The Reticulo-Endothelial System of Teleost Fish with Special Reference to the Plaice (*Pleuronectes platessa*).

by

H. W. Ferguson, BVM&S., MRCVS.

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Abstract

The reticulo-endothelial system of plaice (Pleuronectes platessa) was defined using a variety of different sized particles, both inert e.g. colloidal carbon, and biological, e.g. yeast cells.

From these experiments, it became obvious that the major sites of particle uptake were the kidney, spleen, and atrium, while in complete contrast to the mammalian situation, the liver played virtually no part, Kupffer cells being absent.

On the basis of these findings, it was decided to investigate at an ultrastructural level the following tissues:-

i. the heart, in an attempt to establish the means of uptake by the endocardial lining cells of the atrium.

ii. the ellipsoids or Schweigger-Seidel sheaths, which were the first site of particle trapping in the spleen.

iii. the liver, with special reference to the sinusoidal lining due to the apparent absence of Kupffer cells.

iv. blood, in an attempt to establish the presence of monocytes, the precursors of many macrophages in mammals.

The presence of monocytes was established and the ultrastructural features of the other leucocytes noted. Both monocytes and thrombocytes were able to endocytose intra-venously injected carbon particles. The absence of Kupffer cells was confirmed and the degree of carbon uptake by the fat-storing cells was not considered to be sufficient to be of much importance in natural disease conditions.

The endothelial lining of the axial vessel of the ellipsoids was observed to be comprised of 2 cell types, one of which contained sufficient microfibrillar elements to be considered as possibly contractile. Carbon particles were able to migrate out of the axial vessel into the sheath, between endothelial cells. Here they were endocytosed by the large pale reticular cells which were thought to subsequently migrate out of the sheaths and into the melano-macrophage centres.

Uptake by the endocardial lining cells was restricted to those of the atrium. Macropinocytosis and not phagocytosis, was the mechanism thought to be responsible for regulating particle uptake.

The pathophysiological implications of all of these findings are discussed.

Finally, kinetic studies were performed, involving the clearance of radio-labelled Salmonella gallinarum bacteria from the bloodstream of plaice, kept under various conditions. It was shown that clearance at low temperatures was effected but at a slower rate than normal due probably to a purely metabolic effect. Similarly, clearance

at high temperatures was enhanced, as was the clearance from the bloodstream of fish previously exposed to the antigen.

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Preface

This study, "The reticulo-endothelial system of teleost fish with special reference to the plaice (Pleuronectes platessa)" is divided into 3 major parts:-

i. Mapping out (the definition of) the reticulo-endothelial system (R.E.S.).

ii. Histology and ultrastructure of the R.E.S.

iii. Quantitative clearance studies.

Each of these experimental sections has its own introduction, materials and methods, results, discussion and conclusions. As a result of this method of presentation, many of the more specialised references are to be found only in their relevant section, and not in the general introduction, which confines itself to the major works on the R.E.S. Similarly, the general discussion at the end attempts to draw together the points which are brought to light in each section and there discussed in detail.

Contents

CHAPTER 1 INTRODUCTION

Despite many previous references to mammalian cells capable of phagocytosis of either inert particles (von Kupffer, 1876), or of bacteria (Wyssokowitch, 1886), it was the Russian zoologist Metchnikoff studying the uptake of foreign material by a group of cells in many different animals (1905) who was the first really to appreciate the significance of such a mechanism in the defence of the host against infectious disease. He called the cells he was studying "macrophages", to contrast them with the polymorphonuclear leucocytes or "microphages", which were also capable of uptake, but to a lesser extent.

These cells were later characterised by their ability to ingest particulate matter, and especially colloidal dyes. The classical work on 'vital staining' as this was called, was performed by Cappell (1929), in mice (Mus musculus). The concept arose that vitally staining cells comprised a system, the "reticulo-endothelial system" of Aschoff (1924), so named because of the close association of vitally stained cells with both vascular endothelium, and reticular argyrophilic fibres. These cells had the common ability of phagocytosis, but the degree or capacity varied from cell type to cell type, and they were not necessarily morphologically identical. The reticulo-endothelial system of Aschoff, is shown in Table 1.1.

