IMMUNITY IN THE ALIMENTARY TRACT AND OTHER MUCOSAE

OF THE DOGFISH SCYLIORHINUS CANICULA L.

BY

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of

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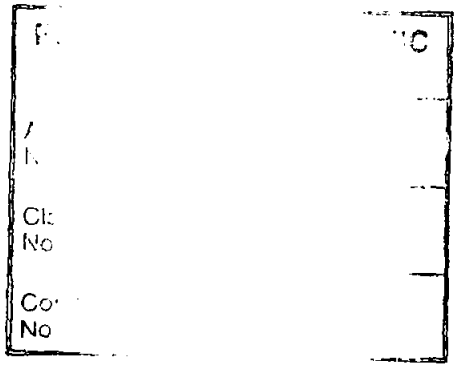
Research was conducted at Plymouth Polytechnic in collaboration with the University of Salford.



DECLARATION

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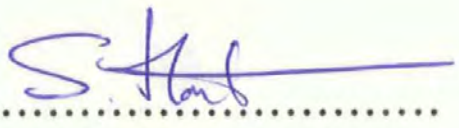

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ABSTRACT

The gut of Scyliorhinus canicula was examined by light and electron microscopy and was found to harbour a large and heterogeneous leucocyte population which occupied three niches: the lamina propria (intralaminal leucocytes), the epithelium (intra-epithelial leucocytes) and as accumulations of leucocytes. The lamina propria had a mixed population of cells including three granulocyte types, lymphocytes, plasma cells and macrophages. The epithelium contained a similar spectrum of cells, with the exception of plasma cells, which were only detected in the lamina propria. The lamina propria and epithelium of the gall bladder and reproductive tract also contained leucocytes, although not in the same quantities. Accumulations of lymphocytes and macrophages were detected throughout the alimentary tract, but were largest and most predominant in the proximal spiral intestine. Leucocyte populations in all three niches were greatest in the proximal spiral valve and virtually absent from the cardiac stomach.

The development of leucocyte population in the spiral intestine was examined. Intralaminal leucocytes were first observed in phase 2 of stage 2 and intra-epithelial leucocytes and lymphoid accumulations in stage 3. The development of the intestinal leucocyte populations occurred after the thymus and kidney and approximately at the same time as the Leydig organ and spleen. Leucocytes were present in all niches of the gut prior to the transition to an exogenous diet.

The epithelium of the spiral intestine of the larval stages was shown to phagocytose particulate material (carbon), while the adult intestine demonstrated no such ability. The epithelium of the spiral intestine of both larval and adults was found to absorb soluble protein material (HGG and ferritin).

Immunoglobulin was detected in the reproductive tract, spiral intestine, and at levels comparable to that of serum in the bile. Biliary immunoglobulin was compared, on the basis of several criteria, with serum immunoglobulin. Sepcific antibodies were detected in the bile after SRBC'S and Vibrio antigens were intubated into the gut by oral and anal routes and injected directly into the peritoneum. No systemic response, however, was elicited to antigens which had been intubated into the gut by either the oral or anal orifice.

I would like to dedicate this thesis

to my parents

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

In fish a continuous exposure to aquatic antigens occurs at the mucosal surfaces of the respiratory, reproductive and gastrointestinal tracts and, in teleost fish, across the unkeratinised skin. These surfaces are constantly subjected to antigenic material from the environment such as commensal and pathogenic organisms, sperm or seminal antigens in the reproductive tract of internally fertilised female fish and dietary antigens in the gut. Some of these antigens will attach to the epithelia, penetrate and may cause local or systemic challenge, from which an infection and disease may result.

The factors which may be involved in the protection of the body surfaces include: specific immunological processes, non-specific defence mechanisms (e.g. phagocytosis, action of lysozyme and proteases etc.) and substances that are primarily involved in digestion but, may control gut pathogens (e.g. gastric acid and proteolytic enzymes). The former category concerns the adaptive mechanisms of host resistance to infection which, in mucous secretions, is likely to be largely mediated by immunoglobulin (Ig). The presence and possible significance of components from the putative non-specific and adaptive immune systems in the mucosae of fish is reviewed in the proceeding pages.

There has been very little work on local immunity of the lower vertebrates and, like the immunology of higher vertebrates, concepts of immunity are largely based on systemic studies. Why such an important area has largely been ignored is not clear but, may be partially explained by the following: a, trends in comparative immunology tend to follow work in higher vertebrates; b, standardisation is difficult, it is hard to define the amount of antigen given by the oral and anal

routes, as uptake will be dependant upon several variables including the amount of food, bile salts and mucus that is present in the gut; c, collection of mucus, unlike sera, for the detection of cells, antibodies and non-specific defence components is imprecise and antibody titres may be radically affected by gut enzymes and bile salts.

The study of local immunity in fish has two major applications, the first of which is the study of phylogeny of the local immune response in vertebrates and the development of the secretory IgA system. Polymeric IgA is the predominant Ig in the secretions of mammals (Tomasi, 1976). However, IgM has retained its capacity for being transported by the liver from serum to bile (Lemaitre-Coelho, Jackson and Vaerman, 1978 and Peppard, Jackson and Hall, 1983) and an increased production of IgM occurs at the mucosal surface of mammals with IgA deficiency (Brandtzaeg, 1975) which may represent the retention of an ancestral mechanism. An IgM-like molecule is found in the bile of the duck Anas platyrhynchos (Ng and Higgins, 1986) and the amphibian Xenopus laevis (Jurd, 1977). Intestinal cells of the latter species were shown to produce an IgM-like molecule by Hsu, Flajnik and Du Pasquier (1985). Biliary IgM-like molecules have also been detected in teleosts (Lobb and Clem 1981a and b and Rombout, Blok, Lamers and Egberts, 1986) and the nurse shark (Ginglymostoma cirratum) (Underdown and Socken, 1978). It is highly likely that Ig is present in the gut of cylostomes, as plasma cells have been detected in the gut of the Atlantic hagfish (Myxine glutinosa) Zapata, Fänge, Mattison and Villena (1984), and the lamprey Petromyzon marinus (Fujii, 1982). The time at which IgA evolved is not clear. Immunoglobulin diversification may have taken place in the teleosts (Lobb, 1986) and chondrihcthyes (Kobayashi, Tomonaga and Kajii, 1984).

The second major application is the potential stimulation of local immunity to agents by oral or bath immersion vaccination.

Recently Ellis (1985a) in an article on the development of fish vaccines, noted that the empirical approach to fish vaccination must be supplemented by a scientific analysis of the conditions favouring the development of protective immunity in fish. An understanding of the significance of local immunity in protection against disease, and development of the best methods of stimulating this system, may allow further applications of bath, and full exploitation of oral vaccination techniques.

While S.canicula is not farmed, and is only of limited economic importance, it has been found to be an excellent subject for experimental purposes. Previous work has dealt with the systemic immune system (Morrow, 1978) and phagocytosis (Parish, 1981). S.canicula survives captivity well and is not particularly disposed to any diseases. Adult fish provide ample supplies of blood and females fertilised before captivity will lay between 20-40 eggs in the first year, providing plenty of material for ontogenic studies.

In this study on S.canicula, the aim of the project was to investigate immune mechanisms operating at the mucosal surfaces of the fish, with particular emphasis on an investigation of these mechanisms in the gut.

The specific objectives were: a, to examine the mucosae of the common dogfish for cells and tissues, which may play a role in immunity, by light and electron microscopy, and compare them with known cell types from the peripheral blood and lymphoid organs; b, to examine the ontogeny of mucosal lymphoid tissues and cells and determine, if possible, the role of antigen in development; c, to determine the distribution and nature of mucosal Ig, and examine local antibody responses; d, to examine the uptake of material from the gut of embryonic, neonatal and adult fish.

1.2 Literature review

This review is confined to work on local immunity in the fishes, i.e. tissues of the gut, reproductive tract, skin and gills. The systemic immune system is not covered as it has recently been reviewed by Lamers (1985) and is a major component of symposia edited by Manning and Tatner (1985) and Stolen and van Muiswinkel (1986).

1.2a Gut immunity

i) Morphological studies

Cyclostomes (hagfishes and lampreys)

Cyclostomes do not possess a true thymus or spleen (Page and Rowley, 1982) but, humoral and cellular immune responses have been described in this group (Hildemann and Thoenes, 1969 and Thoenes and Hildemann, 1969). An Ig-like molecule has been described in the serum of hagfish (Raison, Hull and Hildemann, 1978 and Kobayashi, Tomonaga and Hagiwara, 1985).

Hagfishes

Schreiner (1898) described migrating leucocytes (Wanderzellen) in the basal mucosa and sub-mucosa of the Atlantic hagfish. These cells were called "theliolymphocytes" by Fichtelius, Finstad and Good (1969), although they had difficulty in differentiating these cells from epithelial cells at the light microscopic level. Intraepithelial leucocytes (IEL) were also described (Tomonaga, Hirokane and Awaya, 1973). Lymphoid accumulations were absent from the gut of hagfish (Fichtelius, Finstad and Good, 1968) but, lymphohaemopoietic tissues in the intestinal submucosa of Myxine and Eptatretus may represent a primitive spleen. This tissue consists of granulopoietic tissue arranged in islands around branches of the portal vein separated by fat tissue (Holmgren, 1950; Tomonaga, Hirokane, Shinohara and Awaya, 1973; Tanaka, Saito and Gotoh, 1981; Zapata et al., 1984).

Granulocytes were shown to emigrate into the epithelium of the intestine and bile duct of the Atlantic hagfish (Ostberg, Fänge, Mattisson and Thomas, 1975). These cells were referred to as heterophils or pseudoeosinophils and had periodic acid Schiff (PAS) positive cytoplasm, and contained evenly distributed pleomorphic granules as shown by electron microscopy.

While plasma cells have been detected in the gut of hagfish (Zapata et al., 1984) the entry of pathogens into the epithelium and secretion of Ig into the lumen may be effected by the periotrophic membrane described by Adam (1966).

Lampreys

In the ammocoete larvae of Lampetra and Petromyzon lympho-haemopoietic tissue is found in the spiral valve (or typhlosole) of the intestine (Tanaka et al., 1981). An electron microscopic study of this tissue in L.reissneri showed cells of the erythrocyte and granulocyte lines as well as macrophages, lymphocytes and plasma cells (Fujii, 1982) indicating that the capacity exists to elicit a local immune response. In the adult this tissue in the spiral valve disappears, and is superceded by the supra-neural myeloid organ. Intraepithelial leucocytes were more easily detected in the anadromous sea lamprey (P.marinus) than in the Atlantic hagfish (Fichtelius et al., 1969). Linna, Finstad and Good (1975) and Hansen and Youson (1978) also referred to IEL's.

Chondrichthyes

This class of fish occupies a primitive and fundamental position in vertebrate phylogeny, and the chondrichthyes are the most primitive group of fish which possess an encapsulated thymus (Zapata, 1983). They differ from the bony fish in the possession of two granulopoietic structures; the epigonal tissue and Leydig organ, and in general the absence of a lymphoid kidney (Fänge, 1984).

The immunoglobulins of several shark species have been examined (cf. Rosenshein, Schluter and Marchalonis, 1986) because chondrichthyes represent a primitive stage in phylogeny, which nevertheless have Ig's which are clearly related to those of man and mouse.

While the gut of cartilagenous fish, especially the spiral intestine (Fänge and Grove, 1979) has been well studied, the lymphoid tissue harboured within, however, has received little attention.

Drzewina (1905) and Jacobshagen (1915) found free leucocytes and lymphoid accumulations in the intestine of elasmobranchs. Kanesada (1956) detected single intraepithelial lymphocytes and dense collections of small lymphocytes in the subepithelium of the intestine of the stingray (Dasyatis akajei) which were compared to lymphoid populations in higher vertebrates.

Three species of elasmobranch were investigated by Fichtelius et al. (1968) as part of a search for a bursa equivalent in bursaless vertebrates. The horned shark (Heterodontus francisci) was the most primitive shark examined, it had many accumulations of subepithelial non-pyroninophilic lymphocytes in the mid-gut which were usually found in the crypts of villi. The stingray, a more recent shark, had large collections of lymphocytes encapsulated by connective tissue of the spiral valve. In the eagle ray (Aetobatus narinari) accumulations of lymphocytes were found at the tips of intestinal villi and the overlying epithelium seemed specialised like the dome epithelium of the Peyer's patches in mammals. Fichtelius et al. (1968) proposed that these accumulations and others in the gut of amphibia and reptiles represented an equivalent to the bursa of Fabricius in birds. Whether this is the case is still unclear, and no real evidence of a role in immunity yet exists.

Recent work by Tomonaga, Kobayashi, Hagiwara, Yamaguchi and Awaya (1986) centred upon massive lymphocyte aggregations in the central region of the spiral valve in Mustelus manazo, M.griseus, Heterodontus japonicus and Scyliorhinus torazame, which were regarded as potentially primitive forms of mammalian Peyer's patches.

Osteichthyes

This group has two major divisions, the Actinopterygii (the ray finned fishes) and the Sarcopterygii (the lobe finned fishes). The former group is large, containing the chondrosteans, holosteans and teleosteans, of which the latter has been most extensively studied by comparative immunologists. The Sarcopterygii, contains only the dipnoi [lung fishes, of which there are separate species on the American (Lepidosiren paradoxa), African (Protopterus annectens) and Australian (Neoceratodus forsteri) continents] and Coelacanthes of the genus Latimeria which are found only off South West Africa.

Both ray finned and lobe finned fishes possess Ig, in ray finned fishes the macroglobulin is generally tetrameric whereas in the lobe finned fishes, which are generally considered to be closer to the tetrapod evolutionary line, most groups have a pentameric macroglobulin (reviewed by Litmann, 1984).

The American lungfish (L.paradoxa) appears to possess gut associated lymphoid tissue (Good, Finstad, Pollara and Gabrielsen, 1966). More work, however, has been undertaken in the ray finned fishes.

Chondrostei

The paddle fish (Polydon spathula) has many large lymphoid accumulations in the wall of the midgut, spiral valve and ileocaecal junction and these are in close association with the epithelium (Fichtelius et al., 1968 and Weisel, 1973). The function of these accumulations in chondrostei, like

elasmobranchs, is unknown but, their preponderance in the highly parasitised P.spathula and absence from uninfected Scapirhynchus platorhynchus (Weisel, 1979) indicates a possible role in resistance to parasitaemia or infection. FÄnge (1986) reported the existence of considerable amounts of lymphoid tissue in the gut of the sturgeons (Acipenseridae) and Buddington and Doroshov (1986) described "lymph nodes" in the typhlosole of the spiral valve in the white sturgeon (Acipenser transmonatus).

Holostei

Like the chondrostei the holostei have received little attention. Fichtelius et al. (1968) found that garfish (Lepisosteus platostomus) lacked lymphoid accumulations but that the garfish and the bowfin (Amia calva) both had considerable numbers of theliolymphocytes in the epithelium of the intestine (Fichtelius et al. 1969).

Teleostei

Few authors have referred to lymphoid accumulations in the gut of teleosts. Diconza and Halliday (1971) found diffuse accumulations in the gut of the Australian catfish (Tachysurus australis) and proposed they may be involved in the synthesis of Ig in the intestine. Accumulations were also detected in the roach (Rutilus rutilus) (Zapata, 1979). As in the previous groups there is no evidence for specific roles of lymphoid accumulations, just speculation.

Passing references have been made to mast cells in the gut of teleosts (e.g. Krementz and Chapman, 1975), based mainly on morphology. The tenuous nature of criteria used to classify mast cells is reviewed by Ellis (1977a). While IgE is absent from fish, and "so called" mast cells lack histamine, hypersensitivity reactions nevertheless appear to exist and are possibly mediated

by periodic acid Schiff (PAS) positive granulocytes in cyprinids and eosinophilic granular cells (EGC's) in salmonids (Ellis, 1982 and 1986). Periodic acid Schiff positive cells have been described in the intestine of the common carp (Cyprinus carpio) (Davina, Rijkers, Rombout, Timmermans and van Muiswinkel, 1980). Eosinophilic granular cells first named by Roberts, Young and Milne (1971) are found in considerable numbers in the gut of salmonids and have been studied by many authors (cf. Ezeasor and Stokoe, 1980). There is some argument as to whether the granules of these cells contain basic protein or not (Woodward and Bergeron, 1984). Ezeasor and Stokoe (1980) hypothesised that the ensheathing cells and EGC'S constituted a part of the body's defence mechanism.

Recently Ellis (1985b) found in rainbow trout (Salmo gairdneri), injected IP with Aeromonas salmonicida exotoxins in a dose causing death in 6 hours, a coincidental decrease in the histamine content of the gut, appearance of histamine in the blood, and degranulation of the EGC'S in the gut wall occurred 45 minutes post-injection. Fish developed behavioural patterns similar to those described by other workers in fish undergoing systemic anaphylaxis. The fish also exhibited pale gills, defaecation and widespread vasodilation.

Bullock (1963) described 'wandering cells' in the gut epithelium of the brook trout (Salvelinus fontinalis), the Atlantic salmon (Salmo salar), the rainbow trout and the brown trout (S.trutta). These were of two types; polymorphonuclear cells and lymphocytes; the latter were more frequent, and were often seen to migrate towards the lumen where some appeared to degenerate. The author also described globule leucocytes at the

base of the epithelium. These were large, had round nuclei and prominent large granules. Globule leucocytes were thought to represent degenerating granular cells as apparent intermediates between granular and globule cells were found in the lamina propria.

Chao (1973) found three types of granulocytes and ameobocytes, possible macrophages, in the epithelium of the cunner (Tautogolabrus adspersus). Krementz and Chapman (1975) found a diffuse population including lymphocytes and macrophages in the gut of catfish (Ictalarus punctatus).

Weinberg (1975) found that in the goldfish (Carassius auratus) 40% of cells in the epithelium were lymphocytes, of which some were pyroninophilic blast cells and others plasma cells, confirmed by electron microscopy. Plasma cells were also identified in the intestine of the perch (Perca fluviatilis) (Noaillac-Depeyre and Gas, 1979) and Pontius and Ambrosius (1972) found specific antibody producing cells could be detected in the perch after osophageal challenge with SRBC's.

In a recent comprehensive piece of work Davina et al. (1980) observed mainly heterophilic granulocytes, some lymphocytes and occasional macrophages in the gut of the rosy barb (Barbus conchonius) and the common carp. These cells were present from 6 days after hatching (when feeding commenced) and attained adult population levels by twenty weeks. These cells were investigated using a rabbit antiserum to carp IgM (RAC/IgM) and carp thymocytes (RAC/T). Numerous cells in the lamina propria had cytoplasm that was labelled by RAC/IgM and RAC/T, and in the lamina probably more stained with the RAC/IgM sera. Few cells with RAC/IgM or RAC/T positive membranes were found in the lamina

propria or epithelium. The membrane and cytoplasm of PAS positive granulocytes stained with both antisera in an apparently non-specific manner.

In a later investigation of gut immunity in the rosy barb Davina, Parmentier and Timmermans (1982) showed that the intestine had a regional distribution of intraepithelial lymphocytes and heterophilic leucocytes, with a high proportion in the anterior and posterior gut. PAS positive granulocytes and macrophages were restricted to the lamina propria.

ii) Intestinal immunoglobulin

While Ig light (L) chains are conservative in nature throughout the evolution of vertebrates, the heavy (H) chain has undergone considerable modification, and specialisation to different biological roles although some H chain determinants; most notably J_H and V_H related markers are shared between forms as diverse as sharks and mammals (Rosenshein et al. 1986). Hence in mammals polymeric IgA in the specialised form of Ig found at the mucosal surfaces. Whilst Ig's occur in the mucus of lower vertebrates, the degree of biological specialisation these molecules exhibit has not been investigated.

The term "intestinal or mucosal immunoglobulin" (depending on the site) is probably better than the term "secretory immunoglobulin" as it has not been satisfactorily proven that there is indeed a mechanism for the secretion of Ig in the gut or at other surfaces in the fish.

An Ig has been detected in the intestinal mucus of the Australian catfish (Diconza and Halliday, 1971) and the bile of the sheepshead (Archosargus probatocephalus) (Lobb and Clem, 1981a, b). In both species the intestine and systemic Ig's were

antigenically similar. Lobb and Clem (1981a) found, however, that while the biliary Ig was antigenically similar to the serum Ig, it existed as a non-covalent dimer (molecular weight (M.W.) approx. 320,000 daltons) in physiological buffer. Molecular weight determination in the detergent sodium dodecyl sulphate (SDS) indicated the dimeric protein dissociated into monomeric units (approx. 160,000 daltons) each composed of two heavy and two light chains. Furthermore, the molecular weight of the heavy chain of the biliary Ig (approx. 55,000 daltons) was intermediate between that of the H chains from the high molecular weight (HMW) and low molecular weight (LMW) proteins (approx. 70,000 and 45,000 daltons respectively). These authors proposed that this may be evidence of the existence of a specialised local Ig.

The origin of intestinal Ig is unclear. Harris (1972) demonstrated antibodies in gut mucus to natural infections of the acanthocephalan Pomphoryhnchus in the chub (Leuciscus cephalus) but, the author was uncertain whether the antibody originated locally or systemically. Fletcher and White (1973a) found that in the plaice (Pleuronectes platessa) an oral challenge with Vibrio anguillarum stimulated the production of antibodies in the intestinal mucus but a lower response in the serum. Conversely an intraperitoneal challenge stimulated a high serum and a low intestinal antibody titre. This indicates that exposure of the gut to antigens may preferentially stimulate a local immune response as in mammals.

Potentially conflicting evidence has recently been provided by work on common carp (Rombout et al., 1986) who found that systemic antibodies can be detected after anal priming and boosting with V.anguillarum bacterin.

Lobb and Clem (1981b) found that radiolabelled serum HMW and LMW immunoglobulin was not transported into the bile of the sheepshead. As they did not make a histological investigation of the gut, gall bladder or the liver the origin of this non-systemic biliary Ig is unclear.

iii) Antigen absorption by the gut

Interest in the absorption of material by the gut of teleosts has been generated both by aquaculturists and immunologists with a view to oral vaccine development. Little if any work has been undertaken in other groups of fish, at least to the author's knowledge.

Fänge and Grove (1979) reported that the main mechanism of intestinal absorption of fish appears to be similar to that of mammals and absorption of digested products occurs by both passive and active transfer. In the elasmobranch Squalus acanthias van Slyke and White (1911) found that during digestion of protein di- and tri-peptides appeared in the intestine, although no further work appears to have been accomplished on nutritive absorption in the elasmobranchs.

The intestine of some stomachless teleosts was found to be regionally differentiated (Iwai, 1968; Noaillac-Depeyre and Gas, 1976; Stroband, van der Meer and Timmermans, 1979). In the proximal segments the enterocytes had morphological characteristics associated with lipid absorption; in the second or middle section test proteins such as horse radish peroxidase (HRP) were absorbed by pinocytosis and in the third or distal sections enterocytes with a morphology compatible with water and ion exchange were found. It was proposed by these authors that pinocytosis occurred in fish where the stomach was absent, as

intraluminal protein digestion would have been inefficient. Pinocytosis of macromolecules by the middle intestine was, however, also found in fish with stomachs (Noaillac-Depeyre and Gas, 1979; Ezeasor and Stokoe, 1981; Stroband and Kroon, 1981 and Georgopoulou, Sire and Vernier, 1985).

Shcherbina, Trofimova and Kazlauskene (1976) and Stroband and van der Veen (1981) found that 80% of protein ingestion takes place on the proximal intestine, and the pinocytosis of protein by the mid-intestine was a back-up for when large amounts of food were available.

The findings of Watanabe (1984), that pinocytosis of protein occurs in the rectal epithelia of fish from 12 days to 1 year old, indicates that pinocytosis may be in some case be dependant on age of the species.

Uptake of bacterial antigens has recently been investigated (Davina et al., 1982 and Nelson, Rohovec and Fryer, 1985) both groups having found that V.anguillarum antigens were absorbed in the distal intestine. The former authors showed that macrophages took up some of the material and recognised that pinocytosis may lead to antigenic challenge of lymphoid cells in the gut. The latter authors noted that although antigens were taken up in the gut they did not appear to enter the circulation.

Rombout, Lamers, Helfrich, Dekker and Taverne-Thiele (1985), found that HRP and ferritin are absorbed primarily by the mid-intestine and to a lesser extent by the proximal intestine. The two molecules were processed by the enterocytes in two different ways : HRP was bound to the surface of the enterocytes apparently by receptors, then transported in vesicles to branched endings of the basal and lateral cell membrane. Thus most of the HRP was released into the intracellular space were it made

contact with intraepithelial lymphoid cells. Only small amounts of HRP became localised in secondary lysosomes. Ferritin, in contrast, was absorbed by pinocytosis, present in vacuoles which appear to fuse with lysosome-like bodies. In the mid-intestine the ferritin ended up in large supranuclear vacuoles. While ferritin was absent from the intraepithelial space, macrophages were present which contained this material. As most of the antigen is processed by the mid-intestine region, where many lymphoid and non-lymphoid cells have been located, these authors proposed that the enterocytes of the mid-intestine of carp may have an analogous role to the "M" cells of Peyer's patches in mammals.

Recent work using a sensitive enzyme linked immunoabsorbant assay (McLean and Ash, 1986) showed that HRP was rapidly taken up from the gut of common carp into the circulation.

Material absorbed by pinocytosis in the intestine epithelium may lead to the production of antibodies (which has been previously reviewed) alternatively the gut lymphoid system can induce a state of tolerance which is well documented in mammals (Tomasi, 1980). Udey and Fryer (1978) reported oral tolerance in rainbow trout exposed to A.salmonicida by the alimentary route.

1.2b Skin immunity

i) Cellular

Although a number of cell types and possible cell products have been demonstrated in the skin and mucus no definite function or mechanism of skin immunity exists in fish. In vertebrates the role of skin immunity in the protection of the body is better understood (Bos and Kapsenberg, 1986).

Collections of leucocytes have only been detected in the catfish skin (Diconza and Halliday, 1971). Numerous references exist, however, on the presence of leucocytes in the skin. A few salient references are examined below.

Two of the early authors who recognised free leucocytes were Reid (1894) who called them "Wanderzellen" and Fritsch (1886) (cited by Mittal and Munshi, 1971) who identified these cells as lymphocytes.

It has been reported that lymphocytes occupy lymphatic spaces in the basal epithelium of some fishes (Maurer, 1895; Aust, 1936, cited by Mittal and Munshi, 1971) and the latter author reported that the lymphatic spaces were swollen by immigrating lymphocytes. Later evidence (Mittal, Whitear and Agarwal, 1980) questioned whether these were actually lymphatic spaces. Leonard and Summers (1976), Phromsuthirak (1977) and Peleteiro and Richards (1985) found that lymphocytes were surrounded by a clear space; separating them from the epithelial cells, and lacked junctional complexes or interdigitations with these adjacent cells.

Lymphocytes have also been described in the skin of teleosts by Percy (1970); Roberts, Shearer, Elson and Munro (1970); Bullock and Roberts (1974); Hines and Spira (1974); Mittal and Munshi (1974); Pickering and Macey (1977) and Pickering and Richards (1980).

Lymphocytes have been described in the mucus of fish (Ourth 1980 and Mittal and Whitear, 1979, cited by Peleterio and Richards, 1985), it is not clear whether these cells are capable of carrying out a biological role or were effete cells being discarded from the skin. Pickering and Richards (1980) described occasional lymphocytes crossing the basement membrane of the skin. The authors did not state if this migration might be bi-directional.

Peleterio and Richards (1985) found Ig positive cells in the skin of the rainbow trout, the function of these cells could not be determined as it was unclear of the Ig was on the surface or in the cytoplasm of the cells.

St.Louis-Cormier, Osterland and Anderson (1984) showed that Ig containing plasma cells occurred in the subepithelium, epithelium and mucus of the rainbow trout, indicating that the potential for local Ig synthesis exists.

Neutrophils were described in the skin by Roberts (1972) Hines and Spira (1974); Phromsuthirak (1977); Pickering and Richards (1980) and Ferri and Macha (1982) and increased numbers were seen in Ichthyophthirius infections (Ventura and Paperna, 1985). The role of leucocytes in defence against the fungus Saprolegnia has recently been examined by Wood and Willoughby (1986).

Acidophils/eosinophils were demonstrated in the skin of fish by Roberts et al. (1971); Phromsuthirak (1977) and Blackstock and Pickering (1980).

Macrophages were detected in the skin by Phromsuthirak (1977) and were shown to phagocytose carbon at wound sites.

ii) Immunoglobulin and other components

Immunoglobulin has been detected in the cutaneous mucus of fish, however, the question of its origin is controversial. Fletcher and Grant (1969) found haemagglutinating antibodies in the mucus of the plaice that had a similar carbohydrate and amino acid composition to the serum Ig. Immunoglobulin was also detected in the cutaneous mucus of the garfish (Bradshaw, Richard and Sigel, 1971); the Australian catfish (Diconza and Halliday

(1971), the channel catfish (Ourth, 1980); the rainbow trout (Harrell, Etlinger and Hodgins, 1976) and in the sheepshead Lobb and Clem, (1981c). In each species the cutaneous mucus was found to be antigenically similar to the serum Ig.

The origin of the cutaneous mucus Ig is unclear. Goven, Dawe and Gratzek (1980) hypothesised that protection conferred by IP injection of parasite antigen (Ichthyophthirius multifiliis) in the channel catfish resulted in the concentration of immobilising antibodies in the mucus. This was also reported by St.Louis-Cormier et al. (1984) after IP injection of SRBC's into the rainbow trout. In addition Ourth (1980) postulated that mucus antibodies were derived from the serum.

On the contrary other authors proposed that cutaneous Ig's were derived by local production. Diconza and Halliday (1971) found Ig's in the mucus of fish but not anti-BSA activity in fish that had high serum titres to the antigen. Harrell, Etlinger and Hodgins (1976) found no specific antibodies in the mucus of rainbow trout after passively immunising fish with rainbow trout anti-Vibrio anguillarum serum. Most recently Lobb and Clem (1981b) isolated serum Ig (HMW and LMW) which was radiolabelled with I^{125} and injected it back into the circulation of a fish via the caudal sinus. No significant radioactivity was detected in the cutaneous mucus and the authors concluded that skin mucus Ig was derived from a source other than the serum.

Specific antibody titres to infectious agents have been detected in the skin mucus after sublethal infection by Icthyophthirius (Hines and Spira, 1974 and Wahli and Meier, 1985).

Anti-vibrio activity was detected in the skin of the ayu after oral vaccination with V.anguillarum, which may indicate that immune mechanisms of the skin and gut are linked (Kawai, Kusuda and Itami, 1981).

Besides Ig's, there are several agents which may have, an as yet, undetermined role in defence of the skin.

Complement-like substances, have been detected in the cutaneous mucus (Nelson and Gigli, 1968). Harrell et al. (1976) found stable (presumably antibody) and heat-labile (presumably complement) agents in the mucus of the rainbow trout, which together were capable of inhibiting the growth of V.anguillarum in an in vitro experiment. Higher levels of inhibition occurred with immune mucus indicating that if this system worked in vivo, enhancement of antibody levels by vaccination may increase the level of protective immunity in cutaneous mucus.

Lysozyme causes lysis of gram-positive bacteria by hydrolysing B-1.4-glycosidic linkages in the murein component of the cell wall. In gram-negative bacteria, lysis by lysozyme may be mediated by other factors which can disrupt the outer lipid/protein/polysaccharide complex of the cell wall and unmask the inner murein layer. Lysozyme has been detected in the mucus of fish (Fletcher and Grant, 1968 and Fletcher and White, 1973b).

Protease activity has been detected in the mucus of fish (Hjelmeland, Christie and Raa, 1983), they found the isoelectric point (pH range 4.5 - 5.1), MW (approx. 28,000) and other characteristics were similar to trypsin. The molecule was found to lyse V.anguillarum bacteria at pH 8.0 (close to that of sea water). It was proposed by these authors that this protease enzyme may be involved in natural resistance to infection by bacteria. It was also suggested that antigen fragments stripped by this enzyme may more easily enter the skin and react with lymphoid cells.

A C-reactive protein (CRP) like substance has been detected in the skin mucus of Tilapia mossambica (Ramos and Smith, 1978) during inflammation and necrosis due to local injury. It is thought the immediate hypersensitivity skin reactions in flounder may be mediated by CRP (Baldo and Fletcher, 1975).

Al-Hassan, Afzal, Ali, Thomson, Fatima, Fayad and Criddle (1986) and Al-Hassan, Thompson, Summers and Criddle (1986) examined lipid and protein components which may have a role in skin healing and cell migration in the Arabian gulf catfish (Arius thalassinus).

iii) Antigen absorption

Little information exists on antigen uptake across the skin. Fender and Amend (1978) found BSA was taken up via the lateral line. Bowers and Alexander (1982) presented evidence to the contrary, and most evidence available at the present would suggest that the majority of antigens pass from the aquatic environment into the fish at the head region (Tatner and Horne, 1983), at least when the antigens are administered in a vaccine bath. Evidence for the uptake of antigen by the gill is discussed later. It would seem highly likely, however, that at least some antigen would penetrate the skin under normal conditions, and this would explain the presence of numerous leucocytes in the dermis, epidermis and mucus (see previous section). Peleterio and Richards (1985) proposed that a system analogous to that of mammalian skin immunity may exist (reviewed by Bos and Kapsenberg, 1986); where antigens are trapped by Langerhans cells. They also noted that the loose arrangement of epidermal cells described by Ferri and Sesso (1979) may allow access to antigens from the aquatic medium.

1.2 c) Gill and reproductive tract

Little information exists on immune mechanisms in the gills. Fichtelius et al. (1968) found a lymphoepithelial sheath in the stingray but made no suggestion as to the fundamental significance of this structure on the role of lymphocytes in the gill of fish. The gill of S.canicula has been shown to contain a structure called the corpus cavernosum; which apparently is part of the reticulo endothelial system in this fish (cf. Hunt and Rowley, 1986).

Most information pertains to the gill as a possible site of antigen uptake. As previously discussed the gill has been observed to act as a portal for the entry of antigen during vaccination by immersion (Alexander, Bowers and Shamshoon, 1981; Alexander, Bowers, Ingram and Shamshoon, 1982; Bowers and Alexander 1981 and 1982). The external gill of the embryonic shark Rhizoprionodon terraenovae has also been shown to take up whole protein macromolecules (Hamlett, Allen, Stribling, Schwartz and Didio, 1985).

Tatner and Horne (1983) implicated the head region of rainbow trout in the uptake of carbon 14-labelled V.anguillarum, and found that smaller antigen particles were more avidly taken up.

