THE STRUCTURE AND FUNCTION OF PERIPHERAL BLOOD LEUCOCYTES AND GUT-ASSOCIATED LYMPHOID TISSUE IN THE CICHLID, *OREOCHROMIS MOSSAMBICUS*.

BY

TERESA ANN DOGGETT, HND/TEC (Wolverhampton Polytechnic)
BSc.(Hons) (Trent Polytechnic)

A thesis submitted to the Council for National Academic Awards in partial fulfilment of the requirements for the degree

of

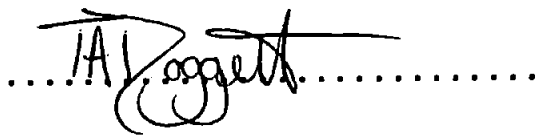
DOCTOR OF PHILOSOPHY

(March 1989)

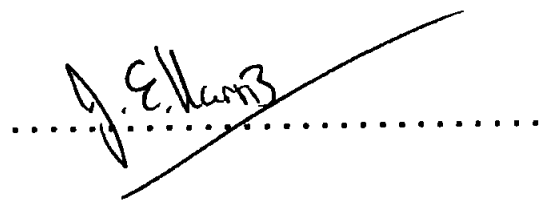
Research was conducted at Plymouth Polytechnic
in collaboration with Wellcome Laboratories, Beckenham

DECLARATION

I hereby declare that thesis has been composed by myself, that it has not been accepted in any previous application for a higher degree, that the work of which it is a record has been performed by myself, and that all sources of information have been specifically acknowledged.



Teresa Ann Doggett



Dr. J.E.Harris

(Supervisor)

PLYMOUTH POLYTECHNIC
LIBRARY
Accn No 5500579-2
Class No T 597.5804113 DOG
Contl X700657877

THE STRUCTURE AND FUNCTION OF PERIPHERAL BLOOD LEUCOCYTES AND GUT-ASSOCIATED LYMPHOID TISSUE IN THE CICHLID, *OREOCHROMIS MOSSAMBICUS*

BY

TERESA ANN DOGGETT BSc, Hons.

ABSTRACT

The peripheral blood of *O.mossambicus* was examined using light and electron microscopy and was found to contain four forms of leucocytes: lymphocytes, thrombocytes, monocytes and three types of granulocytes. The monocyte and two types of granulocyte were found to be phagocytic and ingest colloidal carbon and bacteria.

The alimentary tract was found to contain a number of leucocytes, some showing a morphological similarity to those in the peripheral blood, while others were unique to the gut tissue. These intestinal leucocytes were found mainly as a diffuse cell population in the epithelium and lamina propria, and only occasionally as discrete lymphoid accumulations within the gut tissue. Ontogenic studies showed that a limited number of leucocytes were found in the gut tissue after hatching, however, there was a gradual increase in these numbers once exogenous feeding began.

The intestinal enterocytes of both the anterior and posterior intestine were found to take up intubated macromolecules. An electron microscopical investigation revealed that these macromolecules were absorbed by pinocytosis and were found within large intraepithelial macrophages. These macromolecules were also absorbed and transported into the systemic circulation. In juvenile fish macromolecules were detected in the plasma following both oral and anal intubation, however, in adult fish they were detected in the plasma only after anal intubation, and in smaller quantities. Macromolecular absorption in *O.mossambicus* was compared to that in two other fish species, *Cyprinus carpio* and *Salmo gairdneri*, and it was found that higher levels of absorbed macromolecules were found in the plasma of *O.mossambicus*.

Bovine serum albumin absorption by the gut of the three species revealed that both the 'intact' macromolecule and smaller antigenic fragments, probably resulting from enzymatic modification, were ansorbed and transported into the plasma.

List of publications and conference contributions:

T.A.DOGGETT, A.B.WRATHMELL & J.E.HARRIS.1987. A cytochemical and light microscopical study of the peripheral blood leucocytes in *Oreochromis mossambicus*, CICHLIDAE. J.Fish Biol.31, 147-153.

T.A.DOGGETT & J.E.HARRIS.1987. The ontogeny of gut-associated lymphoid tissue of *Oreochromis mossambicus*. J.Fish Biol. Supp.A. 31, 23-27.

T.A.DOGGETT & J.E.HARRIS. (in press). The ultrastructure of the peripheral blood leucocytes of *Oreochromis mossambicus*.

T.A.DOGGETT & J.E.HARRIS. (in press). Gut-associated lymphoid tissue of the cichlid, *Oreochromis mossambicus*.

T.A.DOGGETT & J.E.HARRIS. (in press). The ultrastructure of gut-associated lymphoid tissue (GALT) in the cichlid, *Oreochromis mossambicus*.

S.HART, A.B.WRATHMELL, T.A.DOGGETT & J.E.HARRIS.1986. Aspects of the immunology of the female genital tract of the elasmobranch, *Scyliorhinus canicula* L. Dev. Comp. Immunol.10, 597-602.

S.HART, A.B.WRATHMELL, J.E.HARRIS & T.A.DOGGETT.1987a. Gut-associated lymphoid tissue (GALT) in the common dogfish *Scyliorhinus canicula* L: an ultrastructural study. J. Mar. Biol. 67, 639-645.

S.HART, A.B.WRATHMELL, T.A.DOGGETT & J.E.HARRIS.1987b. An investigation of the biliary immunoglobulin and the plasma cell distribution in the gall bladder and liver of the common dogfish, *Scyliorhinus canicula* L. Aquaculture. 67, 147-155.

Conference contributions:

T.A.DOGGETT & J.E.HARRIS. Ontogeny of gut-associated lymphoid tissue in the teleost *Oreochromis mossambicus*. A paper presented at the Symposium of Fish Immunology at Plymouth Polytechnic 1987.

T.A.DOGGETT & J.E.HARRIS. A preliminary study of the gut immunology of a lower vertebrate *Oreochromis mossambicus*. A poster presented at the 'Immunology of the Gastrointestinal Tract and the Liver' Conference in Jerusalem, Isreal 1987.

T.A.DOGGETT & A.B.WRATHMELL. Intestinal uptake of Bovine Serum Albumin (BSA) in two fish species, *Oreochromis mossambicus* and *Salmo gairdneri* R. A poster presented at the EAFP Conference in Bergen, Norway 1987.

S.HART, A.B.WRATHMELL, T.A.DOGGETT & J.E.HARRIS. The mucosal immunoglobulin of the dogfish *Scyliorhinus canicula* L. A paper presented at the 2nd International Colloquiom of Pathology and Marine Aquaculture (PAMAQ II), in Oporto, Portugal 1986.

S.HART, A.B.WRATHMELL, T.A.DOGGETT & J.E.HARRIS. The immunology of the female genital tract of the elasmobranch *Scyliorhinus canicula* L. A paper presented at PAMAQ II in Oporto, Portugal 1986.

S.HART, T.A.DOGGETT, A.B.WRATHMELL, G.T.H.GRAYSON & J.E.HARRIS. Fish Immunology: A Review. A poster presented at the 'Immunology of the Gastrointestinal Tract and Liver' Conference in Jerusalem, Isreal 1987.

ACKNOWLEDGEMENTS

I would like to acknowledge the following:

Professor L.A.F. Heath for enabling me to carry out the research for this thesis in the Department of Biological Sciences at Plymouth Polytechnic

Drs. J.E.Harris, A.B.Wrathmell and B.Spacey for their advice and supervision of this project

Drs. R.Ash and E.McLean of Bradford University for their advice concerning the ELISA technique

Dr.R.Moate and Mr.B.Lakey, of the Electron Microscopy suite for the provision of facilities and expert technical assistance

Mr.P.Russell for his help and assistance regarding histology

Mr.A.Smith of media services for providing the plated for this thesis, and Mrs.B.Fox for help concerning the photography

Mr.A.Gough, Mr.S.McMahon and Mr.R.Torr for their care of experimental animals

Mr.P.Jenkins, Mr.R.Barton, Ms.D.Furones, Mr.P.Burgess and Dr.S.Davis for their helpful discussions and enthusiasm

The technical staff of the 4th floor namely, Mrs.A.Bell, Mr.N and Mrs.D. Crocker, Ms.J.Eddy, Ms.L.Cooper and Mrs.M.Pill for their cheerful asistance and friendship

Swansea University, Stirling University, Munton Fisons, Ludbrook Trout Farm and Exe Valley Trout Farm for providing the experimental animals

I would also like to thank the SERC for the provision of my grant and funds to attend a conference. My thanks also to the Fisheries Society of the British Isles and Dr.G.Gregoriadis for their financial assistance while attending conferences.

All experimental work using animals was carried out under Personal Home Office Licence numbers ELA 25/76 and PIL 30/01460

This thesis is dedicated to my parents with thanks for their love and support

CONTENTS

	Page
Title	I
Declaration	II
Abstract	III
List of Publications and conference contributions	IV+V
Acknowledgements	VI+VII
Licence	VIII
Dedication	IX
Contents	X+XI
List of Plates	XII
List of Tables	XIII
List of Figures	XIV+XV
List of Abbreviations	XVI+XVII
CHAPTER 1 Introduction and Literature Review	
1.1 INTRODUCTION	1
1.2 LITERATURE REVIEW: Macromolecular Uptake by the Intestine of Vertebrates	5
1.2.1 **Protein Uptake in Mammals**	5
1.2.1a The Immature or Neonatal Intestine	
i) Selective Absorption	6
ii) Non-selective Absorption	8
1.2.1b The Mature Intestine	11
1.2.2 **Protein Uptake in Fish**	15
1.2.2a Larval Fish	15
1.2.2b Juvenile and Adult Fish	19
1.2.3 **Mechanisms of Macromolecular Absorption**	
1.2.3a Transcellular Pathway	22
1.2.3b Paracellular Pathway	25
1.2.4 **Factors affecting Absorption**	27
1.2.4a Immunological Factors Affecting Absorption	
i) Antigen access to lymphoid tissue	27
ii)Mucosal Immunity	29
1.2.4b Non-immunological Factors Affecting Absorption	
i) Gastric Function	33
ii) Mucous Secretion	34
iii)Peristalsis	36
iv) Membrane Composition	36
1.2.5 **Oral Tolerance**	38
1.2.5a Factors affecting Oral Tolerance	
i) Type of Antigen	39
ii) Dose and Frequency of antigen exposure	40
iii) Age of Animal	41
1.2.5b Mechanisms of Oral Tolerance	42

CHAPTER 2 Morphology, Cytochemistry and Functional studies on the peripheral blood leucocytes

2.1 Introduction 42
2.2 Materials and Methods 44
2.3 Results 46
2.4 Discussion 50

CHAPTER 3 Distribution and Morphology of the Lymphoid tissue in the alimentary tract of adult fish

3.1 Introduction 63
3.2 Materials and Methods 64
3.3 Results 66
3.4 Discussion 72

CHAPTER 4 The ontogeny of the gut-associated lymphoid tissue and major lymphoid tissue

4.1 Introduction 88
4.2 Materials and Methods 89
4.3 Results 90
4.4 Discussion 93

CHAPTER 5 Uptake of Horseradish peroxidase and ferritin by the intestinal epithelium

5.1 Introduction 107
5.2 Materials and Methods 108
5.3 Results 110
5.4 Discussion 113

CHAPTER 6 Intestinal absorption and transport of Ferritin and Horseradish peroxidase into the systemic circulation

6.1 Introduction 126
6.2 Materials and Methods 128
6.3 Results 134
6.4 Discussion 137

CHAPTER 7 Absorption of Bovine serum albumin (BSA) by the intestinal epithelium

7.1 Introduction 155
7.2 Materials and Methods 156
7.3 Results 164
7.4 Discussion 169

CHAPTER 8
DISCUSSION 187

BIBLIOGRAPHY 197

List of Plates

		After Page
CHAPTER 2		
Plate A	Lymphocytes and thrombocytes	61
B	Monocytes and blood granulocyte type 1	62
C	Blood granulocytes type 2 and type 3	63
D	Phagocytosis of bacteria	64
E	Phagocytosis of colloidal carbon	65
CHAPTER 3		
Plate A	Mucosae of the different regions of the GI tract	86
B	Lymphoid accumulations in the GI tract	87
C	Lymphocytes in the intestine	88
D	Plasma cells in the intestine	89
E	Intestinal macrophages and blood granulocyte type 1	90
F	Tissue granulocytes of the intestine	91
CHAPTER 4		
Plate A	Intestinal epithelium, stage 7-P, 14-P and day 1	104
B	Intestinal epithelium, day 1, day 3 and day 5	105
C	Intestinal epithelium, day 10, day 14 and day 84	106
D	Thymic primordia at stage 7-P and intestinal cell complexes	107
E	Intestinal macrophages and blood granulocyte type 1	108
F	Tissue granulocytes	109
G	Plasma cells in the intestine	110
H	Distribution of intraepithelial leucocytes at day 50	111
CHAPTER 5		
Plate A	HRP in the anterior epithelium 5 minutes after intubation	123
B	HRP in the anterior epithelium 2 hours after intubation	124
C	HRP within enterocytes, and blood granulocyte type 1	125
D	Sixteen hours after intubation of HRP	126
E	Ferritin in the anterior intestine 30 min. and 1 hour after intubation	127
F	Ferritin in the anterior intestine 1 hour 4 hours and 16 hours after intubation	128
G	Intestinal macrophages containing ferritin	129
H	Absorption of ferritin 16 and 24 hours after intubation	130

List of Tables

		After Page
CHAPTER 1		
Table 1	Review of Antigen/Protein Uptake in fish	15
CHAPTER 2		
Table 1	Cytochemical techiques used to characterise the peripheral blood leucocytes	47
Table 2	Cytochemistry of the peripheral blood leucocytes	49
CHAPTER 3		
Table 1	Embedding and Histological staining procedures	68
CHAPTER 6		
Table 1	Protocol for the administration of Ferritin and HRP	135
Table 2	Buffers required for RIE and ELISA	136
Table 3	Appearance of HRP in tank water following intubation	138
CHAPTER 7		
Table 1	Effect of state of nutrition on the uptake of BSA in juvenile fish	161
Table 2	Effect of repeat doses of BSA on the uptake in juvenile and adult fish	162
Table 3	Preparation of reagents for SDS-PAGE Gels	163
Table 4	Preparation of Electrophoresis Gels	164
Table 5	Western Blot buffer	164
Table 6	50mM Tris/HCl saline buffer	164
Table 7	Antibody response to BSA	167
Table 8	Buffers for Indirect Haemagglutination	168

List of Figures

	After Page
CHAPTER 1	
Figure 1 Illustration of selective and non-selective absorption	6
Figure 2 Diagram of a M-cell	27
CHAPTER 3	
Figure 1 Distribution of Intra-epithelial leucocytes in the Alimentary Tract	85
CHAPTER 4	
Figure 1 Distribution of Intra-epithelial leucocytes in the developing intestine	102
Figure 2 Schematic representation of the developing intestine	103
CHAPTER 6	
Figure 1 Photomicrograph of a Rocket Immuno-electrophoresis Plate	149
Figure 2 Illustration of the ELISA technique to detect HRP	151
Figure 3 An example of an ELISA test for HRP	152
Figure 4a-c Oral Intubation of Ferritin in the three fish species	154
Figure 5a-c Anal Intubation of Ferrtin in the three fish species	155
Figure 6a-d Oral HRP dose reponse in Plasma and Tissues of Juvenile Tilapia	156
Figure 7a-c HRP levels in the Plasma following repeated Oral intubation	156
Figure 8a-c HRP levels in the Liver following repeated Oral intubation	157
Figure 9a-c HRP levels in the Kidney following repeated Oral intubation	158
Figure 10a-c HRP levels in the Spleen following repeated Oral intubation	159
Figure 11a-d HRP levels in the Plasma and Tissues following Anal intubation	160
CHAPTER 7	
Figure A1-3 Diagrammatic representation of the GI tract in the three fish species	180
Figure 1 Oral and Anal BSA dose response	181
Figure 2a-d Effect of state of nutrition on the absorption of BSA in Juvenile Tilapia	182
Figure 3a-d Effect of state of nutrition on the absorption of BSA in Juvenile Rainbow trout	183
Figure 4a-d Effect of state of nutrition on the absorption of BSA in Juvenile Carp	184
Figure 5a-c Multiple Oral BSA dose in Juvenile Tilapia	185
Figure 6a-c Multiple Anal BSA dose in Juvenile Tilapia	186

Figure 7a-c Multiple Oral BSA dose in Juvenile Rainbow trout 187
Figure 8a-d Multiple Oral and Anal BSA dose in Adult Tilapia 188
Figure 9a-c Multiple Oral BSA dose in Adult Rainbow trout 189
Figure 10a-c Multiple Anal BSA dose in Adult Rainbow trout 190
Figure 11 Photomicrographs of the SDS Gel and Immunoblot 191

List of Abbreviations

AcP- acid phosphatase
AlP- alkaline phosphatase
ANAE- alpha-naphthol acid esterase
BSA- bovine serum albumin
^{0}C- degrees celsius
cm- centimetres
CT- cytochemistry
d- daltons
DAB- 3'- diamino benzidine
ELISA- enzyme linked immuno-sorbent assay
EM- electron microscopy
Fc- fragment crystallisable portion of Ig
FCA- Freund's complete adjuvant
FIA- Freund's incomplete adjuvant
g- gramms
GALT- gut-associated lymphoid tissue
Gth- gonadotrophin
^{3}H- tritiated thymidine
H+E- haematoxylin and eosin
HGG- human gamma globulin
HMW- high molecular weight
HRP- horseradish peroxidase
^{125}I- iodine 125
ICS- intercellular space
ICT- immunocytochemistry
IEL- intraepithelial leucocyte
IF- immunofluorescence
ILL- interlaminal leucocyte
Ig- immunoglobulin
IP- intraperitoneally
IM- immunologically
im- intramuscular
Kd- kilo daltons
KLH- keyhole limpet haemocyanin
LM- light microscopy
mA- milli amps
mg- miiligramms
MGG- May Grunwald Giemsa
MGP- methyl green pyronine
ml- millilitres
MLN- mesenteric lymph node
mm- millimetres
mw- molecular weight
ng- nanogramm
OPD- o-phenylenediamine
OVA- ovalbumin
PAGE- polyacrylamide gel electrophoresis
PAP- peroxidase anti-peroxidase
PAS- Periodic acid-Schiff
PBS- phosphate buffered saline
PO- peroxidase
PP- Peyer's Patches

RER- rough endoplasmic reticulum
RIA- radioimmunoassay
RIE- rocket immunoelectrophoresis
SC- secretory component
SDS- sodium dodecyl sulphate
SRBC- sheep red blood cells
TEM- transmission electron microscope
TEMED- N',N',N',N'-tetramethylethylenediamine
TSB- tryptone soya broth
ug- microgramms
ul- microlitres
um- micrometres
V/V- volume to volume ratio
W/V- weight to volume ratio

CHAPTER 1

1.INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The role of local immune responses and in particular that which operates in the alimentary tract has received increasing interest in mammalian species (Breuton, 1980; Walker, 1980). Only recently has a similar interest become apparent in lower vertebrates and in fish in particular. This has to a degree been stimulated by an increasing awareness of the possibility of oral vaccination in the control of diseases in farmed fish.

The gut is constantly exposed to a variety of antigenic material including a number of microorganisms and dietary antigens. In order to deal with this potentially harmful environment a number of defence strategies have evolved to protect the gastrointestinal (GI) tract. These involve both immunological factors, such as, secretory immunoglobulins and other components of the gut-associated lymphoid tissue, combined with non-immunological factors such as, gastric and pancreatic enzymes, mucous secretions, peristaltic activity of the intestinal muscularis and intracellular digestion by enterocytes and their membrane composition itself.

Gut-associated lymphoid tissue (GALT) has been investigated in a number of lower vertebrates such as, amphibians (Ardavin, Zapata, Villena & Solas, 1982; Jurd, John & Garvey, 1988; Plytcyz & Slezak, 1981; Wong, 1982) and

reptiles (Borysenko & Cooper, 1972; Hussein, Badir, El Ridi & Akef, 1978; Zapata & Solas, 1979). However, relatively few studies have been undertaken on the GALT of fish, with most authors investigating cyclostomes (Good, Finstad, Pollara & Gabrielsen, 1966) and elasmobranchs (Hart, Wrathmell & Harris, 1986a; Tomonaga, Hirokane, Shinohara & Awaya, 1973; Tomonaga, Kobayashi, Kajii & Awaya, 1984). The precise involvement of GALT in the immune system of fish has not been clearly established. It has been speculated that in fish the immunoglobulins found in the gut secretions originate from the plasma cells detected in the gut tissue (Rombout, Blok, Lamers & Egberts, 1985; Tomonaga *et al.*, 1984). In mammals secretory IgA originates from the plasma cells in the lamina propria of the intestine and enters the gut lumen with the secretory component (SC) provided by the intestinal enterocytes. Hart, Wrathmell, Grayson & Harris (1988), from this laboratory, have recently reviewed gut immunology in fish and its role in host defence, and it was thought that as this provided a comprehensive survey of the current literature it was unnecessary to repeat this material in the following literature review.

Tilapians are an economically important fish species in Africa, Asia and South America, where they are farmed on a large scale, and *O.mossambicus* is a good representative species. As an animal model it is ideal for ontogenic studies providing some 150-200 eggs per spawning, and will spawn all the year round in aquaria. Provided with an optimum feeding regime they grow quickly to a size that can

be used for a variety of immunological studies. They are also able to tolerate a wide temperature range, so lend themselves to a number of temperature related investigations.

The initial aim of this study on *O.mossambicus* was to investigate the leucocytes found in the gut and their possible role in gut immunity. To establish whether these cells were unique to the gut tissue they were compared to those found in the peripheral blood. However, as there is a paucity of information concerning the peripheral blood leucocytes of *O.mossambicus* (Sailendri & Muthukarruppan, 1975; Boomker, 1980), an inital investigation of these cells was felt necessary. The functional properties of the peripheral blood leucocytes has been investigated in a number of fish species (Ellis, 1976; Ferguson, 1976; Parish, Wrathmell, Hart & Harris, 1986b; 1986c; Suzuki, 1984; Weinreb & Weinreb, 1969), and has been recently reviewed by Rowley & Ratcliffe (1988).

Having fulfilled the initial aim of the study a second approach was undertaken. This involved the investigation of the absorption of intact macromolecules across the intestinal epithelium and their transport into the systemic circulation.

The principal objectives were therefore:

a) to provide a comprehensive literature survey of macromolecular uptake in mammals and fish, together with the mechanisms of uptake and oral tolerance

b) to examine and characterise the leucocytes of the peripheral blood and gut tissue, and the ontogeny of the lymphoid population of the gut in concert with the development of the major lymphoid organs

c) to investigate the uptake of a number of protein macromolecules by the intestinel epithelium in juvenile and adult *O.mossambicus* and compare this with the uptake of macromolecules in other fish species.

d) to carry out a preliminary investigation of the modification of intubated macromolecules by the intestinal secretions and/or intestinal enterocytes

1.2 LITERATURE REVIEW: MACROMOLECULAR UPTAKE BY THE INTESTINE OF VERTEBRATES

It was generally assumed that the uptake of intact macromolecules was a primitive mechanism for absorbing nutritive material. However, the ability to absorb intact macromolecules has been demonstrated both in the neonate and the adult mammalian intestine. The quantity of intact macrmolecules that escape intracellular digestion is, however, small and nutritionally insignificant (Walker, 1987), but these molecules may remain antigenically intact and be transmitted to the intracellular spaces of the intestinal epithelium and presented to the lymphoid tissue (Bruce & Ferguson, 1986). In the following pages macromolecular uptake in both mammals and fish is reviewed; the proposed mechanisms of uptake and the possible tolerising effect of these molecules are discussed.

1.2.1 Protein Uptake in Mammals

One perspective of the intestinal mucosa is its function as a selective barrier to the luminal contents. Considerable evidence now exists to show, however, that protein macromolecules penetrate and are transported across the intestinal epithelium and gain access to the systemic circulation (Walker, 1976; Walker, 1981). A number of cells in the intestine have been implicated in this permeability, M-cells, absorptive enterocytes, tuft cells and Paneth

cells, all of which occur along the length of the intestine either in discrete associations or as diffuse cells. To combat the potentially harmful components of the luminal contents, such as parasites, toxins, allergens etc., the mucosa is protected by an elaborate system of defence mechanisms. The mucosal barrier of neonates has however, been shown to be incomplete being more permeable than that of adults (Walker, 1980). This permeability may allow the 'bulk' passage of some of the luminal contents, some of which may escape intracellular digestion. The permeability of the neonatal mucosa ceases as the intestinal host defences develop, and intestinal epithelial cells become morphologically and functionally mature; this maturation process being termed 'gut closure'.

1.2.1a The Immature or Neonatal Intestine

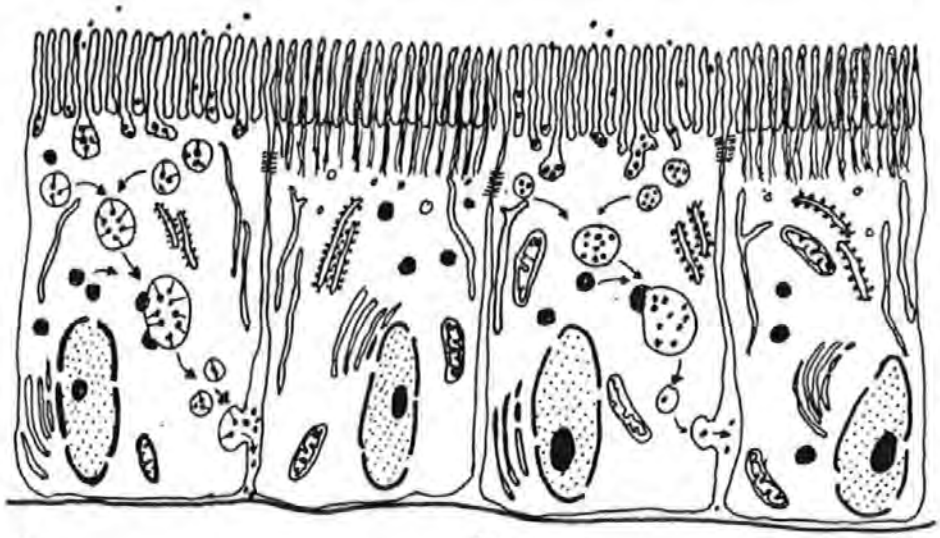
The intestine of the neonate has been shown to be highly permeable to various protein macromolecules with absorption occuring via either 'selective' or 'non-selective' mechanisms.

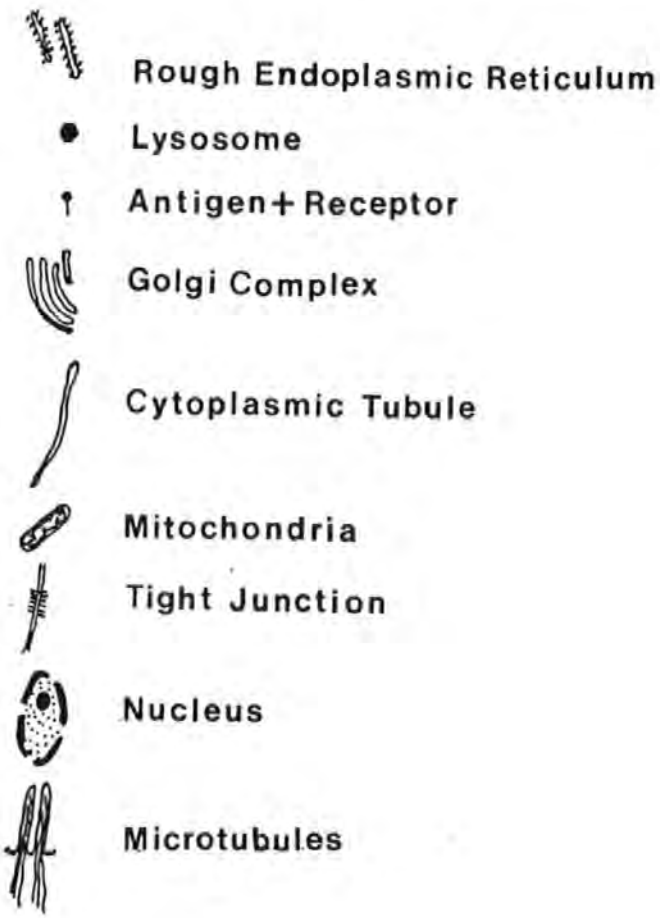
(i) Selective absorption

Selective transport of some proteins across the plasma membrane of cells in the gut epithelium is mediated by receptor molecules located on the membrane (Figure 1a), their function being to bind those molecules for which they have the correct stereospecificty (Bainter,1986). The

Illustration of selective and non-selective absorption

Figure 1





immature small intestine of some neonatal mammals, for example rats, is selectively permeable to maternal IgG present in colostrum. The binding of IgG occurs via Fc receptors on the brush-border membranes of epithelial cells (Brambell, 1958). Most reports indicate that the majority of IgG receptor-mediated uptake occurs in the cells that line the duodenum and proximal jejunum (Abrahamson & Rodewald, 1981), and is pH dependent (Jones & Waldmann, 1972). Hemmings & Williams (1977) have also indicated that the distal jejunum and ileum of the rat is able to transport limited amounts of IgG. In the ileum of the suckling rat Brambell, Halliday & Morris (1958) found a selective IgG absorption, with competition between different IgG subclasses. The binding of immunoglobulin (Ig) to intestinal receptors is highly specific, the epithelium of the suckling rat binding all four subclasses of human IgG but not any other Ig class (Jones & Waldmann, 1972). Peppard, Jackson & Hall (1985) recently demonstrated using ^{125}I-IgG, that there were two types of IgG receptors on the enterocytes of the young rat. These receptors were found to bind the different IgG subclasses at different rates.

Abrahamson & Rodewald (1981) observed that when 10-day old rats were fed horseradish peroxidase (HRP), HRP-IgG or HRP-anti-HRP immune complexes, there was a difference in their localisation within the epithelial cells. They concluded that the intracellular fate of HRP depends on whether it becomes linked to the IgG receptor. This coupling of IgG and HRP having stimulated the absorption of HRP would

suggest that the same receptors are involved in the binding of IgG monomers, aggregates and immune complexes (Morris & Morris, 1976).

These receptors allow the passive transmission of immunity in newly-born mammals via the Ig in the maternal colostrum. In humans this route , however, does not appear to contribute significantly to the serum antibody levels in infants (Walker, 1987). Ogra, Wientraub & Ogra (1979) have shown, however, that human infants fed antibody-rich human colostrum immediately after birth were found to have detectable levels of active antibody in their systemic circulation. In other mammals more appreciable levels of passively transferred Ig can be demonstrated (Jones & Waldman, 1972) which may confer a protective immunity during the first few days of life. Transmission of maternal antibodies from the gut lumen into the circulation ceases after a short period of time, for example in the rat when it is 19-20 days old (Halliday, 1956; Jones, 1972). This coincides with the disappearance of receptors in the enterocyte cell membrane (Bainter & Kocsis, 1984), the maturation of the intestinal epithelial cell (Walker, 1980), and the immune system.

(ii) Non-selective absorption

IgG is mainly absorbed via the Fc receptors present on the epithelial cells but may also be absorbed via non -selective mechanisms (Figure 1b), as seen in the pig and the cow (Brambell, 1970; Kraehenbuhl, Sorbat & Bron, 1979). Calves

are able to absorb molecules of each immunoglobulin class from the colostrum at the same ratios as they are initially present (Blecha, Bull, Olsin, Ross & Curtis, 1981; Brandon & Lascelles, 1971). Colostral Ig concentration decreases rapidly as the colostrum transforms to mature milk, but IgG remains the predominant Ig found in colostrum.

The neonatal intestine is permeable not only to Ig but to smaller amounts of non-IgG proteins. HRP has been shown to be taken up by intestinal villi in significant amounts by endocytosis (Abrahamson & Rodewald, 1981), and Walker (1973) indicated that trace amounts of HRP were transported into the serosal fluid after intubation into neonatal everted gut-sacs, and these quantities increased in proportion to the HRP concentration in the bathing solution. Graney (1968) noted that ferritin was absorbed preferentially by the ileum of suckling rats, with only trace amounts being transported from the intercellular space into the circulation. Bovine gamma-globulin (BGG), ovalbumin (OVA) and colloidal gold administered orally to suckling rats and mice were ingested by the columnar absorptive cells of the jejunum and ileum. At approximately 18 days after birth these cells lost their ability to ingest these proteins and colloids (Clark, 1959). A similar observation is made in the selective absorption of macromolecules across the intestinal epithelium, which may be due to the maturation of the epithelial cells, as seen in morphological studies of enterocytes.

Uptake of non-protein macromolecules has also been demonstrated in the newborn calf, goat, lamb, piglet and

foal (cited from Bainter, 1986) using polyvinylpyrolidone (PVP) which was absorbed undigested. The transmission of PVP into the circulation was only substantial when administered together with colostrum or colostral whey, which may be due to a passive uptake of the PVP with other colostral proteins. Another possibility is that there may be a component in the colostrum which stimulates or enhances the uptake of large molecules by the intestinal epithelial cells.

The mechanism of non-selective transmission is still unclear. Uptake is thought to occur by a pinocytotic mechanism with the accumulation of ingested macromolecules within supranuclear vacuoles. Kraehenbuhl & Campiche (1969) have demonstrated that there is an increase in the lysosomal activity after macromolecule uptake. This would lead to an increase in the digestion of macromolecules and a decrease in their transportation into the systemic circulation. This was clearly demonstrated by Heyman, Ducroc, Desjeux & Morgat (1982) who found that 97% of the HRP intubated into the jejunum of rabbits was intracellularly degraded. The importance of this method of uptake is unclear as the majority of molecules taken up by this method presumably undergo intracellular digestion as described above. The limited amounts that are transported across the epithelial barrier may be involved in tolerance, particularly to possible food/dietary antigens (see Section 1.5).

1.2.1b The Mature Intestine

In previous studies it has been demonstrated that the uptake and subsequent transport of gamma-globulins decreases markedly when the neonatal small intestinal epithelial cell membrane matures (cited from Bainter, 1986). This phenomenon known as 'closure' occurs at a time when the capacity of the absorptive cells to endocytose other macromolecules decreases and coincides with a change in the morphology and physiology of the intestinal cells. The time after birth at which closure occurs is species specific and very variable (eg. 24 hours in the calf; 19-20 days in the rat). It was assumed that all macromolecular absorption ceased after this time, but evidence now exists that small, trace amounts of antigenically intact macromolecules may be transmitted across the mature mammalian gut. Exactly what amounts are normally absorbed into the circulation and what functional significance these macromolecules might have are largely unknown (see Section 1.5).

Bloch, Bloch, Steams & Walker (1979) showed that feeding bovine serum albumin (BSA) to rats resulted in minute (nanogram) quantities of this antigen being detected in the serum. Warshaw, Walker, Cornell & Isselbacher (1971) using tritium-labelled BSA (^{3}H-BSA) infused into the duodenum of adult rats noted that it resulted in the transmittance of 2% ^{3}H-BSA in macromolecular form into the blood and lymph, the amount being directly proportional to the amount

administered. Along with BSA a number of other protein macromolecules have been shown to permeate the adult mammalian intestine, such as, ovalbumin (Dannaeus, Ingrames, Johansson & Foucard, 1979; Brostoff & Challacombe, 1987; O'Hagan, 1988); chymotrypsin (Avakian, 1964); trypsin (Udall, Bloch, Vachne, Feldman & Walker, 1984a); -amylase (Goetz & Rothmann, 1978); lactoperoxidase - (Blok, Mulder-Stropel, Ginsel & Daems, 1981); heparin (Engel & Riggi, 1969); transferrin (Neutra, Ciechanover, Owen & Lodish, 1985) along with many other examples.

After intraluminal injection of HRP into the ligated jejunum or ileum of adult rats, the HRP can be found absorbed to the apical surface membrane, when examined ultrastructurally (Cornell,Walker & Isselbacher, 1971). Walker (1973) observed that the infusion of HRP into rat jejunum resulted in the transmittance of small but 'significant' amounts of the protein tracer across the gut into the intestinal lymph and portal blood. That this endocytic process is energy-dependent and hence an active mechanism has been established using metabolic inhibitors (Walker, Cornell, Davenport & Isselbacher, 1972). Owen (1977) investigated the uptake of HRP by the epithelial cells covering the Peyer's Patches (PP) of the mouse intestine. After 5 minutes HRP was found within vesicles of M-cells and after 1 hour in the extracellular space between M-cells and their enfolded lymphocytes. That these M-cells may be involved with the uptake and transport of luminal molecules was demonstrated by LeFevre, Olivo, van der Hoff &

Joel (1978). They observed the accumulation of latex particles in the PP in mice that were fed latex particles in a water suspension with the subsequent appearance of latex particles in the mesenteric lymph node, a feature similarly reported by Bienenstock (1984) when using latex and carbon particles intubated into the rat intestine. Transport of intestinal luminal material by M-cells and the subsequent uptake by lymphocytes indicates a specific route for antigen uptake into the intestinal lymphoid tissue.

Ferritin, on the other hand, is apparently not internalised by the human epithelial cell although it has a strong affinity for the cell-coat glycoproteins (Blok *et al.*, 1981). A similar affinity has been reported by Casely-Smith (1967) and Bockman & Winborn (1966) in the rat and hamster respectively: however, both these latter authors report that ferritin was taken up by the epithelial cells of their experimental models albeit in only trace amounts.

Thus it would appear that macromolecular uptake does not cease with the maturation of the intestinal enterocytes as small quantities of intact macromolecules, as well as colloidal particles, have been shown to penetrate the mature intestinal epithelium (LeFevre *et al.*, 1978). The access of antigens is facilitated by the presence of specialised intestinal cells, M-cells (Wolf & Bye, 1984), which are in direct contact with the components of the underlying lymphoid tissue. This mechanism of antigen handling would seem to provide a direct access route to the lymphoid tissues of the gut, and a means by which to stimulate the

the local immune system.

Antigen size does not appear to be a major consideration, as far as uptake is concerned, with a wide range in size of macromolecules found to penetrate the intestinal epithelium (eg. OVA, @43,500 and ferritin, @500,000 daltons). It is highly probable that these molecules are affected by the intracellular digestive processes of the enterocytes. Bruce & Ferguson (1986) indicated that OVA that had been processed by the gut of mice could be isolated from the serum by serum fractionation. The fraction that contained components in a molecular weight range that included native OVA also contained immunoreactive components. These authors could not determine conclusively the molecular weight of the OVA present in the serum, or rule out the possibility that the immunoreactive OVA was present as a fragment in association with another molecule.

It has been described that the mature intestine will absorb and transport certain small quantities of macromolecules with no ill-effects. This situation, however, can lead to a pathological state particularly if this 'normal' function is disturbed resulting in an increase in the quantity of antigenic molecules that pass across the mucosal barrier. In the normal state the passage of small quantities of intact macromolecules does not induce an immune reaction but may induce a state of tolerance (see Section 1.5).

1.2.2 Protein Uptake in Fish

The morphology of the digestive tract of teleosts varies a great deal. Most fish species have a complex tract that includes a stomach, pyloric caecae, and intestine. It has been established that approximately 1.5% of all species do not possess a stomach or pyloric caecae (Jakobshagen, 1937). Intact protein absorption across the intestine has been demonstrated in both gastric and agastric species. Georgopoulou & Vernier (1986) have shown that the epithelial cells of the posterior intestine in both young and adult trout are structurally and functionally comparable to those of the ileum of the suckling rat. As to whether this feature dispels the possibility of the phenomenon of 'gut closure' occuring in fish is not known.

1.2.2a Larval Fish

The ability to absorb intact protein macromolecules by larval fishes has been demonstrated by a number of authors (see Table 1). Hart (1987) in his studies of the elasmobranch *Scyliorhinus canicula*, showed that the intestinal epithelial cells of larval fish (Stage II and III) contained carbon and ferritin particles following their injection into the yolk-sac. In this species the yolk-platelets are in direct contact with the epithelial cells of the spiral intestine. He therefore concluded that uptake of the marker protein occurred via pinocytosis along

Table 1 Review of Intestinal Antigen/Protein Uptake in Fish

	Stage of Development	Antigen	Route of Administration	Detection Method	Reference
CYCLOSTOMES					
Lamprey *P.marinus*					
	adult	HRP	oral	EM	Langullle & Youson,1985
CHONDRICHTHYES					
Dogfish *S.canicula*					
	larval	carbon ferritin HGG	injected into yolk-sac	LM EM IF	Hart, 1987
OSTEICHTHYES					
Rainbow trout *S.gairdneri*					
	adult	HRP,ferritin	oral	EM	Georgopoulou *et al.*, 1985
	adult	*Vibrio* vaccine	oral	IF	Nelson *et al.* 1985
	juvenile	HRP	oral	ELISA	McLean & Ash 1987
	adult	HGG	oral	IF	Georgopoulou & Vernier, 1986
	juvenile	ruthenium red	oral	EM	Bergot, 1976
	adult	HRP	oral	ELISA, CT	Georgopoulou *et al.*, 1988
Perch *P.fluviatilis*					
	adult	HRP,ferritin	oral	EM, CT	Noaillac-Depeyre & Gas, 1976
Tench *Tinca tinca*					
	adult	HRP	oral	EM, CT	" ,1979
Goldfish *C.auratus*					
	adult	HRP	oral	EM	Gauthier & Landis, 1972
	larval	HRP	oral	LM	Watanabe,1982
	juvenile	protein	oral	LM	Yamamoto,1972
	larval	fat	oral	LM	Iwai, 1968
	juvenile	HRP	oral	EM	Iida & Yamamoto 1985
	adult	Gth	oral	RIA	Suzuki *et al.* 1988

Table 1 Review of Intestinal Antigen/Protein Uptake in Fish (cont...)

	Stage of Development	Antigen	Route of Administration	Detection Method	Reference
C.carpio					
	adult	HRP	oral	EM,CT	Noaillilac-Depeyre & Gas,1973
	juvenile	HRP	oral	ELISA	McLean & Ash 1986
	adult	HRP,ferritin	oral	EM	Rombout et al. 1985
	juvenile	HRP,ferritin	oral and anal	EM	Lamers, 1985
	adult	ferritin	oral and anal	EM, LM,CT	Rombout & van den Berg, 1985
	young fish	variuos bacteria and viruses	oral	recovery of live organisms	Buras et al. 1985
	adult	V.anguillarum	anal	PAP	Rombout et al. 1985
	larval,juvenile	fat,protein	oral	EM	Iwai, 1969
Rosy barb B.conchonius					
	6 months old	Vibrio bacterin	oral	LM, IM	Davina et al. 1982
Grass carp C.idella					
	larval	HRP	oral	EM	Stroband et al. 1979
Cottus nozawe					
	larval	HRP	oral	LM	Watanabe, 1984a
Pond smelt,Hypomesus transpacificus nipponensis					
	larval,juvenile	HRP	oral and anal	EM	Watanabe, 1984c
White sturgeon A.transmontanus					
	larval	yolk material	-	EM	Buddington & Doroshev, 1986
Clarius lazera					
	larval,juvenile	HRP,food	oral	LM, EM	Stroband & Kroon, 1981

Table 1 Review of Intestinal Antigen/Protein Uptake in Fish (cont..)

	Stage of Development	Antigen	Route of Administration	Detection Method	Reference
Ameca splendens					
	larval	ferritin	–	EM	Wichtrup & Greven,1985
	larval	HRP	–	EM	Wourms & Lombardi,1979
	larval	ferritin	–	EM	Schindler & der Vries,1988
Ayu,Plecoglossus altivelis					
	adult	HRP	injected into gut	LM, EM	Nagai & Fujino,1984
	larval	fat droplets	–	LM	Iwai,1968
Tilapia nilotica					
	young fish	various bacteria and viruses	oral	recovery of live organisms	Buras et al. 1985
	larval	HRP	oral	LM	Watanabe,1981
Cherry salmon O.masou					
	larval	HRP	oral	LM	Watanabe,1984b
Coho salmon O.kisutch					
	juvenile	Gth	oral and anal	RIA	McLean et al. 1988a
Chinook salmon O.tschawytscha					
	juvenile	Gth	oral	RIA	McLean et al. 1988b
Spheroides maculatus					
	(not specified)	proline,glycine	isolated segment of intestine	biochemically	Wilson,1957
Clupea harengus					
	larval	polystyrene spheres	oral	LM	Hjelmeland et al., 1988

List of Abbreviations for Table 1

Abbreviations	
HRP	horseradish peroxidase
HGG	human gamma–globulin
Gth	gonadotrophin
EM	electron microscopy
CT	cytochemistry
ELISA	enzyme linked immuno–sorbent assay
LM	light microscopy
ICT	immunocytochemistry
PAP	peroxidase anti–peroxidase
IM	immunologically
IF	immunofluoresence
RIA	radio immuno–assay

with the closely associated yolk platelets.

In embryonic Goodied larvae the endodermal trophotaenial epithelium acts as a placental exchange site (Schindler & de Vries, 1988). Functional and cytochemical data indicates that the trophotaenial absorptive cells are endocytotically highly active (Wichtrup & Greven, 1985), absorbing HRP (Schindler & de Vries, 1988; Wourms & Lombardi, 1979) and ferritin (Schindler & de Vries, 1988; Wichtrup & Greven, 1985) via pinocytotic vesicles, endosomes and lysosome-like bodies.

The ability of free-swimming larvae to absorb intact protein macromolecules has been demonstrated in studies of post-embryonic development of carp (Iwai,1969) and masu salmon (Watanabe, 1984a), both studies describing protein uptake in the posterior gut. Iwai (1969) suggested that pinocytosis was the method by which the bulk movement of protein occurred in carp larvae. Watanabe (1984a) noted that the rectal epithelial cells of masu salmon retained their ability to ingest protein from 12-13 days after hatching until approximately one year of age. He concluded that intracellular digestion of protein molecules by the rectal cells was not essential to the fry as a means of nourishment. The observation that the epithelial cells of the posterior gut absorb protein molecules has been reported in the larvae of other fish species, such as, goldfish (Iwai, 1968b), rainbow trout (Iwai, 1968a) and the halfbeak (Iwai & Tanaka, 1968).

In a number of studies Watanabe (1981; 1982; 1984a;

1984b; 1984c) demonstrated the uptake of HRP by the rectal epithelial cells of a number of fresh-water and marine teleost larvae, including *Oncorhynchus masou, Hypomesus olidus, Carassius auratus, Tilapia nilotica* and the pond smelt *Hypomesus transpacifus nipponensis*. HRP uptake by the intestinal epithelium has also been found to occur in both larval and juvenile grasscarp, *Ctenopharyngodon idella* (Stroband, van der Veen & Timmermans, 1979). In all these studies the HRP was identified in specific regions of the larval gut, all associated with the rectal epithelium in the supranuclear area of the epithelial cells. The time required for the complete digestion of the HRP varies for each species from 5 hours in *T.nilotica* to 7 days in *O.masou* (Watanabe, 1982). In *H.olidus* Watanabe (1982) also noted that intracellular digestion was greater in the larvae without stomachs than in the juveniles in which the gastric glands had already developed. That the ingestion of intact proteins by intestinal cells was a larval function which disappeared with the development of the adult digestive system, as suggested by Tanaka (1971) would thus appear not to be the case. Both Stroband *et al.* (1979) and Watanabe (1984a) have shown that although uptake is greater in younger fish it does not cease completely in older animals. That the possession of a fully-functional stomach does not preclude the absorption of intact proteins has also been demonstrated by Stroband & Kroon (1981) in *Clarius lazera*. These larvae had a fully-functional stomach 12 days after fertilisation yet the enterocytes of the mid-intestine were

still capable of absorbing HRP.

Hjelmeland, Pederson & Nilssen (1988) studied trypsin release into the intestine of *Clupea harengus* L., using either inert polystyrene spheres or live crustacean prey. Although the polystyrene spheres were non-biodegradable and therefore of no nutritional value they were ingested by the epithelial cells with a subsequent secretion of trypsin. This secretory response was, however, significantly lower than the responses to the ingestion of live prey.

The uptake of protein macromolecules by the intestine is demonstrated by a wide range of species of teleosts, both fresh-water and marine. What exactly then is the function of intact protein absorption? This question has not been satisfactorily answered in mammalian studies. It has been suggested that uptake may be of some benefit to larval fishes for the maximising of protein capture, to compensate for the retardation of intracellular digestion observed in juveniles. This is thought to be related to the loss of nutritional function of the rectal epithelial cells (Watanabe, 1982).

Thus the intestinal enterocytes of larvae of several fish species are capable of absorbing large quantities of intact macromolecules. The ability of the enterocytes to absorb macromolecules is species related. Also the age at which the ability to absorb these macromolecules is impaired is species related, but in general appeared to coincide with the development of a fully-functional stomach.

1.2.2b Juvenile and Adult Fish

Protein uptake also occurs in the posterior intestine of adult fish (Georgopoulou, Sire & Vernier, 1984; 1985; 1986; Georgopoulou, Dabrowski, Sire & Vernier, 1988). The enterocytes responsible are characterised by the presence of numerous invaginations of the plasma membrane into the apical cytoplasm to form pinocytotic vesicles (Iwai, 1969; Langille & Youson, 1985; Yamamoto, 1972).

The majority of studies on absorption of intact protein by the adult fish intestine have been concerned with the localisation of protein within the intestinal cells themselves. However, McLean & Ash (1986; 1987) recently revealed, using an enzyme linked immuno-sorbent assay (ELISA) detection method, that, orally intubated HRP was transported across the intestinal epithelium and ultimately into the blood. Georgopoulou *et al.* (1988) showed that orally administered HRP in rainbow trout resulted in a monophasic clearance with up to 6% of the intubated HRP being transferred to the blood after escaping intracellular lysosomal digestion by the intestinal cells of the posterior segment.

Localisation of intestinally absorbed protein macromolecules has been used to demonstrate the regional differentiation of the intestinal tract. Orally intubated HRP was absorbed primarily by the most distal region of the intestine in the goldfish (Gauthier & Landis, 1972). Noailliac-Depeyre & Gas (1973) observed HRP in the apical

tubulo-vesicular system of the carp intestine after oral administration, which reached the blood circulation via the extracellular spaces, a feature similarly noted in perch (Noalliac-Depeyre & Gas, 1979), tench (Noalliac-Depeyre & Gas, 1976), carp (Ash & Mason, 1983; Rombout, Lamers, Egberts & Taverne- Theile, 1986), ayu (Nagai & Fujino, 1984), goldfish (Iida & Yamamoto, 1985), and rainbow trout (Georgopoulou *et al.*, 1985; Nagai, Aizama, Ono & Naguna, 1983). In *in vitro* experiments on HRP absorption a 5 seconds exposure resulted in the formation of many vesicular structures, including coated vesicles (Iida & Yamamoto, 1985). *In vitro* studies, using isolated carp enterocytes, has indicated that HRP is absorbed by a pinocytotic mechanism (Ash & Mason, 1983). Lamers (1985) speculated that HRP was absorbed via receptors present on the microvillous surface of carp epithelial cells; this assumption being based on the apparent difference in the absorption pattern of HRP and ferritin. He provided no other evidence to substantiate this hypothesis, which contradicts all previous mammalian investigations using the same macromolecules.

Rombout & van den Berg (1985) administered ferritin either orally or anally to carp and found that intact ferritin was transported to and accumulated in the supranuclear vacuoles of the intestinal cells. Although ferritin was not found in the intercellular space it was found within large intraepithelial macrophages, however, the precise route of ferritin transportation has not been demonstrated. Enterocytes in the second gut segment of the

grasscarp contained ferritin both in invaginations of the plasma membrane, which probably represent pinocytotic vesicles, and also in vacuoles in the apical cytoplasm (Stroband *et al.*, 1979).

Further to the investigation of intact protein absorption workers have administered bacteria, bacteriophages and viruses into the intestine via the oral route. Nelson, Rohovec & Fryer (1985) administered *Vibrio* bacterin into the intestine of rainbow trout and could not detect any bacterin in the systemic circulation or in any of the organs. However, lymphoid accumulations were found in the lamina epithelialis of the lower intestine together with evidence of the induction of a mucosal reponse. Administration of the same bacterin perorally into the intestine of the rosy barb resulted in a similar uptake of the bacterin in the posterior intestine with an increase in the number of intraepithelial leucocytes (Davina, Parmentier & Timmermans, 1982).In experiments using carp and tilapia, Buras, Duek & Niv (1985) found that a variety of microorganisms could be recovered from the internal organs after oral intubation. An interesting observation was that bacteria and bacteriophages were recovered from the liver of both fish species in high concentrations. The liver is generally not considered to be an organ of high phagocytic activity (Avtalion & Sharabani, 1975), but phagocytic cells analagous to Kupffer cells have been found in the carp liver (Corbel, 1975; Ellis, Munro & Roberts, 1976).

Intestinal absorption of hormones has recently been

demonstrated in two species of salmonids (McLean, Down, Dye, Souza & Donaldson, 1988a; McLean, Benfey, Dye & Donaldson, 1988b), immunologically active molecules being detected in the blood after oral administration of gonadotrophin (Gth). Similarly Suzuki, Kobayashi, Aida & Hanyo (1988), found that in mature goldfish intubated with Gth maximum absorption occurred after 6 hours.

The intestinal enterocytes of adult fish can thus be shown to absorb and transport intact macromolecules into the systemic circulation. That viable bacteria and viruses may penetrate the mucosal barrier (Buras *et al.*, 1985) has led to the investigation into the possibility of developing oral vaccines to fish pathogens, as it has been demonstrated that oral exposure to bacterial antigens can lead to the stimulation of the local immune system (Davina *et al.*, 1982).

1.2.3 Mechanisms of Macromolecular Absorption

The routes by which macromolecules are absorbed by the intestinal epithelial cells of both fish and mammals are discussed in the following pages. The major routes of entry, by general concensus, are via either the transcellular or paracellular pathway.

1.2.3a Transcellular Pathway

The most common terms used to describe the transcellular

route are either endo- or pino- cytosis. Additionally, two forms of pinocytosis have been identified;

i) fluid phase, where molecules are brought into the cell from the surrounding medium in direct proportion to their concentration without any form of discrimination. As intestinal absorptive cells age it is thought that their cytoplasmic integrity is compromised (Toner, Garr & Winburn, 1971), which may affect the permeability of the cell. A variety of studies with mouse (Hugon & Borgers, 1968) and human enterocytes (Blok *et al.*, 1981) have demonstrated the existence of differential permeability between cells, Hugon & Borgers (1968) also suggested that HRP may gain access to the lamina propria via the goblet cells.

ii) receptor-mediated, where molecules are internalised following combination with specific binding sites. Receptor-mediated pinocytosis requires that a specific ligand binds to a specific receptor molecule prior to initiation of pinocytosis. When a sufficient concentration of molecules come into contact with the cell membrane invagination occurs and small vesicles are formed (Walker, 1981). The sialic acid of the glycocalyx is considered to be an important binding group for the extracellular materials in their uptake by absorptive cells (Yamamoto, 1972).

In teleost larval and adult intestines the absorptive cells show a number of tubular invaginations of the plasma membrane at the bottom of the clefts between the microvilli (Georgopoulou *et al.*, 1986; Watanabe, 1984; Yamamto, 1972). These invaginations penetrate deep into the apical cytoplasm

where they are 'pinched off' with the subsequent formation of vesicles (Georgopoulou *et al.*, 1985; 1986; Iwai, 1968a; 1968b; Noaillac-Depeyre & Gas, 1973; 1976). The time involved varies in different larval teleosts from 30 min. to 3 hours (Watanabe, 1982). These vesicles are then either disrupted or coalesce with the formation of large vesicles (Iwai, 1969; Rombout *et al.*, 1985), in the supranuclear region (Govoni, Boehlert & Watanabe, 1986; Iida & Yamamoto, 1985; Rombout *et al.*, 1985). This process is variable in larval fish taking from 5 hours to 7 days (Watanabe, 1982), while in adult rainbow trout (Georgopoulou *et al.*, 1986) IgG was detected 10 hours after oral administration.

Abrahamson & Rodewald (1981) intubated HRP and an IgG-ferritin complex into the gut of mice and noted that both markers were absorbed by the same cell and that some form of intracellular sorting occurred, involving the segregation and concentration of HRP within lysosomes. It is possible that certain viral and bacterial particles, which are known to gain access to the circulation by pinocytotic mechanisms via M-cells (Owen, Pierce, Apple & Gray, 1986; Wolf, Kaufman, Finberg, Dambrauskas, Fields & Trier, 1983) may avoid proteolysis by either escaping lysosomal fusion via receptor-mediated pathways or by resisting lysosomal acid hydrolases.

In fish, the numerous invaginations of the epithelial cell membrane are closely associated with cytoplasmic tubules (Georgopoulou *et al.*, 1985). In recent ultrastructural studies cytoplasmic tubules have been

observed to contain marker proteins (Georgopoulou *et al.*, 1985; Iida & Yamamoto, 1985; Rombout *et al.*, 1985). Iida & Yamamoto (1985) revealed that 5 sec. after exposure of goldfish hindgut epithelial cells, *in vitro*, the marker protein, HRP, was located in coated vesicles, and after 1 minute 11% of the cytoplasmic tubules contained HRP. Rombout *et al.* (1985) demonstrated the passage of luminally-derived HRP from cytoplasmic tubules to the intercellular space in carp.

Transcellular pathways involve both specific and non-specific absorption of macromolecules in mammals. In fish the transcellular route of macromolecule absorption has been shown to be a highly active process occurring in both larval and, to a lesser extent, in adult enterocytes. As yet there is very little evidence to show the specific absorption of molecules via receptor sites on the enterocyte cell membrane. A feature of fish enterocytes is the involvment of the cytoplasmic tubules in the passage of the ingested macromolecules through the enterocyte which may constitute a major intracellular pathway of intact macromolecule transfer in fish.

1.2.3b Paracellular Pathway

This phenomenon, called persorption, was first demonstrated by Herbst in 1844 (cited by Bainter, 1986) when he administered a starch suspension perorally to dogs and detected starch granules in the blood and lymph. Volkheimer

& Schultz (1968) suggested that starch particles were 'kneaded' between the epithelial cells by a mechanical action of the intestine. Persorption takes place across mucous membranes that are composed of a single cell layer; primarily across the epithelium of the small intestine and at a much slower rate in the stomach, colon and rectum (Volkheimer,1972). The rhythmic contractions of the villi and vascular pulsation, transmitted to the mucosa may contribute to the uptake of macromolecules (Volkheimer, 1972).

Persorption takes place via three possible routes:

i) primarily through the 'extrusion zone' on the tip of the intestinal villi where the intercellular junctions are 'loosened' (Nicklin & Miller, 1984), or

ii) between goblet cells and absorptive enterocytes on the side of the villi where the tightness of the junctions is diminished (Bainter, 1986), or

iii) at a site provided by mechanical lesions due to the intrusion of large paricles around the base of the tightly packed villi (Volkheimer, 1972).

The persorbed particles pass between the epithelial cells, through the subepithelial spaces and enter the lymph and portal blood (Volkheimer, 1972). Stimulation of peristalsis by caffeine, oleum ricci, neostigmine and nicotine (Volkheimer, 1977) increases the rate of persorption. After peroral administration of starch granules ingestion is quite rapid with the first granules appearing in the circulation within 5 min. (Volkheimer, 1977) and with two or three peaks

of absorption (Volkheimer, 1972) . Nutritionally this process seems to lack any importance, and it is unclear whether persorption is of physiological or pathological significance as Volkheimer & Schulz (1968) indicated that certain inert particles gain access to the circulation without initiating any immune response at all.

1.2.4 Factors affecting Absorption

The epithelial surface of the gastrointestinal (GI) tract represents an extensive surface area that is exposed to a hostile environment containing a variety of antigens either dietary or pathogenic (Miller & Nicklin, 1987a; 1987b). Walker (1981) proposed that the local immune system and non-immunological processes were an effective barrier to the attachment and penetration of antigens.

1.2.4a Immunological Factors affecting Absorption

i) Antigen access to lymphoid tissue

In mammals Peyer's Patches (PP) are thought to be the route of entry for a variety of organisms. The mechanism of entry has been shown to be via the specialised M-cells in the epithelium overlying the PP. M-cells appear to selectively sample the environment and pass potentially antigenic material to the closely associated lymphocytes below the epithelium (Owen, 1977), Figure 2. Viable *Vibrio cholerae* injected into the intestinal lumen of rats were

phagocytosed by M-cells, and subsequently discharged among underlying lymphocytes and macrophages (Owen *et al.*, 1986). Wolf *et al.* (1983) showed that M-cells are the sites where rheoviruses penetrate the intestinal epithelium and appear to bind selectively to the M-cell membrane. It may well be that the binding of antigen to the mucosal epithelium is a crucial first step in promoting a secretory immune response.

The presence of an equivalent to the mammalian M-cell in the fish intestine has not been demonstrated. The fish intestinal epithelium has however been shown to absorb orally introduced bacterin. Nelson *et al.* (1984) also showed that the uptake of bacterin by the enterocytes resulted in a local immune response in the gut of rainbow trout. Similarly Fletcher & White (1973) found that the same organism, *Vibrio anguillarum*, induced a mucosal antibody reponse after oral intubation in plaice, of a greater titre than that observed in the serum. The number of intraepithelial leucocytes have also been shown to increase in the intestine of the rosy barb following the oral administration of this bacterin (Davina *et al.*, 1982). This increase was observed after 30 min. and was still apparent 15 hours later. One hour after anal administration of *V.anguillarum* to carp, the macrophages in the intestine were shown to contain bacterin, this reactivity persisted for at least 3 days after intubation (Rombout *et al.*, 1986). The transfer of bacterin from the epithelial supranuclear vacuoles to the intraepithelial macrophages must be an important factor in the intestinal defence system of fish. The significance of

mucosal macrophages their function, derivation and fate needs further investigation.

ii) Mucosal Immunity

An important component of host defences at the epithelial surface is the presence of mucosal antibodies, and the existence of a common mucosal immune system (Montgomery, Lemaitre- Coehlo & Vaerman, 1980; Rombout *et al.*, 1985; St. Louis-Cormier, Osterland & Anderson, 1984). The major Ig in the mucous secretions of mammals is IgA (Ogra & Karzon, 1970; Walker, 1987) and in fish IgM (Hart, Wrathmell, Harris & Doggett, 1987; Lobb & Clem, 1981; Tomonaga *et al.*, 1986). There is evidence that in mammals plasma cells in the intestinal mucosa may specifically secrete IgA, however, little information is available about the origin of these IgA-producing plasma cells within the lamina propria. Several theories exist as to the origins of the precursors for local plasma cells:

1) precursor cells may be of epithelial derivation originating from the primitive gut (Fichtelius, 1968)

2) precursors may be derived from the bone marrow which then seed the lamina propria and differentiate into IgA-producing plasma cells

3) lymphocytes from Peyer's patches under the influence of antigen stimulation differentiate into IgA-producing plasma cells and seed the lamina propria (Craig & Cebra, 1971)

Plasma cells have also been identified in the intestinal

mucosa of several fish species (Doggett, Wrathmell & Harris, 1987; Hart, Wrathmell & Harris, 1986a; Temkin & McMillan, 1986), along with the presence of IgM in mucosal secretions (Hart *et al.*, 1987a; 1987b; Lobb & Clem, 1981; Rombout et al., 1986; St. Louis-Cormier *et al.*, 1984). Tomonaga, Kobayashi, Kajii & Awaya (1984) identified Ig-forming cells in the valvular intestine of the bullhead shark using immunofluoresence techniques. This is analogous to the situation in mammals where the production of secretory Ig is probably mediated by those plasma cells located in the lamina propria close to the epithelial surface (Walker & Isselbacher, 1977).

In mammals secretory IgA (sIgA) found in external secretions is a product of two cell types: plasma cells that produce polymeric IgA with a J-chain, and epithelial cells that produce secretory component (SC) an integral part of the sIgA molecule (Mestecky, McGhee, Crago, Jackson, Kilian, Kiyono, Babb & Michalek, 1980). The dimeric IgA is transported through the epithelial cell, acquiring the SC, and is then secreted as an IgA-J-SC complex (Brandtzaeg, 1974). The proposition that SC-binding immunoglobulins were transported from serum into secretions at a variety of mucosal sites, dependent on SC availability, was shown by Scicchitano, Sheldrake & Husband (1986). They demonstrated that both IgA and IgM were selectively transported into respiratory tract secretions and saliva of sheep. As yet the presence of a similar SC piece in fish has not been demonstrated. Underdown & Socken (1978) have, however,

demonstrated the presence of an SC-binding site for mammalian SC on the high molecular weight (HMW) Ig in amphibia and elasmobranchs. Dobbins (1982) speculated that the SC in mammals conferred a protective function to the dimeric IgA against enzymatic digestion in the intestine.

Recent studies have provided direct evidence for the immune exclusion function of mammalian intestinal antibodies. Williams & Gibbons (1972) found that on exposure to secretory IgA the adherence of *Streptococcus viridans* to epithelial cell surfaces was significantly decreased, and concluded that the IgA blocked specific binding sites on the bacterial cell wall. Intestinal antibodies can also protect against the effects of toxic bacterial by-products, secretory Ig complexing with these molecules in the mucosal coat and enhancing their subsequent degradation by intestinal proteases (Walker & Isselbacher, 1977).

Immunoglobulins are also present in the bile, and Ig-containing cells are seen in the epithelium of the gall-bladder of humans, although fewer in number than in the GI tract (Chen & Tobe, 1974). Plasma cells were also detected in the gall-bladder epithelium of the elasmobranch, *S.canicula* (Hart, Wrathmell, Doggett & Harris, 1987b), and biliary Ig has been detected in a number of fish species in response to antigenic stimulation (di Conza & Halliday, 1971; Engle & Woods, 1957; Rombout *et al.*, 1986; St. Louis-Cormier *et al.*, 1984). Orlans, Peppard, Payne, Fitzharris, Mullock, Hinton & Hall (1983) have shown that in rats IgA is transported across hepatocytes from serum to

bile by a mechanism that involves receptor-mediated endocytosis at the sinusoidal face of the hepatocyte.

Bile collected from rats orally immunised with cholera enterotoxin were shown to contain anti-cholera enterotoxin antibodies (Tamaru & Brown, 1985). It seems likely that IgA in bile consists largely of antibodies to antigens which are encountered in the intestine. Neutralisation of cholera toxin by rat bile secretory IgA antibodies was also shown by Vaerman, Derijck- Langendries, Rits & Delacroix (1985). However,the precise role of the liver in the secretory immune system is not clear. Challacombe, Greenall & Stoker (1987), in humans, demonstrated that antigenic stimulation resulted in an increase in the overall sIgA concentration in the portal blood together with an increase in the concentration of immune complexes. These complexes appear to be , in part, removed from the circulation by transport into the bile (Harmatz, Kleinman, Bunnell, McClethan & Block, 1982). Ligation of the rat bile duct clearly demonstrated the importance of this route for the clearance of IgA immune complexes, delaying but not completely inhibiting their removal (Harmatz, Kleinman, Bunnell, McClethan, Walker & Bloch, 1984). These results would seem to indicate that there may be alternative mechanisms for removing immune complexes from the systemic circulation possibly involving the liver.

1.2.4a Non-immunological Factors affecting Absorption

i) Gastric Function

The effectiveness of the gastric barrier and the acid content of the GI tract are an important factor in the regulation of macromolecular uptake. Neutralisation of gastric acidity with sodium bicarbonate markedly reduces the dose of *Vibrio cholerae* required to infect man (Vaerman *et al.*, 1985), a similar effect was noted by Bloch *et al.* (1979) on the uptake of BSA across the epithelial cells of rats. Walker (1976) reported that pancreatic enzymes adsorbed on the surface of the small intestine facilitated the breakdown of antigen- antibody complexes at that site. Breakdown of ^{125}I-labelled BSA by jejunal gut-sacs from pancreatic duct-ligated rats is significantly decreased in comparison to sham-operated controls (Walker *et al.*, 1972). As previously described in the neonatal rat the intestine is highly permeable to macromolecules, however during the first 21 days of life there is an increase in peptic activity in the stomach which can be correlated with the decrease in the uptake of IgG and maturation of the intestinal epithelial cells (Henning, 1979).

Gastric acidity is an important deterrent to the colonisation of the intestine by Gram positive organisms (Udall, Colony, Fritz, Pang, Trier & Walker, 1981). This together with the interactions between normal flora and potential pathogens reduces the likelihood of enteropathogen colonisation of the GI tract, although there may also be a

form of competition for substrates within the gut by these organisms (Freter, 1962).

Johnson & Amend (1983) have demonstrated that gastric secretions influence the transport of particulate antigens in fish. In rainbow trout vaccinated either orally or anally with *Vibrio anguillarum* and *Yersinia ruckeri* there was an increase in protection against these pathogens when the anally vaccinated group were bath challenged. This would seem to indicate the importance of the stomach and its secretions for protection against pathogens.