Aschoff excluded endothelial cells and fibroblasts from his system because of their very low phagocytic abilities. The fact however,

TABLE 1.1 The reticulo-endothelial system (R.E.S.) of Aschoff

Increase in phagocytic capacity ↓

	Endothelial cells	
	Fibrocytes	
R.E.S. in a wider sense	Reticular cells of spleen and lymph nodes	R.E.S. in a strict sense
	Reticulo-endothelial cells of lymph and blood sinuses, including Kupffer cells	
	Histiocytes	
	Splenocytes and monocytes	

TABLE 1.2 The mononuclear phagocyte system (M.P.S.) of mammals

Cells	Localisation
Precursor cells	bone marrow
Promonocytes	bone marrow
Monocytes	bone marrow and blood
Macrophages	connective tissue (histiocytes)
	liver (Kupffer cells)
	lung (alveolar macrophages)
	spleen (free and fixed macrophages)
	lymph node (free and fixed macrophages)
	bone marrow (macrophages)
	serous cavity (pleural and peritoneal macrophages)
	bone tissue (osteoclasts?)
	nervous system (microglial cells?)

that these cells did take up vital dyes to a degree, by pinocytosis and not phagocytosis, suggested that this criterion alone was unreliable in characterising the members of this system.

Much criticism has since been levelled at the use of the term 'reticulo-endothelial system', as this was considered to be too vague a term, and it was recently suggested that macrophages should be referred to as a group, as "the mononuclear phagocyte system" (van Furth, 1970a). This system was to comprise the tissue macrophages and their precursors, the monocytes and promonocytes. The functional criteria justifying the inclusion of these cells in a single system were avid phagocytosis and pinocytosis plus the ability to adhere firmly to glass (Rabinowitch, 1968). Those cells comprising the mononuclear system of mammals are listed in Table 2.

The weight of evidence points conclusively to the fact that in mammals, tissue macrophages are derived from monocytes circulating in the blood, and their precursors (see review by van Furth, 1970b). There are thus two populations of macrophages, one fixed, the other free to move about.

In deciding on the terminology to be employed in the present work, defining the phagocytic cells of the teleost fish, consideration was given to the recommendations of the International Conference on the mononuclear phagocytes, held in Leiden, The Netherlands, in 1969. It should be noted however that the rate of phagocytosis in a

poikilothermic animal is much slower than in a homeothermic one, and this, together with the completely different locations of the relatively avidly phagocytic cells, compared to the mammalian situation, means that the systems are not identical, and dissimilar mechanisms may be operating in regulating the uptake of particulate matter.

The sole criterion employed here, to justify the inclusion of certain cells within a system, has been the relatively rapid uptake of particulate matter of both an inert, and a biological nature. Although, however, the highly phagocytic cells were the chief initial interest, the less highly phagocytic ones were not excluded from the study. The reticular cells in the ellipsoids of the spleen for example, were considered to be too important in their possible relationship to immunological processes, to be excluded from the study. For these reasons therefore, the present work is, in a broad sense, a study of a group of cells more closely allied to the concept of those comprising the reticulo-endothelial system (R.E.S.) than to those of the mononuclear phagocyte system (M.P.S.), although during the course of the study, certain highly phagocytic cells were encountered which would justify inclusion within the more narrowly defined M.P.S. of the animal. Thus, the work on the quantitative clearance of particulate matter from the bloodstream (radio-labelled Salmonella gallinarum bacteria) will be referred to as reticulo-endothelial clearance, because other mechanisms apart from avid phagocytosis probably come into play e.g. the trapping of material within the reticulin meshwork of the Schweigger-Seidel sheaths of the ellipsoids in the spleen.