In contrast Smith (1982) found that protein was taken up by the gill more readily if it was bound to latex rather than in solution. He did not, however, discount the skin as a possible route for antigen uptake. Branchial phagocytosis has also been demonstrated (Goldes, Ferguson, Daoust and Moccia, 1986).

Recently, Nelson, Rohovec and Fryer (1985) found that after immersion of rainbow trout in V.anguillarum bacterin, antigen was detected on the surface of the gill arches and filaments, and in

the gastrointestinal tract, but whether the gill actually took up material was unclear.

In fish, immune mechanisms in the female urinogenital tract have not been investigated, although some work has been undertaken on materno-foetal transmission of IgM in plaice (Bly 1984 and Bly, Grim and Morris, 1986) and on maternofoetal immunological relations in the viviparous poecilid fish Xiphophorus helleri (Hogarth, 1968; 1972a,b and 1973).

1.2 d) Non-parenteral immunization

The uptake of material across the mucosae of fish has been exploited to administer vaccine by two separate techniques.

Direct immersion vaccination is currently the most widely used method requiring a minimum of handling, a short exposure time and providing good protection (Lamers, 1985). Little is known about the mechanism of antigen uptake, there is some evidence to suggest branchial phagocytosis may play an important role (Smith, 1982; Tatner and Horne, 1983 and Goldes, Ferguson, Daoust and Moccia, 1986).

Oral vaccination, which avoids handling stress and the time/labour costs of other methods, was first investigated by Duff (1942) but, has not yet been exploited commercially. Oral vaccination trials have produced moderate and short lived protection (Lamers, 1985). The fore-gut appears to modify antigens but, when this was bypassed by anal intubation of V.anguillarum and Yersinia ruckeri in rainbow trout, protection was achieved that was greater than by oral and immersion techniques and comparable to protection achieved by injection of antigen (Johnson and Amend, 1983). Work by Rombout et al. (1986) has shown that systemic antibody titres can be raised against

V.anguillarum by anally intubating the bacteria, but cannot be raised by oral intubation. These results have led both groups to suggest the way forward in the development of oral vaccination is to protect the antigen against the anterior gut, possibly by microencapsulation. It is possible, however, that adjuvants may enhance the performance of oral vaccines. The use of adjuvants for oral and bath vaccination is not well documented (Agius, Horne and Ward, 1983 and Ward, Tatner and Horne, 1985). Tatner and Horne (1983) reported that alum improved the uptake of V.anguillarum by immersion, and Anderson, van Muiswinkel and Roberson (1984) reported higher titres to Y.ruckeri O antigen by prior immersion in dimethylsulphoxide (DMSO). Promising results have been reported on the use of cholera toxin as an adjuvant on the local immune response of mice to experimental antigens (Lycke and Holmgren, 1986). Quillaja saponin was found to markedly potentiate the humoral immune responses of mice fed inactivated rabies vaccine, and increased their resistance to subsequent intracerebral challenge with live rabies virus (Maharaj, Froh and Cambell, 1986). The use of such components may be useful in fish.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

a) Fish

(i) Adults

Adult S.canicula of both sexes weighing approximately 600-1000g were obtained from the Marine Biological Association, Citadel Hill, Plymouth and maintained in large (648 litre) polythene tanks in cooled recirculated seawater (12-14°C). The fish were fed on chopped coley.

(ii) Juveniles

Fertilised eggs were collected from the above tanks containing adult experimental fish. Egg cases were attached to a metal framework below the water surface, in 180 litre polythene tanks, and labelled according to the month of collection. Care was taken to keep the eggs well aerated, but physical disturbance was avoided. Handling and removal from the water usually led to the death of larval fish. The young hatched after 6-9 months (at 12-14°C) and were placed in separate tanks, after 3 weeks the fish were fed on finely chopped coley.

Temperature and salinity were monitored regularly by the aquarium staff. For the purpose of idenfification plastic tags were attached to the dorsal fin by nylon line, or indentations were made in the fins.

b) Chemicals

Unless otherwise stated chemical were obtained from the Sigma Chemical Company Ltd., Poole.

c) Antigens

Vibrio anguillarum was cultured in 100ml volumes of tryptone soya broth (TSB) (plus 1.5% NaCl) in 200ml Erlenmeyer flasks under static culture conditions at 20°C. The bacteria were killed by fixing in 0.6% formaldehyde, washed in several changes of phosphate buffered saline (PBS) and stored at -20°C. Bacteria were broken down using a 150 watt sonic disintegrator (MSE, London) using the technique described by Parker (1985).

2.2 Methods

a) Experimental antigenic challenge and the production of antisera

(i) Fish

To investigate the local and systemic humoral responses in the dogfish, antigens were injected into the peritoneum (IP) or intubated into the cardiac stomach or anus. Details are given in Chapter 5.

Routine blood samples were taken from the caudal sinus of fish anaesthetised in MS222 (Sandoz, Basel) with a 19ga needle and 5 or 10ml syringe. Blood was sampled from juvenile fish by severing the tail of anaesthetised fish and collecting blood from the exposed sinus with a capillary tube (Kernick, Cardiff). Blood was allowed to clot at room temperature for 30 minutes, then at 4°C overnight. Complement activity was destroyed by heating at 56°C for 15 minutes (Parish, 1981). Serum was stored at -20°C in the short term (weeks) and at -70°C in the long term (weeks/ months).

Gut mucus was sampled from freshly killed fish by gently scraping the surface of the mucosa with a spatula, bile was aspirated from the gall bladder with a 23ga needle and 1ml syringe. Both samples were stored in a similar fashion to serum.

Routine haematology and cell adherence has been described previously (Parish, 1981).

(ii) Mammals

Several antisera were raised in Dutch rabbits (Hyline Ltd., Cheshire). An antiserum to bovine serum albumin (BSA) was prepared by dissolving 25mg of protein in 0.25ml of phosphate buffered saline (PBS) which was mixed thoroughly with an equal volume of Freund's complete adjuvant (FCA) (BDH, Bristol) and injected subcutaneously. A second injection of 10mg of BSA in Freund's incomplete adjuvant (FIA) was given in a similar manner 6 weeks later. The rabbits were bled from the marginal ear vein approximately 6 weeks after the second injection and the strength and specificity of the antiserum was checked by immunoelectrophoresis against BSA. Blood was clotted and stored as above.

An antiserum was raised in rabbits against whole dogfish serum (WDS) by the technique of Morrow (1978), and an antiserum was raised against bile Ig by substituting bile Ig for WDS. The technique of Ellis (1976) was slightly modified to produce a rabbit antiserum against dogfish IgM. Briefly, an antiserum to sheep red blood cells (SRBC) was first raised in adult dogfish to a titre of approximately 1 : 1000, this was used to agglutinate SRBC's which were then washed exhaustively in PBS and injected subcutaneously into rabbits at 1 month intervals.

b) Morphology and ultrastructure

(i) Acetic acid technique

The acetic acid technique (Cornes, 1965) was used to dissolve the gut epithelium, leaving opaque areas where lymphocytes had accumulated. The gut of adult fish was excised, washed in elasmobranch saline (ES) (Hale, 1965), pinned onto a wax coated board and immersed in 10% acetic acid at 4°C for 24 hours. The gut was examined with the aid of background lighting.

(ii) Paraffin wax histology

This technique was used to make an extensive study of the trends in the distribution of leucocytes and leucocyte accummulations in the gut of adult dogfish. Pieces of tissue (approximately $2cm^3$) were dissected from the gut, washed in ES to remove gut debris, fixed in 10% formol saline for 24 hours at 4°C, dehydrated in graded alcohols, cleared in xylene and finally embedded in molten Fibrowax (BDH, Bristol) at 56/58°C under vacuum for 30-40 minutes. Sections were cut at 5-8μm with a steel knife on a Reichert Jung rotary microtome, and stained by standard procedures (Table 1).

iii) Cryostat histology

Cryostat sections were prepared on a Reichert Jung Fridgocut. Small pieces of tissue (approximately $1cm^3$) were excised and frozen to filter paper on the machine's block face at between -40 to -70°C. Sections were cut at 5-7μm, picked-up on grease-free glass slides, air dried and fixed in pure acetone. Material was stored at -20°C for up to 2 weeks or a month at -70°C prior to use.

(iv) Resin histology

Methacrylate resin (TAAB, Reading).

Small pieces of tissue ($2mm^3$) were fixed and dehydrated as for wax histology, tissues were not cleared in xylene but placed directly into a graded series of alcohol and resin according to the manufacturer's instructions. The resin was polymerised chemically under anaerobic conditions, and 1-2μm sections were cut with glass knives on a Reichert Jung Autocut.

Lowocryl K4M (TAAB, Reading)

This resin is particularly suitable for histochemistry and immunochemistry. Small pieces were fixed by several methods (Table 1) and dehydrated at low temperatures, then infiltrated by resin which was polymerised by ultra violet light at $-18^{o}C$ (TAAB data sheet No.5).

v) Staining

Wax sections were stained with haematoxylin and eosin and Mallory's trichome stain to establish the basic morphology and distribution of diffuse cells and lymphoid accummulations.

Methacrylate sections were stained with Giemsa, periodic acid Schiff and methyl green pyronine stain to establish the basic characteristics of leucocytes in the alimentary tract (Table 1).

To determine the histochemical nature of the cytoplasm of gut leucocytes material was embedded in lowocryl K4M resin and stained by several techniques (Table 1).

TABLE 1 EMBEDDING AND STAINING TECHNIQUES

STAIN	FIXATION	EMBEDDING MATERIAL	AUTHORITY AND MODIFICATIONS
Haematoxylin and Eosin (H&E)	FS	Paraffin wax	Pearse, 1968
Mallory's trichrome	FS	Paraffin wax	" "
Methylene blue	FS,,PF, G., 70%A.,Act.,	Methacrylate and Lowocryl resin	" "
Giemsa .	" " " " "	" "	Parish, 1981
Periodic acid Schiff's (PAS)	" " " " "	" "	Hayhoe and Flemens, 1969
Methyl green pyronine	" " " " "	" "	Pearse, 1968
Sudan black	" " " " "	" "	Hayhoe and Flemens, 1969
Alkaline phosphatase	" " " " "	" "	Culling, 1974 (incubated at room temperature)
Peroxidase	" " " " "	" "	Pearse, 1968 (counter-stained with toluidine blue for 30 sec/1min)
Sulphatase	" " " " "	" "	Austin and Bischel, 1960
Non-specific esterase	" " " " "	" "	Li, Yam and Lam, 1970 (methyl green counter-stain, incubated at room temperature)
Acid phosphatase	" " " " "	" "	Hayhoe and Flemens, 1969

FS : 10% formol saline
PF : paraformaldehyde
G : glutaraldehyde
70%A : 70% alcohol
Act : pure acetone

vi) Photography

Black and white photographs were taken through a green filter with a Pan F, 50 ASA film (Ilford, Essex), and colour slides with Fujichrome 100 ASA film (Fujii Ltd., London) using a Zeiss Photomicroscope II (Zeiss, Herts).

vii) Ultrastructure

Small pieces of tissue ($1mm^3$) were fixed in 2% glutaraldehyde and post-fixed in 2% osmium tetroxide, both solutions were made up in chilled ES, which had an osmolarity of 700 mOsm, to avoid the creation of expansion and shrinkage artifacts caused by osmosis across the cell membrane. After fixation the tissues were washed in ES, dehydrated in graded alcohols, transferred to propylene oxide and then into a graded series of propylene oxide and Spurr resin (TAAB) mixtures. The resin was cured at 70°C for 8 to 9 hours. Gold and silver sections (600-800Å) were cut on a Reichert Jung OM3 ultratome, mounted on copper grids, stained with uranyl acetate and lead citrate then examined using a Philips 300 series transmission electron microscope.

c) Protein separation

i) Gel filtration

Sepharose 6B, an agarose gel with a large pore size was used to separate the high molecular weight immunoglobulin containing fraction of dogfish sera and bile. This technique separated globular proteins with molecular weights of between 1.5×10^4 - 1.5×10^6 daltons.

Gels were degassed, allowed to pack in an 8.5 x 1.5cm column and equilibrated with 20mM phosphate buffer pH 8.0 containing 1% (W/V) sodium chloride and 0.1% (W/V) sodium azide. Fractions of 3.4ml were collected, at a flow rate of 10ml/hr^{-1}, at 4°C. The homogeneity of the gel and void volume were established using blue dextran. Samples were made up with 10% sucrose, chilled to approximately 0°C and injected into a bubble trap. The column effluent was monitored at 280nm with a Uvicord II detector unit (LKB). All equipment with the exception of the Uvicord were supplied by Pharmacia Ltd.

ii) Electrophoresis

Preparative agarose block

This technique was undertaken using the method previously described by Morrow (1978).

Polyacrylamide gel electrophoresis (PAGE)

Gel casting.

Two layer (13% and 4.5% gel)

Sodium doedcyl sulphate (SDS) slab PAGE was carried out according to the procedure of Laemmli (1970) using a 13% separating and 4.5% stacking gel. The composition of the stock solutions and the volumes of each required are given in Tables 2 and 3.

The separating gel was poured into the gel former, over-layered with water saturated n-butanol (BDH, Bristol) and allowed to polymerise in the dark for approximately 45 minutes. When polymerisation was complete the n-butanol was washed off with several changes of distilled water. The stacking gel was then poured onto the separating gel, the well former placed in

position and left to polymerise as above. Gels retained in the gel former, with the well former still in place, were wrapped in cling film and stored at 4°C overnight.

Single layer 3% gel with 1% agarose.

A 3% PAGE slab gel was used to separate unreduced high molecular weight (HMW) proteins. These gels were very fragile, and to allow handling 1% agarose was incorporated into the gel matrix. Briefly, the components necessary to make the 3% gel (Tables 2 and 3), except for the water and the catalyst, were heated to 35°C. Agarose was then added to the appropriate volume of water, dissolved by heating in a microwave oven (Philips Cooktronic 7910, Sweden) for a few seconds, then allowed to cool to approximately 45°C. The molten agar and catalyst were simultaneously added to the other components, quickly mixed, and poured into a prewarmed gel former and a well former immersed in the upper gel. The gel was allowed to cool and polymerise for several hours in the dark.

Sample Preparation

For examination on a 13% slab gel samples were boiled for 5 minutes in equal volumes of loading buffer containing 2 mercaptoethanol, to reduce disulphide bonds and SDS, to neutralise the natural charge of the molecules. Samples to be examined on 3% gels were treated only with SDS at room temperature for two hours prior to electrophoresis.

Sample loading

Samples (20-40µl) were loaded into wells with a Hamilton syringe (Hamilton, Bondaz AG, Switzerland).

TABLE 2 STOCK SOLUTIONS FOR POLYACRYLAMIDE GEL ELECTROPHORESIS

STOCK SOLUTIONS

30% Acrylamide and 0.8% bisacrylamide (filtered and stored at $4^{o}C$)

10% SDS

N,N,N',N'-Tetramethylethylenediamine (TEMED)

10% Ammonium persulphate (made up fresh)

Separation gel buffer 1.5M Tris-HCl pH 8.9

Spacer gel buffer 0.5M Tris-HCl pH 6.7

Electrode buffer	6g	Tris
	28.8g	Glycine
	2g	SDS
Loading buffer	1%	SDS
	10%	Glycerol
	0.1%	Mercaptoethanol
	0.002%	Bromophenol blue (dissolved in /10 strength spacer gel buffer)

TABLE 3 VOLUMES (IN ML) OF STOCKS REQUIRED FOR CASTING POLYACRYLAMIDE GELS

	Separation Gel		Spacer Gel
	3%	13%	4.5%
Acrylamide/bis	2.5	8.7	1.5
Separation buffer	2.5	2.5	-
Spacer buffer	-	-	-
Water	22.4	8.5	5.2
SDS	0.2	0.2	0.1
N,N,N'N'-Tetra-methylethylenediamine (TEMED)	0.01	0.01	0.01
Ammonium sulphate	0.10	0.10	0.1

Electrophoresis

Electrophoresis was carried out using a constant current supplied from a Pharmacia power pack (ECDS 3000/150). A current of 10mA was applied for the first 30 minutes, then 20mA until the bromophenol blue marker was approximately 1cm from the bottom of the gel.

Staining

Gels were stained in Coomassie brilliant blue solution, containing 2g of Coomassie brilliant blue (R-250), dissolved in 1 litre of 50% (V/V) methanol containing 10% (V/V) acetic acid. Gels were destained at 55^{o}C in 10% (V/V) methanol containing 10% (V/V) acetic acid. Bands were observed on a light box and photographed through an orange filter.

d) Immunological techniques

i) Direct Agglutination.

This test was performed in disposable Titertek trays (Flow Laboratories, Ayrshire) using a Titertek single and multipipettes to dispense liquid.

Bacterial agglutination

Doubling dilutions of 50µl aliquots of serum from dogfish immunised with V.anguillarum were made to a dilution of 1:4016 using ES as a diluent. Aliquots of 50µl, containing 1 x 10 bacterial cells per ml were added to each well, gently agitated, incubated at room temperature for 1 hour then at 4°C for 24 hours. After which plates were read. Non-immune fish sera, bile or PBS were used as negative controls. No positive controls were employed.

Haemagglutination

Doubling dilutions of 50µl of heat inactivated sera (56°C for 15 minutes) were prepared as above, 50µl aliquots of 1% (V/V) SRBC's were added to each well then incubated as above using the same controls.

ii) Agar gel precipitation studies

Double diffusion

The method of Ouchterlony (1948) was used to compare the antigenic cross identity between bile and serum Ig's. A 1% (W/V) agarose suspension was made up in PBS and dispensed into petri dishes in 15ml aliquots.

Immunoelectrophoresis

The immunoelectrophoretic technique of Scheidegger (1955) was employed using a Shandon 600mm x 100mm tank and Vokan 400 power pack. Standard microscope slides (76 x 25 x 10mm) were washed in absolute alcohol and mounted on a perspex tray (Shandon, London) and covered with a thin film of 1% agarose (W/V) in 0.8M barbitone buffer at pH 8.2.

Samples (approximately 5-10µl) were loaded into wells with a capillary tube. Bromophenol blue was used as a migration marker. The tank buffer was the same as that used to make up the gels. Electrophoresis was carried out at 30mA for approximately 1-2 hours after which the appropriate antiserum was added to the trough and left overnight in a humidity chamber. The gels were exhaustively washed in PBS, dried, and stained with Coomassie brilliant blue.

Rocket electrophoresis

A procedure based on the technique described by Jurd (1981) was employed. Agarose was made up as for immunoelectrophoresis, cooled to approximately 45°C and while still molten 0.8% (V/V) of the appropriate antisera was added. This was pipetted onto clean grease-free plates (8 x 8cm) in aliquots of 15mls, giving a layer approximately 2mm thick. Once the gel had set a line of holes 4mm in diameter were punched in the gel 1cm from the edge, 5µl samples were loaded with a capillary tube. The plate was then placed in a electrophoresis tank, with holes nearest the cathode, and ran at 20mA, for 2 hours, by which time any insoluble precipitation rockets formed.

f) Immunofluoresence (IF)

The indirect IF test described by Wick, Baudner and Herzog (1978) was used to detect bacteria and protein uptake by the gut and to locate Ig-producing cells in the intestine.

To detect the site of bacteria and protein uptake 5-7µm cryostat section of the gut were fixed in acetone for 15-30 minutes at 4°C and stored at -20°C for up to a week, or at -70°C for up to a month. To detect Ig-producing cells gut sections were not fixed but, washed for 1 hour in PBS, prior to processing, to remove interstitial immunoglobulin which may have given false positive results. The protocol for detection of protein and bacterial uptake is also given in Table 4. Fluorescence was detected on an Olympus BH epifluorescence microscope (Olympus, Leics).

TABLE 4 PROTOCOL FOR IMMUNOFLUORESCENCE

COMPONENT UNDER INVESTIGATION	FIXATION OF SECTIONS	PRE-WASH IN PBS	1° ANTISERA	WASH IN PBS	2° FLUORESCEIN COMPONENT	WASH IN PBS	MOUNT IN GLYCEROL	OBSER-VATION
PROTEIN AND BACTERIA UPTAKE	Acetone(4°C) 15/20 min	3x5 min	Diluted to 1/20, 60 min	3x5 min	Diluted to 1/20, 60 min	3x5 min	✓	✓
IMMUNO-GLOBULIN CONTAINING CELLS	None	"	"	"	"	"	"	"
CONTROL NO.I	(1)	"	"	"	Saline	"		"
CONTROL NO.II	(1)		Saline	" " "	2° Anti-sera, 60 min	"	"	"
CONTROL NO.III	(1)	"	1° Anti-sera raised against another unrelated antigen	"	"	"	"	"

(1) - For bacteria detection material was fixed in acetone, while for immunoglobulin detection material was unfixed.

CHAPTER 3

AN INVESTIGATION OF THE NATURE AND DISTRIBUTION OF LEUCOCYTES IN THE MAJOR MUCOSAL SURFACES

The mucosa of the gut was examined by acetic acid erosion of the epithelium, resin and wax histology and electron microscopy. The reproductive tract was examined by resin histology and electron microscopy, while the gall bladder and gill were examined by resin histology alone.

3.1a The morphology of the alimentary tract

The alimentary tract of 3 male and female dogfish was divided into 6 major regions: the buccal cavity, the oesophagus, the cardiac and pyloric stomachs, the intestine and the rectum (Figure 1). Each region had a characteristic epithelium and subepithelium, which is outlined in Table 5 and amplified in Plate 1.

3.1b The distribution of lymphoid accumulations

To visualise lymphoid accumulations, the dogfish gut was immersed in 10% acetic acid at 4°C for 48 hours. After the epithelium had been dissolved, lymphoid accumulations appeared as white nodules approximately $1mm^3$. These were excised, smeared on glass slides and stained with Giemsa. Amongst the debris of epithelial cells, lymphocytes were detected. Accumulations were absent from the cardiac stomach, and because of the nature of the epithelium, they could not be detected in the spiral valve. The pyloric stomach yielded the highest number of accumulations (Table 6). When visualised by wax histology a similar distribution of lymphoid accumulations was observed, except numerous additional accumulations were detected by wax histology in the proximal spiral valve (Table 7).

FIGURE 1 MAJOR REGIONS OF THE ALIMENTARY TRACT

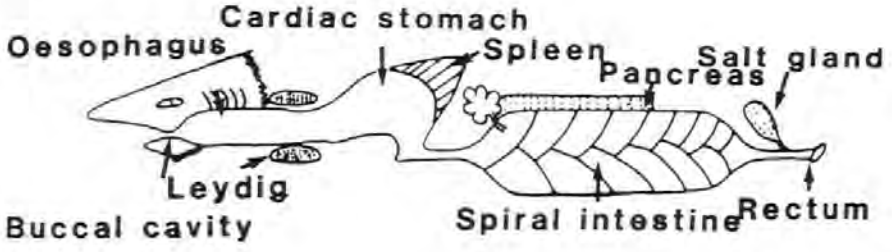


TABLE 5 NATURE OF THE EPITHELIUM AND SUBEPITHELIUM OF THE ALIMENTARY TRACT

REGION	BUCCAL CAVITY	OESOPHAGUS	CARDIAC STOMACH	PYLORIC STOMACH	UPPER SPIRAL INTESTINE	LOWER SPIRAL INTESTINE	RECTUM
NATURE OF THE EPITHELIUM	Stratified epithelium, unicellular mucus glands, taste papillae	Folded ciliated epithelium	Gastric glands with pyramidal secretory	c.f. cardiac stomach	The intestine is essentially a tube containing the spiral valve; which has columnar epithelial cells with numerous microvilli		Stratified epithelium
NATURE OF THE SUBEPITHELIUM	Cartilagenous	Thin	Muscular	Muscular, with a distal sphincter region	Less muscular than stomach region. Vascular		Thin
PLATE NUMBERS	1a	1b	1c	1d	1e	1e	1f

TABLE 6 NUMBERS OF LYMPHOID ACCUMULATIONS VISUALIZED BY ACETIC ACID TREATMENT OF THE DOGFISH GUT

FISH NO.	BUCCAL CAVITY	OESOPHAGUS	CARDIAC STOMACH	PYLORIC STOMACH			UPPER SPIRAL VALVE	LOWER SPIRAL VALVE	RECTUM
				UPPER	MIDDLE	LOWER			
1	1	4	0	20	8	23	0	0	0
2	2	3	0	17	16	30	0	0	1
3	0	2	0	15	12	26	0	0	1

(N.B. Represents data from 3 fish)

TABLE 7 NUMBERS OF LYMPHOID ACCUMULATIONS VISUALIZED BY WAX HISTOLOGICAL ANALYSIS OF THE DOGFISH GUT

FISH NO.	BUCCAL CAVITY	OESOPHAGUS	CARDIAC STOMACH	PYLORIC STOMACH			UPPER SPIRAL VALVE	LOWER SPIRAL VALVE	RECTUM
				UPPER	MIDDLE	LOWER			
1	+	+	-	++	+	+++	++	-	-
2	-	-	-	++	+	+++	++	-	-
3	+	-	-	++	+	++++	++++	-	+
4	++	-	-	++	+	+++	+++++	-	+
5	-	-	-	++	+	+++	+++	-	-
6	-	-	-	++	-	++++	+++	-	-

++++, Maximum number of accumulations recorded; -, No accumulations recorded
(N.B. Represents data from 6 fish)

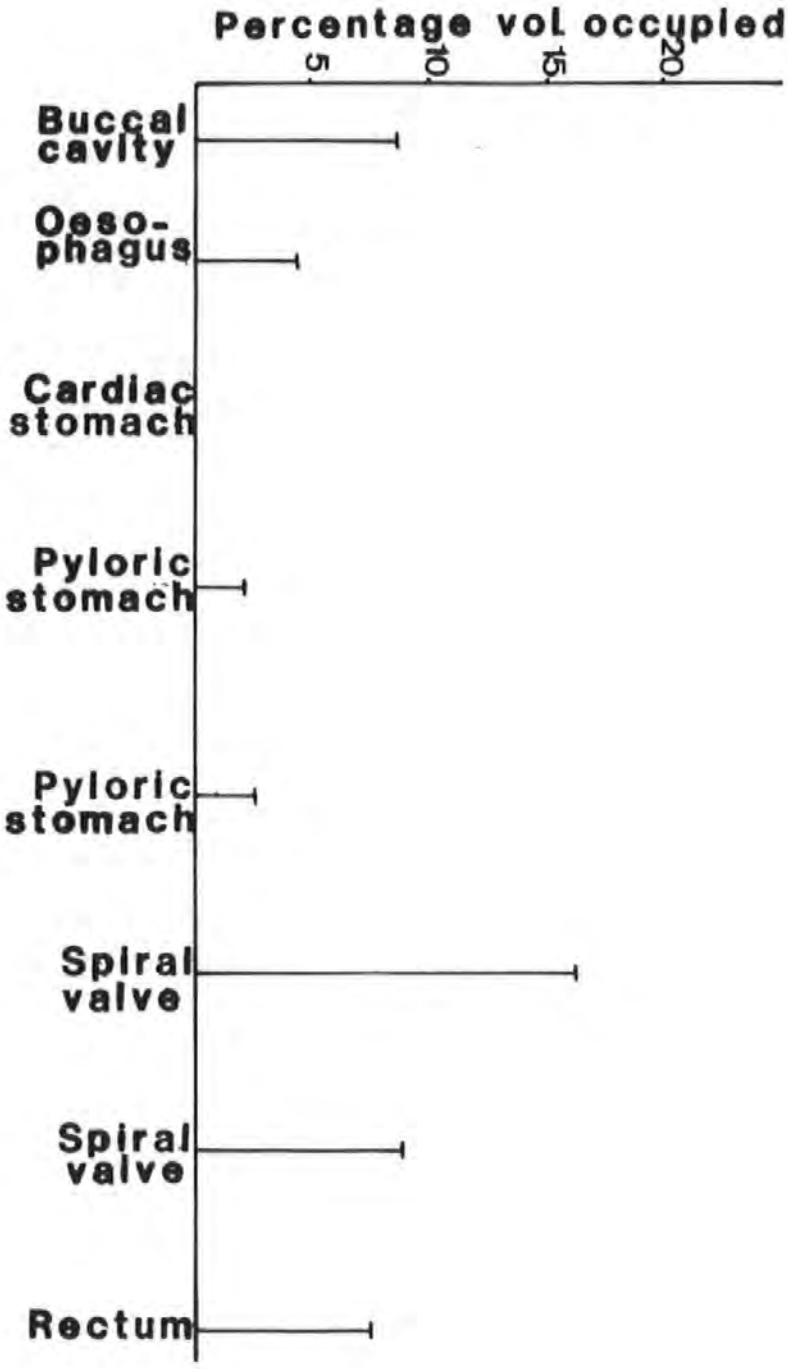
3.1c Examination of accumulations by light and electron microscopy

Two types of lymphoid accumulation were recognised in the alimentary tract: a, as flat infiltrations of cells in the lamina propria and lower epithelium of the upper and mid-pyloric stomach (Plate 2A); b, as larger accumulations, often occupying whole folds of the gut which were predominantly found in the proximal intestine (Plate 2B). The latter form of accumulation was more extensively studied, as it was localised just posterior to the junction of the pyloric stomach and spiral valve and, therefore, quite easy to locate. Small lymphocytes (Plate 2D) were the principal cell type along with a few macrophages (Plate 2C), whereas plasma cells and granulocytes were absent. The bulk of the cells in any accumulation were found in the lamina propria (Plate 2B). The cells were tightly packed and appeared to distort the shape of the intestinal folds (Plates 2B and 3A), compared to folds not containing accumulations of lymphocytes (Plate 3B). At the site of accumulations the basement membrane often appeared to have been breached (Plate 3C) and lymphocytes and macrophages were detected in the epithelial and laminal compartments (Plate 3C).

3.1d Distribution of intra-epithelial and intralaminal leucocytes

Prior to identification of specific cell types, intra-epithelial leucocytes (IEL'S) (defined as any leucocyte-like cell in the epithelium), were counted using a GW2 graticule (Graticules Ltd, Tonbridge, Kent) and expressed as a percentage of the epithelial volume occupied (Figure 2). IEL's were absent from the cardiac stomach and found at highest levels in the proximal spiral valve, where approximately 17% of the total epithelial volume was occupied by leucocytes.

FIGURE 2 PERCENTAGE (%) OF THE EPITHELIAL VOLUME OCCUPIED BY LEUCOCYTES ALONG THE LENGTH OF THE DOGFISH GUT (N.B. DATA REPRESENT MEANS FROM 6 ADULT FISH).


Percentage vol. occupied
5.
10.
15.
20.
Buccal cavity
Oeso-phagus
Cardiac stomach
Pyloric stomach
Pyloric stomach
Spiral valve
Spiral valve
Rectum

The distribution of intralaminal leucocytes (ILL'S) was not quantified because they had an irregular distribution within the lamina propria. Like IEL's, however, most ILL's were detected in the spiral intestine.

3.1e Characterisation of intra-epithelial and intralaminal leucocytes

As identification of the different cell types in all regions of the gut was not practical, only the epithelial and laminal leucocyte populations of the upper spiral valve were examined. No staining reaction was detected with the histochemical stains (Table 1), but the following cell types were recognised using Giemsa, PAS, methyl pyronine and transmission electron microscopy.

Lymphocytes These were small cells, 7-10μm in diameter, with a thin layer of basophilic cytoplasm when stained with Giemsa (Plate 4A) but, which did not stain with PAS or methyl green pyronine. When examined by electron microscopy these cells had a high cytoplasm to nucleus ratio and an abundance of condensed chromatin. The cytoplasm contained a few mitochondria and golgi apparatus (Plate 4B and C). Two types of lymphocytes could be distinguished: granular (Plate 4B) and agranular (Plate 4C) types; the granules of which were small, electron dense and membrane bound. Lymphocytes were detected in both the lamina propria and epithelium.

Plasma cells These were detected only in the lamina propria of the gut, often in groups of up to twenty cells and in close proximity to blood vessels. When stained with methyl green pyronine the cytoplasm, containing RNA, stained pink and the nucleus, containing DNA, green (Plate 5A). The cytoplasm of these cells stained basophilically with Giemsa (Plate 4A), unstained areas, adjacent to the nucleus, occurred in cells treated by both the above stains and were thought to represent the site of the Golgi apparatus. Examination by

electron microscopy revealed that the nucleus contained both peripherally and centrally condensed chromatin (Plate 5C), giving rise to the "clockface" arrangement seen at the light microscopic level (Plate 5B). The cytoplasm contained dilated profiles of endoplasmic reticulum; golgi apparatus; mitochondria and a few electron dense granules (Plate 5C). These cells were further investigated by an indirect immunofluorescent test, in which a rabbit antiserum to dogfish IgM, and a goat anti-rabbit fluorescein-labelled conjugate was employed. Fluorescent cells could be detected in frozen sections. The fluorescence was concentrated in cells located in the lamina propria.

Macrophages Macrophage-like cells were detected in the epithelium and lamina propria at the light microscope level with Giemsa. Macrophage-like cells had a pale basophilic cytoplasm and contained inclusions (Plate 4A), a PAS-positive reaction occurred with Schiff's reagent in cells which also contained inclusions (Plate 6A). The macrophage-like cells appeared to be motile, and consequently morphologically heterogenous, especially when observed by electon microscopy (Plates 6B and C). In the former plate an apparently mobile macrophage has made contact with a plasma cell, and in the latter plate a macrophage is in the process of phagocytosing an effete cell. Macrophage-like cells contained mitochondria, various inclusions and electron translucent vacuoles containing a few particles.

Granular cells Granular cells were identified in the lamina propria and epithelium at the light microscope level (Plate 4A). These cells contained pink staining granules (when stained with Giemsa), which were unlike granules of the peripheral blood, Leydig organ and epigonal tissue granulocytes, which had an orange pigmentation. Cells contained various numbers of granules, they reacted poorly with PAS and produced no staining reaction with a variety of histochemical stains. The granular cells could be separated into three populations after ultrastructural examination:-

Type 1 (Plate 7A) was found in both the lamina propria and the epithelium. The cytoplasm contained numerous mitochondria and electron dense granules, which were regular in shape, mottled and membrane bound (Plate 7B). This cell type appeared to be very mobile.

Type 2 (Plate 7C, E) was found in the lamina propria and epithelium. The granules of this cell type were fibrillar with electron dense areas (7D and F), and were present in the cytoplasm along with mitochondria. This cell type appeared to be less motile than type 1; being located mainly in the basal epithelium with cytoplasm distributed evenly around the nucleus.