Protease inhibitors, such as aprotinin, inhibit trypsin-like activity on the intestinal uptake of BSA in newborn 2- and 4- week old rabbits. This resulted in an increase in the concentration of immunoreactive BSA in the plasma of test animals compared to controls (Udall, Bloch, Vachne, Feldman & Walker, 1984). Similarly Brambell (1970) showed that the presence of proteolytic inhibitors in colostrum has a significant effect upon the activity of both trypsin and chymotrypsin.

ii) Mucus Secretion

Mucus has long been credited with an important role as a physical component of the intestinal defence system, acting both as a lubricant and as protection against digestive enzymes (Walker, 1976). The goblet cell mucus covering the epithelial surface of the GI tract may protect against the penetration of antigens, toxins and microorganisms present within the intestinal lumen. Antigen-antibody complex

formation within the intestinal lumen may trigger the release of goblet cell mucus (Walker, 1981) which decreases the adherence of organisms to the gut surface (Brodsky, 1981). The pathogenicity of strains of *E.coli* is determined by their ability to adhere to the mucosa and hence their capacity to produce enterotoxin (Inman & Canty, 1983). In mammals bacterial adherence has been linked to the presence of genetically determined mucosal receptors (Rutter, Burrows, Sellwood & Gibbons, 1975).

There is some evidence that glycoproteins present in mucus may specifically bind with enterotoxins and thereby limit their interactions with the microvillous receptor (Boedeker, 1982). These glycoproteins may also coat the external surface of bacteria and thus interfere with their attachment to the intestinal surface (Walker, 1985).

Penetration enhancers have been used to improve peptide and protein absorption by one or several mechanisms. All penetration enhancers are capable of increasing membrane fluidity. Four major types of penetration enhancers have been recognised: (1) chelates, such as EDTA and salicylates; (2) surfactants, such as sodium lauryl sulfate; (3) bile salts, such as sodium deoxycholate and sodium glycholate, and (4) fatty acids, such as oleic acid and monoolein. Bile salts have been shown to reduce the viscosity of the mucous layer, facilitating peptide and protein diffusion towards the membrane surface. Some chelates and non-ionic surfactants interfere with tight junctions between epithelial cells, whereas salicylates and some fatty acids

create disorder in the phospholipid domain in the membrane (cited from Lee, 1986). All of these facilitate the absorption of peptides and proteins across the epithelial cell surface.

Possibly the most significant use for penetration enhancers is in the development of oral vaccines. These may be incorporated into the vaccine and stimulate or facilitate the absorption of the vaccine components. The type of enhancer used would depend on the mode of action of the enhancer. Of particular note is that one such enhancer, the saponin Quil-A which also acts as an immunoadjuvant, has been used to produce an animal vaccine for cat flu as well as other experimental vaccines against protozoal diseases (Bomford, 1988).

iii) Peristalsis

In order to colonise the GI tract bacteria must attach to receptors on the microvillous surface. Normal peristalsis of the intestinal muscularis is thought to minimise the possibility of bacterial attachment, and to be an important host defence mechanism (Udall *et al.*, 1981). Pang, Walker & Bloch (1981) provided evidence that antigen-antibody formation in the mucus coat stimulated peristalsis and resulted in the rapid expulsion of antigens from the small intestine.

iv) Membrane Composition

Membrane composition of the epithelial cells of mammals

has in recent studies been shown to change as the cell migrates along the villi and as the animal ages (Quaroni, Kirsch, Herscovic & Isselbacher, 1980). These changes in the membrane composition are thought to affect the ability of bacteria and other toxins to bind to the cell. Bresson, Pang, Udall, Fritze & Walker (1980), using isolated microvillous membranes from the intestine of newborn and adult rats, demonstrated that the membrane protein/phospholipid ratio was dramatically decreased in membranes of newborn animals compared to those of adults. This was accompanied by an increase in the binding of cholera toxin to the membranes.

M-cells which are known to take up macromolecules (Wolf, Rubin, Finberg, Kaufman, Sharpe, Trier & Fields, 1981) and enterocytes share common membrane glyco-conjugates even though their molecular groupings may differ. Lectin binding and anionic charge similarities of M-cells and enterocytes may facilitate antigen sampling by M-cells of particles that adhere to intestinal surfaces in non-Peyer's patch areas (Owen & Bhalla, 1983). M-cells also do not have a well-formed glycocalyx, in addition, the paucity of microvilli and intestinal folds (Madara, Bye & Trier, 1984) and the relative lack of staining of the M-cell apical membrane for alkaline phosphatase (Owen & Bhalla, 1983) may suggest an inability to prevent attachment or secrete relevant enzymes for protection. Madara *et al.* (1984) demonstrated that the apical M-cell membranes were rich in cholesterol, together with a low protein/lipid ratio except

in those domains involved in endocytosis. These properties of the M-cell membrane would seem to indicate that this cell is specially adapted to sampling the luminal environment.

1.2.5 Oral Tolerance

A further feature to be considered when macromolecular uptake in the gut occurs is the phenomenon of oral tolerance or hyporesponsiveness. Oral tolerance is a state of specific systemic immunological unreponsiveness induced by previous oral exposure to an antigen. The induction of oral tolerance after ingestion of antigen has been reported in a number of animal models (Enders, Gottwald & Brendel, 1986; Holt, Vines & Britten, 1988); however, the precise mechanisms responsible for this unreponsiveness are not well understood. Dakin (1829, cited by Mowat, 1987) first described the phenomenon, where South American Indians ate poison ivy leaves in an attempt to prevent contact sensitivity reactions to the plant, demonstrating how the oral ingestion of antigen would modify a systemic immune response.

Oral tolerance affects all aspects of the systemic immune response, a single exposure to an enteric antigen in some instances suppressing the induction of systemic IgM, IgG, and IgE as well as cell-mediated immune responses (Kagnoff, 1978a; 1978b; 1982).The induction of systemic tolerance by oral feeding is influenced by a variety of factors including

type, dose and frequency of administration of antigen and the age of the animal.

1.2.5a Factors affecting oral tolerance

i) Type of antigen

There are demonstrable differences between antigens in their ability to induce oral tolerance which may depend on their physical structure. For example soluble proteins can induce a long-lasting systemic tolerance (in the presence of an active mucosal response) whereas tolerance to more complex antigens such as bacterial cell walls require larger amounts of antigen for tolerance induction which may be shorter lived (Challacombe & Tomasi, 1987).

The experimental administration of the soluble antigen, OVA, into the gut of inbred mice can induce a state of systemic unresponsiveness (Richman, Chiller, Brown, Hanson & Vaz, 1978). which can be long-lasting even after the administration of a single dose of OVA (Vaz, Maia, Hanson & Lynch, 1977). Another soluble antigen, BSA, also readily induces a systemic tolerance in mice (Thomas & Parrott, 1974). However, oral tolerance can be abrogated by agents that activate the reticuloendothelial system, RES (Mowat & Parrott, 1983), such as oestrogen and muramyl dipeptide.

Following the intubation of the complex antigen *Streptococcus mutans*, a significant proliferatve reponse may be found in the Peyer's Patches. With the soluble antigens OVA and KLH however, no such response could be detected

(Challacombe & Tomasi, 1980). The reponse to closely related antigens, such as cholera toxin and cholera toxoid, may also vary in whether they stimulate an immune response or systemic tolerance. Analysis of the mechanisms of tolerance after the feeding of complex antigens, such as bacteria, may be seen as induction of tolerance to one of the surface antigens with enhancement of the immune response by another (Stokes, Newby & Bourne, 1983). Indeed, lipopolysaccharide (LPS) produced by intestinal microflora may play a role in oral tolerance (McGhee, Michalek, Kiyono, Babb, Clark & Mosteller, 1982) perhaps by stimulating the activation of suppressor T cells in Peyer's Patches.

ii) Dose and Frequency of antigen exposure

The dosage of antigen and the feeding protocol appears to be an important parameter in the induction of oral tolerance, this being dependent on both the antigen and the animal species (Nicklin & Miller, 1987).

Oral tolerance to soluble protein antigens has been widely studied. One such antigen is OVA where there are varying reports concerning the response to different doses of OVA. Challacombe & Tomasi (1980) observed that in mice a wide range of OVA doses were capable of inducing tolerance. However, the feeding of small amounts of OVA may prime rather than tolerise mice (Asherson, Perera, Thomas & Zembala, 1979). This priming has been noted in C3-H strains of mice fed low doses of OVA, whereas feeding higher doses was found to induce tolerance (Mowat, Thomas, McKenzie &

Parrott, 1986).

The feeding of a particulate antigen, such as sheep red blood cells (SRBC), can lead to the induction of tolerance when this antigen is re-administered after a 2-3 week period (Andre, Heremans, Vaerman & Cambiaso, 1975). However, the short term feeding of SRBC boosts the IgA and IgG serum antibody titres in mice (Kagnoff, 1980). Andre *et al.* (1975) found that the specific hyporesponsiveness, or tolerance, could be passively transferred to control animals and proposed that this was due to a component in the serum. This serum factor, termed a tolerogen, has a powerful blocking effect on parenteral and enteric challenges suggesting the formation of antibody (IgA)- antigen complexes. Chalon, Milne & Vaerman (1979) however, suggest that the antibody involved is IgG, with the IgG_1 subclass being particularly suppressive. Thus tolerance to SRBC and perhaps other particulate antigens is mediated by the removal of specific antibody moieties from the systemic circulation by antigen that has crossed the intestinal epithelium barrier.

iii) Age of the animal

It has been suggested that low amounts of antigen encountered early in life may predispose the subject to the development of hypersensitivity (Jarret, 1984). Conversely if piglets are exposed to large amounts of dietary antigen before weaning it prevents intestinal hypersensitivity but may induce a state of oral tolerance (Miller & Hanson, 1979). Neonatal mice (1-2 days old) have been shown to be

primed for both delayed type hypersensitivity (DTH) and systemic antibody responses by feeding a dose of OVA which would tolerise older animals (Mowat, 1987). The ability to induce tolerance in mice begins at about 4 days of age (Strobel & Ferguson, 1984), the inability to induce tolerance prior to this may be due to a number of factors such as immaturity of the intestine and/or the immune system.

1.2.5b Mechanisms of oral tolerance

Oral tolerance is a highly complex phenomenon which may involve a wide range of immunoregulatory mechanisms which have not been entirely clarified.

Since antigens absorbed from the intestine travel rapidly to the liver via the portal venous system, this organ was thought to play a crucial role in the induction of tolerance. In those cases where the liver appeared to be critical, it was postulated that the Kupffer cells removed immunogenic aggregates but allowed tolerogenic monomers of fragments to reach the peripheral lymphoid tissues (Tomasi, 1980). However, the induction of tolerance by feeding BSA to rats is not influenced when the liver is by-passed by a portocaval shunt (Thomas, Ryan, Benjamin, Blumgart & MacSween, 1976). The role of the liver in the induction of oral tolerance therefore remains unclear.

Although feeding proteins tolerises both humoral and cellular immunity most workers agree that this is determined

principally by T-cell unresponsiveness, while B-cells remain potentially active. The ingestion of antigenic material results in systemic tolerance and the production of suppressor cells. The exact mechanisms of suppressor-cell activation and function in protein-fed animals have not been clearly demonstrated. Whether this tolerogenic state is the result of activation of T-cells on exposure to antigen during development or suppression by other T-cells is unknown.

Peyer's Patches have been indicated as having a key role in the induction of tolerance, with the production of T suppressor cells in the lymphoid tissue following the ingestion of antigenic material. This inference is due to the presence of specialised M-cells in the epithelium overlying the PP (Owen, 1977), which are capable of ingesting macromolecules that are then brought into direct contact with the lymphoid tissue in the PP. These T suppressor cells may then migrate to other lymphoid tissue such as the mesenteric lymph nodes (MLN) and spleen. Recently however, the direct influence of the PP on the induction of oral tolerance has been investigated in rats that have had their PP surgically removed. In these animals, and those with PP, oral tolerance to SRBC could be demonstrated (Enders *et al.*, 1986), requiring alternative explanations for the mechanism by which tolerance is generated. One such possible explanation is that mammalian intestinal epithelial cells are able to express class II MHC antigens (Curman, Kampfe, Rask & Peterson, 1979) which have

been shown to be important in the presentation of antigen to T cells.

CHAPTER 2.

2. MORPHOLOGY, CYTOCHEMISTRY AND FUNCTIONAL STUDIES ON THE PERIPHERAL BLOOD LEUCOCYTES

2.1 Introduction

Fish are the largest group of vertebrates with over 20,000 known species (Bone & Marshall. 1982), comprising two main types; the agnathans, or jawless fish, and the gnathostomes, or jawed fish which are further divided into the Chondrichthyes and the Osteichtyes. It is commonly accepted that fish possess leucocytes in their peripheral blood consisting of lymphocytes, thrombocytes, monocytes and granulocytes. The area of greatest contention concerns the nomenclature of the various forms of granulocytes that have been identified in the fish species that have been studied. At present this is loosely based on their mammalian equivalents, that is, the neutrophil, eosinophil and basophil. The presence or absence of the different leucocytes are variously reported; Jordan & Speidel (1924) finding no monocyte or basophil-like granulocyte in the two fish species they examined, and Blaxhall & Daisley (1973) who could not identify a blood monocyte in brown trout. Of the peripheral blood leucocytes the basophil-like granulocyte and to a lesser degree the eosinophil-like granulocytes are the most inconsistently observed cells in fish peripheral blood. Most fish haematologists would now

concede that it is no longer feasible or useful to rely exclusively on similarities between fish and mammalian leucocytes in order to provide a comparable terminology. Further investigation of the function of fish leucocytes, together with enzyme cytochemical techniques, is required in many instances where previously only morphological examinations have taken place.

It is known that both environmental and physiological factors can affect many of the parameters of the blood cell composition. Suzuki, Kusakar, Shimzu & Yamada (1983) noted that there was an age-dependent and a seasonal-dependent change in the number of intermediate-type thrombocytes and type 1 lymphocytes in the peripheral blood of the rockfish, *Sebastes schlegeli* HILGENDORF. Ezzat, Shabana & Farghaly (1974) also noted a change in the number of the various leucocyte types in the cichlid *Tilapia zilli* due to seasonal changes.

There is very limited information on the haematology of *O.mossambicus* and tilapians in general. Sailendri & Muthukkaruppan (1975) examined the peripheral blood of this teleost only morphologically, whilst Boomker (1980) confined his examination of blood cell elements to the erythrocyte and thrombocyte series.

Due to the limitations of Giemsa or Romanowsky stains, further analysis using a series of cytochemical techniques and functional phagocytic properties, using colloidal carbon and attenuated bacteria, were employed to confirm and supplement the initial morphological characterisation of the

leucocytes.

2.2 Materials and Methods

2.2.1 Yeast

Brewer's yeast was suspended in phosphate buffered saline (PBS) to a concentration of 1.2 x 10^8 cells /ml. Cells were then heat killed at 100°C for 10 min. The cells were then stained by adding 0.1 ml. of a 2% solution of Trypan blue per ml. of yeast suspension for 10 min. These were then washed three times in PBS by centifugation (700g for 5 min.) and resuspended to the original concentration.

2.2.2 Bacteria

Aeromonas salmonicida (strain 25/77) was cultured in 100ml volumes of tryptone soya broth (TSB) in 200ml Erlenmeyer flasks for 24 hours at 25°C. The bacteria were then killed by fixing in 0.6% formaldehyde, washed in several changes of PBS and stored at -20°C until required.

2.2.3 Tissue Sample Preparation

Blood was sampled from adult fish of both sexes which were kept in recirculating fresh-water systems at 24-26°C. Samples were collected from the caudal sinus in heparinised syringes with a 23g needle.

a) Light Microscopy (LM)

Whole blood smears were prepared in the usual manner,

Table 1. Cytochemical techniques used to characterise the peripheral blood leucocytes

Stains	Fixation	Reference
Sudan Black	Formalin vapour	modified McManus 1947: Hayhoe & Flemens,1969
Periodic Acid Schiff,PAS	Methanol	Hayhoe & Flemens, 1969
May–Grunwald Giemsa,MGG	Methanol	modified Doggett 1985: Pearse 1968
Acid Phosphatase, AcP	2% gluteraldehyde in phosphate buffer	Reynolds,1982: incubate at room temp
Alkaline Phosphatase, AlP	"	"
Non–specific Esterase,ANAE	2% gluteraldehyde in acetate buffer	"
DAB Peroxidase, PO	2% gluteraldehyde	Vacca et al.,1978: counterstain 0.1% toluidine blue

air-dried and fixed as required for the staining technique undertaken (Table 1).

b) Electron Microscopy (EM)

Whole blood was centrifuged at 700g for 10 min. Plasma was removed from above the leucocyte layer and replaced with 2.5% glutaraldehyde in 0.1M cacodylate buffer pH 7.2 and fixed for 1 hour at 4°C. The hard leucocyte layer was removed and cut into 1mm pieces, post-fixed in 1% osmium tetroxide in 0.1M cacodylate buffer for 1 hour. The samples were dehydrated in a graded series of alcohols, infiltrated with Spurr resin/alcohol mixtures and embedded in Spurr resin (TAAB, Berks). The resin was cured at 70°C for 8-9 hours. Gold and silver sections were cut on a Reichert Jung OM3 ultratome and mounted on copper grids. Sections were stained with a triple stain technique, lead citrate- uranyl acetate- lead citrate, and examined using a Philips 300 series electron microscope.

2.2.4 Cytochemistry

The cytochemical procedures employed are outlined in Table 1.

2.2.5 Phagocytosis and Adherence

a) Phagocytic properties were examined *in vivo* by using either 0.2 ml. 20% colloidal carbon suspension (Pelikan Indian Ink) or 0.2 ml. of a 10^8cell/ml suspension of attenuated *A.salmonicida* in 0.85% sterile saline. These were

injected intravenously via the caudal vein and sampled after 4, 6 and 8 hours. Smears were prepared, stained using MG-G and examined. For EM examination samples were prepared as before (2.2.3.b) and viewed by TEM.

b) Adherence and *in vitro* phagocytosis was studied using separated leucocytes. Whole blood (0.2ml.) was centrifuged at 700g for 10 min. The plasma was removed and the leucocyte layer was placed in a plastic LP3 test tube and resuspended in 10 ml. of McCoy's culture medium (BDH). Aliquots of the leucocyte suspension (0.2ml.) were placed on clean glass slides and incubated in a humid atmosphere at room temperature for 1 hour. Non-adherent cells were subsequently removed by washing. 0.2ml. of Trypan blue stained yeast cells in culture medium were then added to the adherent cells for 30 min.; after washing off excess yeasts adherent cells were fixed and stained as for whole blood smears.

2.3 Results

Four types of leucocyte could be identified in the peripheral blood; lymphocytes, thrombocytes, monocytes and granulocytes. The results of the cytochemical procedures are presented in Table 2.

2.3.1 Leucocyte Morphology

Lymphocytes (3-6.6 μm). These cells were morphologically similar to the lymphocytes found in other fish species and mammals with a central nucleus which was surrounded by a

Table 2. Cytochemistry of the peripheral blood leucocytes

Stains	Lymphocyte	Thrombocyte	Monocyte	Granulocyte type 1
Sudan Black	−ve	−ve	−ve	strong +ve
PAS	+ve granules	+ve granules	few +ve granules	strong +ve
AcP	+ve cytoplasm	−ve	+/−	+ve
AlP	−ve	−ve	−ve	+/−
ANAE	+/−	−ve	+/−	+ve
Peroxidase	−ve	−ve	−ve	+ve

-ve no reaction

+ve positive reaction

+/- variable reaction

thin rim of cytoplasm (Plate A1). Occasionally a few small pseudopodia were displayed. Some cells possessed PAS +ve granules in their cytoplasm. At the EM level the most prominent features were the large mitochondria in the cytoplasm (Plate A2). Also observed were small vesicles, free ribosomes, and limited amounts of endoplasmic reticulum (ER). The nucleus contained condensed chromatin, and although usually spherical occasionally cells with cleft nuclei were observed.

Thrombocytes With MG-G the nucleus of these cells stained dark purple and was surrounded by a pale blue cytoplasm. Two forms were observed in the peripheral blood differing in cell shape. A cell with a cytoplasmic 'tail'; often referred to as 'tear-drop' or 'fusiform' (3.5-12.9 µm, Plate A1 & A3) with a vacuole-like structure at the base of the tail or to one side of the nucleus and an oval-shaped cell (3.5-5.6 µm, Plate A1 & A4) in which the nucleus occupied the majority of the cell and possessed a clear vacuole-like structure at either or both poles. In cross-section thrombocytes could be easily confused with lymphocytes.

At EM level smaller vacuoles were observed below the plasma membrane, which as Ferguson (1976) suggested may be analagous to the surface-connecting system of the mammalian platelet (Plate A4). Microtubules were observed in some cells below the plasma membrane running the whole length of the cell (Plate A3). Ribosomes, a few mitochondria and a small Golgi apparatus were observed. At LM PAS +ve granules were found and appeared to be located in the approximate

area of the vacuole-like areas.

Monocytes(11.4-19.1 μm) The largest peripheral blood leucocytes were the monocytes which generally displayed an irregular outline (Plate B1). With MG-G the nucleus stained pink and was reticulated, eccentric and usually ovoid but could be more irregularly shaped or lobed having a darker basophilic, often vacuolated, cytoplasm. The cytoplasm contained several mitochondria with some rough ER (RER) , a Golgi complex and some small lysosomes (Plate B2). A few PAS +ve granules were observed in the cytoplasm and with acid phosphatase (AcP) the reaction product was mainly around the periphery of the cell and at the nucleolus.

Granulocytes. Under the LM only two forms of granulocyte were recognised in the peripheral blood, but further investigation at the EM level revealed a third form.

Granulocyte type 1 (7.2-13.9 μm)

This cell was considered to be analagous to the mammalian neutrophil, being the most abundant granulocyte in the peripheral blood. Usually the cells were spherical but occasionally were found to produce pseudopodial projections. With MG-G the nucleus stained purple, was usually ovoid and eccentric with a pale blue cytoplasm which contained a few fine blue granules (Plate B3). Occasionally the nucleus was observed as a ribbon-like structure across the diameter of the cell, S-shaped or more rarely in a bi-lobed form (Plate B4). The cytoplasm contained numerous small granules which had a central electron dense core of parallel arranged fibrils (Plate B4 inset). A few vacuoles were present in the

cytoplasm also some RER, a Golgi apparatus and several mitochondria.

This cell had a strong positive reaction with PAS, Sudan Black and peroxidase (PO) and had a weak positive reaction with both acid phosphatase (AcP) and alkaline phosphatase (AlP).

Granulocyte type 2 (7.2-11.3 μm)

This granulocyte was observed only rarely in the peripheral blood. The nucleus was often displaced to the periphery of the cell, and with MG-G the granules were acidophilic and filled the cytoplasm (Plate C1 & C2). Ultrastructurally the electron dense granules were spherical and of an homogeneous appearance, some showing a slight granularity (Plate C1). The cytoplasm contained several prominent mitochondria and small lysosomal bodies. Due to its rarity a complete cytochemical survey was not possible but the granules were found to be positive to varying degrees with AcP when examined under the EM (Plate C3).

Granulocyte type 3 (6.9-10.9 μm)

This third type of granulocyte was only rarely observed at the EM level. The most characteristic feature was the granules which had a fibrillar structure (Plate C4). These fibrils were arranged in parallel with inter-connecting fibres (Plate C4i) or they occurred in bundles in different planes within the same granule (Plate C4ii). In some granules the fibrils were arranged in a 'finger-print' fashion. The nucleus was usually ovoid but was occasionally observed irregularly shaped. Several small vesicles and

lysosomes, a few mitochondria,a little RER and small Golgi body were found in the cytoplasm.

2.3.2 Phagocytosis and Adherence

Both colloidal carbon and bacteria were phagocytosed *in vivo* by peripheral blood leucocytes. Granulocyte type 1 (Plate D1 & D2) and moncytes (Plate D3-D5) were found to phagocytose *A.salmonicida.* Both of these cells were also observed to ingest colloidal carbon (Plate E1-E4). The type 3 granulocyte also phagocytosed small quantities of carbon, the carbon becoming associated with the granules (Plate E5).

Monocytes and the type 1 granulocyte were adherent, and the type 1 granulocytes were found to be highly phagocytic towards these yeast cells.

2.4 Discussion

LYMPHOCYTES

Lymphocytes are generally the most commonly observed type of leucocyte in the peripheral blood of fishes although varying in size from species to species. The gross morphology of these lymphocytes in *O.mossambicus* were similar to that reported by other workers in the peripheral blood of amphibians (Daimon, Mizuhira & Uchida, 1979), reptiles (Sypek & Borysenko, 1987), birds (Frazier, 1973) and mammals (Parmley, 1987).

Lymphocytes of *O.mossambicus* gave positive reactions with PAS, AcP and ANAE and unlike mammalian lymphocytes did not stain with Sudan black (Grimaldi, D'Ippolito, Pica & Della

Corte, 1983). PAS positive lymphocytes have been reported in other fish species such as, Spanish mackerel (Pitombeira & Martins, 1970), plaice (Ellis, 1977a), torpedoes (Grimaldi *et al.*, 1983) and conger eel (Hine, Wain & Boustead, 1987). In *O.mossambicus* the reaction was limited to specific 'granule-like' areas in the cytoplasm. AcP and ANAE activity has been reported for mammalian lymphocytes (Cawley & Burns, 1980) but only to a limited extent in fish lymphocytes. AcP activity has been observed in the lymphocytes of the lamprey *Lampetra fluviatilis* (Page & Rowley, 1983) and both AcP and ANAE activity has been found in the lymphocytes of a number of fish species examined by Hine *et al.* (1987); Blaxhall & Hood (1985) used AcP and ANAE in an attempt to identify lymphocyte sub-populations in the brown trout, *Salmo trutta*. Both of these stains are used in mammalian studies to identify T-cell populations in the peripheral blood. Unfortunately, using these techniques, these authors could not conclusively demonstrate the presence of T and B cell populations in the peripheral blood of fish.

THROMBOCYTES

Two forms of thrombocyte were identified in *O.mossambicus*, oval and fusiform. Ellis (1976) identified four morphologically different forms in the blood of plaice, namely spiked, spindle, ovoid and 'lone-nucleus'. The appearance of thrombocytes can apparently be affected by a number of factors when the blood is being sampled which may lead to their aggregation and ragged appearance in blood smears. Stress can lead to the 'rounding off' of

thrombocytes (Mulcahy, Savage & Casey, 1983) which may lead to their being confused with lymphocytes. The presence of granules have been variously reported in the thrombocytes of a number of fish species (Grimaldi *et al.*, 1983; Parish, Wrathmell, Hart & Harris, 1986a) and of birds (Dieterlen-Lievre, 1987). Boomker (1980) reported the presence of a 'specific' acidophilic granule in his study of the thrombocytic series of *O.mossambicus*, but in this study these were not seen.

PAS positive, granule-like inclusions were observed in the cytoplasm of the thrombocytes of *O.mossambicus*. PAS activity has been widely reported in thrombocytes of various fish species, for example, turbot (Burrows & Fletcher, 1986), plaice (Ellis, 1976a), dogfish (Parish *et al.*, 1986a), torpedoes (Grimaldi *et al.*, 1983) and in the large number of fish species examined by Hine *et al.* (1987). Both AlP and AcP have been reported in fish thrombocytes but in general were only weakly positive.

Phagocytosis of colloidal carbon (Ferguson, 1976; Parish, Wrathmell, Hart & Harris, 1986b; 1986c)and bacteria (Suzuki, 1984) by thrombocytes has been reported. The mechanism by which this occurs has been questioned, some workers believing that uptake, particularly of colloidal carbon, may occur via the canicular system which is in direct contact with the environment. Avian thrombocytes have also been shown to phagocytose foreign material; van Alten (1982) indicating that colloidal carbon was actively ingested by the thrombocytes. Like the mammalian platelets,

fish thrombocytes have been shown to be involved in the clotting process, in that thrombocytes of cyprinidontiformes have been seen to extrude long cytoplasmic threads which cross-link forming a fibral network that traps blood cells (Gardner & Yevich, 1969).

MONOCYTES

Monocytes have been reported to be absent from the blood of some species, such as goldfish (Weinreb & Weinreb, 1969) and brown trout (Blaxhall & Daisley, 1973); however, monocytes have now been identified in a number of fish species (Barber & Mills Westermann, 1981; Ferguson, 1976; Hyder, Cayer & Petty, 1983; Linthicum, 1975; Parish *et al.*, 1986a). It may be that in many cases confusion has arisen due to the identification of monocytes as large lymphocytes when using Romanowsky dyes, a problem commented upon by Roubal (1986) when examining the blood of *Acanthopagrus australis* (Gunther). This confusion may be resolved by adherence tests and the phagocytosis of inert particles.

A number of studies using colloidal carbon (Burrows & Fletcher, 1986; Ellis, Munro & Roberts, 1976; Parish *et al.*, 1986b; 1986c), bacteria (Suzuki, 1984; Parish *et al.*, 1986b; 1986c) and yeasts (Hyder *et al.*, 1983) have revealed that monocytes are actively phagocytic. Interestingly, although Weinreb & Weinreb (1969) could find no cell equivalent to a monocyte in the peripheral blood of the goldfish, they did describe a large vacuolated agranular cell that phagocytosed the marker thorotrast. They did not however, believe that this was sufficient evidence to identify this cell as a

monocyte.

GRANULOCYTES

The terminology used to identify blood granulocytes has always proved to be confusing. This is due to the differences which exist between mammalian and fish granulocytes, and even significant differences amongst fish species (Hine & Wain. 1987a; 1987b; 1987c). The neutrophil-type granulocyte is however well-documented in all species, although varying in granule morphology. In this present study granulocyte type 1, showed cytochemical similarities to those of mammalian neutrophils being PO, Sudan black, AcP and AlP positive. This cell type has been reported to possess some or all of these reaction products in other fish species (Blaxhall & Daisley, 1973; Ellis, 1976; Grimaldi *et al.*, 1983; Mulcahy *et al.*, 1983; Parish *et al.*, 1986a).

Morphologically most authors report similar characteristics for this cell type, typically a pale blue cytoplasm and either non-staining or faintly staining granules. Ultrastructurally the granules were small and contained a fibrillar structure similar to those observed in plaice (Ferguson, 1976). Other workers report that neutrophil-like cells have granules of a homogeneous nature (Hine *et al.*, 1987; Linthicum, 1983).

Granulocyte type 1 in *O.mossambicus* is highly phagocytic ingesting intravenously introduced colloidal carbon and bacteria. There is some controversy concerning the phagocytic capability of fish neutrophils; those of rockfish

and rainbow trout (Suzuki, 1984) being able to phagocytose small quantities of colloidal carbon, whereas both Ellis (1976) and Ferguson (1976) reported that plaice neutrophils did not exhibit this function. Phagocytosis of bacteria (Finn & Nielsen, 1971; Parish *et al.*, 1986b; 1986c; Suzuki, 1984) and thorotrast (Weinreb & Weinreb, 1969) by fish neutrophils has also been demonstrated. This capability together with the positive PO reaction would lead to the conclusion that this cell could be considered to be analagous to the mammalian neutrophil.

Granulocyte type 2 bore a morphological resemblance to mammalian eosinophils, its cytoplasm being full of large, spherical acidophilic granules. Reports of this cell in fish blood vary greatly, and even when found it is extremely rare. Eosinophils have been reported to be absent from the blood of brown trout (Blaxhall & Daisley, 1973), plaice (Ellis, 1976), turbot (Burrows & Fletcher, 1986) and pike (Mulcahy *et al.*, 1983) but present in a number of other species (Hine & Wain, 1987a; 1987b; 1987c; Morrow & Pulsford, 1980; Parish *et al.*, 1986a; Roubal, 1986). Unlike mammalian cells the eosinophilic granules of *O.mossambicus* do not possess crystalline inclusions.

Due to the rarity of this cell type , functional characteristics are not well documented, however Parish *et al.* (1986b; 1986c) reported that one of the eosinophilic granulocytes of the dogfish, *Scyliorhinus canicula* L., was able to phagocytose small quantities of colloidal carbon. Weinreb & Weinreb (1969) also claimed that the eosinophils

observed in their study of goldfish blood were phagocytic to thorotrast.

Perhaps the greatest area of contention in fish haematology is the question of the presence of basophils in the peripheral blood. Watson, Shehmaster & Jackson (1963) claimed that basophils were fragile and often disrupted in smear preparations with the subsequent dispersion of their contents. Pitombeira & Martins (1970) also claimed that specific granules of the basophil in the Spanish mackerel were PAS positive. In a previous study of the haematology of *O.mossambicus* by Sailendri & Muthkkaruppan (1975) the presence of a basophil-like cell was reported. This third type of granulocyte was found only under the EM, and was very rare.

Ultrastructurally the most characteristic feature of this granulocyte was the granules which have a very distinctive sub-structure. They bear a similarity to the type 2 granules of the human neutrophil (Daems, 1968) and also the "DB granules" of an eosinophilic cell in *Centrocymnus crepidator* (Hine & Wain, 1987b). Another feature of this cell type was that it was capable of taking up small quantities of colloidal carbon, which becomes associated with granules within the cell. Mammalian basophils have been shown to endocytose particulate material which is transported to specific cell granules (Komiyama & Spicer, 1975). Their involvement with endocytosed material and enzyme content (Christie & Stoward, 1978) identify granules as specialised lysosomes, not just secretory granules. It is not possible

to compare the function of the type 3 granulocyte with that of the mammalian basophil due to its rarity, but the association of endocytosed material with granules is an interesting and possibly salient feature.

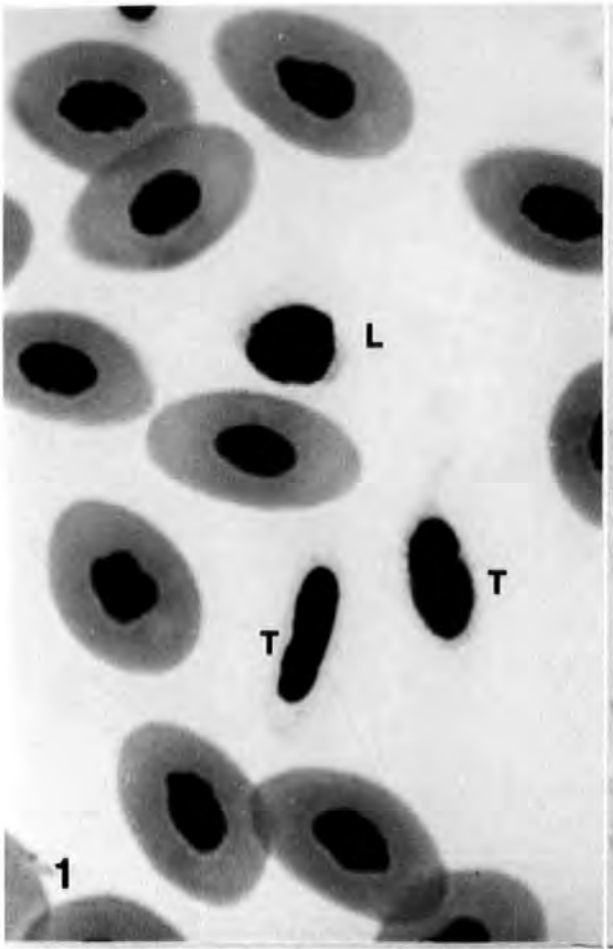
PLATE A Lymphocytes and Thrombocytes

1 Lymphocyte (L) and thrombocytes (T). mag. x1056

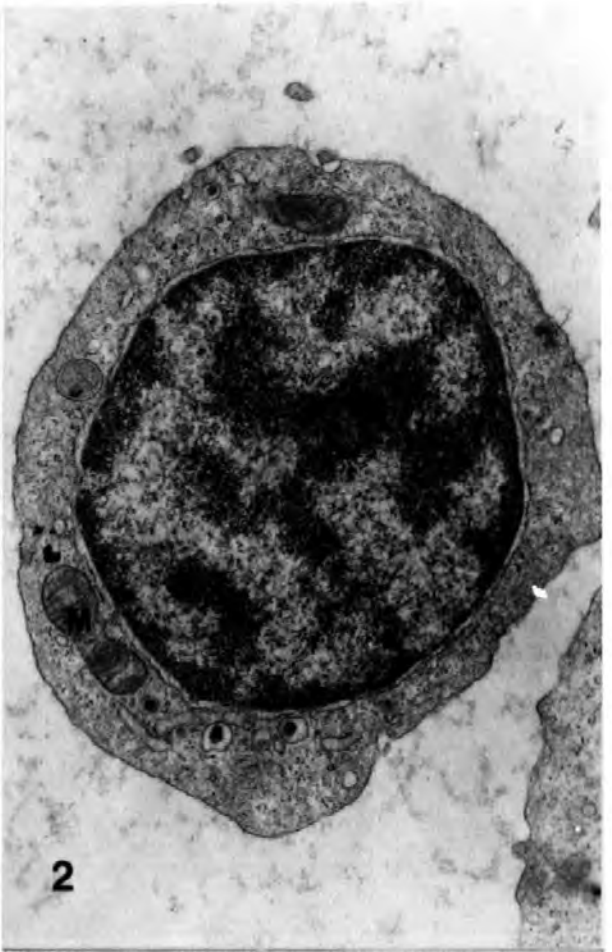
2 Lymphocyte showing characteristic features with nucleus filling the cell and prominent mitochondria. mag. x11,000

3 Tailed thrombocytes with large vacuole (v) and microtubules (Mt) that lie along the length of the cell. mag. x10,000

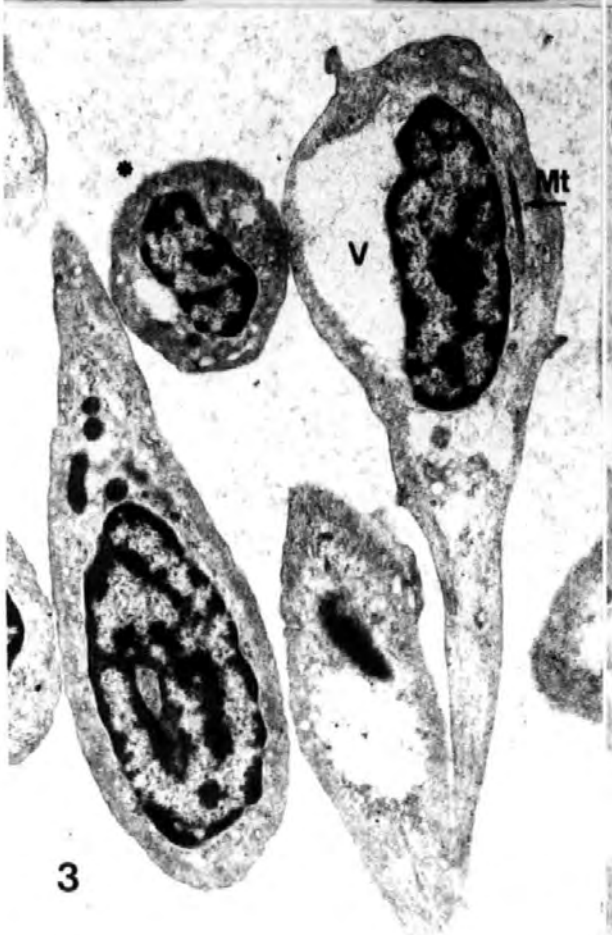
4 Group of thrombocytes mainly oval shaped. mag. x10,000



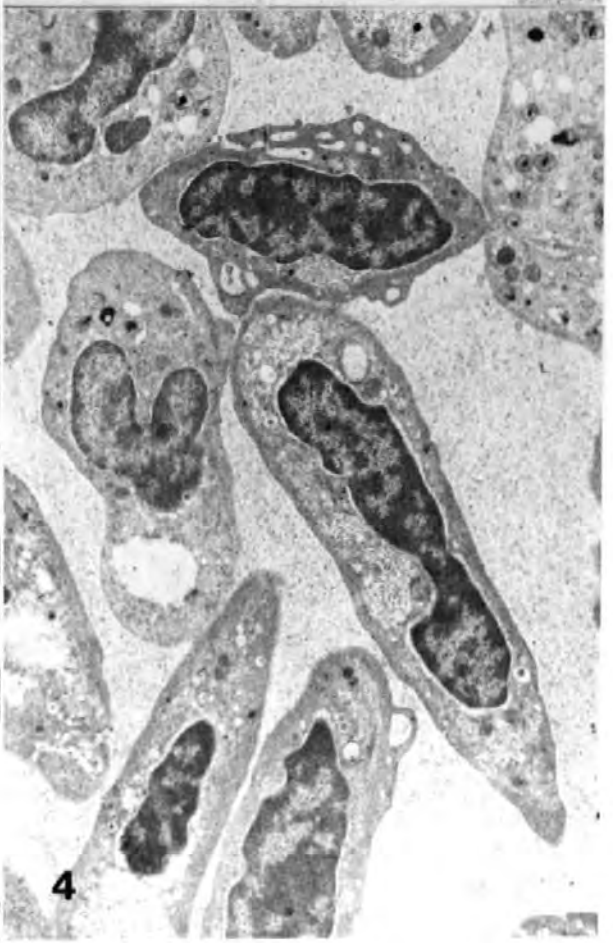
1



2



3



4

PLATE B Monocytes and blood granulocytes type 1

1 Monocyte with several cytoplasmic pseudopodia (arrowed). mag. x1056

2 Monocyte containing several mitochondria (M) and a few lysosomes (ly). mag. x7800

3 Granulocyte type 1 with faintly stained granules (arrowed) and dumb-bell shaped nucleus. mag. x1056

4 Granulocyte type 1 showing the granules (Gr) and a few mitochondria: mag. x8200

Inset: high magnification of a granule showing the fibrils (f) of the electron dense core. mag. x13,000

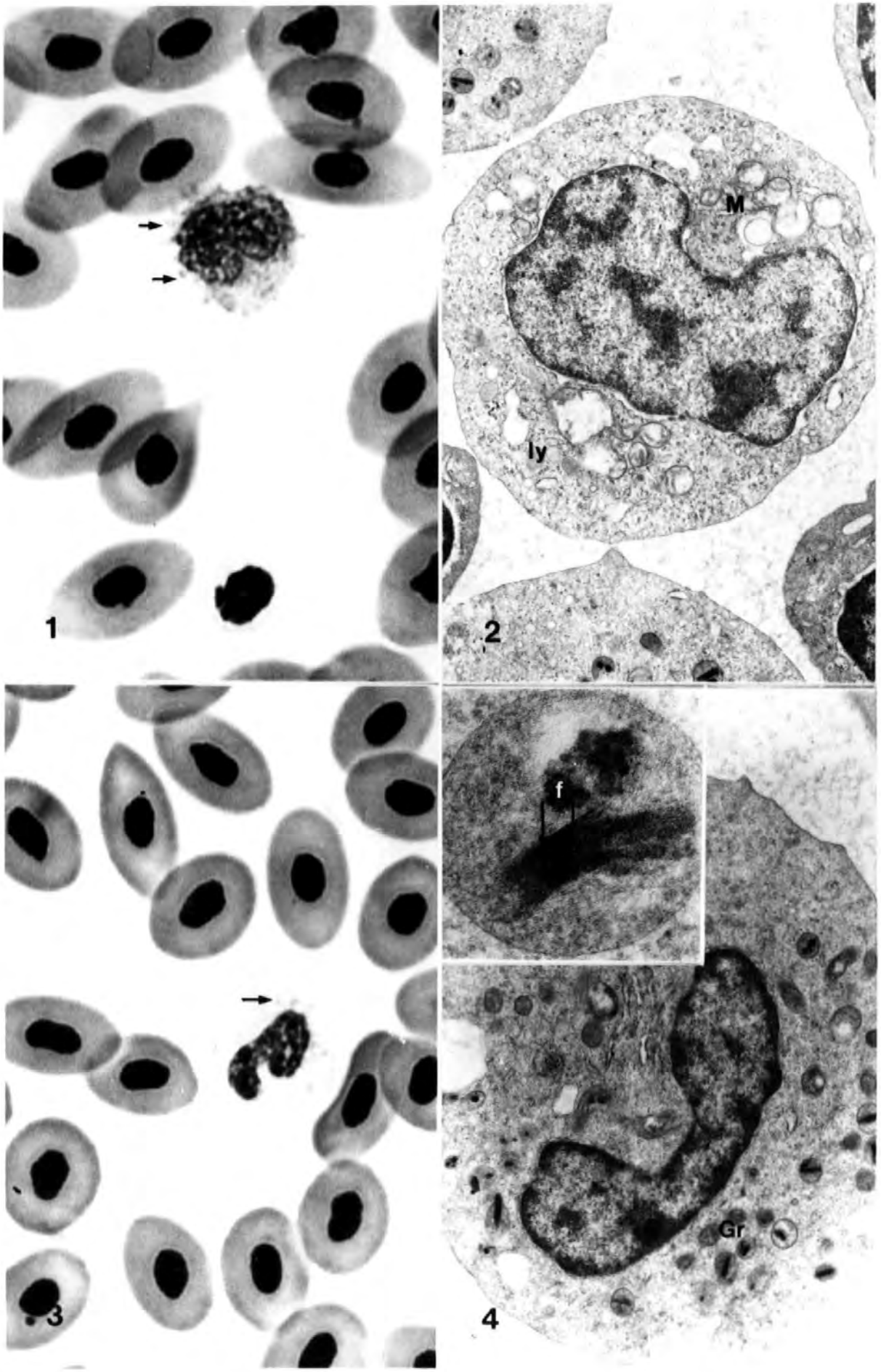

M
ly
f
Gr
1
2
3
4

PLATE C Blood granulocytes type 2 and type 3

1 Granulocyte type 2 with spherical electron dense granules (Gr). mag. x11,000

Inset: higher magnification of one of the granules. mag. x55,000

2 Light micrograph of the type 2 granulocyte. mag. x792

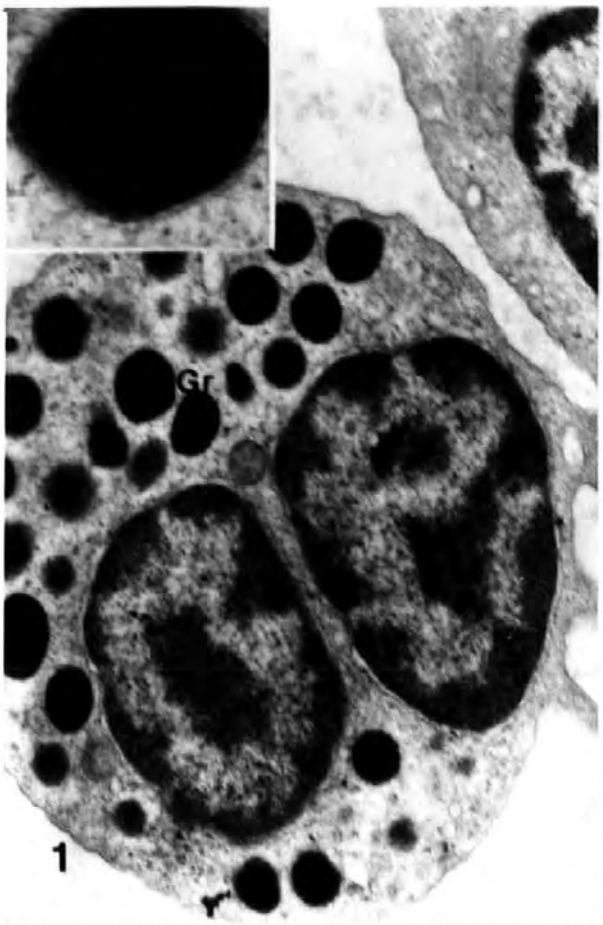
3 Acid phosphatase stained type 2 granulocyte, showing positive reaction in the granules. mag. x13,000

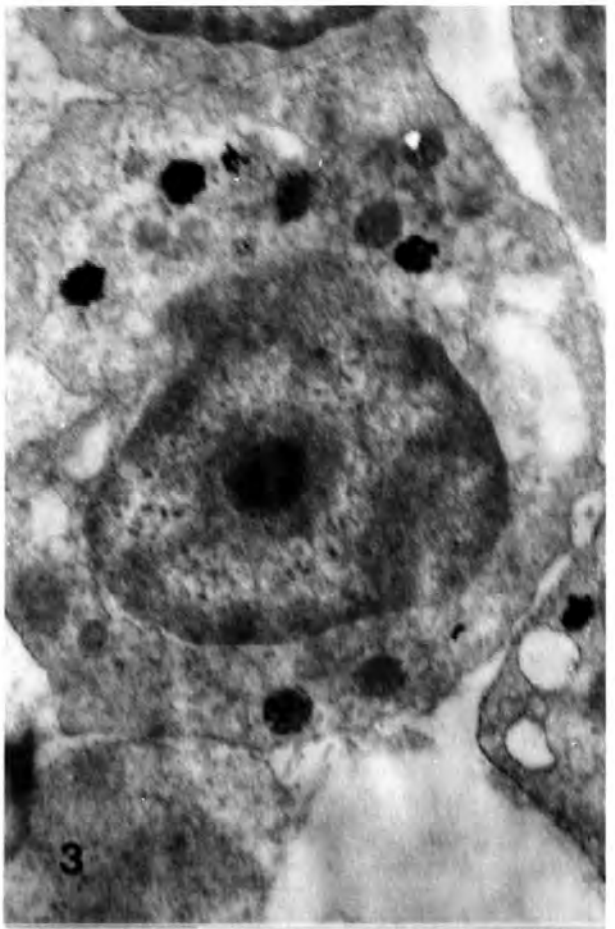
4 Type 3 granulocyte with its characteristic granules. mag. x7800

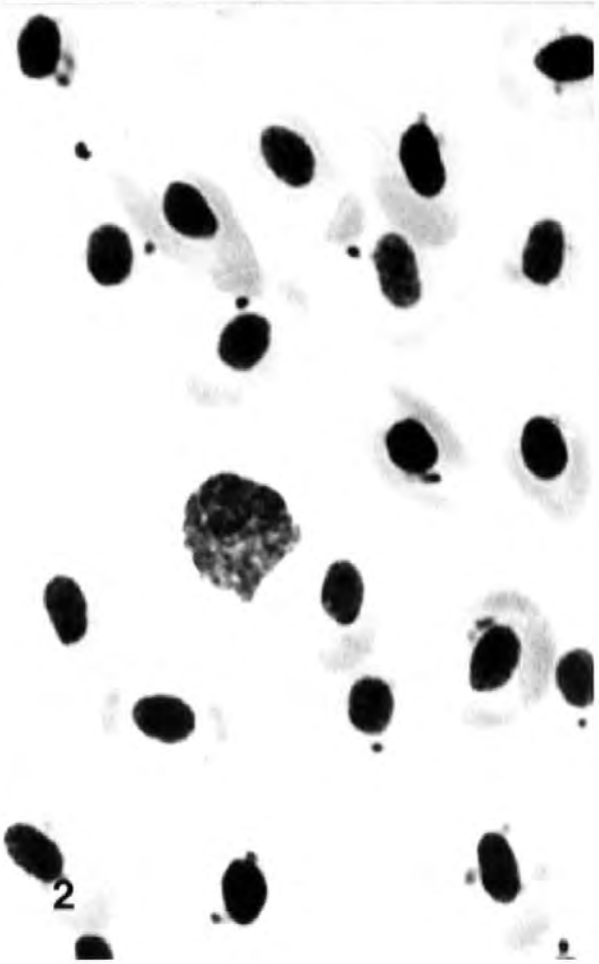
Insets:

i) granule with parallel arranged fibrils. mag. x56,000

ii) granule with fibrils in different planes. mag. x56,000


Gr
1


3


2

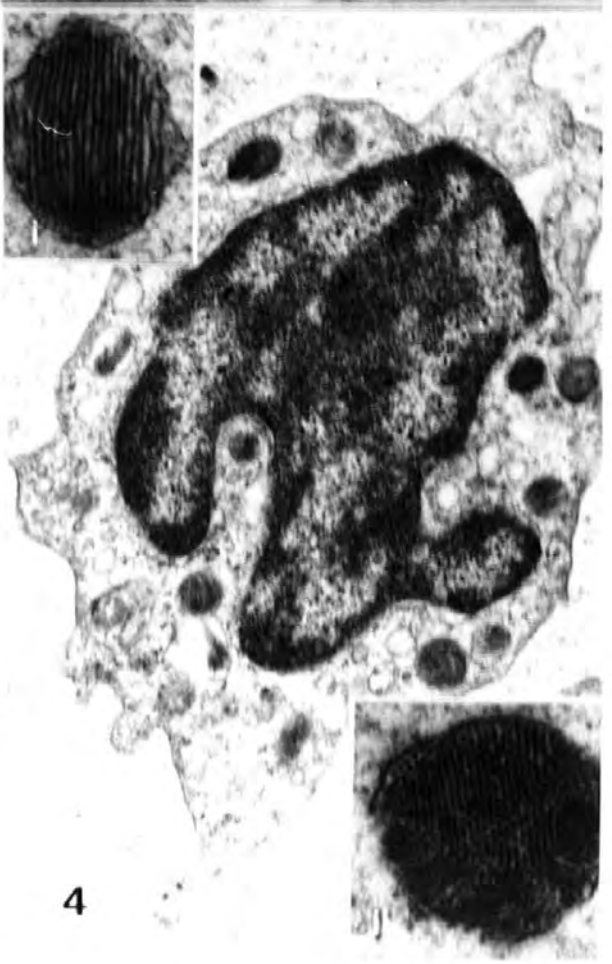

4

PLATE D Phagocytosis of bacteria

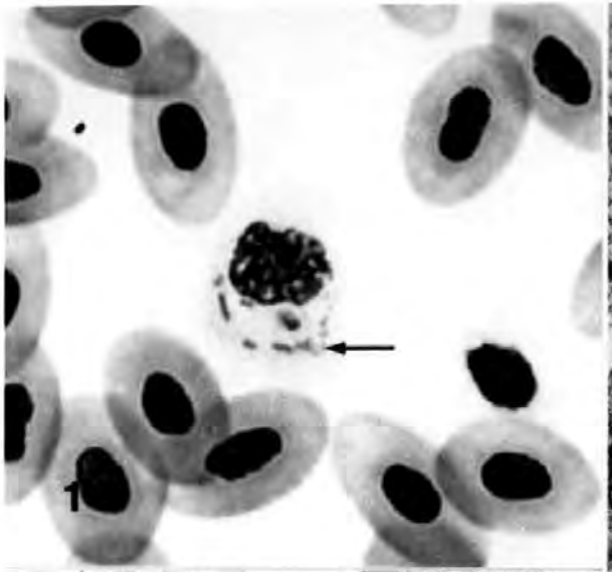
1 Type 1 granulocyte with ingested *A.salmonicida* (arrowed). mag. x1056

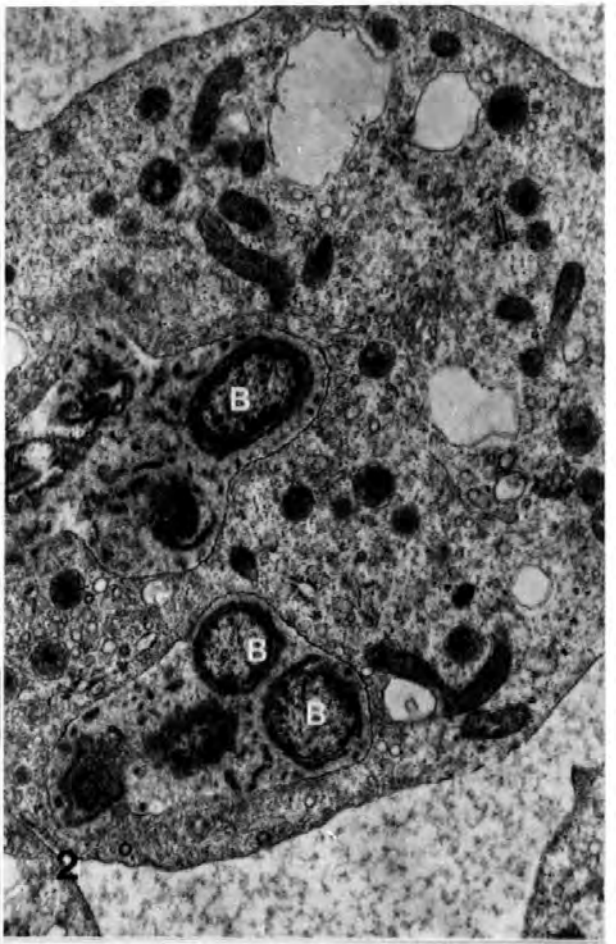
2 Type 1 granulocyte with ingested bacteria (B) within phagosomes. mag. x11,000

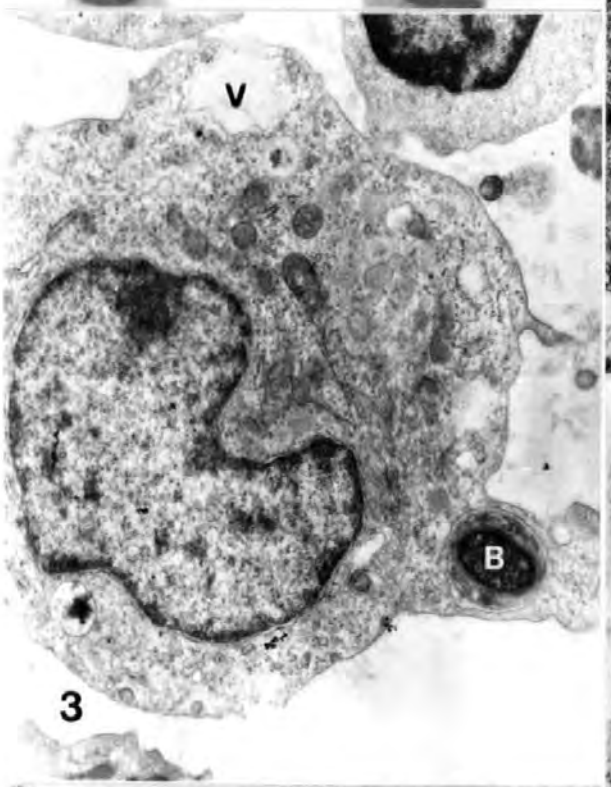
3 Monocyte with phagocytosed bacterium (B); v -vacuole. mag. x6800

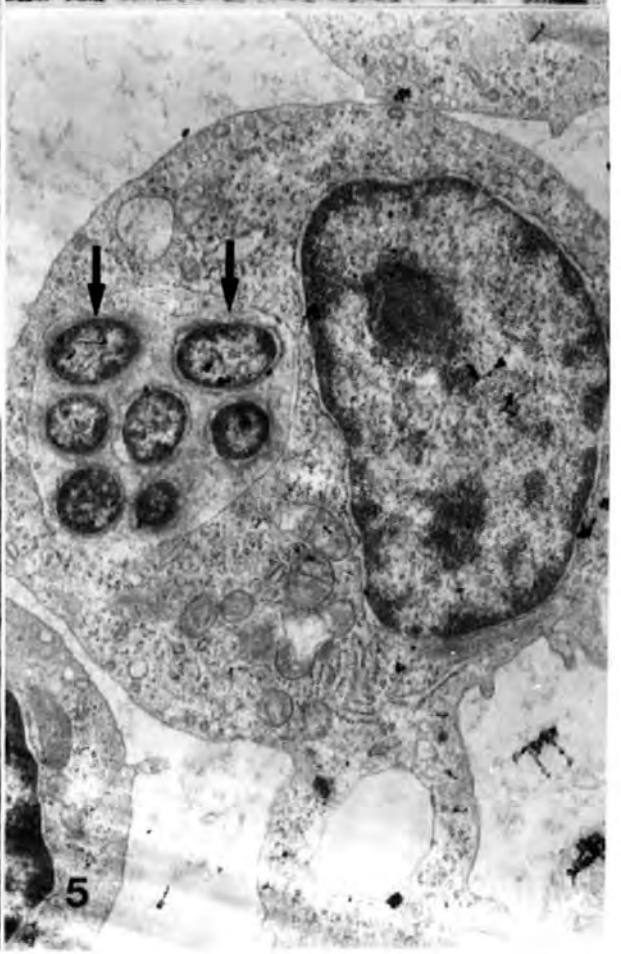
4 Higher magnification of the ingested bacterium in D3. mag. x27,000

5 Monocyte with several ingested bacteria (arrowed) within a large phagosome. mag. x10,000


1


B
B
B
2


V
B
3


5

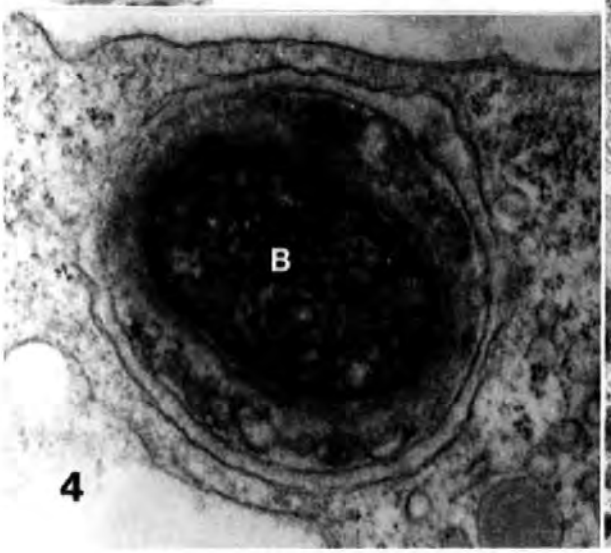

B
4

PLATE E Phagocytosis of colloidal carbon

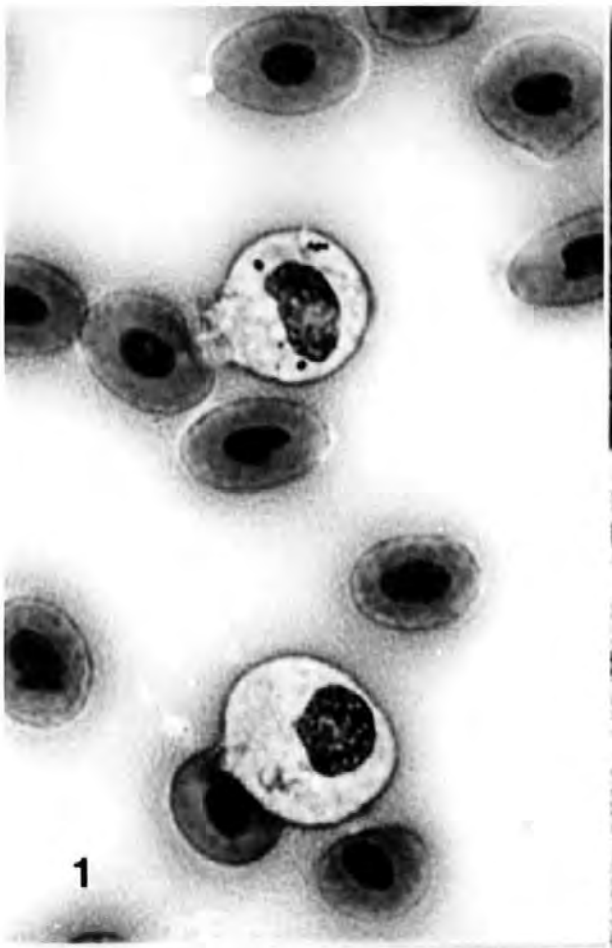
1 *In vivo* phagocytosis of colloidal carbon by type 1 granulocyte. mag. x845

2 Section of type 1 granulocyte showing ingested colloidal carbon in small vesicles. mag. x11,000

3 Monocyte with ingested colloidal carbon. mag. x1056

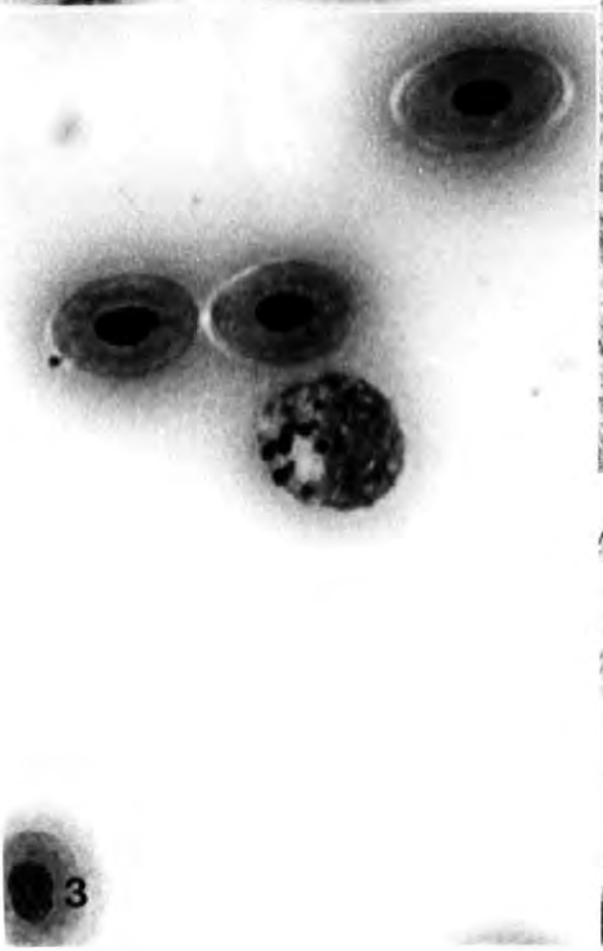
4 Monocyte with colloidal carbon in phagosomes. mag. x11,000

5 Granulocyte type 3 with ingested carbon (C) associated with a granule; Gr -granule. mag. x11,000

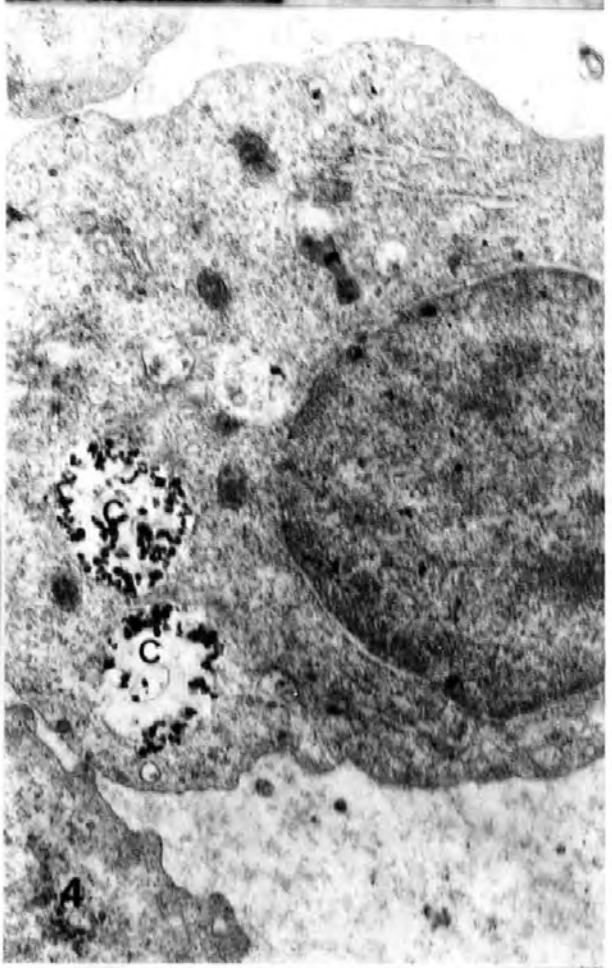


1

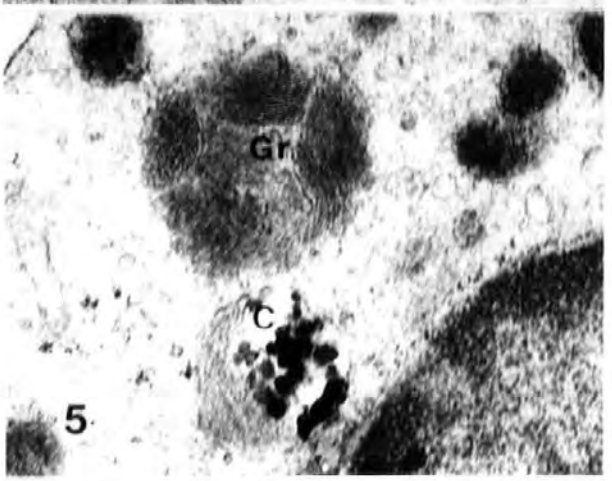
2



3



4



5

CHAPTER 3

3. DISTRIBUTION AND MORPHOLOGY OF THE LYMPHOID TISSUE IN THE ALIMENTARY TRACT OF ADULT FISH

3.1 Introduction

In recent years there has been increasing interest in the role of the gut and gut-associated lymphoid tissue (GALT) in the immune system of fish, particularly the possibility of a common mucosal immune system (Rombout *et al.*, 1985). The role of GALT and its importance as part of the mammalian immune system has long been recognised (Breuton,1980; Reynolds & Morris,1983). Possible roles for the different leucocytes found there have been proposed by several authors, e.g. Ernst, Befus &Bienenstock (1985). However, the origin and function of intestinal leucocytes in lower vertebrates is poorly understood.

In fish, lymphoid tissue has been recognised as consisting of either lymphoid accumulations or as individual cells within the gut tissue. Accumulations and individual leucocytes have been identified in the gut of cyclostomes (Page & Rowley,1982; Tomonaga, Hirokane & Awaya,1973), elasmobranchs (Drzewina,1902; Hart, Wrathmell & Harris,1986a; Tomonaga, Kobayashi, Hagiwara & Yamaguchi,1986a), and teleosts (Bergeron & Woodward,1982; Davina *et al.*,1980; Krementz & Chapman,1975; Peleteiro & Richards,1985; Temkin & MacMillan,1986; Weinberg,1975;

Zaccone,1980). In actinopterygian (Weisel 1979), sarcopterygian (Good, Finstad, Pollara & Gabrielsen, 1966) and chondrichthyan (Hart, Wrathmell, Harris & Doggett, 1986b) fish definitive accumulations have been detected in the spiral valve of the intestine, whereas in teleosts (Davina *et al.*, 1980; Temkin & McMillan, 1986) GALT is composed of a more diffuse population of lymphoid cells.