More recently, attention has been focussed on the importance of reticulo-endothelial cells in relation to immunological processes e.g. their processing of antigen, and its presentation to immuno-competent cells in a suitable form. This, and the many other properties of the R.E.S. e.g. the power of discrimination between old and new, foreign and indigenous, normal or damaged tissue, demonstrate that the approach to the study of the R.E.S. must be a multi-disciplinary one, and accordingly, the present work was designed to be as broadly based as possible, always endeavouring however, to make the prime objective a surer understanding of the initial stages of the disease process in fish.

Investigations on reticulo-endothelial cells of fish, have been few. Metchnikoff (1905), injected guinea-pig erythrocytes into the peritoneal cavity of the goldfish (Carrasius auratus), and noted their rapid phagocytosis by the resident macrophages. Mesnil (1895) showed that pathogenic anthrax bacilli were rendered harmless by macrophages, following their injection into the teleosts Perca fluviatilis, Gobii fluviatilis, and goldfish. Wislocki (1917) studied the action of vital dyes in teleosts, by injecting trypan blue into the peritoneal cavity of goldfish, and noted the distribution of the resulting vitally stained cells or "makrophages". Wislocki noted the presence of Kupffer cells in the liver, and the vitally stained endothelium of the renal portal system. Hoskins and Hoskins (1918), however, in their study of the elasmobranch dog fish (Squalus acanthias), did see Kupffer cells, although a vitally stained renal portal endothelium was absent.

Mackmull and Michels (1932) gave a very complete account of the absorption of colloidal carbon from the peritoneal cavity of a marine teleost, the cunner (Tautogolabrus adspersus), and reviewed the literature up to that date concerning peritoneal absorption in all animals. Since that date, there has been little interest in the R.E.S. of teleosts. Stuart (1970), in his book on the reticulo-endothelial system, devoted one chapter to the phylogenetic aspects, but teleost fish were not mentioned. Ellis (1974) studied the absorption and distribution of indian ink injected intra-peritoneally into plaice (Pleuronectes platessa), but no ultrastructural, or kinetic studies were performed. Indeed, it was the almost total lack of ultrastructural and kinetic information about the R.E.S. of fish, which was one of the stimuli prompting the present investigation.

Following Bisset's work (1946-8) on the effect of temperature on the ability of goldfish to resist infection, Avtalion (1969-74) studied the effect of temperature on the immune response in carp (Cyprinus carpio). He showed that, in complete contrast to Bisset's findings, the initiation of the immune response was the temperature sensitive event, and not the production or release of antibody. This worker also stated (1974) that as part of the initiation of the immune response, phagocytosis itself was unaffected by a drop in temperature. The present work attempts to substantiate this statement for a marine flatfish, the plaice, as opposed to the carp, a relatively warm-water freshwater fish, and to investigate the effect of prior exposure to an antigen on its subsequent clearance from the bloodstream.

'Melano-macrophage centres', 'ceroid centres', or 'pigment nodules', are synonyms for structures associated with the haematopoietic tissue (spleen and kidney) of higher teleost fish, and were first described by Jolly (1923). Their discrete nature is one of the most striking characteristics, although little histological evidence accounting for the separation has been presented. They are very prominent in all flatfish, notably the turbot (Scophthalmus maximus), and have a fine investing cuff of lymphoid tissue. Roberts (1974) proposed the name 'melano-macrophage centres' for these structures because of their high content of melanin type pigments e.g. lipofuscin, and because of their apparent ability to aggregate injected particles e.g. indian ink, presumed to have been brought there by macrophages. Accordingly, ultra-structural investigations were carried out, not only on those organs which proved, from the distribution studies, to contain the major components of the R.E.S., but also on the spleen of plaice and turbot paying particular attention to the melano-macrophage centres, and their relationship to the rest of the components of the spleen.

CHAPTER 2 OBJECTIVES

Objective 1

a) To map out the R.E.S. of plaice, using a variety of injected particles as markers, both of a biological nature e.g. yeast cells and bacteria, and inert e.g. colloidal carbon and trypan blue.

b) To observe these particles by a variety of methods including routine paraffin sectioning, and electron microscopy.

c) To compare and contrast the findings with the mammalian situation.

d) To make some attempt to generalise the major findings by limited repetition in other species of teleosts.