Type 3 (Plate 7G) was very infrequently observed in the epithelium and lamina propria and was characterised by very fine granules and vacuoles in the cytoplasm.

3.1f Movement of cells

While no tracking experiments were undertaken, movement of cells could be inferred by observing the distribution of cells in the gut mucosa. Cells were observed apparently crossing the endothelium of blood vessels in the lamina propria (Plate 8A), moving through the lamina propria (Plate 8B), crossing the basement membrane (Plate 8C) or moving through the epithelium and towards the epithelial surface (Plate 8D).

3.2 The gall bladder and liver

The gall bladder develops from the embryonic gut and is linked to the latter organ in adult fish by the bile duct (Plate 9). A brief histological survey of the liver and the gall bladder was undertaken. The liver contained no lymphoid cells (Plate 10A) while the gall bladder, like the gut, had both intra-epithelial and intra-laminal cell

populations (Plate 10B, C, D); accumulations of lymphocytes, however, were absent. Plasma cells were detected in the lamina propria of the gall bladder by light microscopy (Plate 10D).

3.3 The female reproductive tract

The female reproductive tract was also briefly examined by resin histology and electron microscopy. The tract could be divided arbitrarily into 6 zones (Plate 11, Table 8) and the nature of the epithelium and subepithelium is summarised in Table 4. Intra-epithelial leucocytes (Plate 12A) (specific cell types were not investigated) were observed throughout the reproductive tract but mostly in proximal zone 6 (Table 8). Plasma cells, examined by LM (Plate 12A) and EM (Plate 12C), were located in the subepithelium and were also most frequent in zone 6. Accumulations of leucocytes occurred only in the nidamental gland (Plate 12D).

3.4 The gills

Light microscopy revealed that the gills contained few intra-epithelial leucocytes, although it was very hard to determine accurately whether leucocytes were contained in vacuoles in the epithelium or blood capillaries. At the base of the gill, large spaces were detected, these were the corpus cavernosum (Plate 13A). The spaces were lined with leucocyte-like cells (Plate 13B). Some cells were closely associated with the surface of the cavity, while others were less closely attached (Plate 13C).

N.B. Information contained in this chapter has been published elsewhere (Hart, Wrathmell and Harris, 1986a and b, and Hart, Wrathmell, Harris and Doggett, 1987 and Hart, Wrathmell, Harris and Doggett, In Press).

TABLE 8 HISTOLOGY OF THE FEMALE REPRODUCTIVE TRACT

ZONE	1	2	3	4	5	6
NATURE OF EPITHELIUM	Stratified, 672-900μm many goblet	Folded columnar, 40-50μm	As zone 2	As zone 2	Folded, ciliated 20-40μm	Highly folded, 20-40μm
NATURE OF SUBEPITHELIUM	Muscular	Muscular, numerous subepithelial sinuses in close proximity to the basement membrane	As zone 2	As zone 2	Accumulations of intralaminal leucocytes in the connective tissue of the nidamental organ	Large proportion taken up by blood sinsues
DISTRIBUTION OF SUBEPITHELIAL PLASMA CELLS	-/+	-	As zone 2	As zone 2	-/+	+++
DISTRIBUTION OF INTRA-EPITHELIAL LEUCOCYTES	-/+	+	As zone 2	As zone 2	-/+	+++

(NB. Represents the data from 3 fish)

3.5 Discussion

3.5a Accumulations

The acetic acid technique (Cornes, 1965) was found to be useful in determining the general distribution of lymphoid accumulations in the gut with the exception of the spiral valve. This organ had a complex valvular structure with a great deal of connective tissue and could only be satisfactorily investigated by histology. The pyloric stomach and spiral intestine contained the highest number of lymphoid accumulations, the next most populus region was the oesophagus, then the buccal cavity and rectum. Accumulations were absent from the lower spiral intestine and cardiac stomach.

The distribution of GALT has not been studied in any great detail in other fish. Lymphoid accumulations were found to be absent from the gut of cyclostomes (Fichtelius et al., 1968), but have been detected (if only by brief investigations), in actinopterygian, sarcopterygian and chondrichthyan fishes. Interestingly, primitive actinopterygii (e.g. chondrosteans), the sarcopterygii (e.g. Dipnoi) and chondrichthyes all have spiral valves in the intestine. A considerable amount of lymphoid tissue has been detected in the spiral valve of chondrosteans (Weisel, 1979); the lungfish (Good, Finstad, Pollara and Gabrielsen, 1966) and chondrichthyes (this study). Fichtelius et al. (1968) found large accumulations in the lamina propria associated with the spiral valve of Dasyatis americana and in the intestine of Heterodontus francisci and Aetobatus narinari (all of which are elasmobranchs). Zapata (1977) found accumulations in the intestine of Raja clavata and Torpedo mamorata, and Tomonaga et al. (1986) examined several species of sharks and rays and found that all had large accumulations of lymphocytes in the spiral valves. Teleosts, which have an elongated intestine without a spiral valve have been shown to harbour some lymphoid accumulations (Diconza and Halliday,

1971; Zapata, 1979 and Pontius and Ambrosius, 1972). Telostean accumulations were, however, a lot smaller than those of more primitive fishes.

In amphibia the distribution of GALT has been more thoroughly investigated. The intestine of Pleurodeles waltii contained considerably more lymphoid accumulations than did the rest of the gut (Ardavin, Zapata, Villena, Solas, 1982). A similar situation was found in Bufo marinus and several other anuran species, in which a consistently high population of lymphoid accumulations were detected in the intestine. In marked contrast in this same study, two urodeles : Notophthalmus viridescens and Necturus maculosus contained virtually no GALT (Goldstine, Manickavel and Cohen, 1975).

The reason for the apparent distribution of GALT in S.canicula, other fish species and amphibia is unclear. The structures may deal in some way with ingested antigens, this would explain in part the general paucity of accumulations from non-absorptive regions of the gut. It is interesting that the spiral valve; an ancient feature found in fossil placoderms; primitive actinopterygii; lungfish and elasmobranchs, contains considerable GALT in all of the extant primitive fishes examined.

In this study two types of unencapsulated lymphoid accumulations were observed in the large accumulations of the upper spiral valve and smaller flatter structures found in the rest of the gut. The morphology and ultrastructure of the former when investigated, appeared to be very similar to those found in S.torazame, M.mananzo, M.griseus and H.japonicus (Tomonaga et al., 1986). In this study, and in the investigation of Tomonaga et al. (1986) the accumulations usually occupied a central position, at the core of the spiral.

The majority of cells in the valvular accumulations of S.canicula were lymphocytes and a few macrophages. A similar situation exists in most other lower vertebrates, although granulocytes and plasma cells have been described in amphibian accumulations Ardavin et al. (1982) and Goldstine et al. (1975).

The basement membrane of the epithelium above the lymphoid accumulations appeared to be 'breached' in S.canicula. This was also found to be the case in amphibia (Goldstine et al., 1975) and was apparently caused by leucocytes spreading from the intralaminal accumulations into the epithelium.

While in S.canicula the intestinal folds were expanded and distorted by lymphocytes in the lamina propria, the overlying epithelium did not appear to be reduced in thickness, as described in other elasmobranchs (Tomonaga et al., 1986), nor was the number of goblet cells reduced, as occurs in amphibia (Ardavin et al., 1982 and Goldstine et al., 1975) and reptiles Solas, Leceta and Zapata (1981).

The function of lymphoid accumulations in lower vertebrates has not been established. Several workers have accomplished some limited experimental work. Ardavin (1980), Goldstine et al. (1975) and Solas et al. (1981) found that local stimulation with antigen increased the size of the gut and cloacal lymphoid tissue. El Ridi, El Deeb and Zada (1981) reviewed their group's work on snakes and lizards. They found that the number and size of the majority of gut-associated lymphoid aggregates was affected by seasonal conditions. In Chalcides ocellatus the intestinal lymphoid tissue was unaffected by seasonal change or thymectomy, while reduction in the size and number of oesophageal accumulations was recorded during the winter, and as a result of thymectomy. Removal of the spleen did not drastically effect either graft rejection or the humoral response to injected SRBC's in

Ch.ocellatus. These authors took this to imply that GALT may be a source of T-cells and a site of antibody synthesis, as besides the thymus, the GALT is the only well organised lymphoid structure in snakes and lizards. It is unclear whether the situation is as straight forward as this, or whether other areas may take over the lymphoid function besides the gut. In some fish, splenectomy has been shown to abrogate the antibody response (cf. Fänge, 1985) while in others (marine teleosts and elasmobranchs) splenectomy did not inhibit the ability of the fish to produce antibodies to injected antigens (Ferren, 1967).

El Ridi et al. (1981) also referred to work on Agama stellio and Chamaeleon chamaeleon which apparently had very few gut-associated lymphoid accumulations and did not produce antibodies to human serum albumin (HSA) or human red blood cells (HRBC), but was able to reject grafts. They inferred that GALT of lizards may play a crucial role in humoral immunity i.e. it might be a central lymphoid organ equivalent to the avian bursa of Fabricius. If the gut-associated lymphoid tissue of lizards was equivalent to the bursa of Fabricius it would, however, be hard to envisage these two species being able to survive in the wild without it. The lack of responsiveness to HSA and HRBC may only represent a specific insensitivity to these to antigens.

Lower vertebrate gut immunologists are divided as to the proposed function of GALT. Fichtelius et al. (1968) and several other authors (cf. El Ridi et al., 1981) discussed the possible role of GALT as a bursa equivalent. While other authors (cf. Ardavin et al., 1982) envisaged that GALT was a precursor to the higher vertebrate secretory defence mechanisms, which is manifested in the secretion of IgA in mammals and IgM in some birds (see Chapter 6).

Kobayashi, Tomonaga, Teshima and Kajii (1985) reported that the spleen of the Aleutian skate (Bathyraja aleutica) is the primary lymphoid organ for B lymphocyte differentiation and proliferation, possibly being equivalent to the bursa of Fabricius of birds. Subsequent work by Tomonaga et al. (1986) led them to the conclusion that lymphoid accumulations in several species of elasmobranch may represent primitive Peyer's patches.

Recent work on Peyer's patches in sheep have shown that they can act as primary lymphoid organs (Reynolds, Cahill and Trnka, 1981 and Morris, 1986), that they are different from germinal centres (Reynolds, 1985) and that their development can occur independently of antigen (Reynolds and Morris, 1984). Van Alten and Muehleman (1981) also reported that in addition to its role in the development of the B cell lineage in chickens, the bursa of Fabricius is also involved in the mediation of the local and systemic immune responses.

The latter workers have shown that the understanding of the exact function of higher vertebrate GALT is still uncertain. Little progress will be made towards understanding the role of lymphoid accumulations in the gut of lower vertebrates while workers concentrate on the morphology of these structures, and persist in trying to correlate them with either Peyer's patches or bursa of Fabricii in mammals and birds.

To establish the nature and role of lymphoid accumulations of lower vertebrates, surgical removal of accumulations, the uptake of luminal antigens and the ontogeny of these tissues might best be investigated.

3.5b Diffuse populations of leucocytes in the gut

While lymphoid accumulations were found only in the gut, diffuse populations of cells were revealed in the epithelium (intraepithelial leucocytes, IEL's) and lamina propria (intralaminal leucocytes, ILL's) of the gut, gall bladder and reproductive tract in this study. Small accumulations were, however, occasionally detected in the nidamental gland of the reproductive tract.

Similar populations have been detected in the gut (Ernst, Befus and Bienenstock, 1985), gall bladder (Kent, 1966) and reproductive tract (Ogra, Yamanaka and Losonsky, 1981) of higher vertebrates. A brief examination also revealed the presence of leucocytes in the gill. The latter structure contained large numbers of phagocytic cells lining the endothelium of the corpus cavernosum, which is thought to be part of the reticulo-endothelial system (cf. Hunt and Rowley, 1986). Immunity of the mammalian respiratory mucosa has recently been reviewed (Bergmann, Clancy and Petzoldt, 1985). The skin of S.canicula was not examined in this study but has recently been the focus of considerable attention in higher vertebrates (Bos and Kapsenburg, 1986).

In the gastrointestinal tract of S.canicula the highest number of IEL's and ILL's were detected in the intestine, their distribution, like that of lymphoid accumulations may be associated with the uptake of nutrients in this zone. In the cyprinid Barbus conchonius most IEL's were detected in the intestinal bulb, few in the middle part and a distinct increase noted in the second segment (Davina et al., 1982). Few IEL's were revealed in several elasmobranch species (Fichtelius et al., 1968 and 1969) but, in S.canicula up to 15% of the valvular epithelium was occupied by leucocytes. It was also noticed in S.canicula that considerably more leucocytes were detected in the epithleium than the lamina propria.

A large portion of the leucocytes in the epithelium of the valvular intestine contained granules, this is comparable to the situation which exists in mammals (Ernst et al., 1985). In S.canicula these cells were either granulocytes or granular lymphocytes. The latter differed from agranular lymphocytes solely in the possession of small, membrane bound osmiophilic granules. Granular lymphocytes were not recorded in the peripheral blood of S.canicula by Morrow and Pulsford (1980) and Parish, Wrathmell, Hart and Harris, (1986) but, were found in the blood of the nurse shark (Ginglymostoma cirratum) (Hyder, Cayer and Pettey, 1983). Insufficient evidence is available to determine whether these cells have a separate biological role. It is interesting, however, to note that the epithelium appears to be the preferred habitat of this cell type.

Three types of granulocytes were detected in the gut, Type 1, an apparently mobile cell, with large regular granules, was detected in both the lamina propria and the epithelium. This cell type was morphologically similar to the GI eosinophil of Mainwaring and Rowley (1985) and the Type 1a of Parish et al. (1986) all of whom worked exclusively on the peripheral blood of S.canicula. The Type 1 in the gut was also similar to the phagocytic granular cell of G.cirratum (Hyder et al., 1983) and the eosinophil of Mustelus lenticulatus (Hine and Wain, in press). If this granular gut cell Type 1 is analogous to Parish's Type 1 (Parish et al., 1986), then it may be involved in phagocytosis of material in the gut mucosa. Type 2 had irregular membrane-bound granules with a fibrillar matrix and crystalloid core. This type of cell was not found in the peripheral blood of S.canicula by the previous investigators (Parish et al., 1986). A similar cell, however, described as an eosinophil, was found in G.cirratum (Hyder et al., 1983) and Hine and Wain (in press) described a similar cell type as an eosinophilic granulocytes. Type 3 was typified by the possession

of numerous fine granules and bore no relationship to any cell lineages previously described in S.canicula peripheral blood.

Hine and Wain (in press) proposed that the SD type of M.lenticulatus [which appears to be a synonym of the Type IV granulocyte of S.canicula (Morrow and Pulsford, 1980 and Fänge and Pulsford, 1983) and the thrombocyte Type 2 of S.canicula (Parish et al., 1986)] may belong to the basophil/mast cell lineage. This cell type was not found to be associated with the lamina propria or epithelium of the gut in S.canicula.

Prior to 1977 evidence for the presence of mast cells in fish was sparse (Ellis, 1977a). Immediate hypersensitivity has been reported in fish and the responsive cell may be a PAS positive granulocyte in cyprinids or the eosinophilic granular cell (EGC) of salmonids (Ellis, 1982). EGC's were shown to degranulate in rainbow trout after injection with Aeromonas salmonicida toxins, and a simultaneous decrease in the histamine content of the gut suggesting that the cells are histaminogenic, and therefore similar to mast cells.

Macrophages were revealed in the intestinal mucosa phagocytosing effete cells and it is thought that they may deal with extraneous antigens in the same way.

While a considerable quantitative difference existed between the epithelium and the lamina propria, i.e. more leucocytes were present in the epithelium, an interesting qualitative difference in cell populations also existed. Plasma cells detected by LM and EM, and shown to contain Ig by immunofluorescence, were found only in the lamina propria. Small lymphocytes were located in both the lamina propria and epithelium, but predominately the latter. This may represent evidence that the lamina propria and epithelium are different immune compartments separated by the basement membrane. Immunoglobulin

containing plasma cells have been detected in the gut of other elasmobranchs (Tomonaga, Kobayashi, Kajii and Awaya, 1984) and the lamprey, a primitive vertebrate (Fujii, 1982).

In mammals Ig-synthesising cells are located beneath the secretory epithelium of the gut (Befus and Bienenstock, 1982; McDermott, Clark and Bienenstock, 1982). The precursors of these cells may originate in the Peyer's patches (Cebra, Gearhart, Kamat, Robertson and Tseng, 1977), or possibly from other sources in the alimentary tract of sheep (Morris, 1986). As these progenitor cells mature they become committed to Ig synthesis and migrate to the mesenteric lymph nodes (McWilliams, Phillips-Quagliata and Lam, 1975). Lymphocytes then undergo blastogenesis and seed via the thoracic duct and circulation to the intestinal lamina and other mucosal sites (McWilliams et al., 1975; Befus and Bienenstock, 1982). Why plasma cells in both mammals and this species of elasmobranch (S.canicula) are largely restricted to the lamina propria, and the highest concentrations in this fish are found in the upper spiral valve, is unknown. Undifferentiated B cells may be stimulated in the epithelium, then migrate into the lamina propria and form plasma cells. Alternatively, B cells may be exposed to antigen in the lamina propria and differentiate at the site of exposure. In S.canicula there is no evidence to implicate lymphoid accumulations in the process of plasma cell differentiation, as has been proposed for some mammals (see above).

An obvious method of investigating these hypotheses in fish is not immediately apparent. It seems unlikely that adoptive transfer experiments which have defined the migration of transformed T and B cells in mammals (McDermott, Horsewood, Clark and Bienenstock, 1986) could be used in fish because of the technical difficulties in cannulating vessels draining the gut. The contribution of thymocytes, however, could be achieved by thymectomy of young fish, a technique which has been applied with most encouraging results in salmonids (Grace, 1981).

3.6 The urinogenital tract

Scyliorhinus canicula is ovoviparous but, only the right oviduct is functional in this species. Eggs, released from the ovary, are collected in a ciliated funnel and transported to the nidamental gland where albumin and the eggcase is added. Encased eggs are extruded through the vent.

While some work has been undertaken by Bly (1984), Bly, Grim and Morris (1986) and Hogarth (1968, 1972a, 1972b and 1973) on maternofoetal relationships in fish nothing is known about local immunity in the reproductive tracts of fishes.

In mammals defence against local infection in the reproductive tract has been reviewed (Ogra et al., 1981). In the S.canicula IEL's and intralaminal plasma cells were detected. These were mostly concentrated in the proximal zones of the reproductive tract. The epithelium contained many goblet cells, the mucus from which appears to be moved towards the vent by the action of cilia. This may prevent the attachment of pathogens.

In the absence of any definitive information on local immunity in the reproductive tract of fishes it is possible that internal fertilisation mechanisms and the development of viviparity in osteichthyes and chondrichthyes may have created the necessity for immunocompetence at this mucosa as a result of:- a) infection of this mucosa by the transfer of micro-organisms or other pathogens on the claspers of the male during mating or by the passive entry at other times; b) sperm or seminal antigens stored in S.canicula and other elasmobranchs (Wourms, 1977), which may have the potential to act immunogenically; and c) fertilisation, the zygote being allogenic (although this may have a greater significance in placental sharks than in S.canicula where the zygote is coated by albumin and the eggcase both of which are of maternal origin).

3.7 The gall bladder

The gall bladder and biliary systems are derived from the embryonic gut, and in early phylogeny the liver is thought to be solely a secretory organ (Romer, 1962).

The gall bladder in fish has not been examined histologically for lymphoid tissues or cells, even though Ig has been detected in the bile of catfish (Lobb and Clem, 1981a and b). In Scyliorhinus lymphoid accumulations were absent from the gall bladder, but diffuse IEL and ILL populations were detected. A few plasma cells were found in the lamina propria but, the identity of the cells composing the IEL population was not investigated. It is unclear why plasma cells should exist in the gall bladder, few if any ingested antigens will have access to it, and it is unlikely that many pathogens could survive in the bile. No lymphoid cells were detected in the liver parenchyma, corroborating the findings of Morrow (1978).

3.8 The gills

Few leucocytes were detected in the epithelium of the gill. Numerous fixed leucocyte-like cells, however, were revealed in the corpus cavernosum at the base of the gill filaments. Parish (1981) and Hunt and Rowley (1986) showed that these cells had endocytic properties. The latter authors found that these cells had a more notable pinocytic activity compared to their phagocytic function. After material, especially bacteria, was injected into the bloodstream macrophages collected in the corpus cavernosum. The system may be involved in externalising foreign material (Hunt and Rowley, 1986). Since the gills are susceptible to infection the cells of the corpus cavernosum may alternatively have a role in the defence against infection (Page and Rowley, 1982, Rowley and Page, 1985). Hunt and Rowley (1986) regarded these cells as part of the reticulo- endothelial system, but were not thought to be part of the mononuclear phagocytic system (van Furth et al., 1972).

Plate 1 The mucosae of the alimentary tract

A. Mucosa of the buccal cavity. LP, lamina propria; IEL, intraepithelial leucocyte; E, epithelium.

B. Mucosa of the oesophagus. ILL, intralaminal leucocyte; C, cilia.

C. Cardiac stomach mucosa. B, blood vessel.

D. Pyloric stomach mucosa. SA, small accumulation of leucocytes.

E. Section through part of the spiral intestine.

F. Rectal mucosa.

All sections were of material embedded in wax, cut to 5μm thickness, stained with H and E and examined with a light microscope.

All scale bars = 100μm

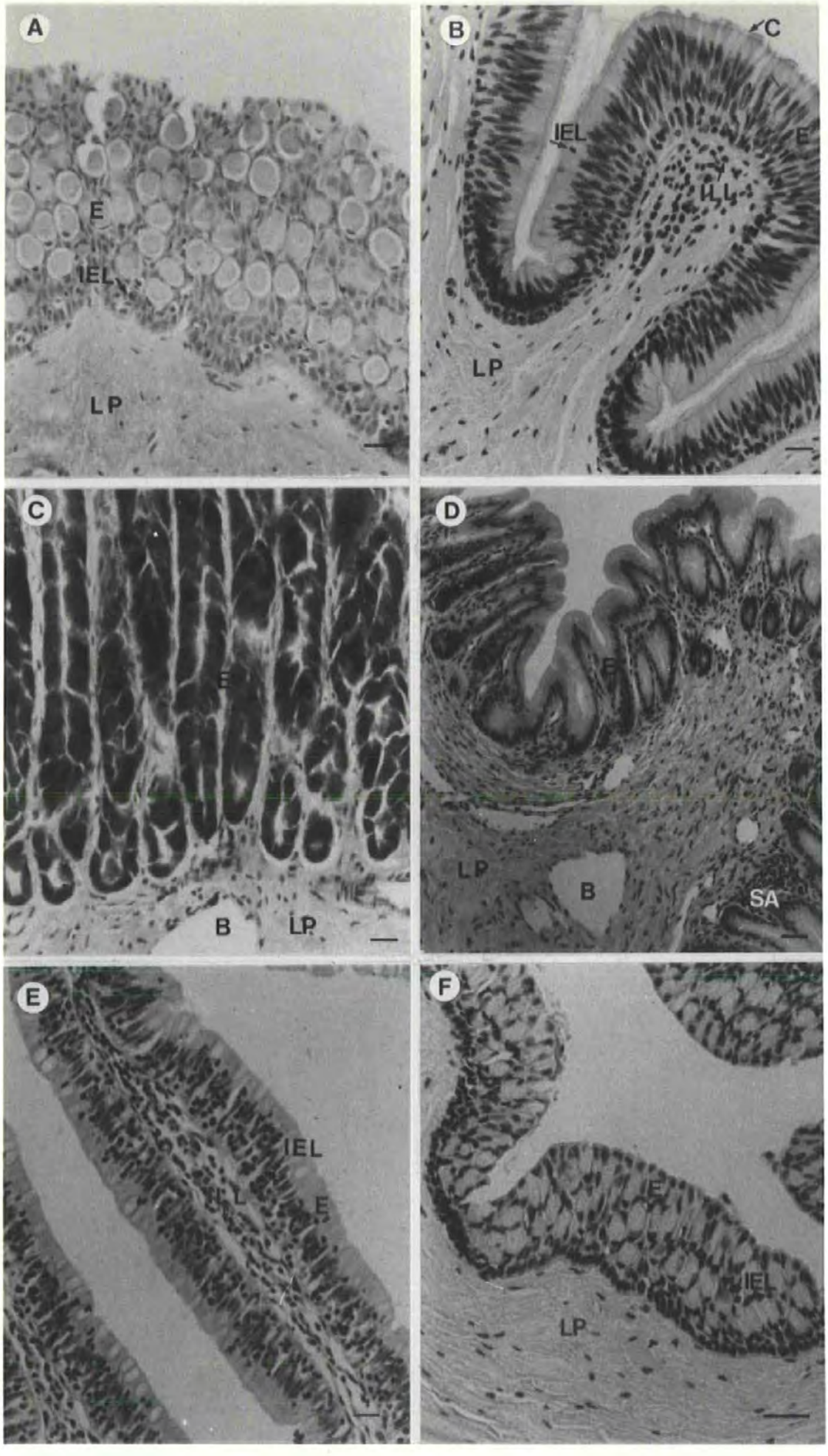

A
E
IEL
LP
B
C
IEL
E
LP
C
E
B
LP
D
E
LP
B
SA
E
IEL
L
E
F
E
IEL
LP

Plate 2 Lymphoid accumulations

A. Accumulation in the upper of middle pyloric stomach. LM, H and E, 5μm wax section. LP, lamina propria; LA, leucocyte accumulation; E, epithelium.

Scale bar = 100μm.

B. Accumulation from the proximal region of the spiral intestine. LM, H and E, 5μm wax section.

Scale bar = 100μm.

C. Macrophage in an accumulation. TEM, N, nucleus; M, mitochondria; V, vacuole.

Scale bar = 1μm

D. Lymphocytes in an accumulation. TEM, C, collagen bundle.

Scale bar = 1μm

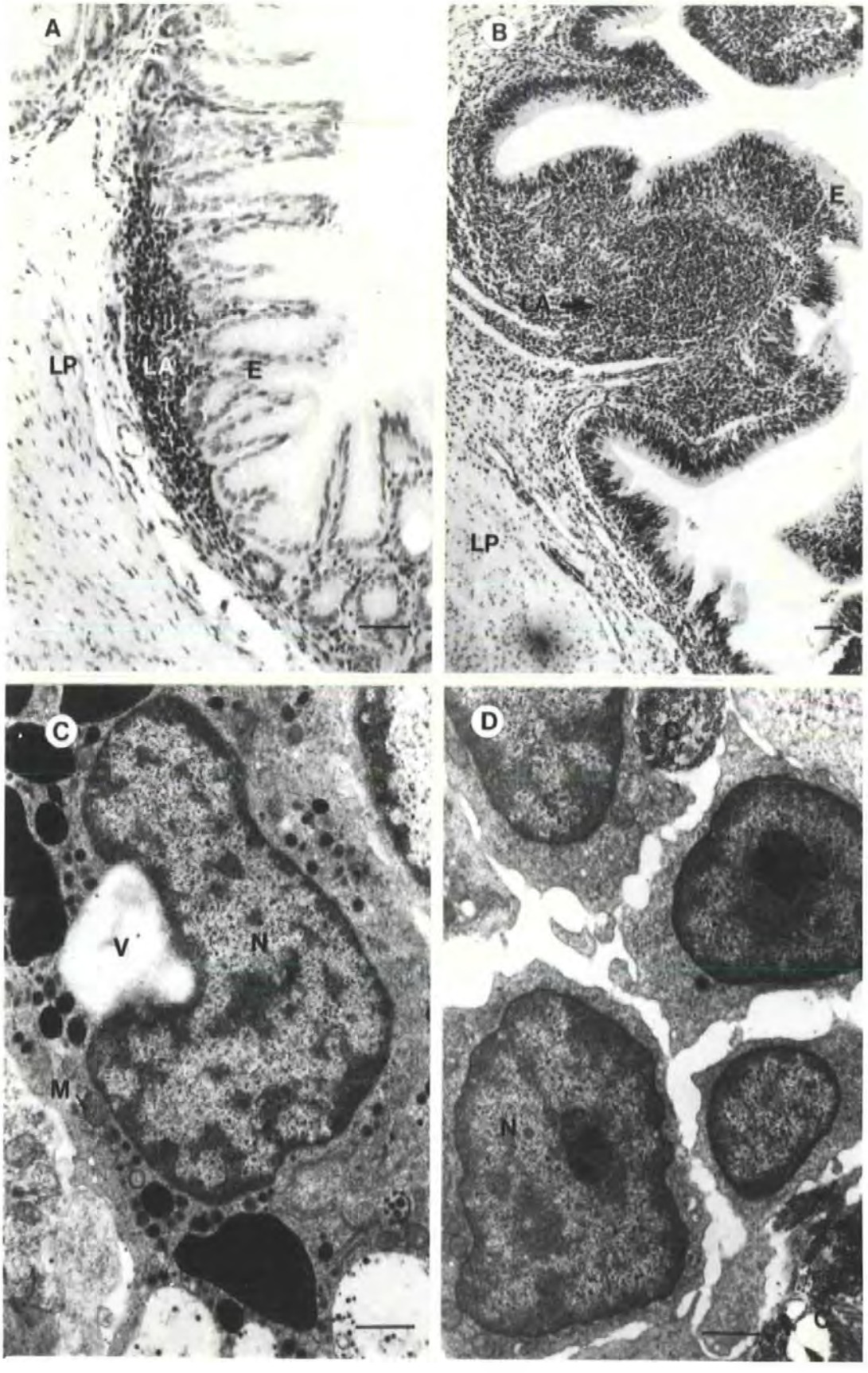

A
LP
LA
E
B
E
CA
LP
C
V
N
M
D
N

Plate 3 Lymphoid accumulations

A. Large area of the proximal spiral valve infiltrated by lymphocytes. LM, H and E, 5µm wax section, L, leucocytes; LP, lamina propria; E, epithelium.

Scale bar = 100µm.

B. Normal fold in the intestinal mucosa. LM, Giemsa, 1µm methacrylate resin section, G, goblet cell; Mi, microvilli.

Scale bar = 100µm.

C. Epithelial/laminal junction. LM, Giemsa, 1µm methacrylate resin section. M, macrophage; BM, basement membrane; B, breach in the basement membrane; L, lymphocyte.

Scale bar = 10µm.

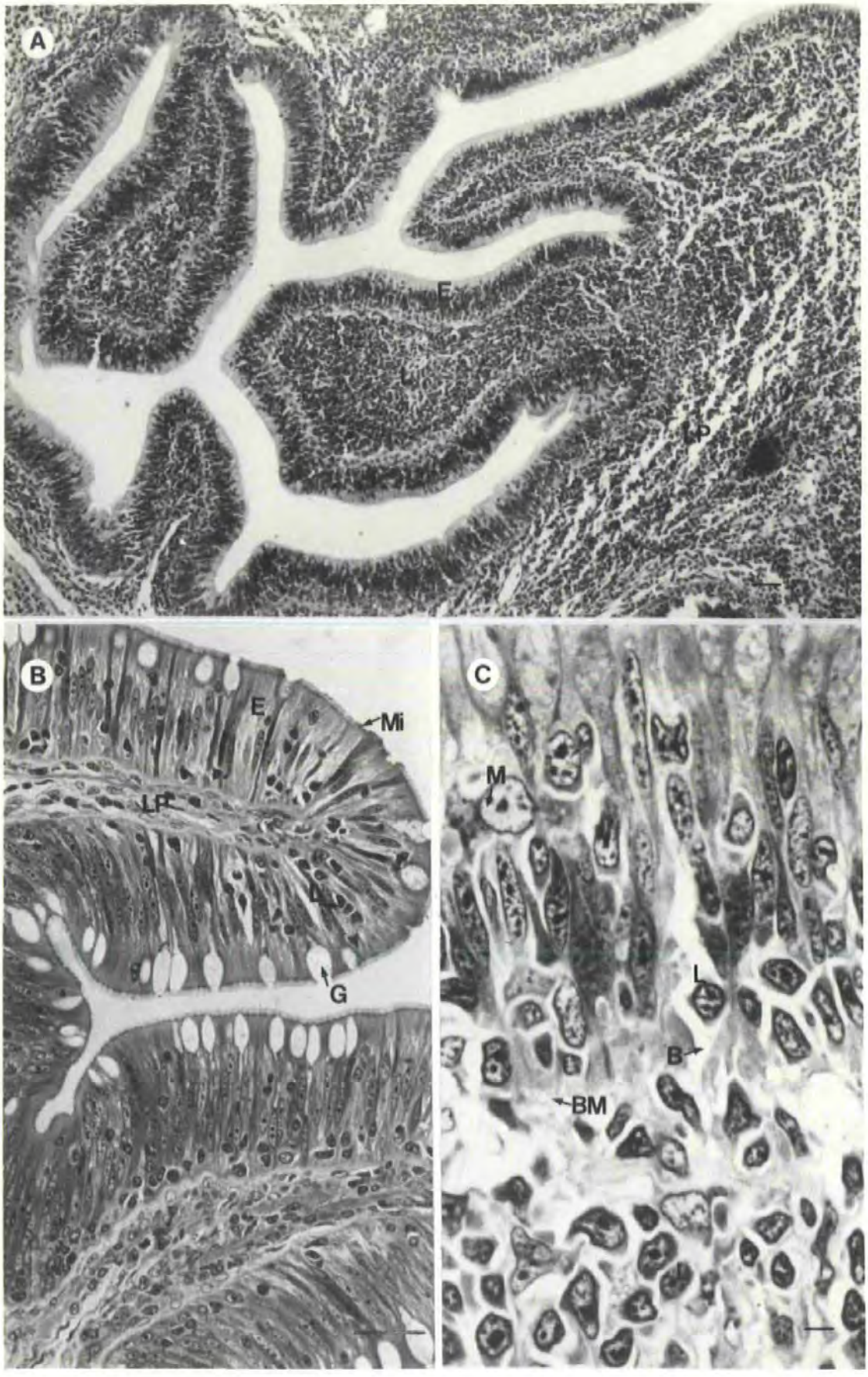

A
E
LP
B
E
Mi
LP
G
C
M
L
B
BM

Plate 4 Lymphocytes

A. Leucocytes in the epithelium and lamina propria. LM, Giemsa, 1µm methacrylate resin section, B, blood vessel; P, plasma cell; F, fibroblast; BM, basement membrane; G, granular cell; L, lymphocyte; N, nucleus of an epithelial cell.

Scale bar = 10µm.