A morphological examination of GALT in the gut of *O.mossambicus* was undertaken to provide a basis for a more comprehensive study into the function of intestinal leucocytes and their role in the immune system.

3.2 Materials and Methods

3.2.1 Fish

Adult *O.mossambicus* of both sexes, 50-100 g, were obtained from Swansea University and kept in a freshwater recirculating system at a temperature of 24-26°C.

3.2.2 Histology

Fish were anaesthetised in MS222 (Sandoz Ltd.) and killed with a sharp blow to the head then pithed. The gut was excised and washed in chilled 0.85% saline prior to fixation.

a) Light Microscopy (LM)

i)Macroscopic visualisation of lymphoid accumulations.

The gut of three fish were treated with 10% acetic acid

for 24 hours at 4°C (Cornes, 1965), washed with chilled 0.85% saline then viewed with a binocular microsope.

ii)Paraffin Wax histology.

This technique was used to examine the whole length of the gut. As the intestine was extremely long so individual sections of 2-3cm were prepared. Tissue was fixed in 10% formol saline for 24 hours at 4°C, dehydrated in graded alcohols, cleared in xylene and embedded in molten Fibrowax (BDH) at 56-58°C under vacuum for 40-50 min. 5-8 μm sections were cut using a steel knife on a Reichert Jung rotary microtome, and stained according to the procedures given in Table 1.

iii)Resin Histology.

Small pieces of tissue (5mm³) were fixed and dehydrated as for wax histology and then placed directly into a series of alcohol/methacrylate resin mixtures and finally pure resin (TAAB, Reading). The resin was polymerised chemically under anaerobic conditions for 10-12 hours. 1-2 μm sections were cut with glass knives on a Reichert Jung Autocut and stained according to the procedures outlined in Table 1.

b) Electron Microscopy (EM)

Small pieces of intestinal tissue (1mm³) were fixed in 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) for 1 hour at 4°C. Tissue preparation was then carried out as in Section 2.2.2b.

Table 1. Embedding and Histological Staining techniques

Stains	Fixation	Embedding Material: Paraffin Wax	Embedding Material: Methacrylate Resin	Reference
Giemsa	Formol saline	+	+	Parish, 1981
Haematoxylin and Eosin (H&E)	"	+	N/D	Pearse, 1968
Methyl Green Pyronine (MGP)	"	+	+	"
Periodic Acid Schiff (PAS)	"	+	+	Hayhoe and Flemens, 1969
1% methylene blue	"	N/D	+	Pearse, 1968

+ technique was used

N/D technique was not used

3.2.3 Enumeration of GALT cells

An approximate enumeration of intraepithelial leucocytes (IEL) under the LM was simply carried out by counting the number of IEL per 100 epithelial cells (IEL/100 EC).

3.3 Results

3.3.1 Morphology of the alimentary tract and leucocyte distribution

The gut was divided into five regions: buccal cavity, oesophagus, pyloric stomach, cardiac stomach and intestine. GALT was observed in all regions of the gut as individual cells and as small aggregations in both the epithelium and the lamina propria. Figure 1 represents the mean number of IEL detected in the mucosae of the gut in the fish sampled.

Buccal Cavity. The epithelium of the buccal cavity (60-85 μm) was pseudostratified and contained numerous taste-buds and mucous cells (Plate A1). Leucocytes were abundant in the thick areolar tissue beneath the epithelium but the intraepithelial leucocytes (IEL) were few.

Oesophagus. The short oesophagus led directly to the pyloric stomach, the epithelium (70-115 μm) being composed of columnar cells and many large mucous secreting cells (Plate A2). A greater number of IEL were observed in this tissue, compared to the buccal epithelium, and also a

greater number of leucocytes in the lamina propria, intralaminal leucocytes (ILL).

Stomach. The stomach had two regions, the pyloric and the cardiac stomach.

i) The pyloric epithelium (110-130 μm) was thrown into numerous, thick, shallow folds the crypts of which were lined with groups of between 10 and 16 secretory-type cells (Plate A3). IEL were observed in the epithelium but were not very numerous.

ii) The cardiac stomach epithelium (220-345 μm) had the appearance of two layers, a superficial layer of columnar cells and a glandular layer with secretory and gland cells lining the crypts (Plate A4). The epithelium was thickly folded with a few IEL and laminal leucocytes in the sub-mucosa.

Intestine. The intestine was arbitrarily divided into three regions; anterior, mid and posterior. Some morphological differences were noted in the height and number of epithelial folds with a decrease in both of these features in the posterior intestine together with a decrease in the size of the microvilli in this region (Plate A5 & A6). The epithelium in all regions (70-95 μm) was composed of striated columnar cells and goblet cells (Plate A5 & A6). IEL were detected in all regions of the GI tract of *O.mossambicus* (Fig.1). From these observations it was noted that the intestinal epithelium was rich in IEL, there being no apparent difference in number along its length. The lamina propria of the intestine was also observed to contain

a more numerous population of leucocytes, compared to the rest of the gut.

3.3.2 Distribution of lymphoid accumulations.

Acetic acid treatment of the gut of *O.mossambicus* did not reveal the presence of lymphoid accumulations. However, using histological methods a few accumulations were detected. These accumulations were not consistent in their location but were generally found in the lamina propria of the intestine.

Leucocytes were observed mainly as individual cells in the epithelium (Plate B1) and the lamina propria (Plate B2). Accumulations were detected in the cardiac stomach (Plate B3), where the leucocytes had infiltrated the epithelium having crossed the basement membrane, and in the anterior (Plate B4) and posterior (Plate B5) intestine. These accumulations were mainly located in the lamina propria (Plate B4 and B5) and could occasionally be seen to cause a distortion of the epithelial folds if a significant area of the sub-mucosa was involved (Plate B5).

3.3.3 Leucocyte morphology

As the intestine was found to have the most numerous population of leucocytes, both in the epithelium (IEL) and lamina propria (ILL), only the intestinal tissue was used to examine leucocyte morphology. These cells were believed to

be representative of the leucocytes found in other regions of the gut.

In the intestine four major cell types were recognised; lymphocytes, plasma cells, macrophages and granulocytes.

Lymphocytes. Lymphocytes were found as single cells or as small aggregations in the epithelium (Plate C1 & C2) and the lamina propria. The nucleus contained condensed chromatin surrounded by a thin rim of cytoplasm which was lightly basophilic when stained with Giemsa and did not stain with either PAS or MGP. When examined by EM the cytoplasm was found to contain a few mitochondria (Plate C1), and in some instances small amounts of RER. Lymphocytes within the epithelium were usually located near the basement membrane (Plate C2) but on occassions were observed to be apparently migrating between epithelial cells (Plate C3). This possible migration from the blood vessels in the lamina propria can be summised by the occasional attachment of blood-borne lymphocytes to the vessel walls (Plate C4).

Plasma cells. These cells were found in both the lamina propria and the epithelium but never in significant numbers. At the LM level these cells possessed the characteristic 'clock-face' nucleus (Plate D1) and a lightly pyroninophilic cytoplasm when stained with MGP, indicative of the presence of RNA. Examination by EM revealed that the cytoplasm contained numerous profiles of RER (Plate D2) often dilated in more mature cells (Plate D3) and in some cases a very

prominent Golgi apparatus (Plate D4). Occasionally plasma cells were observed in close association with lymphocytes in the epithelium (Plate D5).

Macrophages. Macrophages, morphologically similar to the peripheral blood monocytes, with a cytoplasm that contained numerous mitochondria, some RER, a Golgi apparatus and a few lysosomes (Plate E1), were mainly located in the lamina propria. They were also found within the epithelium either in the basal region or occasionally apically near the microvilli, which would seem to indicate that they are motile (Plate E2). Some cells were observed to contain ingested material, possibly debris of effete cells (Plate E3).

Granulocytes. Granular cells were identified in both the epithelium and the lamina propria in all regions of the gut. On examination at the LM level three distinct types of tissue granulocyte were recognised. As well as the unique tissue granulocytes one of the characteristic blood granulocytes was observed in the intestine.

Blood granulocyte type 1. In the peripheral blood three types of granulocyte have been recognised (Chapter 2), of these only the type 1, or neutrophil, was identified in the intestinal tissue. This cell possessed the characteristic small granules with a central fibrillar core (Plate E4), was infrequent in its appearance but was found in both the epithelium and the lamina propria of the intestine.

Granulocyte type I. This was a large cell with large spherical granules of varying sizes with electron dense centres and surrounded by an electron opaque area (Plate F1). In cross-section these granules appeared elongated with a darker core within the electron dense centres (Plate F2). The nucleus was often displaced towards the periphery of the cell. These cells were often found in groups in the lamina propria, particularly at the base of epithelial folds, but were seldom observed within the epithelium itself. At the LM level the granules were lightly basophilic and iridescent, and PAS negative.

Granulocyte type II. Like the type I granulocyte this cell was located mainly in the lamina propria and only rarely within the epithelium. This cell gave the appearance of being motile, its cytoplasm extending through the lamina propria, and was PAS+ve. At the light level the granules of this cell were eosinophilic, and at the EM level were found to be variable in size, shape and electron density (Plate F3). The cytoplasm also contained a few mitochondria and a Golgi body. On occasions the cells were observed to be in various stages of degranulation (Plate F4).

Granulocyte type III. This second type of eosinophilic-like tissue granulocyte was not frequently observed but was always located in the lamina propria, and like the type II, this cell appeared to be motile (Plate F5). The cytoplasm was filled with small, elongated, electron dense granules which appeared to have a central darker core.

A possible fourth type of granulocyte was identified in

the epithelium of the intestine, being similar in structure to that described by Hart (1987), however, this cell was thought to be of an entero-secretory cell lineage.

3.4 Discussion

GALT in most teleosts appears as individual cells or as small aggregations within the epithelium and lamina propria. Using both light and electron microscopical techniques four types of leucocyte could be recognised in the gut tissue; lymphocytes, macrophages, plasma cells and granulocytes. Most of these cells were located mainly in the lamina propria with the exception of the lymphocytes which were the most predominant of the leucocytes in the epithelium.

3.4.1 Accumulations

GALT has not been widely investigated in teleosts, and in those that have been studied no accumulations have been reported (Davina *et al.*, 1980; Weinberg, 1975). In *O.mossambicus* the presence of accumulations was noted but were detected in only a few individuals that were sampled. Lymphoid accumulations have been reported, however, in the gut of elasmobranchs (Hart *et al.*, 1987a; 1987b; Tomonaga *et al.*, 1986a) and cyclostomes (Linna, Finstad & Good, 1975; Ostberg, Fange, Mattisson & Thomas, 1976), all of which seem to be associated with the spiral valve, a feature of the alimentary tract of primitive fish.

Pharyngeal lymphoid accumulations observed in larval lampreys (Page & Rowley, 1982) were found to consist of

lymphocytes which appeared to be held in place by reticular cells. These accumulations were considered to represent a primitve thymus (Good *et al.*, 1966). Hagfish, also cyclostomes, do not appear to possess a definitive thymus (Good *et al.*, 1966) and unlike lampreys no equivalent accumulations have been found in the gastrointestinal (GI) tract. Lymphocytes have been recognised in the epithelial layer of the intestine and also the gill epithelium of the hagfish (Tomonaga *et al.*, 1973), Fichtelius (1968) naming the gut lymphocytes, theliolymphocytes. Haematopoietic foci found in the lamina propria of the gut in *Eptatretus spp.*, believed to be a primitive spleen, contained lymphocytes but no plasma cells (Good *et al.*, 1966).

In the comparatively large number of elasmobranchs studied all have been found to possess considerable lymphocyte aggregations in the central region of the spiral valve, these accumulation being non-encapsulated. As well as these valvular accumulations smaller lymphoid foci are found along the length of the intestine, these being small flat structures in *Scyliorhinus canicula* (Hart, 1987). Tomonaga *et al.* (1986a) and Good *et al.* (1966) commented that the lymphoid tissues of sharks were more highly developed than that of rays.

The distribution of GALT has been more thoroughly examined in amphibians and reptiles. In the amphibian urodele, *Pleurodeles waltlii* (Ardavin, Zapata, Villena & Solas, 1982), lymphoid accumulations were found throughout the lamina propria of the intestine. Jurd, John & Garney

(1988) also found lymphoid aggregations in the lamina propria of two urodeles, *Salamadra tigrinum* and *Ambystoma maxicanum*. In contrast Goldstine, Manickavel & Cohen (1975) and Plytycz & Slezak (1981) found that the GI tracts of the urodeles they examined to be devoid of such accumulations. Anurans have been found to possess nodular, well-organised GALT throughout their GI tracts (Goldstine *et al.*, 1975; Good *et al.*, 1966; Jurd, 1977; Plytycz & Slezak, 1981).

Reptilians also possess numerous dense lymphoid accumulations. In the alligator (Good *et al.*, 1966) and the lizard *Chalcides ocellatus* (Hussein, Badir, El Ridi & Akef, 1978) accumulations are associated with the pharyngeal region. The ileum and cloaca are also found to have abundant lymphoid aggregates. Borysenko & Cooper (1972) concluded that the lymphoid tissue found along the GI tract of the snapping turtle, *Chelydra serpentine*, may represent precursors of the true lymph nodes seen in analogous locations in mammals. However, these accumulations did not have germinal centres, a fundamental characteristic of mammalian nodes.

The GALT of *O.mossambicus* consisted, in the main, of small aggregates of, at most, 5-6 cells within the epithelium of the GI tract, particularly in the intestine, and of numerous individual cells in the lamina propria. Large accumulations were recognised in a few individual animals and were variable in their location. These accumulations were located in the lamina propria and consisted of aggregations of lymphocytes interspersed with

granulocytes, macrophages and plasma cells. Of particular note was that in the large accumulations there would appear to be an absence of the type I granulocyte which was, paradoxically, the most abundant type of granulocyte normally observed in the gut tissue. The largest accumulation was found in the posterior intestine and involved a large region of the intestine (approximately 2cm in length) and appeared to distort the epithelial folds in this region.

An accumulation was also located in the cardiac stomach of *O.mossambicus*. Lymphoid tissue consisting of individual cells has also been reported in the stomach tissue of other fish species (Hart *et al.*, 1986a; 1986b; Temkin & McMillan, 1986). In the stomach accumulation lymphoid cells were observed to have crossed the basement membrane and entered the glandular epithelium. Accumulations in the stomach mucosa are not widely reported in lower vertebrates. However, Plytycz & Slezak (1981) in their examination of lymphoid tissue in several amphibian species identified lymphoid nodules in the stomach of *Rana lessonae*.

The function of lymphoid accumulations in lower vertebrates is not fully understood. El Ridi, El Deeb & Zada (1981) in their investigation of GALT in lizards and snakes noted that the GALT-less lizards, *Agama stellio* and *Chameleon chameleon*, were unable to produce a notable immune response to either HSA or mammalian erythrocytes stimulated by IP, IM or SC routes, whereas GALT-rich lizards, such as *C.ocellatus*, responded well. Splenectomy of *C.ocellatus* did

not drastically impair either allograft rejection or humoral immunity. From these observations they inferred that GALT may be a central lymphoid organ equivalent to the avian bursa of Fabricius. In the urodele *P.waltlii* the perihepatic layer is considered to be an important tissue having been found to contain precursor cells as well as young and mature plasmocytes. Henry & Charlemagne (1977) believe that this may be the urodelean equivalent of the avian bursa. The importance of the urodele amphibian liver has been commented upon by Baldwin & Cohen (1970) who showed that the liver may immunosuppress allograft rejection.

The function of lymphoid accumulations may not be as simple as it was first thought, with reports that there is a seasonal variation in the number and distribution of lymphoid accumulations in both amphibians and reptiles. Both El Ridi *et al.* (1981) and Hussein *et al.* (1978) report that the number, size and occurrence of lymphoid aggregates in the oesophagus of *C.ocellatus* were affected by the ambient seasonal conditions, with an increase in spring and a decrease in winter. In a recent study on the GALT of urodeles (Jurd *et al.*, 1988) seasonal differences in lymphoid accumulations were seen in summer and winter sampled animals. In the case of *Salamandra tigrinum* there were no accumulations in the GI tract of winter sampled animals while in those sampled in the summer lymphoid aggregations were found within the lamina propria of the duodenum and ileum.

The relevance of these observations has not been

adequately evaluated. Do these fluctuations in lymphoid tissue correspond to any other parameter besides the time of year, such as state of nutrition? If the latter is correct it can be correlated to the findings of Ferguson (1977) who has shown the presence of antigens, derived from the environment and the diet, may influence the presence and distribution of the the intestinal lymphoid tissue.

3.4.2 Diffuse leucocytes of the gut

The most significant GALT in *O.mossambicus* consisted of diffuse populations of leucocytes, both in the epithelium (IEL) and lamina propria (ILL) of all major regions of the GI tract. The greatest number of IEL were located within the intestinal epithelium, with no significant differences along the length of the intestine, a similar feature recognised in the intestine of the goldfish *Carassius auratus* (Weinberg, 1975). Weinberg (1975) also reported that these lymphocytes composed 40% of the cells in the epithelial layer, comparable to the value estimated in *O.mossambicus* and the rosy barb (Davina *et al.*, 1980). Davina *et al.* (1980), however, noted that there were regional differences in the distribution of IEL in the rosy barb, a feature not recognised in *O.mossambicus*. It is interesting to compare these results with those of the *S.canicula* (Hart, 1987) where 15% of the valvular epithelium was occupied by leucocytes. In this elasmobranch, and others, the valvular epithelium is the site of large accumulations. Whether there could be a correlation between the presence or absence of

accumulations and the number of IEL is unknown.

As mentioned earlier the majority of the IEL are lymphocytes restricted to the basal part of the epithelium. With Giemsa their nucleus stains dark purple and unlike peripheral blood leucocytes they are not PAS positive (Chapter 2). Ultrastructurally, however, they are morphologically similar to those found in the peripheral blood and to those observed in other species (Hart *et al.*, 1986b; Temkin & McMillan, 1986; Weinberg, 1975). Hart (1987) identified a granular lymphocyte in the epithelium of *S.canicula*, a feature reported in human intraepithelial lymphocytes (Toner & Ferguson, 1971). Aggregations of lymphocytes were common in the epithelium of the intestine of *O.mossambicus*, with groups of 3-6 cells being regularly observed.

In mammals T and B lymphocytes may be distinguished through histochemical staining with α-naphthyl acetate esterase (Hayhoe & Quaglino, 1980), however the presence of such sub-populations in the fish intestine remains uncertain. A similar test was carried out on the intestinal lymphocytes of *O.mossambicus*, however the results were inconclusive and were not incorporated into this investigation. Weinberg (1975) identified a putative T-lymphocyte in the goldfish intestine by the apparent adhesion of viral particles to the surface of specific cells. Davina *et al.* (1980) also identified possible thymus derived cells in the intestine of carp. In man 85-95% of the intraepithelial lymphocytes pan with T-cell markers, in mice

and rats this figure is less than 45% (Wilson, Stokes & Bourne, 1986).

Plasma cells, though not abundant, were identified by their numerous profiles of RER which were often distended, reminiscent of an actively synthesising cell. These cells bore a resemblance to mammalian plasma cells and also to those observed in reptiles (Solas & Zapata, 1980), elasmobranchs (Hart *et al.*, 1986b) and the goldfish (Temkin & McMillan, 1986). They were located mainly in the lamina propria but could also be recognised in the basal region of the intestinal epithelium. Hart (1987) reported that the plasma cells were restricted to the lamina propria of the gut in the dogfish, being closely associated with blood vessels.This is in contrast to earlier work by Good *et al.* (1966) who could not identify plasma cells within the gut tissue of a variety of fish species examined. The use of electron microscopy has allowed for the detailed examination of tissues and the cells they contain. This together with the number of histological techniques available have allowed more thorough investigations. Using immunological techniques Tomonaga *et al.* (1986b) has identified Ig-containing cells within the spiral intestine of *Heterodontus japonicus* and *Scyliorhinus torazame*, as has Hart (1987) in *S.canicula*. These observations confirming the presence of plasma cells in the intestinal tissue of elasmobranchs. Davina *et al.* (1980) have also demonstrated the presence of Ig-containing cells in the lamina propria of carp. In mammals Ig-containing cells are located beneath the

epithelium of the gut (Befus & Bienenstock, 1982). Clough & Dean (1988) noted that in the bovine intestine less than 1% of IEL stained for cytoplasmic Ig while 9% stained positively for surface Ig, with a greater number of both these cell types in the lamina propria. McDermott, Horsewood, Clark & Bienenstock (1986) demonstrated that T blast cells predominated in the epithelium and B blast cells in the lamina propria of the mouse intestine. Although plasma cells have been detected in the epithelium and lamina propria their direct cell lineage has not been determined in *O.mossambicus* in this present study.

Macrophages were located within the epithelium and lamina propria and bore a resemblance to blood monocytes. Occasionally these cells were observed in the apical region of the epithelium between the epithelial cells, in very close proximity to the microvilli. These cells are thought to act as 'scavengers' ingesting effete cells and foreign material (Rombout *et al.*, 1985), and in *O.mossambicus* these cells were occasionally seen as large cells with indistinguishable phagocytosed material within their cytoplasm.

The type 1 blood granulocyte was found within the intestinal tissue in limited numbers. In the blood this cell has been shown to be phagocytic (Chapter 2), ingesting both colloidal carbon and bacteria. It may be that it has a similar function in the gut, but this has yet to be unequivocally proven.

Three types of tissue granulocyte were detected in the

gut in addition to the type 1 blood granulocyte, or neutrophil. Tissue granulocyte type I was a large cell filled with large irridescent basophilic granules. This cell was found only in the lamina propria, often in groups, and was the most numerous leucocyte in the lamina. Of the two types of eosinphilic granulocytes type II bore a marked resemblance to those described in the gut of other fish species (Hart, 1987; Temkin & McMillan, 1986) and also urodelean species (Ardavin *et al.*, 1982), the granules being spherical, sometimes irregular and elongated, and electron dense. Some granules appeared to be in various stages of degranulation. A similar cell has been reported in the peritoneal exudate of *Oreochromis nilotica* (Suzuki, 1986), which was observed to phagocytose zymosan particles, a property not, as yet, attributed to the type II granulocyte of *O.mossambicus*. The eosinophilic type III granulocyte was less commonly found, the granules of which were similar to those of the third type of granulocyte described in the lamina propria of the goldfish (Temkin & McMillan, 1986).

There is evidence that the gut plays a physiological role in sorting the population of migrant lymphocytes that pass into it from the blood (Hall, Hopkins & Orlans 1977). That the presence of leucocytes in the gut epithelium is influenced by the presence of antigens has been demonstrated by Ferguson (1977) and Davina *et al.* (1980), antigenic stimulation having an important role in the establishment of the lymphoid cell populations of the gut.

Figure 1 Distribution of Intraepithelial Leucocytes in the Alimentary Tract

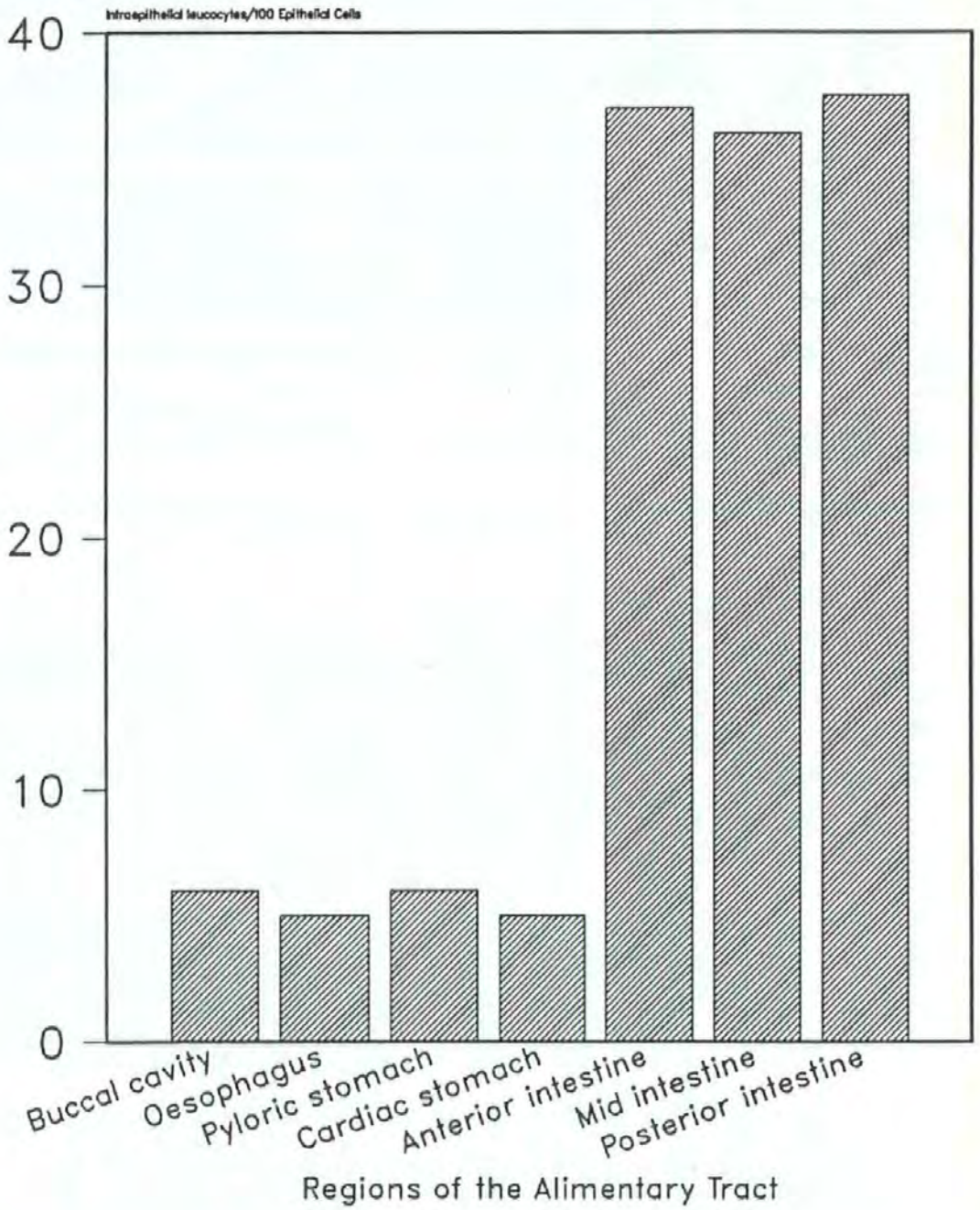


PLATE A Mucosae of the different regions of the GI Tract.

1 Buccal cavity; T- taste-bud, M- mucous cell. mag. x670

2 Oesophagus with psuedostratified epithelium and numerous mucous secreting cells (M); ill- intralaminal leucocytes. mag. x425

3 Pyloric stomach; SE- secretory epithelial cells. mag. x 425

4 Cardiac stomach; GE- glandular epithelium. mag. x425

5 Anterior intestine; iel- intraepithelial leucocytes, E- epithelium, P- plasma cell, arrowed microvilli. mag. x640

6 Posterior intestine, fewer and smaller microvilli compared to anterior intestine (arrowed); G- goblet cell. mag. x640

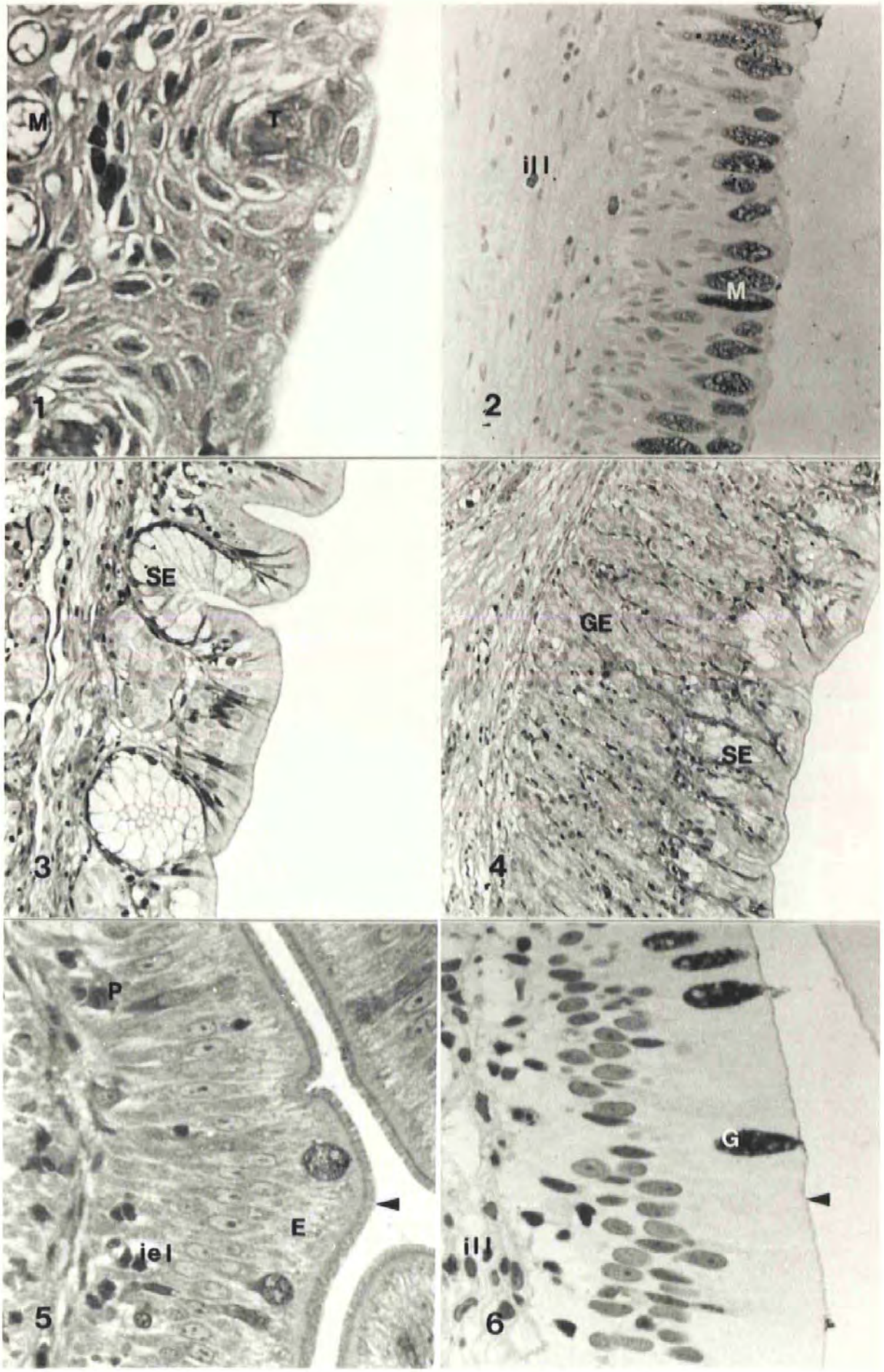

M
T
1
ill
M
2
SE
3
GE
SE
4
P
E
iel
5
G
ill
6

PLATE A Mucosae of the different regions of the GI Tract.

1 Buccal cavity; T- taste-bud, M- mucous cell. mag. x670

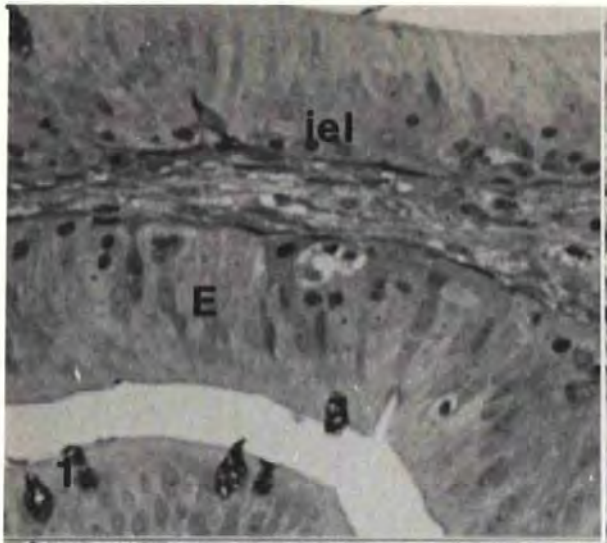
2 Oesophagus with pseudostratified epithelium and numerous mucous secreting cells (M); ill- intralaminal leucocytes. mag. x425

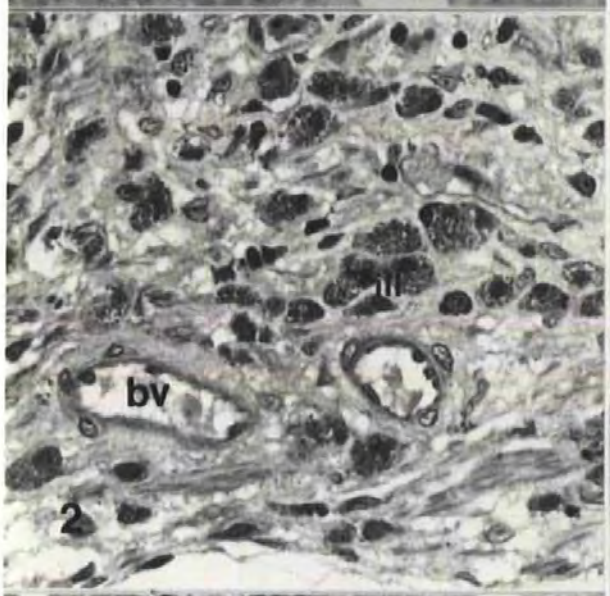
3 Pyloric stomach; SE- secretory epithelial cells. mag. x 425

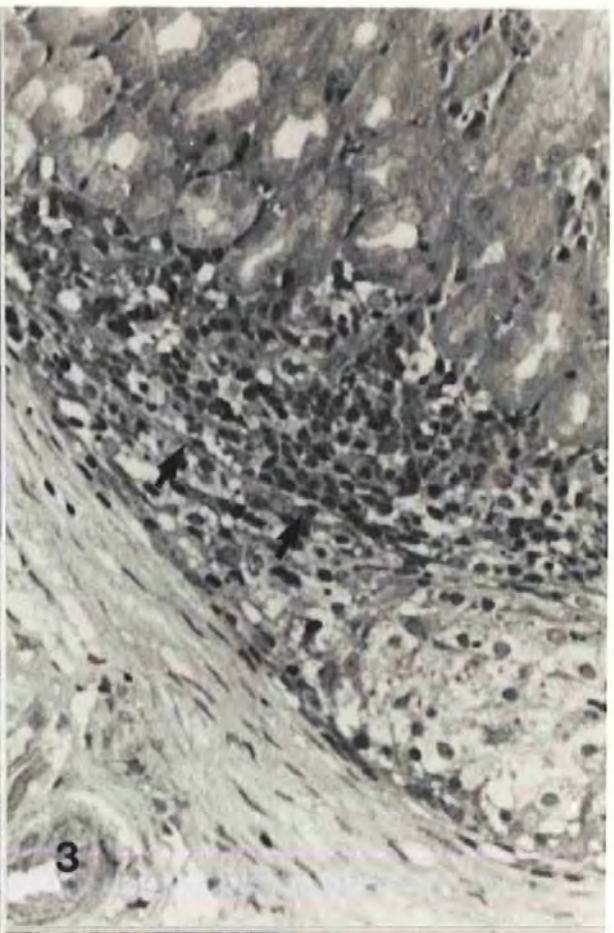
4 Cardiac stomach; GE- glandular epithelium. mag. x425

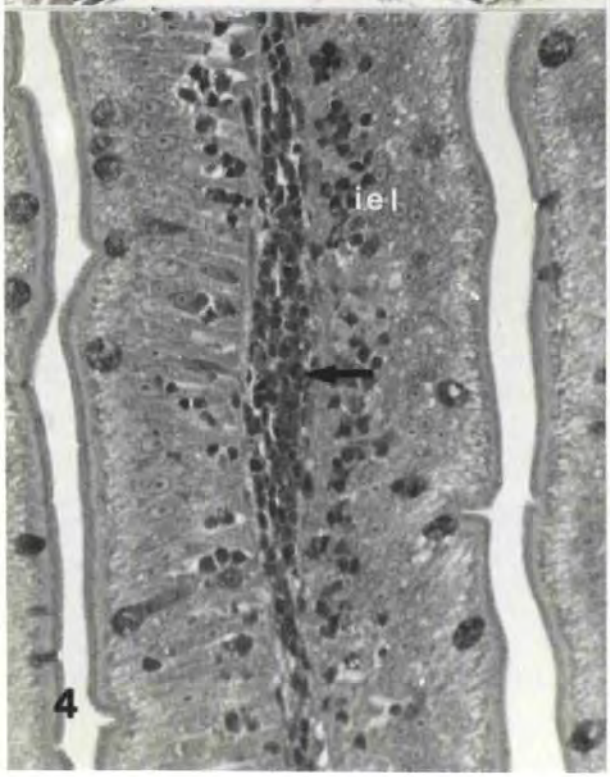
5 Anterior intestine; iel- intraepithelial leucocytes, E- epithelium, P- plasma cell, arrowed microvilli. mag. x640

6 Posterior intestine, fewer and smaller microvilli compared to anterior intestine (arrowed); G- goblet cell. mag. x640


iel
E
g
f


ll
bv
2


3


iel
4

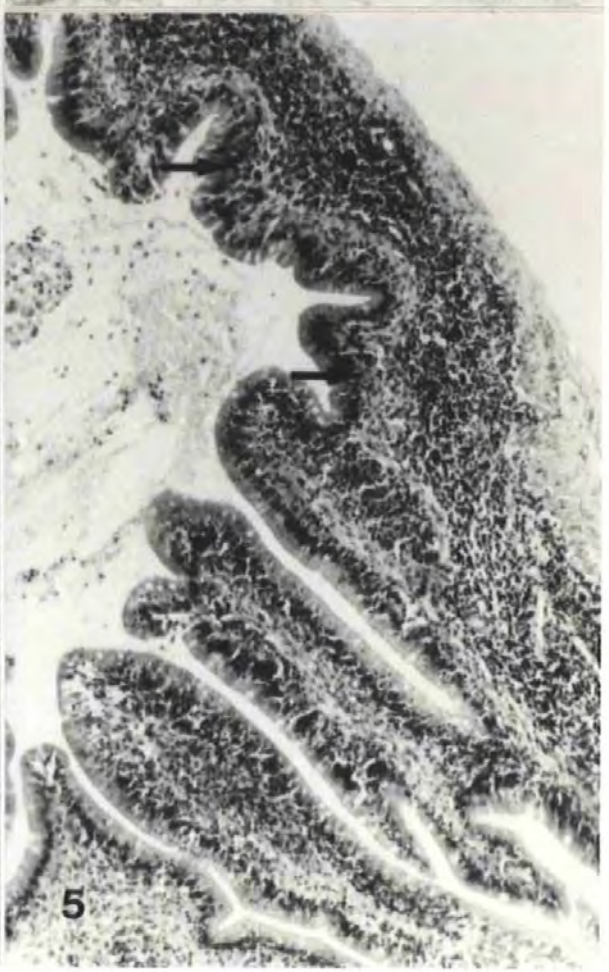

5

PLATE C Lymphocytes in the intestine

1 Two lymphocytes in the epithelium. mag. x8500

2 Group of 5 lymphocytes (L) in the epithelium; BM- basement membrane. mag. x5400

3 Lymphocyte that appears to be passing between epithelial cells. mag. x 3000

4 Blood vessel in the lamina propria with lymphocytes adhering to the vessel walls (arrowed); E- erythrocytes. mag. x6800

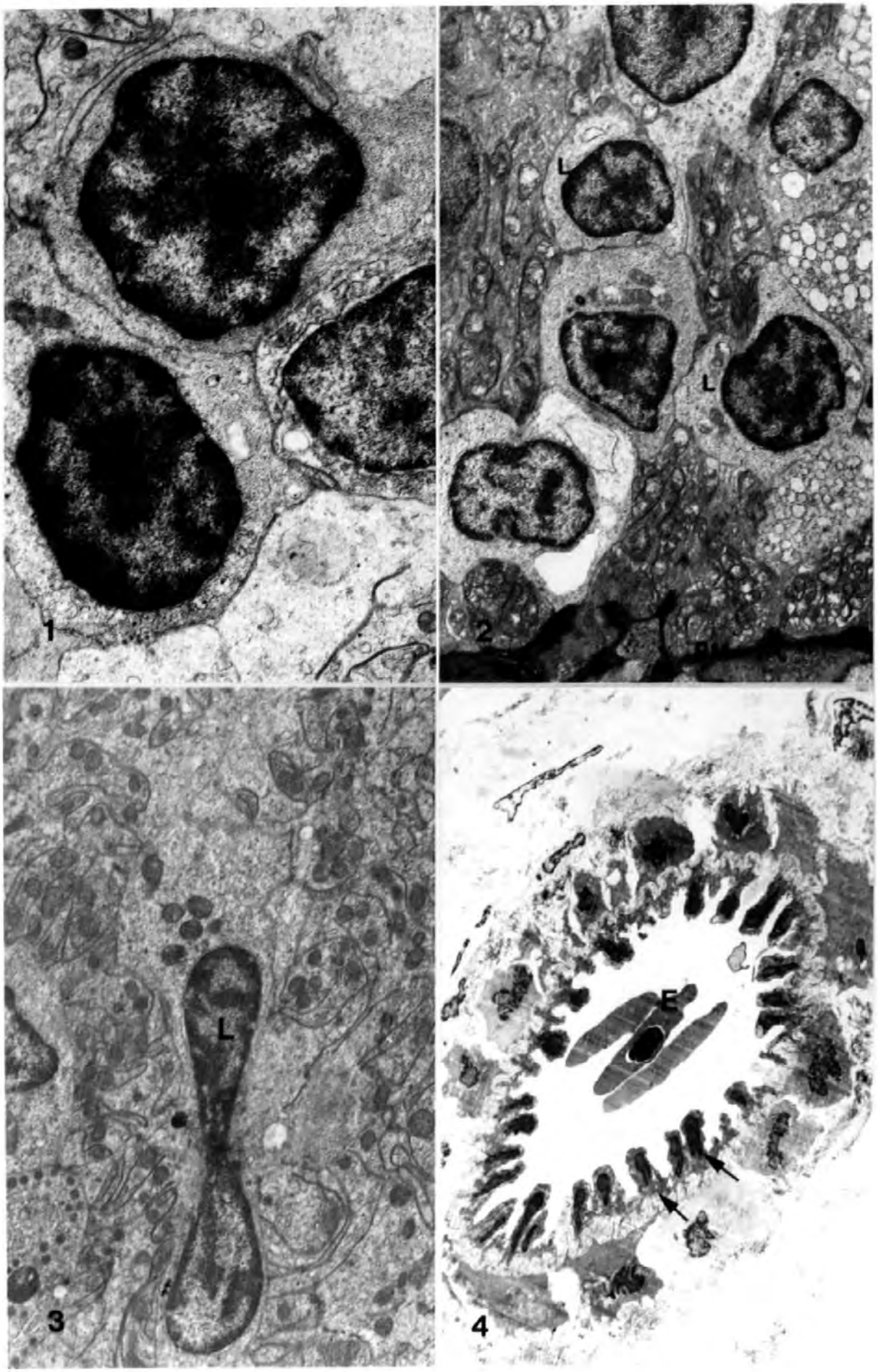

1
L
L
2
L
3
E
4

PLATE D Plasma cells in the intestine

1 Light micrograph of a plasma cell (P) in the lamina propria; GI- tissue granulocyte type I. mag. x800

2 Young plasma cell in the epithelium. mag. x5000

3 Mature plasma cell in the epithelium with numerous profiles of rough endoplasmic reticulum (RER). mag. x8500

4 Mature plasma cell with a prominent Golgi body (Go). mag. x8500

5 Plasma cell from D4 in close association with epithelial lymphocytes (L). mag. x5000

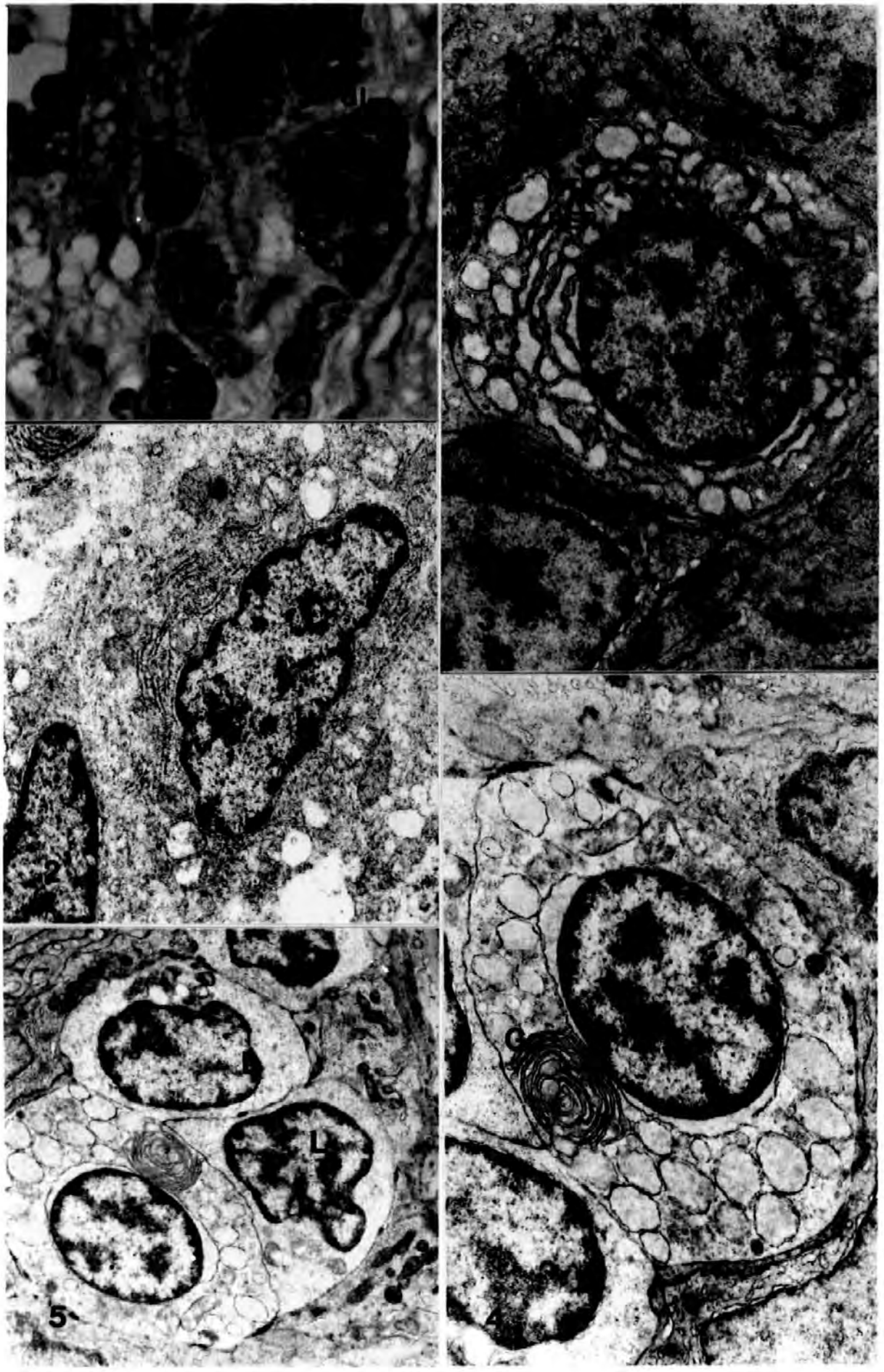


PLATE E Intestinal macrophages and blood granulocyte type 1

1 Macrophage in the intestinal epithelium; M- mitochondria, P- phagosome. mag. x7000

2 Epithelial macrophage with membraneous whorls (arrowed) within its cytoplasm. mag. x12,000

3 Two macrophages in the intestinal epithelium with pseudopodial extensions. mag. x7000

4 Blood granulocyte type 1 in the lamina propria of the intestine; Gr- granules. mag. x10000

Inset:- higher magification of a granule showing fibrils of the electron dense core (arrowed). mag. x21,000

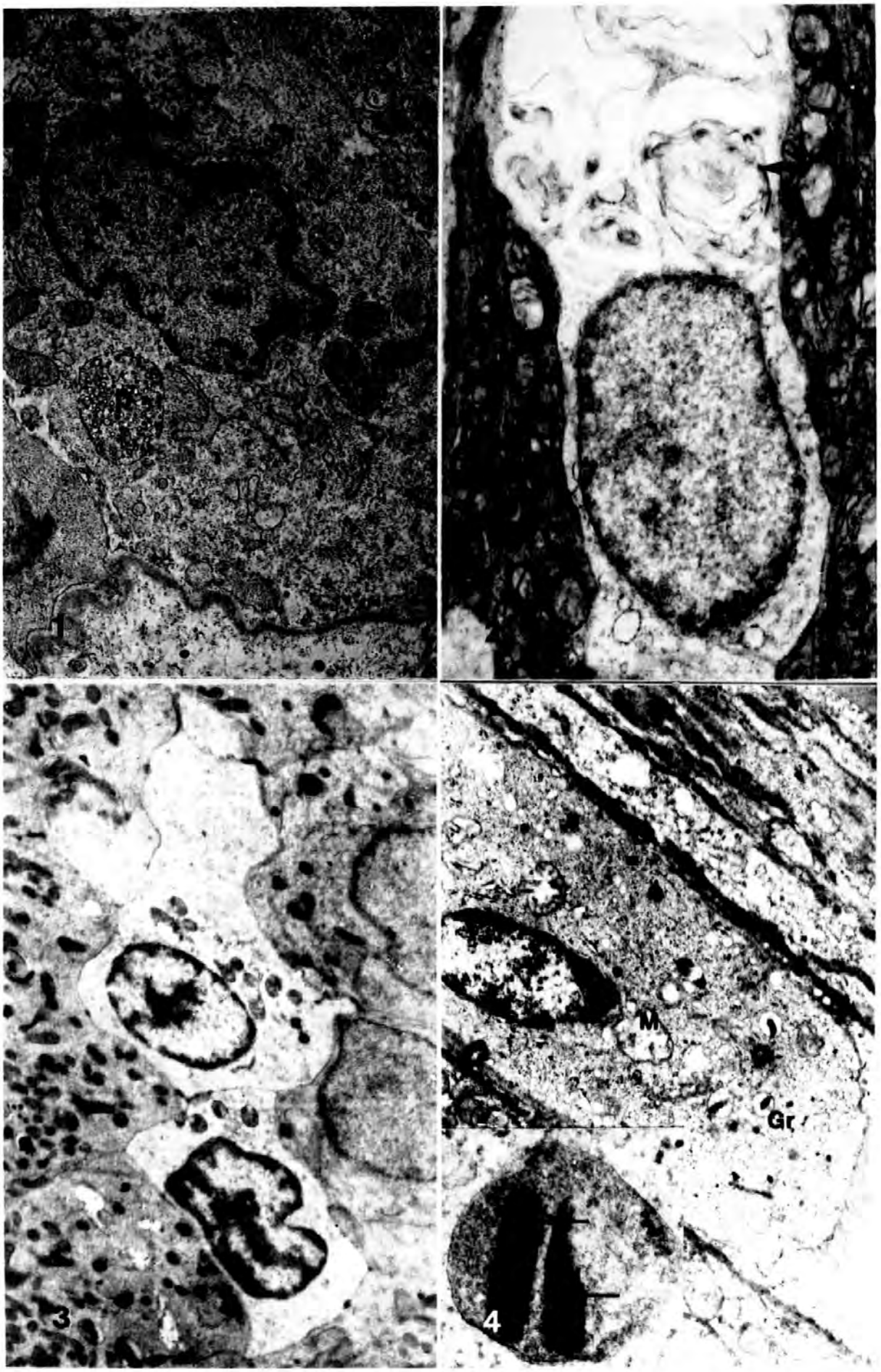

M
Gr
3
4

PLATE F Tissue granulocytes of the intestine.

1 Two type I tissue granulocytes in the lamina propria; Gr- granules. mag. x7000

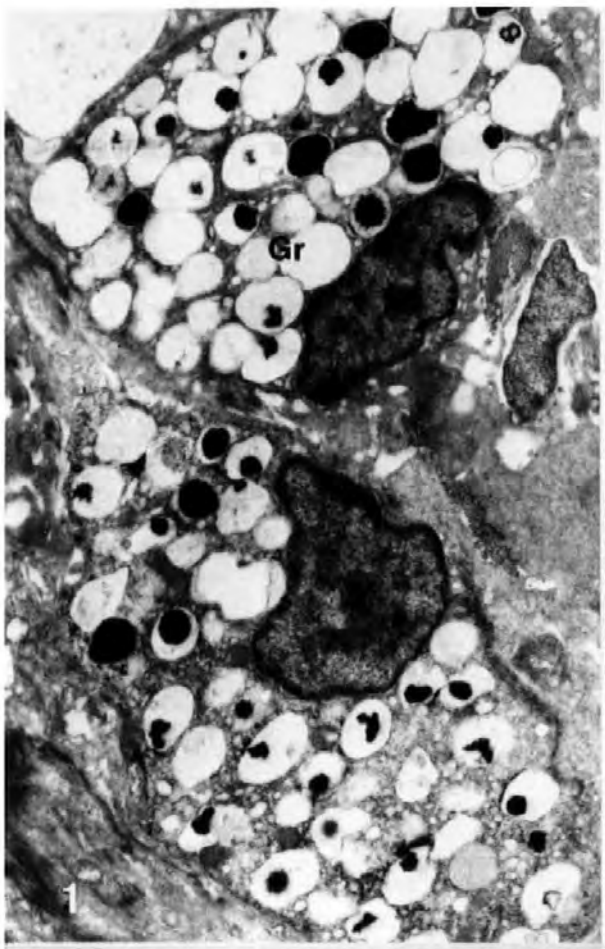
2 Higher magnification of the granule of the type I tissue granulocyte in cross-section; arrow- darker centre to the electron dense core. mag. x23,000

3 Another view of the granule of the type I tissue granulocyte, in this view the core appears homogeneous. mag. x21,000

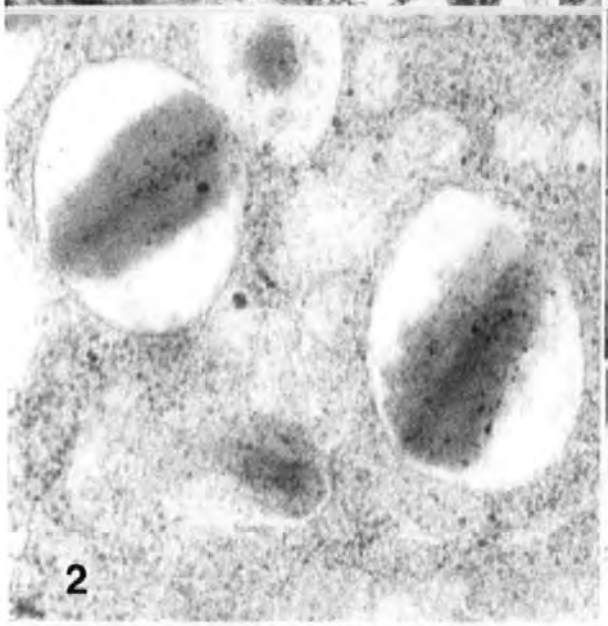
4 Granulocyte type II; M- mitochondria, Gr- granules. mag. x16,000

5 Granules of the type II tissue granulocyte. mag. x28,000

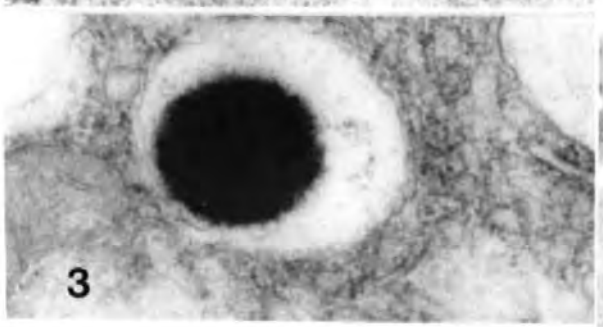
6 Tissue granulocyte type III. mag. x13,000



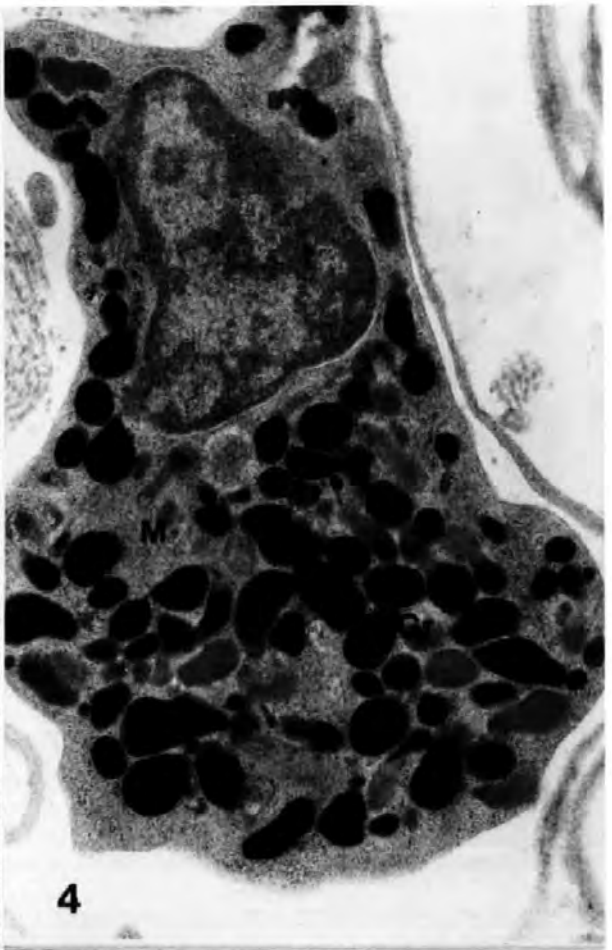
1



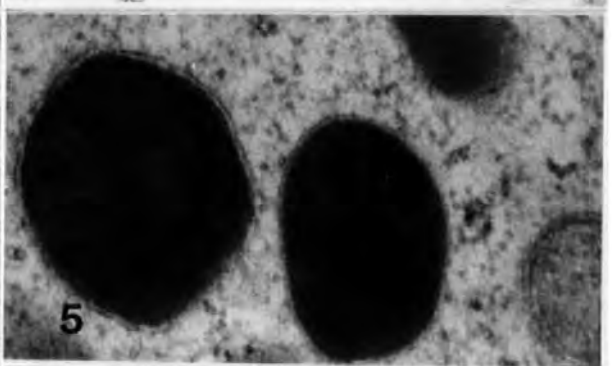
2



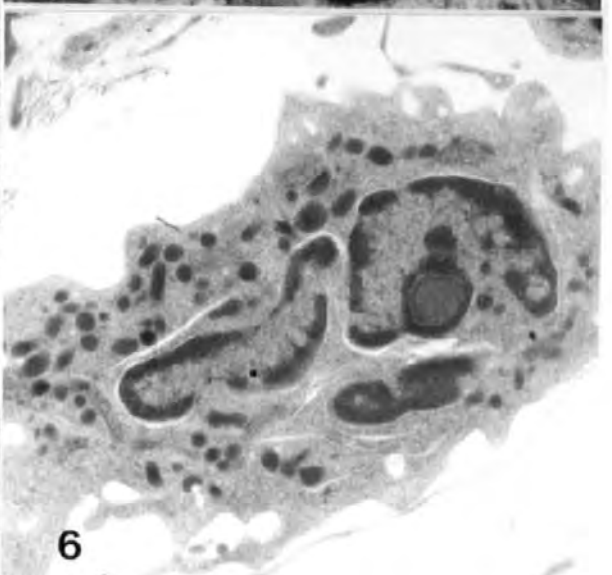
3



4



5



6

CHAPTER 4.

4. THE ONTOGENY OF THE GUT-ASSOCIATED LYMPHOID TISSUE AND MAJOR LYMPHOID ORGANS.

4.1 Introduction

Interest in the possible role of the gut as part of the immune system in fish has increased in recent years. Whilst the importance of the mammalian gut is recognised (Breuton, 1980; Walker, 1980), there is a paucity of information concerning gut-associated lymphoid tissue (GALT) in fish and even less about its ontogeny.

With an increased emphasis placed on the developmental and phylogenetic aspects of immunity, it is surprising that the ontogeny of GALT has been only superficially examined. Even GALT in adult fish has been only touched upon. In comparison there are a number of examinations of the ontogeny of other lymphoid organs in lower vertebrates (Al-Adhani & Kunz, 1976; Ardavin *et al.*, 1984; El Deeb, Zada & El Ridi, 1985; Ellis, 1977; Manning & Collie, 1977; Tatner & Manning, 1983). It is the general concensus that in fish the first lymphoid organ to develop is the thymus then the kidney followed by the spleen. Whether the lymphocytes found within the gut tissue are of thymic origin has not been unequivocally shown but, Davina *et al.* (1980) has demonstrated that cells within the intestinal tissue of carp were recognised by a rabbit anti-carp thymocyte antiserum.

In Chapter 3 preliminary work has described the adult distribution of GALT. In this present study the ontogenesis of GALT was investigated through larval, first-feeding and juvenile stages. The development of GALT was correlated with the ontogeny of the major lymphoid organs namely the thymus, kidney and spleen.

4.2 Materials and Methods

4.2.1 Fish

Adult females were maintained in a freshwater recirculating system at a temperature of 24-26^{0}C. Eggs were fertilised externally then taken up by the female and kept within an oral brood pouch for 13-18 days. After this time the fry were finally released into the environment. Larvae were removed from the buccal cavity of brooding females at various intervals and fry were collected once they were observed free-swimming. From this time onwards 5 fry were sampled daily for 14 days and subsequently every 3 days until day 56, then again on day 84.

4.2.2 Histology

a) Light Microscopy (LM)

Whole fish were fixed in 10% neutralised formol saline, dehydrated in a series of alcohols and embedded in either paraffin wax or methacrylate resin (TAAB, Reading,Berks) as described in Section 3.2.2a. Wax sections (5-8 μm) and resin

sections (1 µm) were stained using Giemsa, PAS, MGP and, in the case of resin sections, 1% methylene blue (Chapter 3).

b) Electron Microscopy (EM)

Using a binocular microscope the whole of the gut was excised from larval and juvenile fish. The intestine was then divided into anterior and posterior regions and fixed in 2% gluteraldehyde in 0.1M cacodylate buffer (pH 7.2) for 1 hour at 4°C. The tissue was then prepared for electron microscopy as described in Section 2.2.3b.

4.2.3 Enumeration of GALT cells

In the adult *O.mossambicus* the intestine contained the most numerous intraepithelial leucoytes (IEL). In this study of young fish only the IEL in the intestine were counted and expressed as the number of IEL per 100 epithelial cells, IEL/ 100EC.

4.3 Results

4.3.1 Morphological development of the gut.

The larval and juvenile fish were sampled over an 84 day (12 weeks) period and the morphological development of the gut was monitored (Figure 2).

The first stage sampled was approximately 7-8 days post-fertilisation and designated as 7-P. The embryo was attached to a large yolk-sac and enclosed within a membrane. The gut was a straight tube, the epithelium being a single

layer of cuboidal cells (Plate A1).

Stage 14-P, at 13-14 days post-fertilisation, was still within the brood pouch but was occasionally observed outside the maternal pouch. The larvae possessed a large yolk-sac and did not feed on exogenous material. The intestinal epithelium now consisted of columnar cells with a brush border (Plate A2), and a few goblet cells.

Day 1, being the first day on which exogenous feeding began, the intestine was found to be a single coil (Plate A3). The epithelial cells of the posterior intestine were found to be highly vacuolated (Plate A4) in the supranuclear region of te cells. In contrast the epithelial cells of the anterior intestine lacked these vacuoles (Plate B1). In Plate B1 the close association of the yolk-sac, liver and intestine can be seen with a well-formed blood supply. In both regions of the gut the microvilli of the epithelial cells were small and sparse (Plate B2), these cells resembling those of the adult posterior intestine. There was also a slight outpushing from the anterior intestine which is the rudimentary stomach (Fig.2). This post-larval or pre-juvenile stage was free- swimming and almost completely independent of the female.

The epithelial cells of the posterior intestine gradually became less vacuolated (Plate B3), with an increase in the number of leucocytes in the lamina propria (Plate B4). As the whole intestine matured there was a gradual increase in the number of goblet cells within the epithelia, with a change in the morphology of the epithelial cells themselves

(Plate C1-C4). The maturation of the enterocytes was seen in the gradual disappearance of the numerous apical invaginations observed in the earlier samples, and the organisation of the cellular organelles.

4.3.2 Development of GALT and major lymphoid organs

The results of the enumeration of the GALT diffuse cell population in the larval intestine are given in Figure 1.

At stage 7-P no IEL or intralaminal leucoytes (ILL) were seen. However, thymic primordia consisting of a few lymphocyte-like cells were observed in the buccal cavity (Plate D1).

Leucocytes were observed within the intestinal tissue at stage 14-P. By this stage the thymus was well-developed and the kidney was located in the anterior region of the body behind the thymus. This organ contained renal and lymphoid tissue together with erythrocytic cells. GALT comprising of lymphocytes and macrophages were found within the epithelium (Plate D2), and also in the lamina propria.

After day 1, the first day of exogenous feeding, there was a rapid increase in the number of IEL. Of particular interest was that at this stage there appeared a cell complex within the epithelium (Plate D3) comparable to that observed in older fish (Plate D4). This complex was composed of 3-6 lymphoid cells, usually a large macrophage with a number of closely associated lymphocytes. They were located in both the anterior and posterior intestine in the basal region of the epithelium. Large macrophages were also found

in the epithelium and appeared to contain debris within their cytoplasm (Plate E1)

The first granulocyte to appear in the intestinal tissue was recognised at the EM level. This was the type 1 blood granulocyte, or neutrophil, which was found in the epithelium and lamina propria at day 1(Plate E2). As well as the appearance of the neutrophil, macrophages were more abundant, and exhibited cytoplasmic extensions between epithelial cells (Plate E3), a characteristic of motile cells. Also at this stage peripheral blood leucocytes were found in abundance within the blood vessels of the lamina propria (Plate E4).

The type I granulocyte was the first type to be observed at day 5 and was found within the lamina propria (Plate F1). From this age onwards granulocytes were found at various stages of intestinal development (Plate F2-F4).

Plasma cells were found in the intestinal tissue from day 4 onwards, both in the epithelium (Plate G1) and the lamina propria (Plate G2).

At day 7 the thymus was extremely prominent (Plate G3) and the kidney was still composed of renal and lymphoid tissue (Plate G4). The spleen was the last lymphoid organ to develop a lymphoid cell population and this occurred at about day 9 (Plate H1). By day 50 the intestinal GALT was equivalent to that of the adult fish with approximately 37 IEL/ 100EC (Plate H2).

4.4 Discussion

Aspects of the ontogenesis of the major lymphoid organs; the thymus, kidney and spleen, was investigated as part of a wider study of the ontogeny of GALT in *O.mossambicus*.

The results of this study show that the thymus was the first major lymphoid organ to develop. This appears to the situation amongst all the vertebrates (Ellis, 1977b; Grace, 1981). The thymus appeared as a few lymphoid cells in the buccal cavity above the gill arches at stage 7-P. In a previous investigation on the ontogeny of lymphoid organs in *O.mossambicus* small lymphocytes were found within the thymus 6-8 days after hatching (Sailendri, 1973). This is slightly later than observed during this investigation where lymphocytes or lymphocyte-like cells were observed at approximately 2-3 days post-hatch. This may be due to: a) it is difficult in many instances to designate a definite age to larvae as they are kept within the brood pouch and may be the result of more than one spawning, b) where the appearance of lymphoid organs may in part be dependent on the environmental temperature at which the animals were kept. In the study by Sailendri (1973) fish were kept at 18-20^{0}C whereas, in this study the experimental animals were kept at approximately 24^{0}C.

The exact origin of thymic lymphocytes in fish has yet to be determined. In *Salmo salar* L. lymphoid differentiation in the thymus takes place at 22 days prior to hatching (Ellis, 1977b). The latter author proposed that stem cells present in the kidney parenchyma migrate to and differentiate in the

thymic anlage. Apart from this contemporary theory of stem cell migration from the kidney, earlier authors believed that lymphocytes originated from the thymus by direct transformation of the epithelial cells (Beard, 1902-03; Maurer, 1886). In foetal sheep the first identifiable lymphocytes appeared in the thymus about 40 days into gestation (Morris, 1986), and the precursors of the stem cells are initially derived from the yolk-sac or foetal liver.

The kidney in the larvae of *O.mossambicus* is not differentiated into pro- and meso-nephros, as it is in adult fish, the larval kidney at this stage containing both renal and lymphoid tissue. Lymphoid cells were observed in this organ after approximately stage 14-P. A similar situation can be found in other teleosts (Botham & Manning, 1981; Grace, 1981).