Objective 2

a) To make a detailed study of the ultrastructure of plaice leucocytes, and especially monocytes, as possible precursors of some reticulo-endothelial cells, and to study their response to colloidal carbon particles.

b) To make a more detailed study of the histology and ultrastructure of those organs which proved to be either sufficiently different from, or similar to the mammalian situation, to warrant further investigation.

Objective 3

To follow the rate of clearance, and organ distribution of chromium ^{51} (^{51}Cr) labelled Salmonella gallinarum bacteria

from the blood stream of plaice, under various conditions, relating primarily to the effect of a variation in environmental temperature, and prior exposure to the antigen.

CHAPTER 3 MAPPING OUT THE R.E.S. I

3.1 Introduction

The uptake of vital dyes by certain cells in the body was the major criterion used by the early workers e.g. Aschoff (1924), to define the cells of the R.E.S. Macrophages take up vital dyes both *in vivo* and *in vitro*, but it is unknown how they are capable of concentrating, within vacuoles, these sometimes very dilute dyes. Vital dyes are usually sulphonated derivatives of triphenylmethane, with molecular weights in the range 600-1000. In water, 3-10 molecules aggregate to form a micelle. Kojima and Imai (1962) concluded that in mammals, the most important feature of vital dyes was that they bound strongly to serum albumin. It is unknown whether pinocytosis of this albumin, taking the vital dye into the cell, is a process which is important to cells of the R.E.S.

In mammals, polymorphonuclear leucocytes phagocytose colloidal carbon, but not vital dyes. This is an important difference. In addition, the carbon must be opsonised before it is endocytosed, but it is very readily taken up by all cells, and as such, has been extensively used, not only as a marker of R.E. cells, but in kinetic studies relating to its clearance from the bloodstream.

Both trypan blue and colloidal carbon however are inert particles, and differences can be expected in the reaction of macrophages to particles of a biological nature. Accordingly, it was decided to use bakers' yeast cells (*Saccharomyces cerevisiae*) and B.C.G. bacilli, as biological markers. The former is a large organism 1μm in diameter, whose high glycogen content renders it easily seen in sections stained with the periodic acid-Schiff method.

These 4 particles therefore represent a wide range of sizes, surface characteristics, and nature.

CHAPTER 3.2 MATERIALS AND METHODS

a) Trypan blue

a.1 Fish used.

The fish used for the trypan blue uptake experiments were adult cultured plaice of approximately 150g body weight. These were obtained from the White Fish Authority's breeding and rearing unit at Hunterston, Ayrshire. The fish were of mixed sex, and were maintained at $10^{o}C$ in a recirculating seawater system.

a.2 Preparation of the inoculum.

A 0.5% weight/volume solution, in sterile teleost ringer (Hale, 1965) was used after filtration through a Whatman's No 1 filter paper.

Three injection procedures were employed:-

i One injection of 0.5 ml/100g body weight, the fish being sacrificed the following day.

ii Two injections, each of 0.5 ml/100g on successive days,

the fish being sacrificed on the third day.

iii Seven injections at 24 hour intervals, each injection being at the rate of 0.5 ml/100g, the fish being sacrificed on the eighth day.

Two fish were used for each of these 3 procedures. Injections were made into the peritoneal cavity, just anterior to the anus, using 25 gauge hypodermic needles, and disposable 1 ml syringes. All injections were made without anaesthesia, the fish lying quite still on the table as long as their heads were covered with a wet cloth. No manipulation lasted longer than a few seconds, and at no time did the fish show any evidence of stress either during, or after the injections.

a.3 Tissues examined.

Virtually all tissues were sampled, and included the following:-

liver (including pancreas and biliary system)
spleen
heart (conus arteriosus, atrium and ventricle)
kidney (anterior and posterior)
muscle (lateral abdominal)

skin

gill

gut (small and large intestine, stomach and mesentery)

gonads

eye

brain

a.4 Fixation, processing and staining procedures.