B. Granular lymphocyte. TEM, G, granule; GM, granule membrane; M, mitochondria.

Scale bar = 1µm.

C. Agranular lymphocyte. TEM.

Scale bar = 1µm.

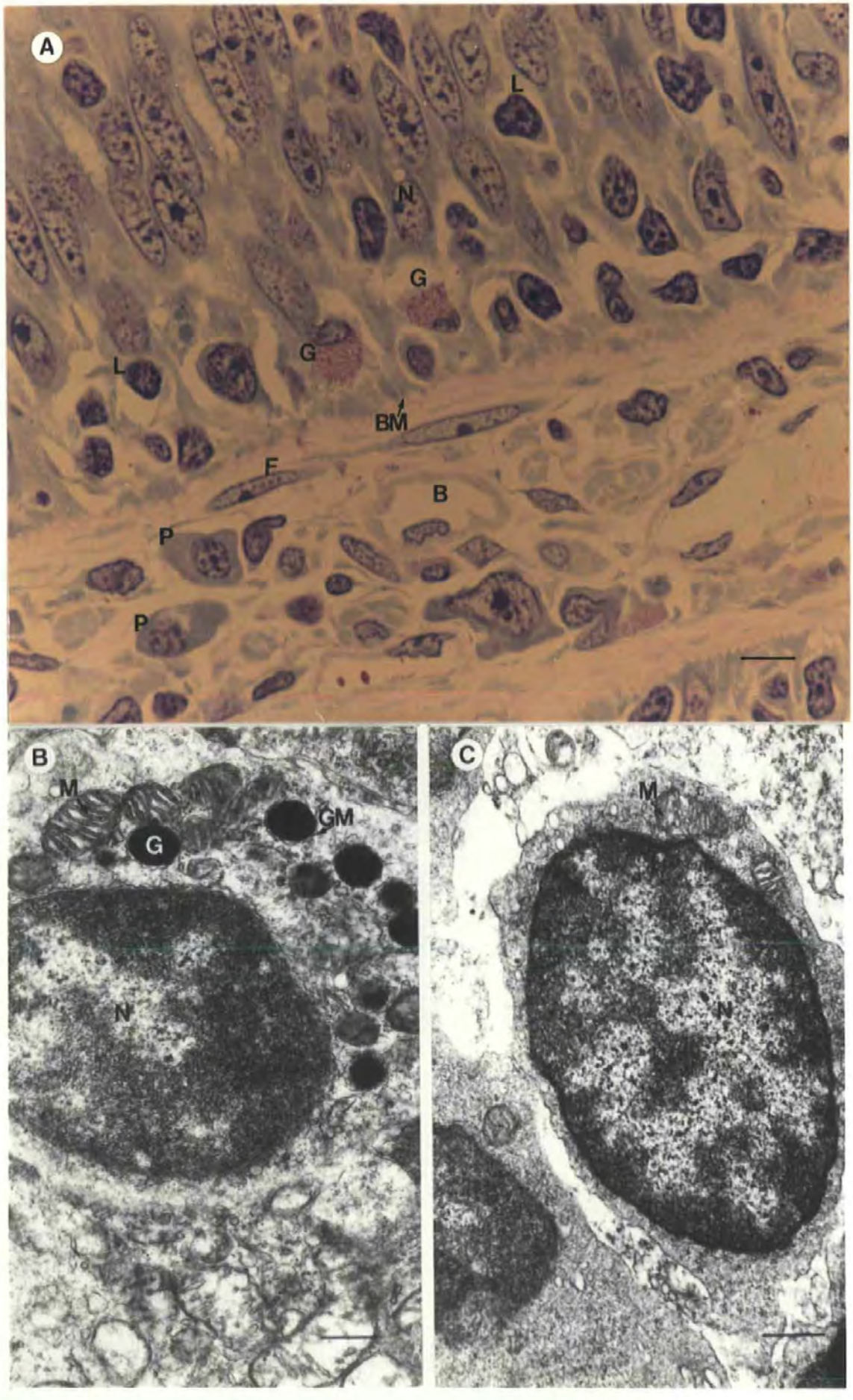

A
L
N
G
G
L
BM
F
B
P
P
B
M
GM
G
N
C
M
N

Plate 5 Plasma cells

A. Lamina propria and epithelium. LM, methyl green pyronin, 1μm methacrylate resin section; P, pyronine positive cell; N, nucleus; B, blood vessel; LP, lamina propria; E, epithelium.

Scale bar = 10μm.

B. Plasma cells adjacent to a blood vessel. LM, Giemsa, 1μm methacrylate resin section; P, plasma cell; NS, non-staining area.

Scale bar = 10μm.

C. Plasma cell. TEM, ER, endoplasmic reticulum; M, mitochondria, G, granule.

Scale bar = 1μm.

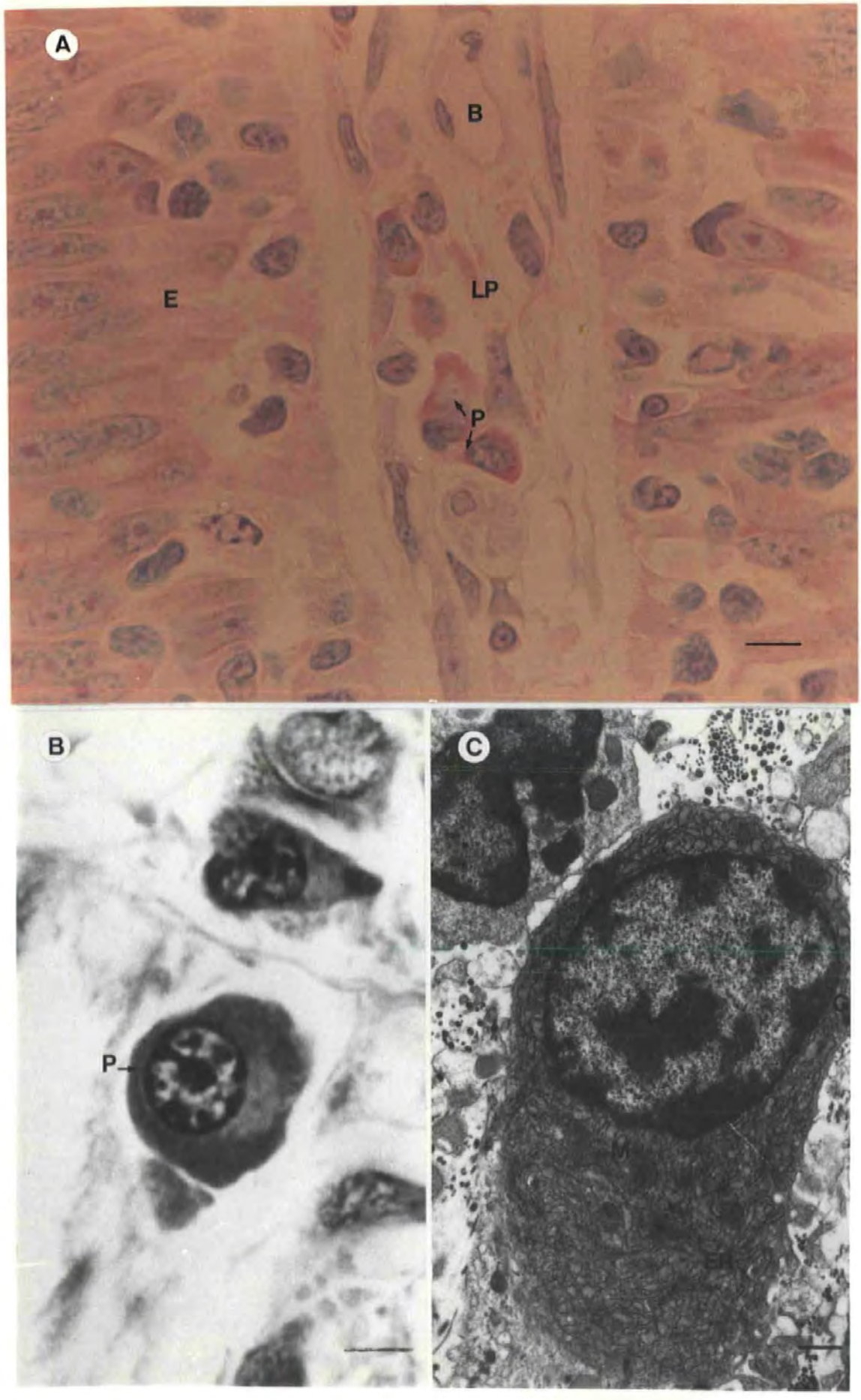

A
B
E
LP
P
B
P
C

Plate 6 Macrophage-like cells

A. Epithelium. LM, PAS, 1μm methacrylate resin section; M, macrophage-like cell; L, lymphocyte; G, goblet cell; Mi, microvilli.

Scale bar = 10μm.

B. A macropahge-like cell in close proximity to a plasma cell. TEM, P, plasma cell; N, elongated nucleus of the macrophage; I, inclusions in the macrophage; C, collagen.

Scale bar = 10μm

C. A macrophage-like cell phagocytosing an effete cell. TEM, E, effete cell; P, pseudopodia.

Scale bar = 1μm.

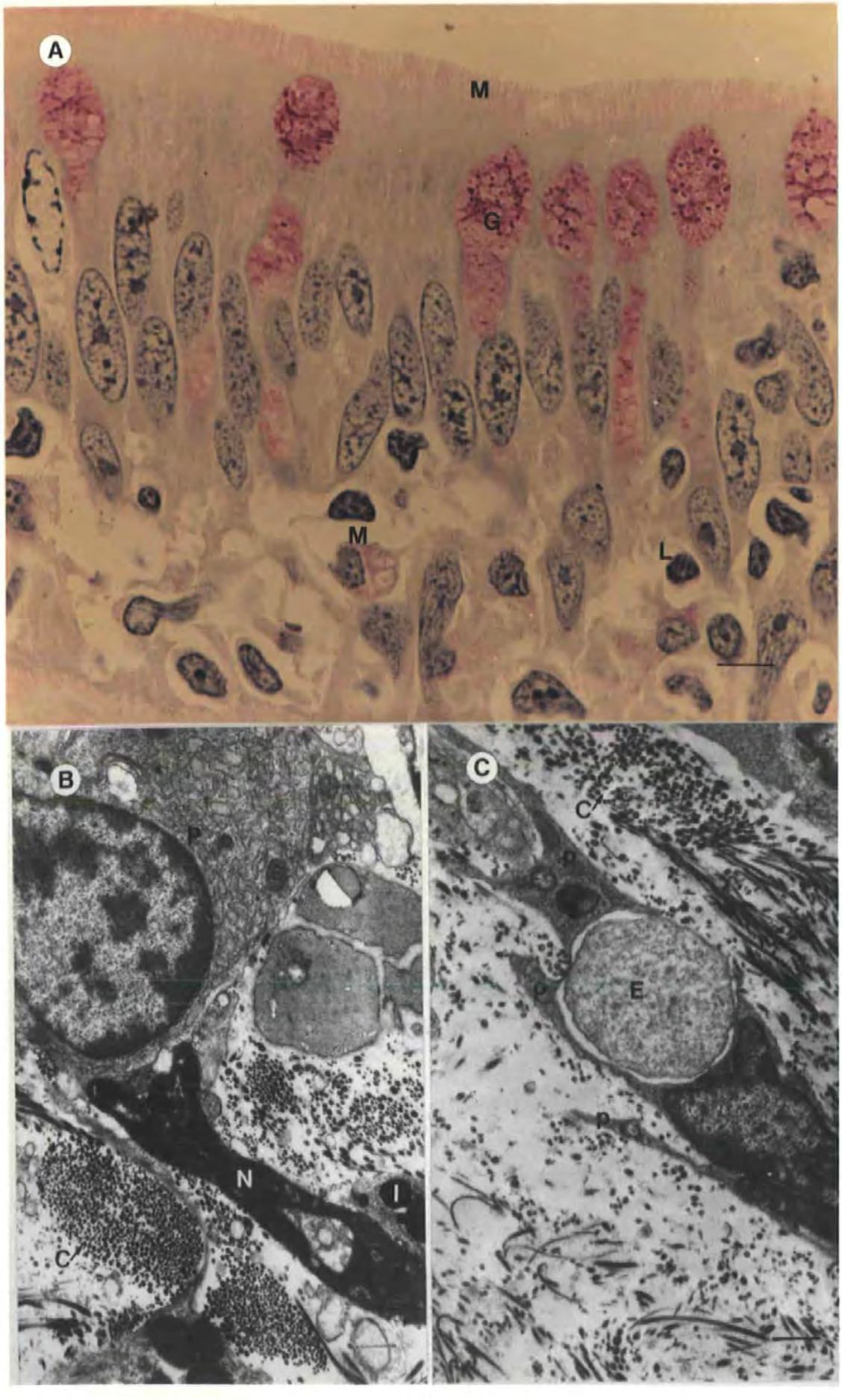

A
M
G
M
L
B
P
I
N
I
C
C
C
P
E
P

Plate 7 Granular gut cells

A. A type 1 granular cell migrating within the epithelium of the spiral valve. N, nucleus of the granular cell; G, granules.
Scale bar = 1μm.

B. Granules of the type 1 granular cell. M, mitochondria.
Scale bar = 0.1μm.

C. A type 2 granular cell.
Scale bar = 1μm.

D. Granules of the type 2 granular cell. F, fibrils; E, electron dense area or crystaloid.
Scale bar = 0.1μm.

E. A type 3 granular cell, with granules in LS.
Scale bar = 1μm.

F. Granules of the type 2 granular cell.
Scale bar = 0.1μm.

G. A type 3 granular cell. V, vacuoles.
Scale bar = 1μm.

All sections were examined using a transmission electron microscope.

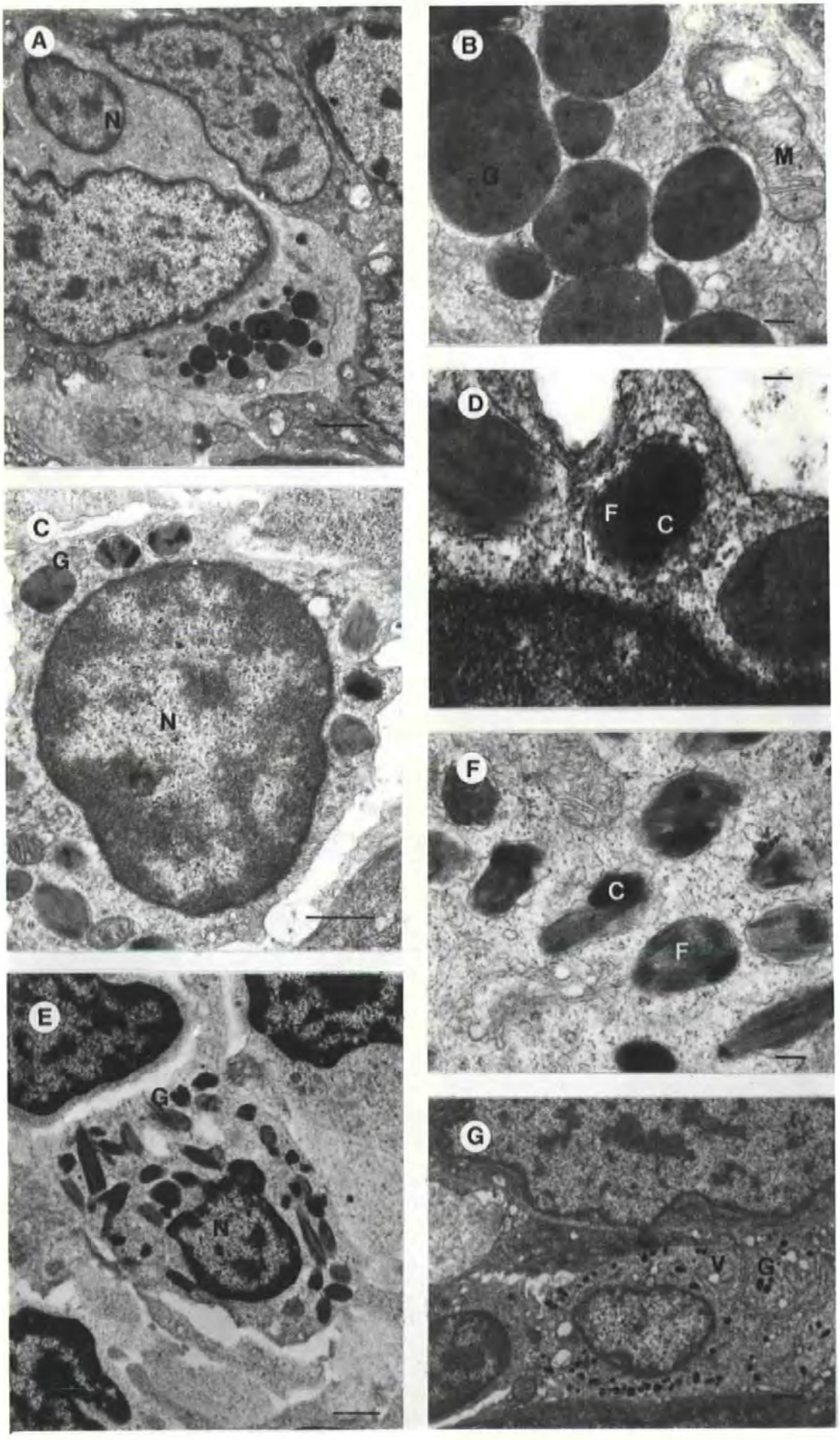

A
N
G
B
G
M
C
G
N
D
F
C
E
G
N
F
C
F
G
V
G

Plate 8 Evidence for cell migration

A. A leucocyte crossing the endothelium of a blood vessel in the lamina propria of the spiral valve. E, endothelium of the blood vessel; LP, lamina propria; C, collagen; L, leucocyte.

Scale bar = 10μm.

B. A macrophage-like cell moving throught the lamina propria. N, nucleus; I, inclusions.

Scale bar = 1μm.

C. A type 2 granular gut cell adjacent to the basement membrane. G, granular cell; NE, nucleus of epithelial cell; BM, basement membrane.

Scale bar = 1μm.

D. A leucocyte in close proximity to the epithelial surface. P, pseudopodial outpushing; M, microvilli.

Scale bar = 1μm.

All sections were examined using a transmission electron microscope.

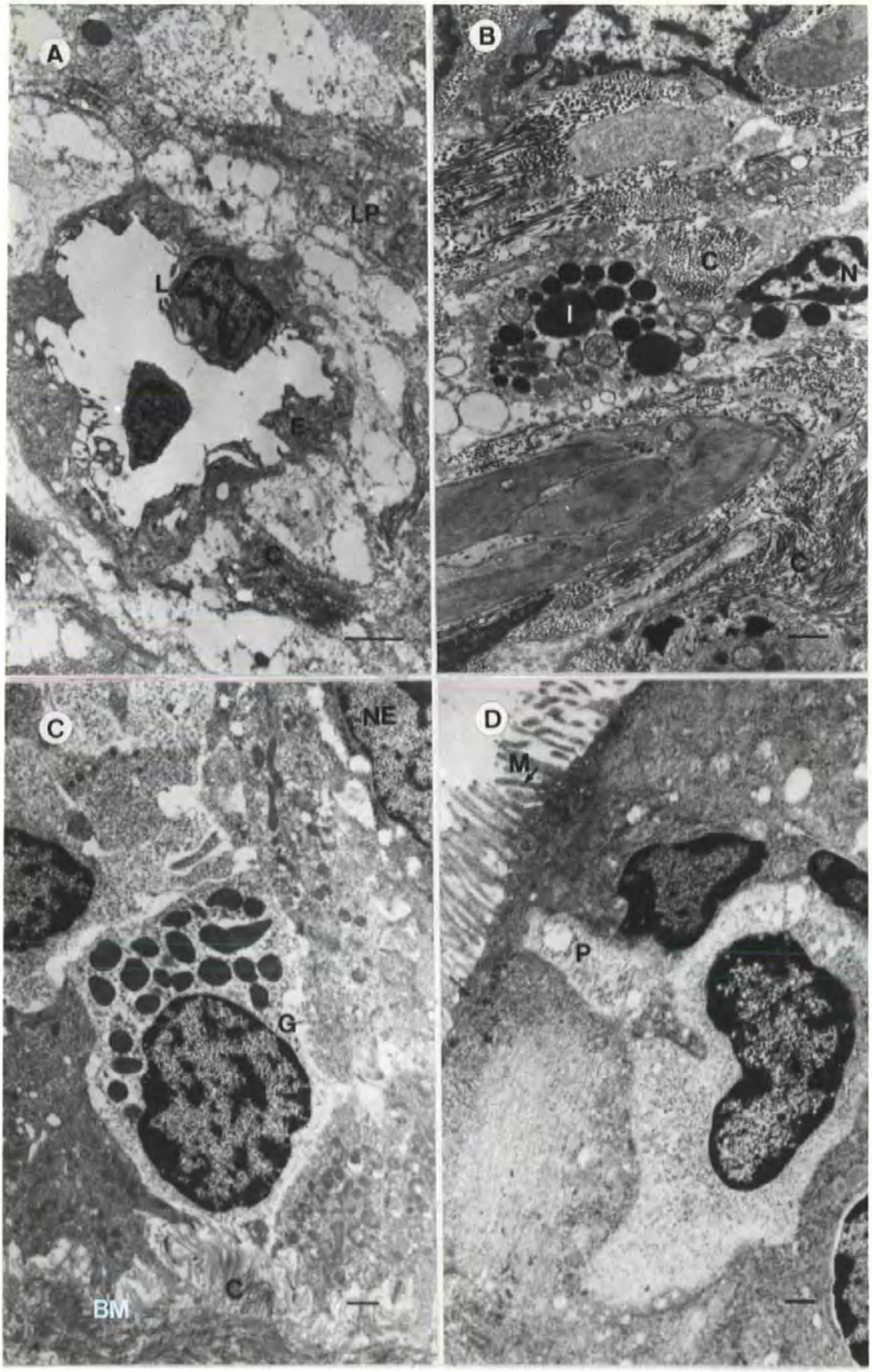

A
LP
L
E
C
B
C
N
I
C
C
NE
G
BM
C
D
M
P

Plate 9 The liver and bilary system

L, liver; GB, gall bladder; B, bile duct; S, spleen; SV, spiral valve.

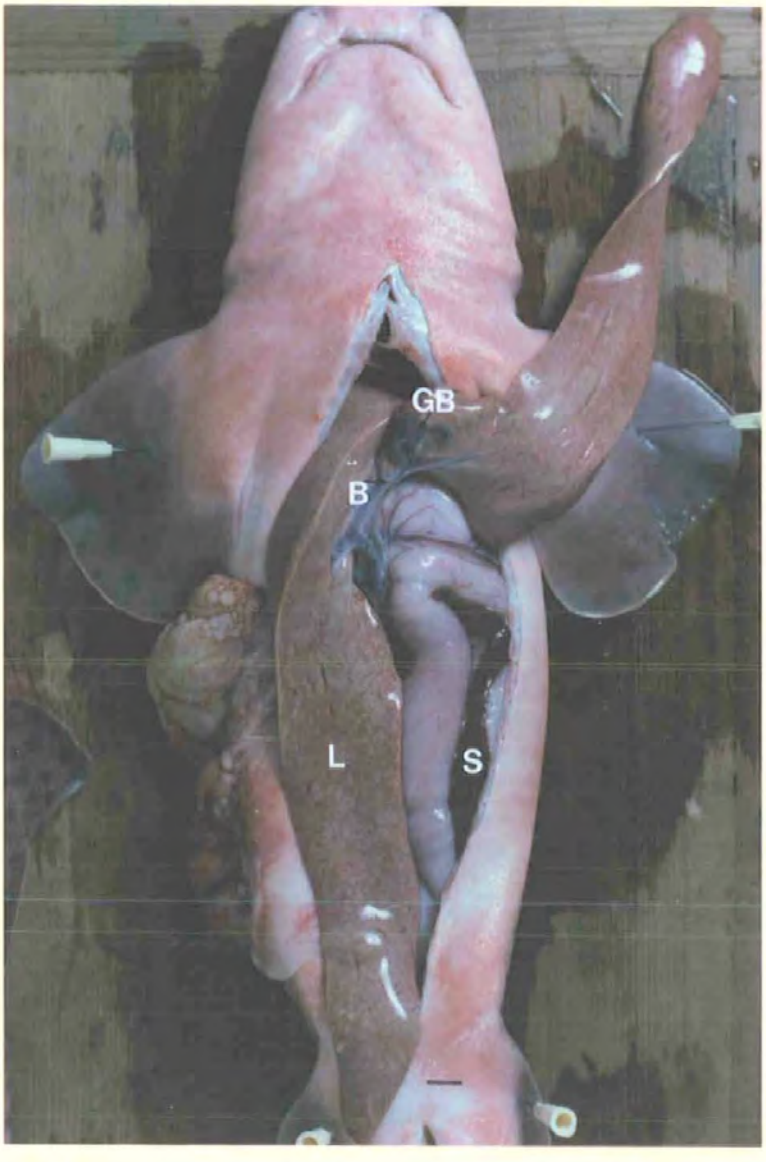

GB
B
L
S

Plate 10 The liver and gall bladder

A. Liver parenchyma. M, melanomacrophage; RBC, red blood cell.

Scale bar = 10μm.

B. Epithelium and lamina propria of the gall bladder. C, cilia; N, nucleus of epithelium; IEL, intra-epithelial leucocyte.

Scale bar = 10μm.

C. Epithelium and lamina propria of the gall bladder. E, epithelium.

Scale bar = 15μm.

D. Lamina propria of the gall bladder. P, plasma cell; V, blood vessel.

Scale bar = 10μm.

All material was embedded in methacrylate resin, sections were cut to 1μm in thickness, stained with Giemsa and examined by light microscopy.

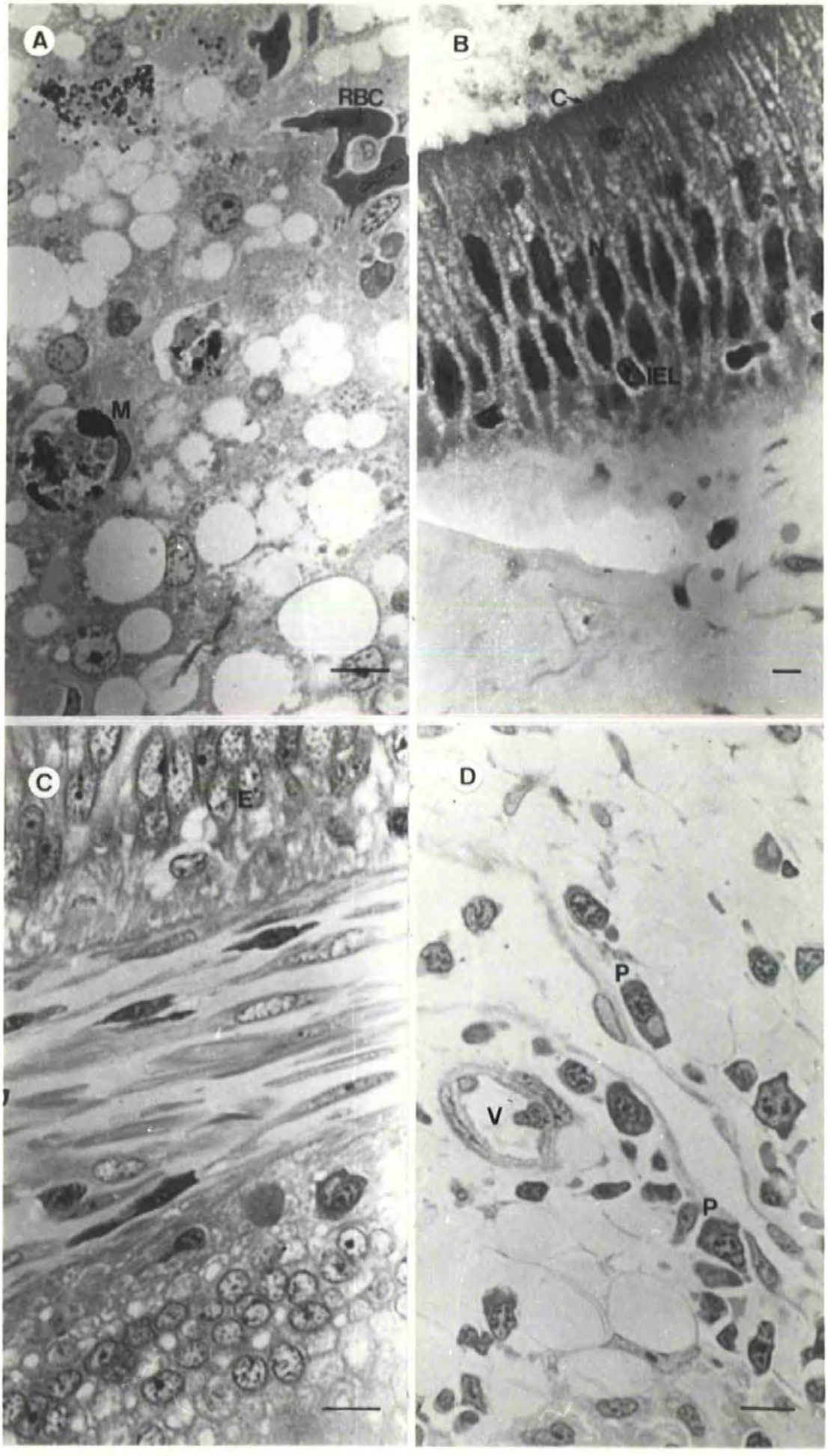

A
RBC
M
B
C
N
IEL
C
E
D
P
V
P

Plate 11 The female reproductive tract.

1-6 arbritrary zones; N, nidamental gland; E, epigonal tissue; V, vent.

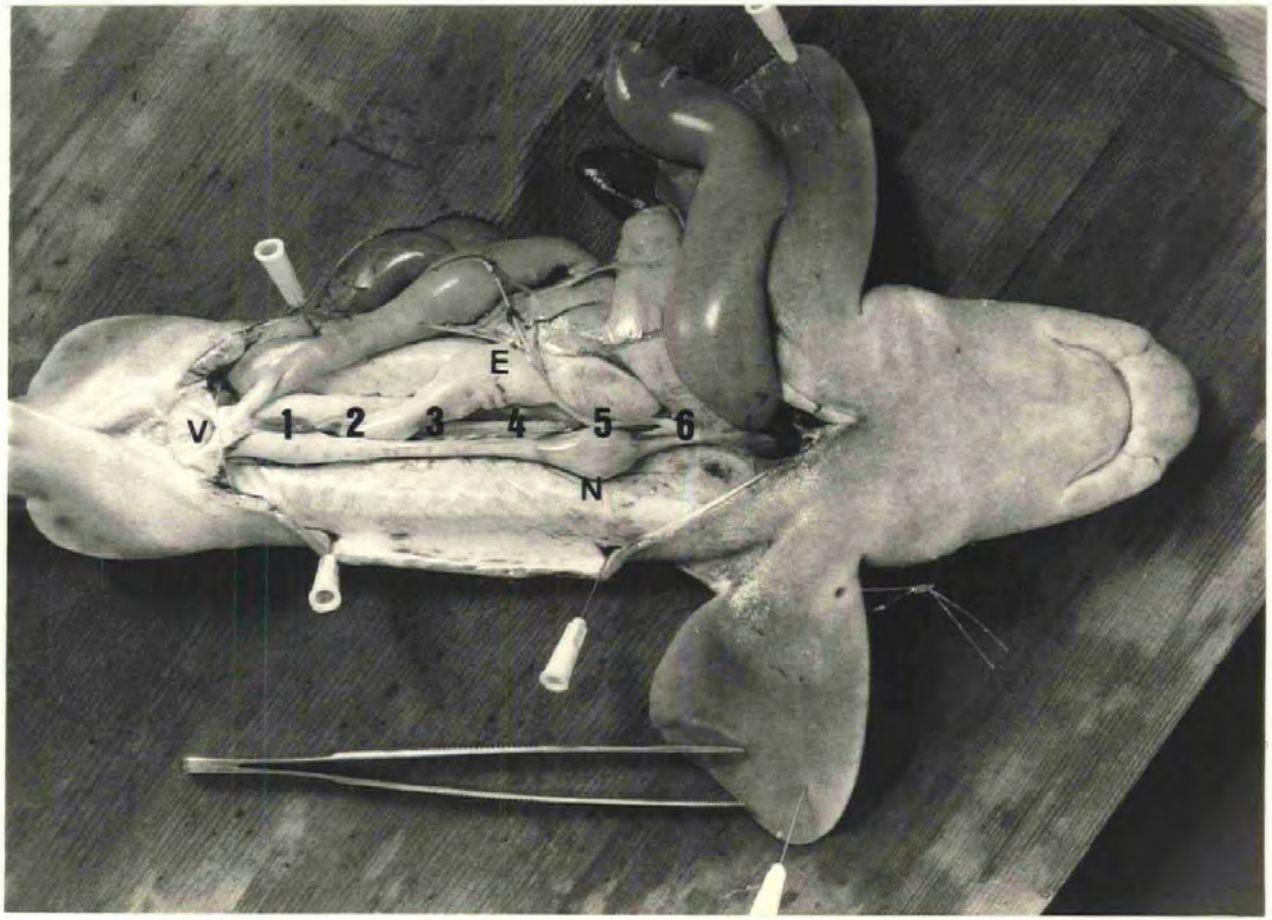

E
V
1
2
3
4
5
6
N

Plate 12 The female reproductive tract

A. Epithelium of zone 2-5. LM, Giemsa, 1µm methacrylate resin section, IEL, intra-epithelial leucocyte; LP, lamina propria; G, goblet cell; C, cilia.

Scale bar = 10µm.

B. Plasma cells in zone 6. LM, Giemsa, 1µm methacrylate resin section, P, plasma cell.

Scale bar = 10µm.

C. Plasma cell in zone 6. TEM, ER, endoplasmic reticulum; M, mitochondria; C, collagen.

Scale bar = 1µm.

D. Nidamental gland. LM, Giemsa, 1µm methacrylate resin section. G, glandular tissue; LA, lymphoid accumulation.

Scale bar = 50µm.

