma antibody generation curves that were very similar to those observed after the immunisation of the same antigen in an earlier study (see Chapter 6).

Intraperitoneal (IP) immunisation with HGG, alone, resulted in levels of antigen specific antibodies in the plasma that were significantly greater than those achieved after the enteric delivery of the same immunising dose. IP immunisation resulted in peak levels of plasma antibodies 28 days post-immunisation that were similar to those achieved earlier (see Chapter 6). IP immunisation of other soluble protein antigens in *O. mossambicus* also resulted in similar levels of antibodies to those observed in this study (Doggett, 1989) and to those achieved after immunisation in other teleost species (Mughal et al., 1986; Davidson, 1991).

The oral and anal delivery of HGG alone also resulted in similar levels of antibodies to those observed earlier (see Chapter 6), that were lower by comparison with those achieved after IP immunisation. As discussed in detail previously (Chapter 6) the enteric delivery of immunising doses of protein antigens in teleost fish generally results in lower plasma antibody titres than the IP immunisation of the same antigen (Doggett, 1989; Rombout et
Conflicting evidence exists for the efficacy of the repeated enteric delivery of protein antigens. Thus, either the elevation of the resultant antibody response to levels approximating those achieved after parenteral immunisation occurs (Rombout et al., 1989b; Davidson, 1991) or no increased response is demonstrated (Doggett, 1989).

The immunisation of HGG in combination with cholera toxin β-subunit resulted in an increase in the peak antibody levels observed after IP immunisation and an overall increase in the levels of plasma antibodies after oral and anal delivery. The parenteral delivery of HGG with cholera toxin β-subunit resulted in the peak levels of plasma antibodies being detected 21-28 days after both oral and anal delivery which were of a similar magnitude. The antibody generation responses detected after enteric delivery of HGG plus cholera toxin β-subunit were similar to those observed without adjuvant but appeared to be tenable at least 35 days post-immunisation.

The delivery of cholera toxin β-subunit mixed or conjugated to soluble protein antigens, such as HRP, is reported to cause an increase in the antigen-specific antibody response in the bile, intestinal mucus and serum (if delivered enterically), in humans (Langevin-Perriat, Lafont, Vincent, Revillard, Mazert, Gerfaux and Tron, 1988) and other mammals (Mckenzie and Halsey, 1984; Czerkinsky et al., 1987; Czerkinsky et al., 1991). The delivery of cholera toxin β-subunit (or cholera holotoxin) mixed with Sendai virus primed for increased mucosal immunity as evidenced by increased levels of sIgA in the gastrointestinal tract and increased levels of IgA, IgM and IgG in the serum but did not induce protective immunity in the upper respiratory tract of mammals (Liang et
al., 1988). In contrast, the oral delivery of *Streptococcus mutans* protein I and II with cholera toxin β-subunit resulted in good mucosal responses (in the salivary glands) in the form of antibody titres and populations of antibody-secreting cells in the spleen, mesenteric lymph nodes, salivary glands and circulating in the blood (Czerkinsky et al., 1987; Czerkinsky et al., 1991).

The enteric delivery of cholera toxin β-subunit has several direct immunological effects that may have consequences for both the quantitative (magnitude) and qualitative (type) resultant immune response (Lycke and Holmgren, 1986; Kay and Ferguson, 1989a, 1989b; Lycke et al., 1989a, 1989b; van der Heijden et al., 1989; Clarke et al., 1991; Lycke et al., 1991; Quiding et al., 1991; van der Heijden et al., 1991). The oral delivery of cholera toxin and cholera toxin β-subunit causes an increase in gut-permeability and specifically influences the T and B lymphocytes and antigen presenting cells in the intestine. It also affects the immune regulatory pathways via its action on cAMP levels, IL-1 and cell-associated IL-1, early B-cell isotype differentiation (Lycke et al., 1991) and its inhibition of some T-cell functions (Clarke et al., 1991). Similar increases in antigen-specific local immunity were observed in humans, with increases in specific IgA, IgG and gamma-interferon levels and exhibited a classical secondary response (on antigen boosting) that was attributed to either natural killer or T-cells (Quiding et al., 1991).