The spleen developed after both the thymus and the kidney, and was the tissue first seen as a site of erythropoiesis. After day 9 lymphoid-like cells were recognised within the haemopoietic tissue.

Leucocytes were observed within the lamina propria at stage 9-P but there were no IEL at this stage. Fish at this stage were no longer enclosed within a membrane but were still within the maternal brood pouch. After first-feeding, day 1, when the larvae were fed an exogenous diet, there was a rapid increase in the number of IEL in the intestine. By day 50 (7 weeks) the adult complement of IEL was reached. The fry were finally released into the environment at day 1,

but were seen to return, intermittently, to the brood pouch for up to one week after this time.

In the adult fish three types of granulocytes have been identified in the intestine, and all types were found during the examination of the young stages. In contrast to those found in the adult intestine, granulocytes in the larval intestine were found at various stages of maturation. Davina *et al.* (1980) observed that in *Barbus conchonius* , the adult complement of epithelial leucocytes was achieved by 20 weeks of age. In *O.mossambicus* the rate of leucocyte infiltration of the gut epithelium is more rapid with adult equivalents being reached by 7 weeks of age.

Once the membrane enclosing the larvae and yolk-sac has been broken the larvae are then exposed to the environment, and hence to potential antigens present in the aquatic habitat. The transition from yolk-sac dependence to an exogenous diet exposes the GI tract to antigens in the food and from the environment. In mammals the location and distribution of leucocytes is independent of antigen exposure (Ferguson & Parrot, 1972; Husband & Gowans, 1978; Reynolds, 1981), but an increase in the number of IEL can be correlated with the presence of antigens (Ferguson, 1977). This increase in the number of IEL in the presence of antigen has been noted in several fish species including the goldfish (Weinberg, 1975), carp (Davina *et al.*, 1980) and the rosy barb (Davina *et al.*, 1982).

The increase in the number of leucocytes within the intestinal epithelium of the larvae may therefore be due to

the increase in antigenic exposure experienced by the intestine. The epithelium of the larvae appears highly pinocytotic, with numerous membrane invaginations in the apical region of the epithelial cell. This corresponds with the observations by Watanabe (1982; 1984a; 1984b; 1984c) who demonstrated a marked uptake of the protein HRP by the intestinal epithelial cells of both larval and juvenile fish. Ultrastructurally the epithelial cells of the early *O.mossambicus* larvae were similar to those of suckling rats which have been shown to allow the passage of intact protein macromolecules (Rodewald, 1970).

An interesting observation was the presence of a cell complex in the epithelium of the larvae. These cellular configurations were not observed in the adult intestine of *O.mossambicus*. These complexes were composed of 3-5 lymphoid cells, generally lymphocytes surrounded by a large macrophage, and were always located in the basal region of the epithelium. Whether they are a form of M-cell complex as observed in mammals (Owen, 1977) is uncertain as they are not associated with a specialised epithelial cell. However, this does not rule out their having a similar function, with the macrophage presenting possible antigenic material to the closely associated lymphocytes.

Figure 1 Distribution of Intraepithelial Leucocytes in the Alimentary Tract

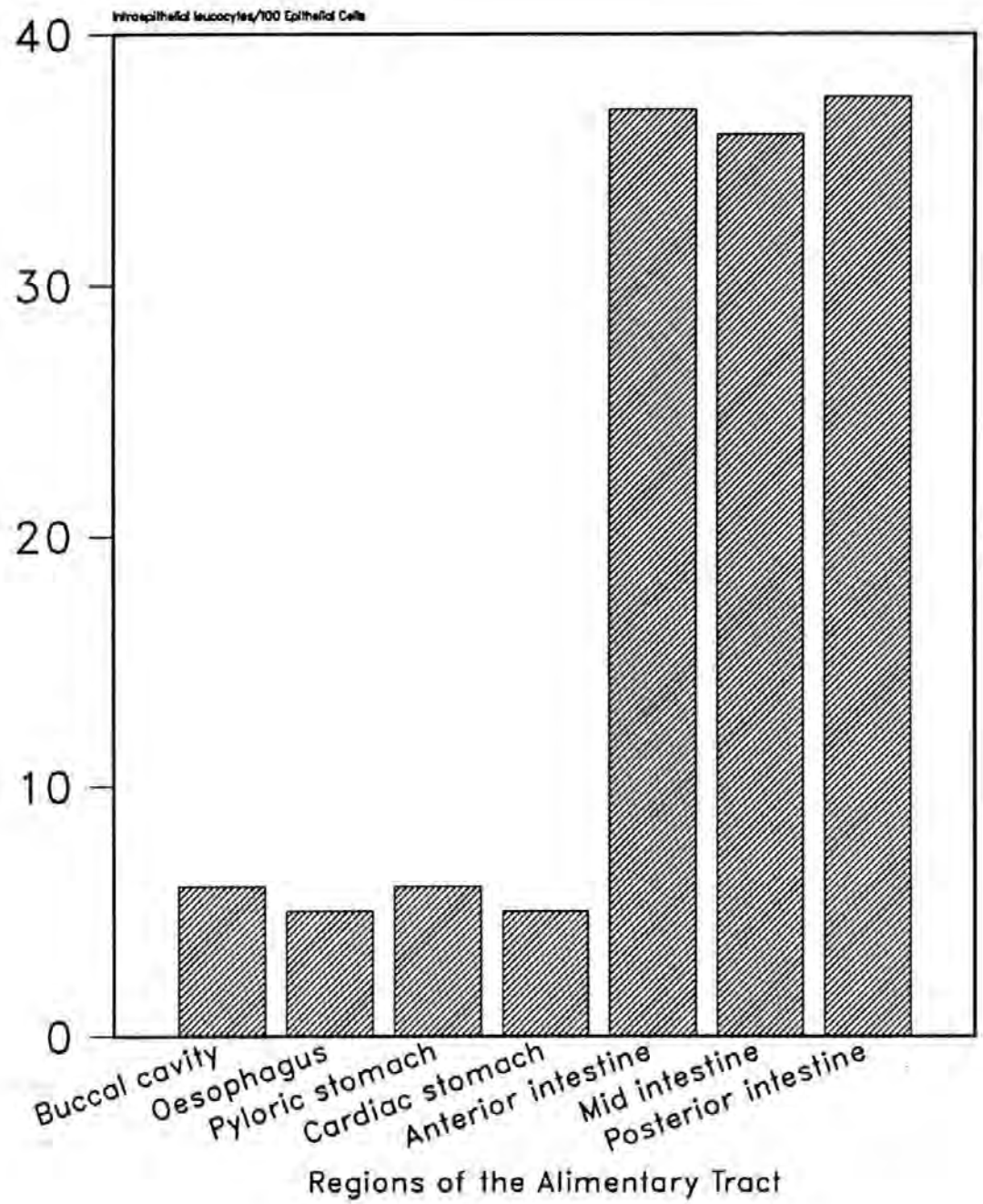


FIGURE 2. SCHEMATIC REPRESENTATION OF THE DEVELOPMENT OF THE GUT.

1 Stage 14-P; h- heart, ys- yolk-sac, gb- gall bladder, g- gut, s- spleen

2 Day 1; st- stomach.

3 Day 2

4 Day 3

5 Day 4

6 Day 5

7 Day 6

8 Day 7

9 Day 10

10 Day 14

Figure 2. Schematic representation of the developing intestine

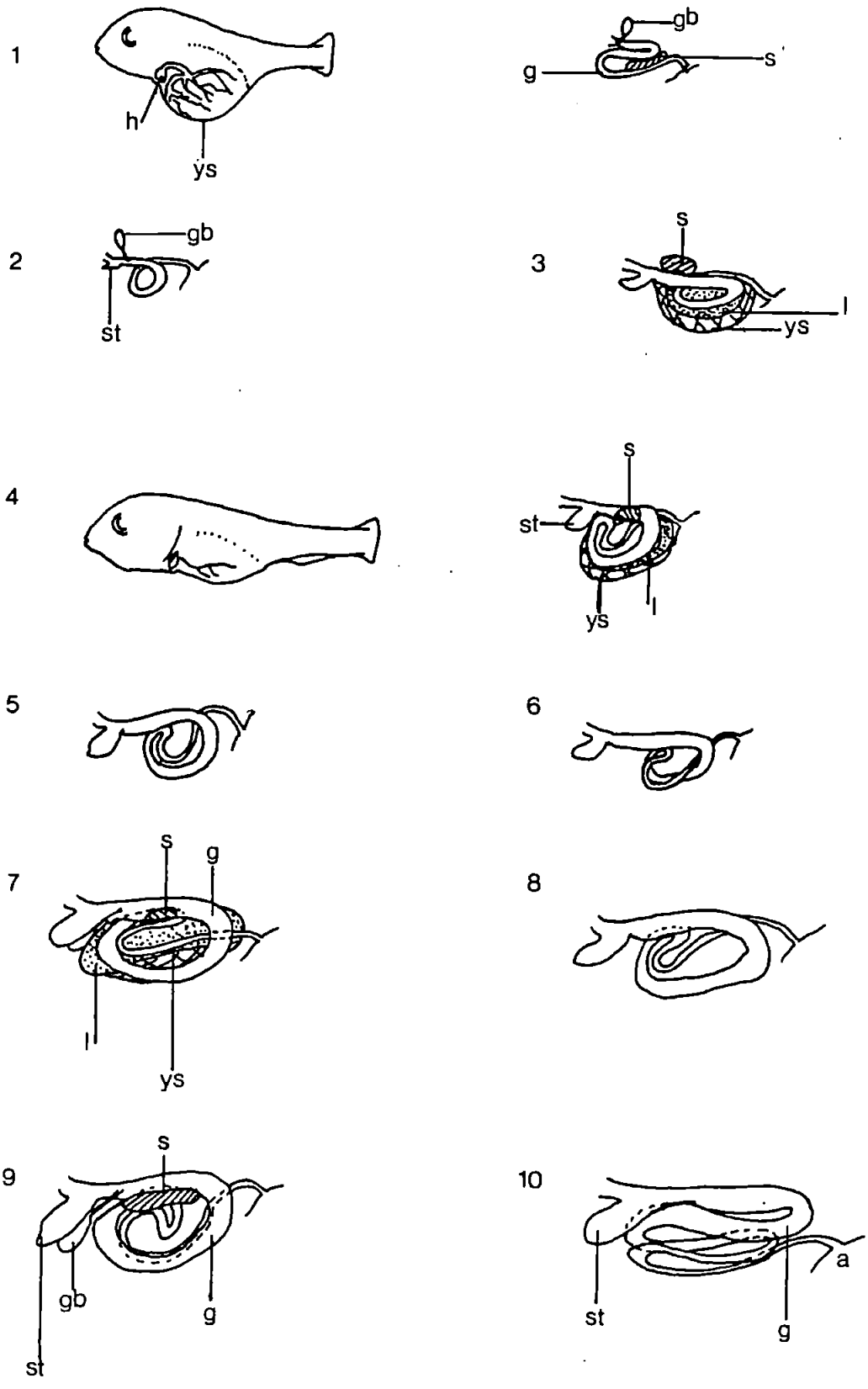


PLATE A Intestinal epithelium, stage 7-P, 14-P and day 1

1 Gut of fry at stage 7-P; In- intestine, L- liver, YS- yolk-sac, YP- yolk platelet. mag. x1056

2 Stage 14-P; E- epithelium. mag. x950

3 Coiled intestine at day 1, with vacuolated posterior intestinal epithelium (arrowed). mag. x105

4 Vacuolated epithelium of the posterior intestine, day 1. mag. x1056

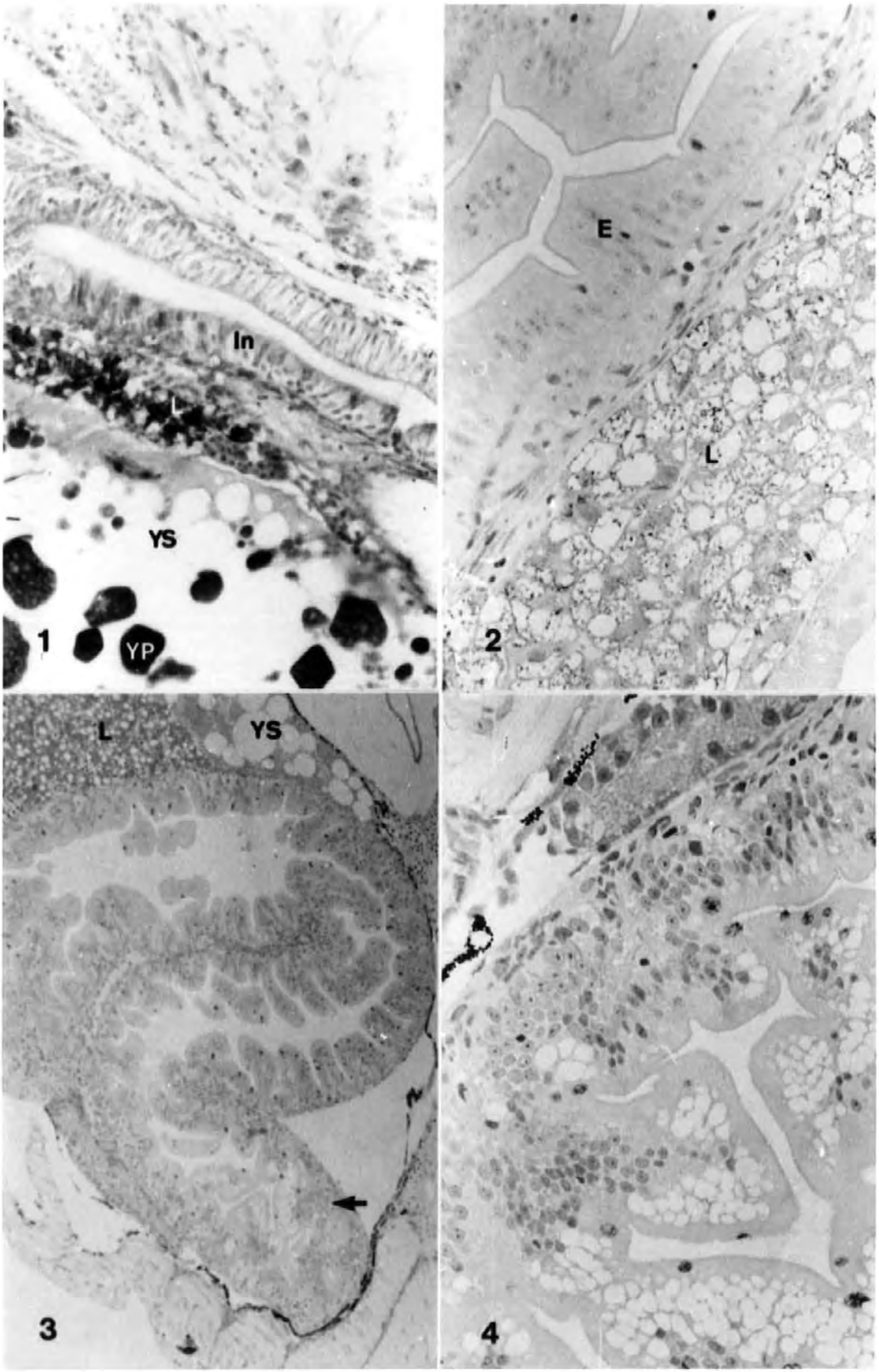

In
L
YS
YP
1
E
L
2
L
YS
3
4

PLATE B Intestinal epithelium, day 1, day 3 and day 5

1 Light micrograph of the anterior intestinal epithelium, day 1; iel- intraepithelial leucocytes. mag. x1056

2 An electron micrograph of the epithelium of the anterior intestine, day 1. mag. x2600

3 Posterior intestine day 3; GC- goblet cell, Er- erythrocytes in blood vessel in the lamina propria. mag. x2200

4 Leucocytes in the lamina propria of the anterior intestine, day 5; E- epithelium. mag. x3000

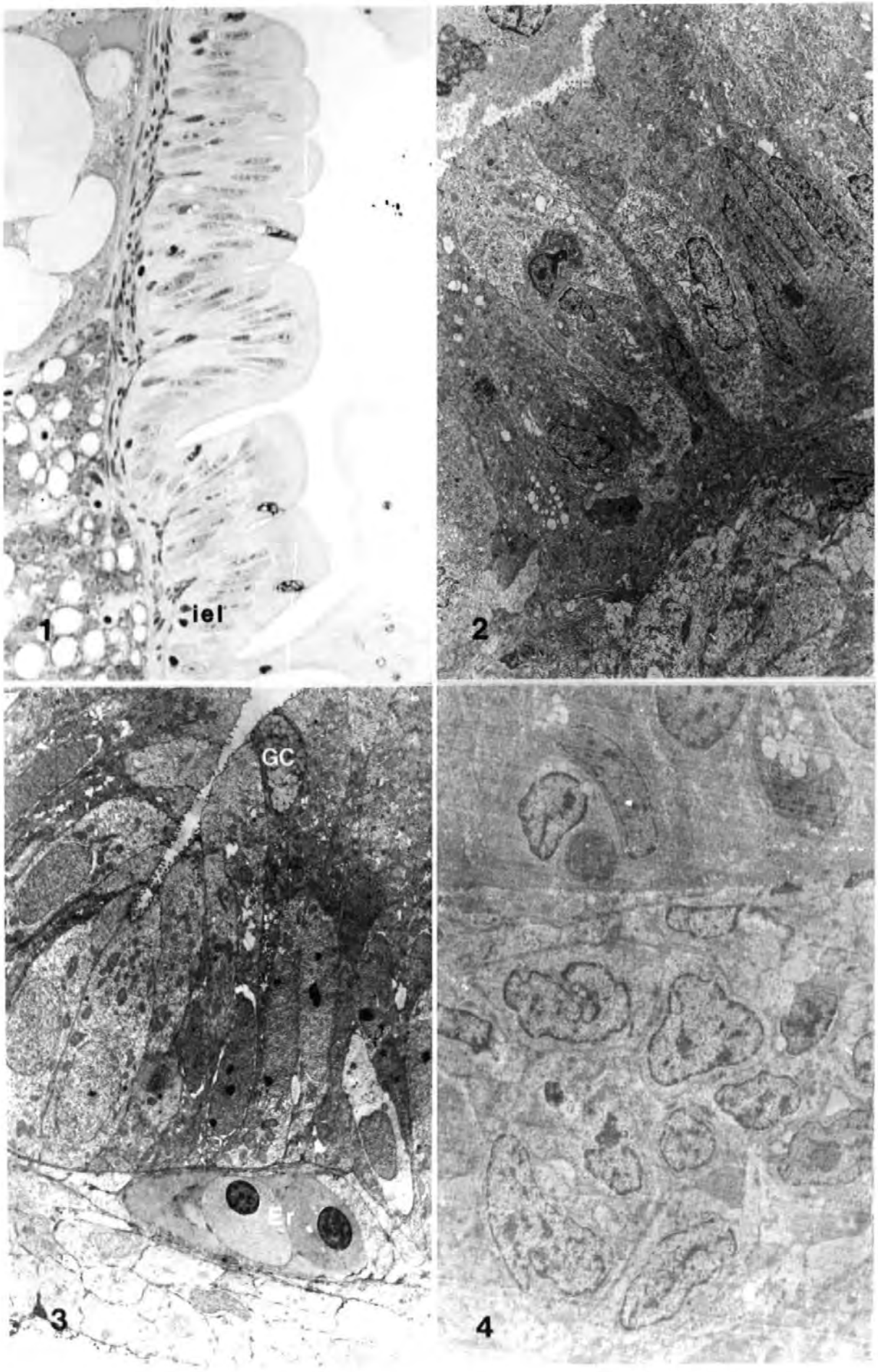

iel
1
2
GC
3
4

PLATE C Intestinal epithelium, day 10, day 14 and day 84

1 Epithelium of the anterior intestine day 10. mag. x8000

2 Epithelium of the anterior intestine day 14. mag. x7000

3 Epithelium of the anterior intestine, day 14, after feeding. Note the large number of vacuoles in the enterocytes; v- vacuole, arrow- apical cytoplasm invaginations. mag. x7000

4 Anterior intestinal epithelium at day 84; iel- intraepithelial leucocytes, lp- lamina propria. mag. x1056

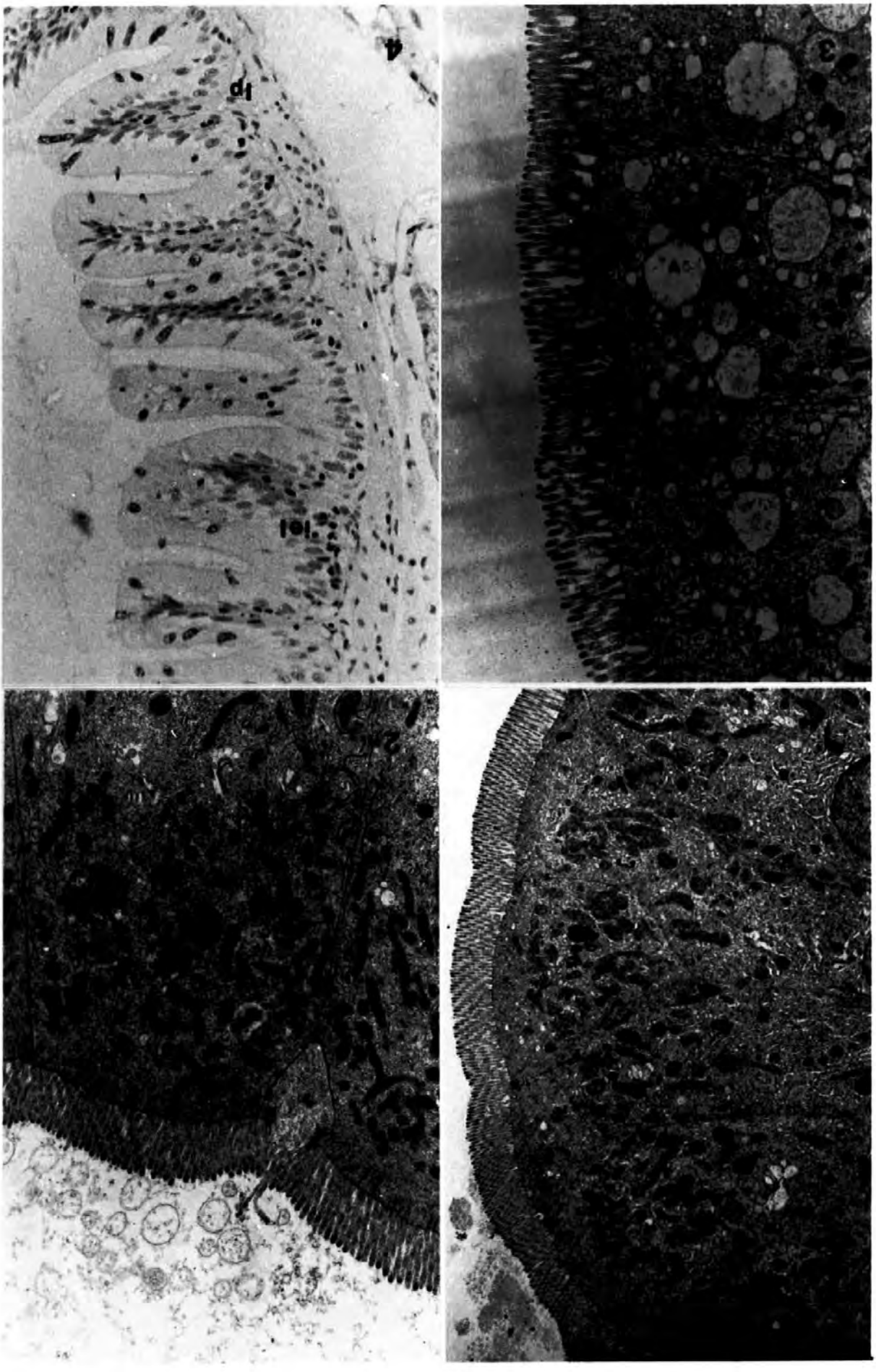

d1
101

PLATE D Thymic primordia at stage 7-P and intestinal cell complexes

1 Thymic primordia (T) in the buccal cavity stage 7-P. mag. x640

2 Lymphocyte (L) in the anterior intestinal epithelium, day 5. mag. x3800

3 Cell complex in the anterior intestinal epithelium, day 5. mag. x4400

4 Two cell complexes (arrowed) in the anterior intestinal epithelium, day 38. mag. x1056

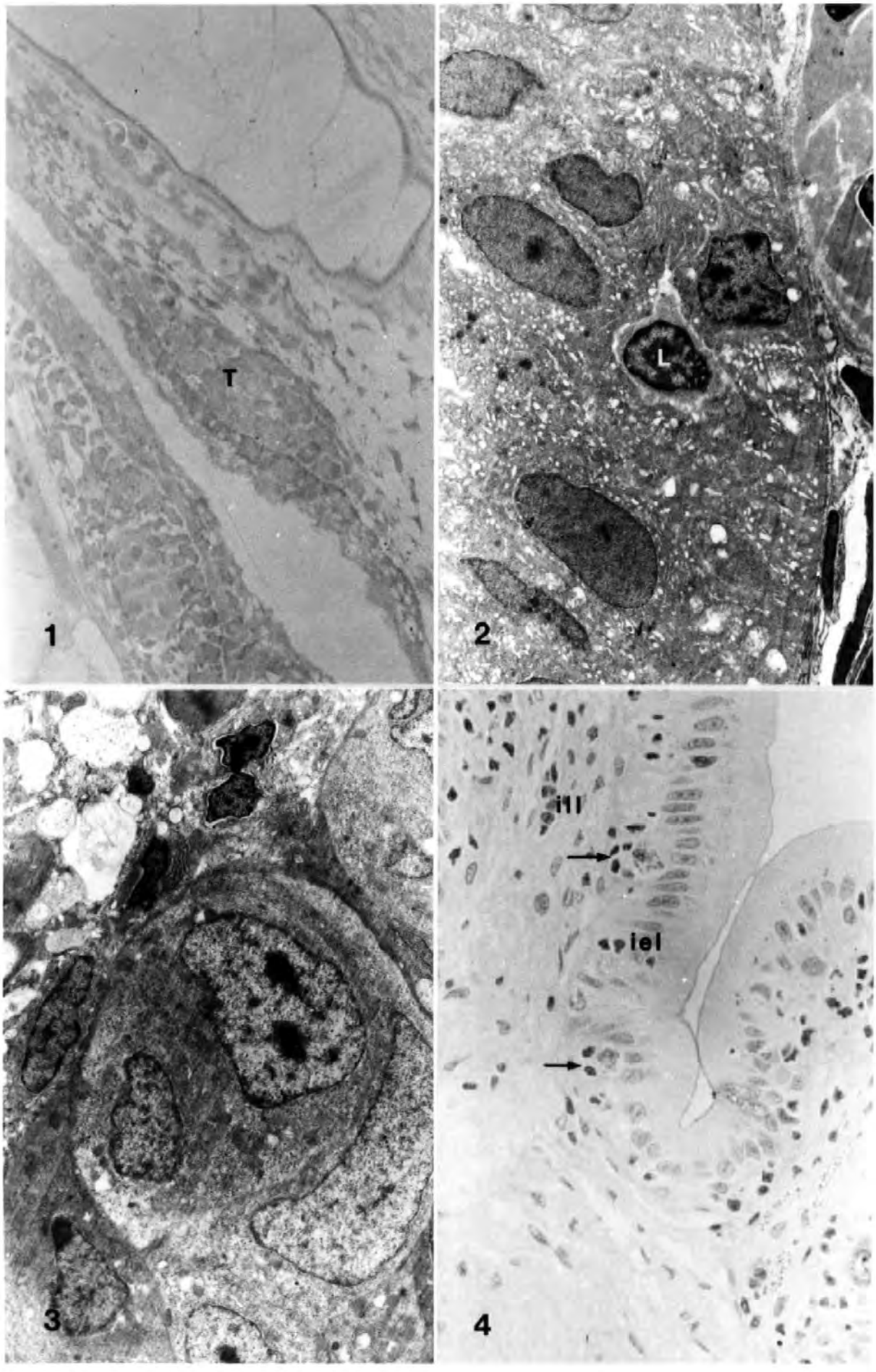

T
1
L
2
3
ill
iel
4

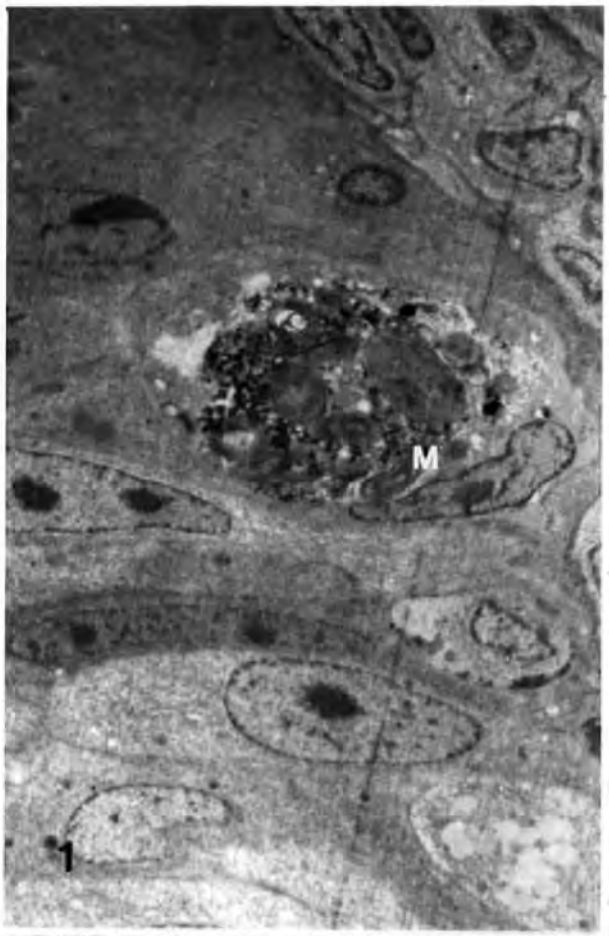
PLATE E Intestinal macrophages and blood granulocyte type 1

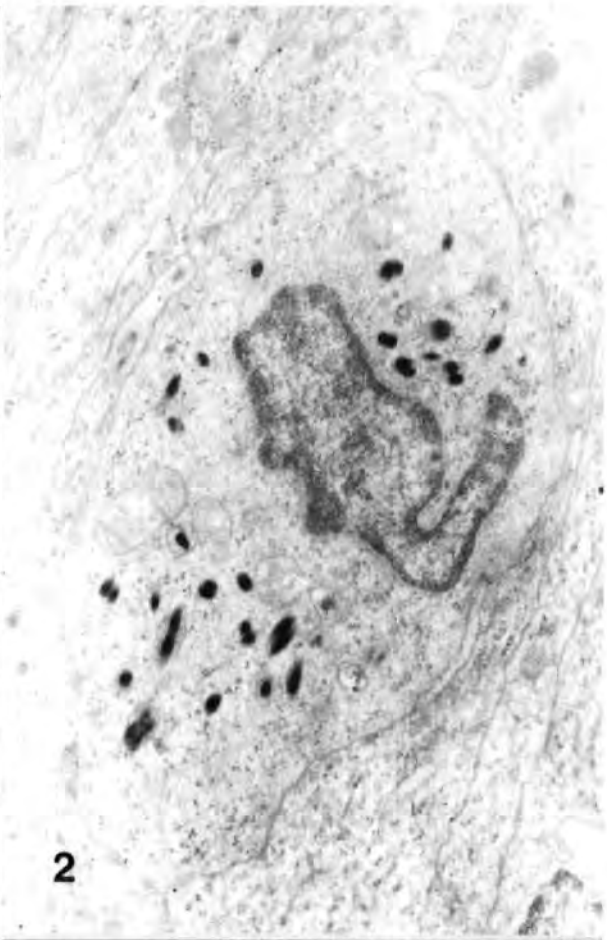
1 Macrophage (M) in the anterior intestinal epithelium. mag. x3600

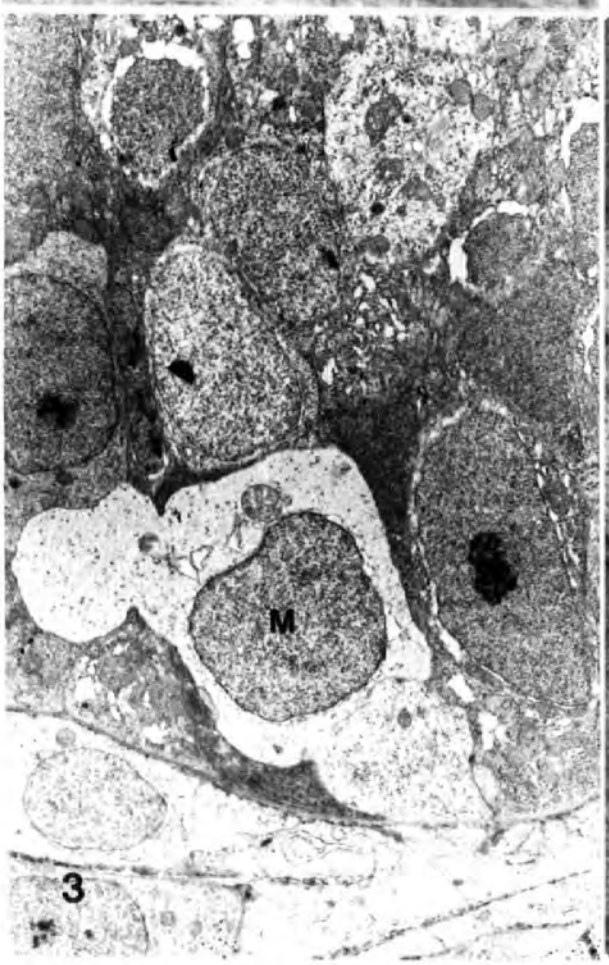
2 Type 1 blood granulocyte in the intestinal epithelium, day 1. mag. x8200

3 Macrophage in the anterior intestinal epithelium, day 1. mag. x3600

4 Blood vessel (BV) in the lamina propria (lp); L- lymphocyte, T- thrombocyte. mag. x4500


M
1


2


M
3

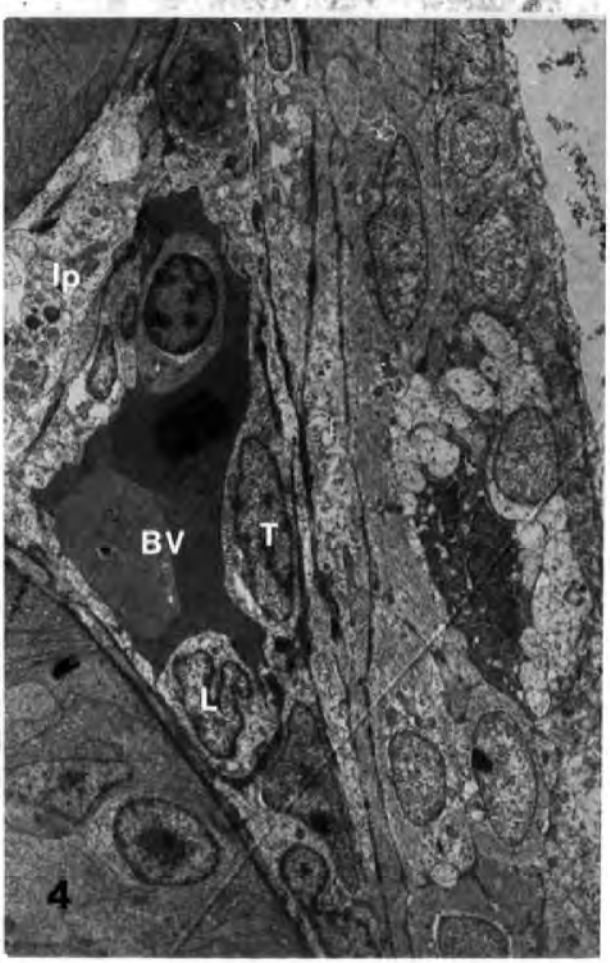

lp
BV
T
L
4

PLATE F Tissue granulocytes

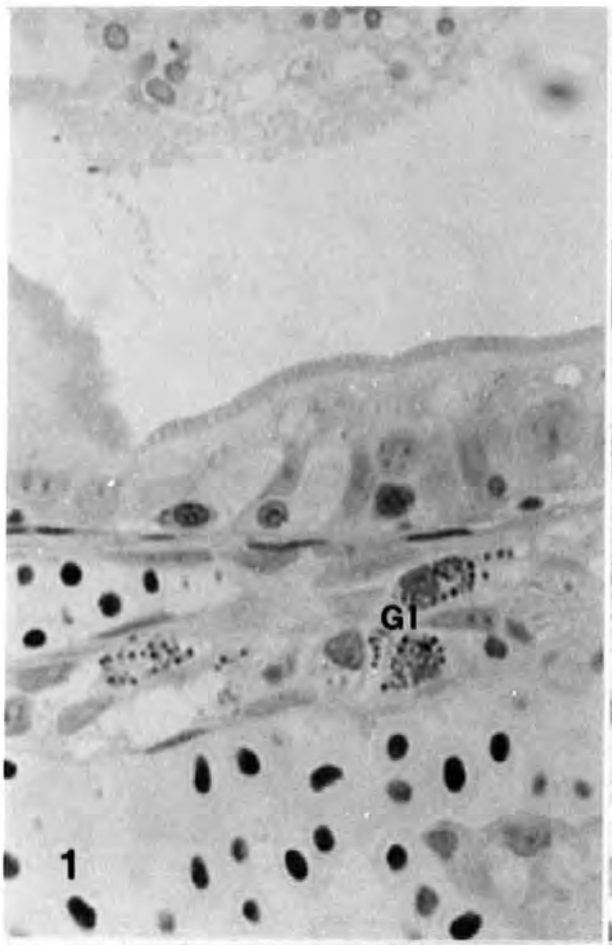
1 Type I tissue granulocytes (GI) in the lamina propria of the intestine. mag. x660

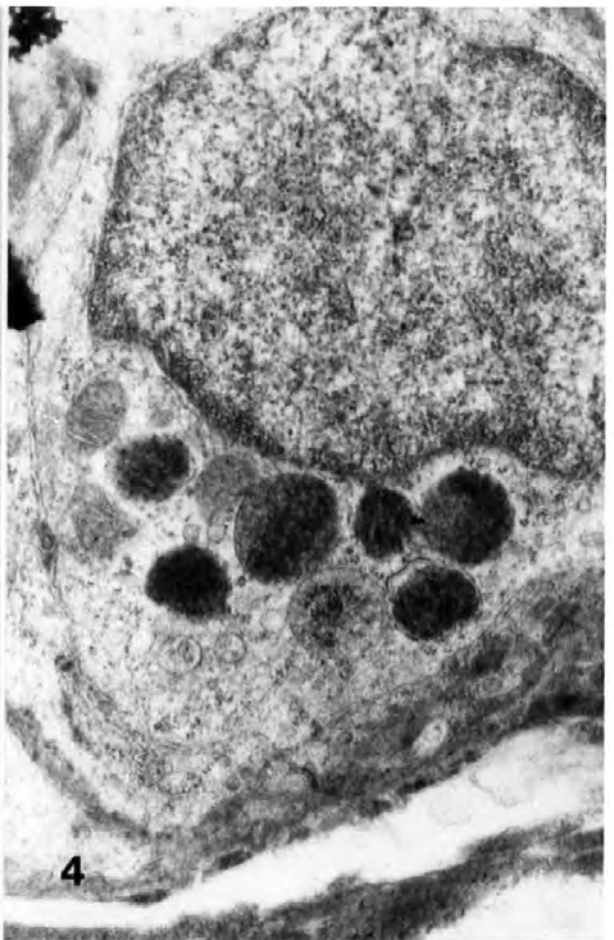
2 Pro-granulocyte type I, with a granule showing the electron dense core (arrowed) found in matire cells. mag. x13000

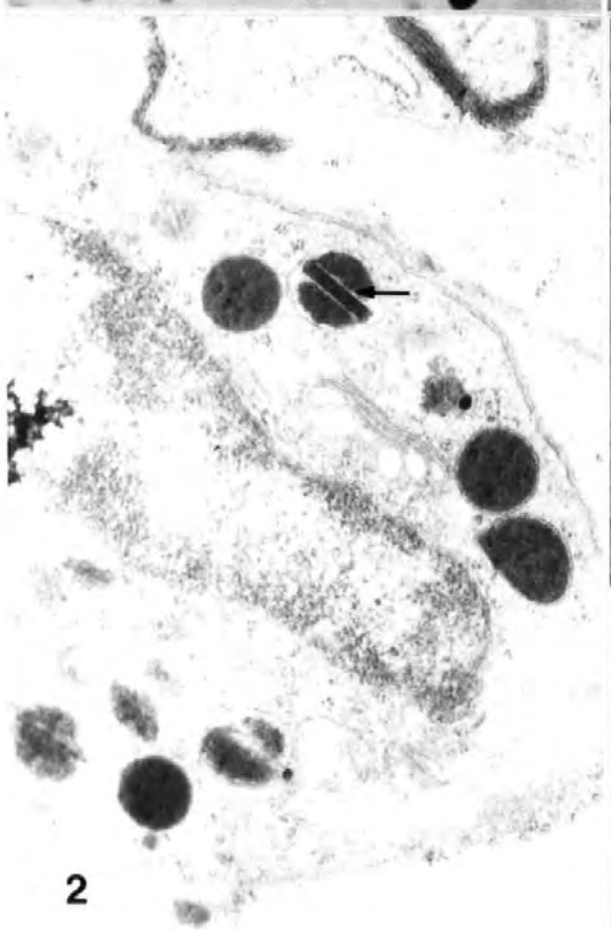
3 Higher magnification of a granule with an electron dense core, arrowed. mag. x23,000

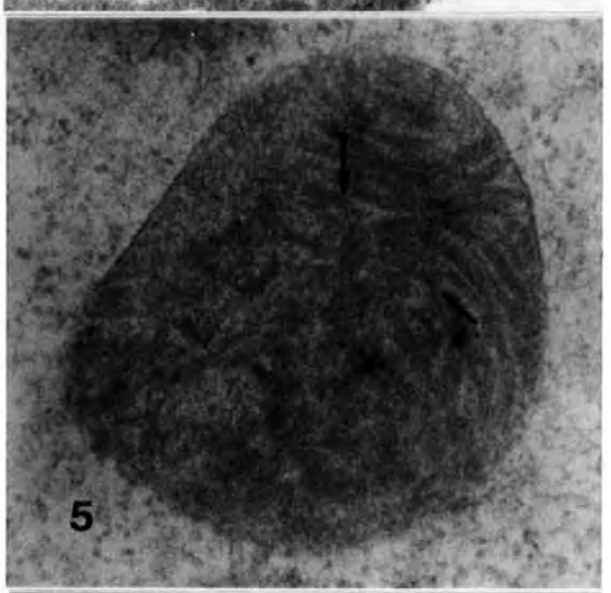
4 Progranulocyte type II. mag. x17000

5 High magnification of a granule of the cell in F4, showing its fibrillar substructure (arrowed). mag. x28,000


GI
1


4


2


5

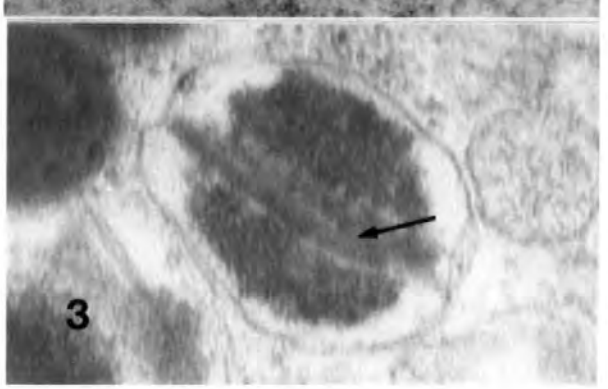

3

PLATE G Plasma cells in the intestine

1 Plasma cell in the epithelium of the anterior intestine; RER- rough endoplasmic reticulum. mag. x11,000

2 Plasma cell (P) in the lamina propria of the intestine. mag. x500

3 Thymus at day 14; E- buccal cavity epithelium, Oz- outer zone, Iz- inner zone. mag. x400

4 Pronephros at day 9 containing lymphoid tissue and nephrons (N); S- stomach. mag. x400

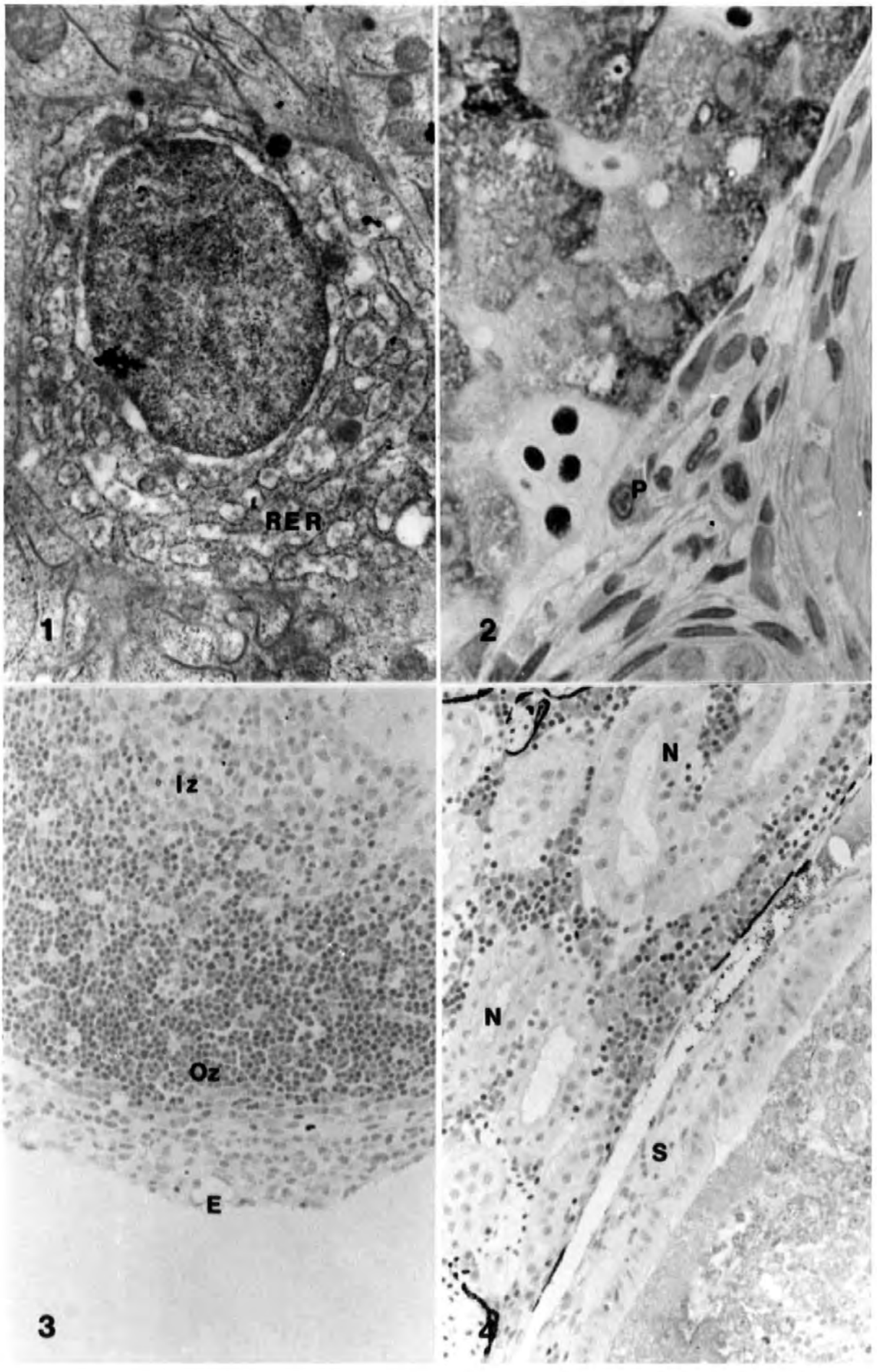
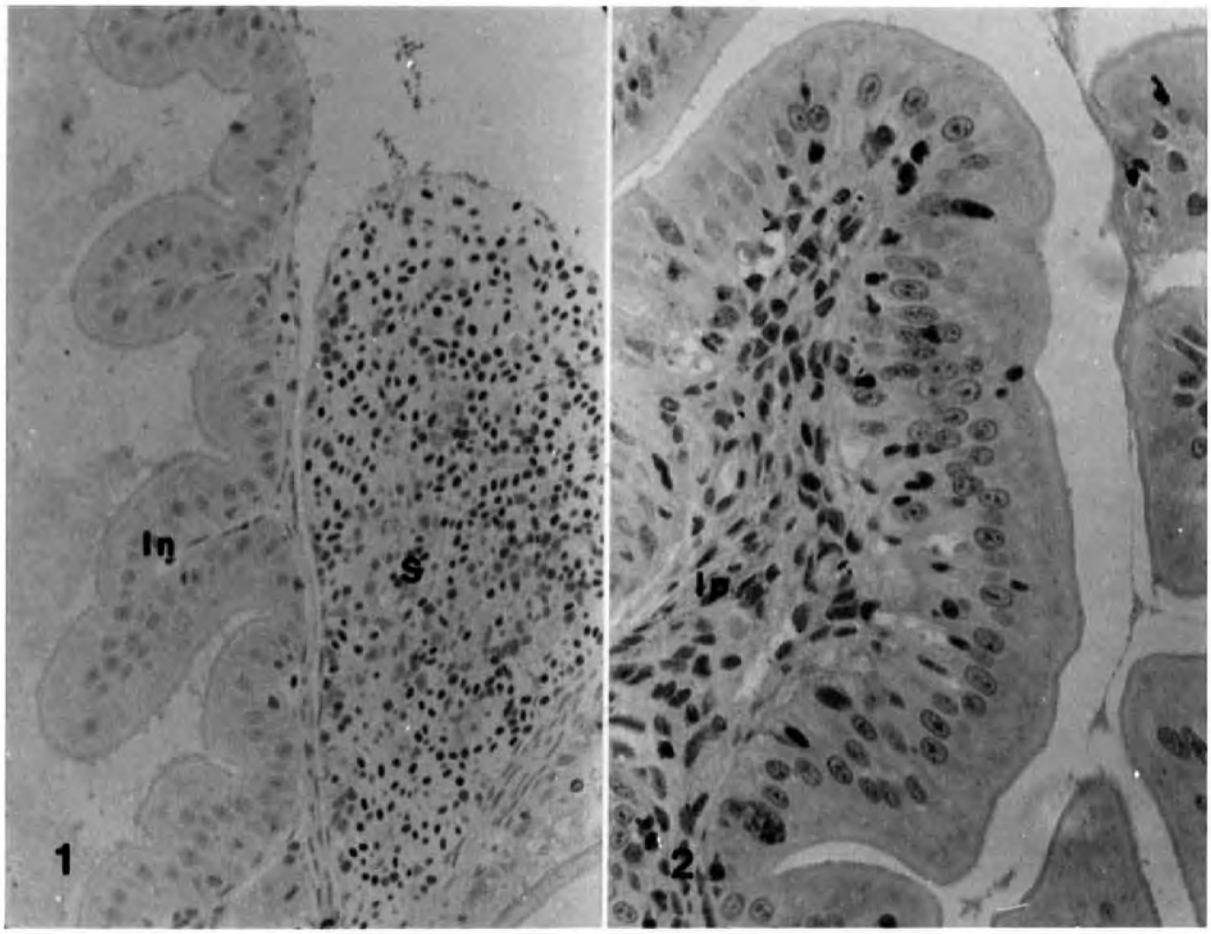

RER
1
P
2
Iz
Oz
E
3
N
N
S
4

PLATE H Distribution of intraepithelial leucocytes at day 50

1 Spleen (S) at day 9; In -intestine. mag. x400

2 The distribution of intraepithelial leucocytes at day 50, equivalent to levels found in adult epithelium. mag. x416


In
S
1
2

CHAPTER 5.

5. UPTAKE OF HORSERADISH PEROXIDASE AND FERRITIN BY THE INTESTINAL EPITHELIUM.

5.1 Introduction

The morphology of the digestive tract of teleosts varies a great deal, with approximately 1.5% of all species not possessing a stomach or pyloric caecae (Jakobshagen, 1937) as well as regional differences in the intestinal epithelium which have been noted in a number of fish species (Gauthier & Landis, 1972; Noaillac-Depeyre & Gas, 1979; Stroband *et al.*, 1979). The absorption of intact macromolecules has been demonstrated in both gastric and agastric species (Georgopoulou *et al.*, 1984; Hart, 1987; Lamers, 1985). This contradicts earlier work where it was postulated that macromolecule absorption was related to the lack of a stomach, and the need to absorb undigested protein in this type of gut. Georgopoulou *et al.* (1986) demonstrated that the epithelial cells of the posterior intestine in both young and adult trout were structurally and functionally similar to those of the ileum of the suckling rat. This feature, together with the pinocytotic capacity of the enterocytes of the posterior intestine have led to their comparison with the mammalian M-cell. These specialised cells found in the intestinal epithelium of mammals take up macromolecules by pinocytosis (Owen, 1977).

Lymphoid cells which are present in the lamina propria and the epithelium of the intestine may also be involved in the processing of macromolecules. McLean *et al.* (1988a; 1988b) have demonstrated that gonadotrophin absorbed by the intestinal enterocytes of the chinook and sock-eye salmon was still immunologically active.

This present study visualises the uptake and transport of two different macromolecular marker proteins horseradish peroxidase (mw.@40 000 daltons) and ferritin (mw. @500 000 daltons) by the intestinal epithelial cells of *O.mossambicus*.

5.2 Materials and Methods

5.2.1 Fish

Juvenile *O.mossambicus*, of both sexes (8-12g), were kept in a freshwater recirculating system at a temperature of 24-26^{0}C.

5.2.2 Administration of Macromolecules

Prior to the intubation of the macromolecules all fish were deprived of food for 48 hours. The macromolecules were orally intubated into the stomach using a polyethylene tube (diameter 1.6mm) attached to a 23g needle and 1ml. syringe.

a) Horseradish Peroxidase, HRP (Grade I, Sigma)

0.1ml. of an HRP solution (4% HRP in PBS) was orally intubated into each of a group of nine fish. Fish were

sampled at 5 min., 2 h. and 16 h. after the intubation. Three fish were sampled at each sample time.

The gut from each fish was excised and sections of the anterior and posterior intestine were fixed for 1 hour at 4°C in 2% gluteraldehyde in 0.1M cacodylate buffer, pH 7.2. These were then rinsed for 15 min. in 0.05M Tris/HCl buffer, pH 7.6.

To demonstrate the presence of HRP small pieces of the fixed tissue were incubated for 1 hour at room temperature in 0.5% 3,3- diaminobenzidine -tetrahydrochloride (DAB grade II, Sigma). This was followed by incubation in the same solution containing 0.01% H_2O_2 for 1 hour. The tissue was then rinsed for 10 min. in 0.05M Tris (pH 7.6) and 10 min. in 0.1M cacodylate buffer (pH 7.2) and subsequently prepared for electron microscopy as described in Section 2.2.3b, with a uranyl acetate counterstain.

The tissue of two control animals was examined for any endogenous peroxidase activity.

b) Ferritin (Horse Spleen, Sigma)

0.1 ml/fish of a ferritin solution (10% ferritin in PBS) was orally intubated into a group of fifteen fish. Three fish were sampled at 30 min., 1, 4, 16 and 24 hours after the intubation. The gut was excised and parts of the anterior and posterior intestine were fixed in 2% gluteraldehyde in 0.1M cacodylate buffer (pH 7.2) for 1 hour at 4°C. The tissue was then prepared for electron microscopy as described in Section 2.2.3b,with a uranyl acetate

counterstain.

5.3 Results

5.3.2 Absorption of horseradish peroxidase (HRP)

Five minutes after oral intubation the HRP could be visualised 'coating' the microvilli of the anterior intestine (Plate A1-A4). Invaginations of the membrane between the microvilli extended into the apical cytoplasm (Plate A2 & A4), forming sacular invaginations. Some of these invaginations were seen to have pinched off to form small coated vesicles (Plate A1).

Two hours after intubation HRP was not seen around the microvilli but was present in small vesicles throughout the apical cytoplasm of the enterocytes of the anterior intestine (Plate B1 & B2). Lamellar infoldings were found to contain limited amounts of HRP (Plate B3 & B4) running parallel to the intercellular space (ICS) between adjoining cells. In some instances these infoldings could be seen to be joining with the lateral membrane of the cell, connecting with the ICS. These infoldings were also found to be closely associated with small multivesicular bodies (MVB), formed by the coalescing of smaller membrane bound vesicles. HRP was observed in large quantities in the ICS and at the basement membrane.

Macrophages were found to contain copious quantities of HRP within their cytoplasm (Plate B5) appearing as large cells within the epithelium and the lamina propria.

Membraneous whorls, a characteristic of phagocytic cells, were observed in their cytoplasm coated with HRP (Plate C1-C3). These membraneous whorls were also found in the enterocytes, the inner membranes of which were coated with HRP. These membraneous whorls in the enterocytes were also observed to contain effete organelles (Plate C3).

In the previous chapter the blood granulocyte type 1, or neutrophil, was identified as being present in the intestinal tissue. This cell was shown to possess endogenous peroxidase activity associated with its granules (Chapter 2), however in this investigtion this cell was found to contain peroxidase activity within cytoplasmic vesicles (Plate C4). This activity is probably due to the ingestion of HRP that has been sequestered from the intestinal enterocytes into the lamina propria.

Sixteen hours after intubation HRP had almost disappeared from the enterocytes of the anterior intestine, although some small HRP containing vesicles were still apparent. The large macrophages were still seen in the lamina propria and the epithelium. Some macrophages were seen endocytosing small quantities of HRP that was present in the intracellular space (Plate D1). After 16 hours HRP was also detected in the posterior enterocytes (Plate D2), in small vesicles in the apical cytoplasm, and in macrophages in the epithelium (Plate D3 & D4) and lamina propria (Plate D5). HRP was detected in the posterior intestine although in smaller quantities than were present in the anterior intestine.

5.3.2 Absorption of Ferritin

Ferritin, being electron dense, was easily visualised and was detected after 30 min. between the microvilli (Plate E1) and within invaginations in the apical cytoplasm (Plate E2) of the anterior enterocytes. Of particular note was the clathrin coat of the plasma membrane invaginations (Plate E2), which eventually pinch-off to form small clathrin coated vesicles (Plate E3). Ferritin was also found accumulated within larger vesicles in the supranuclear region in the epithelial cells of the anterior intestine (Plate E4). These vesicles were still apparent in the anterior intestinal epithelial cells after 1 hour (Plate E5), however at this time ferritin could no longer be detected at the microvillous surface. These large vesicles, in which the ferritin was found, varied in structure. They were either large membrane bound structures (Plate F1), multi- membraneous vesicles (Plate F2) or small multi-vesicular structures (Plate F3). Four hours after intubation ferritin-containing vesicles were very numerous (Plate F4 & F5) but were still found in the supranuclear region. Sixteen hours after the intubation of ferritin only small amounts of this macromolecule was found within the anterior enterocytes.

Macrophages were observed to contain varying quantities of ferritin within their cytoplasm (Plate G1-G4). At 4 hours post-intubation macrophages containing ferritin (Plate G1) and others containing no ferritin (Plate G2) were present at

the apical region of the epithelial cells. In some instances these macrophages displayed pseudopodial extensions between epithelial cells. Macrophages containing ferritin were also recognised in the lamina propria (Plate G3) and closely associated with lymphocytes (Plate G4). After 16 hours macrophages were still seen with ferritin in their cytoplasm (Plate H1 & H2) but less than was detected earlier.

An enterosecretory cell found within the intestinal epithelium was seen to contain membraneous whorls with ferritin particles associated with these structures (Plate H3).

Twenty-four hours after intubation small quantities of ferritin were still apparent in vesicles in the intestinal epithelial cells of the anterior intestine (Plate H4).

During the course of this examination no ferritin was detected in the posterior intestinal epithelial cells or in the intra- epithelial macrophages of this region.

No particles resembling ferritin were detected in the control samples in either the anterior or posterior intestine.

5.4 Discussion

Several authors have described the absorption of both HRP and ferritin by the absorptive cells of the posterior intestine of fish (Noailliac-Depeyre & Gas, 1973; Stroband *et al.*, 1979; Watanabe, 1984). However, relatively few have described this phenomena in the anterior intestinal cells of

fish (Lamers, 1985). In this present study considerable amounts of both these marker proteins were absorbed by the enterocytes of the anterior intestine of *O.mossambicus*. The absorption of macromolecules by the mammalian small intestine has been demonstrated (Walker, 1973; 1980) and in neonates is involved with the passive transfer of immunity via the maternal colostrum (Bainter, 1987).

The enterocytes of carp (Lamers, 1985) and tench (Noailliac-Depeyre & Gas, 1973) are ultrastructurally similar to the anterior intestinal enterocytes of *O.mossambicus*. These cells being thought to be specifically adapted for the transport of macromolecules. In some studies absorptive enterocytes have been characterised by the presence of lamellar infoldings in the apical cytoplasm. These structures are also called cytoplasmic tubules by some authors (Iida & Yamamoto, 1985).

In previous studies HRP has been shown to coat the invaginations of the cell membrane between the microvilli (Georgopoulou *et al.*, 1985; Iida & Yamamoto, 1985). Some of these invaginations form HRP- coated pits that are 'pinched-off' to form small coated vesicles. In *O.mossambicus* HRP was found coating the microvilli of the anterior enterocytes 5 min. after the oral intubation of HRP. Small coated vesicles were also observed in close proximity to shallow invaginations in the apical membrane. Some of these vesicles appeared to migrate to the lateral cell membrane and were occasionally associated with cytoplasmic tubules. These tubules were found to contain

only limited amounts of HRP, whereas other studies have suggested that these structures were responsible for the transport of the majority of the absorbed HRP to the intracellular space (Lamers,1985).

Lamers (1985) suggested that the uptake and transport of HRP in the absorptive enterocytes of carp was receptor-mediated, protecting HRP containing vesicles from fusion with lysosomes. This would correlate with the type of transport of HRP to the ICS that was observed in that study, however, HRP is considered to enter mammalian enterocytes via fluid phase endocytosis which does not require receptors. This has also been demonstrated to some extent in fish by Schindler & de Vries (1988). Owen (1977) believed that HRP did not reflect the usual pathways of macromolecule uptake because of diffusion due to its small molecular size and low molecular weight (4x 10^{1}d). To qualify this observation of HRP transport to the ICS, Owen (1977) proposed that HRP in vesicles does coalesce with lysosomes but, if lysosomal capacity is exceeded then these vesicles moved to the lateral cell wall and discharged their contents into the ICS. This might account for Lamers (1985) suggesting that HRP uptake was receptor- mediated, as HRP was detected in the ICS.That HRP eludes lysosomal activity is apparent as HRP was detected in the ICS in *O.mossambicus*, and within large intraepithelial macrophages.

Ferritin (mw @5x 10^{1}d) was absorbed by the anterior intestinal epithelial cells and was visualised between the microvilli and at the base of the invaginations thirty

minutes after oral intubation. These invaginations were observed to pinch-off to form small clathrin coated vesicles. Larger vesicles, formed by the fusion of smaller vesicles, contained large amounts of ferritin and were multimembraneous. These multi-membraneous whorls were also recognised during the investigation of HRP uptake. These structures are associated with the melanomacrophages of fish (Agius & Agbede, 1984) and other phagocytic cells in fish (Parish, 1981), but in enterocytes may be the result of fusion of small membrane bound vesicles. No ferritin was detected in cytoplasmic tubules or intra-cellular space, a feature also recognised by Rombout & van den Berg (1985) and Georgopoulou *et al.* (1985). This raises the question as to how ferritin passes to the intraepithelial macrophages, as no exocytosis of ferritin into the ICS was observed.

The mechanism by which ferritin is absorbed by the intestinal enterocytes of fish has been commented upon; Lamers (1985) believed that this was a non-selective process of fluid phase absorption. However, cationised ferritin has a strong affinity to accessible negative charges on cell surfaces, being a specific ligand -receptor probe. The latter being one of the four recognised pathways in receptor -mediated endocytosis (Schindler & de Vries, 1988). Ferritin has been demonstrated as being possibly attached to such receptors on fish epithelial cells (Schindler & de Vries, 1988; Wichtrup & Greven,1985). However, the possibility of such ligand formation was not demonstrated in *O.mossambicus*. The presence of a clathrin coat, supposed to be indicative

of selective transfer of macromolecules (Abrahamson & Rodewald, 1981), may also support this hypothesis of ligand formation. This does not, however, explain why ferritin-containing vesicles are not seen to exocytose their contents into the ICS.

The anterior intestine of *O.mossambicus* is thus capable of absorbing macromolecules. This is perhaps surprising as this species possesses a fully functional stomach, and macromolecular uptake has been proposed as being a function of stomachless fish. Macromolecular absorption has also been demonstrated in the epithelial cells of the posterior intestine of fish (Georgopoulou *et al.*, 1986; Noailliac-Depeyre & Gas, 1973; 1976; 1979) and they have been compared to mammalian M-cells. These specialised epithelial cells are specially adapted for antigen uptake and transport to the underlying lymphoid cells (Owen, 1977). In this study intraepithelial macrophages were observed closely associated with lymphocytes in the epithelium and lamina propria. This may allow antigen presentation to the lymphocyte by the macrophage and thus initiate a local immune response as observed in mammalian studies.