Ten per cent formalin in 0.9% sodium chloride solution (formol-saline) was the routine fixative of the laboratory, and this was found to give satisfactory fixation in most cases. The criteria used to judge if fixation had been adequate were:-

i Excessive shrinkage of the tissue. This was particularly noticeable in liver sections, around the hepatic sinusoids, leading to an abnormally wide Space of Disse. The spleen was another organ which proved to be particularly sensitive to shrinkage, which was often so severe as to render it extremely friable on sectioning.

ii Autolysis, resulting in poor histological detail. This was not generally a problem, provided that tissues were quickly fixed after death, and that the size of the blocks did not exceed 0.5 cm^3.

Liver fixation was improved by using a buffered formalin fixative (Culling, 1974). Optimal fixation of the spleen was achieved by removal of the major part of the blood within the organ by perfusion with formol-saline, prior to its immersion fixation. Perfusion for 2-3 minutes was simply accomplished by injecting fixative with a syringe, into the capsule of the divided organ, until the fixative emerging from the cut face was no longer discoloured with blood.

The tissues were left in approximately 30 ml fixative, in clear plastic screw-top jars, for 16-24 hours, but longer periods than this did not adversely affect the quality of the fixation, or the hardness of the tissue.

The tissues were processed in an automatic tissue processor*, employing the routine outlined in Table 3.1.Following processing, the tissues were embedded in paraffin wax at 60^{o}C.

Five or six µm thick sections were cut on a rotary microtome*,[1] and mounted on chemically clean microscope slides.

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*Shandon Elliot Ltd

*[1] Leitz Wetzlar

Table 3.1 Processing routine for the automatic tissue processor.

50% Methylated spirit	1 hour
80% Methylated spirit	2 hours
8% Phenol meths	3 hours
8% Phenol meths	2 hours
8% Phenol meths	2 hours
Absolute alcohol	2 hours
Absolute alcohol	1 hour
Chloroform	1 hour
Chloroform	1 hour
Paraffin wax	2 hours
Paraffin wax	2 hours
Paraffin wax	5 hours

The sections were stained with neutral red which acted as a counter-stain (Culling, 1974) to the delicate vital staining by the trypan blue.

b) Colloidal carbon

b.1 Fish used.

As in the trypan blue experiments, plaice were used for the greater part of the investigation. In order however to study further certain features observed in plaice, rainbow trout (Salmo gairdneri) and turbot, were also examined in order to confirm that the phenomena observed in the plaice were of more general significance.

b.2 Preparation of the inoculum.

This was prepared from Pelikan ink C11/1431/A*, according to the method of Biozzi et al.(1953), with the exceptions that firstly, the 1% gelatin level used by these workers was reduced to 0.5% w/v, in order to retain fluidity at the lower experimental temperature required for a poikilothermic animal, and secondly, that teleost ringer was used.

Suspensions were prepared to contain approximately 2, 8, 16 and 32 mg carbon per ml. The injected dose levels were:-

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*Gunther Wagner Ltd., Hanover

i 0.5 mg per 100g body weight

ii 4 mg per 100g body weight

iii 8 mg per 100g body weight

iv 16 mg per 100g body weight

Injections were made into the renal portal vein (R.P.V.) of unanaesthetised fish (Wardle, 1970). The size of the inoculum in all cases was 0.5 ml per 100g body weight.

Plaice were sacrificed at the following times post injection, for each of the 4 dose levels:-

15 minutes	12 hours	7 days
30 minutes	24 hours	2 weeks
1 hour	2 days	3 weeks
3 hours		
6 hours		

Two fish were used for each time, for each dose level, i.e. 2 x 12 x 4 = 96 plaice in all.

b.3 Tissues examined.

In the initial experiments, using a dose level of 4 mg/100g, organs were sampled, as for the trypan blue injections, and in

addition blood smears were prepared.

For the other dose levels, it was subsequently found necessary to sample the following organs:-

liver (including pancreas and biliary system)

spleen

heart (conus arteriosus, atrium and ventricle)

kidney (anterior and posterior)

gills

blood - samples were taken only up to 3 days post injection.

Sampling for the turbot and rainbow trout also followed this pattern.

b.4 Fixation, processing and staining procedures.