The oral and anal delivery of antigen plus cholera toxin or its β-subunit may also result in the generation of antibody-secreting cells that are migratory and eventually seed distant mucosae (van der Heijden et al., 1991) and furthermore may be associated with efficient immunological memory induction.
(Lycke and Holmgren, 1986).

In the current study it appears that the increase in the antibody response observed after parenteral, and especially the enteric delivery of HGG with cholera toxin β-subunit is not exclusively due to the increased levels of antigen absorption in this delivery regime. It is likely that cholera toxin and its β-subunit act, as they appear to do in mammals, in a manner such that they directly influence the immune regulatory pathways and enhance the immune response (Wilson, Clarke and Stokes, 1989). It is also likely that such profound influences in antigen absorption, antigen presentation and general immune status will also manifest itself in the configuration of the antigen that reaches the plasma, which as evidenced earlier (see Chapter 5), may be important in the generation of an immune response and the abrogation of tolerance (as discussed in Chapter 6).

The adjuvant effect of cholera toxin β-subunit on (the increase of) specific antibody levels is less by comparison with the increases in the plasma antibody levels after the delivery of HGG in various forms with Quill-A (see Chapter 6). However, several factors must be taken into account when making such direct comparisons including the increased detail and optimisation of the delivery systems in the Quill-A study and the possible substantial modification of aspects of the immune response by cholera toxin that were not monitored in this preliminary investigation (e.g. mucosal antibody responses, antibody-secretory cell responses, potential immunological memory induction and broad range alterations in cytokine levels). Further studies on the potential use of cholera toxin and cholera toxin β-subunit as adjuvants/targeting agents for enteric delivery systems in fish are merited, on the basis of the moderate enhancement
effects observed in this study.

The parenteral immunisation of HGG adsorbed with aluminium hydroxide did not result in an increase in the levels of antibodies elicited. Antibody titres peaked after 35 days post-immunisation and were not significantly different from those observed after the IP immunisation of HGG without adjuvant.

The oral delivery of HGG adsorbed with aluminium hydroxide resulted in peak levels of antibodies in the plasma after 28 days that were slightly greater than those achieved with HGG alone. Anal administration of HGG adsorbed with aluminum hydroxide resulted in peak levels of antibodies 35 days post-immunisation that were greater than those obtained after immunisation with HGG alone, but levels did not differ between these two groups until this time. The antibody response after parenteral and enteric immunisation with HGG adsorbed with aluminium hydroxide were lower than the comparable groups immunised with cholera toxin β-subunit. The levels of antibodies generated were lower after enteric immunisation than expected if there was a direct correlation between the levels of antigen absorbed and the eventual immune response.

Mammalian studies indicate that aluminium hydroxide is a moderate enhancer of humoral immune responses when adsorbed with the delivered antigen correctly (Edelman, 1980; Warren et al., 1986; Warren and Chedid, 1988; Lise and Audibert, 1989). Aluminium hydroxide causes slow release of antigen at the site of delivery, the subsequent attraction of immunocompetent cells to the site of introduction of the antigen and the eventual stimulation of
production of both antibody forming cells in the regional lymph nodes (for subsequent dissemination throughout the body) and local plasma cell containing granulomas (Edelman, 1980). Primary clinical immunisation with tetanus and diphtheria toxoid preparations is considerably enhanced by adsorption of these antigens to aluminium hydroxide (Aprile and Wardlaw, 1966). Similarly the use of aluminium hydroxide was found to significantly increase the antibody response and clinical protection to disease for Salk polio vaccine preparations, rabies vaccine preparations and subunit influenza vaccines (consisting of haemagglutinin and neuraminidase) (Potter, Jennings, McLaren, Edey, Stuart-Harris and Brady, 1975; Saroso, Bahrawi, Witjaksono, Budianto, Bencic, Dewitt and Gomez, 1978; Kuwert, Menzel, Marcus and Majer, 1978). They were effective, however, in vaccines that are likely to rely on a mucosally directed immune response to achieve significant clinical protection (Cvejtanovic and Uemura, 1965).

It seems likely from the relatively moderate increases in the antibody titres after the enteric delivery of HGG adsorbed with aluminium hydroxide that this mode of delivery is not optimised in this study with respect to the ratio of the antigen to the adjuvant. There remains considerable scope for future analysis of aluminium hydroxide or the new generation of aluminium based adjuvants such as algammulin (Cooper and Steele, 1991) in enteric or parenteral antigen delivery systems in fish.