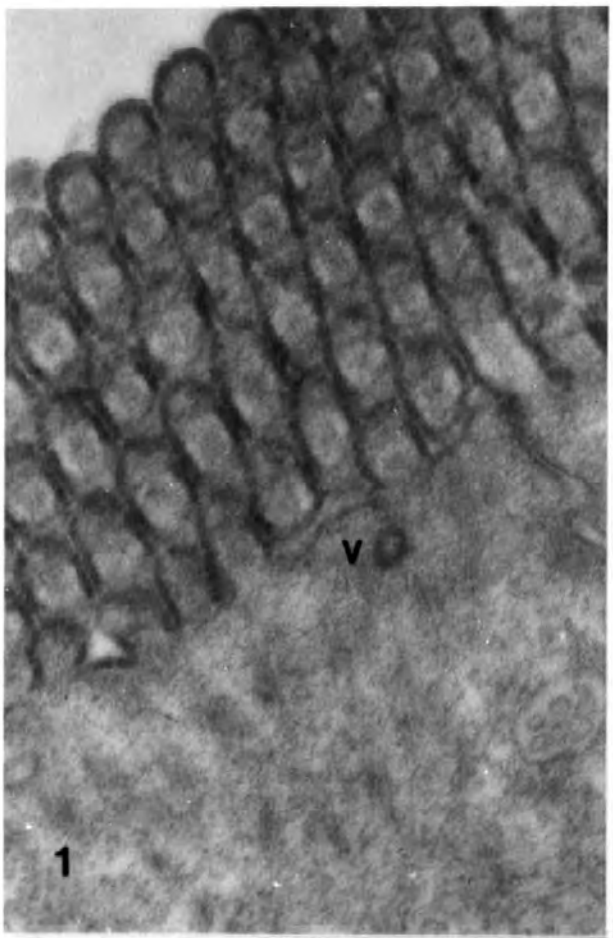
PLATE A HRP in the anterior epithelium 5 minutes after intubation

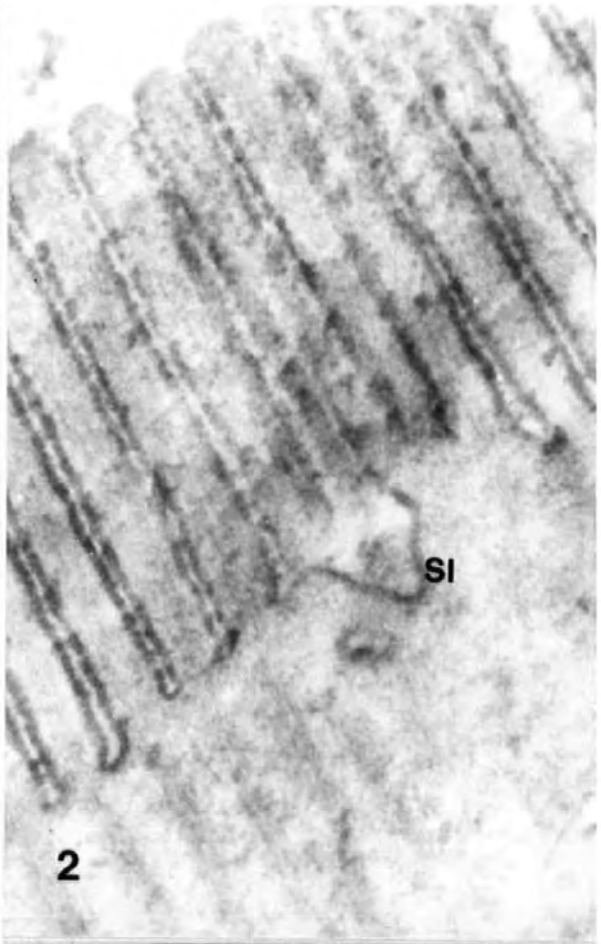
1 Microvilli coated with HRP; v- small HRP coated vesicle. mag. x36,000

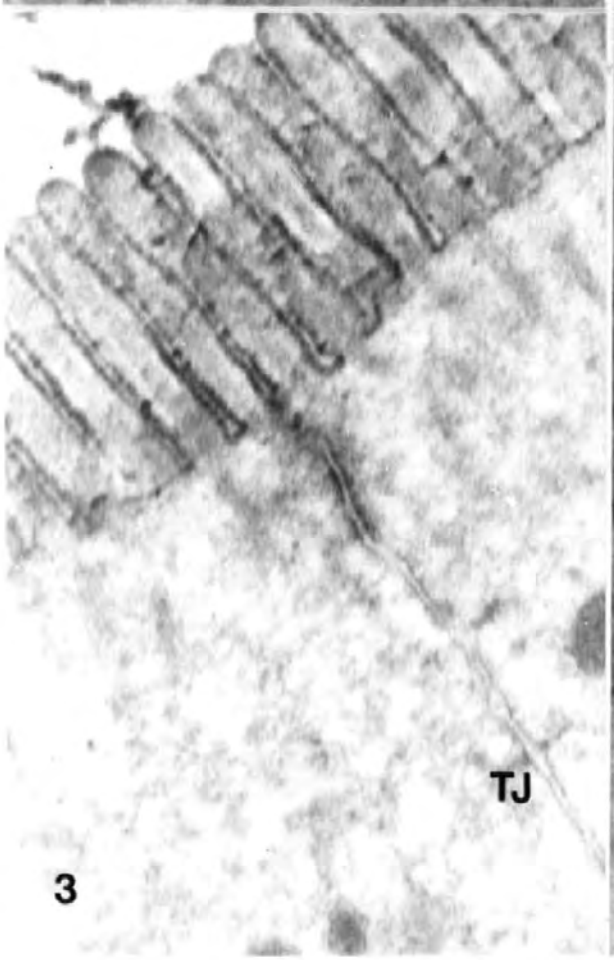
2 HRP-coated microvilli with a sacular invagination (SI) coated with HRP. mag. x45,000

3 HRP passing between epithelial cells (ICS); TJ- tight junction. mag. x28,000

4 Invaginations into the apical cytoplasm coated with HRP. mag. x36,000


V
1


SI
2


TJ
3

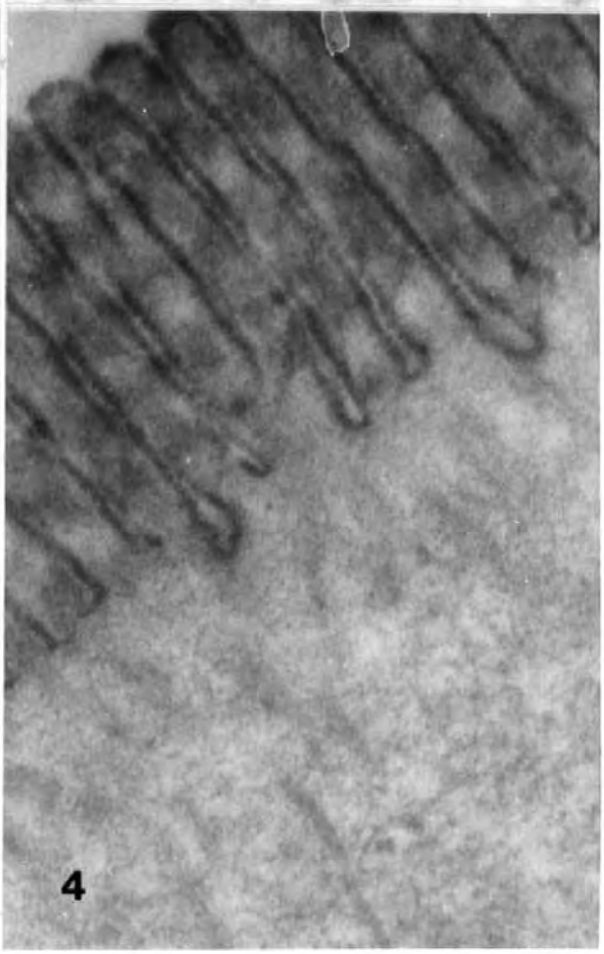

4

PLATE B HRP in the anterior epithelium 2 hour after intubation

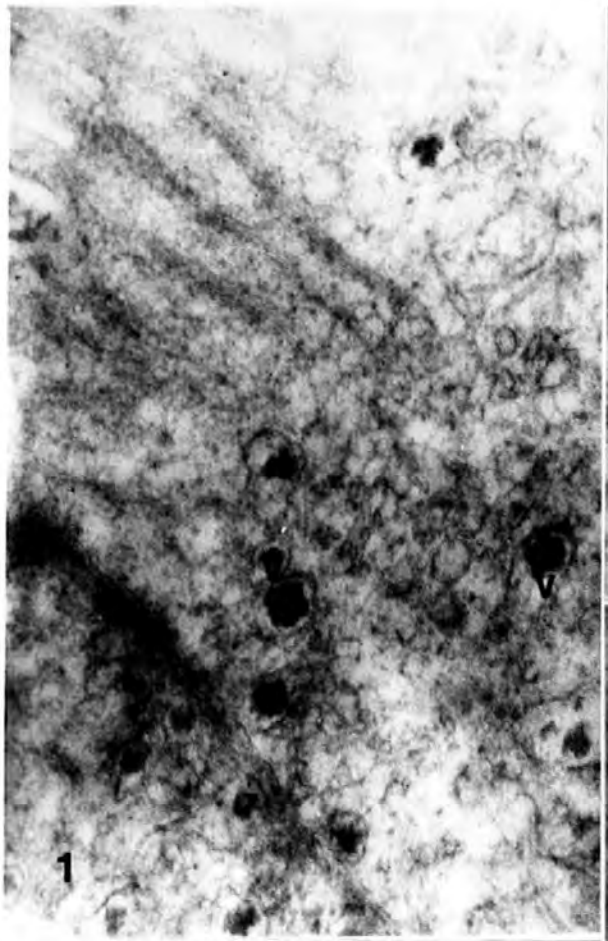
1 Small HRP-containing vesicles (V) in apical cytoplasm of the anterior intestinal enterocytes. mag. x45,000

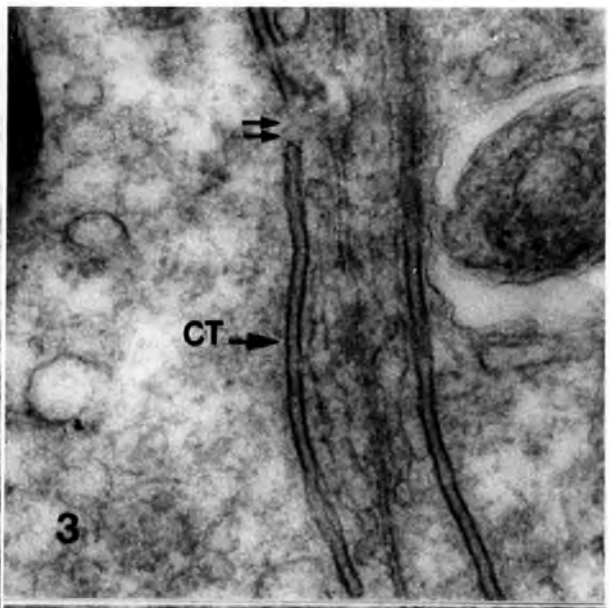
2 Distribution of HRP vesicles in the anterior enterocytes after 2 hours; TJ- tight junction, CT- cytoplasmic tubules. mag. x13,000

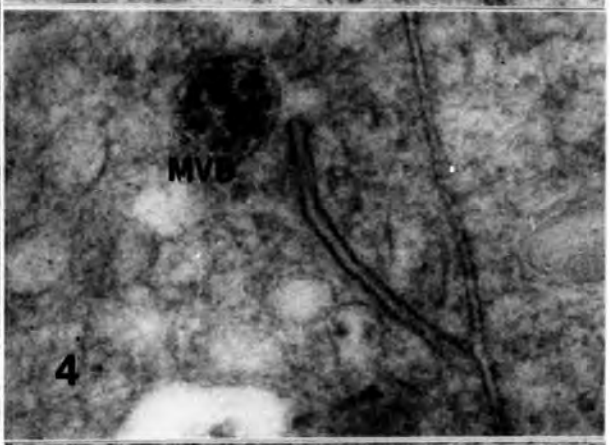
3 HRP coated cytoplasmic tubules (CT) showing fenestrations (double arrow). mag. x45,000

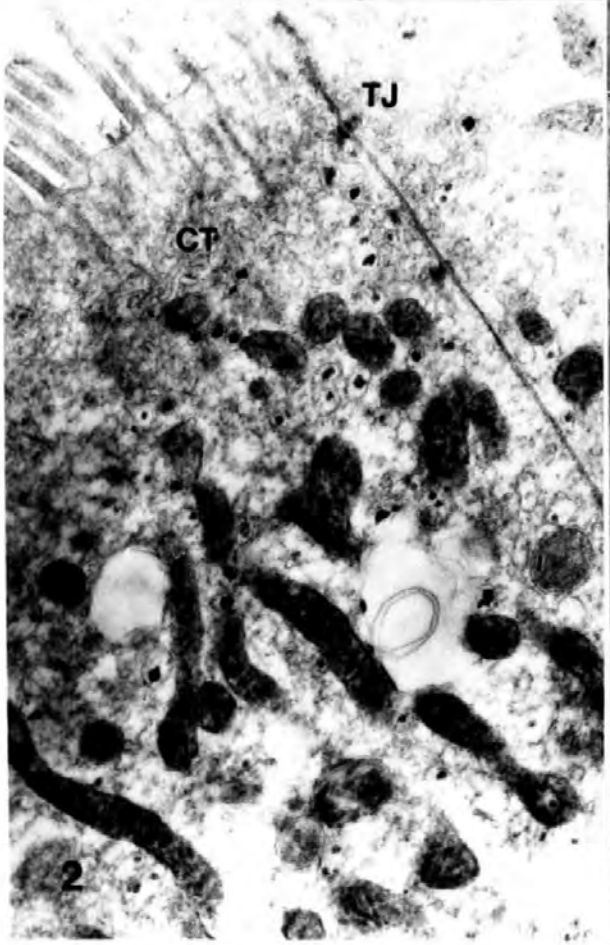
4 Small multivesicular body (MVB) containing HRP in close association with a cytoplasmic tubule. mag. x45,000

5 Macrophage in epithelium containing HRP. mag. x6800


V
1


CT
3


MVB
4


TJ
CT
2

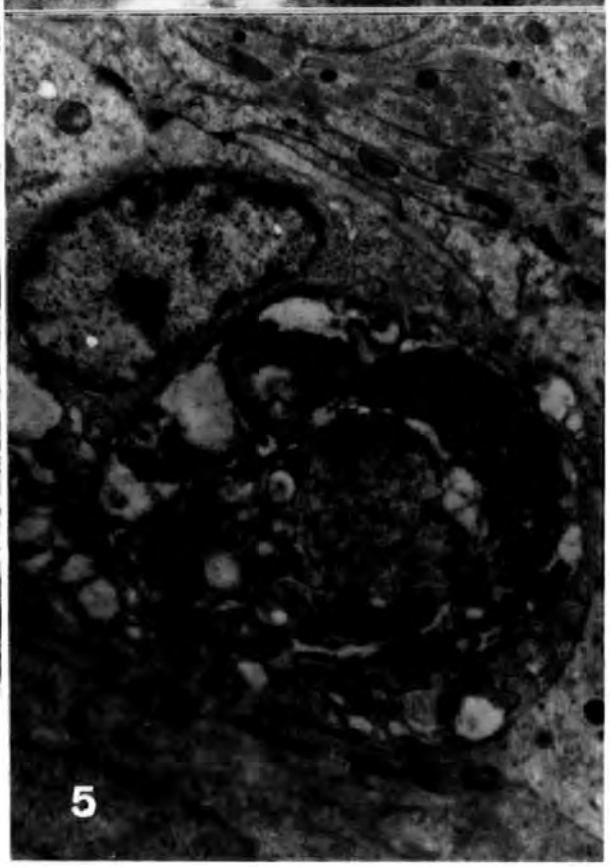

5

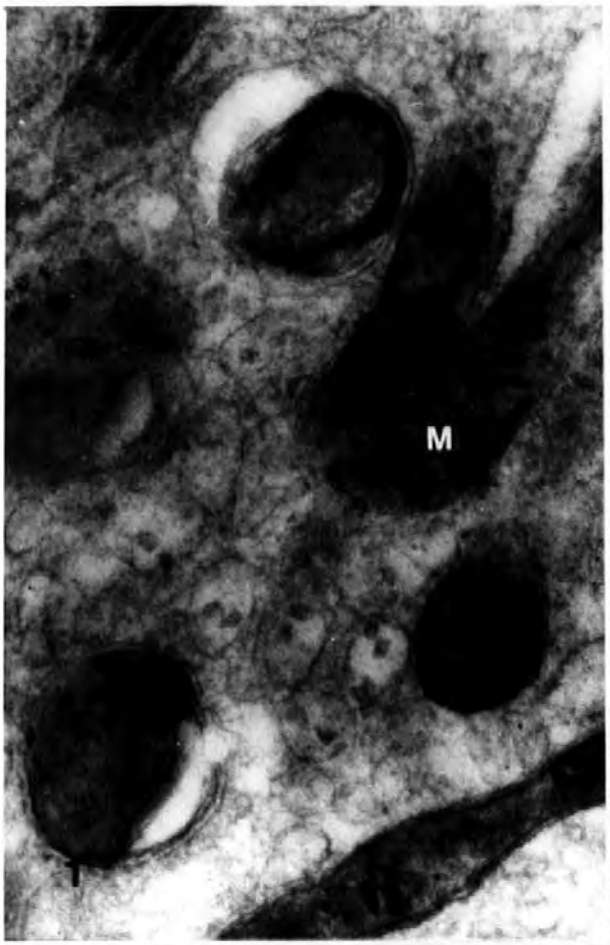
PLATE C HRP within enterocytes and blood granulocyte type 1

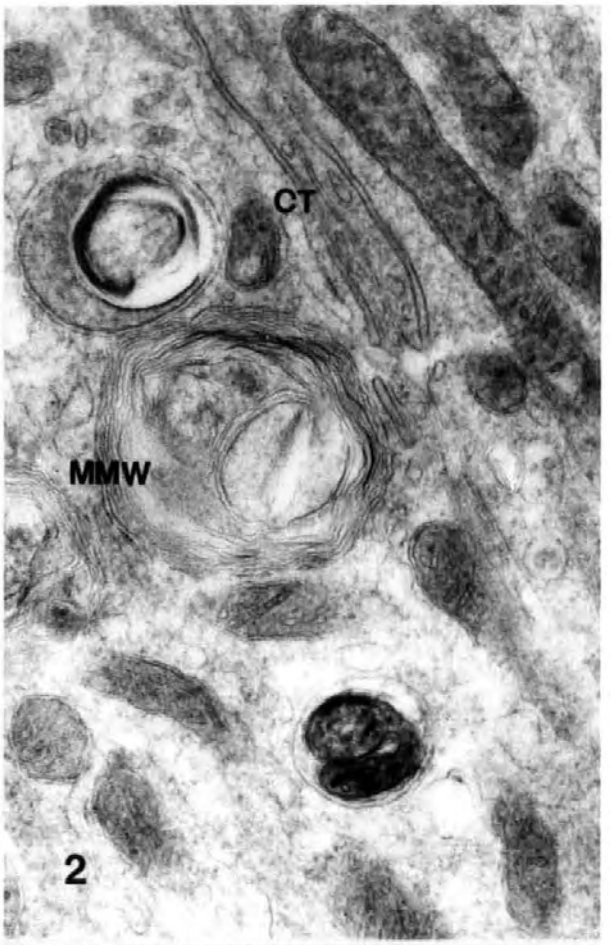
1 Membraneous whorls (MW) coated with HRP; M- mitochondria. mag. x 36,000

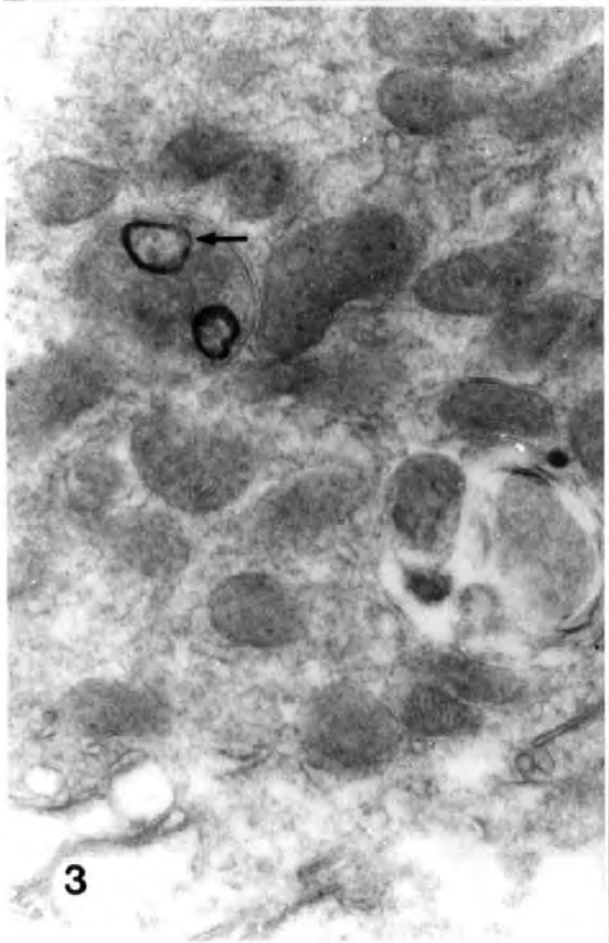
2 Multi-membraneous whorls (MMW) in epithelial enterocyte; CT- cytoplasmic tubule. mag. x22,000

3 MMW containing two HRP coated vesicles, arrowed. mag. x22,000

4 Type 1 blood granulocyte in lamina propria; Gr- peroxidase positive granule (endogenous activity), arrow- HRP containing phagosome. mag. x22,000


M
1


CT
MMW
2


3

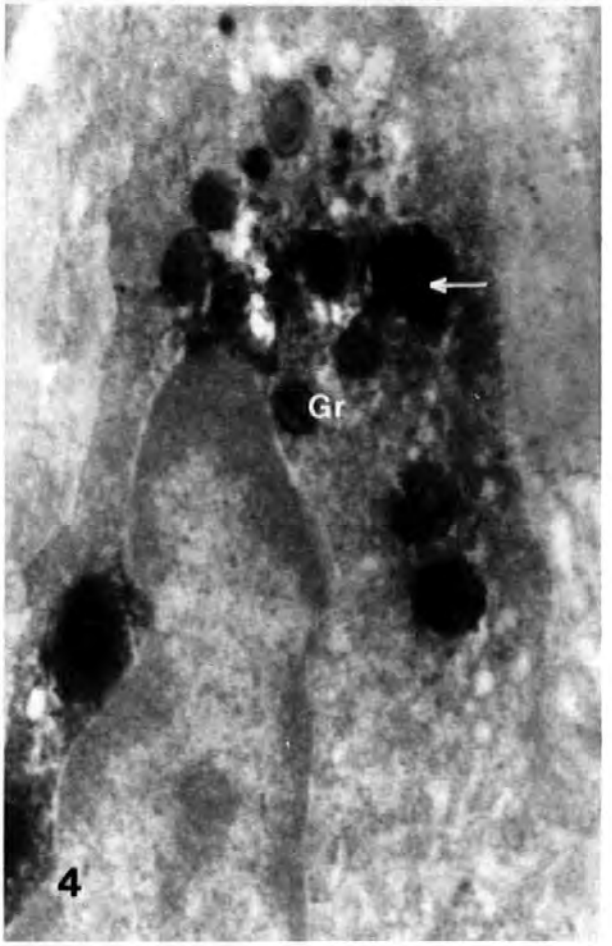

Gr
4

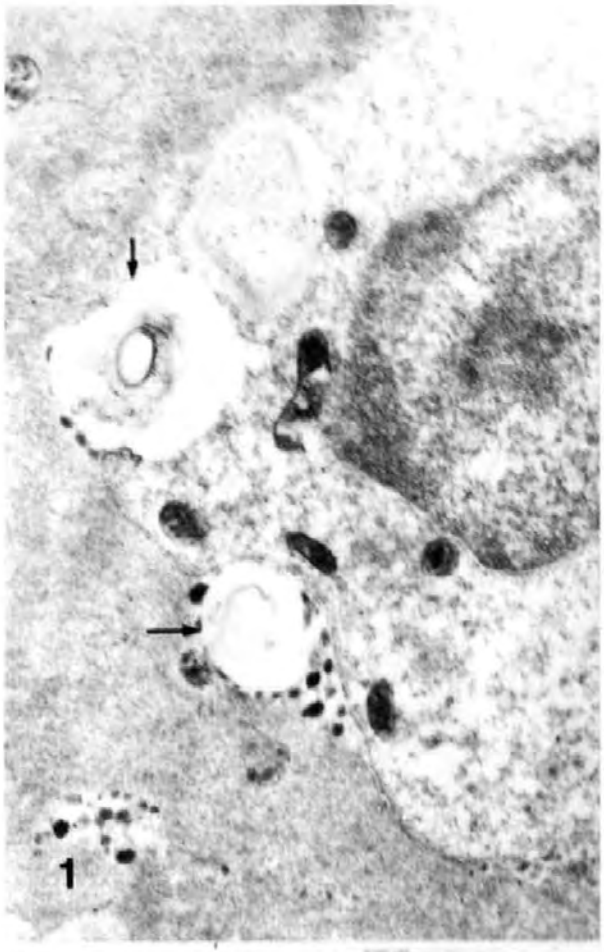
PLATE D Sixteen hours after intubation of HRP

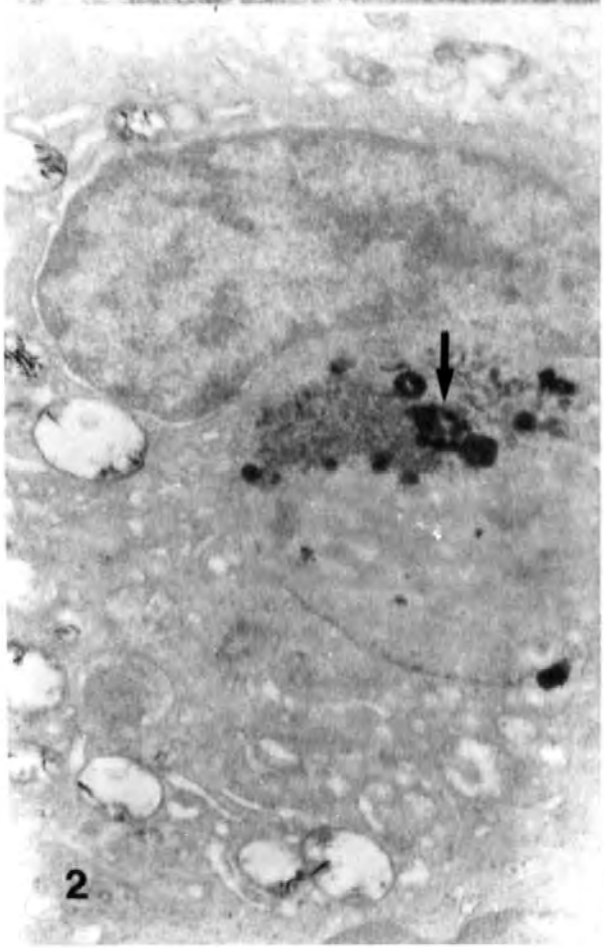
1 Macrophage between epithelial cells ingesting HRP in the intercellular space (arrowed). mag. x13,000

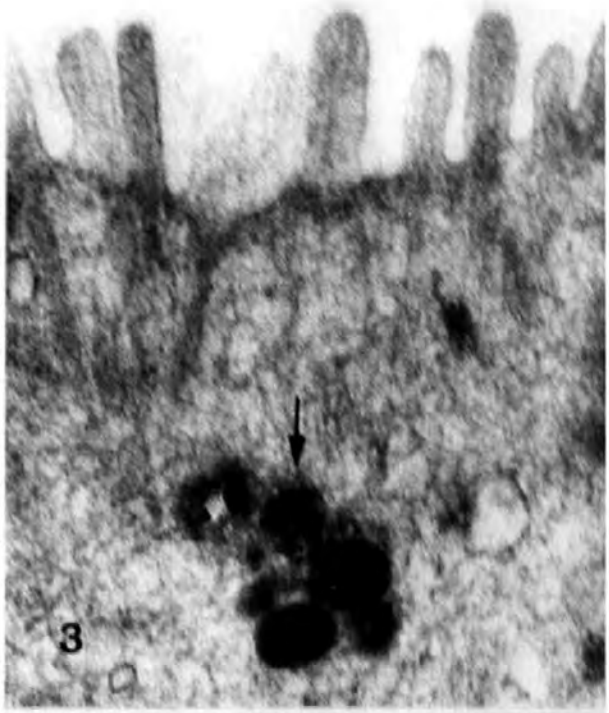
2 Macrophage containing small amounts of HRP, arrowed. mag. x7800

3 Posterior epithelial enterocytes with several small HRP vesicles (arrowed). mag. x11,000

4 Macrophage in the lamina propria containing small amounts of ingested HRP (arrowed). mag. x28,000


1


2


3

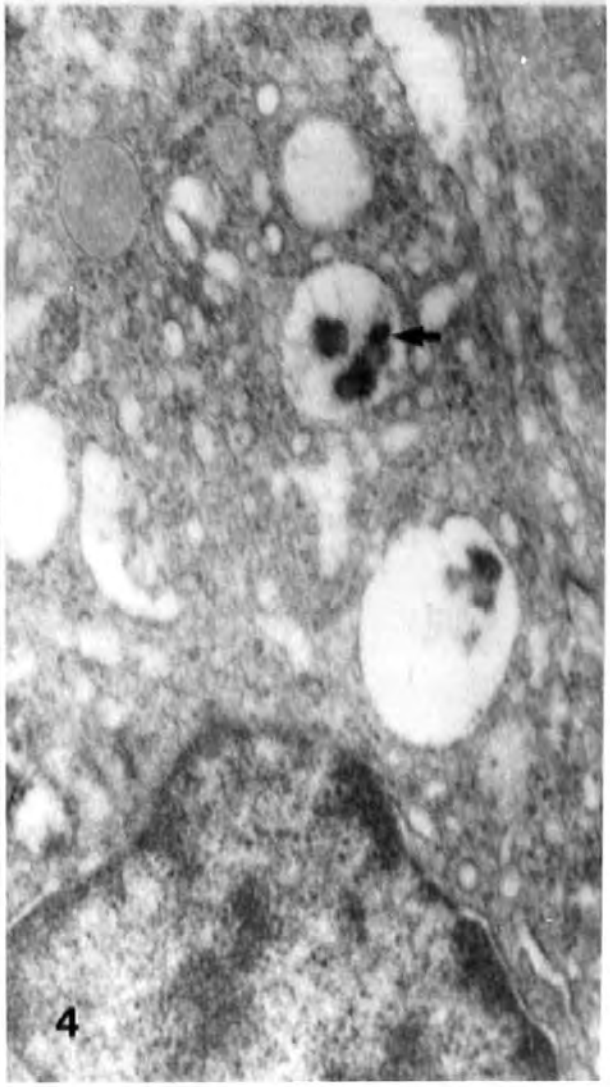

4

PLATE E Ferritin in the anterior intestine 30min. and 1 hour after intubation

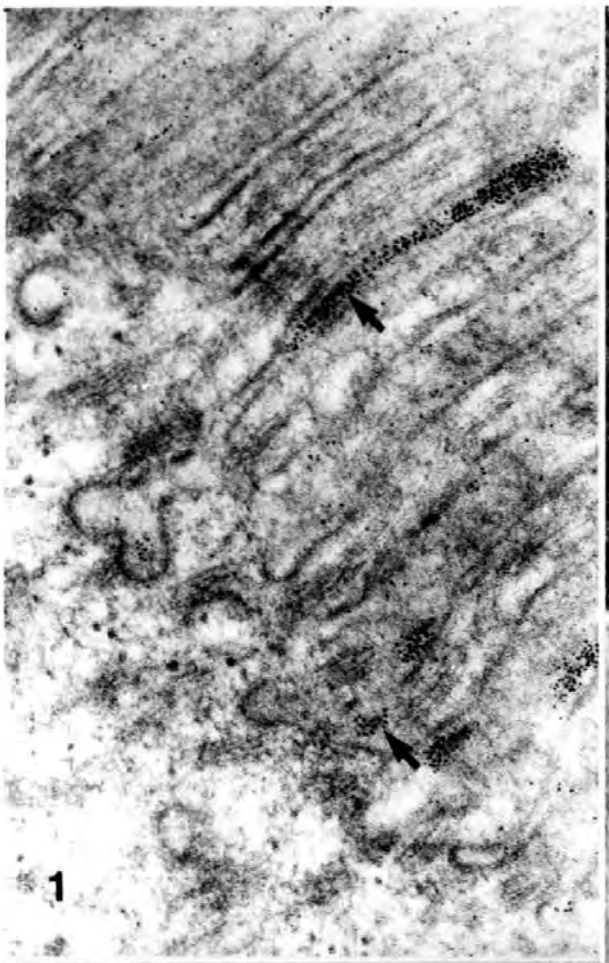
1 Ferritin particles (arrowed) between the microvilli 30 min. after intubation. mag. x56,000

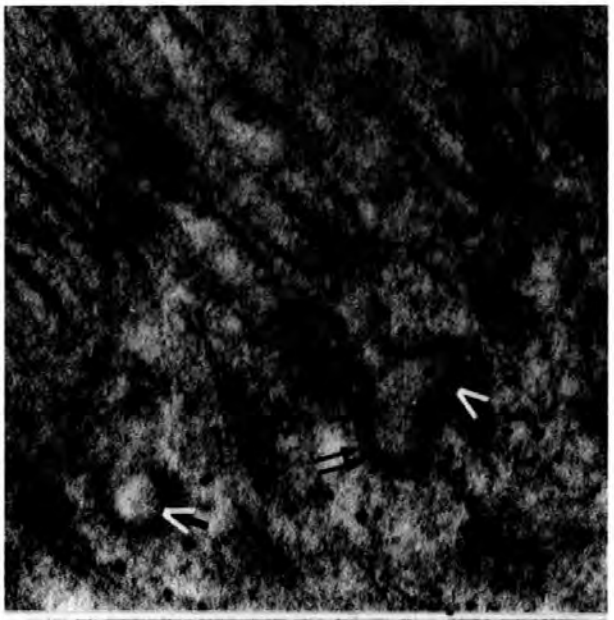
2 Ferritin in small vesicles and at the base of invaginations (thick arrow); double arrow- clathrin coat. mag. x68,000

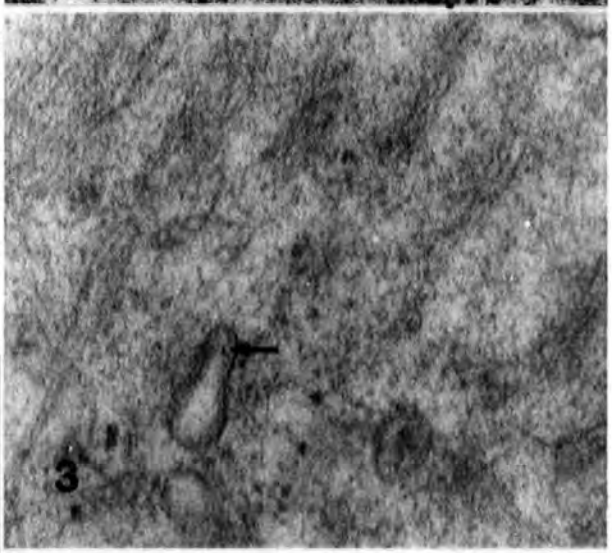
3 Ferritin in a small clathrin- coated vesicle (arrowed). mag. x68,000

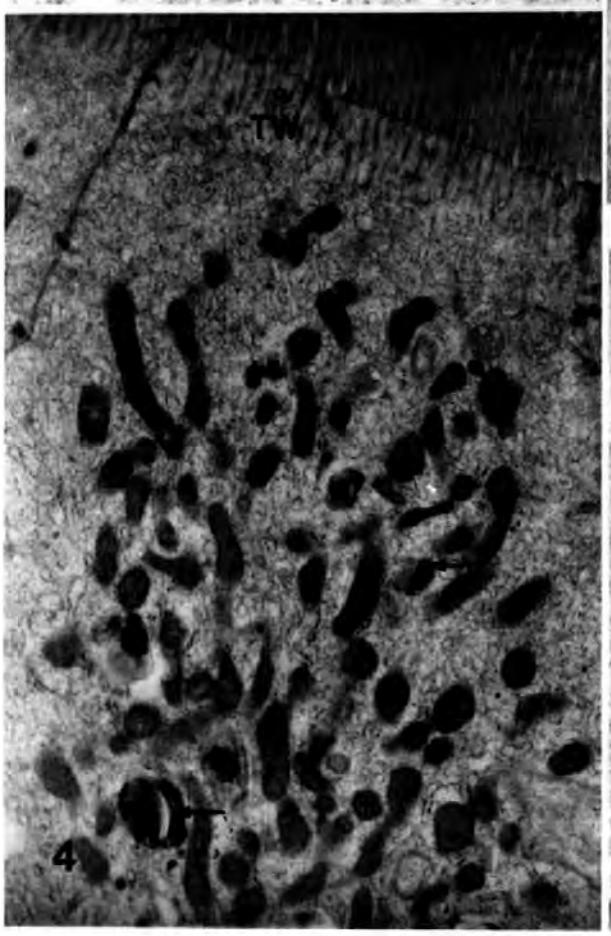
4 Apical cytoplasm of enterocyte showing ferritin in larger vesicles; TW- terminal web, MV- microvilli. mag. x21,000

5 Ferritin (F) in large vesicles, 1 hour after intubation. mag. x21,000


1




3


TW
4

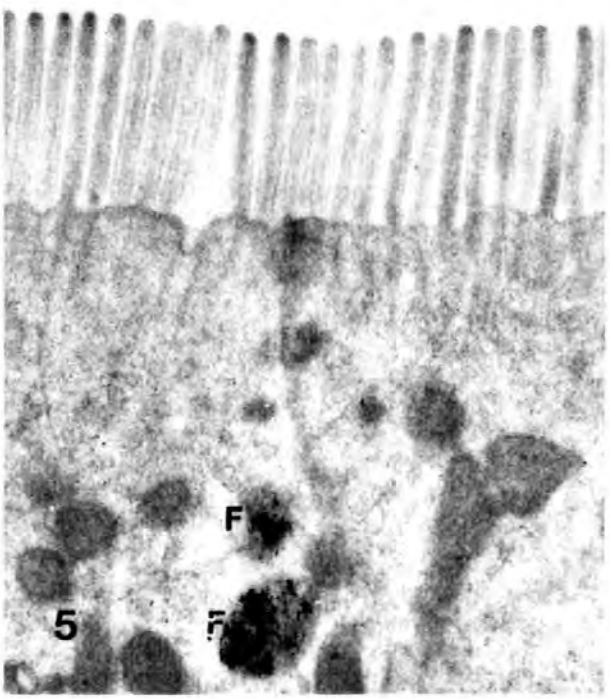

F
5

PLATE F Ferritin in the anterior intestine 1 hour, 4 hours and 16 hours after intubation

1 Membrane bound vesicle containing ferritin (F), 1 hour after intubation. mag. x36,000

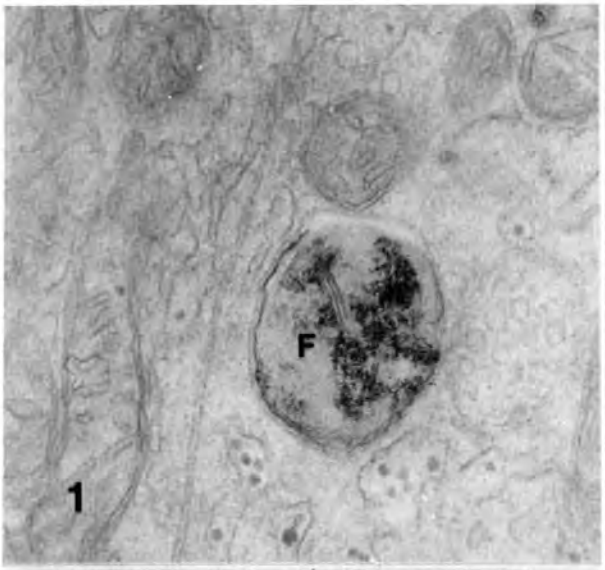
2 Multimembraneous vesicle (MMV) containing ferritin (F); ly- lysosome. mag. x27,000

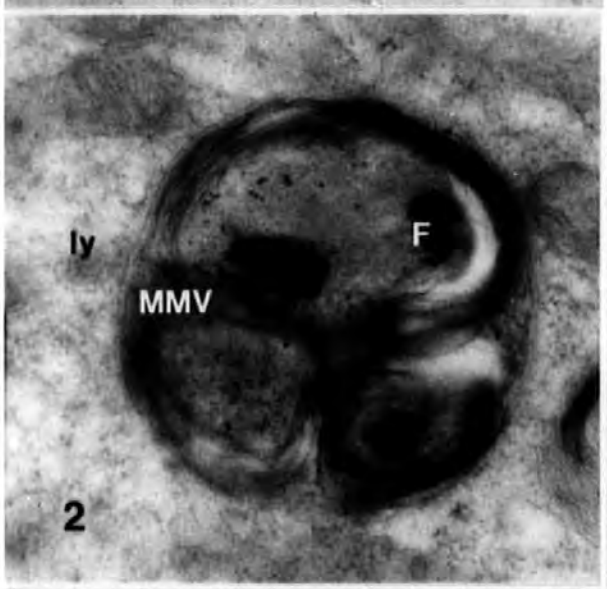
3 Multivesicular structure containing a small amount of ferritin, arrowed. mag. x80,000

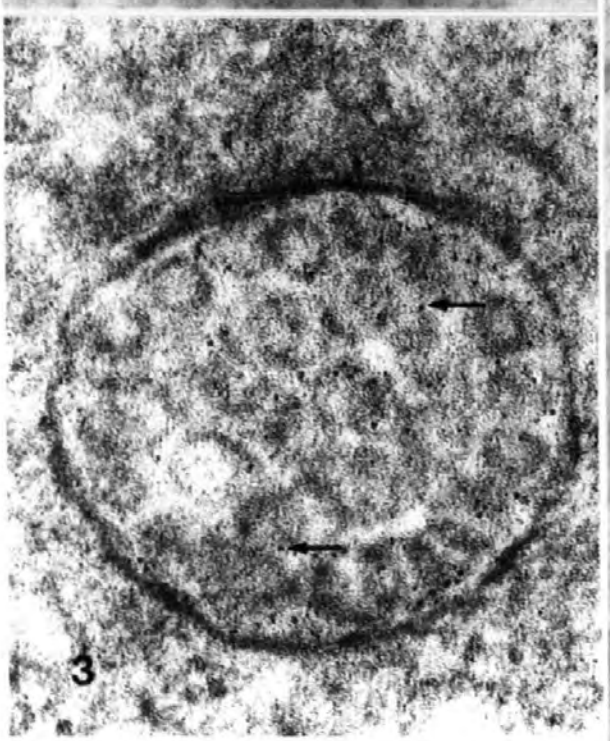
4 Numerous ferritin containing MMV, 4 hours after intubation; M- mitochondria. mag. x28,000

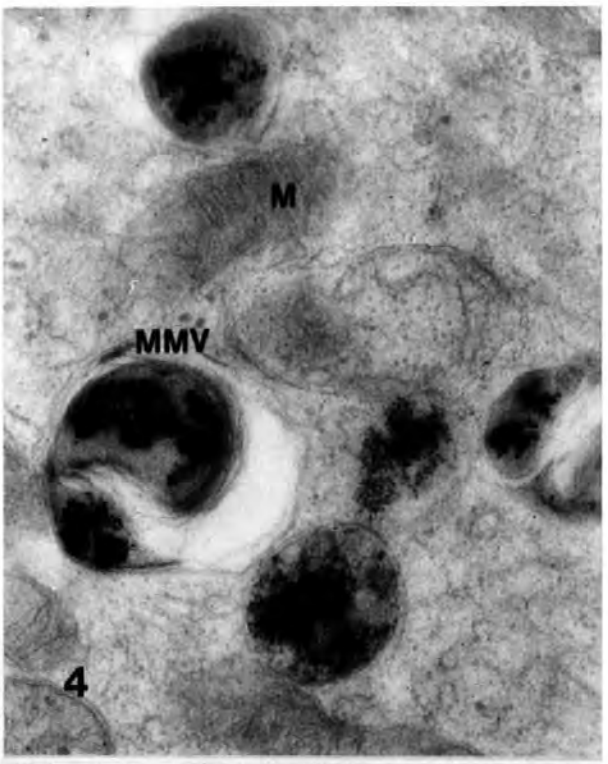
5 Ferritin containing vesicles in enterocyte (arrowed), 16 hours after intubation. mag. x11,000

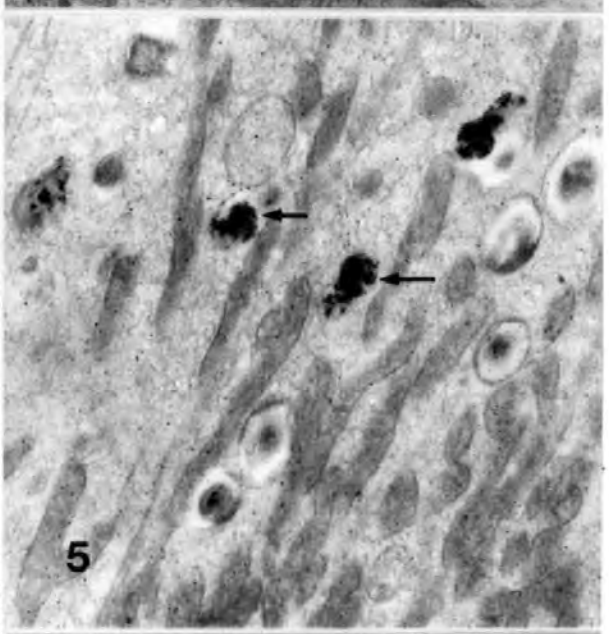
6 Vesicle containing ferritin, arrowed. mag. x22,000


F
1


ly
F
MMV
2


3


M
MMV
4


5

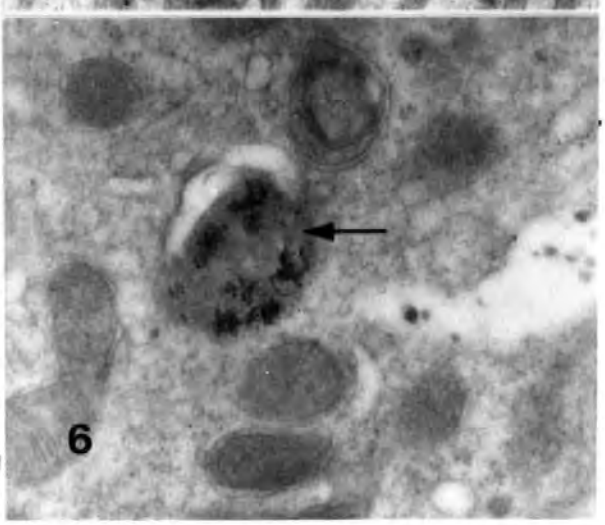

6

PLATE G Intestinal macrophages containing ferritin

1 Large macrophage containing ferritin (F) within large phagosome (arrowed) close to microvilli (MV), 4 hours after intubation. mag. x6800

2 Macrophage at microvillous (MV) surface; G- golgi body, F- ferritin. mag. x6300

3 Macrophage containing ferritin (double arrow) in the lamina propria; single arrow- multimembraneous structures. mag. x7800

4 Macrophage (M) in close association with an epithelial lymphocyte (L), 16 hours after intubation. mag. x6800

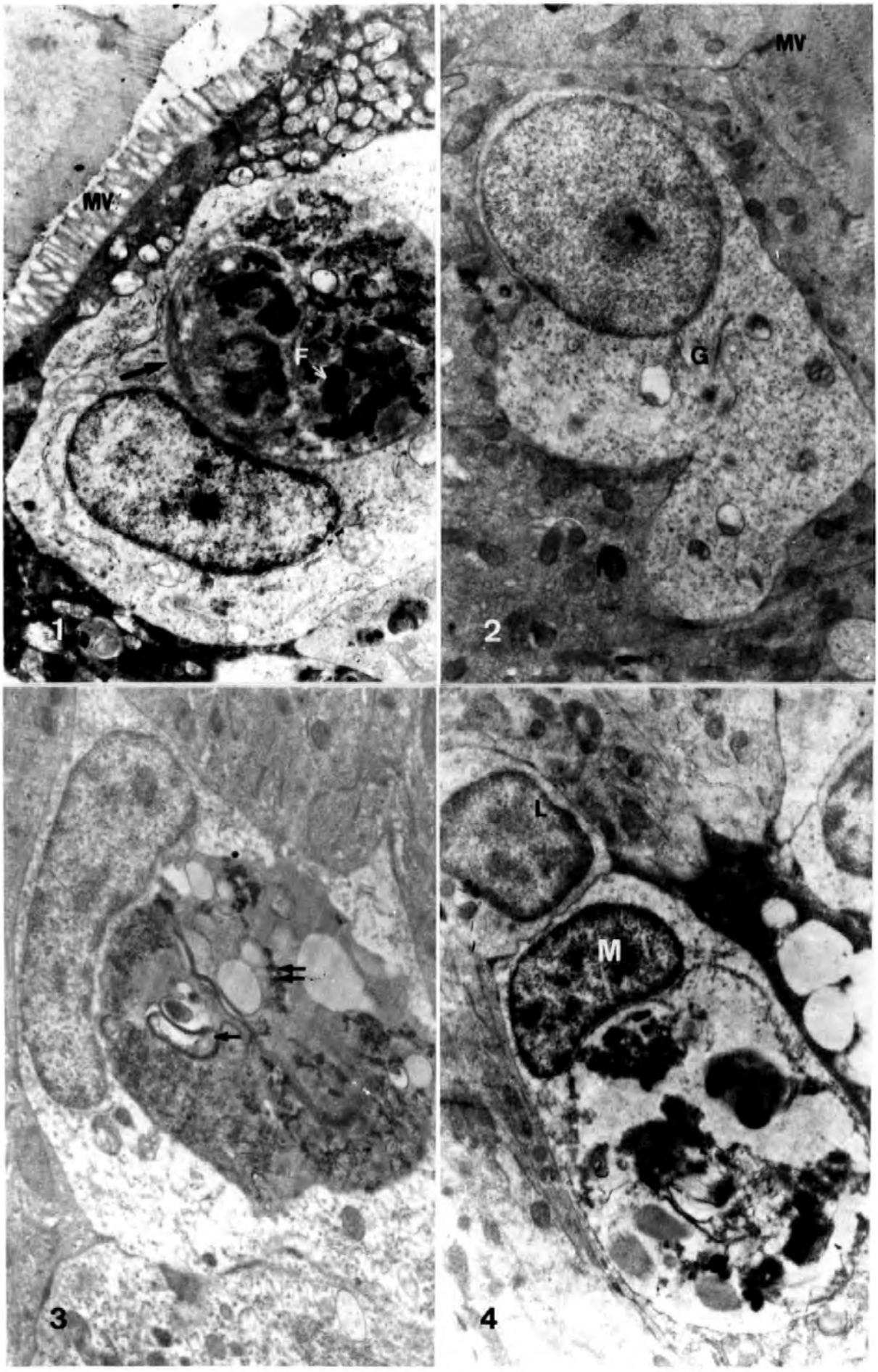

MV
F
1
MV
G
2
3
L
M
4

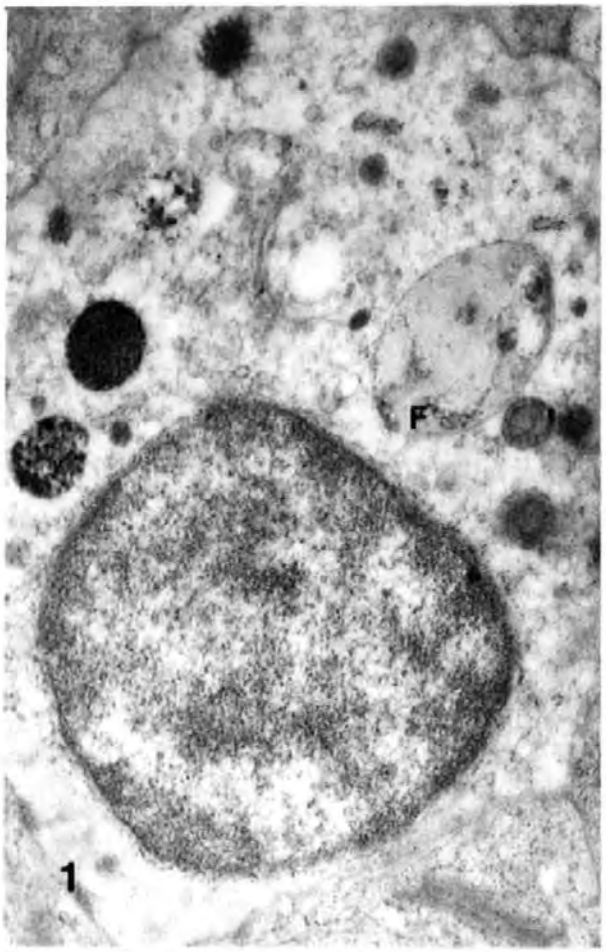
PLATE H Absorption of ferritin 16 and 24 hours after intubatiion

1 Macrophage containing ferritin (F) after 16 hours. mag. x6800

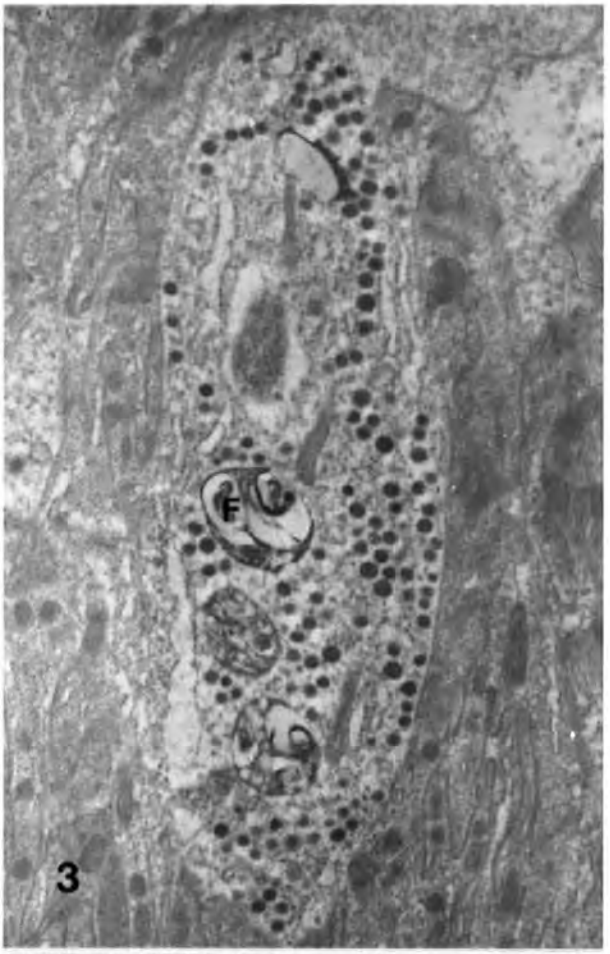
2 Macrophage in the lamina propria containing ferritin (F) . mag. x13,000

3 Enterosecretory cell in the epithelium containing ferritin (F). mag. x11,000

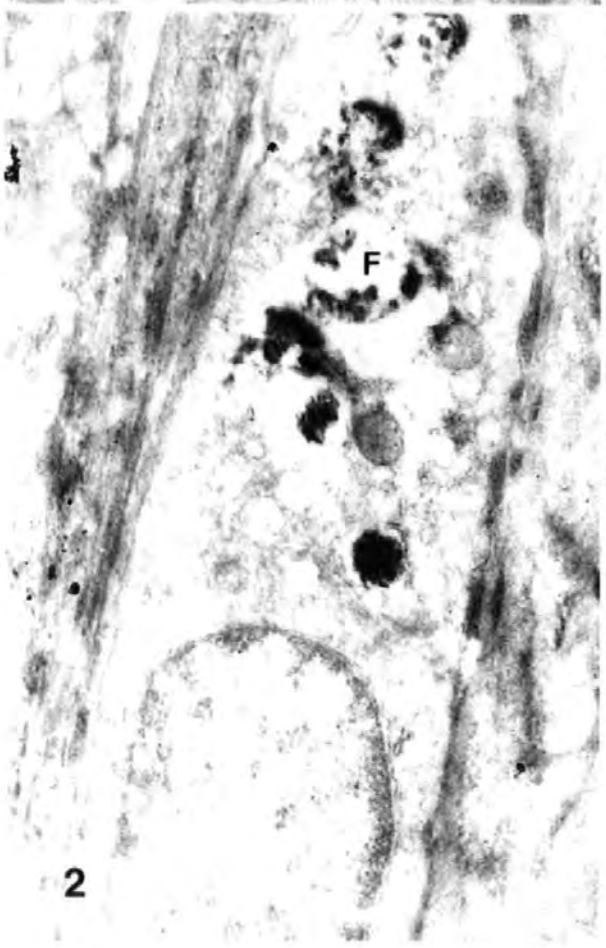
4 Ferritin (F) in vesicles in an anterior intestinal enterocyte 24 hours after intubation; LL- large lymphocyte. mag. x6800



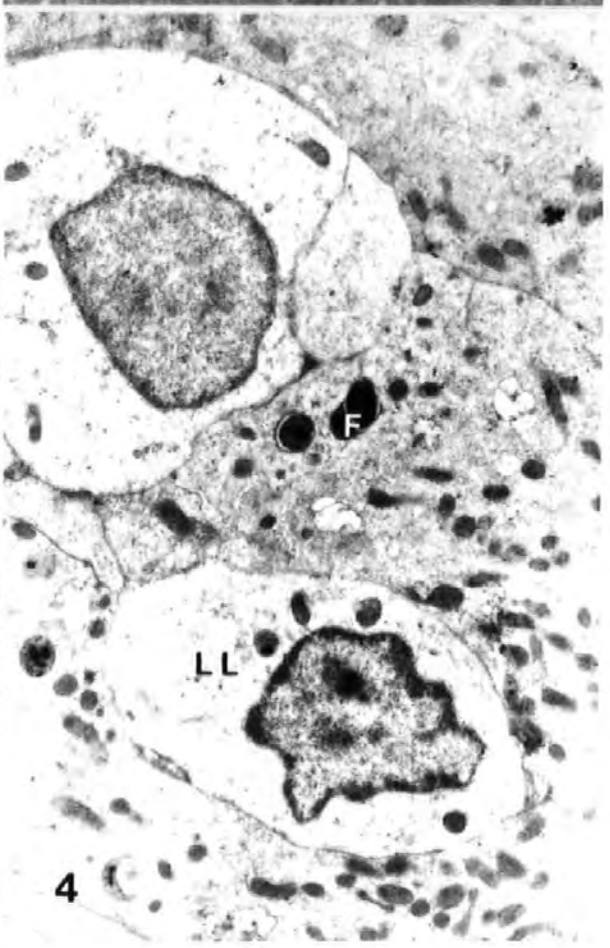
1



3



2



4

CHAPTER 6

6. INTESTINAL ABSORPTION AND TRANSPORT OF FERRITIN AND HORSERADISH PEROXIDASE INTO THE SYSTEMIC CIRCULATION

6.1 Introduction

The ability of mammmalian enterocytes to absorb intact macromolecules has been widely reported (Abrahamson & Rodewald, 1981; Walker, 1973; 1976). Protein fragments arising from intracellular degradation as well as intact proteins (Telemo, Westrom & Karlson, 1982) which are potentially antigenic may pass from the intestinal lumen into the lymph and the blood (Walker, 1981). According to Jeurissen, David & Sminia (1985) particulate proteins pass through the specialised M-cells and stimulate a local IgA immune response. This is in contrast to soluble proteins which pass through the absorptive cells and initiate a systemic IgG and IgM response.

In fish the epithelial cells of the posterior intestine in both young and adult animals have been shown to take up macromolecules (Georgopoulou *et al.*, 1986; 1988; Watanabe, 1981; 1982; 1984a; 1984b; 1984c). McLean & Ash (1986; 1987) have shown that large quantities of the protein marker HRP is absorbed by the intestinal epithelial cells and transported into the systemic circulation. In some instances the absorptive cells of the posterior intestine, particularly of young and adult trout, have been shown to be structurally and functionally comparable to those of the suckling rat which are particularly permeable to

macromolecules (Georgopoulou & Vernier, 1986).

A number of hypotheses have been proposed in an attempt to explain the physiological significance of protein absorption in fish; (i) that the non-selective uptake of intact proteins may be an extension of the normal digestive capacity of the fish gut (Ezeasor & Stokoe, 1981, Georgopoulou *et al.*, 1986), (ii) that this mechanism provides a standby facility during periods of food deprivation (Stroband & van der Veen, 1981), (iii) that this phenomenon may be linked to the provision for the absorption and recirculation of digestive enzymes (Beynon & Kay, 1976; Xu, 1985).

In the previous chapter it was demonstrated, by microscopy, that both HRP and ferritin were absorbed by the anterior and posterior intestinal enterocytes of *O.mossambicus*. Here an attempt to quantify the absorption and transport of these macromolecules across the intestinal epithelium into the systemic circulation and tissues has been carried out.

The absorption of HRP across the intestinal epithelium into the systemic circulation has been quantified in rainbow trout and carp by McLean & Ash (1986; 1987). In this study the absorption of HRP in *O.mossambicus* was monitored and compared with these earlier investigations. Ferritin has been used in several studies to investigate the uptake of macromolecules by fish intestinal enterocytes (Rombout *et al.*, 1985; Schindler & de Vries, 1988). Although these ultrastructural investigations have shown that absorption of

this macromolecule occurs, quantification of ferritin levels in the systemic circulation has not been reported. In this study an attempt has been made to quantify the ferritin levels in the systemic circulation in *O.mossambicus*, together with a comparison of the absorption of ferritin in two other fish species, rainbow trout and carp and allow comparison of the absorption of these two distinct macromolecules in the three species to be made.

6.2 Materials and Methods

6.2.1 Fish

a) Juvenile *O.mossambicus*, 10-15g, approximately 4 months old, were bred at Plymouth Polytechnic from brood stock obtained from Swansea University. Fish were kept in a freshwater recirculating system at a temperature of 24-26°C.

b) Juvenile *Salmo gairdneri* R., 10-15g, were obtained from Exe Valley Trout Farm. They were maintained in a freshwater flow-through system at a temperature of 11-13°C.

c) Juvenile *Cyprinus carpio* L., 8-12g, were obtained from Munton Fisons, Stowmarket. They were kept in a freshwater recirculating system at a temperature of 15-18°C.

6.2.2 Administration of Macromolecules

Prior to intubation all fish were deprived of food for 48 hours. The macromolecules were intubated either orally or

or anally into the intestine using a polyethylene tube (diameter 1.6mm) attached to a 23g needle and 1ml. syringe.

a) Ferritin

Ferritin was intubated either orally, or anally into the intestine of the three fish species. 1mg of ferritin in 0.85% sterile saline was administered to each fish, as used in the ultrastructural investigation. Blood samples were collected at 15, 30, 60, 90 180 and 360 min. after intubation. Three fish were sampled at each sample time and the plasma samples pooled (see Table 1).

Three control fish, intubated either orally or anally with 0.85% sterile saline, were sampled at the same time.

b) HRP

i) Dose Response- To establish a suitable HRP dose, the dose reponse was investigated using 2.5, 5.0, 10, 12.5 and 15 mg of HRP per fish. Five fish (*O.mossambicus*) were sampled for each dose 45min. after oral intubation of HRP. Plasma, liver, kidney and spleen samples respectively, were pooled for each dose and prepared as in Section 6.2.3b.

Three control fish were orally intubated with 0.85% sterile saline and were similarly sampled at 45min.

ii) Oral Intubation- Having established from (i) a suitable HRP dose (10mg HRP/fish) seventy-two *O.mossambicus* were orally intubated with 10mg HRP/fish in 0.85% sterile

saline, and divided into three groups of 24 fish (Table 1) which received the following:

Group 1- one dose of HRP

Group 2- two doses at two week intervals

Group 3- three doses at two week intervals

In each group samples of plasma, liver, kidney and spleen were taken from three fish at each of the eight time points. The samples from the three fish were subsequently pooled and evaluated by ELISA for their HRP content.

iii) Anal Intubation- A similar dose of 10mg HRP per fish was anally intubated into the posterior intestine of 24 *O.mossambicus*. Three fish were sampled at the same sample times as after oral intubation and the plasma, liver, kidney and spleen tested for the presence of HRP. Tissue samples from three control fish were prepared and tested with the experimental samples for peroxidase activity.

6.2.3 Sample Preparation

a) Rocket Immunoelectrophoresis (RIE)

For the detection of ferritin blood samples were collected in heparinised capillary tubes after severing the caudal sinus. The samples were kept overnight at 4°C and the plasma removed. Plasma samples were tested either undiluted or diluted in barbitone buffer, pH 8.2 (Table 2).

b) ELISA

Plasma was collected as above and tested in a series of

Table 1 Protocol for administration of Ferritin and HRP

	Antigen	Antigen dose	Number of doses	Route of administration	Tissue sample	Sample time (min)
O.mossambicus						
	ferritin	1 mg/fish	1	oral	plasma	15,30,60 90,180,360
	"	"	1	anal	"	"
Rainbow trout						
	ferritin	1 mg/fish	1	oral	plasma	"
	"	"	1	anal	"	"
Common carp						
	ferritin	1 mg/fish	1	oral	plasma	"
	"	"	1	anal	"	"
O.mossambicus						
	HRP	10 mg/fish	1	oral	plasma,liver kidney,spleen	15,30,45 60,75,90 180,360
	"	"	2	"	"	"
	"	"	3	"	"	"
	"	"	1	anal	"	"

doubling dilutions in PBS/Tween, pH 7.4 (Table 2). Tissue samples of liver, kidney and spleen were removed and weighed. PBS/Tween was added to each tissue sample to give a final concentration of 100mg tissue/ml. buffer. Tissue was disrupted using a sonicator, 4 x 6sec. bursts, to release the HRP from within the tissues. Tissue preparations were tested using a series of doubling dilutions in PBS/Tween.

6.2.4 Rocket Immunoelectrophoresis (RIE)

This technique was adapted from that described in Hudson & Hay (1980), and was used to detect ferritin. 1% (w/v) agarose in 0.08M barbitone buffer pH 8.2 (Table 2), was dispensed into 6ml. aliquots in small glass bijoux and kept molten at 50°C until required.

To a 6ml. volume of molten agarose 20µl. of anti-horse spleen ferritin (Sigma,U.K.) was added and mixed. This was then carefully poured onto clean, grease- free glass plates (8 x 8cm.), giving a layer approximately 2mm. thick. Once the gel had set a line of 12 wells, 2mm. in diameter, were punched in the gel 1cm. from one edge (Fig. 1) To the wells a 10ul. volume of either a ferritin standard (10 - 100µg ferritin/ml.) or a test sample was added using a capillary tube. The prepared plates were then placed in an electrophoresis tank with the wells nearest the cathode and run at a constant current of 30mA for 2 hours.

Plates were then washed for 45 minutes in 0.85% saline to remove excess protein. They were then removed from the wash bath and covered with wet filter paper, a layer of tissue

Table 2. Buffers required for RIE and ELISA

	Quantity
0.08M Barbitone Buffer pH 8.2	
a) Sodium barbitone	12g
b) Diethylbarburic acid	4.4g
dissolve (a) in 800 ml. distilled water and (b) in 150 ml. dis. water at 95°C. Mix (a) and (b) and pH to 8.2	
Phosphate Buffered Saline (PBS) pH 7.4	
Sodium chloride	8g
Disodium hydrogen orthophosphate Na_2HPO_4	2.9g
Potassium dihydrogen orthophosphate KH_2PO_4	0.2g
Potassium chloride	0.2g
make up to 1 litre in dis. water and pH	
Bicarbonate/Carbonate Buffer pH 9.6	
Disodium carbonate Na_2CO_3	1.59g
Sodium hydrogen carbonate $NaHCO_3$	2.93g
make up to 1 litre in dis. water and pH	
Citratre/Phosphate Buffer pH 5.0	
Citric acid	4.67g
Disodium hydrogen phosphate.$2H_2O$ $Na_2HPO_4.2H_2O$	7.29g
make up to 1 litre in dis. water and pH	
0.1% Tween/PBS	
Tween 20	1 ml
make 0.1% Tween v/v in PBS pH 7.4	

paper and a heavy weight, and left for 45 min. to remove excess moisture from the gels.

To enhance the visualisation of rockets, plates were stained in Coomassie brilliant blue solution; 0.2g Coomassie brilliant blue (R 250) dissolved in 1 litre of buffer containing 40% (v/v) methanol, 10% (v/v) glacial acetic acid and 50% (v/v) distilled water. Plates were destained in 10% (v/v) methanol, 10% (v/v) glacial acetic acid and 80% (v/v) distilled water (Figure 1). The height of the rockets for the standards were measured and used to construct a calibration curve whereby the height of a sample rocket could be converted to quantify the amount of ferritin in the sample, and divided by the number of fish in the sample.

6.2.5 Enzyme Linked Immunosorbent Assay (ELISA)

The technique used to detect HRP was first described by Ambler & Peters (1984). The buffers required are detailed in Table 2 and the procedure in Figure 2.

To all the wells in a 96 well flat-bottomed ELISA plate (L.I.P., Yorks), a 100µl. of a 1/1000 dilution of goat anti-HRP (Sigma, UK) in bicarbonate/carbonate buffer (pH 9.6) was added. The plate was then incubated for 2 hours at room temperature in a humid container to allow antibody adsorption to the well surface. The plate was then washed three times with phosphate buffered saline, pH 7.4, and the plate shaken dry.

To the first column a series of HRP standards (0.1 - 10µg HRP/ml) in PBS/Tween buffer (pH 7.4) was added to give a

final volume of 100µl., plus one well containing only PBS/Tween (blank). To the other wells the test samples were added in a series of doubling dilutions (in duplicate). The plate was then incubated at room temperature for 2 hours in a humid container, washed three times with PBS/Tween and shaken dry.

Bound HRP was detected using o- phenylenediamine, OPD (Sigma, UK) as the chromagen. 20mg. OPD was added to 50ml. of citrate/phosphate buffer (pH 5.0) and to this was added 20 µl of H_2O_2. 100ul. of this substrate solution was added to each well and the colour allowed to develop (5-15 min.). The reaction was stopped by adding 50ul. of 1M H_2SO_4, and the results read against the control blank at 490nm using a Dynatech ELISA MR590 Minireader (Dynatech, UK). A mean value was calculated and divided by the number of fish in the sample. An example of the reaction is shown in Figure 3.

6.2.6 Regurgitation of Intubated Material

An investigation into the possible regurgitation or expulsion of intubated HRP was carried out. This macromolecule was chosen due to the sensitivity of the detection method. The protocol for this investigation is presented in Table 3. *O.mossambicus*, both starved and fed, were intubated either orally or anally with 10mg HRP/fish and placed in individual small holding tanks. Tank water was sampled prior to the addition of the fish. Fish were sampled at 15min. and 60min. after intubation together with a water sample from each tank.

Table 3 Appearance of HRP in tank water following intubation

State of nutrition	Route of administration	Sample time (min)	HRP levels detected in plasma (ug/ml)	HRP levels detected in water after intubation	
				ug HRP per tank volume	% of HRP intubated
starved 1	oral	15	188.3	147.88	1.48
" 2	"	"	0.078	296.96	2.97
" 3	"	"	0.13	332.8	3.32
starved 1	oral	60	3165	7.12	0.07
" 2	"	"	0.174	268.49	2.69
starved 1	anal	15	1625	19.64	0.11
" 2	"	"	2905	11.84	0.12
starved 1	anal	60	1775	114.42	1.14
" 2	"	"	3170	229.45	2.30
fed 1	oral	15	32.5	168.96	1.69
" 2	"	"	0.036	18.56	0.19
fed 1	oral	60	0.201	23.53	0.24
" 2	"	"	0.108	41.28	0.41
fed 1	anal	15	3480	78.08	0.78
" 2	"	"	2985	5.36	0.05
fed 1	anal	60	2695	4.51	0.04
" 2	"	"	4390	13.7	0.14

Samples were tested using the ELISA technique described in Section 6.2.5.

6.2.7 Statistical Analysis

An analysis of variance was used to determine if there was a significant difference between the levels of ferritin detected in the serum of the three species, at a 0.05% probability level. A Microtab computer programme was used to calculate the ANOVA.