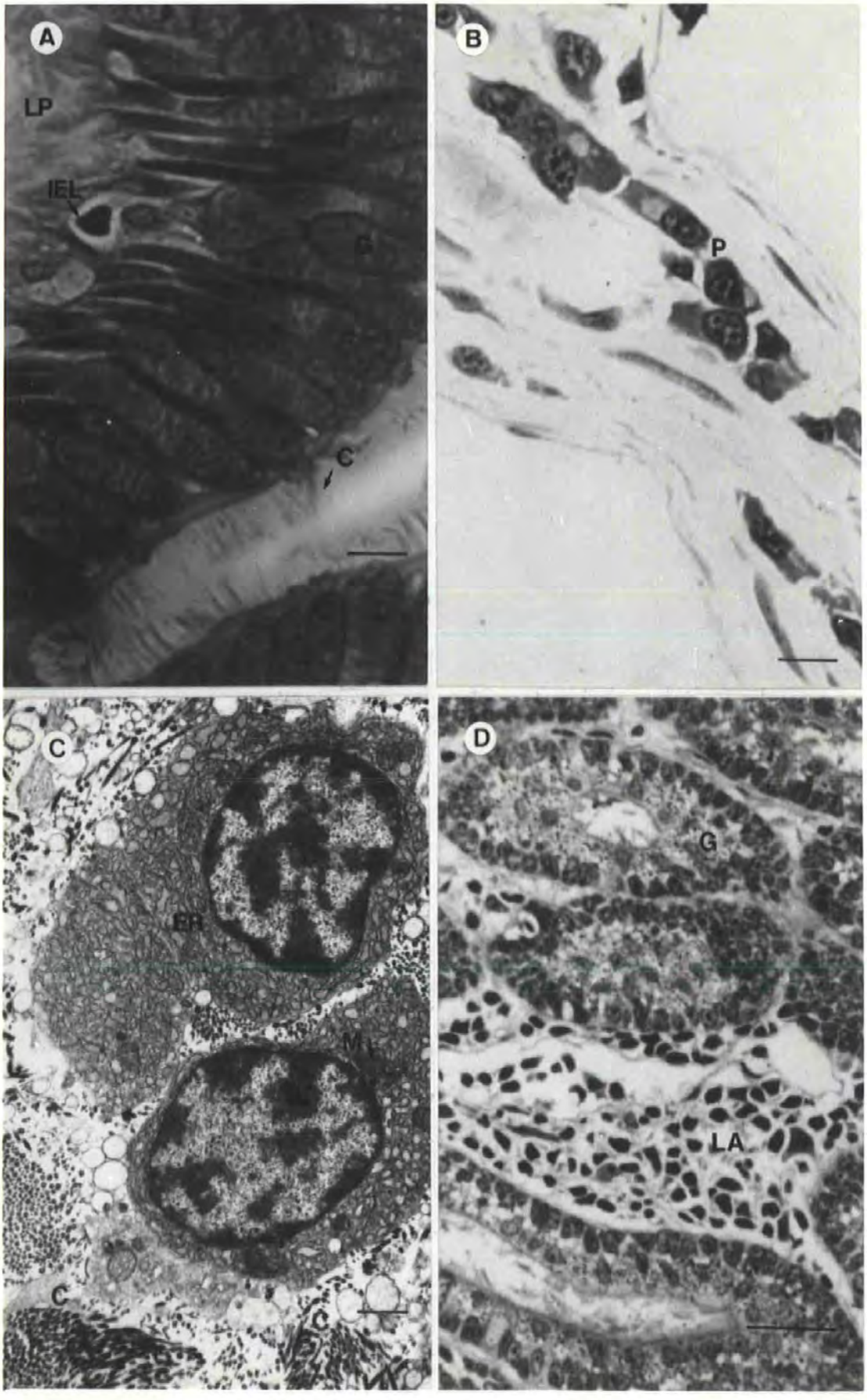

A
LP
IEL
C
B
P
C
ER
M
C
C
D
G
LA

Plate 13 The gill

A. The gill. G, gill filament; S, secondary lamallae; T, thymus; C, corpus cavernosum.

Scale bar = 100μm.

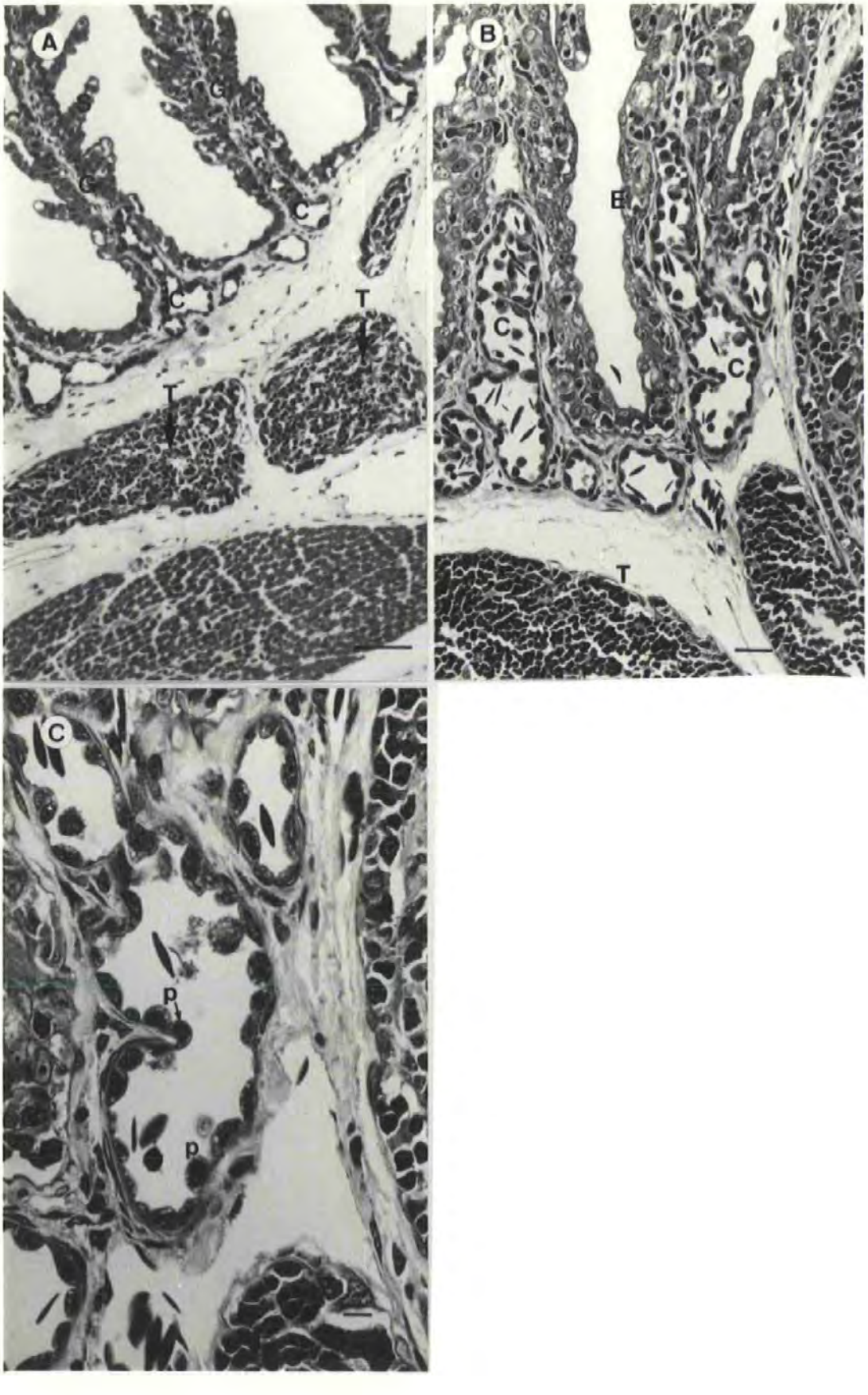
B. The corpus cavernosum. E, epithelium.

Scale bar = 50μm.

C. Cells of the corpus cavernosum. P, fixed phagocytes.

Scale bar = 15μm.

All sections were embedded in methacrylate resin, sectioned to 1μm in thickness, stained with Giemsa and examined with a light microscope.


A
C
C
C
C
T
T
B
E
C
C
T
C
p
p

CHAPTER 4

THE ONTOGENY OF GUT-ASSOCIATED LYMPHOID TISSUE AND THE MAJOR LYMPHOID AND LYMPHOMYELOID ORGANS

Work on the distribution of lymphoid cells in the adult gut revealed that the spiral intestine was found to harbour the highest leucocyte population in the gut (Chapter 3). In this study on the ontogeny, only the spiral intestine was examined as examination of the rest of the gut would have been prohibitively time consuming. Development was examined by resin histology and compared to the ontogeny of the spleen, thymus, Leydig organ, epigonal tissue and kidney. Prior to this investigation the general features of development in S.canicula were established.

4.1 General morphological development

Surprisingly little information exists on the development of S.canicula therefore the general pattern of development and differentiation was first established by observation of the internal and external gross morphology. After fertilisation the telolecithal egg of sharks is enclosed within an albumen coat and eggcase by the nidamental gland. Scyliorhinus canicula is ovoviparous, and after expulsion from the vent, the eggcase was found to contain embryos at varying stages of development. Some embryos consisted merely of a disc of cells in the yolk mass, in others development was quite advanced and the embryos were already at stage 1 (Table 9). These had a well differentiated yolk sac and embryo with eyes and gill slits, connected to it by a stalk (Table 9).

Stage 2 fish were slightly bigger, their principle characteristic was the possession of external gills which were well vascularised and bright red. Approximately one third of the way through stage 2 the eggcase became ventilated by seawater.

TABLE 9 GENERAL MORPHOLOGICAL DIFFERENTIATION OF S.CANICULA

STAGE AND APPROXIMATE AGE	DEVELOPMENTAL FEATURES	SCHEMATIC REPRESENTATION OF STAGES (NOT TO SCALE)
STAGE 1 1-2 months (5)	Albumin coated stage 1-2cm long	
STAGE 2 2-5 months (7)	External gill stage 2-5cm long	
STAGE 3 5-7 months (6)	Internal yolk sac stage 5-10cm long	
STAGE 4 Post-hatch up to 3-4 weeks (8)	Post-hatch stage 16cm long	

TABLE 10 DEVELOPMENT OF THE GUT AND LYMPHOID CELL POPULATIONS, TISSUES AND ORGANS IN S.CANICULA

STAGE	GUT DEVELOPMENT (NOT TO SCALE)	GALT			LYMPHOID TISSUE AND ORGANS				
		ILL	LYMPHOID ACCUMU-LATION	IEL	THYMUS	KIDNEY	SPLEEN	EPIGONAL TISSUE	LEYDIG ORGAN
STAGE 1		Absent	Absent	Absent	√	√	Absent	Absent	Absent
STAGE 2 PHASE 1			"	"	√	√	"	"	"
PHASE 2		√	"	"	√	√	√	√	√
PHASE 3		√	"	"	√	√	√	√	√
STAGE 3		√	√	√	√	√	√	√	√
STAGE 4		√	√	√	√	√*	√	√	√

* - absent from adult

Stage 3 was characterised by the loss of external gills (which appeared to retract into the body), the development of internal gills and the formation of the internal yolk sac.

Stage 4, the post-hatch, free-swimming stage had only a small vestige of the external yolk sac left; as a small nodule on the anterioventral surface. The internal yolk sac, however, was at its maximum size on hatching and was consumed during the first few weeks of free swimming life. The relationship between the decrease in wet weight of the external yolk sac and increase in weight of the external yolk sac and carcass, prior to hatching, are shown in Figure 3.

4.2 Development of GALT and other lymphoid organs

As the development of the lymphoid organs in S.canicula has not been previously investigated a brief examination of the thymus, Leydig organ, epigonal tissue, spleen and kidney were undertaken, and compared with the ontogeny of leucocyte populations in the gut (Table 10). Material was embedded in TAAB methacrylate resin, cut to 1µm thickness and stained with Giemsa and observed by light microscopy unless otherwise stated.

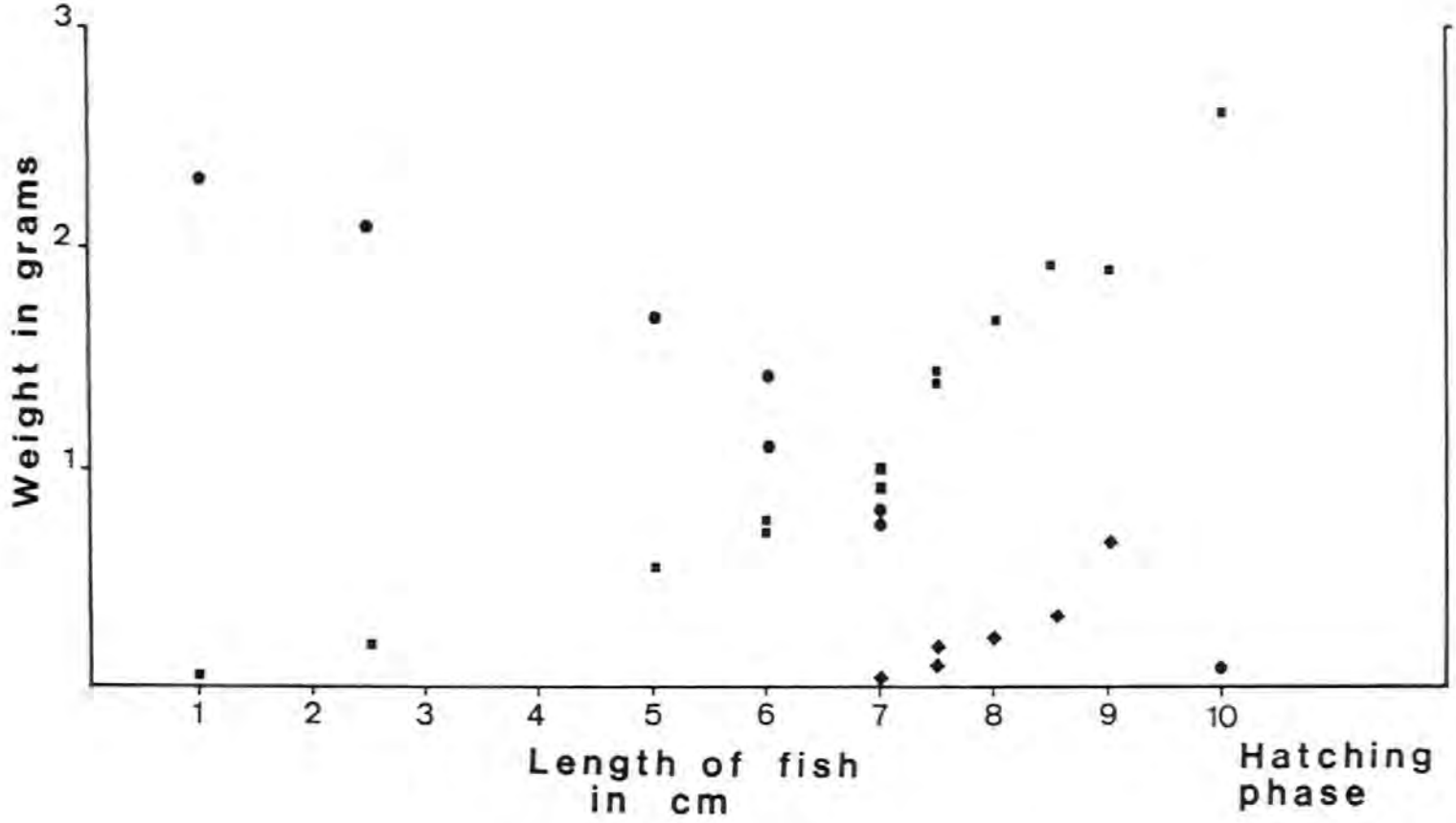
By Stage 1 (Table 9 and 10) the tissue destined to become the spiral valve was present as an outpushing of the intestinal wall (Plate 14A). Cilia were present at the surface of the epithelium (Plate 14A and B); which was thin an unfolded, and also in the yolk sac. The cilia appeared to be involved in the movement of yolk platelets from the yolk sac to the spiral intestine (Plate 14A and B). The gut was completely occluded in the sub-oesophageal region (Plate 14C). While GALT, IEL's and ILL's were absent from the intestine, lymphocyte-like cells had begun to develop around the renal tubes in the proximal and distal kidney (Plate 14D), and the encapsulated thymus had begun to differentiate. The thymus appeared to develop from the epithelium of the pharyngeal pouch (Plate 15A and B) and expand dorsally adjacent to

FIGURE 3 GRAPH SHOWING THE RELATIONSHIP BETWEEN THE WEIGHT (IN GRAMS) OF THE EXTERNAL AND INTERNAL YOLK SACS COMPARED TO CARCASS WEIGHT IN FISH OF DIFFERENT LENGTHS

KEY: ● - weight of external yolk sac in grams

■ - weight of the fish carcass in grams

◆ - weight of the internal yolk sac in grams


Weight in grams
3
2
1
1
2
3
4
5
6
7
8
9
10
Length of fish in cm
Hatching phase

the blood vessels (Plate 15C and D). Initially a large portion of the cells appeared to be epithelial in nature but, darkly staining lymphocyte-like cells were also present.

Stage 2 was recognised as having 3 phases. In phase I the spiral valve began to differentiate (Plate 16A) but, GALT was still absent from the gut. The size of the population of cells in the kidney, around the renal tubes and vessels, expanded (Plate 16B) and the thymus was larger, and by now, separated from the pharyngeal epithelium (Plate 16C). Leucocytes were absent, however, from the Leydig organ (Plate 16D), and the epigonal tissue and spleen had not developed.

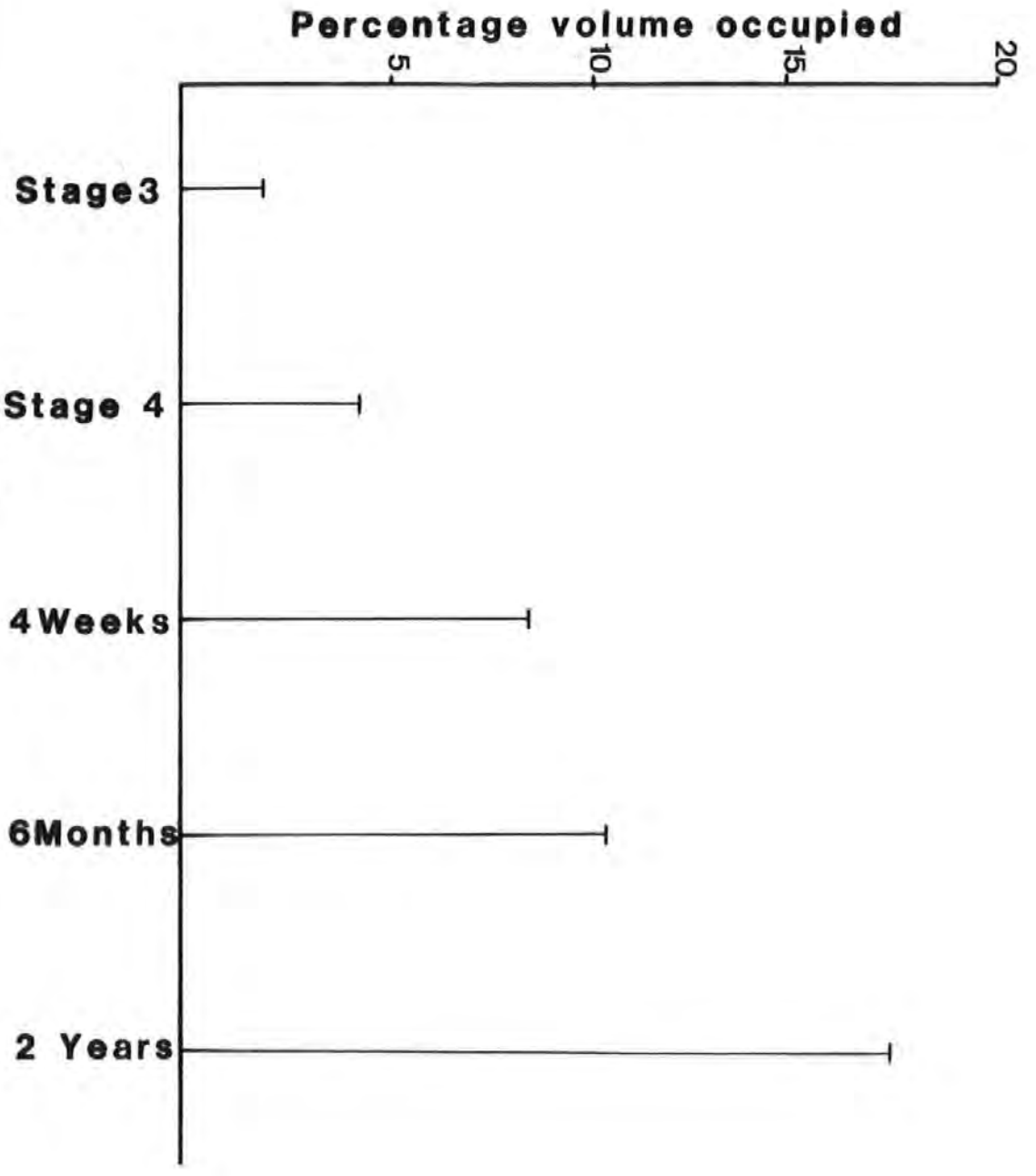
By phase II the eggcase was no longer sealed and the larvae were freely ventilated by seawater. The spiral valve and intestine had differentiated quite considerably; the spiral valve had developed several coils, the epithelium was folded (Plate 17A and B), and was compatible to the spiral valve of a 1 year old fish (Plate 17D), except that the epithelium was highly vacuolated and had cilia at its surface (Plate 17C). The kidney contained a considerable number of cells (Plate 18A) but, it was still unclear if these were of a lymphocyte lineage (Plate 18B and C). The thymus had expanded and developed a lobed morphology (Plate 18D, 19A and B) and contained many small lymphocytes with a high nucleus to cytoplasm ratio. The Leydig organ had developed in the wall of the oesophagus (Plate 19C and D) and cells may have appeared as early as phase I, but a primordial Leydig organ was not recognisable until late phase III. The epigonal tissue (Plate 20A and B) contained lymphocyte-like cells and the spleen (Plate 20C) developed populations of lymphocyte-like, and red blood cells in phase II or III.

In phase II, but, particularly in phase III, intralaminal lymphocyte and macrophage-like cells were detected in the spiral intestine (Plate 17C).

Stage 3 was characterised by the loss of external gills and the development of a shark-like external morphology. All the lymphoid and lymphomyeloid organs were well developed by this stage. The thymus was multilobed and was located over the 4th and 5th gill arches. The kidney contained numerous lymphocyte-like cells and the epigonal tissue and Leydig organ developed further, the latter bordering the typically highly folded oesophageal mucosa (Plate 20D). Accumulations of lymphocytes were observed in the gut for the first time (Plate 21A) along with IEL's (Plate 21B).

During the stage 4, the free swimming stage the contents of the internal yolk sac were metabolised, the spiral valve lost its vacuolated appearance and took on an adult morphology. The number of IEL's increased (Figure 4) in the free swimming fish, as did the size of lymphoid accumlations (Plate 21C). The thymus also appeared to increase in size, at least up to 9 months; when the last fish was killed. The kidney did not contain any detectable leucocytes one month after hatching (Plate 21D). The first granulocytes were observed in the gut 1 month after hatching and plasma cells were first detected in the lamina propria at 6 months post-hatch.

FIGURE 4 PERCENTAGE (%) OF EPITHELIAL VOLUME OCCUPIED BY LEUCOCYTES ALONG THE LENGTH OF THE DOGFISH GUT AT STAGES IN THE DEVELOPMENT OF THE FISH (N.B. DATA REPRESENT MEANS FROM 4 STAGE 3; 4 STAGE 4; 5 FISH OF 4 WEEKS OF AGE; 2 FISH OF 6 MONTHS OF AGE AND 6 ADULTS)


Percentage volume occupied
5
10
15
20
Stage3
Stage 4
4Weeks
6Months
2 Years

4.3 Discussion

The results show that the thymus and kidney were the first organs to develop leucocyte populations (stage 1), followed by the Leydig organ, epigonal tissue, spleen and intralaminal leucocytes (stage 2), lymphoid accumulations and IEL's in the spiral intestine (stage 3).

Since an early study by Beard (1902-03) little work has been published on the ontogeny of lymphoid organs in elasmobranchs. However, considerable work has been undertaken on teleosts most recently by Botham and Manning (1981); Grace (1981) and Ellis (1977b).

In this study the thymus and the kidney were the first organs to contain haematopoietic cell populations in stage 1. It was not clear from this brief investigation which of these two structures were the first to contain blood cells, nor what specific lineage the lymphocyte-like cells of the kidney belonged to. Ellis (1977b) proposed that the haemopoietic cells in the kidney of salmon (Salmo salar) seeded the thymus at a very early stage in development. A similar hypothesis was proposed by earlier workers, suggesting that the stem cells may have migrated from other sources (Hammar, 1909; Maximow, 1912; Hill, 1935 and von Hagen, 1936). Others suggested that lymphoid cells were derived directly from the pharyngeal epithelium (Beard 1902-03; Maurer, 1886 and Nusbaum and Prymak, 1901). While Deansley (1927); Lele (1933) and Hafter (1952) suggested the complete thymus was derived in part from the pharyngeal epithelium and also by immigration from surrounding tissues. In mammals, for example sheep, the lymphocytes originate in the yolk sac or liver (Morris, 1986) and the first lymphoid organ to develop is the thymus, at approximately 40 days gestation (Al Salami, Simpson-Morgan and Morris, 1985). The thymus in S.canicula soon became detached from the pharyngeal epithelium which is the normal situation in most vertebrates (Grace, 1981) but, contrasts with teleosts, where the thymus has a superficial position until involution (Grace 1981; Botham and Manning, 1981 and Ellis, 1977b).

Involution appears to occur about the time of sexual maturity in some fish (Deansly, 1927; Hill, 1935; Lele, 1933; von Hagen, 1936 and Hafter, 1952). Pulsford, Morrow and Fänge (1984) reported that the thymus involutes at 3 weeks post-emergence in S.canicula. In this study, however, the thymus was still present at 9 months post-emergence, and in some species of elasmobranchs the thymus is present in sexually mature fish (cf. Fänge, 1984). While it was unclear if the thymus of S.canicula was divided into a cortex and medulla, central areas of mainly epithelial cells could be recognised in some parts of the thymus.

The kidney contained a population of lymphocyte-like cells from stage 1; these cells occured in all regions of the kidney with maximum populations detected prior to hatching from the eggcase. Unlike teleosts (Botham and Manning, 1981; Grace, 1981 and Ellis, 1977b) the kidney of free-swimming dogfish were devoid of these lymphoid-like cells. Their absence from the adult explains the general premise that the kidney of elasmobranchs is not lymphoid (Fänge, 1984). Little work has been undertaken on the ontogeny of lymphoid organs in elasmobranchs and consequently there is no information on the presence of lymphoid cells in the kidney of other chondrichthyans. In a recent report on embryonic stages of the Aleution skate (Bathyraja aleutica) no reference was made to the importance of the kidney as a lymphoid organ (Teshima and Tomonaga, 1985).

The spleen, epigonal tissue and Leydig organ developed at about the same time as seawater ventilated the eggcase. The spleen had a population of lymphocytes and red blood cells at late stage 2. The role of the adult spleen of elasmobranchs and teleosts in immunity is unclear. Yu, Sarot, Filazzola, Perlmutter (1970) found that the spleen was essential for the antibody forming response in Trichogaster trichopterus. In contrast, however, Ferren (1967) reported that

antibody production was unaffected by splenectomy in teleosts and elasmobranch representatives. Morrow (1978) claimed that the spleen was the chief site for antibody production in S.canicula. Kobayashi et al. (1985) found two types of Ig producing cells in the adult Aleutian skates, one produced the HMW Ig and the other the LMW Ig. In embryonic Aleutian skates single cells produced both types of Ig, a phenomenon not encountered in the adult, these authors suggested that this was evidence that the spleen was a primary lymphoid organ for B-lymphocyte differentiation and proliferation, possibly equivalent to the bursa of birds.

The epigonal tissue and Leydig organ are involved in granulopoiesis in elasmobranchs (Zapata, 1981). Comparable organs are not found in teleosts, and elasmobranchs have many granulocytes in their circulation e.g. S.canicula (Parish et al., 1986). The Leydig organ began to differentiate while the oesophageal epithelium was simple and unfolded. The origin of the cells in these organs is unclear although they both developed after the kidney and thymus.

The first ILL's appeared at mid-stage 2, athough IEL's and lymphoid accumulations did not occur until stage 3. The role of extraneous antigen in the development of leucocyte populations has been investigated in mammals. Ferguson (1977) found that in rats the number of leucocytes was increased by the presence of antigens. However, Ferguson and Parrot (1972) and Husband and Gowans (1978) found that, in the same species, the distribution of leucocytes was unaffected by antigen.

Scyliorhinus canicula possessed lymphoid accumulations, IEL's and ILL's prior to feeding. Yolk was discharged directly into the spiral valve, and in stage 1, at least, the anterior gut was occluded preventing the fish swallowing water possibly containing antigens. From stage 2 onwards, however, the fish were bathed in seawater. This

would expose the skin, gill and buccal cavity to antigens, and the posterior parts of the gut may also be exposed to antigens once the alimentary tract is fully differentiated. Experimentally, it has been shown that antigens may enter the external gills of embryonic sharks (Hamlett et al., 1985) and the internal gills of adult teleosts (Tatner and Horne, 1983). Recent work by Wrathmell (unpublished data) showed the anally intubated BSA gains access to the blood serum and retains its antigenicity. In view of this information, the embryonic dogfish may receive, in addition to dietary antigens of maternal origin, extraneous antigens suspended or dissolved in the seawater bathing the fish.

In mammals the gut of the foetus is sterile (Morris, 1986). Peyer's patches have been found to develop in the foetal gut prior to antigenic challenge (Reynolds, 1981; Reynolds et al., 1981 and Morris, 1986). These findings have led to a reassessment of the role of Peyer's patches in the development of the B cell lineage, at least in sheep. Recently, Tomonaga et al. (1986) found lymphoid accumulations in the upper spiral valve of sharks prior to parturition, indicating these accumulations in lower vertebrates may initially develop prior to stimulation by dietary or other ingested antigens. The agnatha, fish and amphibia, however, do not possess an amnion (a membrane enclosed fluid space), necessary for the survival in the terrestial environment, which is found in birds, reptiles and mammals. Placental sharks in embryonic stages within the uterus are therefore possibly challenged by a variety of antigens from the microflora and fauna of the reproductive tract of female viviparous fishes.

PLATE 14 The gut and kidney of a stage 1 fish

A Early development stage of the spiral intestine; B, body cavity; I, intestine; Y, yolk platelets; S, primordial spiral valve; C, cilia.

Scale bar = 100μm

B Intestinal cilia. TEM, M, microvilli.

Scale bar = 1μm

C A partial gut occlusion. O, occluded tissue; L, liver

Scale bar = 100μm

D Kidney region. T, kidney tubule; L, lymphoid-like cells; SC, spinal column.

Scale bar = 60μm

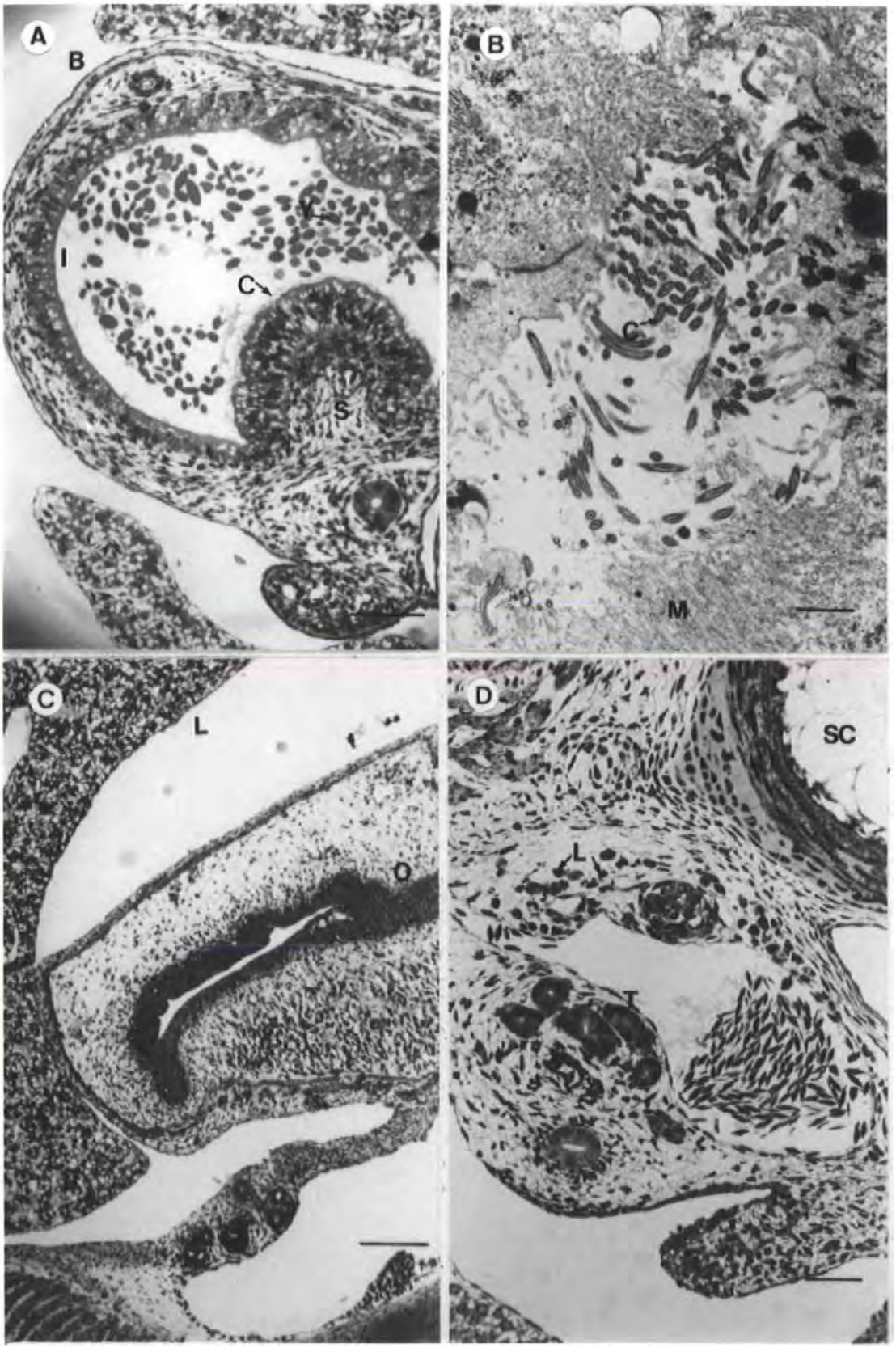

A
B
I
C
S
B
C
M
C
L
O
D
SC
L
I

PLATE 15 The thymus of a stage 1 fish

A&B Area from which the thymus may differentiate. G, gill; B, blood vessel; OT, possible origin of thymus; P, pharyngeal cavity; E, pharyngeal epithelium; S, skin.