The parenteral immunisation of HGG in combination with ammonium chloride resulted in an antibody response in the plasma that peaked 21 days post-immunisation with titres that were lower by comparison with the peak
specific titres achieved using cholera toxin B-subunit or without adjuvant. However it was similar to the response after the IP immunisation with HGG adsorbed to aluminium hydroxide.

The oral delivery of HGG plus ammonium chloride resulted in levels that were statistically unchanged from control levels but were lower than the antibody titres generated after the oral delivery of HGG with either cholera toxin B-subunit or aluminium hydroxide. The anal delivery of HGG plus ammonium chloride resulted in considerably lower levels of antibodies than after oral and IP delivery of HGG with ammonium chloride and also than those achieved with the other delivery systems. The degree of reduction of the plasma antibody response was considerably less, after oral delivery of HGG with ammonium chloride, by comparison with the decrease in the antibody titre after anal delivery. This may be a reflection of the proposed decreased absorption of orally delivered ammonium chloride, after its passage through the stomach by comparison with anal delivery which avoids this and may be the reason for the disparity between antigen absorption after oral and anal delivery. An increased absorption of ammonium chloride, as evidenced from mammalian studies, caused a considerable reduction in the antigen presentation capacity of the enterocytes and the resultant immune response (Bland and Whiting, 1990). The passage of the antigen through the enterocytes appears to be considerably affected by the lysosomotrophic action of ammonium chloride which subsequently affects the function of antigen presenting cells in the GALT and it is this function of the enterocytes which is increasingly considered to be a fulcrum of immune responses initiated in the gut (Mayer, 1991).
The current study represents the first, preliminary, investigation on the effects of three specific adjuvants, cholera toxin B-subunit, aluminium hydroxide and ammonium chloride, on the enteric and parenteral delivery of HGG in *O. mossambicus*. The requirement for the testing of these substances in oral delivery systems has been recently highlighted (McLean, 1987; Ellis, 1988; Davidson, 1991) and further work is required to ascertain their exact modes of action *in vivo* and *in vitro* and to expand their use in experimental systems with other species.
FIGURE 7.1

Figure 7.1 shows the temporal absorption of HGG into the plasma after the oral and anal delivery of 10mg/ml (0.2ml dose) without adjuvant. Error bars are 75% limits.
Fig. 7.1 HGG absorption after oral and anal delivery without adjuvant.

Level of HGG in the plasma (μg/ml).

Control HGG.

- Orb delivery.     - Δ Anal delivery.
FIGURE 7.2.

Figure 7.2 shows the temporal absorption of HGG into the plasma after the oral and anal delivery of 10mg/ml (0.2ml dose) in combination with 50μg cholera toxin β-subunit. Error bars are 75% limits.
Fig. 7.2  HGG absorption after oral and anal delivery in combination with chol.tox. B.

Level of HGG in the plasma (μg/ml).

Time after delivery (Hours).

HGG plus cholera toxin β-subunit.

-□- Oral delivery.  -△- Anal delivery.
FIGURE 7.3.

Figure 7.3 shows the temporal absorption of HGG into the plasma after the oral and anal delivery of 10mg/ml (0.2ml dose) adsorbed 1:1 w/w with aluminium hydroxide (AlOH₃). Error bars are 75% limits.
Fig. 7.3  HGG absorption after oral and anal delivery in combination with AIOH.

Level of HGG in the plasma (μg/ml).

Time after delivery (Hours).

HGG plus aluminium hydroxide (AIOH).

-☐- Oral delivery.  -△- Anal delivery.
FIGURE 7.4.

Figure 7.4 shows the temporal absorption of HGG into the plasma after the oral and anal delivery of 10mg/ml (0.2ml dose) 1:1 w/w with ammonium chloride (NH₄Cl). Error bars are 75% limits.
Fig. 7.4  
HGG absorption after oral and anal delivery in combination with NHCl.

Level of HGG in the plasma (μg/ml).

Time after delivery (Hours).
HGG plus ammonium chloride (NHCl).

- □ - Oral delivery.  - ▲ - Anal delivery.
FIGURE 7.5.