6.3 Results

6.3.1 Absorption of Ferritin

Ferritin appeared in the systemic circulation in the three species after both oral (Fig.4a-c) and anal (Fig.5a-c) intubation.

a) In *O.mossambicus* the greatest levels of ferritin were detected after anal intubation (Fig.5a), with a maximum level of 700ug/ml serum being detected at 90 min. Ferritin was also detected in the plasma after oral intubation (Fig.4a), in this instance a maximum level of 300ug/ml plasma was detected at 60 min.

b) In rainbow trout after oral intubation low levels of ferritin only were detected at the 15min sample time (Fig.4b). Greater levels of ferritin were detected in the systemic circulation after anal intubation (Fig.5b).

c) In carp, as in rainbow trout and *O.mossambicus*, overall greater levels of ferritin were detected in the

plasma after anal intubation (Fig.5c) than after oral intubation (Fig.4c).

Statistically there was no significant difference between the levels of ferritin in the plasma of the three species after oral intubation. After anal intubation there was a significantly higher level detected in the plasma of tilapia than in carp and rainbow trout plasma. There was however, no significant difference between the levels detected in the plasma of carp and rainbow trout.

6.3.2 Absorption of HRP

a) Dose Response (Fig.6a-d)

From the results of the dose response it was decided that a 10mg HRP dose gave acceptable detectable levels in the tissues and plasma and this was used in subsequent experiments.

b) Multiple Oral Intubation

HRP was detected in all four tissues tested after oral intubation with the highest HRP levels detected in the plasma followed by the liver, spleen and kidney. Endogenous peroxidase was detected in the control tissues but it was minimal. These control values were deducted from the levels detected in the test samples.

Plasma (Fig. 7a-c). Absorption appeared to be greatest after dose 1 (Fig.7a) with levels of 690 µgHRP/ml. plasma being detected. There was a marked decrease in HRP levels after the second dose (Fig.7b) with an increase in

detectable levels of HRP after the third dose (Fig.7c). This increase after dose 3 still gave values approximately three times less than was detected after dose 1.

Liver (Fig.8a-c). The absorption pattern in the liver was similar to that in the plasma, the greatest levels of HRP being detected after dose 1, then a decrease with dose 2 followed by an increase after dose 3. HRP levels detected in the liver after dose 3 were almost equivalent to those after dose 1.

Kidney (Fig.9a-c). Levels of HRP detected in the kidney were approximately half of those detected in the liver. The pattern of absorption after subsequent doses corresponded to those in the liver and plasma. HRP levels in the kidney after dose 3 were almost equivalent to those after dose 1.

Spleen (Fig.10a-c). Absorption of HRP into the splenic tissue was slightly different to that observed in the plasma and other tissues, with the greatest levels of HRP being detected after dose 2, these levels being a third greater than after dose 1. Levels of HRP after dose 2 were almost at the level found in the liver tissue.

c) Anal Intubation (Fig.11a-d) - Single administration

The levels of HRP detected in the tissues after anal intubation followed a similar pattern to that observed after oral intubation, that is, highest levels of HRP being detected in the plasma followed by the liver, spleen and kidney.

6.3.3 Regurgitation/Evacuation of Intubated HRP

This investigation demonstrated that HRP was regurgitated after oral intubation and evacuated from the posterior intestine after anal intubation (Table 3). However, the maximum levels of HRP detected in the tank water at the two time points did not exceed 3.2% of the total amount of HRP that was intubated into each fish.

There was no apparent difference in the amount of HRP found in the tank water after oral or anal intubation. The results did however, show the high degree of variability that occurs between fish.

6.4 Discussion

In Chapter 5 it was demonstrated that both ferritin and HRP were taken up by the intestinal enterocytes of *O.mossambicus*. A similar mode of absorption has been reported in other fish species including carp (Lamers, 1985) and rainbow trout (Georgopoulou *et al.*, 1984; 1986).

Ferritin has been used as a marker protein for macromolecule absorption in a variety of studies of both mammals (Casely- Smith, 1967) and fish (Georgopoulou *et al.*, 1984; Hart, 1987; Lamers, 1985, Rombout & van den Berg, 1985). These experiments have concentrated on the visualisation of this protein at the EM level and ferritin has been recognised within large macrophages in the epithelium (Rombout & van den Berg, 1985; Lamers, 1985). Similar large macrophages were also observed in the intestinal epithelium of *O.mossambicus* (see Chapter 5).

However, here it was demonstrated serologically that ferritin is transported across the intestinal epithelium into the systemic circulation in the three fish species examined.

Ferritin was detected in the systemic circulation of each of the three species after both oral and anal intubation. A peak level of 300µg/ml plasma was detected in the plasma of *O.mossambicus* after oral intubation. There would appear to be no significant difference between the ferritin levels detected in the plasma of the three species although there were differences in the peak levels of ferritin detected.

Following anal intubation of ferritin there were higher levels of this macromolecule in the systemic circulation of the three species than following oral intubation. Highest levels of ferritin were detected in the plasma of *O.mossambicus*, these levels being statistically significantly higher than levels detected in the serum of carp and rainbow trout. Although the peak levels of ferritin were 156µg/ml and 117µg/ml in carp and rainbow trout respectively, the overall levels were not significantly different.

The examination of HRP uptake involved the detection of HRP in the systemic circulation and its subsequent appearance in the liver, kidney and spleen. It is apparent from previous studies that HRP is taken up by the intestinal enterocytes of fish (Georgopoulou *et al.*, 1984; Lamers, 1985; Schindler & de Vries, 1988; Watanabe, 1982), and is rapidly transported across the epithelium into the systemic

circulation (McLean & Ash, 1986; 1987). This study sought to investigate the absorption of this macromolecule, HRP, and to compare its absorption by the intestinal enterocytes of *O.mossambicus* with the other quantitative absorption studies in other fish species.

In *O.mossambicus*, HRP, like ferritin, was detected in the systemic circulation after both oral and anal intubation, with peak levels being detected within the first 90 min. of intubation. This apparent 'bulk' absorption observed after both oral and anal intubation may, in part, be due to the starved state of the animal, where the presence of protein may stimulate uptake by the enterocytes after the period of food deprivation. This rapid transport of HRP into the systemic circulation is further reflected in the rapid appearance of HRP in the tissues after intubation.

The rapid appearance of HRP in the tissues is consistent with the clearance of antigens from the peripheral blood observed in other studies (Maas & Bootsma, 1982; Rijkers, 1980), although Georgopoulou *et al.* (1988) was apparently unable to detect HRP in the plasma until 7-8 hours after intubation. The spleen and the kidney are recognised as being sites of antigen 'trapping', however, the detection of HRP in the liver is an interesting feature. In the mammalian liver there are present highly phagocytic Kuppfer cells (Laskin, Sirak, Pilaro & Laskin, 1988), which are believed to deal with antigenic material that may be present in the hepatic portal circulation. The presence of such cells in the teleost liver is uncertain, however, similar cells have

been reported in the hagfish liver which are able to phagocytose latex particles (Tomonaga, Yamaguchi, Ihara & Awaya, 1986b). A histological examination of the liver of *O.mossambicus* was not carried out, but the ability of the teleost liver to take up, or accumulate, antigenic material has also been shown by Buras *et al.* (1985). The role of the liver is very interesting especially as the mammalian liver has been shown to be involved with the clearance of immune complexes from the systemic circulation (Walker & Bloch, 1983).

The effect of repeated oral doses on the uptake of HRP was also investigated in this study. After the second dose of HRP there was a dramatic decrease in the amount of HRP that could be detected in the plasma and in the liver and kidney samples. This was in contrast to the levels detected in the spleen where there was an increase in the levels of HRP. This may be due to the more effective clearance of the HRP from the systemic circulation by this tissue on secondary exposure, or possibly this tissue retains this macromolecule longer than the other tissues after the first dose. In support of the former hypothesis the more rapid clearance of the HRP by the spleen would be mirrored in the decreased levels found in the plasma and other tissue samples. Ellis (1980) indicated that antigenic material (BSA) was retained within the metaphil fibres of the ellipsoids in the plaice spleen in large quantities up to 37 days after administration.

After the third dose the HRP levels were found to have

increased. The amount of HRP detected in the plasma was approximately six times less than after the first dose, whereas HRP levels in the liver and kidney were found to be equivalent to those after the first dose with a corresponding decrease in the spleen. The antibody response to HRP during the repeated HRP exposures was not investigated, as the primary objective was to investigate the effect of repeated doses on the absorption of this macromolecule.

After anal intubation of HRP the plasma was found to contain the highest levels of HRP, followed by the liver, spleen and kidney. Plasma levels of HRP in anally intubated fish were approximately equivalent to those detected in orally intubated fish, whereas HRP levels in the liver and kidney were almost twice those detected after oral intubation. This would appear to indicate a more rapid clearance of HRP after anal intubation compared to oral intubation. This may reflect the highly absorptive capability of the posterior intestinal enterocytes as reported in other fish species (Lamers, 1985; Watanabe, 1981; 1982).

The levels of HRP detected in the plasma and tissues of tilapia following oral intubation are greater than those detected in carp and rainbow trout (McLean & Ash, 1986; 1987). The latter authors observed that there was a 1000 fold difference in the uptake between carp and rainbow trout, with uptake being greater in the carp. Similarly greater levels of ferritin were detected in the systemic

circulation of tilapia compared to rainbow trout and carp, however, there was no significant difference between the levels detected in the plasma of rainbow trout and carp. This may be a function of the size of the ferritin macromolecule or that the intestinal enterocytes of the older fish used by McLean & Ash (1986; 1987) may be selectively permeable, compared to the enterocytes of the young fish used in this study as observed in mammalian absorption studies (Walker, 1977).

The regurgitation of orally intubated material or expulsion of anally intubated material has always been recognised as significant, as has the possibility of 'gulping' during immersion vaccination. This latter effect has been noted by Tatner, Johnson & Horne (1984) where after immersion of trout in a bath of *Aeromonas salmonicida*, bacteria were isolated from the gut. McLean, Ash & Westcott (1987) commented on the fact that HRP levels in the plasma and tissues of rainbow trout after immersion in a bath containing 2.5mg HRP/ml were equivalent to those in fish intubated with an oral load eight times greater. Both oral and anal intubation of HRP resulted in the absorption of large amounts of this macromolecule across the intestinal epithelium and its subsequent detection in the plasma, liver, spleen and kidney, but did not equal the total amount of HRP that was intubated. The exact location of the residual HRP is not known, it may be present in other tissues not sampled, in the intestinal lumen, it may have been degraded by the various digestive enzymes that are

present in the gut or may have been expelled from the gut after intubation. In this study it was shown that a maximum of 3.32% of the total HRP intubated into the intestine of *O.mossambicus* was expelled into the tank water. As HRP was detected in the tank water it does not rule out the possibility that some of the HRP is absorbed across the gills and/or skin by osmotic penetration, and so contribute to the overall levels of HRP detected.

A variety of factors may influence the absorption of macromolecules by the gut, many of which would prove difficult to measure and correlate with macromolecular absorption, which is itself very variable. Repeated doses of HRP did not appear to enhance the absorption of this macromolecule and would appear, from the vaccination viewpoint to be of no value. However, this is only one antigen and may not represent the whole picture of antigen absorption. The important feature to arise is that intact macromolecules are absorbed across the intestinal epithelium of fish in potentially the correct amounts to stimulate an immune response.

Figure 1. Photomicrograph of a Rocket immunoelectrophoresis Plate

Figure 1. Photomicrograph of a Rocket Immunoelectrophoresis Plate

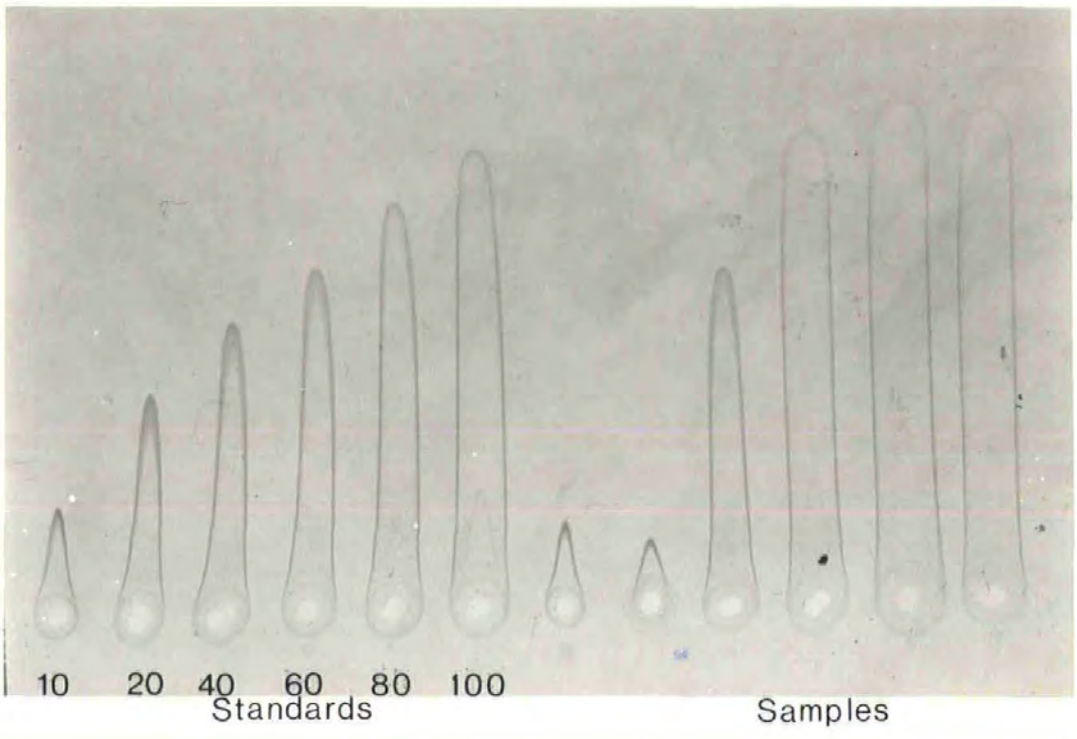


Figure 2. Illustration of the ELISA technique to detect HRP

Figure 2 Illustration of the ELISA technique to detect HRP

1. Adsorb the goat anti-HRP in bicarbonate/ carbonate buffer to the well for 2 hours at room temperature in a humid container

2. Add sample to the well in PBS/Tween and incubate for 1 hour in humid container at room temperature

3. Wash three times with PBS/Tween

4. Add chromagen (OPD) in citrate buffer plus hydrogen peroxide

Stop colour reaction with sulphuric acid

Read absorbance at 490 nm.

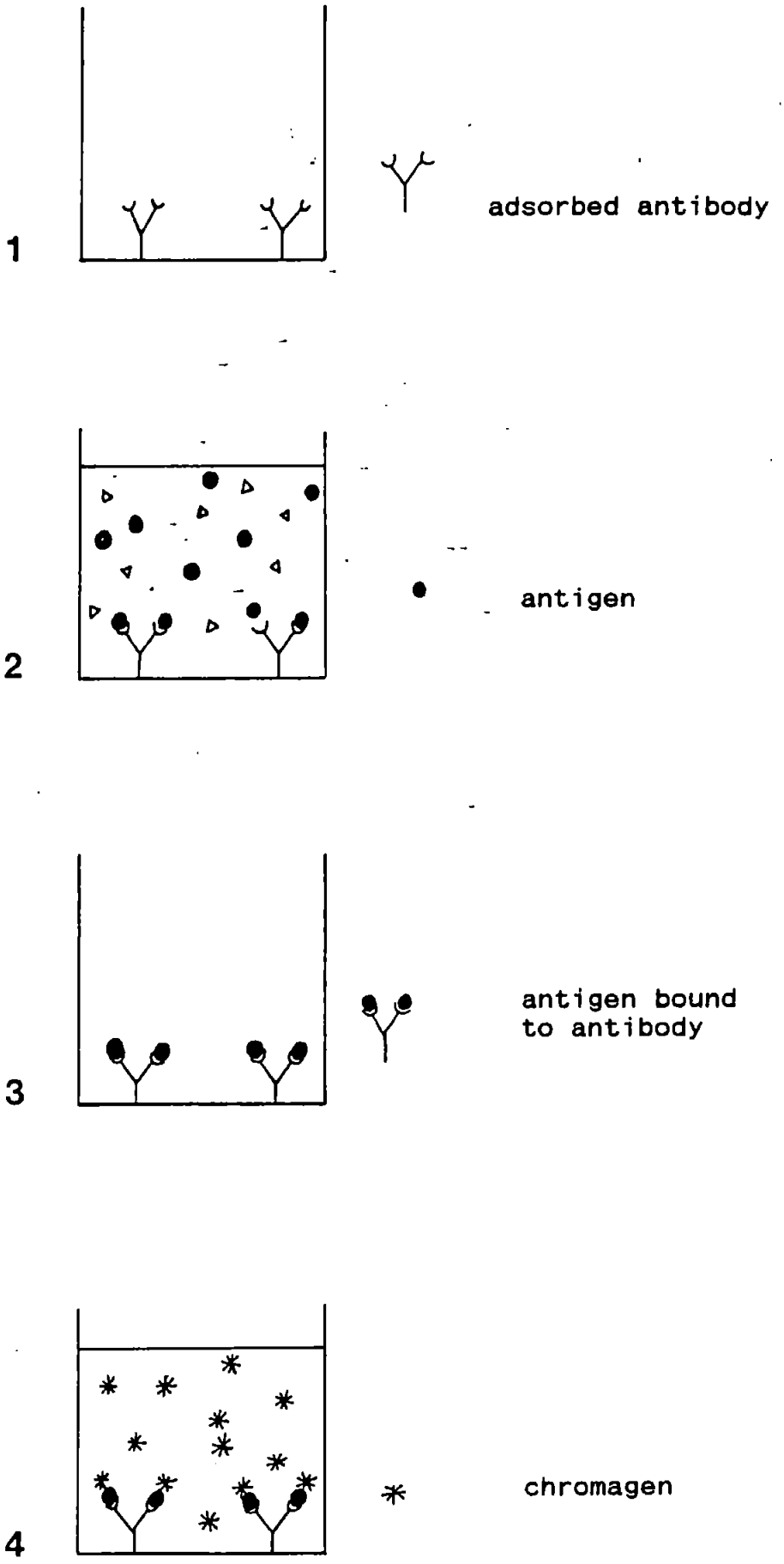

1
adsorbed antibody
2
antigen
3
antigen bound
to antibody
4
chromagen

Figure 3. An example of an ELISA test for HRP

Figure 3 An example of an ELISA test for HRP

This figure illustrates the results in plasma and tissue samples after the third oral intubation of 10mg HRP/fish.

Samples were taken at 15, 30, 45, 60, 75, 90, 180 and 360 min. after intubation.

All samples were tested in a series of doubling dilutions starting at 1/40 for test samples and 1/2 for control samples.

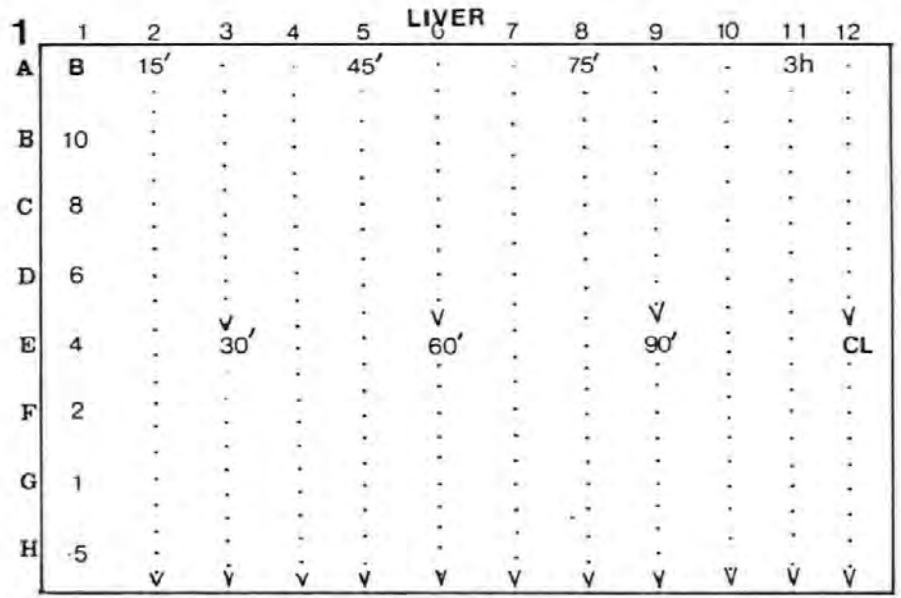
Column 1 on all plates contained HRP standards from 0.5-10 ug HRP/ml and a blank B.

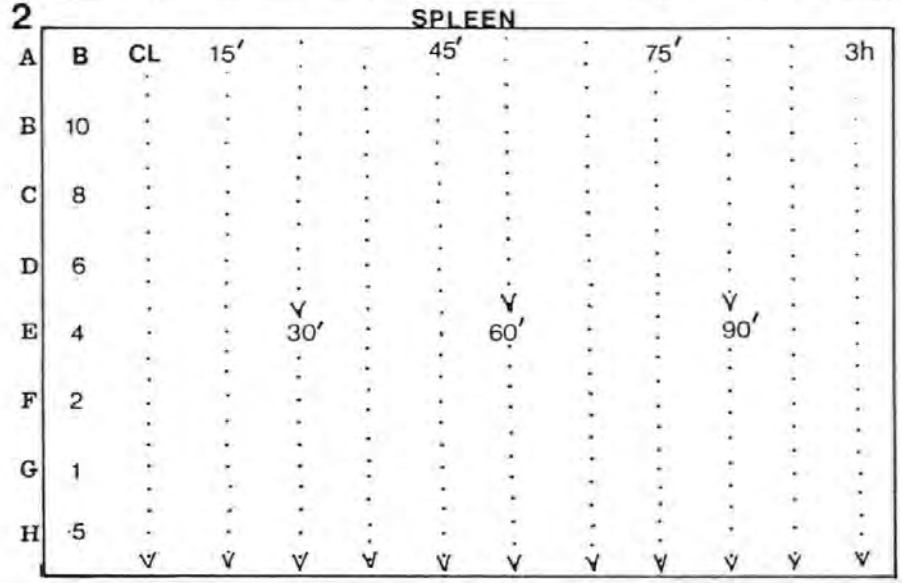
CL- control liver sample

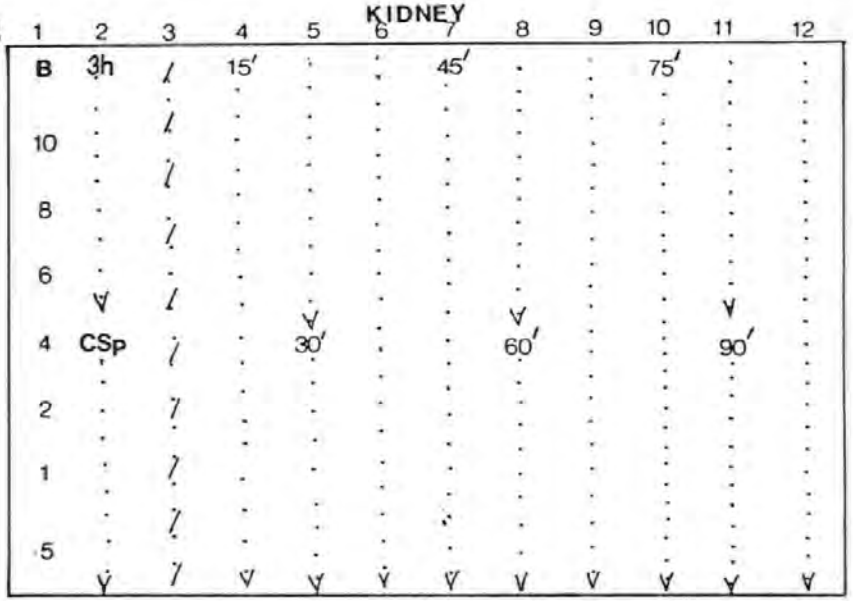
CSp- control spleen sample

CK- control kidney sample

CP- control plasma sample


1
LIVER
1 2 3 4 5 6 7 8 9 10 11 12
A B 15′ 45′ 75′ 3h
B 10
C 8
D 6
E 4 30′ 60′ 90′ CL
F 2
G 1
H 5


2
SPLEEN
A B CL 15′ 45′ 75′ 3h
B 10
C 8
D 6
E 4 30′ 60′ 90′
F 2
G 1
H 5


3
KIDNEY
1 2 3 4 5 6 7 8 9 10 11 12
B 3h 15′ 45′ 75′
10
8
6
CSP 30′ 60′ 90′
4
2
1
5

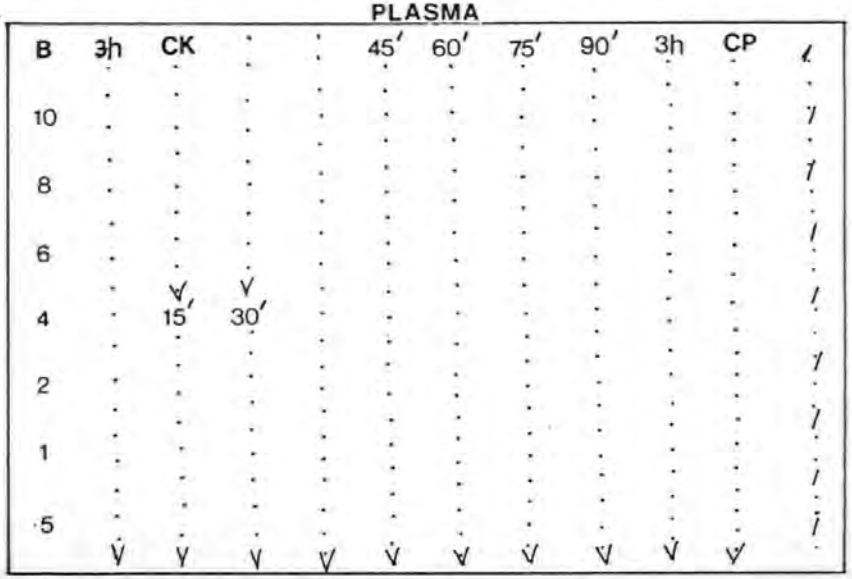

4
PLASMA
B 3h CK 45′ 60′ 75′ 90′ 3h CP
10
8
6
15′ 30′
4
2
1
5

Figure 3. An example of an ELISA test for HRP

Figure 4a-c. Oral intubation of Ferritin in the three species

= less than zero

Figure 5a-c. Anal intubation of ferritin in the three species

Fig.5a Juvenile Tilapia:ANAL FERRITIN

Fig.5b Juvenile Rainbow trout:ANAL FERRITIN

Fig.5c Juvenile Carp:ANAL FERRITIN

— less than zero

Figure 6a-d. Oral HRP dose reponse in Plasma and Tissues of Juvenile Tilapia

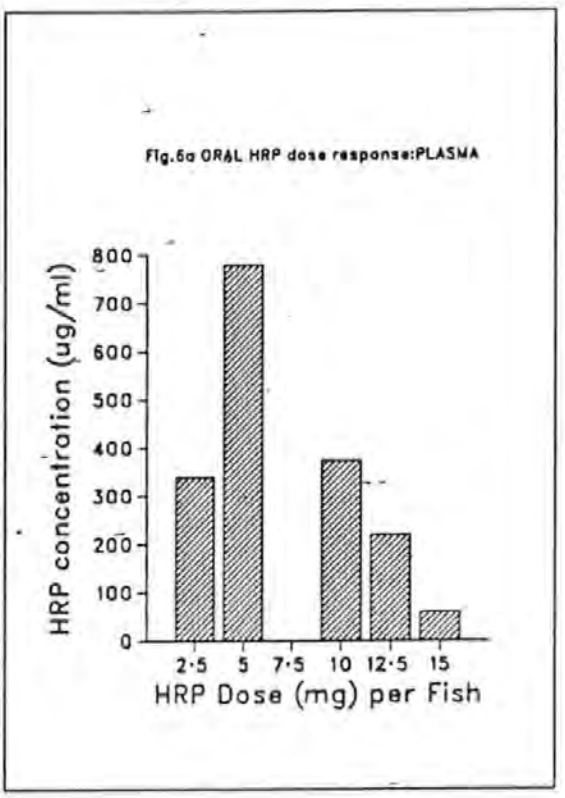


Fig.6a ORAL HRP dose response:PLASMA
HRP concentration (ug/ml)
800
700
600
500
400
300
200
100
0
2·5 5 7·5 10 12·5 15
HRP Dose (mg) per Fish

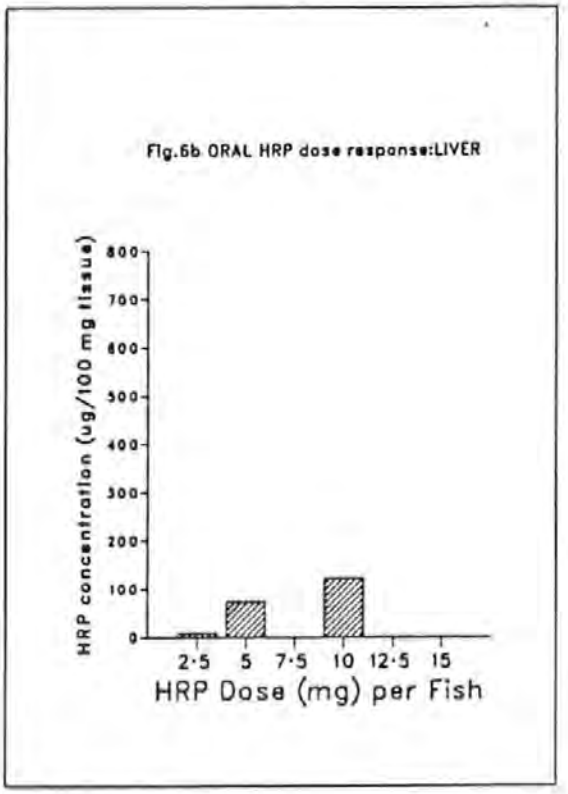


Fig.6b ORAL HRP dose response:LIVER
HRP concentration (ug/100 mg tissue)
800
700
600
500
400
300
200
100
0
2·5 5 7·5 10 12·5 15
HRP Dose (mg) per Fish

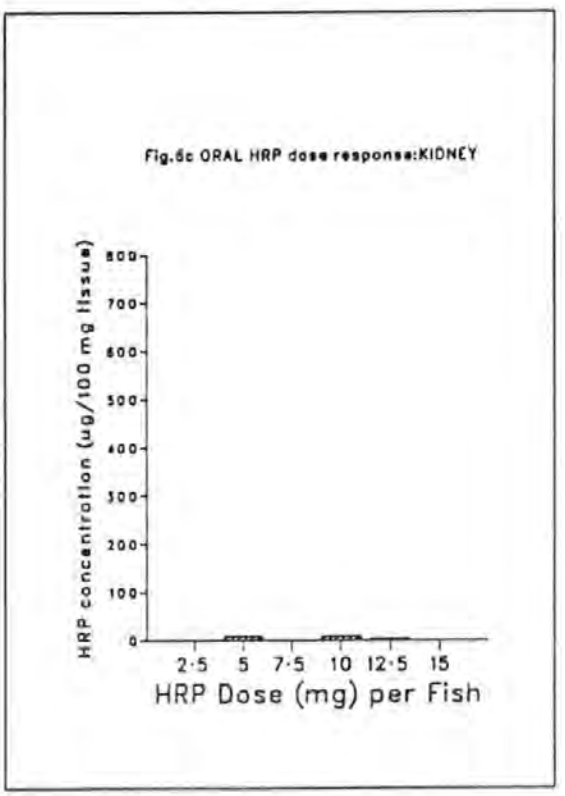


Fig.6c ORAL HRP dose response:KIDNEY
HRP concentration (ug/100 mg tissue)
800
700
600
500
400
300
200
100
0
2·5 5 7·5 10 12·5 15
HRP Dose (mg) per Fish

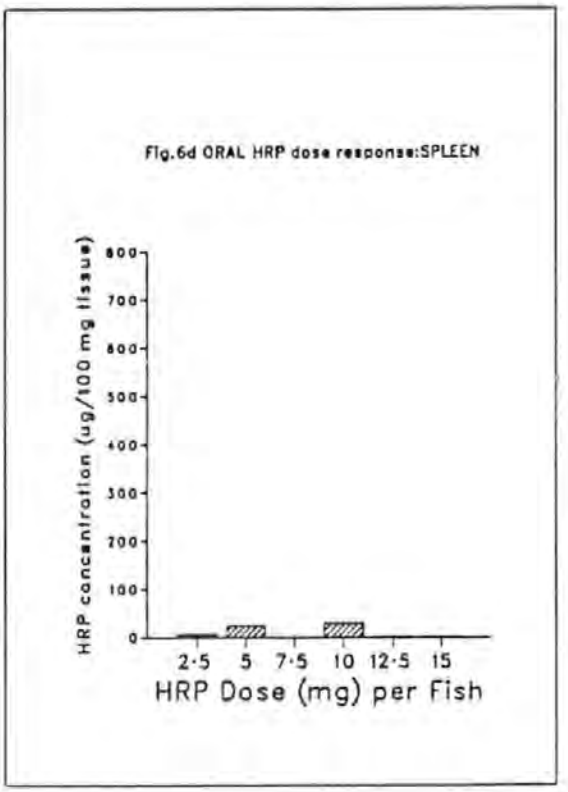


Fig.6d ORAL HRP dose response:SPLEEN
HRP concentration (ug/100 mg tissue)
800
700
600
500
400
300
200
100
0
2·5 5 7·5 10 12·5 15
HRP Dose (mg) per Fish

Figure 7a-c. HRP levels in the Plasma following repeated Oral intubation

Fig.7a HRP levels in PLASMA:ORAL DOSE 1

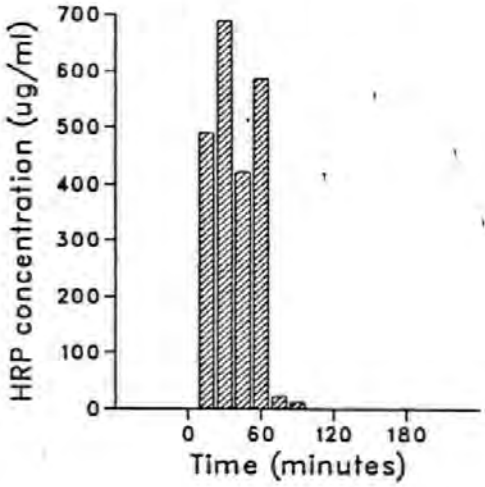


Fig.7b HRP levels in PLASMA:ORAL DOSE 2

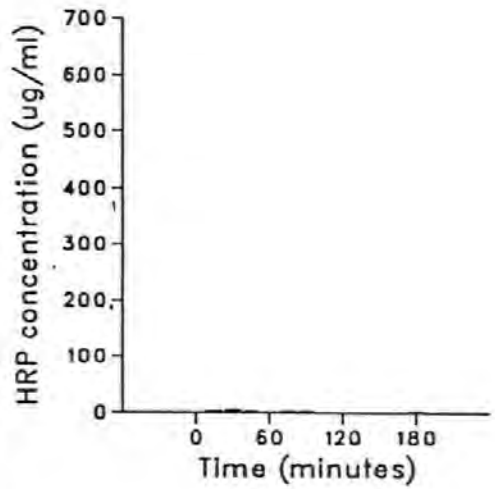


Fig.7c HRP levels in PLASMA:ORAL DOSE 3

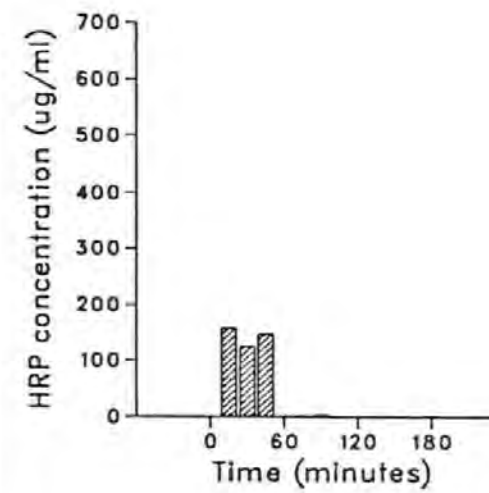


Figure 8a-c. HRP levels in the liver following repeated Oral intubation

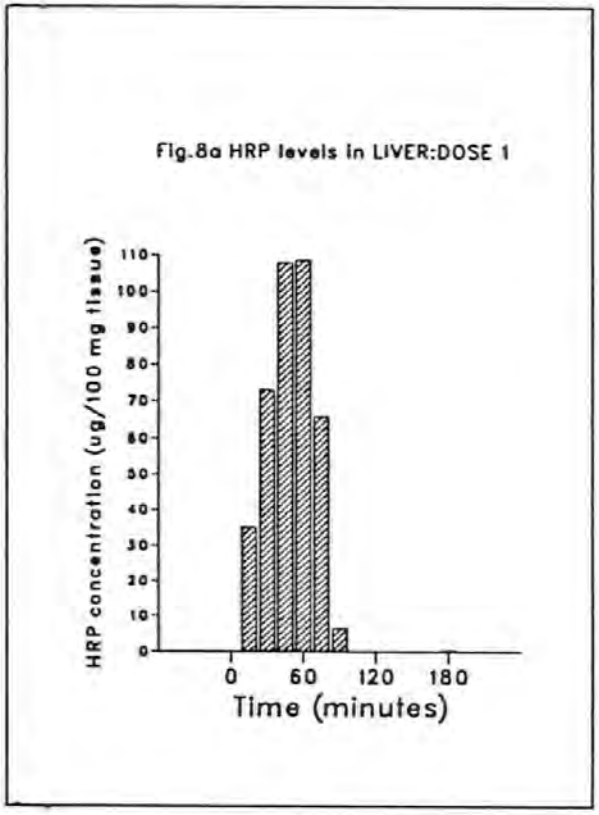


Fig.8a HRP levels in LIVER:DOSE 1
HRP concentration (ug/100 mg tissue)
110
100
90
80
70
60
50
40
30
20
10
0
0
60
120
180
Time (minutes)

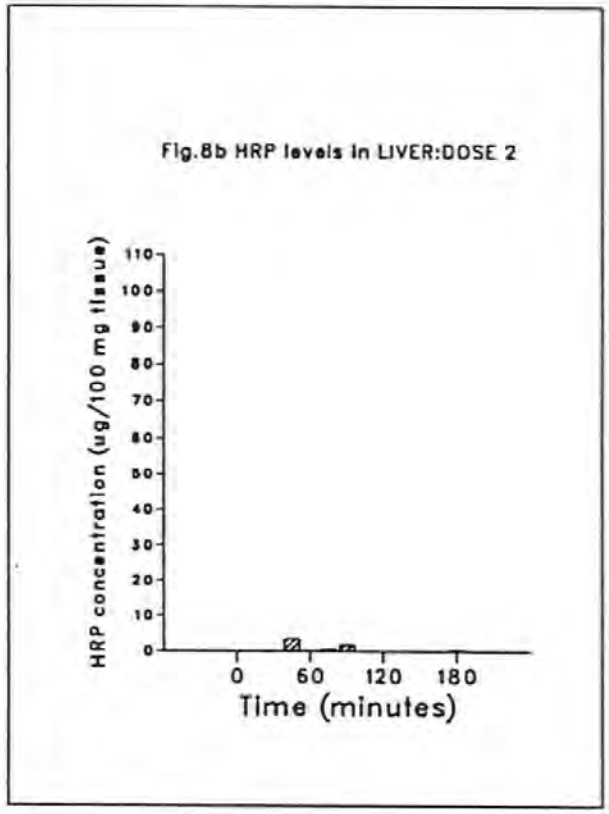


Fig.8b HRP levels in LIVER:DOSE 2
HRP concentration (ug/100 mg tissue)
110
100
90
80
70
60
50
40
30
20
10
0
0
60
120
180
Time (minutes)

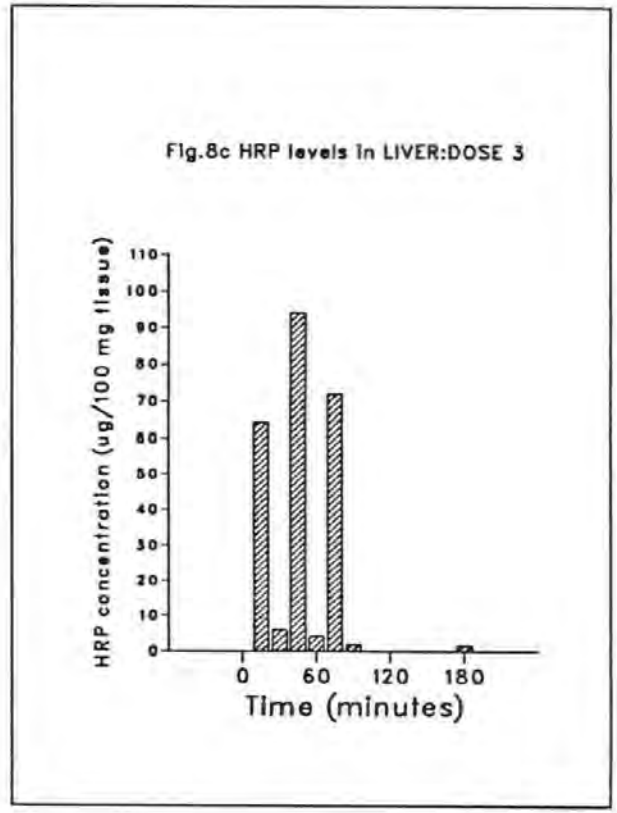


Fig.8c HRP levels in LIVER:DOSE 3
HRP concentration (ug/100 mg tissue)
110
100
90
80
70
60
50
40
30
20
10
0
0
60
120
180
Time (minutes)

Figure 9a-c. HRP levels in the Kidney following repeated Oral intubation

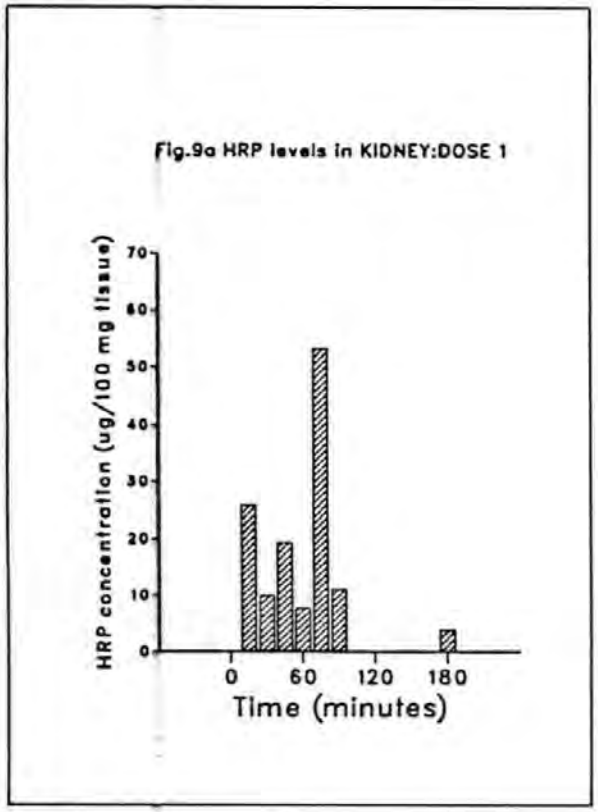


Fig.9a HRP levels in KIDNEY:DOSE 1
HRP concentration (ug/100 mg tissue)
70
60
50
40
30
20
10
0
0
60
120
180
Time (minutes)

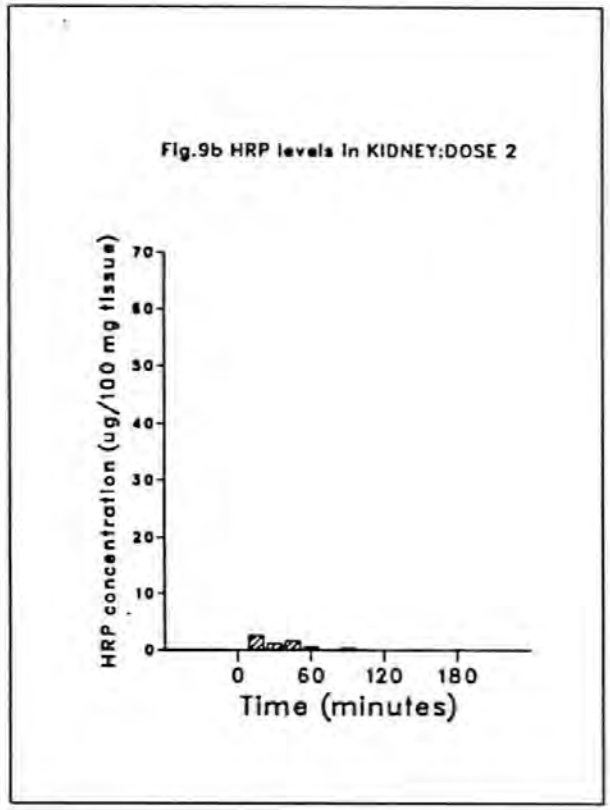


Fig.9b HRP levels in KIDNEY:DOSE 2
HRP concentration (ug/100 mg tissue)
70
60
50
40
30
20
10
0
0
60
120
180
Time (minutes)

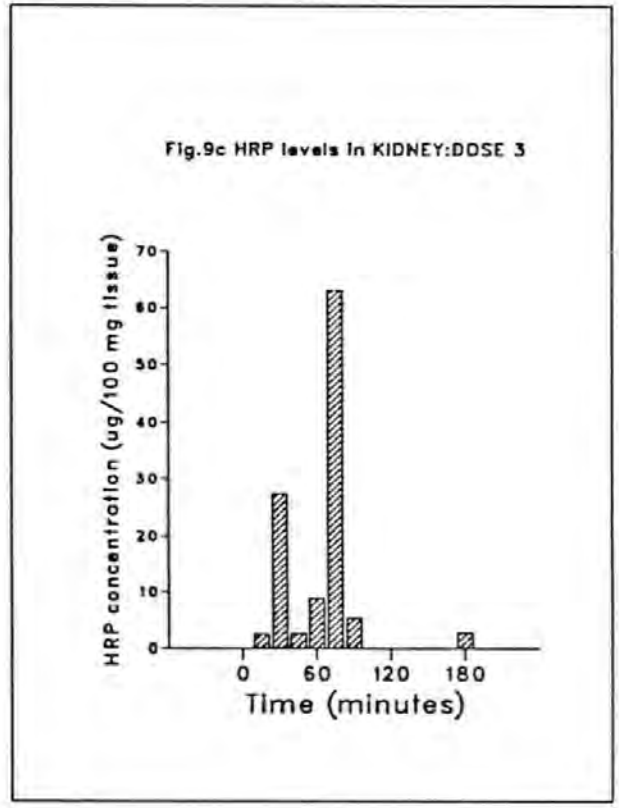


Fig.9c HRP levels in KIDNEY:DOSE 3
HRP concentration (ug/100 mg tissue)
70
60
50
40
30
20
10
0
0
60
120
180
Time (minutes)

Figure 10a-c. HRP levels in the Spleen following repeated Oral intubation

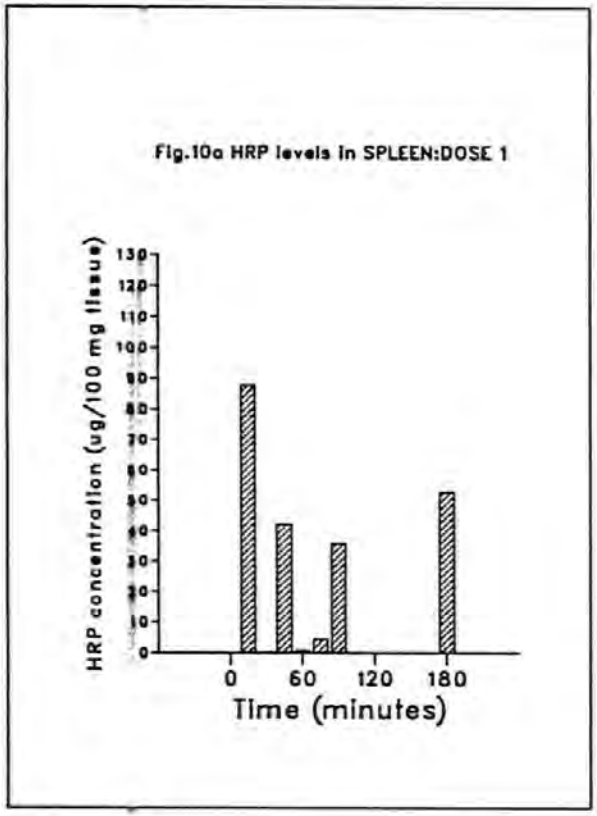


Fig.10a HRP levels in SPLEEN:DOSE 1
HRP concentration (ug/100 mg tissue)
0
60
120
180
Time (minutes)

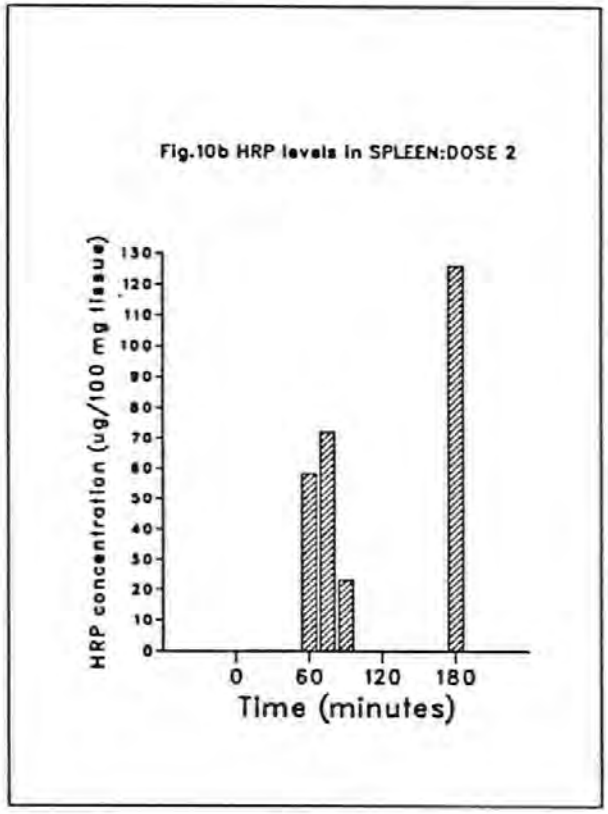


Fig.10b HRP levels in SPLEEN:DOSE 2
HRP concentration (ug/100 mg tissue)
0
60
120
180
Time (minutes)

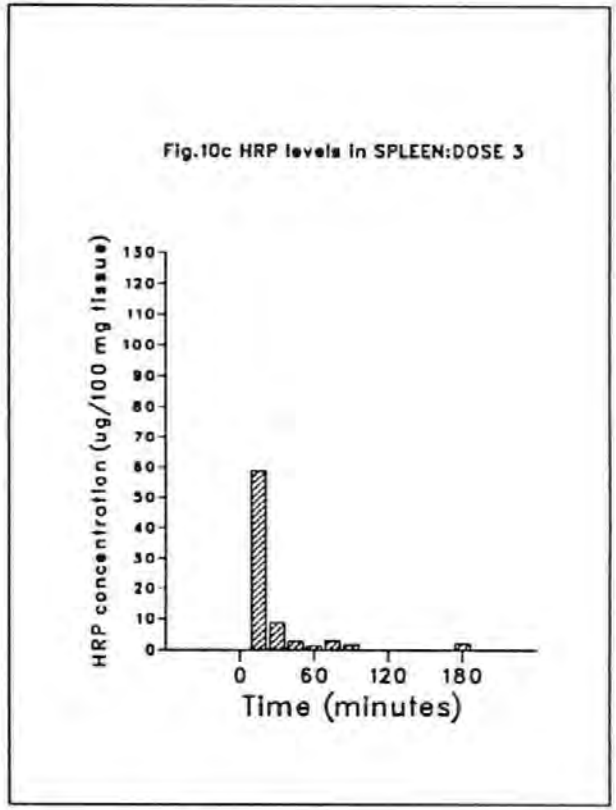


Fig.10c HRP levels in SPLEEN:DOSE 3
HRP concentration (ug/100 mg tissue)
0
60
120
180
Time (minutes)

Figure 11a-c. HRP levels in the Plasma and Tissues following Anal intubation

Fig.11a HRP levels in PLASMA:ANAL

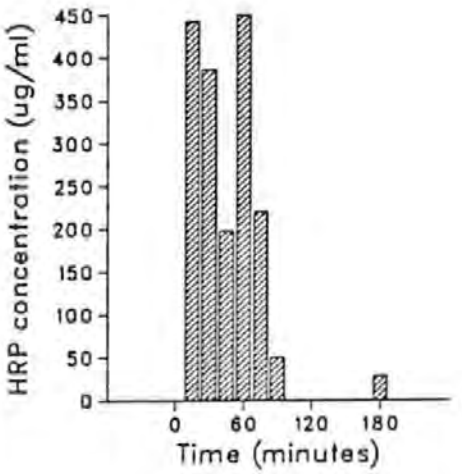


Fig.11b HRP levels in LIVER:ANAL

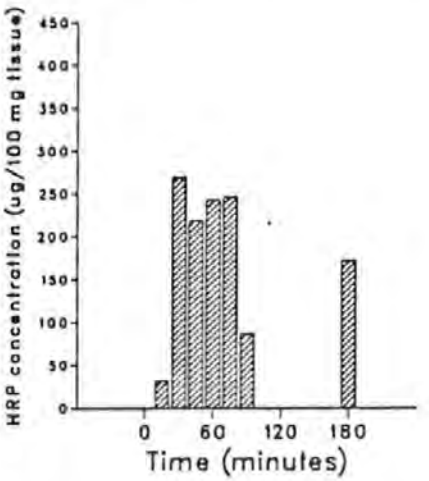


Fig.11c HRP levels in KIDNEY:ANAL

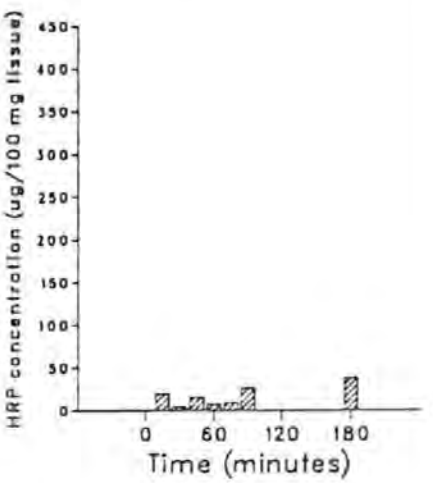
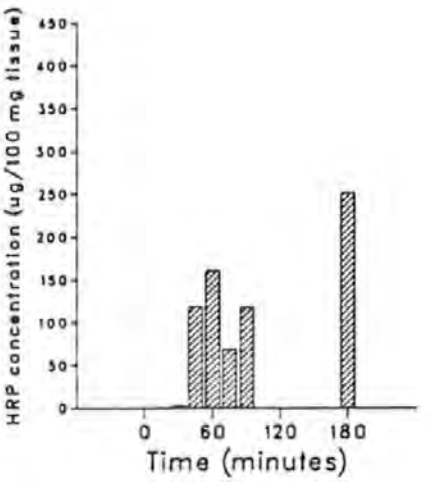


Fig.11d HRP levels in SPLEEN:ANAL



CHAPTER 7

7. ABSORPTION OF BOVINE SERUM ALBUMIN (BSA) BY THE INTESTINAL EPITHELIUM

7.1 Introduction

Bovine serum albumin (BSA) has been frequently used as a 'model' protein in investigations of macromolecular absorption by the mammalian intestinal epithelium (Gruskay & Cooke, 1955; Rothberg, 1969; Walker & Bloch, 1983) and more recently this macromolecule has been used to investigate T-cell responses to complex protein antigens (Benjamin & Holaday, 1987). As BSA is widely used in mammalian studies this led to its choice as a suitable antigen for an investigation of macromolecular absorption in fish.

The absorption of BSA in two gastric fish species; *O.mossambicus* and rainbow trout (*Salmo gairdneri* R), and one agastric species, carp (*Cyprinus carpio*) was compared using rocket immunoelectrophoresis. Differences between juvenile and adult fish, and also the route of administration, the effect of the state of nutrition and the effect of repeated doses on the absorption of BSA were investigated. A brief examination of both the systemic antibody response to BSA after repeated exposure and also the possible modification of the absorbed BSA molecule by the intestine and its secretions was also undertaken.

7.2 Materials and Methods

7.2.1 Fish

a) Juvenile (8-10g) and adult (50-100g) *O.mossambicus* were kept in freshwater recirculating systems at a temperature of 24-26°C. Juvenile fish were bred from adult brood stock obtained fron Swansea University.

b) Juvenile (10-15g) and adult (100-150g) rainbow trout, *Salmo gairdneri* R., were obtained from Ludbrook Trout Farm, Ermington, Devon. They were kept in freshwater flow- through systems at a temperature of 11-13°C.

c) Juvenile carp (8-12g), *Cyprinus carpio* L., obtained from Munton Fisons, Stowmarket, were kept in freshwater recirculating systems at a temperature of 15-18°C.

All fish were fed on a formulated, pellet diet supplied by Dr.S. Davies (Plymouth Polytechnic) and were fed twice a day at 4-5% of their body weight.

7.2.2 Administration of BSA

All fish were deprived of food for 48 hours prior to the intubation of BSA unless otherwise stated. The BSA was intubated either orally or anally using a polyethylene tube (diameter 1.6mm) attached to a 23g needle with either a 1ml or 2ml syringe.

Table 1 Effect of state of nutrition on the uptake of BSA in juvenile fish

	Age of Fish	Nutritional Status	Route of Administration	Times of Sampling (min)
O.mossambicus				
	juvenile	fed	oral	30,60,90, 120,150,360
	"	fed	anal	"
	"	starved	oral	"
	"	starved	anal	"
Rainbow Trout				
	juvenile	fed	oral	30,60,90 180,360
	"	fed	anal	"
	"	starved	oral	"
	"	starved	anal	"
Common carp				
	juvenile	fed	oral	30,60,90 180,360
	"	fed	anal	"
	"	starved	oral	"
	"	starved	anal	"

a) Dose Response- To establish a 'working' oral and anal BSA dose in juvenile *O.mossambicus*, an initial dose response was determined using 2.5, 5.0, 7.5, 10, 12.5 and 15mg BSA per fish. Five fish were sampled for each dose, 45 min after oral and anal intubation of 0.1ml. of the BSA solution. Blood samples collected from the caudal sinus were pooled and tested by rocket immunoelectrophoresis (RIE). Control fish were orally and anally intubated with 0.1ml. of 0.85% saline and sampled at the same time interval as BSA intubated fish.

An optimum dose (10mg/fish) was established and used in the further investigation of the absorption of this macromolecule in juvenile fish.

b) Effect of nutrition on the absorption of BSA- The effect of the state of nutrition on absorption after a single oral or anal intubation of BSA was investigated in juvenile *O.mossambicus*, rainbow trout and carp. Fish were either deprived of food for 48 hours or fed as usual.

After the administration of BSA three fish were sampled at each of the sample times indicated in Table 1. Blood was collected in heparinised capillary tubes from the caudal sinus and the plasma pooled.

Control fish were intubated either orally or anally with 0.85% saline.

c) Multiple intubation of BSA- The protocol is outlined in Table 2.

Table 2 Effect of repeat doses of BSA on the uptake in Juvenile and Adult Fish

	Age of Fish	Dose	Number of Doses	Route of Administration	Sample Times (min)
O.mossambicus					
	Juvenile	10 mg/fish	1	oral & anal	15,30,45,60 75,180,360
	"	"	2	"	"
	"	"	3	"	"
Rainbow trout					
	Juvenile	10 mg/fish	1	oral	15,30,45,60 75,180,360
	"	"	2	"	"
	"	"	3	"	"
O.mossambicus					
	adult	20 mg/fish	1	oral & anal	30,90 180,360
	"	"	2	"	"
Rainbow trout					
	adult	20 mg/fish	1	oral & anal	30,60,90 180,360
	"	"	2	"	"
	"	"	3	"	"

(i) Juvenile Fish- Each fish was intubated with 0.1ml. of a BSA solution equivalent to 10mg BSA/fish.

O.mossambicus: sixty-three fish were orally intubated and sixty-three anally intubated with BSA.

Rainbow trout: sixty-three fish were orally intubated only with BSA.

Each group of sixty-three fish was further divided into three groups:-

Group 1- one dose of BSA

Group 2- two doses of BSA after a two week interval

Group 3- three doses of BSA at two week intervals

Three fish were sampled at each sample time (Table 2) and the plasma pooled.

Three control fish intubated with 0.85% saline were sampled 45min. after intubation.

(ii) Adult Fish- 0.2ml. of a BSA solution (20mg/fish) was intubated either orally or anally into the intestine of *O.mossambicus* and rainbow trout.

To *O.mossambicus* BSA was intubated once, and then again after a two week interval. Blood samples were taken from the caudal sinus of two fish at each sample time.

To rainbow trout BSA was intubated three times at two week intervals. Three fish were sampled at each sample time and their plasma pooled.

Control fish were intubated with 0.85% saline and sampled 45min. after each intubation.

Table 3 Preparation of Reagents for SDS-PAGE Gels

A. Separating Gel Buffer

Tris	36.3g
TEMED	0.32ml

Dissolve and dilute to 90ml with distilled water, and pH to 8.9

B. Stacking Gel Buffer

Tris	5.98g
TEMED	0.46ml

Dissolve and dilute in 80ml dis. water, and pH to 6.7

C. Separating Gel Solution

Acrylamide	28.0g
N',N'-Methylenebisacrylamide	0.74g

Dissolve in dis. water to a final volume of 100ml., and filter.

D. Stacking Gel Solution

Acrylamide	10.0g
N,N-Methylenebisacrylamide	2.5g

Dissolve in dis. water to a final volume of 100ml, and filter.

E. SDS Solution

Sodium dodecyl sulfate, SDS	0.21g

Dissolve in 100ml dis. water.

F. 2x Sample Buffer

Tris	1.51g
Glycerol	20.0ml

Dissolve in 35ml dis.water, adjust pH to 6.5, then add:

SDS	4.0g
Bromophenol blue	0.002g

Dilute to a final volume of 100ml.

G. Electrode Buffer

Tris	6.05g
Glycine	28.8g
SDS	2.0g

Dissolve in 2 litres of dis. water, pH 8.3

H. Fixing Solution

Methanol	400ml
Glacial acetic acid	70ml
Dis. water	530ml

I. Staining Solution

Coomassie Brilliant Blue R	2.5g

Dissolve in 500ml of Fixing Solution

7.2.3 Sample Preparation

Blood samples were taken from the caudal sinus and collected in either heparinised capillary tubes (juvenile fish) or heparinised 23g needles and syringes (adult fish). Blood was left overnight at 4°C and the plasma removed. Plasma samples were tested either undiluted or diluted in 0.08M barbitone buffer, pH 8.2, by RIE (Section 6.2.3c).

7.2.4 Production of Antisera

An antiserum to bovine serum albumin, BSA, was raised in two Dutch Rabbits (Hyline Ltd., Chesire). 2.5mg of BSA in 0.25ml of phosphate buffered saline was mixed with an equal volume of Freund's complete adjuvant, FCA (BDH, Bristol) and injected subcutaneously. A booster injection of 10mg of BSA in Freund's incomplete adjuvant, FIA, was given via this route 6 weeks later. Blood was taken from the marginal ear vein approximately 6 weeks after the second injection, allowed to clot overnight at 4°C, the serum removed and stored at -70°C until required. The specificity of the antiserum was demonstrated by immunoelectrophoresis against BSA.

7.2.5 Immunoelectrophoresis

This technique was based on that described by Hudson & Hay (1980). Clean, grease- free microscope slides (76 x25 x10mm) were placed in a perspex tray (Shandon, London) over which molten 1% agarose (w/v) in 0.08M barbitone buffer, pH8.2 (Section 6.2.3) was poured to form a thin gel.

Table 4 Preparation of Electrophoresis Gels

1. **Preparation of 11% Separation Gel**

   3.0 ml. Separating Gel Buffer
   9.5 ml. Separating Gel Solution
   11.5 ml. SDS Solution containing 17mg of Ammonium Persulfate, freshly prepared.

2. **Preparation of 3% Stacking Gel**

   1.0 ml. Stacking Gel Buffer
   2.0 ml. Stacking Gel Solution
   4.0 ml. SDS Solution
   1.0 ml. water containing 8mg of Ammonium Persulfate, freshly prepared

Table 5 Western Blot Buffer

Tris/Glycine Buffer

Tris	2.42g
Glycine	11.26g
Methanol (AnalAr)	200 ml.

Make up to 1 litre with distilled water. Final pH 8.3

Table 6 50mM Tris/HCl Saline Buffer

A.

Tris	6.05g
Sodium chloride	16.80g

Dilute in 800ml. dis. water and pH to 7.4 with 1M HCl. Make up to 1 litre.

B. Blocking/Incubating Buffer

Tween 20 0.1 ml.
Make up to 100ml. with Tris/HCl Saline buffer

Using a template two wells with a central trough were cut in the gel on each slide. Samples (5-10µl) were loaded into the wells with a capillary tube. To one well a migration marker, Bromophenol blue, was added. The samples were run at a constant current of 30mA for 1-2 hours. The test antiserum was placed in the central trough, gels were left overnight in a humid container for the precipitation to develop, then washed in PBS for 2 hours, dried and finally stained with Coomassie brilliant blue.

7.2.6 Detection of BSA

BSA present in plasma samples was detected and quantified using rocket immunoelectrophoresis, RIE (Section 6.2.4). Rabbit anti-BSA was incorporated into 1% agarose in 0.08M barbitone buffer (pH8.2). BSA standards (10-100µg BSA/ml) were used to construct a calibration curve to enable the quantification of BSA in the test samples.

7.2.7 Statistical Analysis

An analysis of variance (ANOVA) was carried out on the results to establish whether there was a significant difference between the results at a 0.05% probability level.

7.2.8 Modification of BSA by the intestine

An attempt to determine whether intact or partially degraded BSA was absorbed by the intestinal epithelium of each of the three fish species was carried out using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and

Western Blotting.

a) SDS-PAGE

An 11% separating gel and 3% stacking gel were prepared using the reagents listed in Table 3 and the volumes as in Table 4. The separating gel was poured into the gel former and overlaid with water-saturated n-butanol (BDH,Bristol) and allowed to polymerise (45-60 min.). The butanol was then washed off with several changes of distilled water. The stacking gel was carefully poured onto the separating gel, the well former positioned and left to polymerise for a further 45 min. The ten well comb was carefully removed and the wells washed with distilled water. The gel forming unit was placed in the electrophoresis tank, which was then filled with the electrode buffer (Table 3).

Plasma samples from BSA intubated fish and a standard BSA sample (1mg/ml) were mixed in a 1:4 ratio with the 2x sample buffer (Table 3) and left at room temperature for 1 hour. A range of molecular weight markers, @14,200-66,000 daltons (SDS-7, Sigma,UK) were also prepared. 5µl. of each of the samples was loaded into the wells using a microsyringe. Electrophoresis was carried out at a constant voltage of 200V for 40-50 min. in a Mini-Protean II system (BioRad, Herts.). The gels were then stained for 45min. in the staining solution and destained in the fixing solution (Table 3) until all excess stain was removed.

Western Blotting.

a) SDS-PAGE

An 11% separating gel and 3% stacking gel were prepared using the reagents listed in Table 3 and the volumes as in Table 4. The separating gel was poured into the gel former and overlayed with water-saturated n-butanol (BDH,Bristol) and allowed to polymerisation (45-60 min.). The butanol was then washed off with several changes of distilled water. The stacking gel was carefully poured onto the separating gel, the well former positioned and left to polymerise for a further 45 min. The ten well comb was carefully removed and the wells washed with distilled water. The gel forming unit was placed in the electrophoresis tank, which was then filled with the electrode buffer (Table 3).

Plasma samples from BSA intubated fish and a standard BSA sample (1mg/ml) were mixed in a 1:4 ratio with the 2x sample buffer (Table 3) and left at room temperature for 1 hour. A range of molecular weight markers, @14,200-66,000 daltons (SDS-7, Sigma,UK) were also prepared. 5µl. of each of the samples was loaded into the wells using a microsyringe. Electrophoresis was carried out at a constant voltage of 200V for 40-50 min. in a Mini-Protean II system (BioRad, Herts.). The gels were then stained for 45min. in the staining solution and destained in the fixing solution (Table 3) until all excess stain was removed.

b) Immunoblotting

(i) Western Blotting- To detect the presence of 'intact' or fragmented BSA in the plasma samples a second 11% gel was run and the proteins blotted onto nitrocellulose in a Trans-Blot Cell (BioRad, Herts.).

Three pieces of 3mm Whatman filter paper (Maidstone, Kent), one piece of nitrocellulose (Hybond-C 0.45um, Amersham, Bucks.) and two sponge pads were presoaked in bolt buffer (Table 5) and put together in the following order; sponge pad, two pieces of filter paper, nitrocellulose, gel, one piece filter paper, sponge pad. This 'sandwich' was sealed into the blot holder then placed in the Trans-Blot Cell with the gel nearest the cathode. The cell was then filled with blot buffer and protein transfer was carried out at a constant current of 30mA for 16-19 hours.