Scale bar = 100μm

C&D The primordial thymus. C, connective tissue capsule; T, thymus.

Scale bar = 100μm

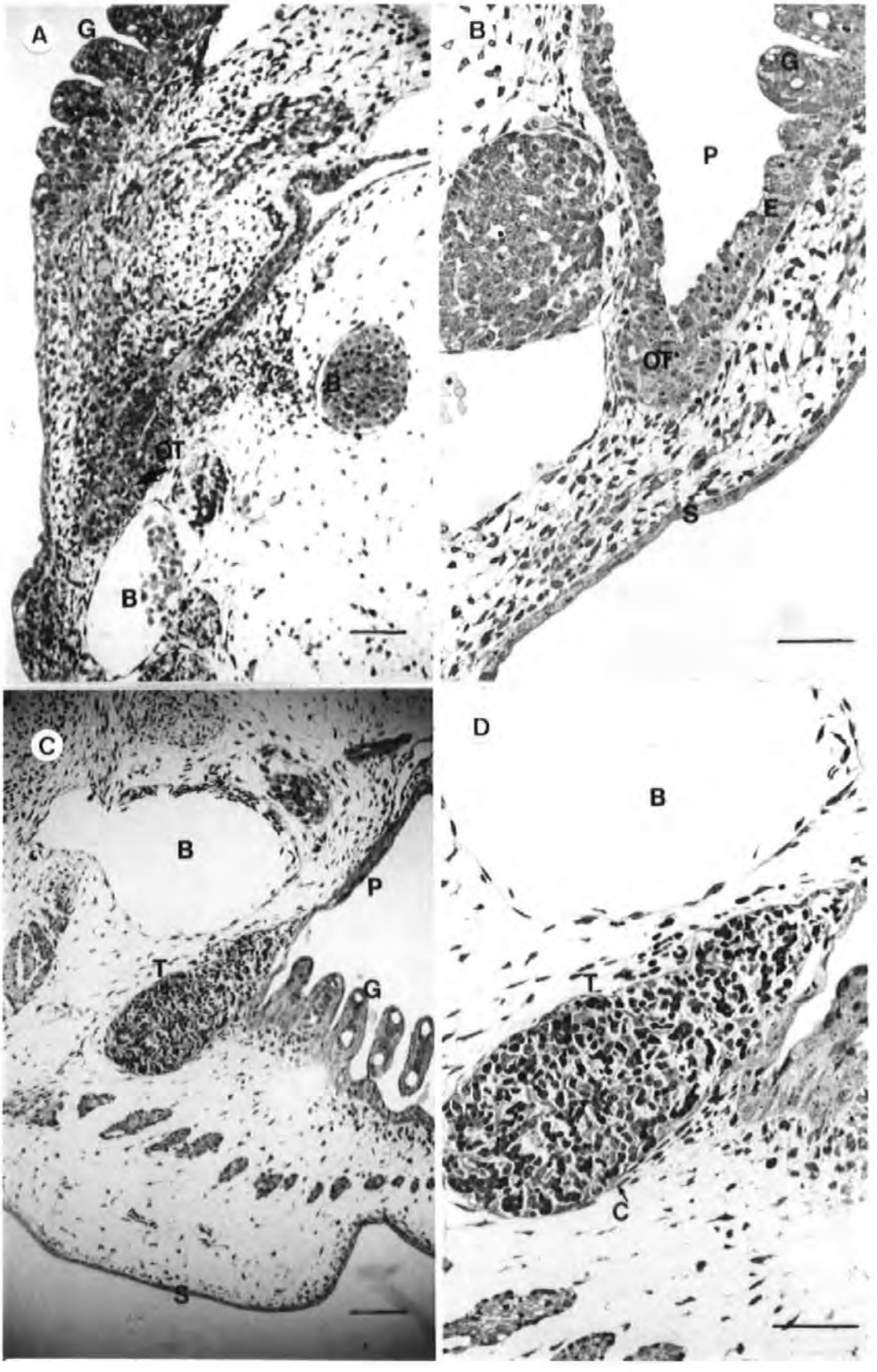

A
G
B
OT
B
B
G
P
E
OT
S
C
B
P
T
G
S
D
B
T
C

PLATE 16 The gut, kidney and thymus of a stage 2 fish

A Partially differentiated spiral valve. S, spiral valve; Y, yolk platelets, Li, liver.

Scale bar = 10μm

B Lymphoblast-like cells in the kidney region. G, glomerulus; Ca, cartilage; L, lymphoblast-like cells; K, kidney tubules; B, blood vessel; BC, body cavity.

Scale bar = 100μm

C Thymus. T, thymus; C, connective tissue capsule.

Scale bar = 25μm

D Oesophageal region of the gut. E, enfolded epithelium; L, primordial component of the Leydig organ.

Scale bar = 100μm

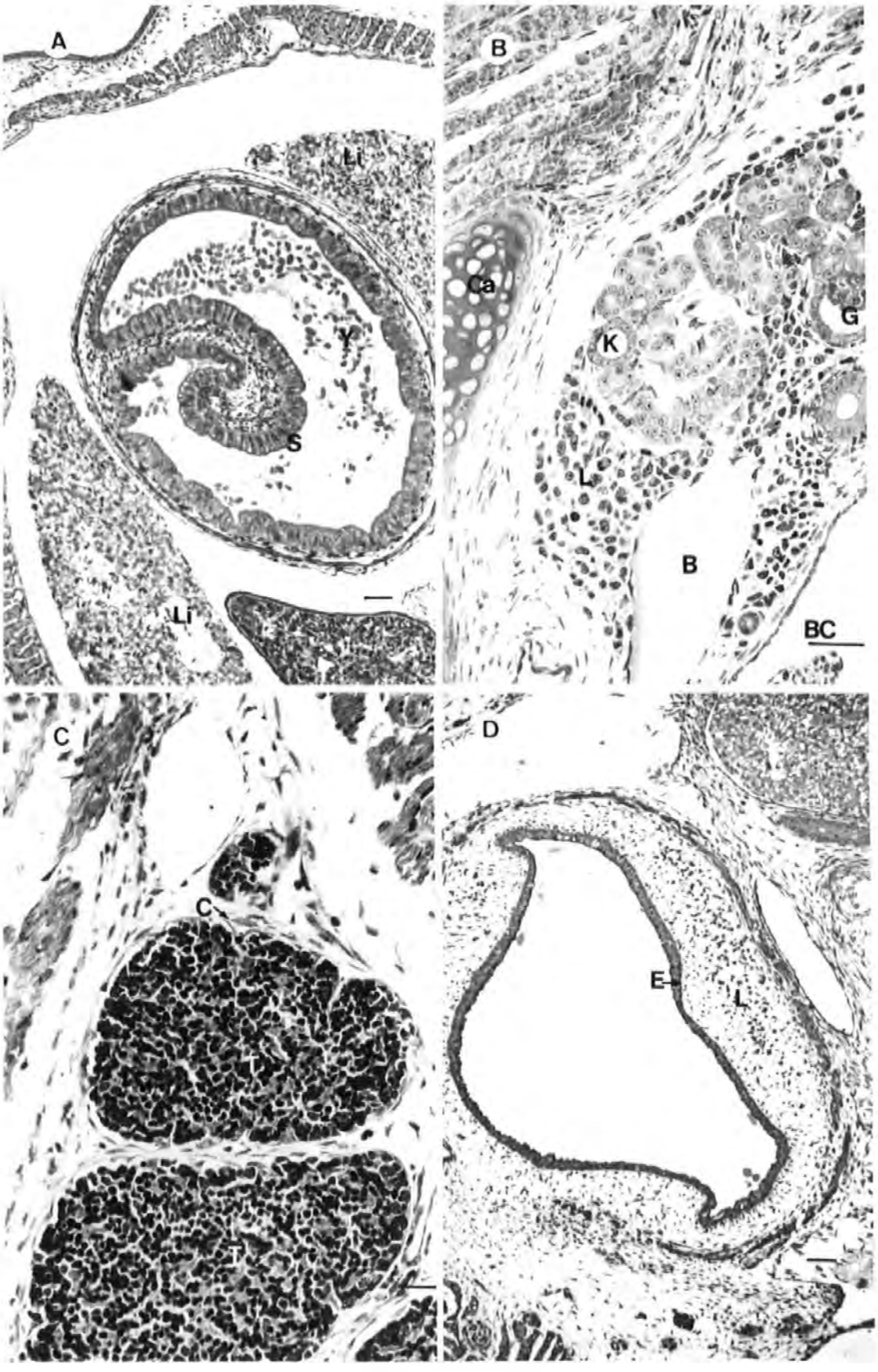

A
Li
Y
S
Li
B
Ca
G
K
L
B
BC
C
C
D
E
L

PLATE 17 Spiral intestine of stage 2 and 1 year old fish

A Spiral intestine of a stage 2 fish exhibiting a well folded epithelium. E, epitheial fold; B, blood vessel; Y, yolk platelet.

Scale bar = 50μm

B High power micrograph of an epithelial fold of a stage 2 fish. IEL, intraepithelial leucocyte.

Scale bar = 10μm

C The vacuolated nature of the epithelium of a stage 2 fish. N, nucleus of the epithelium; C, cilia; N, nucleus; ILL, intralaminal leucocyte.

Scale bar = 10μm

D The spiral valve of a one year old fish. LP, lamina propria.

Scale bar = 100μm

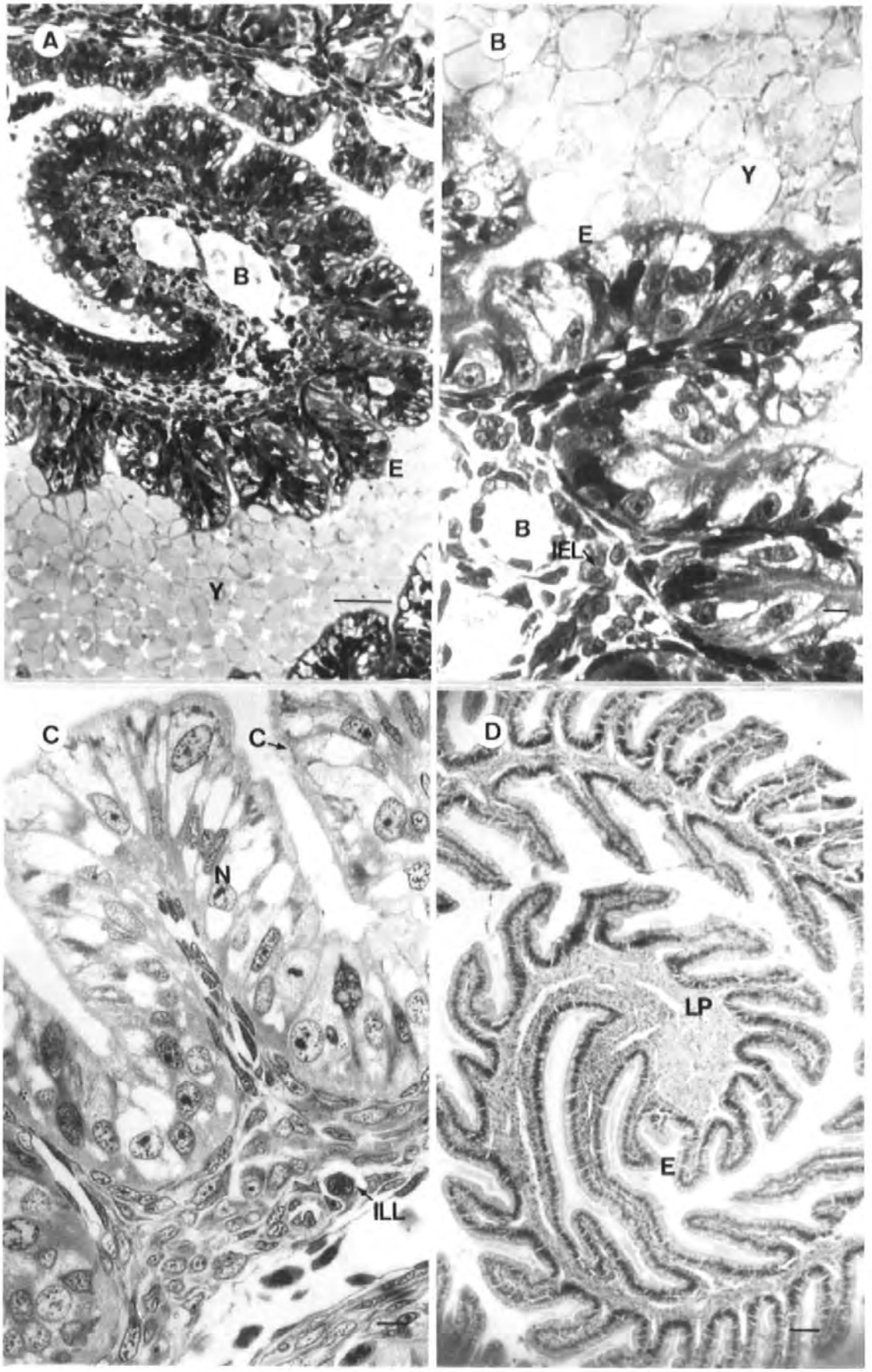

A
B
E
Y
B
Y
E
B
IEL
C
C
N
ILL
D
LP
E

PLATE 18 Kidney and thymus of stage 2 fish

A Cross section through the whole body. L, lymphocyte-like cells in the kidney; E, epigonal; S, spinal column; I, intestine.

Scale bar = 100μm

B High power micrograph of lymphocyte-like cells in the kidney region; C, cartilage.

Scale bar = 50μm

C High power micrograph of the kidney. G, glomerulus; K, kidney tubule; PL, putative lymphocytes.

Scale bar = 20μm

D Multilobed thymus. C, connective tissue capsule; T, thymus.

Scale bar = 100μm

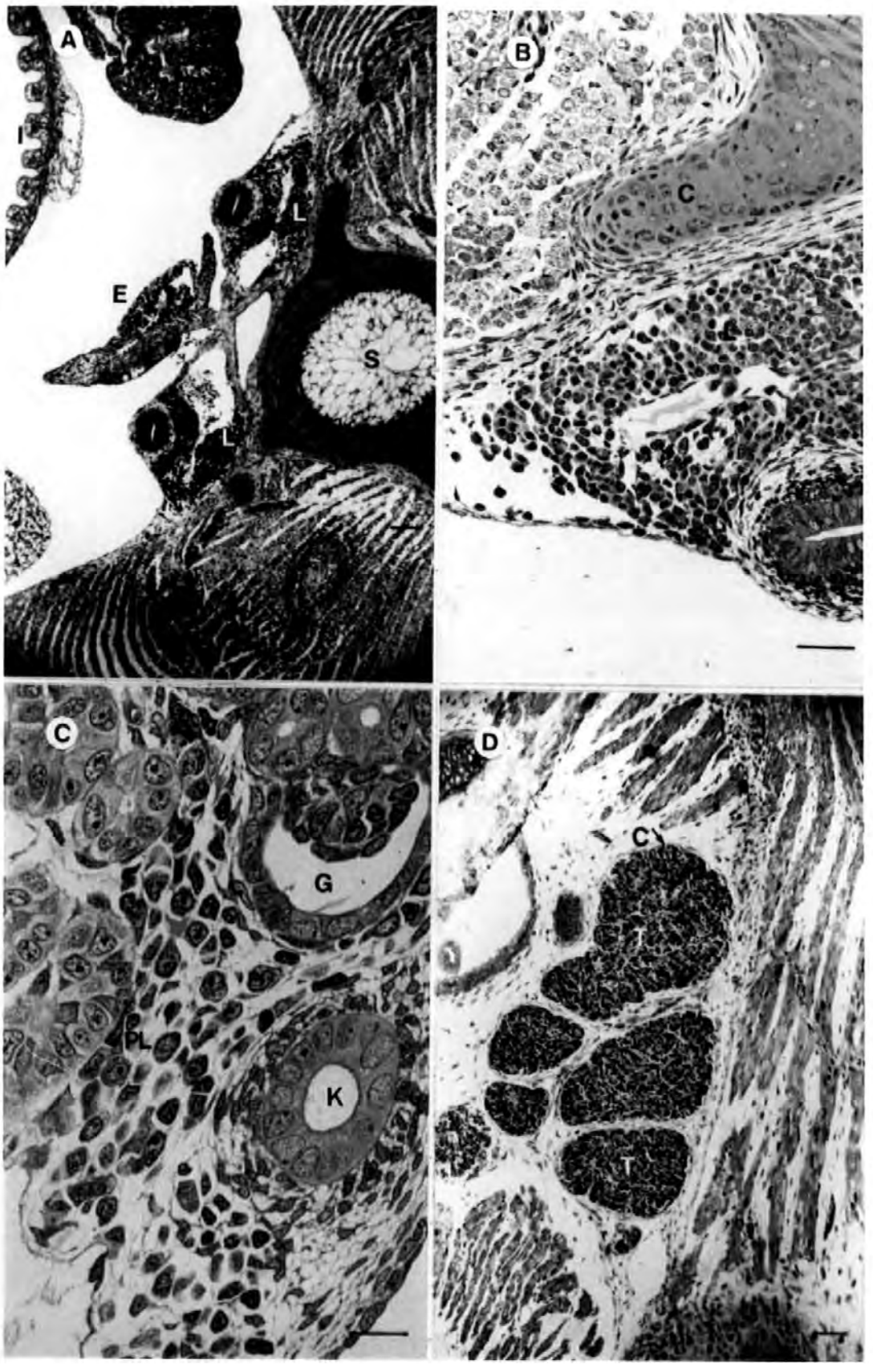

A
I
L
E
S
L
B
C
C
G
PL
K
D
C
T

PLATE 19 The thymus and Leydig organ of stage 2 fish

A A multilobed thymus, which is present in late stage 2 till at least 9 months post-hatch. T, thymus; C, connective tissue capsule; B, blood vessel.

Scale bar = 100μm

B A high power micrograph of the thymus. Th, thymocytes.

Scale bar = 10μm

C A low power micrograph of the developing Leydig organ. S, spinal column; A, dorsal aorta; L, Leydig organ; E, enfolded epithelium.

Scale bar = 100μm

D A high power micrograph of the developing Leydig organ. B, blood vessel; Le, leucocytes.

Scale bar = 20μm

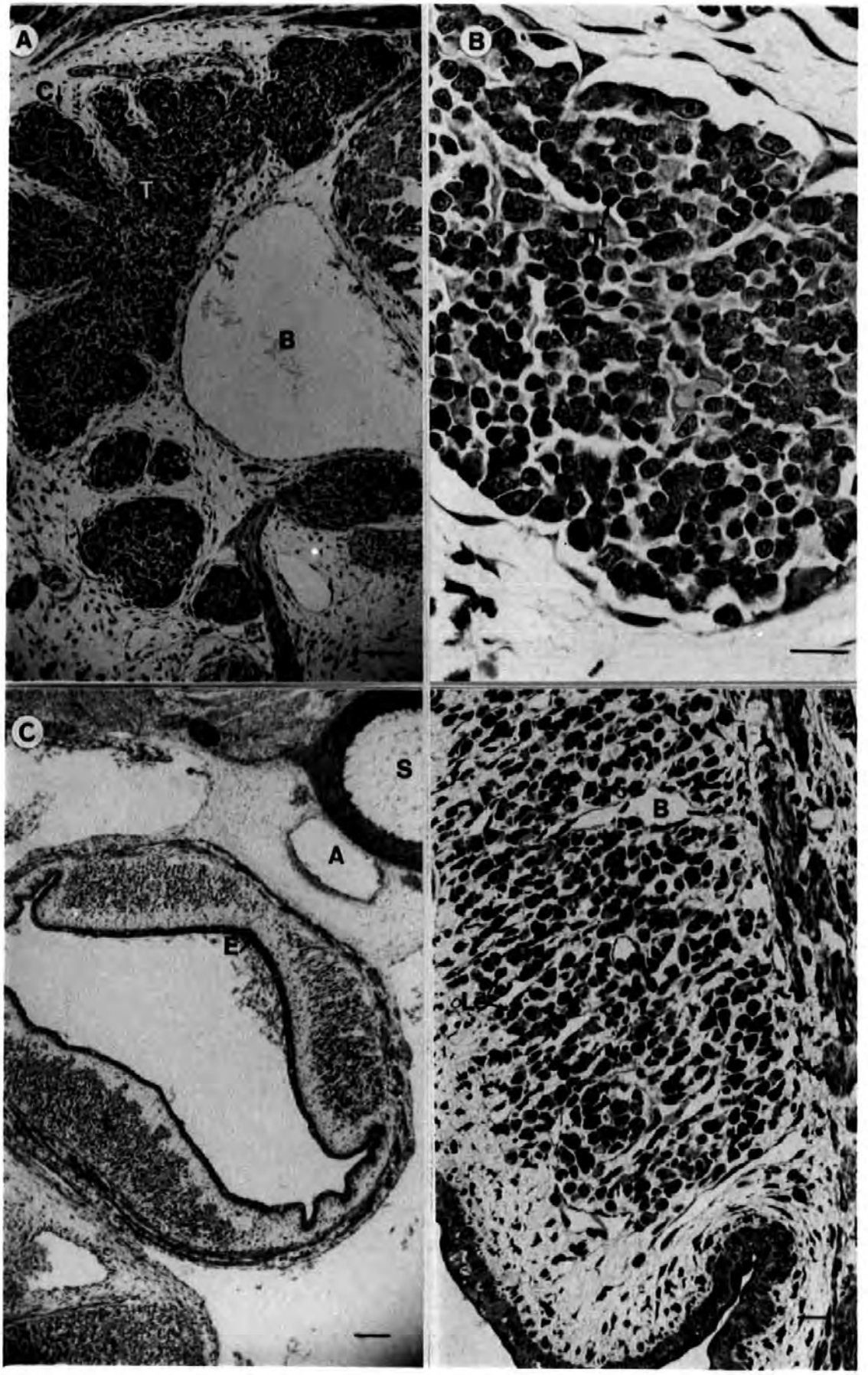

A
C
T
B
B
C
S
A
E
B

PLATE 20 Epigonal, spleen and Leydig organ

A A low power micrograph of the developing epigonal tissue region. K, kidney region, E, epigonal tissue; L, leucocytes.

Scale bar = 50μm

B A high power micrograph of the epigonal tissue leucocyte population.

Scale bar = 50μm

C The spleen containing red blood cells and leucocytes, R, red blood cells; LY, lymphocytes.

Scale bar = 50μm

D Leydig organ of a stage 3 fish. L, leydig organ; O, folded oesophageal epithelium; C, cilia; B, blood vessel

Scale bar = 50μm

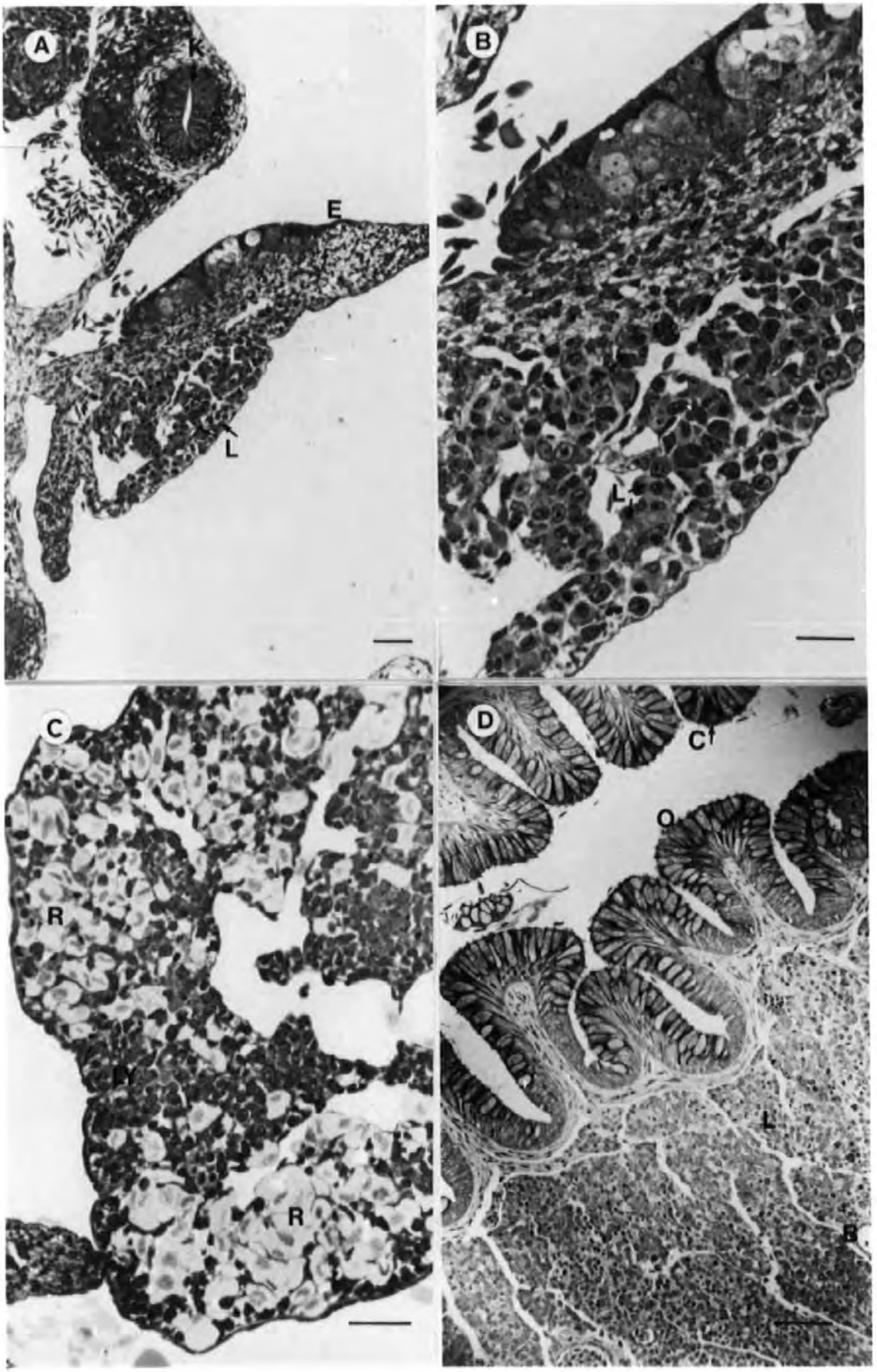

A
K
E
L
B
L
C
R
R
R
D
C
O
L
B

PLATE 21 The intestine, leucocyte populations and kidney

A A lymphoid accumulation at the centre of the spiral valve of a stage 3 fish. LA, lymphoid accumulation; V, vacuolated epithelium.

Scale bar = 100μm

B Intraepithelial leucocytes of a stage 3 fish. IEL, intraepithelial leucocyte; BM, basement membrane, B, blood vessel.

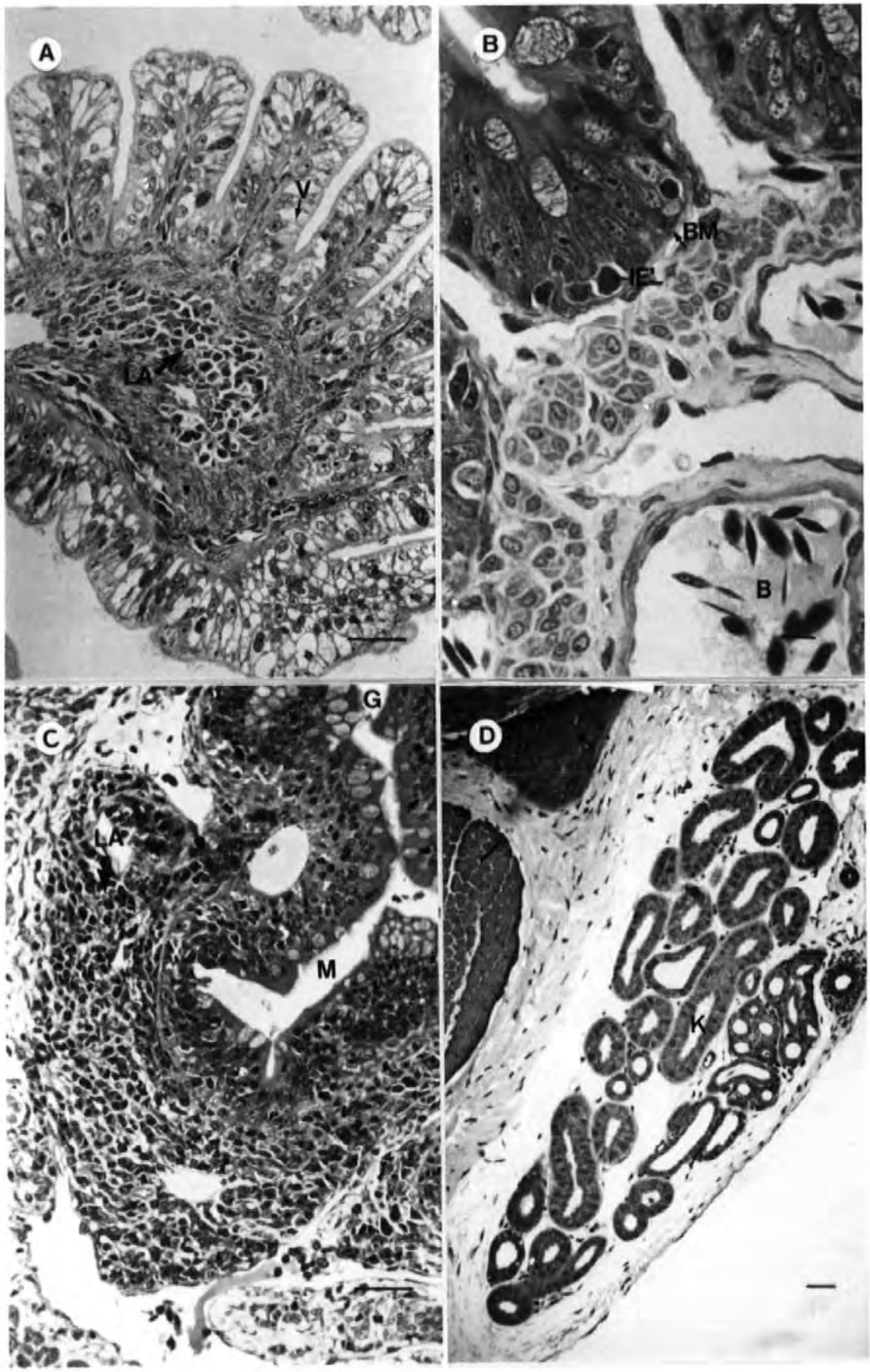
Scale bar = 20μm

C Lymphoid accumulation of a 9 month - 1 year old fish. LA, lymphoid accumulation; M, microvilli; G, goblet cells.

Scale bar = 100μm

D Kidney of a 1 month post-hatch fish. K, kidney tubules.

Scale bar = 100μm


A
V
LA
B
BM
IEL
B
C
G
LA
M
D
K

CHAPTER 5

AN INVESTIGATION OF THE ABSORPTION OF MATERIAL BY THE GUT

The results of investigations on the absorption of particulate and soluble materials by the gut of larval (Stage II & III), neonatal (Stage IV) and adult fish are presented in this chapter.

5.1 The absorption of particulate material

5.1a Absorption of carbon from the gut of Stage III fish

0.1ml of carbon suspension (Pelican Ink, W.Germany), made up 50:50 with whole dogfish serum, was injected into the yolk sac near the yolk sac stalk (Figure 5A) using a 25 ga needle and a 1ml syringe. After approximately 20 hours the spiral intestine took on a dark appearance as carbon particles were moved by cilia from the yolk sac, along the yolk sac stalk into the spiral intestine. Control fish were injected with whole dogfish serum (WDS) only (Table 11).

Fish were killed 6, 22, 48 and 72 hours later and the spiral intestine, spleen, liver, Leydig organ, epigonal tissue and gill were examined by methacrylate resin histology. Blood was also aspirated from the caudal sinus and smears made.

Carbon was absent from the blood of all stages in this experiment (Table 11) but was detected in the spiral epithelium of 22, 48 and 72 hour fish (Plate 22A and B). The carbon appeared to be taken up in quite a diffuse fashion, although more was detected in the vacuolar regions of the epithelium. As carbon was absent from the internal organs and the corpus cavernosum (Table 11) and was not found in the blood the ultimate destination of this material was unclear.

TABLE 11 ABSORPTION OF CARBON FROM THE GUT OF STAGE III FISH

TIME	NO. OF EXPERIMENTAL FISH	NO. OF CONTROL FISH	G	BC	O	CS	PS	SV	R	T	S	LO	ET	L	BLOOD SMEAR	GLASS ADHERENCE
6	2	1														
22	3	1						+								
48	3	1						+								
72	3	1						+								

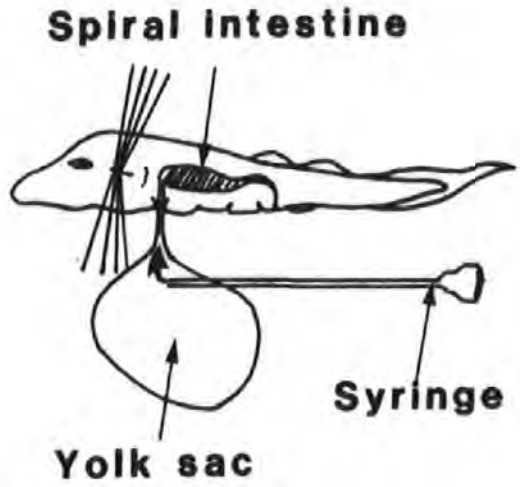
TABLE 12 ABSORPTION OF CARBON FROM THE GUT OF NEONATAL (STAGE IV) AND ADULT FISH

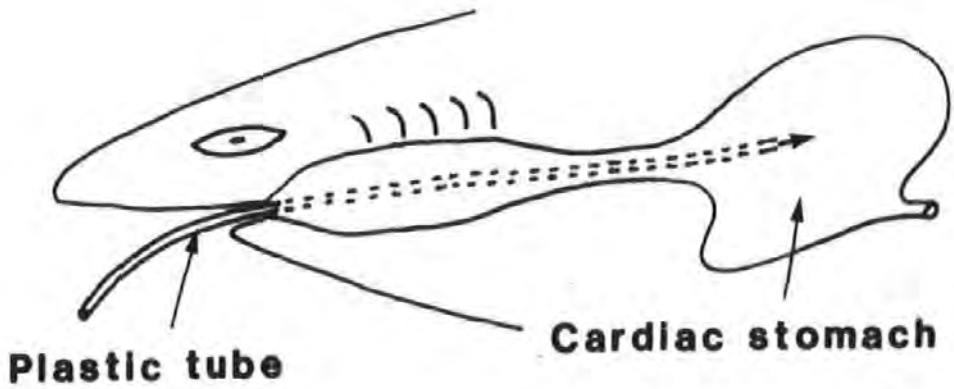
STAGE	TIME OF KILLING	NO. OF EXPERIMENTAL FISH	NO. OF CONTROL FISH	G	BC	O	CS	PS	SV	R	T	S	LO	ET	L	BLOOD SMEAR	GLASS ADHERENCE
4																	
"	6	3	1														
"	22	2	1													+/-	+/-
"	48	4	1													+/-	+/-
"	72	3	1													+/-	+/-
Adult	6	4	1														
"	22	4	1														
"	48	4	1													+/-	+/-
"	72	4	1													+/-	+/-

KEY: G - Gill; BC - Buccal cavity; O - Oesophagus; CS - Cardiac Stomach; PS - Pyloric stomach; SP - Spiral valve; R - Rectum; T - Thymus; S - Spleen; LO - Leydig organ; ET - Epigonal tissue; L - Liver.