Figure 7.5 shows the plasma antiserum titration 21 days after the oral and anal delivery of 10mg/ml (0.2ml dose) HGG in combination with cholera toxin β-subunit.
Fig. 7.5 Antibody response in the plasma after the oral and anal delivery of HGG in combination with chol.tox.B.

Absorbance 492nm.

Immunisation with HGG plus cholera toxin B-subunit.

21 day response.

- IP immunisation.
- Oral immunisation.
- Anal immunisation.
FIGURE 7.6.

Figure 7.6 shows the temporal antibody response in the plasma after the oral, anal and intraperitoneal (IP) immunisation with 10mg/ml (0.2ml dose) HGG without adjuvant.
Fig. 7.6  
Plasma antibody response after immunisation with HGG without adjuvant.

$-\log_{10}$ antibody titre.

Time post-immunisation (Days).

Control HGG:
- □ Oral immunisation.
- △ Anal immunisation.
- ⊙ IP immunisation.
FIGURE 7.7.

Figure 7.7 shows the temporal antibody response in the plasma after the oral, anal and intraperitoneal (IP) immunisation with 10mg/ml (0.2ml dose) HGG in combination with 50μg cholera toxin β-subunit.
Fig. 7.7  Plasma antibody response after immunisation with HGG plus chol.tox.B.

-1\ Log 10 antibody titre.

Time post-immunisation (Days).
Immunisation with HGG plus cholera toxin $\beta$-subunit.

- - Oral immunisation.  \triangle - Anal immunisation.
\textbullet - IP immunisation.
Figure 7.8 shows the temporal antibody response in the plasma after the oral, anal and intraperitoneal (IP) immunisation with 10mg/ml (0.2ml dose) HGG adsorbed 1:1 w/w with aluminium hydroxide (AlOH₃).
Fig. 7.8 Plasma antibody response after immunisation with HGG plus AlOH.

-1/ Log 10 antibody titre.

Time post-immunisation (Days).
Immunisation with HGG plus aluminium hydroxide (AlOH).

- - Oral immunisation.
- - Anal immunisation.
- - IP immunisation.
FIGURE 7.9.

Figure 7.9 shows the temporal antibody response in the plasma after the oral, anal and intraperitoneal (IP) immunisation with 10mg/ml (0.2ml dose) HGG mixed 1:1 w/w with ammonium hydroxide (NH₄Cl).
Fig. 7.9  
Plasma antibody response after immunisation with HGG plus NHCl.

-1/ Log 10 antibody titre.

Time post-immunisation (Days).
Immunisation with HGG plus ammonium chloride

- □ - Oral immunisation.
- △ - Anal immunisation.
- • - IP immunisation.
CHAPTER 8.
CHAPTER 8. CONCLUDING REMARKS.

There exists a requirement for the development of oral delivery systems for the administration of macromolecules and antigens in clinical, veterinary and aquacultural situations (Ellis, 1988; Ogilvie, 1988; Ebrahim, 1990). The enteric delivery of antigens of pathogens is the most logical route of immunisation to achieve a response specifically designed to maximise immunological protection at mucosal surfaces, and to induce the active expression of immunity in the secretions of the mucosae involved with the transmission of the pathogen into the host body. Oral delivery of antigens, potentially, combines relative ease of administration and opportunity for the rapid boosting of any immune response by the secondary delivery of antigens. Recently it has become increasingly evident that the delivery of antigen to the gut-associated lymphoid tissue may allow, under optimal conditions, the generation of a locally produced immune response rather than (or in addition to) the direct stimulation of a systemic (antibody) immune response that may be less effective (Mestecky and Eldridge, 1991). Additionally, the enteric delivery of antigens has also been shown to be able to disseminate the specificity of a generated immune response to diverse mucosae, distanced from the site of antigen introduction (gut), via antigen-specific B-cells committed to secretory immunoglobulin production (Czerkinsky et al., 1991).

However, the direct enteric delivery of antigen to the intestine often results in either a very poor immune response locally, or systemic
hyporesponsiveness to the antigen or both. The immunological mechanisms that affect such tolerogenic processes are complicated and only partially understood, even in mammalian investigations. Thus the oral administration of antigens requires the development of rationalised delivery systems that incorporate antigens specifically designed, or in such quantities, to avoid such tolerogenic responses. Incorporation of immunomodulatory substances (adjuvants) may overcome some of the barriers that exist to the efficient generation of a protective immune response. The design of oral vaccination regimes has also concentrated on the use of adjuvants not only to increase the overall (quantitative) level of the immune response but to also possibly induce an aspect of the immune response that would otherwise remain unstimulated such as aspects of cell-mediated immunity.