Fixing and processing was as for the trypan blue injected fish. Blood smears were prepared from un-heparinised blood. Fixation was with 80% methanol for 10 minutes, and the smears were then stained for 10 minutes with Giemsa's stain diluted 1:3 with teleost ringer.

Following rinsing with distilled water, they were air dried and then mounted in synthetic resin with a cover-slip.

Staining of all the sections from carbon injected fish, was by routine Mayer's haematoxylin and eosin (Culling, 1974).

c) Yeast Cells

c.1 Fish used.

Only plaice were used.

c.2 Preparation of the inoculum.

Half a gram of freeze-dried baking yeast was suspended in 10 ml of teleost ringer. This was injected at the level of 0.5 ml/100g body weight, into the R.P.V. of unanaesthetised fish. Eight fish in all were injected, and 2 were killed at each of the following times post injection:-

24 hours

48 hours

7 days

14 days

c.3 Tissues examined.

The same tissues were sampled as listed for carbon. Blood smears were not prepared.

c.4 Fixation, processing and staining.

Fixation and processing was as for trypan blue. Staining of sections was by periodic acid-Schiff (P.A.S.) (Culling, 1974), which stained the polysaccharide-containing capsule of the yeast a particularly bright red. Some heart tissue was processed for toluidine blue stained epon embedded sections (see Ch. 5.2).

d) B.C.G. bacilli

d.1 Fish used.

Only plaice were used, as for trypan blue.

d.2 Preparation of the inoculum.

Freeze-dried human B.C.G. vaccine bacilli were prepared at 2 dose levels - 2 mg/ml and 4 mg (dry weight) per ml., using teleost ringer. Half an ml was the size of the inoculum for each dose level. Fish were sacrificed at the following times post injection:-

24 hours

48 hours

7 days

Two fish were sacrificed for each dose level, at each of the above times.

d.3 Tissues examined.

The same tissues were sampled as for the yeast injection studies.

d.4 Fixation, processing and staining procedures.

Fixation and processing was the same as for the trypan blue. The sections were stained by Ziehl-Neelsen's stain for acid-fast bacilli (Culling, 1974).

CHAPTER 4 MAPPING OUT THE R.E.S. II

4.1 Results

A Trypan blue

Macroscopic appearances.

All fish, regardless of the injection-routine, had an overall blue colouring, although some of the paler tissues showed this better than the darker ones.

Microscopic appearances.

Only those fish which received 7 injections are described here. The lower dose levels did not adequately mark any cells.

1. Liver (including pancreas and biliary system).

The most prominently marked cells were seen around the pancreatic tissue. They were long spindle-shaped cells disposed between the surrounding hepatic tissue and the outer connective tissue of the tubes of pancreas. These were referred to by Mackmull and Michels (1932) as the "periglandular capillary" areas.

A small amount of blue was seen in the region of the hepatic sinusoids, but it was too diffuse to be certain of its precise location.

2. Spleen.

The reticular cells comprising the Schweigger-Seidel sheaths, or ellipsoids, were especially well marked. Although these cells were more rounded and larger than the spindle-shaped cells of the "periglandular capillaries", the trypan blue was not aggregated into large vacuoles. Some of the endothelial lining cells of the splenic sinuses were marked in a similar manner to the cells of the ellipsoids, although they were smaller and the vacuoles were larger. Other cells containing trypan blue were scattered throughout the stroma, not related to either the ellipsoids, or the sinuses. 'Melano-macrophage centres' (see later) were a conspicuous feature of the spleen.

3. Heart (conus arteriosus, atrium and ventricle)

a. conus arteriosus

No phagocytic cells were seen, the only colouration being of a very pale, diffuse nature.

b. atrium

The endocardial lining cells of the atrial muscle trabeculae were very prominent, and virtually every one contained large amounts of trypan blue in the form of both large and small dark blue vacuoles. Very occasionally, some of these cells, replete with trypan blue, were seen to be only tenuously attached to the

endocardium, or else free, giving the impression that they were in the process of rounding up and floating off, similar to Kupffer cells in mammalian liver.

c. ventricle

Only the occasional endocardial lining cell contained any trypan blue, and such cells were never as prominent as those of the atrium.