(ii) Immunostaining-

The blot was washed in Tris/HCl saline (Table 6A) for 30 min., then in blocking buffer (Table 6B) for 30 min., and finally placed in a solution containing a 1/200 dilution of sheep anti-BSA peroxidase conjugate (Binding Site, Birmingham University) in blocking buffer, and incubated at room temperature for 3 hours.

The blot was then washed in several changes of Tris/HCl saline for 30 min. then placed in the substrate solution containing 3mg 4-chloro-1-naphthol (Sigma,UK) in 1ml. methanol, diluted in five volumes of Tris/HCl saline plus 5ul of H_2O_2. The blot was incubated in the substrate

Table 7 Antibody response to BSA

	Antibody Titres			
Day Number	Oral (Group 1)	Anal (Group 2)	IP– BSA+FCA (Group 3)	Control (saline) (Group 4)
*0	–	–	–	–
7	–	–	1/10	–
10	–	–	1/32–1/64	–
*14	–	1/4	1/30720	1/2
17	1/4–1/8	1/4	ND	ND
21	1/8	1/8	1/81920	–
26	1/32	1/16	ND	ND
*28	1/16	1/16	1/209600	–
33	1/8	1/4	ND	ND

* day of intubation

\- no antibody detected

ND no sample taken

solution for 10-15 min. then washed in Tris/HCl saline.

7.2.9 Antibody Response to Intubated BSA

The antibody response to BSA was measured in juvenile *O.mossambicus* in parallel with the investigation of multiple orally and anally administered doses of BSA. The antibody levels were monitored in intubated fish over a period of 33 days (Table 7) and measured using indirect haemagglutination.

a) Protocol

Eighty-four *O.mossambicus* were divided into four groups:

Group 1: 24 fish were orally intubated with 10mg BSA/fish

Group 2: 24 fish were anally intubated with 10mg BSA/fish

Group 3: control group one, 18 fish were injected intra-peritoneally (IP) with 1mg BSA in FCA (0.1ml.)

Group 4: control group two, 18 fish were orally intubated with 0.1ml. 0.85% saline

Fish in Group 1 and 2 received multiple doses of BSA following the protocol in 7.2.2c. Fish were sacrificed and sampled on the day of intubation prior to the intubation of BSA to the other fish in these groups.

Three fish from each of the 4 groups were sampled at each sample time and their plasma pooled.

b) Indirect Haemagglutination

Complement in the plasma samples was heat inactivated (20min. at 45°C). A suspension of 2.5% SRBC in PBS pH 7.2

Table 8 Buffers for Indirect Haemagglutination

Phosphate buffered saline (PBS) pH 7.2

Sodium chloride	8g
Potassium chloride	0.2g
Disodium hydrogen phosphate.12H_2O	2.89g
Potassium dihydrogen phosphate	0.2g

PBS pH 6.4

0.15M potassium dihydrogen phosphate	67.8ml
0.15M disodium hydrogen phosphate	32.2ml
0.85% saline	100.0ml

(Table 8) v/v was mixed with an equal volume of tannic acid in 0.85% saline (0.005% w/v), and incubated at room temperature for 30min. The tanned SRBC were washed twice in PBS pH 7.2, 5min. at 700g, and resuspended to 2.5% in PBS pH6.4. One volume of BSA (1mg/ml) was mixed with 1 volume 2.5% tanned SRBC and 4 volumes of PBS pH 6.4 (Table 8). For control tanned SRBC 0.85% saline was used instead of BSA. These cells were left at room temperature for 30min.

The coated SRBC were washed twice with serum diluent (1% pooled serum in PBS pH6.4) and resuspended to 1% v/v in serum diluent. To each well in the microtitre plate 50µl of serum diluent was added, and for each sample a series of doubling dilutions in the wells of each row, in duplicate, was carried out. 50ul of coated SRBC or control SRBC respectively were added to the duplicate sample dilutions and left overnight at 4^0C.

7.3 Results

Figure A1-3 illustrates the gut of the three species used in these investigations. Figure A1 shows the gut of *O.mossambicus* with its long, coiled intestine (In) and well defined stomach (St). Figure A2 shows the gut of rainbow trout, *Salmo gairdneri* with a shorter intestine (In) and pyloric caecae (Pc). Figure A3 represents the gut of the agastric carp, *Cyprinus carpio*, with a slightly longer intestine (In) than the rainbow trout.

7.3.1 Absorption of BSA in Juvenile Fish

From the results of the initial dose reponse experiment (Fig.1a & 1b) it was decided to choose a dose of 10mg BSA per fish for future administration. This gave a detection level of between 50% and 70% of the initial amount of BSA intubated.

a) Effect of the state of nutrition on the uptake of BSA.

In *O.mossambicus* (Fig.2a-d) BSA was detected in the plasma after both oral and anal intubation of this protein. 'Normal' feeding did not appear to affect the absorption of BSA and it was observed that significantly greater levels of BSA were detected in the oral-fed group (Fig.2a) as compared to the oral-starved group (Fig.2b). In the anally intubated groups slightly higher levels were detected in the starved group (Fig.2d) compared to the fed group (Fig.2c).

In rainbow trout (Fig.3a-d) no BSA was detected in the systemic circulation of either the oral-fed (Fig.3a) or oral-starved (Fig.3b) groups. However, BSA was detected in the plasma of anally intubated fish (Fig.3c & 3d).

As in *O.mossambicus* BSA was detected in the plasma of carp after oral intubation (Fig.4a & 4b). Greater levels of BSA were detected in the plasma of anally intubated fish (Fig.4c & 4d) with the highest levels of BSA being detected in the plasma of anal-fed fish (Fig.4c).

Overall it was found that the BSA levels in the plasma of carp and rainbow trout were considerably lower than those in *O.mossambicus*. Statistical analysis indicated that for all

three species there was no significant difference between the BSA levels detected in the fed-anal and the starved-anal fish. There was also no significant difference between the BSA levels in the plasma of carp and rainbow trout either in the starved or fed groups. There was however, a significant difference between the detectable BSA levels of both the fed and starved tilapia and the other two species.

b) Multiple intubation of BSA

In the juvenile tilapia the overall trend of the effect of repeat doses of BSA was a subsequent increase in the levels of BSA detected in the plasma of these fish. This increase was observed in both the orally (Fig.5a-c) and anally (Fig.6a-c) intubated fish, with significantly greater levels of BSA being detected in the plasma of anally intubated fish.

In juvenile rainbow trout repeat oral intubation of BSA produced a marked increase in the levels of BSA detected in the systemic circulation (Fig.7a-c). This was more pronounced after dose 3 where levels were approximately 300 times greater than after the previous two exposures.

7.3.2 Absorption of BSA after multiple intubation in adult fish

In adult tilapia there was a difference in the levels of BSA detected in the plasma after the first and second dose of BSA in both orally (Fig.8a & 8b) and anally (Fig.8c & 8d) intubated fish. After the first oral dose no BSA could be detected in the systemic circulation, however after the

second dose a peak level of 1.44mg BSA/ml. plasma was detected. Similarly after the second anal dose much larger quantities of BSA were detected in the plasma.

Similar findings were observed after multiple oral and anal BSA doses into the intestine of adult rainbow trout. After both the first and second oral dose no BSA was detected in the plasma (Fig.9a & 9b), however after the third dose, BSA was detected in the plasma (Fig.9c) with a peak level of 53.5ug BSA/ml. plasma being detected.

BSA was detected in the plasma after the first anal dose (Fig.10a), but a second dose resulted in a decrease in detectable levels in the plasma. (Fig.10b). However, the third dose of BSA raised the levels of BSA detected in the plasma (Fig.10c).

7.3.3 Modification of BSA by the Intestine

Test and control plasma samples together with a BSA sample and molecular weight markers were run on an 11% SDS gel, the BSA sample (fraction V) was the same as that intubated into the three fish species. The main BSA protein band had a molecular weight of between @47,500- 60,100 daltons. Also present in the BSA sample were a number of proteins of higher molecular weight, between 74,300 and 95,800 daltons (Fig.11A).

A similar gel to that in Fig. 11A was blotted onto nitrocellulose and the proteins detected using sheep anti-BSA peroxidase conjugate (Fig.11B). In lane 10, containing the BSA sample, several bands were found to be

antigenic. There was no background activity in the control sera (not shown). Lane 7, containing plasma from anally intubated tilapia, showed several protein bands that were antigenic, similar to those detected in the BSA sample together with a band of @40,100 d. This lower molecular weight protein was not detected in the other plasma samples. In orally intubated tilapia, lane 6, and orally (lane 2) and anally (lane 3) intubated carp a protein of @52,000 d was identified, equivalent to the intact BSA molecule. In orally and anally intubated rainbow trout (lane 4 & 5) a protein band of @72,000 d was detected, equivalent to one of the high molecular weight proteins in the BSA sample and anally intubated tilapia plasma sample.

7.3.4 Antibody Response to BSA

The systemic response to orally and anally intubated BSA was minimal compared to the immune response following the IP injection of BSA.

Antibody to BSA was not detected in the plasma of orally or anally intubated fish until after day 14 (Table 7). On this day the fish were given a second dose of BSA and plasma titres were seen to increase slightly over the next two week period, with the highest titre on day 26 (1/32) in the orally intubated fish. The third dose on day 28 did not result in an increase in circulating antibody by day 33.

7.4 Discussion

In the first experiment, to determine any effects of the state of nutrition on the absorption of BSA, there was no significant difference in the detectable levels of BSA in starved and fed fish at least in the case of carp (Fig.4a-d) and rainbow trout (Fig.3a-d). However, in tilapia there was a significant difference between the fed-oral and the starved-oral groups (Fig.2c & d) but not the fed-anal and the starved-anal groups (Fig.2a & 2b). The difference in this species between the oral fed and starved groups may be due to a more rapid clearance of the BSA from the systemic circulation, that is before the first sample time of 30min. Overall, higher levels of BSA were detected in the plasma of tilapia compared to the other two species. From these observations it may be concluded that the state of nutrition is perhaps not an important factor in the absorption of BSA in these three species. It was also found that anal intubation resulted in higher levels of BSA in the systemic circulation compared to oral intubation in all three fish species.

In fed fish, once digestion has been initiated, parts of the meal are passed along the gut which stimulate the secretion of digestive enzymes. The rate of the passage of food through the GI tract varies for each species and is dependent on a number of factors including type of diet and temperature (Fange & Grove, 1979). In starved fish it has been shown that there is a basal rate of trypsin-like activity (Hjelmeland, Huse, Jorgensen, Molvik & Raa, 1984)

and gastric acid secretion (Holstein, 1977). In view of this it is surprising that there appeared to be no significant difference between the detectable levels of BSA in starved and fed carp and rainbow trout. This may also be the case in *O.mossambicus* as the levels detected may be due to a more rapid clearance of BSA from the blood, as mentioned earlier.

An interesting feature is that most ultrastructural investigations of macromolecular absorption involves the deprivation of food prior to the intubation of the macromolecule. Investigations concerning oral vaccination however, are usually based on the incorporation of antigenic material into foodstuffs. In this study it was found that feeding of the fish prior to the intubation of BSA did not have a significant effect on the levels of BSA detected in the plasma. This would seem to be encouraging from the point of view of possibly incorporating antigenic material into fodstuffs, where normal feeding would not affect the amount of antigen that would be transported into the systemic circulation.

The effect of repeated intubation was investigated in both juvenile and adult tilapia and rainbow trout. In juvenile *O.mossambicus* there was no significant difference between the levels of BSA in the plasma after repeated oral intubations, however repeated anal intubations resulted in greater levels of detectable BSA after the second and third administrations. In juvenile rainbow trout there was a significant increase in the levels of BSA in the systemic circulation after the third dose. In adult *O.mossambicus* and

rainbow trout the detectable levels of BSA were considerably lower than those detected in the juvenile fish. This is comparable to observations in mammalian studies where there is a significant decrease in the detectable levels of antigen in the systemic circulation of orally intubated adult mammals compared to neonates (Walker, 1987). Following repeated oral and anal intubation of BSA in adult *O.mossambicus* BSA levels were greater after the second dose in both orally and anally intubated fish. In orally intubated adult rainbow trout BSA was detected in the plasma only after the third dose, whereas in anally intubated fish circulating BSA levels were equivalent after the first and third dose, with a decrease after the second. As in juvenile fish greater levels of circulating BSA were detected after anal intubation.

Anal intubation demonstrated that if the hydrolytic enzymes were 'bypassed' relatively large amounts of antigenic material were absorbed and transported into the systemic circulation. That there is a decrease in the proteolytic activity in the hindgut of fish has been demonstrated (cited from Ash, 1985) and may possibly be due to the reabsorption of enzymes in this region (Hofer & Scheimer, 1981). A decrease in the quantity of proteolytic enzymes in the lumen of the posterior intestine would increase the amount of BSA left intact to be absorbed by the absorptive enterocytes of this region, hence the higher levels of BSA detected in the plasma of anally intubated fish. If orally intubated antigens could be protected

against proteolytic degradation by such methods as microencapsulation or incorporation of protective ingredients in the diet during their passage through the gut, they could be made available to the absorptive cells of the posterior intestine.

Antibody titres in juvenile *O.mossambicus* following repeated oral and anal intubation of BSA were low when compared to a single IP injection of BSA+FCA. In general it has been demonstrated that the systemic immune response to orally intubated antigens is poor compared to antigen introduced IP (Hart, 1987; Rombout *et al.*, 1986). Even repeated oral doses of *Vibrio* bacterin did not elicit a systemic immune response in carp (Rombout *et al.*, 1986). However, the mucosal immune response to oral antigens can be quite substantial (Fletcher & White, 1973; Rombout *et al.*, 1986). Rombout *et al.* (1986) did however demonstrate that if the stomach and anterior intestine is 'bypassed' by anal intubation a systemic response almost equivalent to that following an intramuscular injection can be elicited. An estimation of the Ig levels in the mucus of *O.mossambicus* following oral and anal administration of BSA was attempted using a number of techniques. However, due to the difficulties in sampling such small fish and the contamination of mucus samples with blood the mucosal antibody response was not evaluated.

There are three possible reasons why the levels of BSA detected in the plasma of the three fish species are higher than those following intubation of HRP these are; firstly,

it has been indicated that proteolysis of protein antigens leads to a decrease in the absorption of the antigen, it must therefore be questioned whether the macromolecules are absorbed and transported into the systemic circulation intact or as partially digested fragments. In this investigation, after the intubation of BSA, low molecular weight protein antigens (@ 40 Kd) were found in the plasma of tilapia, which were antigenic. These low molecular weight proteins were not found in the 'native' BSA sample and must therefore be partially digested products of the BSA protein that was intubated into these fish. Benjamin & Holaday (1987) cited evidence that shows that enzymatic cleavage of BSA yields a number of fragments which retain their antigenic activity. Walker & Bloch (1983) found that BSA was processed by the intestine of rats that were orally immunised with this macromolecule. They found intact protein, smaller fragments and high molecular weight components which they believed to be characteristic of antigen-antibody complexes. It may be that not only intact BSA is detected in the systemic circulation using RIE but also distinct antigenic fragments, which contribute to the overall levels of BSA detected in the plasma.

Secondly, BSA may not only pass through, but also between intestinal epithelial cells, suggesting that both transcellular and paracellular routes of absorption may occur in the adult rat gut (Warshaw *et al.*, 1974). Warshaw, Walker & Cornell (1971) indicated that in orally intubated rats the percentage of BSA absorbed was more than 100-1000

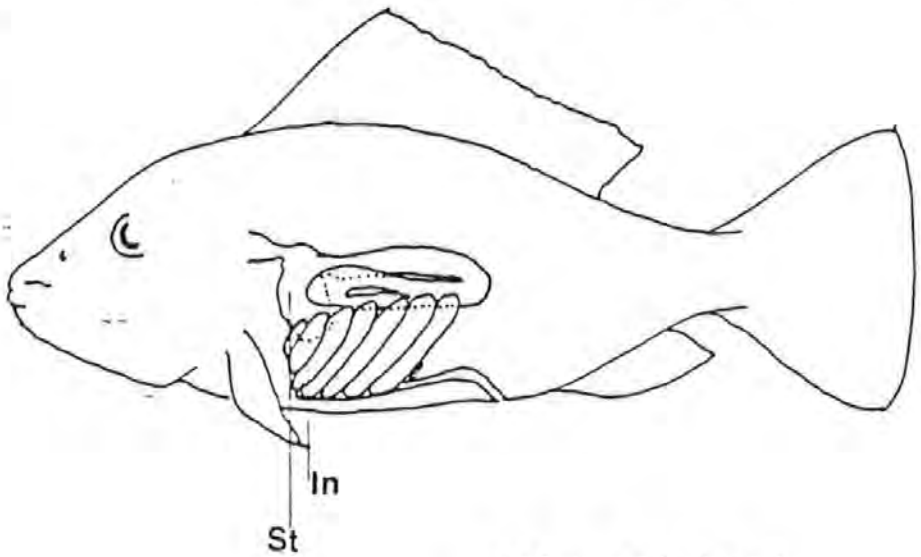
times greater than the percentage of HRP absorbed, which was shown to be transported intracellularly (Rhodes & Karnovsky, 1971). It may be that the mode of absorption of these two macromolecules may, in part, account for the different levels of detectable protein. These observations are mirrored in fish, where greater levels of BSA were detected in the systemic circulation after oral intubation compared to those after oral intubation of HRP. This was found to be the case for all three species investigated. A third factor which may explain the difference in the levels of these two macromolecules is that BSA preparations are known to be contaminated with endotoxins and phospholipases (Dvorak & Rast, 1970) which may effect the tight junction between cells, and hence the paracellular route of absorption may be more pronounced (Warshaw *et al.*, 1974).

Figure A. Diagramatic representation of the GI Tract of the three fish species

Figure A. Diagramatic representation of the GI Tract of the three fish species

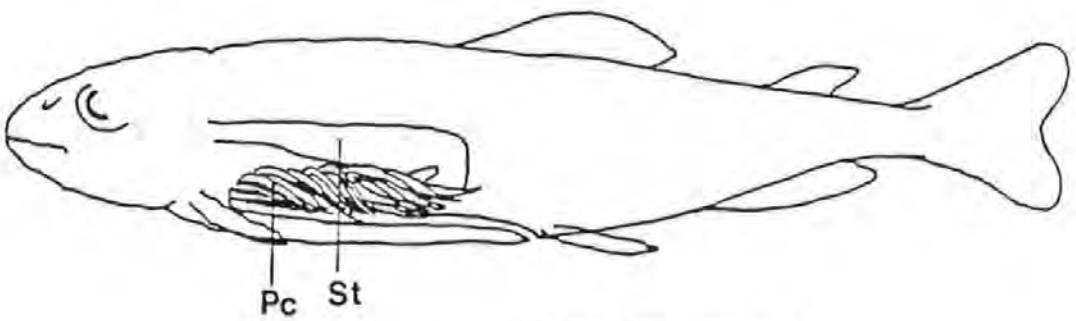
Oreochromis mossambicus

1



Salmo gairdneri

2



Cyprinus carpio

3

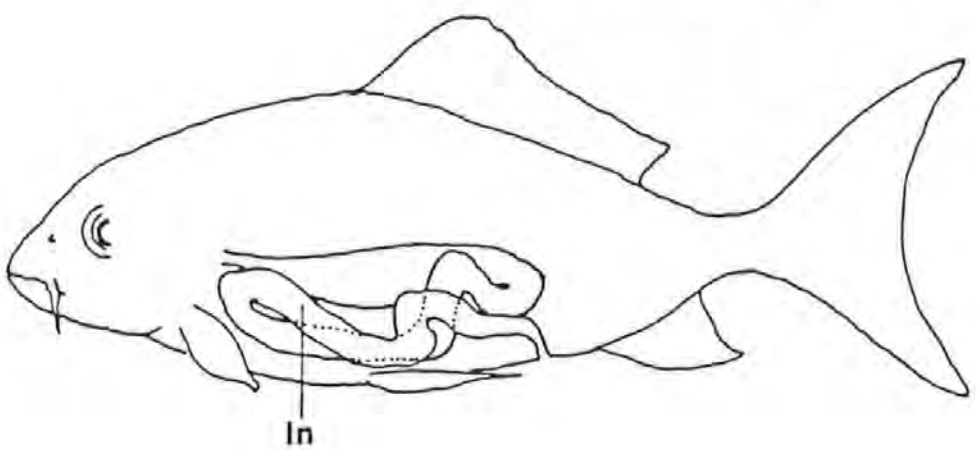


Figure 1. Oral and Anal BSA dose response

Fig.1a ORAL BSA dose response

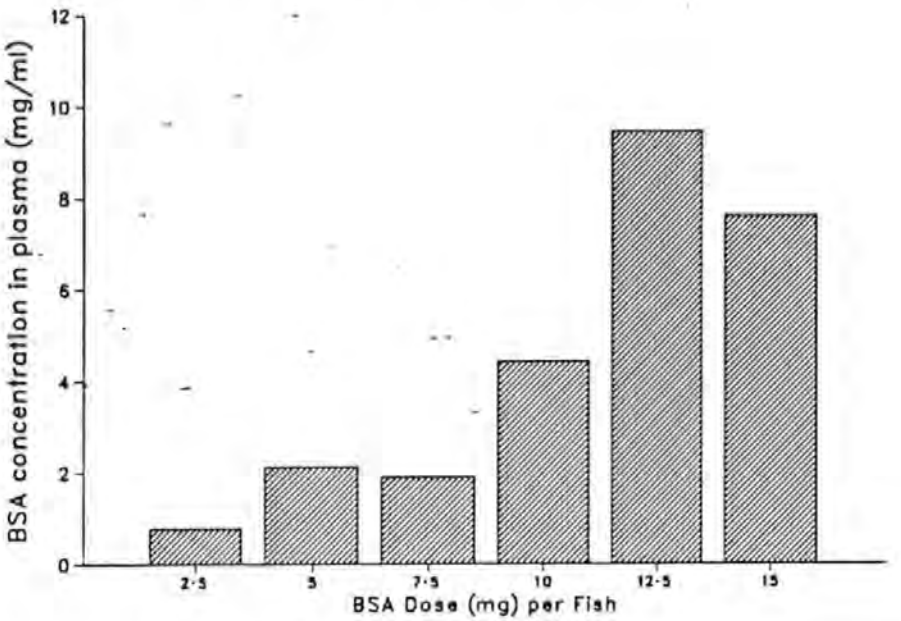


Fig1b ANAL BSA dose reponse

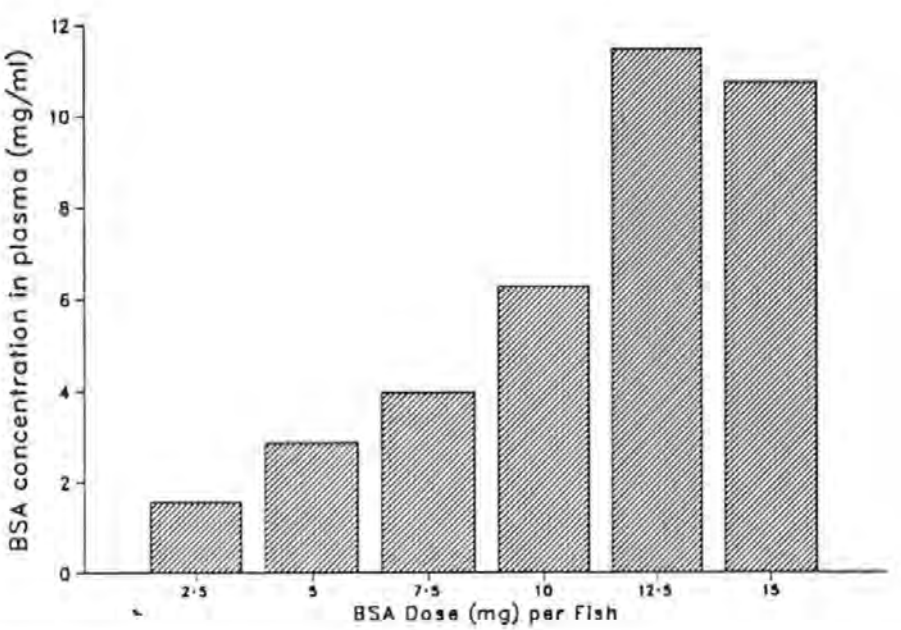


Figure 2a-d. Effect of the state of nutrition on the absorption of BSA in Juvenile Tilapia

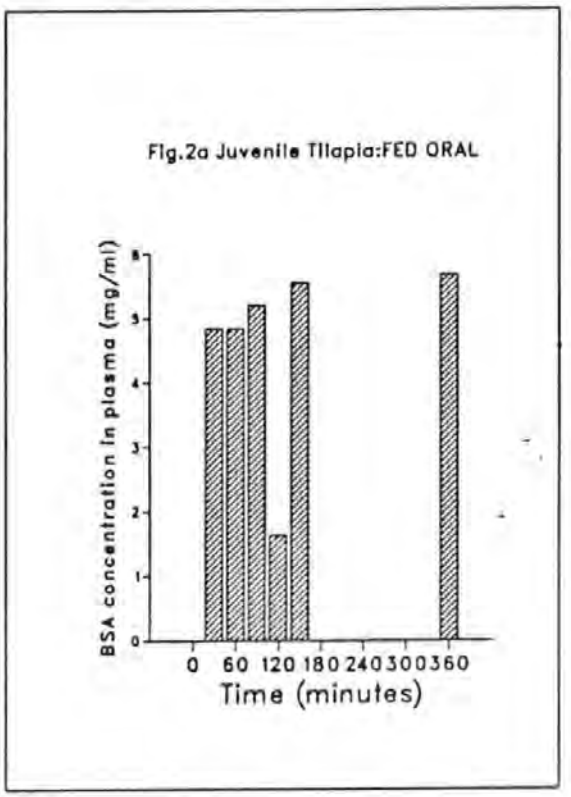


Fig.2a Juvenile Tilapia:FED ORAL
BSA concentration in plasma (mg/ml)
0 1 2 3 4 5 6
0 60 120 180 240 300 360
Time (minutes)

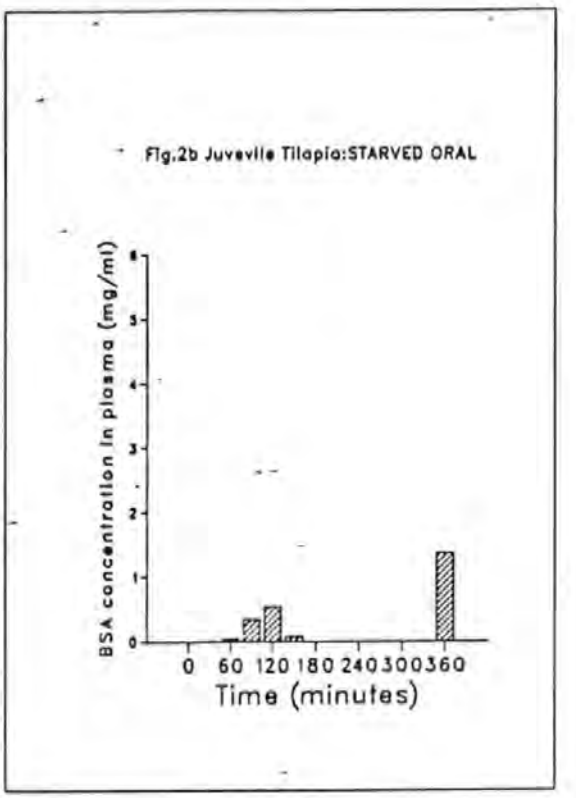


Fig.2b Juvenile Tilapia:STARVED ORAL
BSA concentration in plasma (mg/ml)
0 1 2 3 4 5 6
0 60 120 180 240 300 360
Time (minutes)

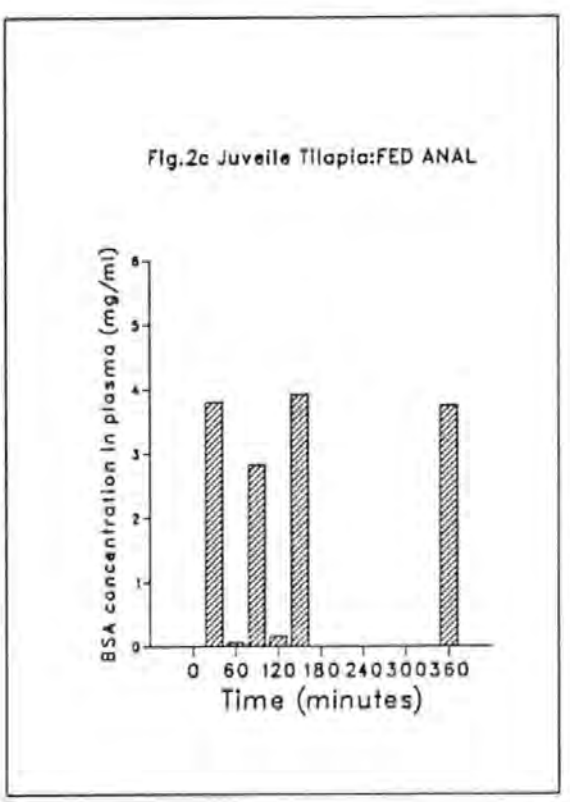


Fig.2c Juvenile Tilapia:FED ANAL
BSA concentration in plasma (mg/ml)
0 1 2 3 4 5 6
0 60 120 180 240 300 360
Time (minutes)

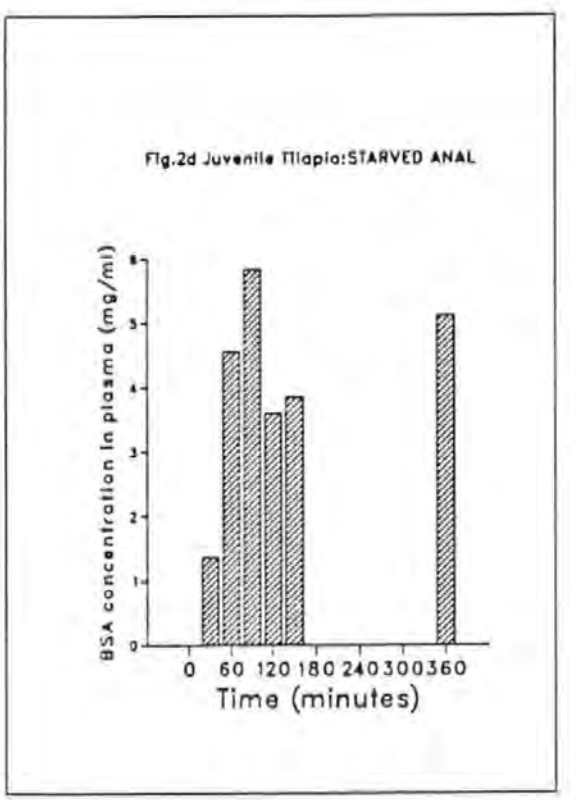
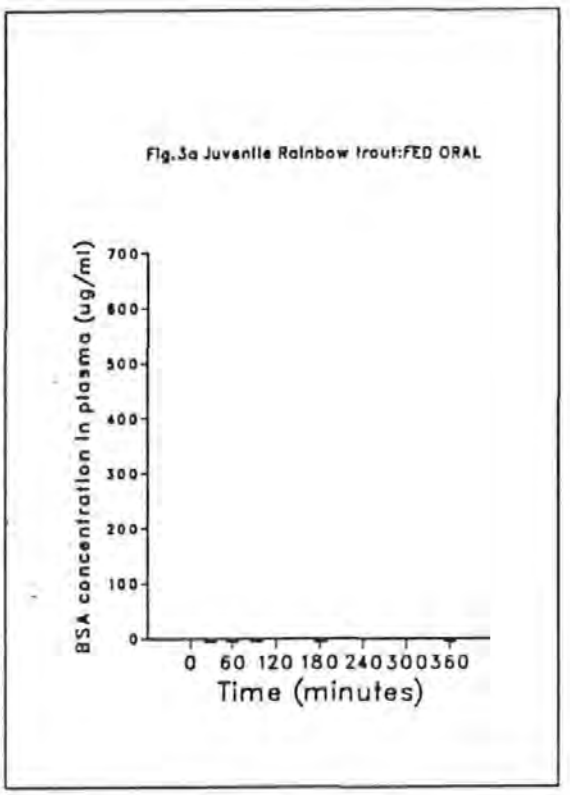
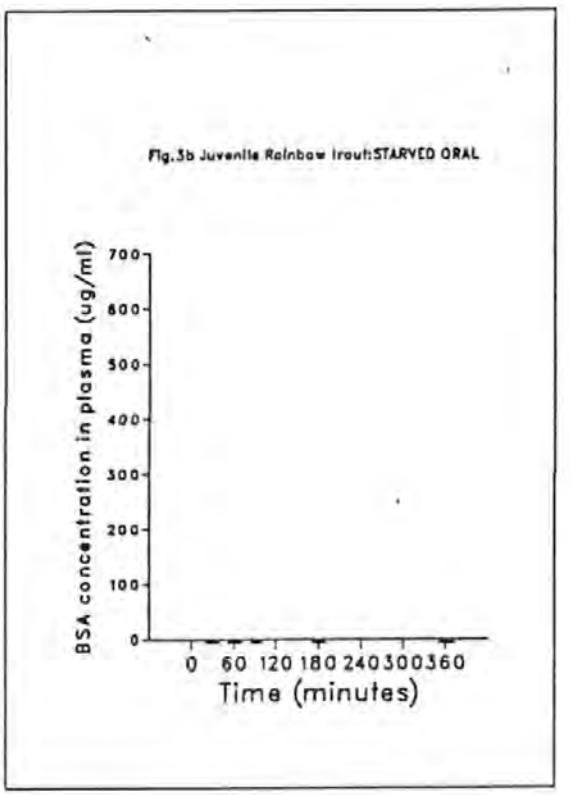
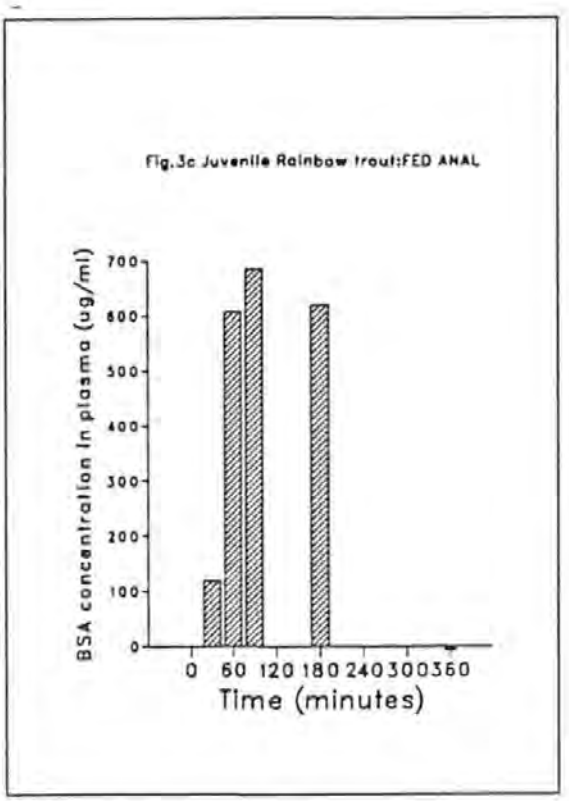


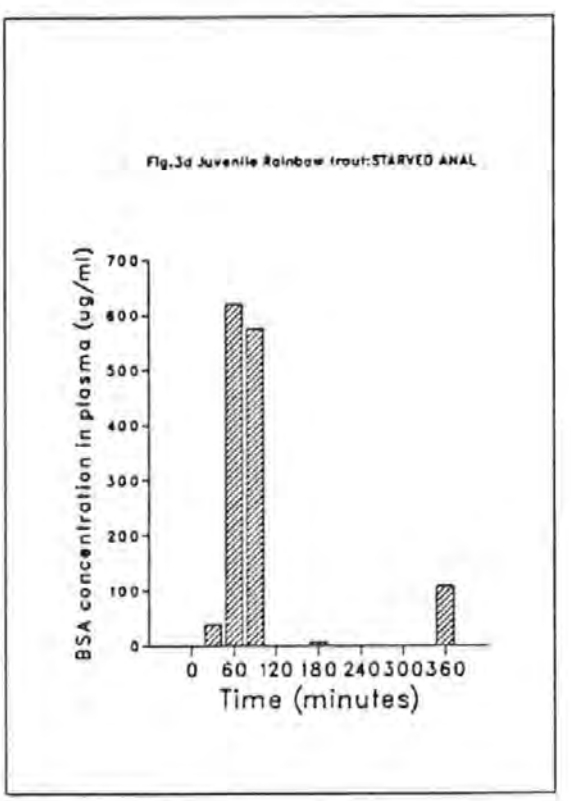
Fig.2d Juvenile Tilapia:STARVED ANAL
BSA concentration in plasma (mg/ml)
0 1 2 3 4 5 6
0 60 120 180 240 300 360
Time (minutes)

Figure 3a-d. Effect of the state of nutrition on the absorption of BSA in Juvenile Rainbow trout



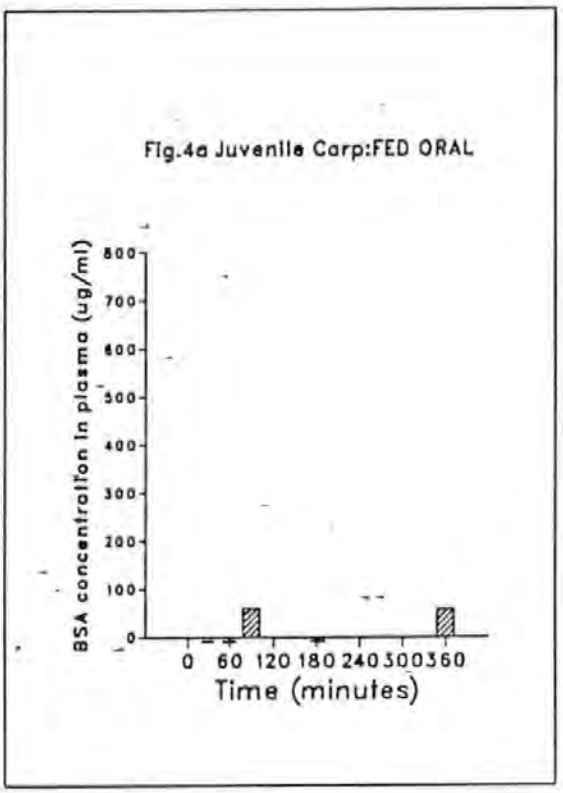


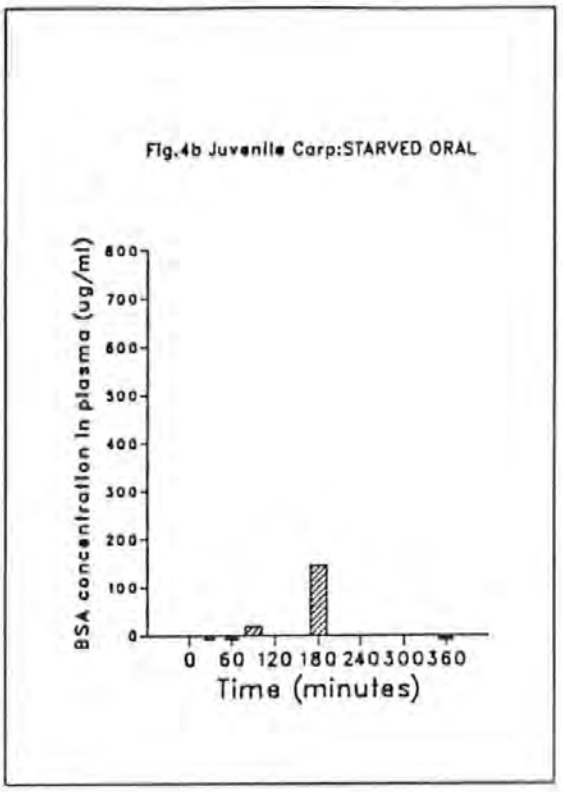


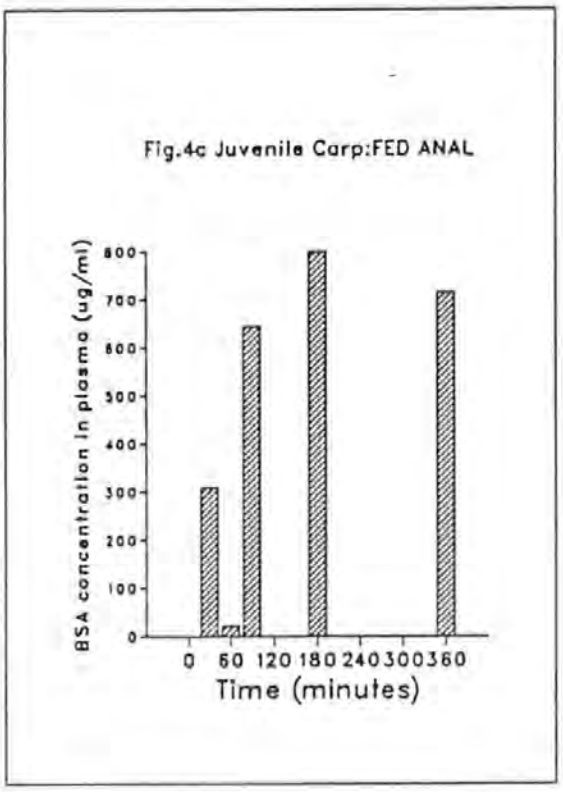


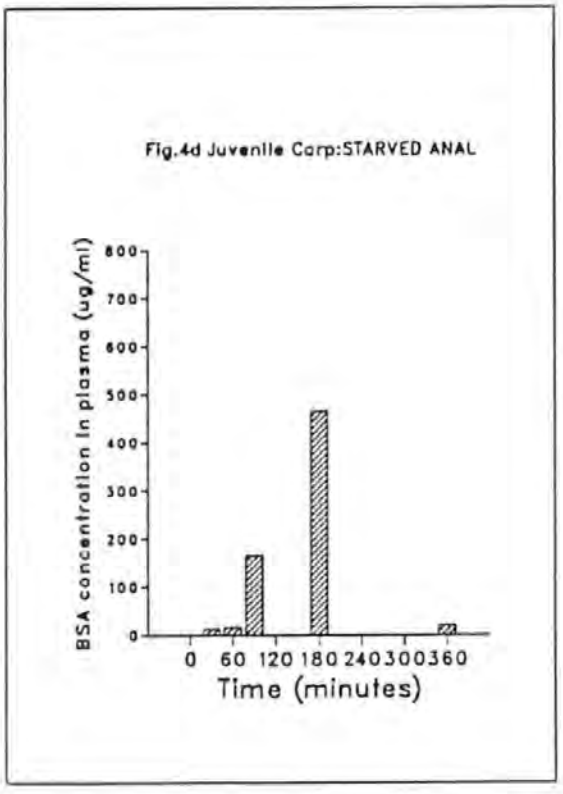
— less than zero

Figure 4a-d. Effect of the state of nutrition on the absorption of BSA in Juvenile Carp









— less than zero

Figure 5a-c. Multiple Oral dose in Juvenile Tilapia

Fig.5a Juvenile Tilapia:ORAL DOSE 1

Fig.5b Juvenile Tilapia:ORAL DOSE 2

Fig.5c Juvenile Tilapia:ORAL DOSE 3

— less than zero

Figure 6a-c. Multiple Anal dose in Juvenile Tilapia

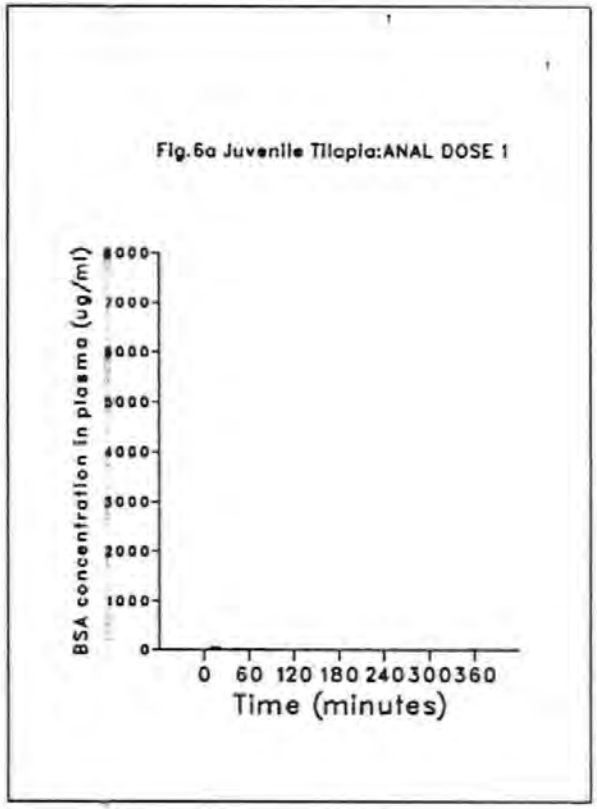


Fig.6a Juvenile Tilapia:ANAL DOSE 1
BSA concentration in plasma (ug/ml)
8000
7000
6000
5000
4000
3000
2000
1000
0
0 60 120 180 240300360
Time (minutes)

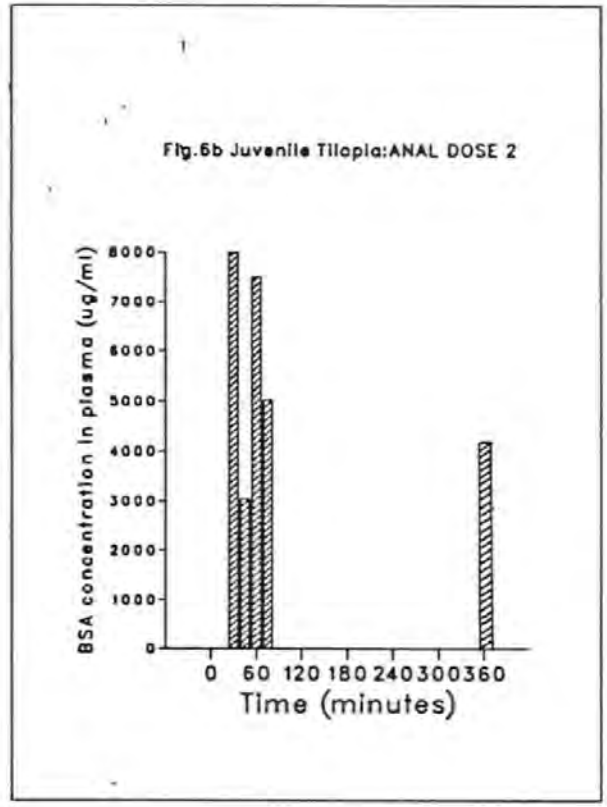


Fig.6b Juvenile Tilapia:ANAL DOSE 2
BSA concentration in plasma (ug/ml)
8000
7000
6000
5000
4000
3000
2000
1000
0
0 60 120 180 240300360
Time (minutes)

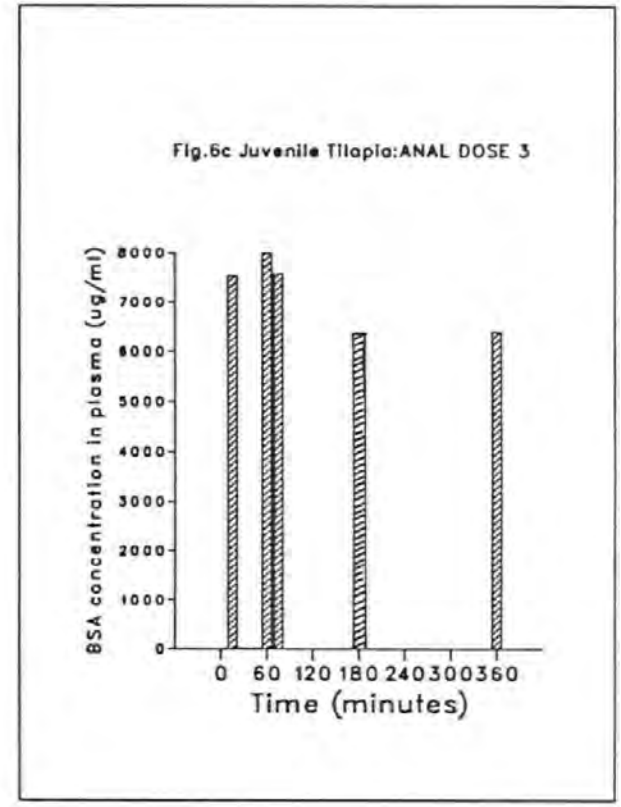


Fig.6c Juvenile Tilapia:ANAL DOSE 3
BSA concentration in plasma (ug/ml)
8000
7000
6000
5000
4000
3000
2000
1000
0
0 60 120 180 240300360
Time (minutes)

Figure 7a-c. Multiple Oral dose in Juvenile Rainbow trout

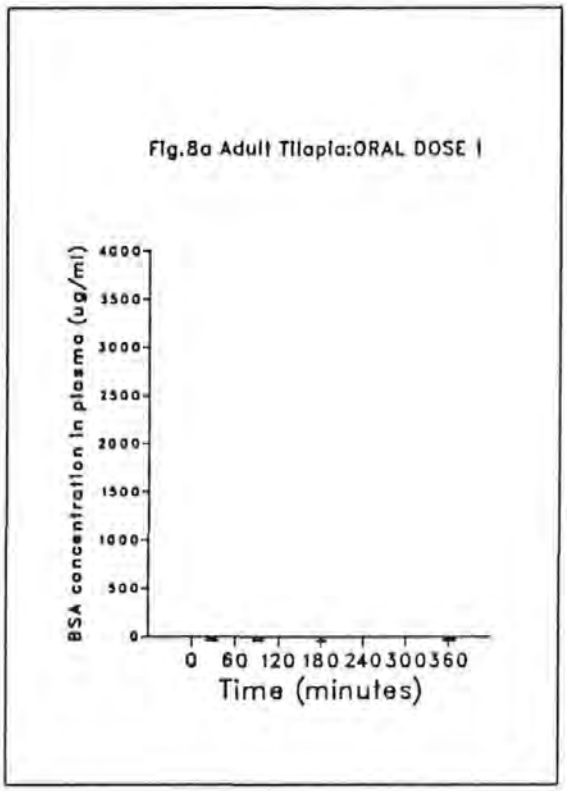
Fig.7a Juvenile Rainbow trout:ORAL DOSE 1

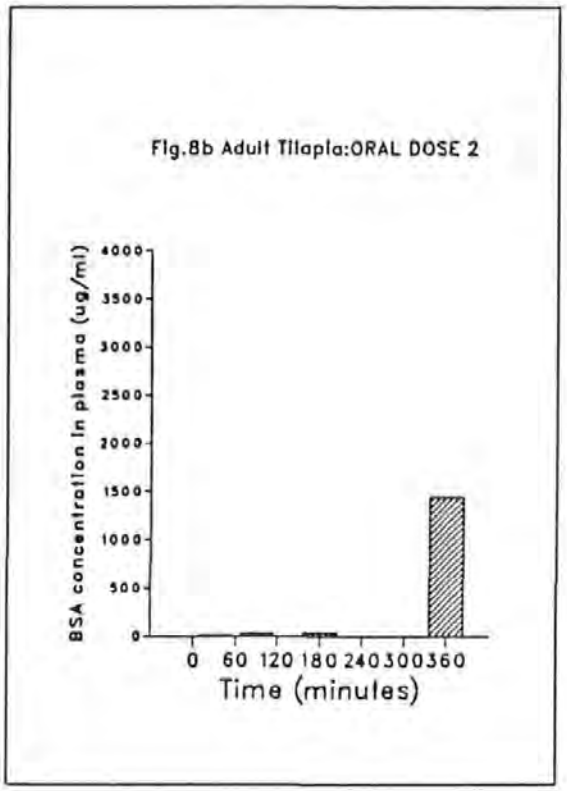
Fig.7b Juvenile Rainbow trout:ORAL DOSE 2

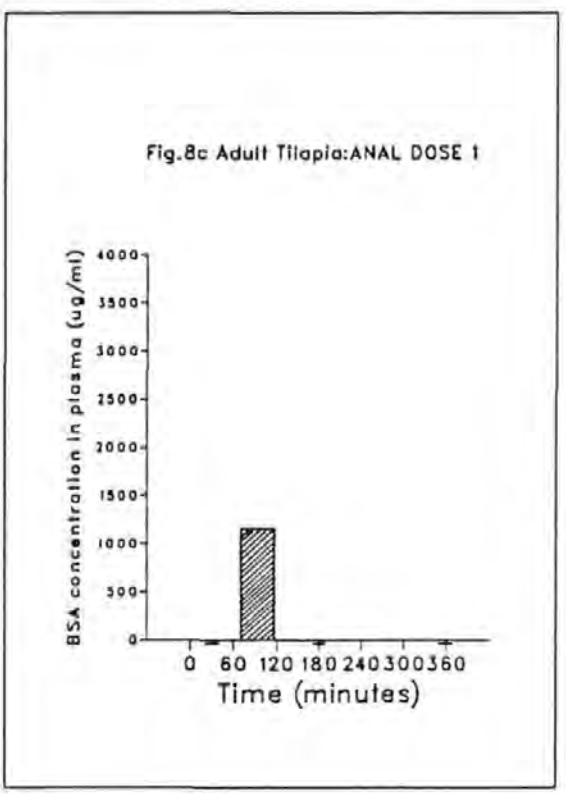
Fig.7c Juvenile Rainbow trout:ORAL DOSE 3

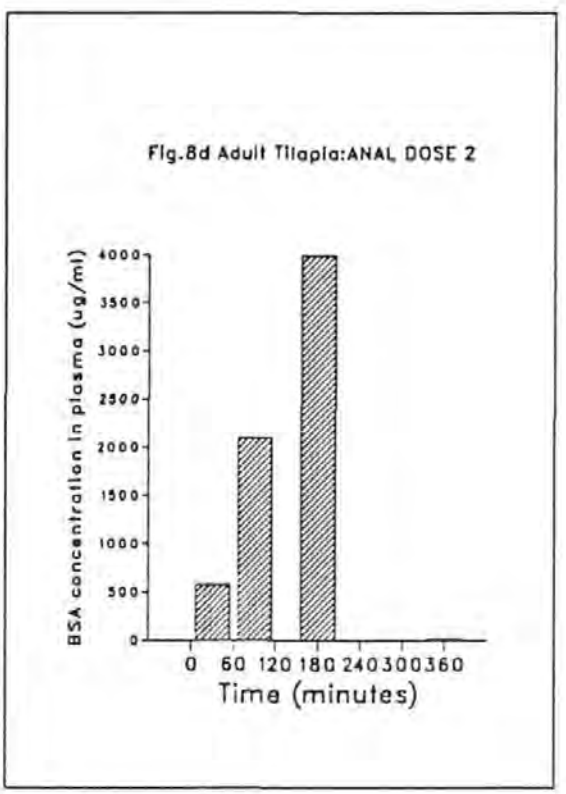
— less than zero

Figure 8a-d. Multiple Orail and Anal dose in Adult Tilapia









— less than zero

Figure 9a-c. Multiple Oral dose in Adult Rainbow trout

Fig.9a Adult Rainbow trout:ORAL DOSE 1

Fig.9b Adult Rainbow trout:ORAL DOSE 2

Fig.9c Adult Rainbow trout:ORAL DOSE 3

— less than zero

Figure 10a-c. Multiple Anal dose in Adult Rainbow trout

Fig.10a Adult Rainbow trout:ANAL DOSE 1

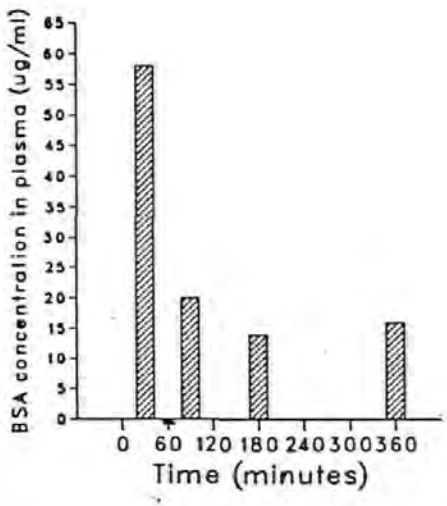


Fig.10b Adult Rainbow trout:ANAL DOSE 2

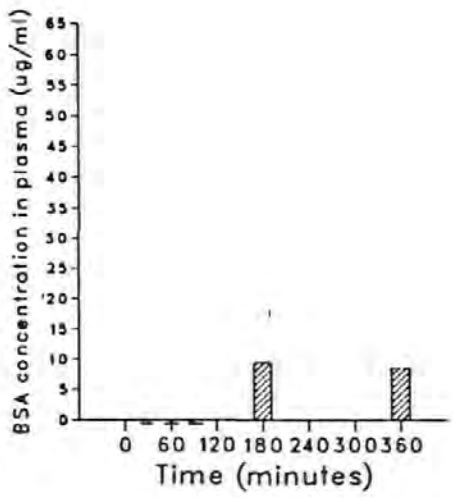
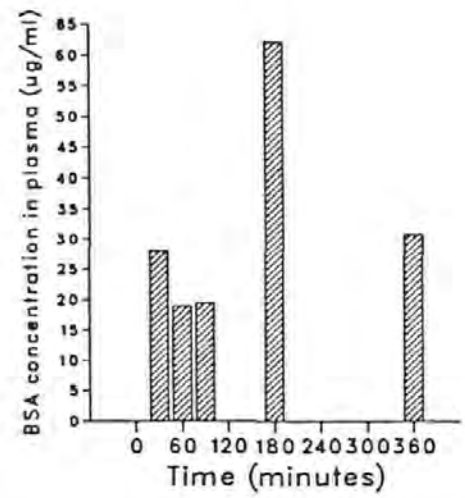


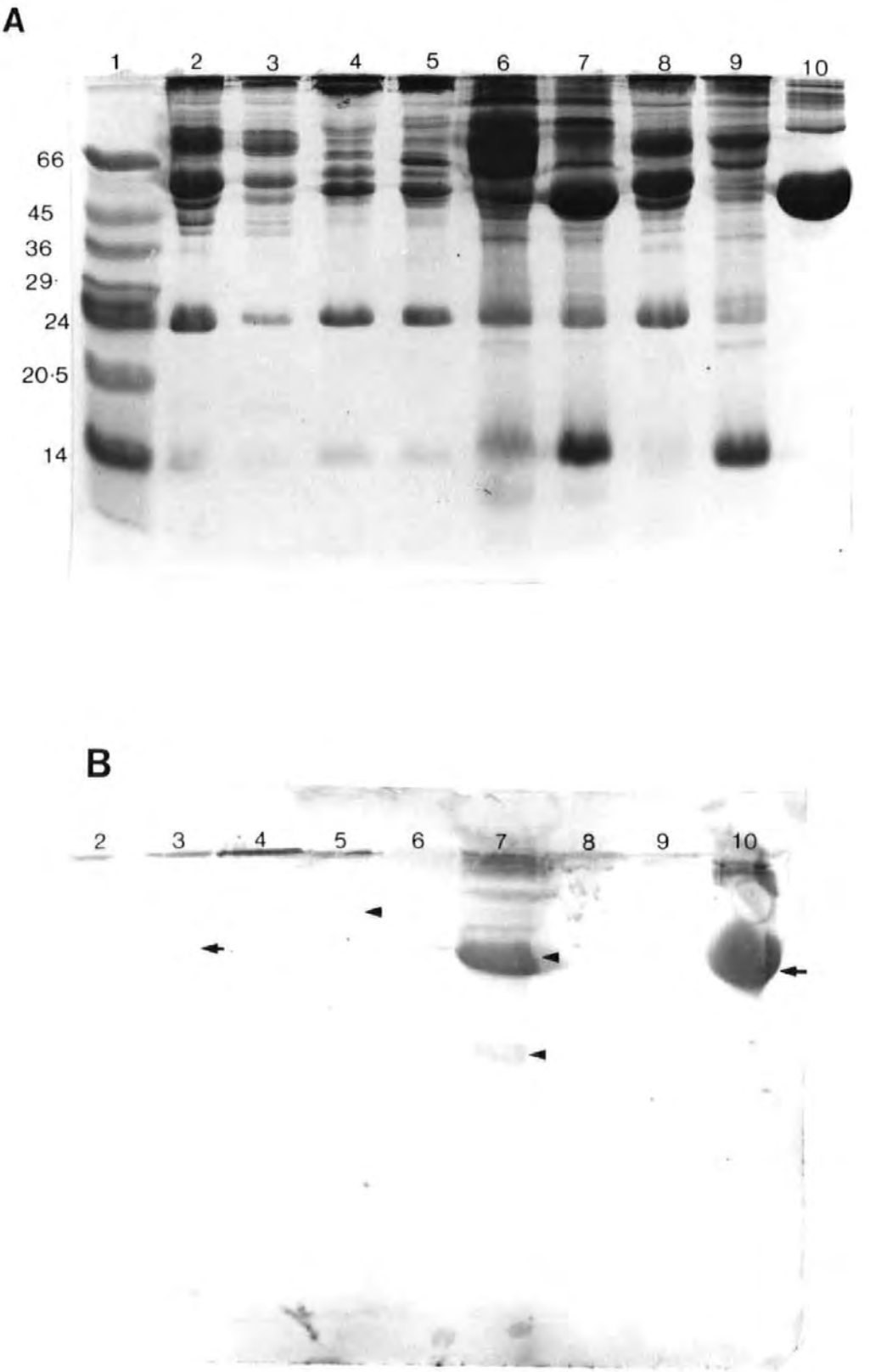
Fig.10c Adult Rainbow trout:ANAL DOSE 3



— less than zero

Figure 11. Photomicrograph of the SDS gel and Immunoblot

Figure 11 Photomicrographs of the SDS gel and Immunoblot



DISCUSSION

The alimentary tract of *O.mossambicus* was found to contain a number of leucocytes, some showing a morphological similarity to those in the peripheral blood, while others were unique to the gut tissue. These intestinal leucocytes were found mainly as a diffuse cell population, and only occasionally as discrete lymphoid accumulations within the intestinal tissue. Ontogenic studies showed that a limited number of leucocytes were found in the gut tissue after hatching, however, there was a gradual increase in these numbers once exogenous feeding began.

An acceptable uniform nomenclature of fish leucocytes, particularly granulocytes, has often proved difficult especially when the major criteria is their morphological analogy with mammalian leucocytes. Most authors examining leucocytes attempt to devise a specific nomenclature which is exaggerated by the fact that there is such morphological variation between species (Hine *et al.*, 1987) and even within species (Hine & Wain, 1987a; 1987b; 1987c). The techniques used to enumerate the leucocytes within the gut tissue also vary but in the majority of cases the distribution of IEL in the gut of lower vertebrates is not evaluated.

It is believed that antigenic stimulation of the gut plays an important role in the establishment of the lymphoid

cell populations in the gut mucosa; plasma cells being absent from the tissues of foetal lambs where antigen is not present (Reynolds, 1981). In *O.mossambicus* it was found that there was an increase in the number of IEL found in the intestine of developing larvae, particularly at the onset of exogenous feeding. In mammals the location and distribution of leucocytes in the gut is independent of antigen exposure (Ferguson & Parrott, 1972; Husband & Gowans, 1978), however, there is an increase in the number of IEL in the presence of antigens (Ferguson, 1977). That the number of IEL in the fish gut epithelium is influenced by the presence of antigen has been demonstrated by Davina *et al.* (1980; 1982). They found that following the administration of bacterial antigens into the gut of carp and rosy barb there was an increase in the number of IEL in the intestinal epithelium of both fish species. It is therefore important to examine the interaction between and the presentation of antigens in the gut lumen and the intestinal leucocytes.

The mammalian alimentary tract has been shown to be permeable to macromolecules which are then transported into the systemic circulation. A number of studies have indicated that fish intestinal enterocytes were capable of absorbing macromolecules (Georgopoulou & Sire, 1986; Georgopoulou *et al.*, 1984, 1985, 1988; Lamers, 1985; Rombout *et al.*, 1985; Watanabe, 1981; 1982; 1984a: 1984b: 1984c). Only recently has an attempt to quantify these absorbed macromolecules that are transported into the systemic circulation been carried out (McLean & Ash, 1986; 1987; McLean *et al.*, 1988a;

1988b; Suzuki *et al.*, 1988). In this study it was shown that a variety of macromolecules varying in size, @40,000-500,000 daltons, were absorbed and transported into the systemic circulation by the intestine of *O.mossambicus*, a gastric species and that greater amounts were detected in the plasma of juvenile fish compared to adult fish. A comparison of the absorption of macromolecules by the intestine of another gastric fish, rainbow trout, and an agastric species, carp, revealed that greater levels of intubated antigen were detected in the plasma of *O.mossambicus*. This would seem to indicate that the ability of the intestine to absorb macromolecules may not necessarily depend on whether or not fsih possess a stomach and that it varies from species to species. It is known that a number of digestive enzymes are secreted by the gut, and that these differ from species to species, also that the pH required for the optimum action of these enzymes varies (Ash, 1985; Fange & Grove, 1979). Perhaps of more relevance is the type, and quantity of enzymes that are secreted by the stomach and intestine and also the pH of those secretions.

Macromolecules were detected in the plasma of juvenile *O.mossambicus* and carp after oral intubation but not in juvenile and adult rainbow trout or adult tilapia, however, after repeated oral intubation BSA was detected in the plasma of these fish. Results of studies on the effect of oral administration of bacterial antigens also show species variation. Buras *et al.* (1985) showed that live bacteria and

viruses orally intubated into carp and *T.nilotica* could penetrate the intestinal epithelium, these organisms being subsequently isolated from a number of internal organs, whereas Tatner *et al.* (1984) could not detect either the live or formalin-killed orally intubated bacteria within the internal organs of rainbow trout. Davina *et al.* (1980; 1982) demonstrated that *Vibrio* bacterin was taken up by the intestinal enterocytes of the rosy barb and remained there for up to 6 days, resulting in an increase in the number of IEL in this area. The reported levels of protection following oral immunisation also varies, but the majority of studies seem to agree that protection is greater when there is at least 8 weeks between the immunisation and challenge, resulting in levels of 50-85% protection (Agius, Horne & Ward, 1983; Johnson & Amend, 1983), the duration of protection lasting, in some cases, up to 113 days (Kawano, Aoki & Kitao, 1984).

One feature which has emerged from the macromolecule absorption investigations undertaken in this study was that greater levels of absorbed macromolecules were detected in the plasma of anally intubated fish. This was true for both juveniles and adults of all three fish species. It was also found that the levels of macromolecules detected in the plasma after anal intubation were lower in adult fish. Johnson & Amend (1983) found that a single anal intubation of either *Yersinia ruckeri* or *Vibrio anguillarum* in rainbow trout resulted in better protection against a subsequent challenge, compared to either oral intubation or immersion

vaccination. Hofer & Scheimer (1981) commented on the disppearance of proteolytic activity in the hind-gut of fish, possibly due to the reabsorption of digestive enzymes in this region. In tilapia there is a gradual decrease in the pH along the length of the gut (Moriarty, 1973) which may also play a part in the higher levels detected in the plasma following anal intubation. This observation may, in part, explain the observations made during this study and by Johnson & Amend (1983). Also the absorption of macromolecules may be influenced by the maturity of the fish intestinal enterocytes, as observed in mammals (Walker, 1987), there being a decrease in the detectable levels of macromolecules in the plasma of adult fish compared to the levels in juvenile fish.

It was also found in this investigation that the feeding of the experimental animals prior to the intubation of BSA did not significantly affect the levels of this macromolecule transported into the systemic circulation. This is interesting as some chemotherapeutics used to treat infections in fish are delivered to starved fish, however if the chemotherapeutic does not have an adverse effect on fed fish, there would seem to be no advantage in starving fish prior to treatment but to continue with a 'normal' feeding regime. Two other considerations that must be taken into account in the development of oral vaccine are, providing a diet that will not impair or depress the immune response and seriously affect disease resistance (Blazer & Wolke, 1984) and, the palatability of the diet-vaccine formulation.

An understanding of how the macromolecules may be modified by the gut secretions also needs to be investigated. It was shown in this study that the BSA molecule was partially degraded either by extracellular gut secretions or intracellular digestion. This resulted in the detection of low molecular weight proteins that were antigenic, and which were not found in the original BSA sample. This has also been reported in mammalian studies (Walker & Bloch, 1983; Warshaw *et al.*, 1974). Benjamin & Holaday (1987) indicated that the site of cleavage of BSA and the resulting fragments, depended on the type of enzymes used to generate these fragments. Each fragment bears antigenic determinants for at least one of the three domains in the BSA macromolecule. Although enzymatic cleavage by the gut enzymes does result in the formation of some antigenic fragments, it is clear from this study and mammalian investigations that essentially 'intact' macromolecules are absorbed and trans ported into the systemic circulation by the intestine. To determine which fragment or fragments are generated would require raising monoclonal antibodies to these fragments.

In general it has been shown that a mucosal immune response is generated following oral intubation of antigens, these being higher than those found in the serum (Fletcher & White, 1973; Hart, 1987; Rombout *et al.*, 1986). This would correlate with the increase in the number of Ig secreting cells found within the intestinal tissue that is observed following antigen intubation (Hart, 1987; Tomonaga *et al.*,

1984). Rombout *et al.* (1986), however, demonstrated that anal intubation of bacteria resulted in systemic antibody titres almost equivalent to those following i.m. injection. This observation together with those of Johnson & Amend (1983) seems to indicate that if bacteria, given orally, could reach the posterior unaffected by the digestive processes of the gut, systemic immunity may be elicited. Antigens that are absorbed by the intestinal enterocytes have been found within intestinal macrophages (Lamers, 1985; Rombout *et al.*, 1985), however, the exact interactions between the absorptive enterocytes, intestinal leucocytes and the absorbed antigens needs to be examined.