The oral delivery of macromolecules, and specifically of stimulatory antigens, in fish has also been recognised as a primary requirement, especially for commercially important diseases such as furunculosis, salmon lice, vibriosis and bacterial kidney disease. It has also been viewed as the most convenient mode of vaccination, for much the same reasons as those identified in clinical and veterinary situations as well as for the fact that it is a logistically convenient method for the delivery to large numbers of fish in aquaculture systems. Recent studies have localised and characterised the GALT in various fish species (Hart et al., 1988) and have also shown, as in mammals, it is probably one of the major arms of the immune system. Phenotypically, the GALT of fish is very complicated and ultrastructural and functional studies have shown that all the component cell types required for the active generation of a primary immune
response are present (Hart et al., 1988; Rombout et al., 1989a, 1989b; Davidson, 1991). The enteric delivery of antigens, therefore, may potentially be able to induce an immune response, generated at the GALT, that can be protective for antigens from pathogens (Rombout et al., 1989b). Recent investigations have also demonstrated substantial evidence towards this, with intestinally delivered antigens being able to induce an antibody response and the possibility of the existence of a common mucosal immune system, in fish, being postulated (Rombout et al., 1989b; Davidson, 1991). The absorption of orally delivered protein antigens may, physiologically, have roles that are divorced from those required for an oral vaccination regime per se. Absorbed protein antigens may contribute nutritionally to fish physiology although the significance of this, quantitatively, has been recently disputed (reviewed by McLean and Donaldson, 1990). It has also been suggested that protein absorption may be associated with some mechanism of entero-pancreatic enzyme recirculation that operates in the fish alimentary tract.

However, the current status of oral delivery systems for antigens from important pathogenic organisms remains, as it does in mammals, in a state of flux with rationalised delivery systems and adjuvants particularly suited for these delivery systems being largely unrealised and the availability of oral vaccination systems being poor (the most widely studied, and available, data from oral vaccination regimes remains those carried out with Salmonella typhi Ty21a and its derivatives, in humans) (Black et al., 1990).

The current study represents a detailed investigation of the design and use of oral delivery systems for the administration of a protein antigen, human
gamma globulin (HGG), to the cichlid teleost *Oreochromis mossambicus*. The enteric (oral and anal) delivery of HGG was monitored using a sensitive immunocytochemical detection system which allowed a chronological assessment of the absorption of the intact molecule, or its epitopes, across the intestine of these tilapia. The administration of HGG was altered by the incorporation of the saponin adjuvant *Quillaja saponaria* [Molina] into the delivery system as either a direct adjuvant, as a three-dimensional immune stimulatory complex (ISCOM) or as a micellar delivery vehicle for the antigen. The incorporation of the adjuvant, in the delivery system, caused an increase in the absorption of HGG across the intestine of tilapia and an increase in the interaction of the absorbed antigen with GALT cells by a variety of mechanisms mostly visualised as alterations in the integrity of the lumenal membrane of the intestinal enterocytes. The observed (largely) transcellular absorption of the antigen corresponds to the observed effects of the delivery of Quil-A with antigens in mammalian studies (Maharaj *et al.*, 1986; Mowat *et al.*, 1991).

The absorption of HGG into the blood plasma was monitored by the use of a sensitive ELISA assay system. Enterically delivered HGG was absorbed in a temporally complex manner into the plasma in amounts that were immunologically significant. The incorporation of Quil-A into the delivery system prior to, concurrently with, and as a delivery vehicle for the antigen significantly increased the levels of absorbed HGG over a narrow range of concentrations. This evidence represents a considerable improvement in the development of useful oral delivery systems, albeit for a test antigen, in fish where the circumvention of the reduced effective dose inherent after oral vaccination
procedures is a prerogative for the potential generation of an immune response.