FIGURE 5 A. METHOD OF INJECTING LARVAL FISH WITH EXPERIMENTAL MATERIAL

B. METHOD OF INTUBATING EXPERIMENTAL MATERIAL INTO THE CARDIAC STOMACH OF EXPERIMENTAL FISH


Spiral intestine
Syringe
Yolk sac


Plastic tube
Cardiac stomach

5.1b Absorption of carbon from the gut of neonatal stage IV and adult fish

This investigation was undertaken to determine if there was a functional correlation between the absorption of material and the morphological differentiation of the spiral valve epithelium recorded in Chapter 4.

One ml of carbon suspension (see Section 5.1a) was intubated directly into the cardiac stomach of adult fish and 0.1ml into the gut of neonates (Figure 5B). Control fish were exposed to the same volume of WDS (Table 12).

The gut and other organs were sampled and examined as previously described. Blood samples were taken and in addition to smearing the blood, blood monocytes were examined after being allowed to adhere to glass.

Carbon was not detected in the gut or other body organs including the corpus cavernosum, although occasional monocyte-like cells on blood smears and adherent cells from the blood contained a few carbon particles (Plate 23).

5.2 The absorption of soluble material

The absorption of soluble material was investigated using 3 techniques. In Stage II and adult dogfish HGG was injected into the yolk sac, and in the adult was given orally and anally by intubation. The absorption of HGG was detected by immunofluorescence. Ferritin was also injected into the yolk sac of stage II fish and detected by electron microscopy. In adult dogfish the level of BSA in the blood was measured by rocket immunoelectrophoresis after oral and anal intubation.

5.2a Absorption of HGG in Stage II and adult fish

One ml of 1% HGG solution was intubated directly into the cardiac stomach (orally) and the lower spiral valve (anally) of adult dogfish (approximately 1kg), and 0.1ml injected into the yolk sac of stage II fish. Control fish were treated with saline. Fish were killed at 22, 48 and 72 hours (Tables 13 and 14) and tissues were examined for the presence of HGG using a fluorescein labelled rabbit anti-HGG serum (Wellcome Biotech Ltd., Beckenham) on 8μm cryostat sections. HGG was taken up in the spiral intestine of both stage II and adult fish (Tables 13 and 14), in the latter however, only a weak fluorescent reaction was observed and fluorescent activity could not be detected in the other organs (Table 13).

5.2b Absorption of ferritin in the spiral valve of stage II fish

The purpose of this experiment was to develop an experimental system to investigate the mechanism of absorption of soluble proteins in the gut.

Three stage II fish were injected with 0.1ml of 1% colloidal equine spleen ferritin (Sigma Biochem Ltd.) into the yolk sac. A control fish was injected with a similar volume of ES. Fish were killed at 24 hours and the spiral intestine was excised, cut into small pieces ($1mm^3$) and prepared for electron microscopy (see methods). The following alterations to the normal post-fixation regime were employed:-

TABLE 13 UPTAKE OF HGG BY ADULT FISH AFTER ORAL AND ANAL INTUBATION

STAGE	ROUTE OF ADMINI-STRATION	TIME OF KILLING	NO. OF EXPERI-MENTAL FISH	NO. OF CONTROL FISH	G	BC	O	CS	PS	SV	R	T	S	LO	ET	L	BLOOD SMEAR	GLASS ADHERENCE
Adult	Oral	22	1	-						+/-								
"	"	48	1	-						+/-								
"	"	72	1	1														
"	Anal	22	1	-						+/-*								
"	"	48	1	-						+/-*								
"	"	72	1	1														

* Uptake was detected only in the lower spiral intestine

TABLE 14 UPTAKE OF HGG BY STAGE II FISH AFTER INJECTION INTO THE YOLK SAC

STAGE	ROUTE OF ADMINI-STRATION	TIME OF KILLING	NO. OF EXPERI-MENTAL FISH	NO. OF CONTROL FISH	G	BC	O	CS	PS	SV	R	T	S	LO	ET	L	BLOOD SMEAR	GLASS ADHERENCE
		22	1	-						+								
		48	1	1						+								
		72	1	1						+								

KEY: G - Gill; BC - Buccal cavity; O - Oesophagus; CS - Cardiac stomach; PS - Pyloric stomach; SP - Spiral valve; R - Rectum; T - Thymus; S - Spleen; LO - Leydig organ; ET - Epigonal tissue; L - Liver.

FERRITIN CHALLENGED FISH

Osmicated		Non-Osmicated	
Stained	Unstained	Stained	Unstained

CONTROL (ES) FISH

Osmicated		Non-Osmicated	
Stained	Unstained	Stained	Unstained

Ferritin was detected in the lumen of the spiral intestine (Table 15) and was often associated with yolk material (Plate 24A), which appears to be moved by cilia (24B), and was most easily detected in unosmicated, unstained material where its intrinsic electron density made it easily visible (Plate 24C), avoiding the confusion sometimes encountered with osmicated and stained material.

While some material was detected free in the cytoplasm of the epithelium (Plate 24D) most ferritin was detected at the periphery of vacuoles containing yolk material (Plate 25A and B), which must have been taken up by a phagocytic process.

5.2c Absorption of BSA from the gut of adult fish

In this experiment after oral and anal intubation of a 10% BSA solution the serum was tested by rocket electrophoresis to determine if this molecule was taken up by the gut in an antigenic form.

TABLE 15 UPTAKE OF FERRITIN IN THE SPIRAL VALVE OF STAGE II FISH

STAGE	ROUTE OF ADMINI-STRATION	TIME OF KILLING	FISH NO.	CONTROL	PRESENCE OF FERRITIN IN THE SPIRAL VALVE
2	Yolk sac	24 hrs	1	1*	√
2	" "	"	2	-	√
2	" "	"	3	-	√

* Ferritin was absent from the control fish

One ml of a 10% solution was given orally, anally and intravenously (into the caudal sinus). In control fish 1ml of ES was introduced into the fish by the same routes. Blood samples were taken from the caudal sinus at timed intervals (Table 16). BSA was not detected in the serum after oral or anal exposure to antigen. BSA introduced into the blood sinus by injection remained for at least three days. No BSA was detected in the serum of control fish.

TABLE 16 DETECTION OF BSA IN THE SERUM OF FISH AFTER ANAL, ORAL AND INTRAVENOUS INTRODUCTION OF THE ANTIGEN

STAGE	NO. OF EXPERI-MENTAL FISH	NO. OF CONTROL FISH	ROUTE OF ADMINI-STRATION	TIME OF BLEEDING	DETECTION OF BSA IN SERUM
ADULT	2	1	Oral	4	-
			"	6	-
			"	12	-
			"	24	-
			"	48	-
			"	72	-
ADULT	2	1	Anal	4	-
			"	6	-
			"	12	-
			"	24	-
			"	48	-
			"	72	-
ADULT	2	1	Intravenous	4	√
			"	6	√
			"	12	√
			"	24	√
			"	48	√
			"	72	√

DISCUSSION

These results show that there is a clear difference between the way that the spiral valve absorbs material in the developing dogfish, which is dependent upon yolk material, and the adult which has an exogenous diet. A similar phenomenon has been observed in the sturgeons, where an increase in intraluminal enzyme levels is correlated with a concomitant decrease in intestinal pinocytic activity during the transition to an exogenous diet in late larval stages (cf. Buddington and Doroshov, 1986). Yolk material is phagocytosed by the spiral intestine, of the developing dogfish, and under experimental conditions the intestine was found to absorb carbon, HGG and ferritin. The latter protein, investigated by electron microscopy, was associated with yolk particles phagocytosed by the intestine.

In the adult phagocytosis of carbon by the spiral intestine epithelium did not occur. Some HGG uptake was detected after oral and anal intubation, but after exposure to BSA in a similar fashion none was detected by immunoelectrophoresis in the bloodstream. Low levels of absorption may be caused by the breakdown of proteins intubated by the oral route, and by the restriction of access to the intestine by the anal route, caused by the spiral valve. Using the same protocol, uptake of BSA from the intestinal lumen into the bloodstream has been demonstrated in S.gairdneri (Wrathmell, unpublished data). Uptake only occurs after anal intubation, not oral, and appears to be correlated with the nutritional status of the fish.

The loss of phagocytic activity in the intestine can be correlated with a change in morphology. In Chapter 4 the epithelium of the spiral intestine of fish dependent upon yolk was found to have a highly vacuolated morphology and possess cilia at the surface. In the adult such vacuolation, or the presence of cilia was not detected. The mechanism by which the gut morphology and function was transformed from

Stage II to adult, and what stimuli initiates and controls it is in S.canicula has not been investigated. However, an appreciation of the complexity of this transformation may be gained from reference to work on the rat model (cf. Buts and Delacroix, 1985).

The gut of larval teleosts may show enhanced pinocytosis of proteins (cf. Georgopoulou et al., 1985) although the occurence of phagocytosis by the gut has not been investigated. There is considerable evidence to show that irrespective of the state of maturity or gut morphology there are absorptive enterocytes localised with specific regions which are capable of absorbing macromolecules in teleosts (Ash, 1985 and Georgopoulou et al., 1985). Carbon particles were not taken up in the common carp (Davina et al., 1982).

Why are adult dogfish incapable of absorbing carbon? A possible answer is that if phagocytosis was retained as the chief mechanism of absorbing material from the intestine, pathogens may gain entry to the the body. The Peyer's patches of adult rats are capable of phagocytosing material (Levre, Olivo, van der Hoff and Joel, 1978) and this is a particular route of infection by Salmonella typhi (Carter and Collins, 1974). The lymphoid accumulation of lower vertebrates have been likened to Peyer's patches (cf.Chapter 3),in this brief investigation, however, they were not found to be a focus for the uptake of material under experimental conditions.

The gut of adult higher vertebrates is now regarded as being permeable to macromolecules probably not in amounts of nutritional importance (Rothberg, Kraft, Farr, Kriebel and Goldberg, 1971 and Warshaw, Walker and Isselbacher, 1974) but, certainly in immunogenic quantities (Volkeimer and Schulz, 1968; Schreiber, 1974; Warshaw, Walker and Isselbacher, 1974 and Cook and Olson, 1979). Neonates, however, are capable of absorbing much larger quantities of macromolecules across the intestinal wall. Specific Fc receptors for

IgG have been described on the microvilli of the proximal intestine of the neonatal rat up to day 20 (Peppard, Jackson and Mackenzie, 1985), while in the distal intestine, internal digestion of milk substances occurs (Ono and Satoh, 1981). Premature human infants were found to absorb large quantities of lactoglobulin compared to the adult (Levinsky, Pagnelli, Robertson and Atherton, 1980)

As previously mentioned, the mechanism of carbon and ferritin uptake appears to be by phagocytosis along with yolk material. Some material was observed free in the cytoplasm of epithelial cells. It is unclear how this material gained access to the cytoplasm, diffusion from the lumen across the epithelial surface (Williams, 1978) is thought to be improbable. Ferritin may have been originally contained in vacuoles which lost their integrity during fixation and processing, or the vacuoles may have disintegrated and liberated their contents naturally.

The mechanism by which macromolecules, including proteins gain access to the epithelium and are processed by teleost fish is unclear (McLean and Ash, 1986). Access may be transcellular (Yamamato, 1966 and Rombout et al., 1985), intracellular (Volkeimer, 1972) or by pathways made available during the sloughing off of epithelium or localised tissue damage (Mclean and Ash, 1986). Rombout et al. (1985) proposed that HRP may be transported by a specific receptor system largely avoiding interaction with lysosomes, and gaining rapid access to the intercellular space. This work appears to be corroborated by McLean and Ash (1986) who found that orally intubated HRP is rapidly taken up into the bloodstream. The uptake of ferritin appeared to be by non-specific liquid phase pinocytosis in which material was digested in phagolysosomes; some of which was taken up in intercellular macrophages (Rombout et al., 1985).

The situation described above has some similarities to that in neonatal and adult rats. In the neonates specific Fc receptors occur in the proximal intestine which transport maternal IgG from the lumen to the systemic circulation (Peppard et al., 1985). In the distal intestine material is taken up by liquid phase pinocytosis and is subject to intracellular digestion mediated by lysosomes (Ono and Satoh, 1981). The latter mechanism is retained to a degree in the adult and has previously been dealt with in this discussion.

It is likely that HGG uptake, detected by immunofluorescence in adult dogfish in this study, is by pinocytosis as cyclostomes (Langille and Youson, 1985), teleosts and higher vertebrates (mentioned earlier in this chapter) all exhibit pinocytosis, the exact mechanism has yet to be investigated.

While the phagocytic mechanism exhibited by the enterocytes of stage II dogfish is likely to have a nutritional role, the function of pinocytosis of protein macromolecules of adult teleosts is, however, less certain. McLean and Ash (1986) cited three hypotheses which dealt with the significance of protein absorption by the distal intestine of teleosts: 1) Stroband and van der Veen (1981) proposed that this phenomenon may provide a standby facility whenever the normal capacity of the digestive enzymes are overloaded (e.g. during larval and post-starvation periods). 2) Hofer and Schiemer (1981) suggested pinocytosis may allow enteropancreatic recirculation of enzyme, while 3) Davina et al. (1982) proposed that absorption enterocytes may be an antigen sampling system similar to "M"-cell specialisation in higher vertebrates.

The function of macromolecular uptake in higher vertebrates is also unclear. As previously discussed, amounts taken up are nutritionally insignificant. However, antigens in the lumen do stimulate the production of secretory IgA, which may prevent toxins and pathogens entering the enterocytes by immuno exclusion (Walker, 1985).

Feeding antigens has also been found to suppress delayed hypersensitivity responses and create a state of systemic tolerance in mammals, mediated by T-suppressor cells or immunoglobulin (Enders, Gottwald, and Brendel, 1986).

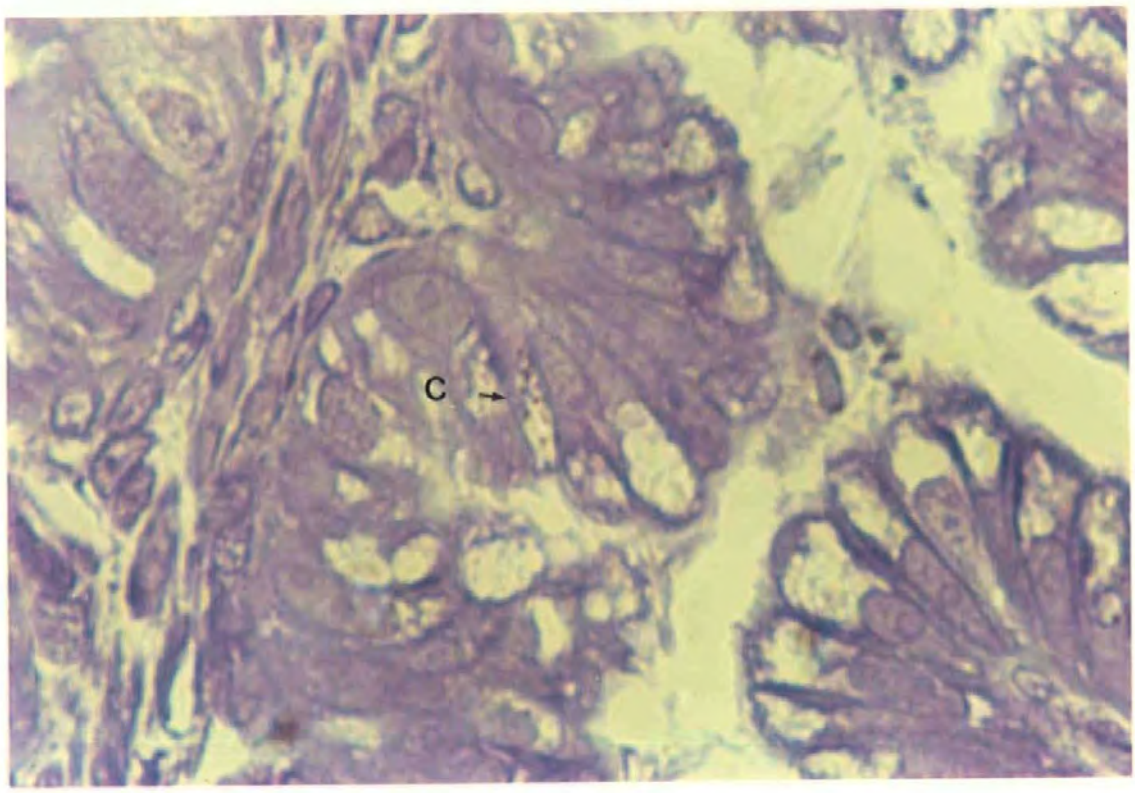
A breakdown in the control of antigen uptake in mammals may lead to a state of hypersensitivity, a common example of which is food allergy (Soothill, 1980). Large populations of cells have been detected in the gut of teleosts and elasmobranchs (Chapter 3) which appear to be involved in the production of antibody to luminal antigens (Chapter 6). The role of these cells in oral tolerance and hypersensitivity is unknown. However, oral tolerance was reported in S.gairdneri after feeding V.anguillarum (Udey & Fryer, 1978) and a hypersensitivity-like reaction may be mediated by EGC's in the gut of the same fish (see literature review and Chapter 3).

The pinocytotic activity of the distal intestine in teleosts may be exploited as a route for vaccination (Lamers, 1985 and Rombout et al., 1986). For a vaccine to be effective, however, it must be protected from or be resistant to, gastric hydrolysis and enzyme digestion, and be taken up in sufficient quantities to stimulate a protective response. Similar problems still present a stumbling block in the development of oral vaccines to mammalian diseases such as cholera, shigellosis and enterotoxigenic E.coli disease (Lycke and Holmgren, 1986).

PLATE 22 Carbon uptake in the intestine of stage III fish

A and B Particles of carbon detected in the epithelium of the spiral valve. LM, Giemsa, 1μm methacrylate resin section; C, carbon; B, blood vessel; L, lumen.

Scale bar = 10μm


C

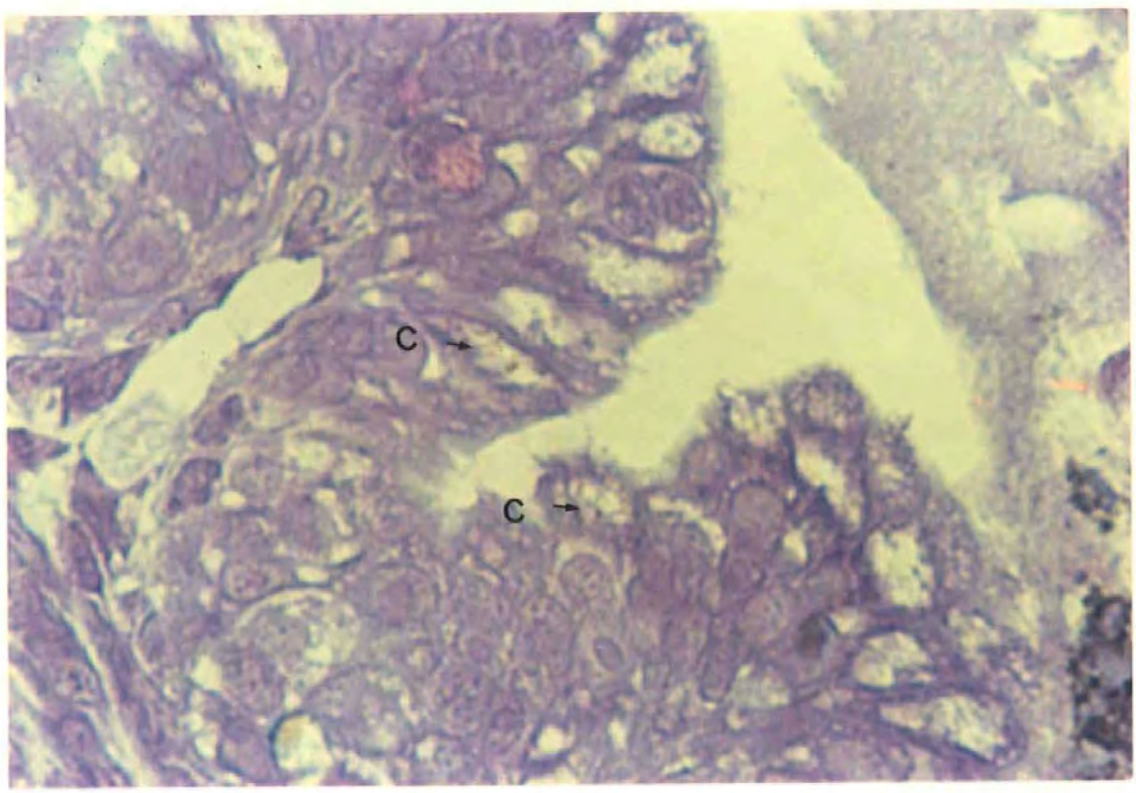

C
C

PLATE 23 Adherent blood monocytes containing carbon particles

Monocytes containing carbon particles which have adhered and spread on glass. Giemsa; M, monocyte; C, carbon grains.

Scale bar = 10μm

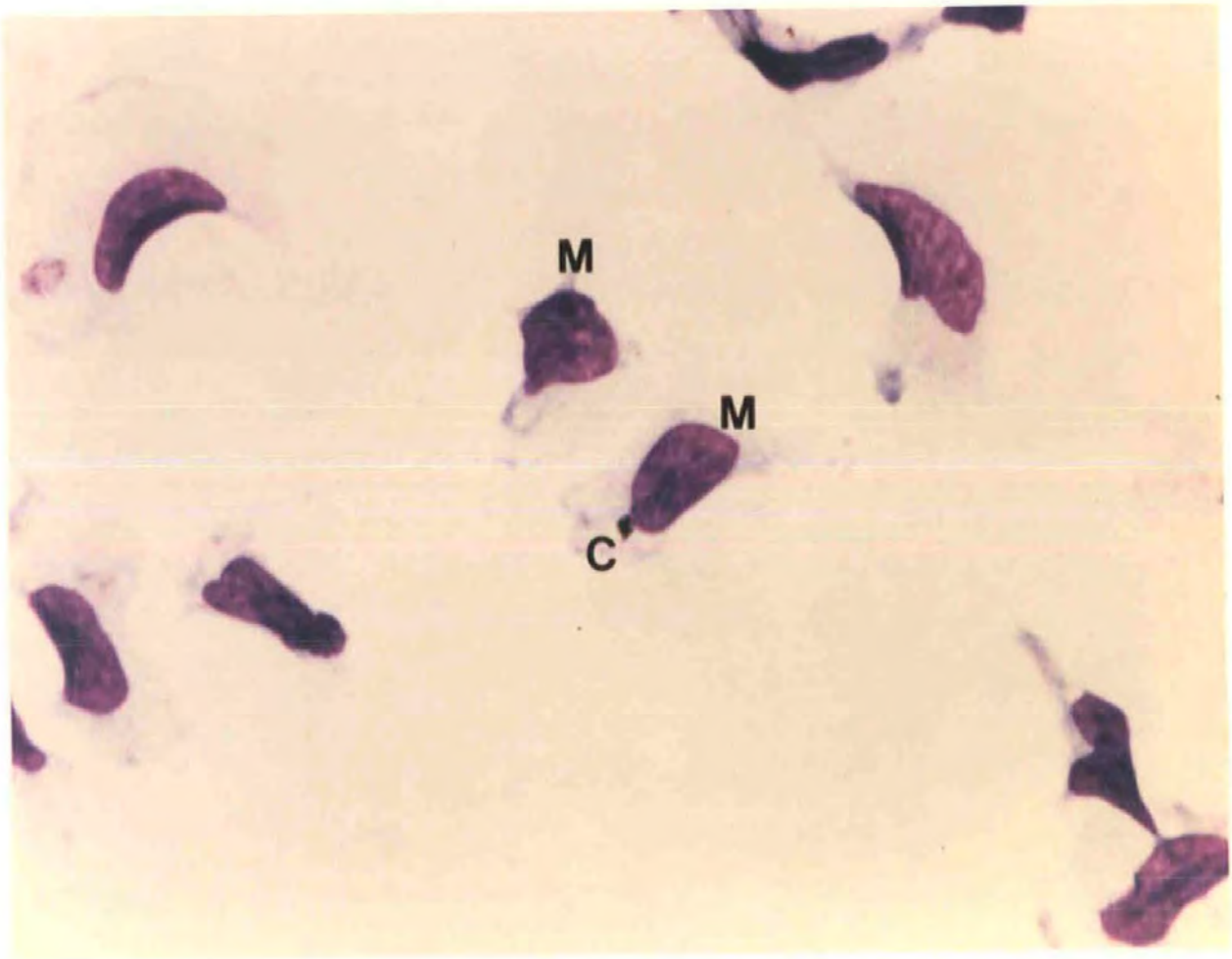

M
M
C

PLATE 24 Ferritin uptake in the intestine of stage II dogfish

A. A yolk platelets adjacent to the epithelial surface of the spiral valve. Y, yolk platlet, E, epithelial surface.

Scale bar = μÅ

B. Ciliated surface of the spiral intestine epithelium C, cilia; M, microvilli; L., lumen.

Scale bar = μÅ

C&D. Unstained unosmicated sections of the epithelium of the spiral valve. F, ferritin; Mi, mitochondria.

Scale bar = μÅ

All sections were examined using a transmission electron microscope.

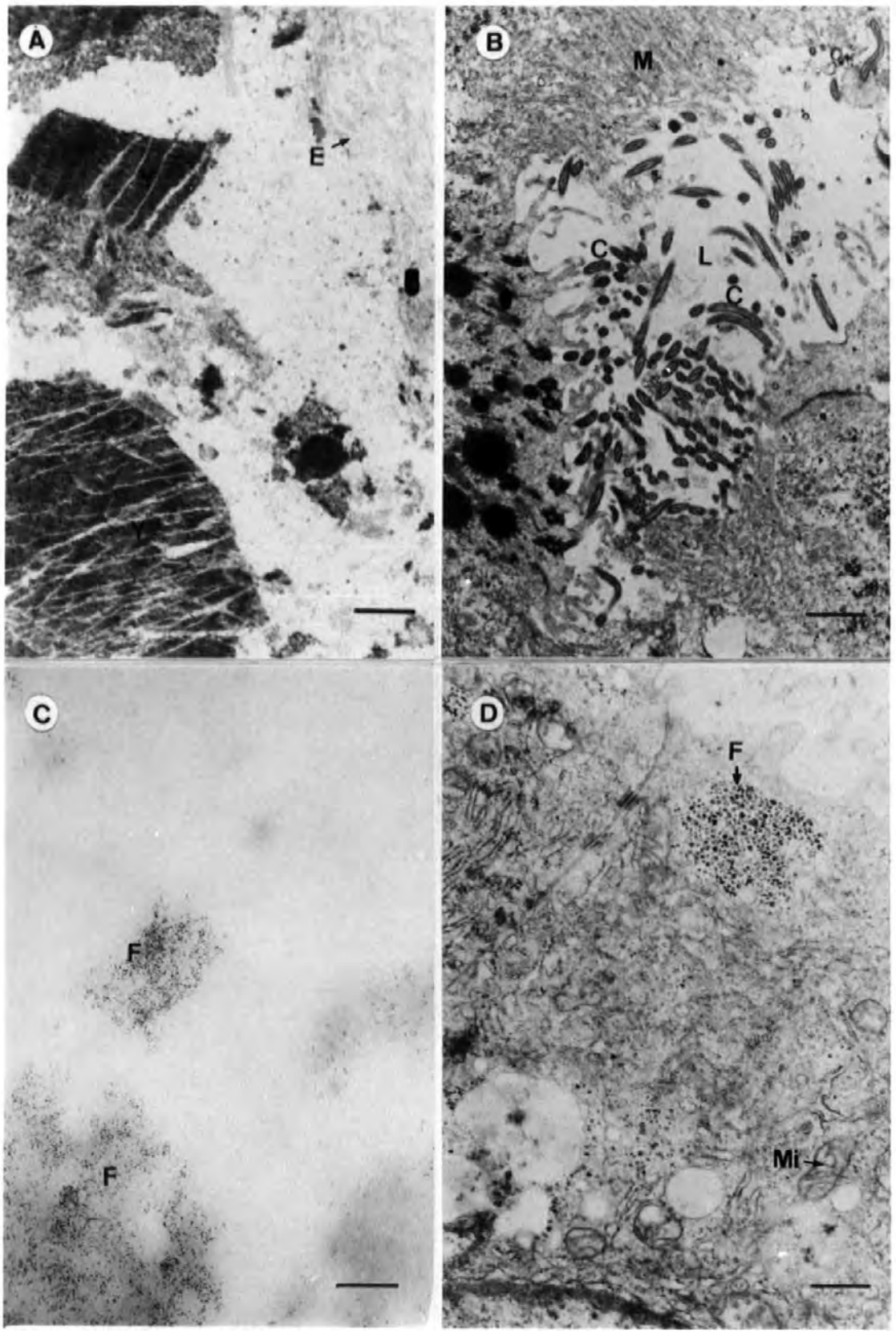

A
E
B
M
C
L
C
C
F
F
D
F
Mi

PLATE 25 Ferritin uptake by the intestinal enterocytes of stage II dogfish

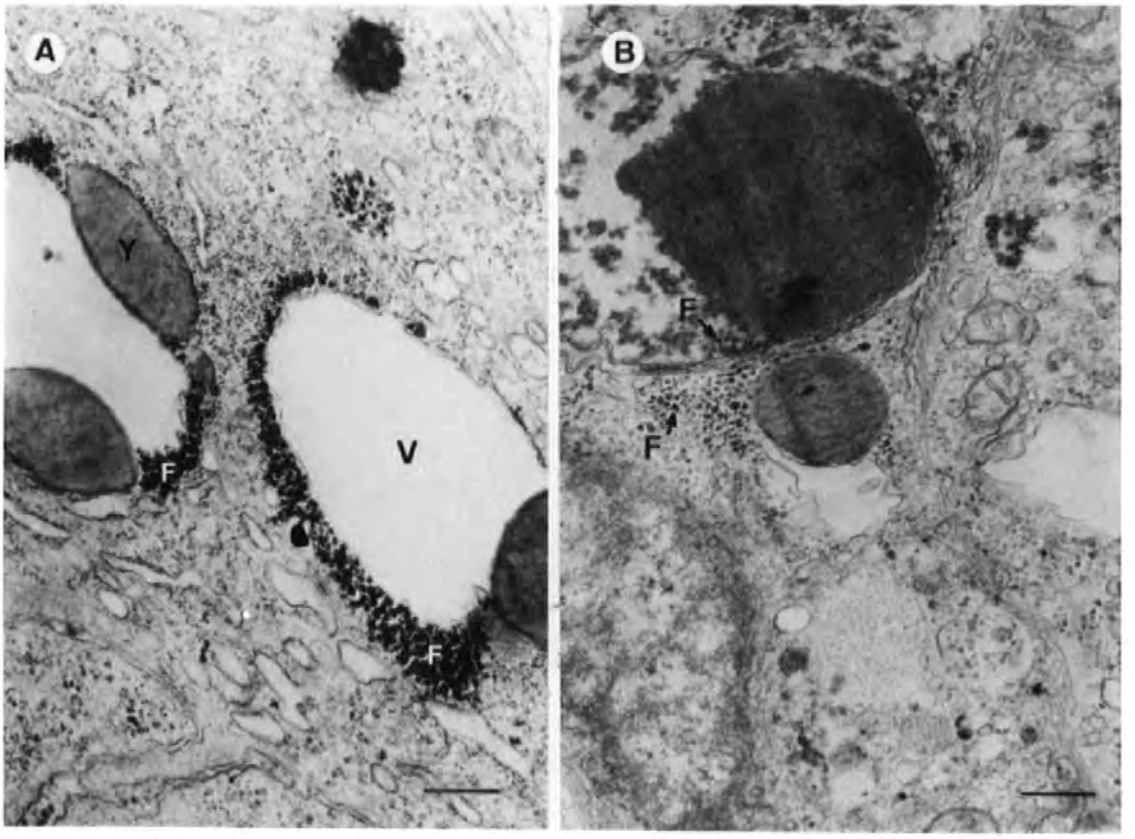
A. Ferritin at the periphery of a vacuole containing part of a yolk platelet. Y, yolk platelet; F, clump of ferritin; V, vacuole.

Scale bar = μÅ

B. Large accumulation of ferritin around a vacuole containing partially digested yolk platelets.

Scale bar = μÅ

Sections were examined using a transmission electron microscope.


A
Y
V
F
F
B
F
F

99

CHAPTER 6

A BRIEF INVESTIGATION OF THE ANTIBODY RESPONSE IN THE GUT AND THE NATURE AND DISTRIBUTION OF MUCOSAL IMMUNOGLOBULIN

This chapter describes investigations on the local antibody response to killed and sonicated V.anguillarum, and sheep red blood cells in the gut and biliary system of adult fish (Figure 6 and Plate 9). The distribution, ontogeny and some aspects of the nature of the mucosal immunoglobulin was also investigated.

6.1a Antibody responses to antigens presented orally and anally by intubation, and by injection into the peritoneum

Fish were exposed to 10^9 formalin-killed, sonicated V.anguillarum or 10^9 SRBC's by oral and anal intubation, or IP injection at weekly intervals for 1 month. Antibody levels were measured in the bile and the serum 60 days after the final administration of antigen by direct agglutination (see Methods). Intestinal mucus was also tested for the presence of antibodies.