Oral vaccines for fish have not yet been totally successful (Hart *et al.*, 1988) although this mode of immunisation would be potentially the most useful, reducing the stress, due to handling and anaesthesia, on fish that is imposed in the techniques that are now available. The possible large scale immunisation regime that may be employed if the vaccine could in some way be incorporated into the food would be welcomed by fish farmers. However, this method of vaccination would depend on the protective immunity generated by consumption of this diet-vaccine product, and perhaps more on ensuring that each individual animal obtains an optimum dose. The correlation between the amount of antigen absorbed and the amount that is needed to generate protective immunity needs to be investigated.That the antigen must escape enzymatic hydolysis within the intestinal lumen has led investigators in mammalian

immunology to examine the use of penetration enhancers, such as polyoxythylene-9- lauryl ether and sodium glycholate (Davis, 1987), and immunoadjuvants, such as saponins, aluminium hydroxide (Al OH) (Bomford, 1988) and liposomes (Gregoriadis, 1988).

The gastrointestial tract has extremely efficient mechanisms to restrict the entry of macrocolecules, including enzymatic hydrolysis which degrades proteins and peptides, also varying levels of immunoglobulins which neutralise antigens before and after they are absorbed. The use of penetration enhancers to alter membrane permeability and coadministration of inhibitors to restrain the activity of proteolytic enzymes at the absorption site are strategies that may be employed to enhance absorption by the intestine. However, totally harmless absorption enhancers have yet to be discovered, and the implications of long term therapy on possible damage to the mucosa need to be investigated. Also these modifiers delivered by the oral route are usually unstable and are degraded by a number of digestive enzymes themselves. The approach of using absorption enhancers to enhance the uptake of peptides and proteins in the GI tract, albeit empirical, could lead to successful clinical applications in mammalian immunology.

The use of immunoadjuvants is an ongoing strategy in the development of mammalian vaccines. In the literature it has been stated that they should not induce hypersensitivity to host tissue nor to the adjuvant, should not possess cross-reactive antigens nor carcinogenic, teratogenic or

abortogenic activities and that they should not be toxic for myelolymphoid cells (Stewart-Tull, 1988). Both saponin and aluminium hydroxide are used in veterinary and human vaccines and have recently been examined for their adjuvant activity in fish. Cossarini-Dunier (1985) reported that a combination of saponin and aluminium hydroxide given IP with SRBC had no adjuvant properties in rainbow trout. Grayson, Williams, Wrathmell, Munn & Harris (1987) also reported that the immersion of rainbow trout in a mixture of saponin (Quil-A) and ERM vaccine did not result in a conclusive improvement of the immune response as compared to immersion in vaccine alone. Agius *et al.* (1983) however, reported that the addition of alum as adjuvant to either an antigen extract of or whole, formalin- killed *V.anguillarum* enhanced the response to antigen administered by both IP and oral routes. The possible use of antigen-adjuvant combinations together with the incorporation of penetration enhancers into these formulations for oral vaccines, to enhance the immune reponse requires further investigation

Macromolecules express a large number of potential antigenic epitopes at their surface, not all of which are immunogenic. This has led to the investigations into the development of synthetic peptide vaccines. The most recent advances though involve using a replicating vector by the insertion of a genome of an infectious agent into a harmless, infectious vector, such as vaccinia and *Salmonella* type 21a (Steward & Howard, 1987), providing a means to stimulate all arms of the immune response.

The crucial aspects which must be considered for the future development of oral vaccines both in mammalian and fish immunology are: the form in which the antigen is to be administered, the protection of the antigen from the hydrolytic enzymes in the GI tract by such methods as microencapsulation and enteric coating, whether proteolytic inhibitors, immunopotentiating agents or penetration enhancers are used, and possibly the controlled release of antigen which may reduce transit time through the gut.

BIBLIOGRAPHY

Abrahamson,D.R. & Rodewald,R. (1981) Evidence for the sorting of endocytic vesicle contents during the receptor-mediated transport of IgG across the newborn rat intestine. J. Cell Biol., 91, 270-280.

van Alten,P.J. (1980) Ontogeny of RES function in birds. In *The Reticuloendothelial System: a Comprehensive Treatise. Vol. 3. Ontogeny and Phylogeny.* Ed. N.Cohen & M.M.Sigel. Plenum Press, New York. pp 659-685.

Agius,C. & Agbede,S.A. (1984) An electronmicroscopical study on the genesis of lipofucsin, melanin and haemoseridin in the haemopoietic tissues of fish. J. Fish Biol., 24, 471-488.

Agius,C., Horne,M.T. & Ward,P.D. (1983) Immunisation of rainbow trout *Salmo gairdneri* Richardson against vibriosis: comparison of an extract with whole cell bacterins by oral and intraperitoneal routes. J. Fish Dis., 6, 129-134.

Al-Adhani,M.A. & Kunz,Y.W. (1976) Haemopoietic centres in the developing Angelfish *Pterophyllum scalare* (Cuvier & Valenciennes). Wilhelm Roux's Arch. Dev. Biol., 199, 393-401.

Andre,C., Heremans,J.F., Vaerman,J.P. & Cambiaso,C.L. (1975) A mechanism for the induction of immunological tolerance by antigen feeding antigen-antibody complexes. J. Exp. Med., 142, 1509-1519.

Ambler,L. & Peters,G.E. (1984) An immunospecific enzyme assay for horseradish peroxidase. Analyt. Biochem., 137, 66-68.

Ardavin,C.F., Zapata,A., Villena,A. & Solas,T. (1982) GALT in the amphibian urodele *Pleurodeles waltlii*. J. Morph., 173, 35-41.

Ash,R. (1985) In *Nutrition and Feeding.* Ed. C.B.Cowey, A.M.Mackie & J.G.Bell. Academic Press, London. pp 69-98.

Ash,R. & Mason,S.L. (1983) Preparation, characterisation and utilisation of isolated enterocytes from carp (*Cyprinus carpio*) for the study of intact protein absorption. IVth. International Symposium Protein Metabolism and Nutrition. (Unpublished conference contribution)

AshersonG.L., Zembala,M., Perera,M.A.C.C., Mayhew,B. & Thomas,W.R. (1977) Production of immunity and unresponsiveness in the mouse by feeding contact sensitising agents and the role of suppressor cells in the Peyer's Patches, mesenteric lymph nodes and other lymphoid tissues. Cell. Immunol., 33, 145-153.

Avakian,S. (1964) Further studies on the absorption of chymotrypsin. Clin. Pharmacol. Theory, 5, 712-715.

Avtalion,R.R. & Sharabani,K. (1975) Studies on the phagocytosis in fish. I. *In vitro* uptake and killing of living *Staphylococcus aureus* by the peripheral leucocytes of carp, *Cyprinus carpio*. Immunology, 29, 1181-1187.

Bainter,K. (1986) *Intestinal Absorption of Macrmolecules and Immune transmission from Mother to Young*. CRC Press, Florida.

Bainter,K. & Kocsis,Gy. (1984) Occurence and some characteristics of the IgG-receptors of the small intestinal mucosa. *In vitro* experiments. Acta. Vet. Hung., 32, 76-81.

Baldwin,W.M. & Cohen,N. (1970) Liver-induced immuno-suppression of allograft immunity in urodele amphibians. Transplantation, 10, 530-534.

Barber,D.L. & Mills Westermann,J.E. (1981) The blood cells of the Antarctic icefish *Chaenocephalus aceratus* Lonnberg: light and electron microscopic observations. J. Fish Biol., 19, 11-28.

Beard,J. (1902-3) The origin and histogenesis of the thymus in *Raja batis*. Zool. Jahrbuch., 17, 403-480.

Befus,A.D. & Bienenstock,J. (1982) Mucosal Immunity. Immunology, 41, 249-270.

Benjamin,D.C. & Holaday,B.J. (1987) T-cell responses to a complex antigen. Ir-gene control, fine specificity and helper potential. In *Immunogenicity of Protein Antigens: Repertoire and Regulation*. Ed. E.E.Sercarz & J.A.Berzofsky. CRC Press, Florida. pp 83-93.

Bergeron,T. & B.Woodward (1983) Ultrastructure of the granule cells in the small intestine of the rainbow trout (*Salmo gairdneri*) before and after stratum granulosum formation. Can. J. Zool., 61, 133-138.

Bergot,P. (1976) Demonstration par le rouge de Ruthenium d'invaginations profondes de la membrane plasmique apicale des enterocytes dans l'intestin posterieur chez la truite arc en ciel. Ann. Biol. Biochem. Biophysiol., 16, 37-42.

Beynon,R.J. & Kay,J. (1976) Enteropancreatic circualtion of digestive enzymes. Nature, 260, 78-79.

Bienenstock,J. (1984) Mucosal barrier functions. Nutrit. Rev., 42, 105-108.

Blaxhall,P.C. & Daisley,K. (1973) Routine haematological methods for use with fish blood. J. Fish Biol., 5, 771-781.

Blaxhall,P.C & Hood,K. (1985) Cytochemical staining of fish lymphocytes separated on a Percoll gradient. J. Fish Biol., 27, 749-755.

Blazer,V.S. & Wolke,R.E. (1984) Effect of diet on the immune response of rainbow trout (*Salmo gairdneri*). Can. J. Fish Aquat. Sci., 41, 1244-1246.

Blecha,F., Bull,R.C., Olsin,D.P., Rosa,R.H. & Curtis,S. (1981) Effects of preparation restriction in the beef cow on immunoglobulin content in the blood and colostral whey and subsequent immunoglobulin absorption by the foetal calf. J. Anim. Sci., 53, 1174-1177.

Bloch,K.J., Bloch,D.B., Steams,M. & Walker,W.A. (1979) Intestinal uptake of macromolecules. VI.Uptake of protein antigen *in vivo* in normal rats and rats infected with *Nippostrongulus brasiliensis* or subjected to mild systemic anaphylaxis. Gastroenterology, 77, 1039-2044.

Blok,J., Mulder-Stropel,A.A., Ginsel,L.A. & Th Daems, W. (1981) Endocytosis in absorptive cells of the cultured human small intestinal tissue: HRP, lactoperoxidase and ferritin as markers. Cell Tissue Res., 216, 1-13.

Bockman,D.E. & Winborn,W.B. (1966) Light and electron microscopy of intestinal ferritin absorption. Observations in sensitised and non-sensitised hamsters (*Mesocricetus auratus*). Anat. Rec., 155, 603-622.

Boedeker,E.C. (1982) Enterocyte adherence of *Escherichia coli*: its relation to diarrhoeal disease. Gastroenterology, 83, 489-492.

Bomford,R. (1988) Aluminium salts: perspectives in their use as adjuvants. NATO-ASI *Immunological Adjuvants and Vaccines*. Cape Sounion, Greece. (Unpublished conference contribution)

Bone,Q. & Marshall,N.B. (1982) In *Biology of Fishes*. Blackie Publications, London. pp 80-82.

Boomker,J. (1980) The haematology and histology of the haemopoietic organs of South African fresh-water fish. II.Erythrocytes and Thrombocytes of *Clarius gariepinus* and *Sarotherodon mossambicus*. J. Vet. Res., 47, 95-100.

Borysenko,M. & Cooper,E.L. (1972) Lymphoid tissue in the Snapping turtle, *Chelydra serpentina*. J. Morph., 138, 487-498.

Botham,J.W. & Manning,M.J. (1981) The histogenesis of the lymphoid organs in the carp *Cyprinus carpio* L. and the ontogenic development of allograft reactivity. J. Fish Biol., 19, 403-414.

Brambell,F.W.R. (1958) The passive immunity of the young mammal. Biol. Rev., 33, 488-531.

Brambell,F.W.R. (1970) The transmission of passive immunity from mother to young. In *Frontiers of Biology.* Vol. 18. North Holland, Amsterdam. pp 20-41.

Brambell,F.W.R., Halliday,R. & Morris,I.G. (1958) Interference by human and bovine serum protein fractions with the absorption of antibodies by suckling rats and mice. Proc. R. Soc. Lond. Ser. B, 149, 1-12.

Brandon,M.R. & Lascelles,A.K. (1971) Relative efficiency of absorption of IgG_1, IgG_2, IgA and IgM in the newborn calf. J. Exp. Biol. Med. Sci., 49, 629-633.

Brandtzaeg,P. (1974) Mucosal and glandular distribution of immunoglobulin components: differential localisation of free and bound secretory component in secretory epithelial cells. J. Immunol., 112, 1553-1555.

Bresson,J.L., Pang,K., Udall,J., Fritze,L. & Walker,W.A. (1980) Evidence for increased enterotoxin binding to newborn microvillous membranes: A possible explanation for enhanced toxigenic diarrhoea in infancy. Gastroenterology, 78, 1145-

Breuton,M.J. (1980) Immune mechanisms and the gut. Eur. J. Pediatr. 133, 201-205.

Brodsky,F.M. (1981) The intracellular traffic of immunologically active molecules. Immunol. Today, 5, 350-357.

Brostoff,J. & Challacombe,S.T. (1987) *Food Allergy and Intolerance.* Bailliere/Tindall, London.

Bruce,M.G. & Ferguson,A. (1986) The influence of intestinal processing on the immunogenicity and molecular size of absorbed, circulating ovalbumin in mice. Immunology, 59, 293-300.

Buddington,R.K. & Doroshev,S.I. (1986) Structural and functional relations of the white sturgeon (*Acipenser transmonatus*). J. Morph., 190, 201-213.

Buras,N., Duek,L. & Niv,S. (1985) Reactions of Fish to Microorganisms in Wastewater. App. Enviro. Microsc. October, 989-995.

Burrows,A.S. & Fletcher,T.C. (1986) Blood Leucocytes of the turbot, *Scophthalmus maximus* L. PAMAQ II, Oporto, Portugal. (Unpublished conference contribution)

Casely-Smith,J.R. (1967) The passage of ferritin into jejunal epithelial cells. Experientia, 23, 370-371.

Cawley,J.C. & Burns,G.F.(1980) The cytochemistry of human lymphoreticular sub-populations. Immunol. Today, 1, 85-86.

Challacombe,S.J., Greenall,C. & Stoker,T.A.M. (1987) A comparison of IgA in portal and peripheral venous blood. Immunology, 60, 111-116.

Challacombe,S.J. & Tomasi,B. (1980) Systemic tolerance and secretory immunity after oral immunisation. J. Exp. Med., 152, 1459-1462.

Challacombe,S.J. & Tomasi,T.B. (1987) Oral Tolerance. In *Food Allergy and Intolerance.* Ed. J.Brostoff & S.J.Challacombe. Bailliere/Tindall, London. pp 255-268.

Chalon,M-P., Milne,M.W. & Vaerman,J-P. (1979) *In vitro* immunosuppressive effect of serum from orally immunised mice. Eur. J. Immunol., 9, 797-751.

Chen,S-T. & Tobe,T. (1974) Cellular sites of immunoglobulin. V.An immunological study of the human gall bladder. Digestion, 10, 184-190.

Christie,K.N. & Stoward,R.J. (1978) Endogenous peroxidase in mast cells localised with a semi-permeable membrane technique. Histochem. J., 10, 435-433.

Clark.S.L. (1959) The ingestion of proteins and colloidal materials by columnar absorptive cells of the small intestine of suckling rats and mice. J. Biophys. Biochem. Cytol., 5, 41-50.

Clough,E.R. & Dean,H.J. (1988) Isolation and characterisation of lymphocytes from bovine intestinal epithelium and lamina propria. Vet. Immunol. Immunopathol., 19, 39-49.

diConza,J.J. & Halliday,W.T. (1971) Relationship of catfish antibodies to immunoglobulin in mucous secretions. Austr. J. Exp. Biol. Med. Sci., 49, 517-519.

Corbel,M.J. (1975) The immune response of fish: a review. J. Fish Biol., 7, 539-563.

Cornell.R., Walker,W.A. & Isselbacher,K.J. (1971) Small intestinal absorption of HRP. A cytochemical study. Lab. Invest., 25, 42-48.

Cornes,J.S. (1965) Number, size and distribution of Peyer's Patches in the human small intestine I. The development of Peyer's Patches. Gut, 6, 225-229.

Cossarini-Dunier,M. (1985) Effect of different adjuvants on the humoral immune response of rainbow trout. Dev. Comp. Immunol., 9, 141-146.

Craig,S.W. & Cebra,J.J. (1971) Peyer's Patches: An enriched source of precursors for IgA-producing immunocytes in the rabbit.J. Exp. Med., 134, 188-193.

Curman,B., Kampfe,O., Rask,L. & Peterson,P.A. (1979) Presence of alloreactive Ia antigens on murine intestine epithelial cells. Scand. J. Immunol., 10, 11-14.

Daems,W.Th. (1968) On the fine structure of human neutrophilic leucocyte granules. J. Ultrastr. Res., 24, 343-348.

Daimon,T., Mizuhira,V. & Uchida,K. (1979) Fine structural distribution of the surface-connected canicular system in frog thrombocytes. Cell Tissue Res., 201, 431-440.

Dakin,R. (1829) Remarks on a cutaneous infection produced by certain poisonous vegetables. Am. J. Med. Sci., 4, 98.

Danneaus,A., Ingrames,M., Johansson,S.G.O. & Foucard,T. (1979) Intestinal uptake of ovalbumin in malabsorption and food allergy in relation to serum IgG antibody and orally administered sodium cromoglycate. Clin. Aller., 9, 263-270.

Davina,J.H.M., Rijkers,G.T., Rombout,J.H.W.M., Timmermans,L.P.M. & van Muiswinkel,W.B. (1980) Lymphoid and non-lymphoid cells in the intestine of cyprinid fish. In *Development and Differentiation of Vertebrate Lymphocytes.* Ed J.B.Horton. Academic Press, Amsterdam. pp 129-140.

Davina,J.H.M., Parmentier,H.K. & Timmermans,L.P.M. (1982) The effect of oral administration of *Vibrio* bacterin on the intestine of cyprinid fish. Dev. Comp. Immunol., Supp.2, 157-166.

Dieterlen-Lievre,F. (1987) In *Vertebrate Blood Cells: Chapter 5, Birds.* Ed. A.F.Rowley & N.A.Ratcliffe. Cambridge University Press. pp 139-175.

Dobbins,W.O. (1982) Gut immunophysiology: a gastro-enterologists view with emphasis on pathophysiology. Amer. J. Physiol., 242, G1-G8.

Doggett,T.A., Wrathmell,A.B. & Harris,J.E. (1987) Ontogeny of the gut-associated lymphoid tissue in *Oreochromis mossambicus.* J. Fish Biol., Supp. A, 31, 23-27.

Drzewina,A. (1905) Contribution a l'etude du tissue lymphoide des Ichthyopsides. Arch. Zool. Exp. Gen., 4, 145-338.

Dvorak,A.F. & Rast,R.C. (1970) Nature of the immunogen in crystalline serum albumins. Immunochemistry, 7, 118-124.

El Deeb,S., El Ridi,R. & Zada,S. (1986) The development of lymphocytes with T and B membrane determinants in the lizard embryo. Dev. Comp. Immunol., 10, 353-364.

El Ridi,R., El Deeb,S. & Zada,S. (1981) The gut-associated lymphoepithelial tissue (GALT) of lizards and snakes. In *Aspects of Developmental and Comparative Immunology.* Ed. J.B.Solomon. Pergamon Press, Oxford. pp 233-239.

Ellis,A.E. (1976) Leucocytes and related cells in the plaice *Pleuronectes platessa.* J. Fish Biol., 8, 143-156.

Ellis,A.E. (1977a) The leucocytes of fish: a review. J. Fish Biol., 11, 453-491.

Ellis,A.E. (1977b) Ontogeny of the immune response in *Salmo salar.* Histogenesis of the lymphoid organs and appearance of membrane immunoglobulin and mixed leucocyte activity. In *Developmental Immunobiology.* Ed. J.B.Solomon & J.D.Horton. Elsevier/North Holland Biomedical Press, Amsterdam. pp 225-231.

Ellis,A.E. (1980) Antigen-trapping in the spleen and kidney of the plaice *Pleuronectes platessa* L. J. Fish Dis., 3, 413-426.

Ellis,A.E., Munro,A.L.S. & Roberts,R.J. (1976) Defence mechanisms in fish.I. A study of the phagocytic system and the uptake of intraperitoneally injected particulate material in the plaice. J. Fish Biol., 8,67-78.

Enders,G., Gottwald,T. & Brendel,W. (1986) Induction of oral tolerance in rats without Peyer's Patches. Immunology, 58, 2, 311-314.

Engel,R.H. & Riggi,S.J. (1969) Effect of sulfated and non-sulfated surfactants on the intestinal absorption of heparin. Proc. Soc. Exp. Biol. Med., 130, 879-884.

Engle,R.L.Jnr. & Woods,K.R. (1957) Phylogenesis of plasma proteins and plasma cells. II. Observations on the occurrence of plasma cells in marine invertebrates and fishes. Biol. Bull., 113, 363-365.

Ernst,P.B., Befus,A.D. & Bienenstock,J. (1985) Leucocytes in the intestinal epithelium Immunol. Today, 6, 50-55.

Ezeasor,D.N. & Stokoe,W.H. (1981) Light and electron microscopic studies on the absorptive cells of the intestine, caeca and rectum of the adult rainbow trout, *Salmo gairdneri* Richardson. J. Fish Biol., 18, 527-544.

Ezzat,A.A., Shabana,M.B. & Farghaly,A.M. (1974) Studies on the blood characteristics of *Tilapia zilli* (Gervais). I. Blood cells. J. Fish Biol., 8, 1-12.

Fange,R. & Grove,D. (1979) Digestion. In *Fish Physiology. Vol. VIII. Bioenergetics and Growth.* Academic Press, London. pp 161-260.

Ferguson,A. (1977) Intraepithelial lymphocytes of the small intestine. Gut, 18, 921-937.

Ferguson,H.W. (1976) The ultrastructure of plaice (*Pleuronectes platessa*) leucocytes. J. Fish Biol., 8, 139-142.

Ferguson,A. & Parrott,D.M.V. (1972) The effect of antigen deprivation on thymus-dependent and thymus-independent lymphocytes in the small intestine of the mouse. Clin. Exp. Immunol., 12, 477-480.

Fichtelius,K.E. (1968) Bursa equivalents of bursaless vertebrates. Lab. Invest., 19, 339-351.

Fichtelius,K.E., Finstad,J. & Good,R.A. (1969) The phylogenetic occurrence of lymphocytes within the gut epithelium. Int. Arch. Aller., 35, 119-133.

Finn,J.P. & Nielsen,N.O.(1971) The inflammatory response of rainbow trout. J. Fish Biol., 3, 463-478.

Fletcher,T.C. & White,A. (1973) Antibody production in the plaice *Pleuronectes platessa* after oral and parenteral immunisation with *Vibrio anguillarum* antigens. Aquaculture, 1, 417-428.

Frazier,J.A. (1973) Ultrastructure of the chick thymus. Z. Zellforsch. Mikrosk. Anat. 136, 191-205.

Freter,R. (1970) Mechanisms of action of intestinal antibody in experimenal cholera. II. Antibody-mediated antibacterial reaction at the mucosal surface. Infect. Immunol., 2, 556-562.

Gardner,G.R. & Yevich,P.P. (1969) Studies on the morphology of three estuarine Cyprinidontiform fishes. J. Fish Res. Bd. Can., 26, 433-447.

Gauthier,G.F. & Landis,S.C. (1972) The relationship of ultrastructural and cytochemical features to absorptive activity in the goldfish intestine. Anat. Rec., 172, 675-702.

Georgopoulou,U., Sire,M. & Vernier,J. (1984) Macromolecular absorption of proteins by the epithelial cells of the posterior segment and their intracellular digestion in the rainbow trout, ultrastructural and biochemical study. Cell Tissue Res., 215, 397-401.

Georgopoulou,U., Sire,M. & Vernier,J. (1985) Macromolecular absorption of proteins by epithelial cells of the posterior intestinal segment and their intracellular digestion in the rainbow trout. Ultrastructural and Biochemical Study. Biol. Cell, 53,269-282.

Georgopoulou,U., Sire,M. & Vernier,J. (1986) Immunological demonstration of intestinal absorption and digestion of protein macromolecules in the trout. (*Salmo gairdneri*). Cell Tissue Res., 245, 387-395.

Georgopoulou,U. & Vernier,J.M. (1986) Local immunological response in the posterior intestinal segment of the rainbow trout after oral administration of macromolecules. Dev. Comp. Immunol., 10, 529-537.

Georgopoulou,U, Dabrowski,K., Sire,M.F. & Vernier,J.M. (1988) Absorption of intact proteins by the intestinal epithelium of trout, *Salmo gairdneri*. Cell Tissue Res., 251, 145-152.

Goldstine,S.N., Manickavel,V. & Cohen,N. (1975) Phylogeny of gut-associated lymphoid tissue. Am. Zool., 15, 107-118.

Goetz,H. & Rothmann,S.S. (1978) Amylase transport across the ileal epithelium *in vitro*. Biochem. Biophys. Acta., 512, 214-220.

Good,R.A., Finstad,J., Pollara,B. & Gabrielsen,A.E. (1966) Morphologic studies on the evolution of the lymphoid tissues among the lower vertebrates. In *Phylogeny of Immunity*. Ed. R.T.Smith, P.A.Meischer & R.A.Good. University of Florida Press, Gainesville. pp 149-168.

Govoni,J.J., Boehlert,G.W. & Watanabe,Y. (1986) The physiology of digestion in fish larvae. Environ. Biol. Fish, 16, 59-77.

Grace,M.F. (1981) The functional histogenesis of the immune system in rainbow trout, *Salmo gairdneri*. PhD Thesis, University of Hull, U.K.

Graney,D.O. (1968) The uptake of ferritin by ileal absorptive cells in suckling rats. An electron microscope study. Am. J. Anat., 123, 227-254.

Grayson,T.H., Williams,R.J., Wrathmell,A.B., Munn,C. & Harris,J.E. (1987) Effects of immunopotentiating agents on the immune response of rainbow trout, *Salmo gairdneri* Richardson, to ERM vaccine. J. Fish Biol., Supp. A, 31, 195-202.

Gregoriadis,G. (1988) Liposomes as immunological adjuvants. Optimisation studies. NATO-ASI *Immunological Adjuvants and Vaccines*. Cape Sounion, Greece.(Unpublished conference

Grimaldi,M.C., D'Ippolito,S., Pica,A.M & Della Corte,F. (1983) Cytochemical identification of the leucocytes of torpedoes (*Torpedo marmorata* Risso and *Torpedo ocellata* Rafinesque). Basic Appl. Histochem., 27, 311-317.

Gruskay,F.L. & Cooke,R.E. (1955) The gastrointestinal absorption of an unaltered protein in normal infants and in infants recovering from diarrhea. Pediatrics, 16, 763-768.

Hall,J.G., Hopkins,J. & Orlans,E. (1977) Studies on the lymphocytes of sheep.III. Destination of lymph-borne immunoblasts in relation to their tissue of origin. Eur. J. Immunol., 7, 30-37.

Halliday,R. (1956) The termination of the capacity of young rats to absorb antibody from milk. Proc. R. Soc. Lond. Ser. B, 145, 179-182.

Harmatz,P.P., Kleinman,R.E., Bunnell,B.W., McClethan,D.T., Walker,W.A. & Block,K.J. (1982) Hepatobiliary clearance of IgA immune complexes formed in the circulation. Hepatology, 2, 328-333.

Harmatz,P.P., Kleinman,R.E., Bunnell,B.W., McClethan,D.T., Walker,W.A. & Block,K.J. (1984) The effect of bile duct obstruction on the clearance of circulating IgA immune complexes. Hepatology, 4, 96-100.

Hart,S. (1987) Immunity in the alimentary tract and other mucosae of the dogfish *Scyliorhinus canicula* L. PhD Thesis, Plymouth Polytechnic, U.K.

Hart,S., Wrathmell,A.B. & J.E. Harris (1986a) Gut-associated lymphoid tissue in the dogfish *Scyliorhinus canicula*: a light microscopic study. J. Mar. Biol. Assoc. (UK)., 66, 721-729.

Hart,S., Wrathmell,A.B., Harris,J.E. & Doggett,T.A. (1986b) Ontogeny of gut-associated lymphoid tissue (GALT) in the dogfish *Scyliorhinus canicula*: a light microscopicic study. Vet. Immunol. Immunopathol., 12, 107-116.

Hart,S. Wrathmell,A.B., Harris,J.E. & Doggett,T.A. (1987a) Aspects of the immunology of the urinogenital tract of the elasmobranch, *Scyliorhinus canicula* L. Dev. Comp. Immunol., 10, 597-602.

Hart,S., Wrathmell,A.B., Doggett,T.A & Harris,J.E. (1987b) An investigation of the biliary and intestinal immunoglobulin and plasma cell distribution in the gall bladder and liver of the common dogfish, *Scyliorhinus canicula* L. Aquaculture, 67, 147-155.

Hart,S., Wrathmell,A.B., Grayson,T.H. & Harris,J.E. (1988) Gut Immunology: A review. Dev. Comp. Immunol., 12, 453-480.

Hayhoe,F.G. & Flemens,J. (1969) *An Atlas of Haematological Cytology*. Second Edition. Wolfe Publications, London.

Hayhoe,F.G.J. & Quaglino,D. (1980) *Haematological Cytochemistry*. Churchill Livingstone, Edinburgh.

Hemmings,W.A. & Williams,E.W. (1977) Quantitative and visualisation studies of the transport of rat and bovine IgG and ferritin across the segments of the small intestine of the suckling rat. Proc. R. Soc. Lond. Ser. B, 197, 425-440.

Henning,S.J. (1979) Biochemistry of intestinal development. Enviro. Health Per., 33, 9-16.

Henry,M. & Charlemagne,J. (1977) Plasmocytic series in the perihepatic layer of the urodele amphibian *Pleurodeles waltlii* Micah (Salamandridae). Dev. Comp. Immunol., 1, 23-32.

Herbst,G. (1844) Das lymphagefassystem und sine verrichtung. Vandenhoeck & Ruprecht, Gottinham. pp 333-342.

Heyman,M., Ducroc,R., Desjeux,J.F. & Morgat,J.L. (1982) Horseradish peroxidase transport across adult rabbit jejunum *in vitro*. Am. J. Physiol., 242, G558.

Hine,P.M. & Wain,J.M. (1987a) Composition and ultrastructure of elasmobranch granulocytes. I. Dogfishes (Squaliformes). J. Fish Biol., 30, 547-556.

Hine,P.M. & Wain,J.M. (1987b) Composition and ultrastructure of elasmobranch granulocytes. II. Rays (Rajiformes). J. Fish Biol., 30, 557-566.

Hine,P.M. & Wain,J.M. (1987c) Composition and ultrastructure of elasmobranch granulocytes. III. Sharks (Lamniformes). J. Fish Biol., 30, 567-576.

Hine,P.M., Wain,J.M. & Boustead,N.C. (1987) The leucocyte enzyme chemistry of fish. N.Z. Fish Res. Bull., 28, 1-74.

Hjelmeland,K., Huse,I., Jorgensen,T., Molvik,G. & Raa,J. (1984) Trypsin and trypsinogen as indices of growth and survival potential of cod (*Gadus morhua* L.) larvae. Flod. rapportser., 1, 189-202.

Hjelmeland,K., Pederson,B.M. & Nilssen,E.M. (1988) Trypsin content in intestines of herring larvae, *Clupea harengus*, ingesting inert polystyrene spheres or live crustacea prey. Mar. Biol., 98, 331-335.

Hofer,R. & Scheimer,F. (1981) Proteolytic activity in the digestive tract of several species of fish with different feeding habits. Oceologia, 48, 342-345.

Holt,P.G., Vines,J. & Britten,D, (1988) Suppression of IgE responses by antigen inhalation failure of tolerance mechanisms in newborn rats. Immunology, 63, 591-593.

Holstein,B. (1975) Gastric secretion in a teleostean fish: A method for the continuous collection of gastric effluence from a swimming fish and its response to histamine and pentagastrin. Acta. Physiol. Scan., 95, 417-423.

Hudson,L. & Hay,F.C. (1980) *Practical Immunology*. Second Edition. Blackwell Publications, London.

Hugon,J.S. & Borgers,M. (1968) Absorption of HRP by the mucosal cells of the duodenum of mouse. I.The fasting animal. J. Histochem. Cytochem., 16, 229-236.

Husband,A.J. & Gowans,J.L. (1978) The origin and antigen dependent distribution of IgA containing cells in the intestine. J. Exp. Med., 148, 5, 1146-1160.

Hussein,M.F., Badir,N., El Ridi,R. & Akef,M. (1978) Differential effect of seasonal variation on lymphoid tissue of the lizard, *Chalcides ocellatus*. Dev. Comp. Immunol., 2, 297-310.

Hyder,S.L., Cayer,M.L. & Pethy,C.L. (1983) Cell types in the peripheral blood of the nurse shark: An approach to structure and function. Tiss. Cell, 15, 437-455.

Iida,H. & Yamamoto,T. (1985) Intracellular transport of horseradish peroxidase in the absorptive cells of goldfish hindgut *in vitro*, with special reference to the cytoplasmic tubules. Cell Tissue Res., 240, 553-560.

Illum,L. (1986) Microspheres as a potential controlled release nasal drug delivery system. In *Delivery Systems for Peptide drugs*. Ed. S.S.Davis, L.Illum & E.Tomlinson. NATO ASI Series, Plenum Press, London. pp 205-211.

Inman,L.R. & Cantey,J.R. (1983) Specific adherence of *Escherichia coli* (Strain RDEC-1) to membraneous (M) cells of the Peyer's patches in *E.coli* diarrhoea in the rabbit. J. Clin. Invest., 71, 1-8.

Iwai,T. (1968a) Fine structure and absorption patterns of the intestinal epithelial cells in rainbow trout alevins. Z. Zellforsch Mikrosc. Anat. Dev. Biol., 91, 368-379.

Iwai,T. (1968b) The comparative study of the digestive tract of teleost larvae. V.Fat absorption in the gut epithelium of goldfish larvae. Bull. Jpn. Soc. Sci. Fish., 34, 973-978.

Iwai,T. (1969) Fine structure of gut epithelial cells of larval and juvenile carp during absorption of fat and protein. Arch. Histol. Jpn., 30, 183-199.

Iwai,T. & Tanaka,M. (1968) The comparative study of the digestive tract of teleost larvae. III.Epithelial cells in the posterior gut of the halfbeak larvae. Bull. Jpn. Soc. Sci. Fish., 34, 44-48.

Jakobshagen,E. (1937) Darmystem IV. Mittel und Enddarm. Rumpfdarm. In *Handbuch der vergleichenden Anatomie der Wirbeltiere*. Vol.III. Ed. L.Bolk, E.Cooper, E.Kallius, & W.Lubosch. Urban und Schwarzenberg, Berlin und Wien. pp 563-724.

Jarrett,E.E. (1984) Immunoregulation of IgE responses: the role of the gut in perspective. Ann. Aller.,53, 550-556.

Jeurissen,S.H.M., David,S. & Sminia,T. (1985) Uptake of particulate and soluble antigens in the small intestine of the rat. Cell. Biol. Int. Rep., 9, 523-559.

Jones,R.E. (1972) Intestinal absorption and gastrointestinal digestion of protein in the young rat during the normal and cortisone-induced post-closure period. Biochem. Biophysiol. Acta., 274, 412- ..

Jones,E.A. & Waldmann,T.A. (1972) The mechanism of intestinal uptake anf transcellular transport of IgG in the new born rat. J. Clin. Invest., S1, Z916-Z927.

Johnson,K.A. & Amend,D.F. (1983) Efficacy of *Vibrio anguillarum* and *Yersinia ruckeri* bacterins applied by oral and anal intubation to salmonids. J. Fish Dis., 6, 473-476.

Jordan,H.E. & Speidel,C.C.(1924) Studies on Lymphocytes II. The origin, function and fate of the lymphocytes in fishes. J. Morph., 38, 529-549.

Jurd,R. (1977) Secretory immunoglobulins and gut-associated lymphoid tissue of *Xenopus laevis*. In *Developmental Immunobiology*. Ed. J.B.Solomon & J.D.Horton. Elsevier/North-Holland, Biomedical Press, Amsterdam. pp 307-313.

Jurd,R., John,I., and Garney,L. (1988) Gut-associated lymphoid tissue (GALT) in Urodeles. ISDCI Proceedings, Nottingham. (Unpublished conference contribuion)

Kagnoff,M.F. (1978a) Effects of antigen feeding on intestinal and systemic responses. III. Antigenic-specific serum mediated suppression of humoral antibody responses after antigen feeding. Cell. Immunol., 40, 186-198.

Kagnoff,M.F. (1978b) Immunological unresponiveness after enteric antigen administration. In *Recent advances in mucosal immunity*. Ed. W.Strober, L.Hanson & K.Sell. Raven Press, New York. pp 91.

Kagnoff,M.F. (1980) Effects of feeding on intestinal and systemic immune responses. II Similarity between the suppressor factor in mice after erythrocyte lysate injection and erythrocyte feeding. Gastroenterology, 49, 54.

Kagnoff,M.F. (1982) Immunological unresponsiveness after enteric antigen administration. In *Recent Advances in Mucosal Immunity*. Ed. W.Strober, L.Hanson & K.Sell. Raven Press, New York. pp 95.

Kawano,K., Aoki,T. & Kitao,T. (1984) Duration of protection against vibriosis in Ayu, *Plecoglossus altivelis*, vaccinated by immersion and oral administration with *Vibrio anguillarum*. Bull. Jpn. Soc. Sci. Fish., 50, 771-774.

Komiyama,A. & Spicer,S.S. (1975) Acid phosphatase demonstrated ultrastructurally in mast cell granules altered by pinocytosis. Lab. Invest., 32, 485-491.

Kraehenbuhl,J.P., Bron,C. & Sorbat,B. (1979) Transfer of humoral, secretory and cellular immunity from mother to offspring. Curr. Topics Pathol., 66, 105-157.

Kraehenbuhl,J.P. & Campiche,M.A. (1969) Early stages of intestinal absorption of specific antibodies in the newborn. An ultrastructural, cytochemical and immunological study in the pig, rat and rabbit. J. Cell Biol., 42, 345-365.

Krementz,A.B. & G.B. Chapman (1975) Ultrastructure of the posterior half of the intestine of the channel catfish, *Ictalurus puctatus*. J. Morph., 145, 441-448.

Lamers,C.H.J. (1985) The reaction of the immune system of fish to vaccination. PhD. Thesis, University of Wageningen, The Netherlands.

Languille,R.M. & Youson,J.H. (1984) Morphology of the intestine of prefeeding and feeding adult lampreys *Petromyzon marinus* L.: The mucosa of the diverticulum anterior intestine and transition zone. J. Morph., 182, 39-61.

Laskin,D.L., Sirak,A.H., Pilaro,A.M. & Laskin,J.D. (1988) Functional and biochemical properties of rat Kuppfer cells and peritoneal macrophages. J. Leuco. Biol., 44, 71-78.

Lee,V.H.L. (1986) Enzymatic barriers to peptide and protein absorption and the use of penetration enhancers to modify absorption. In *Delivery Systems for Peptide Drugs*. Ed. S.S.Davis, L.Illum & E.Tomlinson. NATO ASI Series, Plenum Press, London. pp 87-101.

LeFevre,M.E., Olivo,R., van der Hoff,J.W. & Joel,D.D. (1978) Accumulation of latex in Peyer's Patches and its subsequent appearance in villi and mesenteric lymph nodes. Proc. Soc. Exp. Biol. Med., 159, 298-304.

Linna,J.T., Finstad,J. & Good,R.A. (1975) Cell proliferation in epithelial and lympho-haemopoeitic tissues of cyclostomes. Am. Zool., 15, 29-33.

Linthicum,D.S. (1975) Ultrastructure of the Hagfish Blood Leucocytes. Adv. Exp. Med. Biol., 64, 241-250.

Lobb,C.J. & Clem,L.W. (1981) The metabolic relationships of the immunoglobulins in fish serum, cutaneous mucus and bile. J. Immunol., 127, 4, 1525-1529.

Maas,M.G. & Bootsma,R. (1982) Uptake of bacterial antigens in the spleen of carp (*Cyprinus carpio* L.). Dev. Comp. Immunol., Supp.2, 47-52.

Madara,J.L., Bye,W.A. & Trier,J.S. (1984) Structural features of and cholesterol distribution in M-cell membranes in guinea-pig, rat and mouse Peyer's Patches. Gastroenterology, 87, 1091-1103.

Manning,M.J. & Collie,M.H. (1977) The ontogeny of thymic dependence in the amphibian *Xenopus laevis*. In *Developmental Immunobiology*. Ed. J.B.Solomon & J.D.Horton. Elsevier/North Holland Biomedical Press, Amsterdam. pp 291-298.

Maurer,F. (1886) Schilddruse und Thymus der Teleostier. Morphol. Jahrbuch., 11, 129-135.

McDermott,M.R., Horsewood,P., Clark,D.A. & Bienenstock,J. (1986) T lymphocytes in the intestinal epithelium and lamina propria of mice. Immunology, 57, 213-218.

McGhee,J.R., Michalek,S.M., Kiyono,H., Babb,J.L., Clark,M.P. & Mosteller,I.M. (1982) Lipopolysaccharide regulation of the IgA immune response. In *Recent Advances in Mucosal Immunity*. Ed. W.Strober, L.A.Hanson & K.W.Sell. Raven Press, New York. pp 57-72

McLean,E. & Ash,R. (1986) The time course appearance and net accumulation of HRP presented orally to juvenile carp, *Cyprinus carpio*. Comp. Biochem. Physiol., 84, 687-691.

McLean,E. & Ash,R. (1987) Absorption of HRP into the hepatic portal vein of free-swimming rainbow trout (*Salmo gairdneri* R). Comp. Biochem. Physiol., 88, 507-510.

McLean,E., Ash,R. & Westcott,P.A.B. (1987) The appearance of soluble antigen, horseradish peroxidase (HRP), in tissues of the rainbow trout (*Salmo gairdneri* R), subsequent to variable dip-immersion procedures. International Symposium on Feeding & Nutrition in Fish, Bergen, Norway. (Unpublished conference conribution)

McLean,E., Down,N.E., Dye,H.M., Souza,L.M. & Donaldson,E.M. (1988a) The absorption of rbGth by the gastrointestinal tract of coho salmon parr (*Oncorhynchus kisutch*, Walbaum,1792): Comparison between oral and rectal routes of administration. First International Symposium on Fish Endocrinology, Edmonton, Canada. (Unpublished conference conribution)

McLean,E., Benfey,T.J., Dye,H.M. & Donaldson,E.M. (1988b) Gastrointestinal absorption and passage of gonadotrophin into the circulation of juvenile chinoock salmon, *Oncorhynchus tschawytscha* (Walbaum, 1792). Western Regional Conference on Comparative Endocrinology, University of Washington, Seattle. (Unpublished conference contribution)

Mestecky,J., McGhee,J.R., Crago,S.S., Jackson,S., Kilian,M., Kiyono,H., Babb,J.L. & Michalek,S.M. (1980) Molecular-Cellular interactions in the secretory IgA response. J. Retic. Soc., 28, 45s-59s.

Miller,S.D. & Hanson,D.G. (1979) Inhibition of specific immune responses by feeding protein antigens. II. Evidence for tolerance and specific active suppression of cell-mediated immune response to ovalalbumin. J. Immunol., 123, 2344-2350.

Miller,K. & Nicklin,S. (1987a) *Immunology of the Gastrointestinal Tract.* Vol.I. CRC Press, Florida.

Miller,K. & Nicklin,S. (1987b) *Immunology of the Gastrointestinal Tract.* Vol.II. CRC Press, Florida.

Montgomery,P.C., Lemaitre-Coelho,I. & Vaerman,J.P. (1980) A common mucosal immune system. Antibody expression in secretions following gastrointestinal stimulation. Immunol. Commun., 9, 705-713.

Moriarty,D.J.W. (1973) The physiology of digestion in the cichlid fish *Tilapia nilotica*. J. Zool. Lond., 171, 25-39.

Morris,B. (1986). The ontogeny and compartment of lymphoid cells in foetal and neonatal sheep. Immunol. Rev., 91, 219-233.

Morris,B. & Morris,R. (1976) Quantitative assessment of the transmission of labelled protein by the proximal and distal regions of the small intestine of young rats. J. Physiol., 255, 619-634.

Morrow,W.J.W. & Pulsford,A. (1980) Identification of the peripheral blood leucocytes of the dogfish (*Scyliorhinus canicula* L.) by electron microscopy. J. Fish Biol., 17, 461-475.

Mowat,A.McI. (1987) The regulation of immune responses to dietary protein antigens. Immunol. Today, 8, 93-98.

Mowat,A.McI. & Parrott,D.M.V. (1982) Immunological responses to food protein antigens in mice. IV. Effects of stimulating the reticuloendothelial system on oral tolerance and intestinal immunity to ovalbumin. Immunology, 50, 547.

Mowat,A.McI., Thomas,M.J., MacKenzie,S. & Parrott,D.M.V. (1986) Divergent effects of bacterial lipopolysaccharide on immunity to orally administered protein and particulate antigens in mice. Immunology, 58, 677-683.

Mulcahy,M.F., Savage,A. & Casey,N. (1983) The leucocytes of the pike, *Esox lucius* L. Ir. Fish Invest. Ser. A, 28, 81-86.

Nagai,A., Aizama,Y., Ono,S.I. & Naguna,H. (1983) Antigenic protein uptake in the gut of rainbow trout *Salmo gairdneri*. J. Fac. Mar. Sci. Technol. Tokai Univ., 16, 234-247.

Nagai,A. & Fujino,Y. (1984) A study of the ingestion of horseradish peroxidase into the mucosal epithelial cells in the gut of Ayu fish, *Plecoglossus altivelis*. J. Fac. Mar. Sci. Tokai Univ., 18, 253-263.

Nelson,J.S., Rohovec,J.S. & Fryer,J.L. (1985) Tissue localisation of *Vibrio* bacterin delivered by intraperitoneal injection, immersion and oral routes to *Salmo gairdneri*. Fish Pathol., 19, 4, 263-269.

Neutra,M.R., Ciechanover,A., Owen,L.S. & Lodish,H.F. (1985) Intracellular transport of transferrin and Asialo-orosomucoid colloidal gold conjugates to lysosomes after receptor- mediated endocytosis. J. Histochem. Cytochem., 33, 11, 1134-1144.

Nicklin,S. & Miller,K. (1984) Effect of orally administered food-grade carrageenans on antibody- mediated and cell-mediated immunity in the inbred rat. Food Chem. Toxicol., 22, 615-628.

Noalliac-Depeyre,J., & Gas,N. (1973) Absorption of protein macromolecules by the enterocytes of the carp (*Cyprinus carpio*). Z. Zellforsch., 146, 523-537.

Noailliac-Depeyre,J. & Gas,N. (1976) Electron microscopic study of the gut epithelium of the tench (*Tinca tinca*) with respect to its absorptive funtions. Tiss. Cell., 8, 511-530.

Noailliac-Depeyre,J. & Gas,N. (1979) Structure and function of the intestinal epithelial cells in the perch (*Perca fluviatilis*). Anat. Rec., 195, 621-640.

Ogra,P.L. & Karzon,D.T. (1970) The role of immunoglobulins in the mechanism of mucosal immunity to virus infection. Pediatr. Clin. N. Am., 17, 2, 386-400.

Ogra,S.S., Weintraub,D. & Ogra,P.L. (1979) Immunologic aspects of human colostrum and milk.III. Fate and absorption of cellular and soluble components in the gastrointestinal tract of the newborn. J. Immunol., 119, 245-248.

O'Hagan,K. (1988) Polymeric particulate carriers as potential orally active immunological adjuvants. NATO ASI *Immunological Adjuvants and Vaccines*. Cape Sounion, Greece. (Unpublished conference contribution)

Orlans,E., Peppard,J.V., Payne,A.W., Fitzharris,B.M., Mullock,B.M., Hinton,R.H. & Hall,J.G. (1983) Comparative aspects of the hepatobiliary transport of IgA. Ann. N.Y. Acad. Sci., 411-427.

Ostberg,Y., Fange,R., Mattisson,A. & Thomas, N.W. (1975) Light and electron microscopical characterisation of heterophilic granulocytes in the intestinal wall and islet parenchyma of the hagfish, *Myxine glutinosa* (Cyclostomata). Acta Zool., 57, 89-102.

Owen,R.J. (1977) Sequential uptake of horseradish peroxidase by the lymphoid follicle epithelium of Peyer's Patches in the normal unobstructed mouse intestine: An ultrastructural study. Gastroenterology, 72, 440-451.

Owen,R.L. & Bhalla,D.K. (1983) Cytochemical analysis of alkaline phosphatase and esterase activities and lectin-binding and anionic sites in rat and mouse Peyer's Patch M-cells. Am. J. Anat., 168, 199-212.

Owen,R.L., Pierce,N.F., Apple,R.T. & Cray,W.C. (1986) M-cell transport of *Vibrio cholerae* from the intestinal lumen into Peyer's Patches. A mechanism for antigen sampling and for microbial transepithelial migration. J. Infect. Dis., 153, 1108-1118.

Page,M. & Rowley,A.F. (1982) A morphological study of pharyngeal lymphoid accumulations in larval lampreys. Dev. Comp. Immunol., Supp.2, 35-40.

Page,M. & Rowley,.A.F. (1983) A cytochemical, light and electron microscopical study of the leucocytes of the adult river lamprey, *Lamptera fluviatilis* (L. Gray). J. Fish Biol., 22, 503-518.

Pang,K-Y., Walker,W.A. & Bloch,K.J. (1981) Intestinal uptake of macromolecules. Differences in distribution and degradation of protein antigen in control and immunised rats. Gut, 22, 1018-1024.

Parish,N. (1981) A study of the structure, distribution and function of phagocytic cells in the immune system of fish. PhD. Thesis, Plymouth Polytechnic, U.K.

Parish,N., Wrathmell,A., Hart,S. & Harris,J.E. (1986a) The leucocytes of the elasmobranch *Scyliorhinus canicula* L.: A morphological study. J. Fish Biol., 28, 545-561.

Parish,N., Wrathmell,A., Hart,S. & Harris,J.E. (1986b) Phagocytic cells in the peripheral blood of the dogfish *Scyliorhinus canicula* L. I. *In vitro* studies. Acta. Zool., 67, 215-224.

Parish,N., Wrathmell,A., Hart,S. & Harris,J.E. (1986c) Phagocytic cells in the peripheral blood of the dogfish *Scyliorhinus canicula* L. II. *In vivo* studies. Acta. Zool., 67,225-234.

Parmley,R.T. (1987) In *Vertebrate Blood Cells: Chapter 6, Mammals.* Ed. A.F.Rowley & N.A.Ratcliffe. Cambridge University Press. pp 176-205.

Pearse,A.G.E. (1968) *Histochemistry.* Third Edition. Churchill, London.

Peleteiro,M.C. & Richards,R.H. (1985) Identification of lymphocytes in the epidermis of the rainbow trout, *Salmo gairdneri* Richardson. J. Fish Dis., 8, 161-172.

Peppard,J.V., Jackson,L.E. & Hall,J.G. (1985) Comparison of *in vitro* binding to enterocyte brush borders of rat IgG subclasses and their transmission *in vivo* in the rat. Immunology, 56, 51-55.

Pitombeira,M.S. & Martins,J.M. (1970) Haematology of the Spanish mackeral, *Scomberomorus maculatus.* Copeia, 182-186.

Plytycz,B. & Slezak,J. (1981) Gut-associated lymphoid tissue in several amphibian species. Folia Biol. (Krakow), 29, 2.

Quaroni,A., Kirsch,K., Herscovic,S. & Isselbacher,K.J. (1980) Surface membrane biogenesis in rat intestinal epithelial cells at different stages of maturation. Biochem. J., 192, 133-144.

Reynolds,G.T (1982) *Lymphoid Tissue.* Inst. Med. Lab. Sci, Wright PSG, London.

Reynolds,J.D. (1981) Peyer's patches and the ontogeny of B lymphocytes in sheep. In *The Immune System.* Vol.I. Ed. C.M.Steinberg & I.Lefkovitz. Karger, Basel. pp 95-128.

Reynolds,J.D. & Morris,B. (1983) The evolution and involution of Peyer's patches in foetal and post-natal sheep. Eur. J. Immunol., 13, 627-635.

Rhodes,R.S. & Karnovsky,M.J. (1971) Loss of macromolecular barrier function associated with surgical trauma to the intestine. Lab. Invest., 25, 280-289.

Richman,L.K., Chiller,J.M., Brown,W.R., Hanson,D.G. & Vaz,N.M. (1978) Enterically induced immunologic tolerance. I. Induction of suppressor T lymphocytes by intragastric administration of soluble proteins. J. Immunol., 121, 2429.

Rijkers,G.T. (1980) The immune system of cyprinid fish. PhD. Thesis, Agricultural University, Wageningen, The Netherlands.

Rodewald,R.D. (1970) Selective antibody transport in the proximal small intestine of the neonatal rat. J. Cell Biol., 45, 635-640.

Rombout,J.H.W.M. & van den Berg,J. (1985) Uptake and transport of ferritin in the epithelium of carp (*Cyprinus carpio* L) and the possible immunological implications. Cell. Biol. Int. Rep., 9, 516.

Rombout,J.H.W.M., Blok,L.J., Lamers,C.H.J. & Egberts,E. (1985) Immunisation of carp (*Cyprinus carpio*) with a *Vibrio anguillarum* bacterin: Indications for a common mucosal immune system. Dev. Comp. Immunol., 10, 341-351.

Rombout,J.H.W.M., Lamers,C.H.J., Helfrich,M.H., Dekker,A. & Taverne-Thiele,J.T. (1986) Uptake and transport of intact macromolecules in the intestinal epithelium of carp, *Cyprinus carpio*, and the possible immunological implications. Cell Tissue Res., 239, 519-530.

Rothberg,R.M. (1969) Immunoglobulin and specific antibody synthesis during the first weeks of life of premature infants. J. Pediatr., 75, 391-399.

Roubal,F.R. (1986) Blood and other possible inflammatory cells in the sparid *Acanthopagrus australis* (Gunther). J. Fish Biol., 28, 573-594.

Rowley,A.F. & Ratcliffe,N.A. (1988) *Vertebrate Blood Cells*. Cambridge University Press.

Rutter,J.M., Burrows,M.R., Sellwood,R. & Gibbons,R.A. (1975) A genetic basis for resistance to enteris disease caused by *E.coli*. Nature, 257, 135-136.

Sailendri,K. (1973) Studies on the development of lymphoid organs and immune responses in the teleost *Tilapia mossambica* (Peters). PhD. Thesis, Madurai University, India.

Sailendri,K. & Muthukkaruppan,V.R. (1975) Morphology of lymphoid organs in a cichlid teleost *Tilapia mossambica*. J. Morph., 147, 109-122.

Schindler,J.F. & de Vries,U. (1988) Maternal embrionic relationships in the goodeid teleost, *Xenophorus captivus*. Cell Tissue Res., 253, 115-128.

Scicchitano,R., Sheldrake,R.F. & Husband,A.J. (1986) Origin of immunoglobulins in respiratory tract sercretion and saliva of sheep. Immunology, 58, 315-321.

Solas,M.T. & Zapata,A. (1980) Gut-associated lymphoid tissue (GALT) in reptiles: intraepithelial cells. Dev. Comp. Immunol., 4, 87-97.

St.Louis-Cormier,E.A., Osterland,C.K. & Anderson,P.D. (1984) Evidence for a cutaneous secretory immune system in rainbow trout, *Salmo gairdneri*. Dev. Comp. Immunol., 8, 71-80.

Steward,M.W. & Howard,C.R. (1987) Synthetic peptides: a next generation of vaccines? Immunol. Today, 8, 51-57.

Stewart-Tull,D.E.S. (1988) Guidelines for testing the biological activities of immunopotetiators. NATO-ASI *Immunological Adjuvants and Vaccines*. Cape Sounion, Greece. (Unpublished conference contribution)

Stokes,C.R., Newby,T.J. & Bourne,F.J. (1983) The influence of oral immunisation on local and systemic responses to heterologous antigens. Clin. Exp. Immunol., 52, 399.

Stroband,H.J.W. & Kroon,A.G. (1981) The development of the stomach in *Clarius lazera* and the intestinal absorption of protein macromolecules. Cell Tissue Res., 215, 397-415.

Stroband,H.W.J. & van der Veen,F.H. (1981) Localisation of protein absorption during transport of food in the intestine of the grass carp (*Ctenopharngodon idella*). J. Exp. Zool., 218, 149-154.

Stroband,H.W.J., van der Veen,F.H. & Timmermans,L.P.M. (1979) Regional functional differentiation in the gut of the grass carp (*Ctenopharyngodon idella*). Histochemistry, 64, 235-249.

Strobel,S. & Ferguson,A. (1984) Immune response to fed protein antigens to mice. III Systemic tolerance or priming is related to age at which antigen is first encountered. Pediatr. Res., 18, 588-594.

Suzuki,K. (1984) A light and electron microscope study of leucocytes in rockfish and rainbow trout. Bull. Jpn. Soc. Sci. Fish., 50, 1305-1315.

Suzuki,K. (1986) Morphological and phagocytic characteristics of peritoneal exudate cells in tilapia, *Oreochromis nilotica* (Trewavas), and carp *Cyprinus carpio* L. J. Fish Biol., 29, 349-364.

Suzuki,K., Kusakar,M., Shimzu,M. & Yamada,J. (1983) Haematological studies on Rockfish, *Sebastes schlegeli*. II.Seasonal changes of blood cell counts in cultured fish. Sci. Rep. Hokkaido Fish Exp. Stn. 25, 201-216.

Suzuki,Y., Kobayashi,M., Aida,k. & Hanyo,I. (1988) Transport of physiologically active salmon gonadotrophin into the circulation in goldfish following oral administration of salmon pituitary extract. J. Comp. Physiol., 157, 753-758.

Sypek,J. & Borysenko,M. (1987) In *Vertebrate Blood Cells: Chapter 4, Reptiles*. Cambridge University Press. pp 104-138.

Tamuru,T. & Brown,W.R. (1985) IgA antibodies in rat bile inhibit cholera toxin-induced secretion in ileal loops *in situ*. Immunology, 55, 579.

Tanaka,M. (1971) Studies on the structure and function of the digestive system in teleost larvae. III. Development of the digestive system during post-larval stage. Jpn. J. Ichthyol., 18, 164-174.

Tatner,M.F., Johnson,C.M. & Horne,M.T. (1984) The tissue localisation of a live and a formalinised vaccine preparation of *Aeromonas salmonicida* in rainbow trout, *Salmo gairdneri* Richardson, following intraperitoneal injection, per-oral administration and direct immersion exposure. J. Fish Biol., 24, 95-108.

Tatner,M.F. & Manning,M.J. (1983) The ontogeny of cellular immunity in the rainbow trout, *Salmo gairdneri*, in relation to the stage of development of the lymphoid organs. Dev. Comp. Immunol., 7, 69-75.

Telemo,E., Westrom,B.R. & Karlson,B.W. (1982) Proteolytic activity as a regulator of the transmission of orally fed proteins from the gut to the blood serum in the suckling rat. Clin. Chem., 31, 1335-1341.

Temkin,R.J. & McMillan,D.B. (1986) Gut-associated lymphoid tissue (GALT) of the goldfish, *Carassius auratus*. J. Morph., 190, 9-26.

Thomas,M.C. & Parrott,M.V. (1974) The induction of tolerance to a soluble protein antigen by oral administration. Immunology, 27, 631-639.

Thomas,H.C., Ryan,C.J., Benjamin,I.S., Blumgart,L.H. & MacSween,R.N.M. (1976) The immune response in cirrhotic rats. The induction of tolerance to orally administered protein antiges. Gastroenterology, 71, 114-126.

Tomasi,T.B. (1980). Oral Tolerance. Transplantation, 29, 353-356.

Tomonaga,S., Hirokane,T. & Awaya,K. (1973) Lymphoid cells in the hagfish. Zool. Mag., 82, 133-135.

Tomonaga,S., Kobayashi,K., Kajii,T. & Awaya,K. (1984) Two populations of immunoglobulin-forming cells in the skate *Raja kenojei*; their distribution and characterisation. Dev. Comp. Immunol., 8, 801-813.

Tomonaga,S., Kobayashi,K., Hagiwara,K., Yamaguchi,K. & Awaya,K. (1986a) Gut-associated lymphoid tissue in Elasmobranchs. Zool. Sci., 3, 453-458.

Tomonaga,S., Yamaguchi,K., Ihara,K. & Awaya,K. (1986b) Mononuclear phagocytic cells (Kuppfer cells) in Hagfish liver sinusoids. Zool. Sci., 3, 615-620.

Toner,P.G. & Ferguson,A. (1971) Intraepithelial cells in the human intestinal mucosa. J. Ultrastruc. Res., 34, 329-344.

Toner,P., Garr,K.E. & Winburn,G.M. (1971) *The digestive system an ultrastructural atlas and review*. Butterworths, London.

Udall,J.N., Colony,P., Fritz,L., Pang,K., Trier,J.S. & Walker,W.A. (1981) Development of gastrointestinal mucosal barrier. II. The effect of natural versus artificial feeding on intestinal permeability of macromolecules. Pediatr. Res., 15, 245-249.

Udall,J.N., Bloch,K.J., Vachne,G., Feldman,P. & Walker,W.A. (1984) Development of the gastrointestinal mucosal barrier. IV. The effect of inhibition of proteolysis on the uptake of macromolecules by the intestine of the newborn rabbit, before and after weaning. Biol. Neonate, 45, 289-295.

Underdown,B.J. & Socken,D.J. (1978) A comparison of secretory component in immunoglobulin interactions amongst different species. Adv. Exp. Med. Biol., 107, 503-511.

Vacca,L.L., Hewett,D. & Woodson,G. (1978) A comparison of methods using DAB to localise peroxidase in erythrocytes and neutrophils and peroxidase-antiperoxidase complex. Stain Technol., 53, 331-338.

Vaermann,J.P., Derijck-Langendries,A., Rits,M. & Delacroix,D. (1985) Neutralisation of cholera toxin by rat bile secretory IgA antibodies. Immunology, 54, 600-601.

Vaz,N.M., Maia,L.C.S., Hanson,D.G. & Lynch,J.M. (1977) Inhibition of homocytotrohic antibody responses in adult inbred mice by previous feeding of the specific antigen. J. Aller. Clin. Immunol., 60, 110-114.

Volkheimer,G. (1972) *Persorption.* Georg Thieme Verlag, Stuttgart.

Volkheimer,G. (1977) Persorption of particles: physiology and pharmacology. Adv. Pharmacol. Chemother., 14, 163-187.

Volkheimer,G. & Schultz,F.H. (1968) The phenomenon of persorption. Digestion, 1, 213-218.

Walker,W.A. (1973). Immunology of the gastrointestinal tract. Part 1. J. Pediatr., 83, 517-530.

Walker,W.A. (1976) Host defence mechanisms in the gastrointestinal tract. Pediatrics, 57, 901-916.

Walker,W.A. (1980) Gastrointestinal host defence: importance of gut closure in control of macromolecular transport. In *CIBA Foundation Symposium 70. Development of mammalian absorptive processes.* Excerpta Med, Amsterdam. pp 201-216.

Walker,W.A. (1981) Intestinal transport of macromolecules. In *Physiology of the gastrointestinal tract.* Ed. L.R.Johnson. Raven Press, New York. pp 1271-1289.

Walker,W.A. (1985) Role of the mucosal barrier in toxin/microbial attachment to the gastrointestinal tract. In *Microbial Toxins and Diarrhoea Disease.* Ed. D.Evered. & J.Whelan. Pitman, London. pp 34-56.

Walker,W.A. (1987) Role of the mucosal barrier in antigen handling by the gut. In *Food Allergy and Intolerance.* Ed. J.Brostoff & S.J.Challacombe. Bailliere/Tindall, London. pp 209-222.

Walker,W.A. & Bloch,K.J. (1983) Intestinal uptake of macromolecules *in vitro* and *in vivo* studies. Ann. N.Y. Acad. Sci., 593-602.

Walker,W.A., Cornell,R., Davenport,L.M. & Isselbacher,K.J. (1972) Macromolecular absorption. Mechanism of horseradish peroxidase uptake and transport in adult and neonatal rat intestine. J. Cell. Biol., 54, 195-205.

Walker,W.A. & Isselbacher,K.J. (1977) Origin and distribution of mucosal plasma cells and their immuonglobulins. Physiol. Med., 297, 767-773.

Warshaw,A.L., Walker,W.A., Cornell,R. & Isselbacher,K.J. (1971) Small intestinal permeability to macromolecules: transmission of horseradish peroxidase into mesenteris lymph and portal blood. Lab. Invest., 25, 675-684.

Warshaw,A.L., Walker,W.A. & Isselbacher,K.J. (1974) Protein uptake by the intestine: evidence for absorption of intact macromolecules. Gastroenterology, 66, 978-992.

Watanabe,Y. (1981) Ingestion of horseradish peroxidase by the intestinal cells in larvae or juveniles of some teleosts. Bull. Jpn. Soc. Sci. Fish., 47, 1299-1307.

Watanabe,Y. (1982) Intracellular digestion of horseradish peroxidase by the intestinal cells of teleost larvae and juveniles. Bull. Jpn. Soc. Sci. Fish., 48, 37-42.

Watanabe,Y. (1984a) Postembryonic development of intestinal epithelium of masu salmon (*Oncorhyncus masou*). Bull. Jpn. Soc. Sci. Fish., 46, 1-14.

Watanabe,Y. (1984b) Morphological and functional changes in rectal epithelial ells of pond smelt during postembryonic development. Bull. Jpn. Soc. Sci. Fish., 50, 808-814.

Watanabe,Y. (1984c) An ultrastructural study of intracellular digestion of horseradish peroxidase by the rectal epithelium cells in larvae of a freshwater cottid fish, *Cottus nozawe*. Bull. Jpn. Soc. Sci. Fish., 50, 409-416.

Watson,L.J., Shehmeister,I.L. & Jackson,L.L. (1963) The Haematology of Goldfish, *Carassius auratus*. Cytologia, 28, 118-130.

Weinberg,S. (1975) Occurence of lymphoid cells in the intestine of the goldfish. Biodrag. Tot. dierKunde., 45, 195-294.

Weinreb,E.L. & Weinreb,S. (1969) A study of experimentally induced endocytosis in a teleost. I. Light microscopy of peripheral blood cell response. Zool. N.Y., 54, 25-34.

Weisel,G.F. (1979) Histology of the feeding and digestive organs of the shovelnose sturgeon *Scapirhynchus platorhyncus*. Copeia, 518-525.

Wichtrup,L.G. & Greven,H. (1985) Uptake of ferritin by the trophotaeniae of the goodeid fish *Ameca splendens* Miller & Fitzsimmons 1971 (Teleostei, Cyprinidontiformes). Cytobios, 42, 33-40.

Williams,R.C. & Gibbons,R.J. (1972) Inhibition of bacterial adherence by secretory immunoglobulin A: a mechanism of antigen disposal. Science, 177, 696-699.

Wilson,T.H. (1957) *In vitro* studies on intestinal absorption in fish. Mar. Biol. Bull., 113, 363.

Wilson,A.D., Stokes,C.R. & Bourne,F.J. (1986) Morphology and functional characteristics of isolated porcine intraepithelial lymphocytes. Immunology, 59, 109-113.

Wolf,J.L. & Bye,W.A. (1984) The membraneous epithelial (M) cell and the mucosal immune system. Ann. Rev. Med., 35, 95-112.

Wolf,J.L., Rubin,D.H., Finberg,R., Kaufmann,R.S., Sharpe,A.H., Trier,J.S. & Fields,B.N. (1981) Intestinal M-cells. A pathway for entry of reovirus into the host. Science, 212, 471-472.

Wolf,J.L., Kaufmann,R.S., Finberg,R., Dambrauskas,R., Fields,B.N. & Trier,J.S. (1983) Determinants of reovirus interaction with the intestinal M-cells and absorptive enterocytes of murine intestine. Gastroenterology, 85, 291-300.

Wong,W.C. (1982) Lymphoid aggregations in the oesophagus of the toad (*Bufo melanostictus*). Acta. Anat., 83, 461-465.

Wourms,J.P. & Lombardi,J. (1979) Cell ultrastructure and protein adsorption in the trophotaenial epithelium, a placental analogue of vivparous fish embryos. J. Cell Biol., 83, 399.

Yamamoto,T. (1972) Absorption across the plasma membrane of the intestinal absorptive cells. Acta. Histochem. Cytochem., 5, 266-268.

Xu,G. (1985) Enteropancreatic circulation of digestive enzymes. Shenghi Kexue Jinzhain., 16, 282-283.

Zaccone,G. (1980) On the occurence of eosinophilic granular cells in the teleost epidermis: A review. Zool. Jam. Abt. Anat. Ontog. Tiere., 14, 139-143.

Zapata,A. & Solas,M.T. (1979) Gut-associated lymphoid tissue (GALT) in Reptiles: structure of mucosal accumulations. Dev. Comp. Immunol., 3, 477-487.