The qualitative nature of the delivered antigen reaching the intestine and the GALT is another major factor determining the eventual (immunological) fate of orally delivered antigens. The administration of HGG, alone, results in the appearance of the intact molecule in the plasma and a range of epitopes of various molecular weights. The gastric degradation of orally delivered antigen often results in the production of epitopes that mediate tolerogenesis by being in configurations that are particularly suited to the stimulation and induction of T-suppressor cells in the epithelium of the intestine (Michael, 1989). The delivery of Quil-A resulted in alterations in the qualitative nature of the HGG absorbed into the plasma and in increasing the number and complexity of its fragmentation profiles. Quil-A delivery probably altered the nature of the processing undertaken of the antigen in the intestinal lumen and/or the intestinal enterocytes. The processing of orally delivered antigen by the intestinal enterocytes is increasingly being realised as a primary factor in the determination of the efficacy of the eventual immune response and is susceptible, in vivo, to the addition of adjuvants (Bland and Whiting, 1990).

The immune response, as measured by the antibody titres in the plasma, bile and cutaneous mucus, was considerably lower following enteric delivery of HGG alone, by comparison with the titres achieved after intraperitoneal immunisation. The inclusion of Quil-A in the delivery system, enterically, resulted in dramatically increased antibody responses in the plasma, bile and cutaneous mucus by comparison with immunisations without adjuvant. These increases in the immune response were probably mediated by
the aforementioned alterations in the levels of absorbed HGG, the alterations in its interactions whilst in the intestinal tissue, the changes in its fragmentation and by immunomodulatory affects of the adjuvant that were not studied and remain hypothetical in fish (e.g. possible an increase in the levels of interleukin release). Levels of antibody were also dramatically increased in the bile and cutaneous mucus suggesting that the incorporation of optimised Quil-A into the delivery system greatly elevated the specific mucosal immune response and lends a body of data towards the conclusion that there is a common mucosal immune response mechanism in teleosts that may be initiated by the judicious delivery of enteric antigens.

The present study has also shown that cholera toxin and its B-subunit are potentially useful as targeting agents for the oral delivery of antigens in fish as their use caused both an increase in the levels of HGG absorbed and the subsequent plasma antibody response. Preliminary studies have also indicated that the enteric use of both aluminium hydroxide and ammonium chloride, at least in this test system, had little beneficial immunomodulatory effect.

The current study illustrates that the immune response, to well characterised antigens, in teleost fish can be manipulated by the incorporation of the antigen in specifically tailored delivery systems. The precise composition of any potential oral delivery system will be dependent on the nature of the protective antigens from the specific pathogen and the requirement of the immune response to that pathogen i.e. the site of the immune response, the nature of the response (e.g. a cellular response is likely to be more efficacious than a humoral response for intracellular pathogens) and the degree to which
the delivered antigen will be absorbed and acted upon by the intestinal enterocytes.

In order to achieve these aims future studies will have to generate considerably more basic information on the mucosal immune responses in fish with respect to the requirements for its efficient induction through a combination of *in vivo* and *in vitro* experiments and also to the nature of its potential dissemination from an immunisation site of the intestine *i.e.* the degree of its similarity to the mucosal immune response of mammals. In addition to this the extent of the degradation of the delivered antigen in both the lumen and in the intestinal enterocytes should be studied with the aim of discerning the role that the enterocytes have on antigen processing and presentation. Such information will lend itself to being able to avoid tolerogenic epitopes of vaccine antigens or to the manipulation of antigen processing functions so that the endocytic pathways which reveal them are diverted. The use of vaccine adjuvants will probably be essential to overcome some of the other problems inherent in oral vaccination such as the reduction in delivered antigen load due to gastric degradation. The practical use of oral vaccines for a particular fish pathogen will require a rationalised delivery system utilising specific antigens (*"classically"*, synthetically or recombinantly derived) and specific adjuvants tailored towards the induction of a useful, protective and sustained immune response.
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APPENDIX I, REAGENTS USED.

A). REAGENTS FOR IMMUNOHISTOCHEMISTRY AND IMMUNOCYTOCHEMISTRY (all volumes are per litre, unless stated).

1) Phosphate buffered saline, PBS (pH 7.2-7.4).
   Sodium chloride, NaCl - 8g.
   Disodium hydrogen orthophosphate, Na$_2$HPO$_4$ - 1.15g (anhydrous).
   Potassium dihydrogen orthophosphate, KH$_2$PO$_4$ - 0.2g.
   Potassium chloride, KCl - 0.2g.