Using a second protocol, fish were immunised with SRBC's, as above, i.e. by oral, anal and IP routes and after 60 days all fish, including controls were exposed to 10^9 SRBC's (in 0.5ml PBS) in an equal volume of FCA by IP injection. The bile, serum and intestinal mucus were tested for antibodies after a further 60 days when a good systemic response was previously encountered. Too few fish were available to determine the temporal nature of the biliary antibody response and for the purpose of this study was presumed to be similar in nature to the systemic response.

Biliary antibodies against V.anguillarum and SRBC's were detected after peroral and peranal exposure to these antigens (Table 17); no systemic response was elicited by these routes. Parenteral (i.e. IP) immunisation with these antigens, however,

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FIGURE 6 DIAGRAMATIC REPRESENTATION OF THE DOGFISH ALIMENTARY TRACT, LIVER AND GALL BLADDER

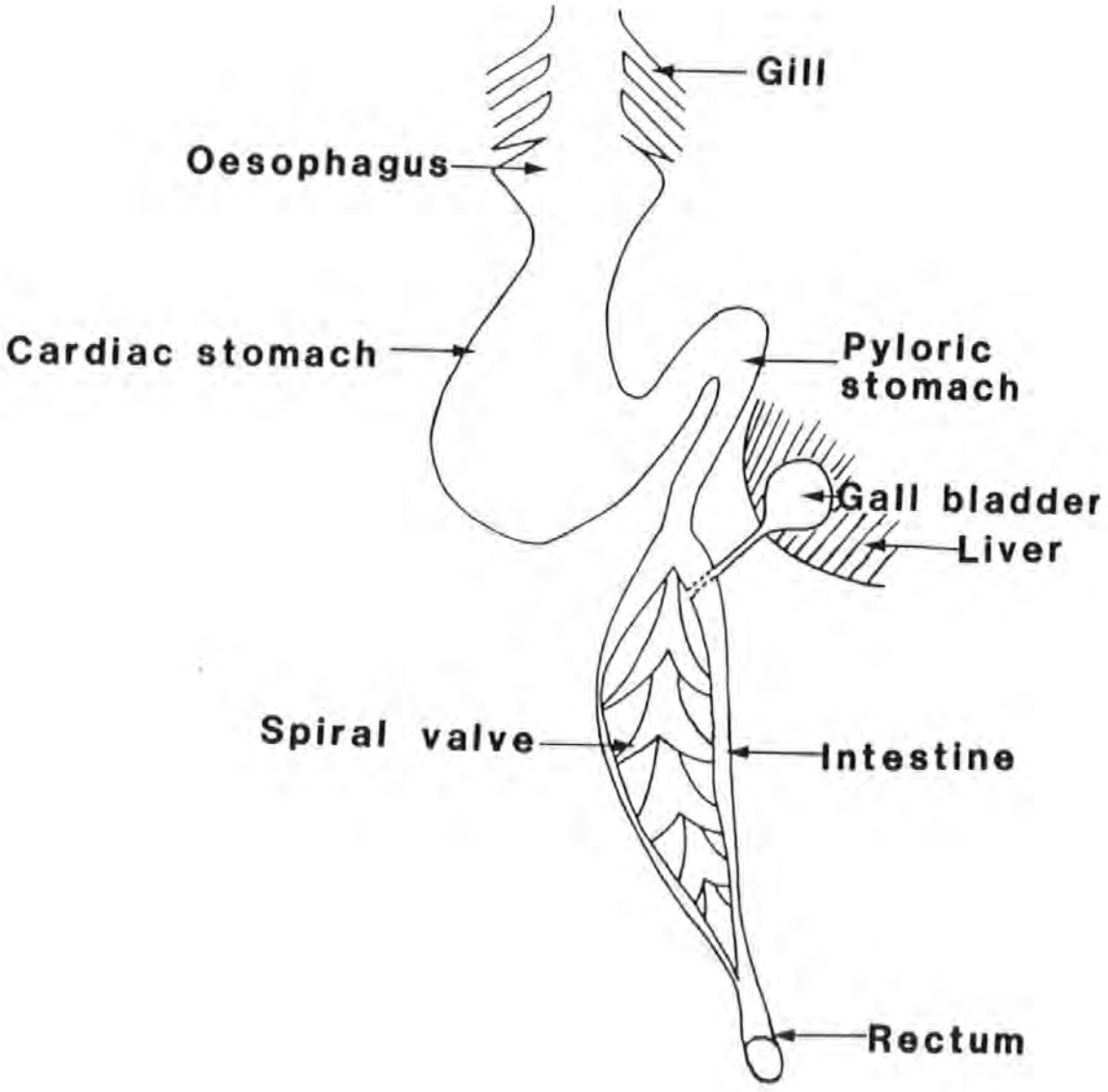

Gill
Oesophagus
Cardiac stomach
Pyloric stomach
Gall bladder
Liver
Spiral valve
Intestine
Rectum

TABLE 17 AGGLUTINATION TITRES OF DOGFISH SERUM AND BILE TO ORALLY, ANALLY AND INTRAPERITONEALLY ADMINISTERED VIBRIO AND SRBC ANTIGENS

ANTIGEN	ROUTE OF ADMINISTRATION	NO. OF FISH		ANTIBODY TITRE			
		EXPT.	CONTROL	EXPT.		CONTROL	
				SERUM	BILE	SERUM	BILE
Vibrio	OR	3	1	-	1/4	-	-
"	AN	2	1	-	1/8	-	-
"	IP	3	1	1/512	1/16	-	-
SRBC	OR	3	1	-	1/2	-	-
"	AN	3	1	-	1/4	-	-
"	IP	3	1	1/1024	1/16	-	-
SRBC/FCA	OR	3	1	1/512	1/206	1/512	1/16
"	AN	3	1	1/1024	1/256	1/128	0
"	IP	3	1	1/4096	1/256	1/512	1/4

NB Controls were exposed to PBS by oral (OR), anal (AN) and intraperitoneal (IP) routes

elicited both a systemic and a biliary response, the latter being greater than that occurring after the introduction of antigens via the alimentary tract. Injection of SRBC's with the adjuvant FCA enhanced the haemagglutination titre in both the bile and serum irrespective of the initial route of exposure (Table 17).

6.1b Detection of IgM and other proteins in the mucus and exogenous secretions

In order to detect serum and mucus IgM, antisera to dogfish serum IgM were prepared in rats and rabbits as described in the materials and methods. The specificity of the antisera were tested by immunoelectrophoresis against whole dogfish serum and bile. The antisera to bile and serum Ig were both tested against whole dogfish serum and bile. Both produced a strong precipitin arc in the gamma region which was thought to be the 19s Ig (Plate 26A and B). A second, much fainter precipitin arc, was detected in the same region which may have been the 7s Ig, and not due to cross reaction with an unrelated molecule (Plate 26B). A strong reaction was also elicited against WDS.

Mucus was collected by gently scraping the surface of the gut, gill and female reproductive tract with a spatula. Bile was aspirated from the gall bladder with a 23g needle and a 1ml syringe. Examination of biliary and urinogenital samples with a rabbit anti IgM serum usually yielded two precipitin arcs migrating cathodically (Plate 26B). Some Ig was detected in the spiral intestine while none was found in the anterior gut or gill mucus.

A third protein was detected by immunoelectrophoresis of urinogenital mucus with rabbit anti-WDS. This protein migrated anodically (Plate 26C). The nature of this protein was not elucidated. Only IgM was detected in the bile, while in the serum many proteins were present (Plate 26D).

IgM was first detected in the bile and serum at stage 2 of development but, it was difficult to collect mucus from the urinogenital tract and gut at this stage which was not contaminated by blood.

6.1c Isolation of dogfish biliary and serum immunoglobulin

Separation of serum IgM was undertaken by a two-step process, in which proteins were first subjected to gel filtration then agarose block electrophoresis. Bile IgM, however, was separated by gel filtration alone.

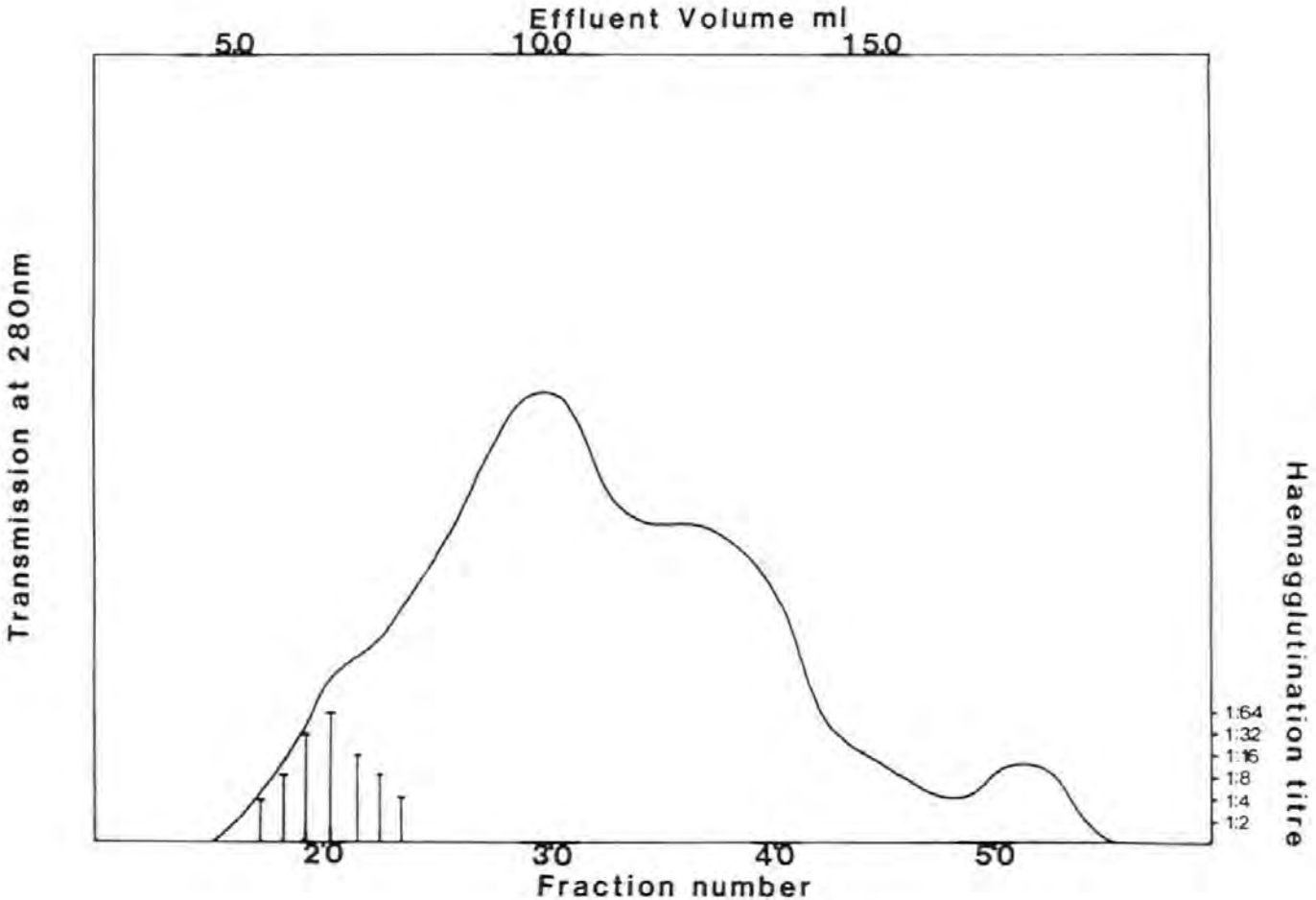
i) Gel filtration

Two mls of serum from dogfish immunised with SRBC (with a titre of approximately 1:1000) and the same volume of bile from unimmunised fish were applied to separate Sepharose 6B columns and the elution of proteins monitored (Figures 7 and 8). The serum fractions were tested for haemagglutinating antibody activity, and for Ig by immunoelectrophoresis with a rabbit anti-dogfish IgM serum. Bile fractions were tested by the latter technique only. Peak anti-SRBC activity was detected in serum fraction 21 (Figure 7) and this also produced a strong reaction on immunoelectrophoresis with a rabbit antiserum to dogfish IgM (Plate 26B). Bile Ig, detected by immunoelectrophoresis with rabbit anti-dogfish IgM, was eluted at approximately the same position (Figure 8).

Fraction 21, in addition to serum immunoglobulin, was shown to contain a second protein, by immunoelectrophoresis with a rabbit anti WDS, which migrated cathodically at a faster rate. In order to separate these two proteins fraction 21 was concentrated ten fold in Aquacide (Calbiochem, Ltd) and a

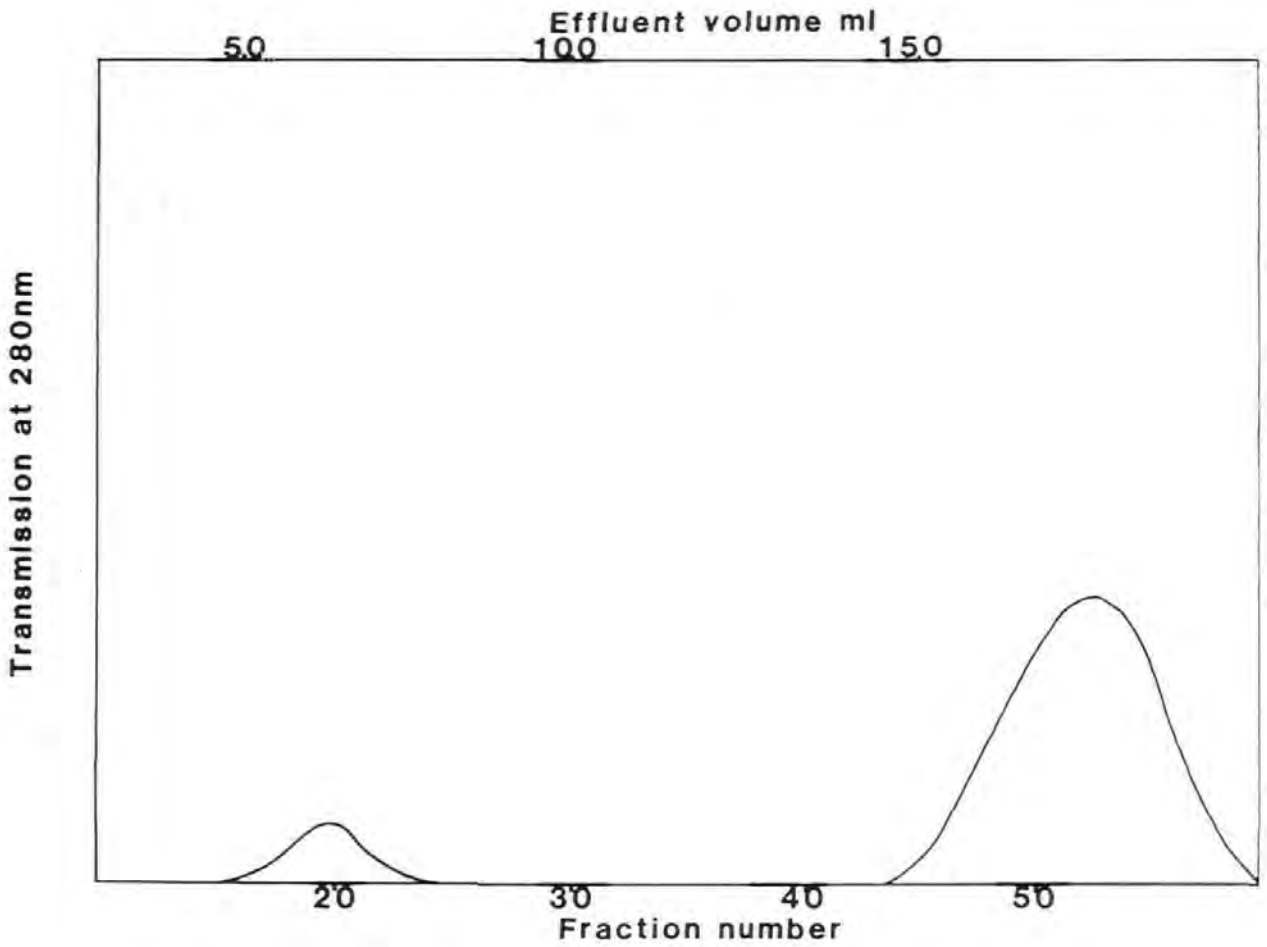
103

FIGURE 7 PROFILE OF DOGFISH WHOLE SERUM CONTAINING ANTIBODIES AGAINST SRBC, SEPARATED ON A SEPHAROSE 6B COLUMN.


Effluent Volume ml
5.0
10.0
15.0
Transmission at 280nm
Haemagglutination titre
1:64
1:32
1:16
1:8
1:4
1:2
20
30
40
50
Fraction number

104

FIGURE 8 SEPARATION OF AN IG-CONTAINING BILE SAMPLE ON A SEPHAROSE 6B COLUMN.


Effluent volume ml
50
100
150
Transmission at 280nm
20
30
40
50
Fraction number

further ten fold by Amicon filtration (Danvers, MA). The concentrated samples were separated by agarose block electrophoresis. The position of the two proteins was detected by staining with Coomassie brilliant blue, the specific regions of the gel were cut out and the proteins eluted in ES. The slowest migrating protein was identified as IgM by immunoelectrophoresis with a rabbit antisera to dogfish IgM, and the fraction was shown to be pure by producing only one precipitin arc with a rabbit antiserum to IgM.

Fraction 21, produced after separating bile on a Sepharose 6B column, contained only IgM shown by immunoelectrophoresis with rabbit antisera to dogfish IgM and WDS. This fraction was therefore regarded as a pure source of biliary Ig.

6.1d Comparison of serum and mucosal IgM

Serum and biliary immunoglobulins were compared on the basis of electrophoretic mobility, molecular weight of the whole molecules and subunit light and heavy chains, the antigenicity of the two molecules and finally the relative concentrations of the two molecules were determined.

i) Electrophoretic mobility

Bile, intestinal and urinogenital IgM had a marked cathodic migration (Plate 26B), while serum IgM was bimigrational about the origin (Plate 26A).

ii) Molecular weight of unreduced IgM

IgM partially purified from serum and bile was separated on a 3% SDS polyacrylamide gel and both proteins migrated to a similar position (Plate 26F), this together with the similar elution profiles on Sepharose 6B suggests they had a similar molecular weight.

iii) Molecular weight of light and heavy chains

Serum and biliary IgM were reduced with 2ME, run on a 13% polyacrylamide gel and the migration of the protein subunits compared with high and low molecular weight protein standards ranging from 14,000 to 200,000 daltons (Sigma Biochemicals Ltd). Protein bands were detected in the upper region of the gel corresponding to a molecular weight of approximately 66,000 daltons and in the lower region of the gel corresponding to a molecular weight of approximately 20,000 daltons (Plate 26E). There appeared to be multiple protein bands in the high molecular weight region of the serum IgM separation, and 3 bands in the low molecular weight region of the biliary IgM separation.

iv) Antigenicity of serum and biliary immunoglobulin

A reaction of identity was produced when rabbit antisera to bile and serum Ig's were cross-reacted with serum and bile in an Ouchterlony double diffusion test (Plate 26H and I).

v) Concentration of serum and biliary immunoglobulins

Using a similar technique to that above, rabbit antisera to serum Ig was reacted against doubling dilutions of bile and WDS from three fish, to determine, approximately the comparative concentration of Ig from the two sources. A similar end point of between 1:128 and 1:512 was found in the bile and serum of the 3 fish examined (Plate 26G), indicating that biliary and serum Ig were present at approximately similar concentrations.

N.B. Information from this chapter has been published elsewhere (Hart, Wrathmell, Doggett and Harris, In Press).

DISCUSSION

Immunoglobulin was absent from the mucus of the proximal gut and gill but, present in the fluid bathing the spiral valve and female reproductive tract. As plasma cells were absent from the former and present in the latter regions (Chapter 4) a straight forward relationship, in which plasma cells secrete directly into the lumen of the reproductive and alimentary tracts, might be expected to exist. This is unlikely to be the case in the gall bladder, as in this structure Ig was found in the bile at concentrations comparable to the serum Ig level, the mucosa of the gall bladder, however, contained very few plasma cells.

Peroral and peranal administration of Vibrio bacterins and whole SRBC elicited a biliary antibody response, but did not stimulate the production of serum antibodies. This would appear to indicate that the biliary system plays a role in the local antibody response in the gut. This is a novel finding in fish, although biliary Ig has been detected previously in elasmobranchs (Underdown and Socken, 1978) and teleosts (Rombout et al., 1986 and Lobb and Clem, 1981a and b). These results indicate that some antigens may reach the absorptive regions of the intestine, under experimental conditions, without being destroyed in the proximal gut, and the spiral valve does not exclude all antigens from the absorptive regions after anal intubation. The absence of serum antibodies may indicate that the production of biliary antibodies in mediated only via the gut and gall bladder. The spleen is thought to be the site of systemic antibody production in S.canicula (Morrow, 1978). Such a local response after oral exposure to Vibrio bacterin has been reported in plaice (Fletcher and White, 1973a) and carp (Rombout et al., 1986). The latter authors found, however, that by priming and boosting by the oral route high systemic titres could be elicited. Whether this is a normal phenomenon in cyprinids, or represents the result of overloading the lysosomal system in the distal intestine is unclear.

In S.canicula IP injection of antigen elicited both a systemic and biliary response, even after prior oral and anal challenge. This indicates that antigens do not first have to cross the epithelium of the intestine, or other regions of the gut, before a biliary response can be initiated. A similar situation was shown to exist in rats following intravenous injection of V.cholerae antigens (Jackson and Cooper, 1981). Prior exposure to antigen by the oral routes causes tolerance to antigens injected systemically in mammals (cf. Tomasi, 1980). This may be mediated by humoral (Chalon, Milne and Vaerman, 1979) or cellular mechanisms (MacDonald, 1983). In S.canicula prior exposure to SRBC introduced into the gut did not inhibit the subsequent systemic response to antigen presented by IP injection. Mughal (1984) was also unable to demonstrate oral tolerance. Udey and Fryer (1978), however, found that oral tolerance to A.salmonicida could be induced in the rainbow trout.

As mentioned above the presence of Ig in the bile at a similar concentration to serum Ig, and the detection of specific antibodies in the bile strongly implicate the biliary system in local immunity in the gut. To date, the Ig levels measured in other fish have been much lower in the bile than in the serum. In carp serum Ig was found at 1732µg/ml and bile Ig at 12µg/ml (Rombout et al., 1986), in the sheepshead serum HMW Ig was found at 2.90mg/ml and in bile at 0.09mg/ml (Lobb and Clem, 1981b).

The origin of biliary IgM was not determined in this study. Many plasma cells were detected in the spiral valve, few were detected in the gall bladder and none were found in the liver (Chapter 3). It is possible that biliary Ig may be derived from the intestinal plasma cells, in a similar fashion to some mammals (Vaerman, Lemaitre-Coelho, Limet and Delacroix, 1982). Using labelling experiments Lobb and Clem (1981b) found that the LMW or HMW serum Ig's of the sheepshead were

transported into the bile. As these authors did not examine the liver, gall bladder or gut the origin of the biliary Ig is unknown.

The mechanism by which Ig enters the gall bladder of S.canicula cannot be elucidated from this current work. The high molecular weight of Ig's and the high concentration of the molecule in the bile suggests, however, that it is actively transported. This transport mechanism must be specific as no other serum proteins are present in the bile. A secretory component was not detected in the bile of S.canicula in this study, or in the sheepshead (Lobb and Clem, 1981a), but may have been present in the cutaneous mucus of the latter species (Lobb and Clem, 1981c).

In mammals polymeric IgA is the chief immunoglobulin found in the external body secretions (Tomasi, 1976), although IgM appears to fulfil this role in IgA deficient human patients (Brandtzaeg, 1975). IgM is generally accepted as the sole Ig of fish (Nisonoff, Hopper and Spring, 1975) and recent work (Rossenshein et al., 1986) has shown that using several criteria caracharhine elasmobranch Ig closely resembled mammalian IgM. Other authors have noted marked heterogeneity in piscean Ig's. Lobb (1986) reviewed data on the light chain subclasses of serum Ig's in the catfish. In previous work (Lobb and Clem, 1981a), a separate type of Ig was detected in the bile, which was a non-covalently linked dimer with a heavy chain intermediate in MW between the heavy chains of the serum HMW and LMW Ig's. These authors proposed that this Ig was destined to function in the secretions of the gut of the sheepshead. In S.canicula, as previously mentioned, biliary Ig was found at levels comparable to serum Ig, and was predominately of the high molecular weight type, previously identified as a pentamer (Morrow, 1978). A second Ig was detected by immunoelectrophoresis in both the serum and bile, and may correspond to the 7S identified by radial immunodiffusion (Morrow, 1978).

In S.canicula it is uncertain whether the differences in electrophoretic mobility, and apparent heterogeneity in the light and heavy chain molecular weights represents the characteristics of two separate Ig populations. Heterogeneity has been shown in other elasmobranch Ig's. Kobayashi et al. (1984) found a non-covalent dimer with an H chain of 40-50 ,000 daltons compared to a μ chain of pentameric Ig which had a MW of 70,000 daltons. These Ig's were also shown to be produced by separate cell populations (Kobayashi et al., 1984). Further work might be best directed to examining H chain heterogeneity in serum and biliary Ig and determining the origin and secretory mechanism of biliary and other mucosal Ig in S.canicula.

This initial work implicates the biliary route in local secretion of IgM into the intestine of S.canicula. This route also appears to be important in amphibia (Jurd, 1977), chickens (Hadge and Ambrosius, 1983), ducks (Ng and Higgins, 1986) and mammals (Vaerman et al., 1982). In all but the latter group non-IgA-mediated mechanisms were found.

The role of secretory Ig in fish has received no attention in elasmobranchs but was found to inhibit the attachment of V.anguillarum to the gut of rainbow trout in an in vitro binding experiment (Horne and Baxendale, 1983). A similar function was described in mammals (Walker, 1985) where IgA may combine with dietary and pathogenic antigens preventing them binding to and entering the epithelium.

The role of immunity in the female reproductive tract may be more complex. Besides the possible control of pathogens spread by copulation and passive entry at other times, immune mechanisms may respond to seminal antigens which are stored in the reproductive tract of elasmobranchs (Wourms, 1977) and also the zygote which is allogeneic.

During the course of this experimentation the author found bile much easier to work with than mucus i.e. it was easier to collect and assay for antibodies. To accurately investigate and develop oral vaccines by non-empirical methods, a teleost species with a high biliary Ig level may be a convenient model. Recent evidence from Wrathmell (unpublished data) shows that mullet (Chelon labrosus) bile contains Ig, but in more variable quantities than in S.canicula.

PLATE 26 Immunochemical analysis of bile and body mucus

A. Example of an immunoelectrophoresis gel of WDS, against specific rabbit antisera to dogfish Ig.

B. Example of an immunoelectrophoresis gel of bile against a rabbit antiserum to dogfish IgM or WDS, or mucus against a rabbit antiserum to dogfish IgM.

C. Example of an immunoelectrophoresis gel of mucus from the reproductive tract of a female dogfish, using a rabbit antiserum to WDS.

D. Example of an immunoelectrophoresis gel of WDS against a rabbit antiserum to WDS.

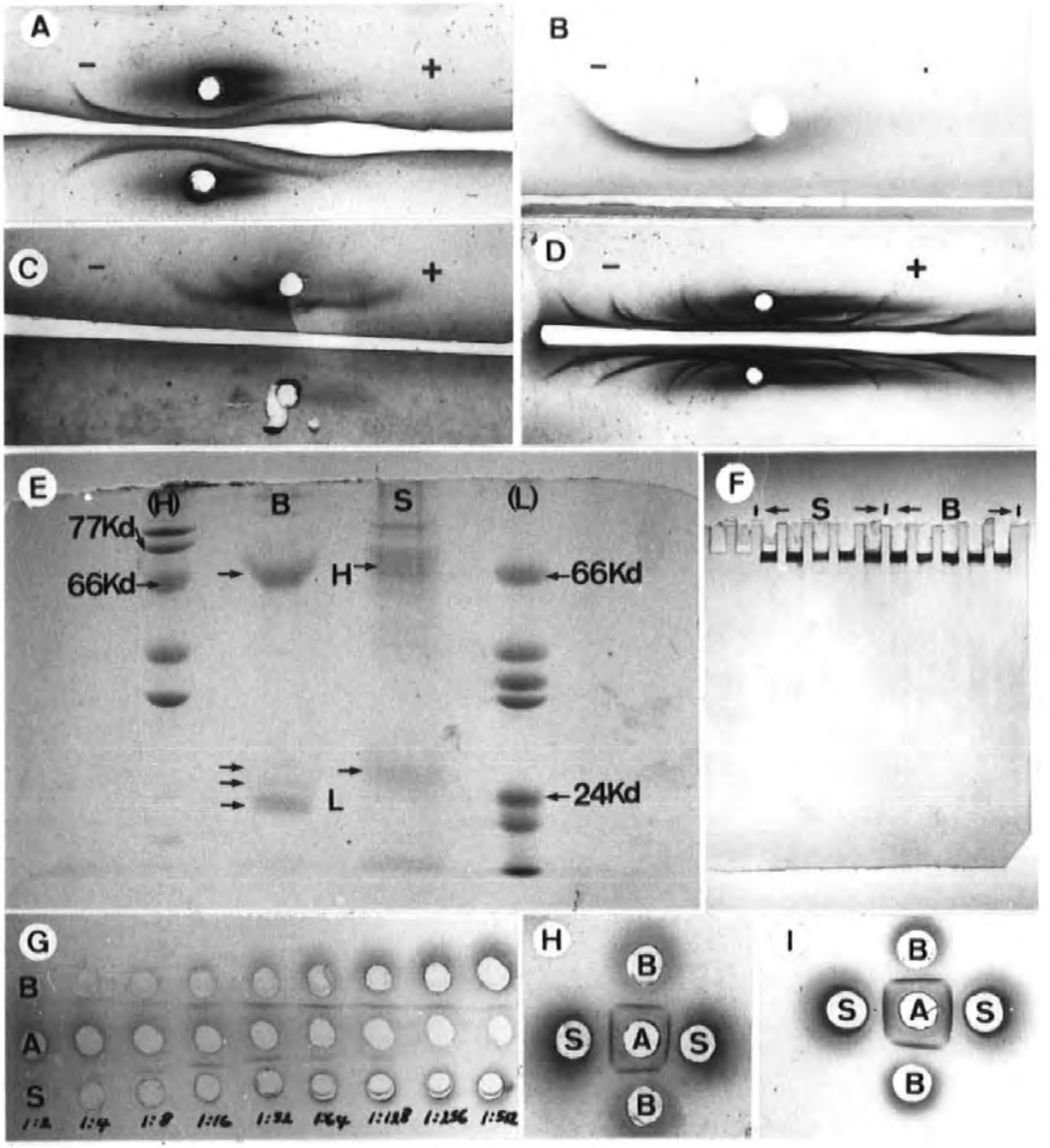
E. Separation of partially purified dogfish serum and biliary Ig on a 13% SDS/PAGE with 2 mercaptoethanol (H), HMW marker; (L) LMW marker; B, biliary Ig sample; S, serum Ig sample; H, heavy chain; L, light chain.

F. Separation of partially purified dogfish serum and biliary Ig on a 3% non-reducing SDS/PAGE.

G. Doubling dilutions of bile (B) and serum (S) in upper and lower wells, with undiluted rabbit anti-dogfish serum Ig in the central wells (A).

H. Ouchterlony reaction of rabbit anti-dogfish biliary Ig (A) against whole bile (B) and whole dogfish serum (S).

I. Ouchterlony reaction of rabbit anti-dogfish serum Ig (A) against whole bile (B) and whole dogfish serum (S).


A
B
C
D
E
F
G
H
I
77Kd
66Kd
(H)
B
S
(L)
H
L
24Kd
S
B
B
A
S
B
S
A
S
B
B
S
A
S

CHAPTER 7

CONCLUSIONS

The alimentary tract of S.canicula was found to harbour a large and heterogenous cell population, which occupied three niches: the epithelium, lamina propria and as large accumulations. While some cell types were positively identified (e.g. plasma cells, macrophages and lymphocytes) and others classified in less well defined manner (e.g. granulocytes), little was discovered about the function of leucocytes in the gut. More knowledge may be obtained about the origin of lymphocytes by thymectomy (Grace, 1981) or in vivo labelling of thymocytes (Tatner, 1985). An investigation of T-cell subsets can not be undertaken until the appropriate technology for T-cell typing has been established in elasmobranchs. Useful information might result from investigation of antigen handling in the epithelium lamina propria and lymphoid accumulations using high resolution immunocytochemistry. Due to the nature and location of the lymphoid accumulation in S.canicula it is unlikely that surgical removal will become an option for investigating the functions of these structures.

Ontogenic work showed that the diffuse leucocyte populations and accumulations in the gut of larval S.canicula developed prior to feeding on an exogeneous diet. By culturing larval fish in sterile conditons it may be possible to examine the role of environmental antigens on the development of GALT in this species.

The epithelium of the spiral intestine of the larval stages was shown to phagocytose carbon, while this material was not taken up in adult fish. Both larval and adult fish were shown to take up soluble proteins, although in the adult this was at very low levels. S.canicula appears to be a poor model for the investigation of antigen uptake, as after oral intubation material appeared to be digested or hydrolysed, and access via the anus was restricted by the spiral valve.

Recent work on the tilapian Oreochromis mossambicus (Doggett, unpublished data) showed that BSA was rapidly absorbed into the serum, in considerable quantities after oral intubation. Wrathmell (unpublished data), in contrast, found that in rainbow trout no BSA was detectable in the serum after oral intubation but, if the protein was intubated through the anus uptake occurs. It would appear that if uptake is to be studied using the anal route species without a spiral valve are best employed.

High levels of immunoglobulin were detected in the bile of S.canicula and while the molecule appeared to be similar to serum immunoglobulin no information about its origin and mechanism of transport into the bile was established. Further work might best be directed towards looking for a secretory piece-like molecule, and determining whether biliary immunoglobulin is produced by a separate 'local' plasma cell population. It might be interesting to examine other chondrichthyian species, cyclostomes and primitive bony fishes to determine if biliary immunoglobulin occurs widely in these primitive vertebrates.

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