2) Tris-Saline (pH 7.4) for the preparation of ISCOMS.
   0.1M Tris (Trishydroxymethylaminomethane) - 12.11g.
   0.05M Sodium chloride, NaCl - 2.92g.

3) Ammonium acetate for the dialysis of ISCOMS.
   0.05M Ammonium acetate - 3.854g.

4) Tris-saline (pH 7.6) for streptavidin-biotin-HRP immunocytochemistry.
   0.05M Tris - 6.055g.
   0.15M Sodium chloride, NaCl - 8.766g.

5) PBS (pH 7.2) for immunogold silver staining.
   Sodium chloride, NaCl - 8.0g.
   Potassium chloride, KCl - 0.2g.
   Potassium dihydrogen orthophosphate, KH$_2$PO$_4$ - 0.2g.
   Disodium hydrogen orthophosphate, Na$_2$HPO$_4$·2H$_2$O - 1.44g.

6) Silver enhancement solution for immunogold-silver staining.
a) 1M Citrate buffer pH 3.5.

12.75g Citric acid.H₂O

11.75g Trisodium citrate.2H₂O in 100ml D.Water.

then:

20ml Citrate buffer pH 3.5.

30% w/v gum Arabic in D.Water - 33ml.

17ml D.Water.

Silver lactate - 0.11g in 15ml D.Water.

Hydroquinone - 0.85g in 15ml D.Water.

B). REAGENTS FOR ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA).

1) PBS pH 7.4 as in A).

2) PBS-TWEEN.

PBS (as above) plus 0.5% v/v TWEEN 20 (polyoxyethylene sorbitan monolaurate).


Disodium carbonate, Na₂CO₃ - 1.59g.

Sodium hydrogen carbonate, NaHCO₃ - 2.93g.

4) Citrate/Phosphate conjugate/developing buffer,pH 5.0.

Citric acid, - 4.23g (Anhydrous).

Disodium hydrogen orthophosphate, NaH₂PO₄ - 5.80g anhydrous.

5) Heparin - Anti coagulant for plasma collection.

10mg/ml Heparin in saline.

6) Development substrate.
Orthophenyldiamine (OPD) - 20mg in
5ml Citrate/phosphate buffer plus
20μl H₂O₂.

C. REAGENTS FOR SODIUM DODECYL SULPHATE (SDS)
POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE).

1) Separating gel buffer (all volumes are 100ml, unless stated).
   Tris - 36.3g.
   N',N',N',N' - Tetra methylethylenediamine (TEMED) - 0.23ml.
   plus 90ml D.Water.
   pH to 8.9.
   Volume to 100ml.

2) Stacking gel buffer.
   Tris - 5.98g.
   TEMED - 0.46ml.
   to 80ml with D.Water.
   pH to 6.7.
   Volume to 100ml.

3) Separating gel solution.
   Acrylamide - 28.0g.
   N',N' - Methylenebisacrylamide - 0.74g.

4) Stacking gel solution.
   Acrylamide - 10.0g.
   Bis-Acrylamide - 2.5g.

5) SDS-Solution.
Sodium dodecyl sulphate, SDS, lauryl sulphate - 0.21g.

6) 2X Sample buffer (reducing).

Tris - 1.51g.
Glycerol 20ml in 35ml D.Water.
Adjust to pH 6.75.
Add SDS - 4g.
β-Mercaptoethanol - 10ml.
Bromophenol blue - 0.002g.
Volume to 100ml.

7) Electrode buffer (2.0 Litres).

Tris - 6.05g.
Glycine - 28.8g.
SDS - 2g.
pH at approximately 8.3 (unadjusted).

D) REAGENTS FOR COOMASSIE BLUE STAINING OF ELECTROPHORESIS GELS.

1) Coomassie blue solution.
Coomassie brilliant blue-R - 1.25g.
in 500ml of:
Methanol - 400ml.
Glacial acetic acid - 70ml.
D.Water - 530ml.
Filter (Whatman number 1 paper).

2) Destain/Fixer.
Methanol - 400ml.

Glacial acetic acid - 70ml.

D.Water - 530ml.

3) Coomassie brilliant blue-G colloidal concentrate (BBG).

A working solution of 800ml plus BBG.

Add 4:1 working solution BBG : ANALAR methanol.

Destain in 10% v/v acetic acid in 25% v/v Methanol.

4) Fixing solution for BBG staining.

A 1:5 solution of 60% w/v trichloroacetic acid (TCA) plus 17.5% w/v Sulphosalicylic acid : D.Water.

E) REAGENTS FOR NEUTRAL SILVER STAINING OF ELECTROPHORESIS GELS.

1) Fixer solution.

30% v/v Ethanol - 300ml.

10 v/v Acetic acid - 100ml.

D.Water - 600ml.

2) Silver equilibration solution.

Silver nitrate concentrate - 7.5ml.

D.Water - 292.5ml.

3) Development solution.

Sodium carbonate - 30ml.

D.Water - 120ml.

Formaldehyde - 0.17ml.

4) Stop solution.
Acetic acid solution 1% v/v in D.Water.

5) Reducer solution.

Potassium ferricyanide - 2ml.
Sodium thiosulphate - 4ml
Sodium carbonate - 0.7ml.
D.Water - 293.3ml.

F). REAGENTS FOR IMMUNOBLOTTING.

1) Transfer buffer (2 Litre volume).

Tris - 6.1g.
Glycine - 28.8g.
Methanol - 400ml.
D.Water - 1600ml.

2) Tris-saline (pH 7.5) for immunoblotting.

Sodium chloride, NaCl - 11.68g.
Tris - 6.05g.

3) Tris-saline-milk (TSM) pH 7.4.

Tris-saline (as in 2) plus 3% w/v "Marvel" very low-fat Dried milk.

4) Amido Black.

Amido Black - 0.1% v/v.
Methanol - 45% v/v.
Acetic acid - 10% v/v.

5) Amido Black destain.

Methanol -90% v/v.
Acetic acid - 2% v/v.
D. Water - 8% v/v.

6) India ink.

India ink - 100μl.

PBST 100ml.

Destain in PBS pH 7.2.

7) Peroxidase blot development solution:

50mg 3’3 Diamino benzidine tetrahydrachloride (DAB) in 100ml D.Water plus 100μl H₂O₂.

G). SDS-PAGE GEL MOLECULAR WEIGHT MARKERS.

1) 6H High Molecular Weight markers.

Carbonic anhydrase (Bovine erythrocytes) - 29kDa.

Ovalbumin (Egg) - 45kDa.

Bovine albumin (Plasma) - 66kDa.

Phosphorylase B (Rabbit muscle) - 97.4kDa (subunit).

β-Galactosidase (E.coli) - 116kDa (subunit).

Myosin (Rabbit muscle) - 205kDa (subunit).

2) 7H Low Molecular Weight Markers.

α-Lactalbumin (Bovine milk) - 14.2kDa.

Trypsin inhibitor (soybean) - 20.1kDa.

Trypsinogen (Bovine pancreas) - 24.0kDa.

Carbonic anhydrase (Bovine erythrocytes) - 29kDa.

Glyceraldehyde-3-phosphate dehydrogenase (Rabbit muscle) - 36kDa.

Ovalbumin (Egg) - 45kDa.

Albumin (Bovine plasma) - 66.0kDa.
3) Pre-stained molecular weight markers.

Triose phosphate isomerase (Rabbit muscle) - 26.6kDa.
Lactic dehydrogenase (Rabbit muscle) - 36.5kDa.
Fumarase (Porcine heart) - 48.5kDa.
Pyruvate kinase (Chicken muscle) - 58kDa.
Fructose-6-phosphate kinase (Rabbit muscle) - 84kDa.
β-Galactosidase (E.coli) - 116kDa.
α₂-Macroglobulin (Human plasma) - 180kDa.

4) Native (Non-denaturing) Molecular Weight Markers.

α-Lactalbumin (Bovine milk) - 14.2kDa.
Carbonic anhydrase (Bovine erythrocytes) - 29kDa.
Ovalbumin (Chicken egg) - 66kDa (Monomer).
Jack bean urease (Jack Bean) - 132kDa (Dimer).

- 272kDa (Trimer).
- 545kDa (Hexamer).