4. Kidney (anterior and posterior).

a. anterior kidney

The endothelial cells lining the sinuses, and other cells scattered throughout the stroma of this haematopoietic organ, were the only ones which contained any trypan blue.

b. posterior kidney

The cells of the tubules, exhibited only a very faint blue tinge, whereas the lining cells of the "peritubular capillaries" (Mackmull and Michels, 1932) were very prominent. These "peritubular capillaries" were in fact more like sinuses surrounding the renal tubules. Again, some of these endothelial lining cells were in the process of rounding up and floating off, possibly to migrate into the inter-tubular tissue, where other free macro-

phages containing trypan blue were situated, in addition to the melano-macrophage centres.

5. Muscle.

This had an overall diffuse blue, but only in the connective tissue between the muscle bundles were there any cells containing concentrated trypan blue. These were spindle-shaped, and resembled fibroblasts.

6. Skin.

The fibroblast-like cells in the connective tissue of the sub-epidermal layers (Roberts et al., 1971) were the only cells which contained any trypan blue. The melanin-containing macrophages (Roberts et al., loc cit.) did not contain any.

7. Gill.

a. primary lamellae

The spindle-shaped fibroblast-like cells surrounding the axial cartilage, were well marked with trypan blue, as well as melanin granules.

Fig. 1 Paraffin section of small intestine from a plaice which had received 7 intra-peritoneal injections of trypan blue. Observe the vitally stained cells in the lamina propria and in the epithelium (arrows).

Neutral red x 1,250

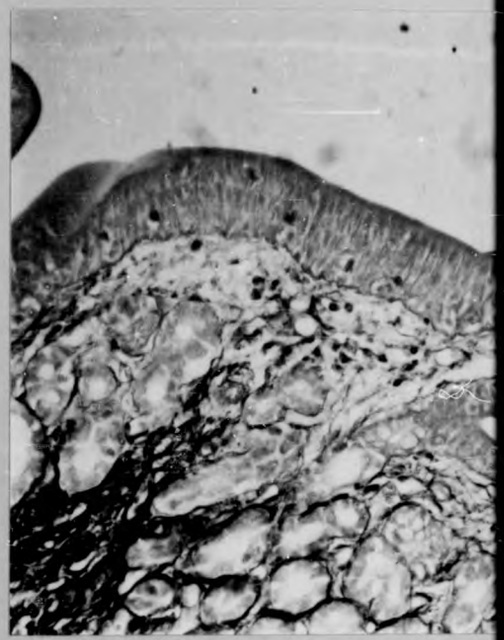
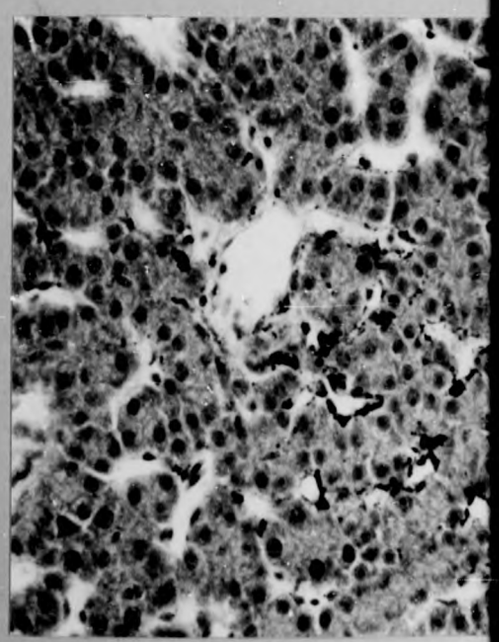


Fig. 2 Paraffin section of liver from a plaice which had received a large intra-venous injection of colloidal carbon. Observe that the carbon lies mainly in a peri-sinusoidal position, possibly within the Space of Disse. Kupffer cells are not visible.

H & E x 1,800



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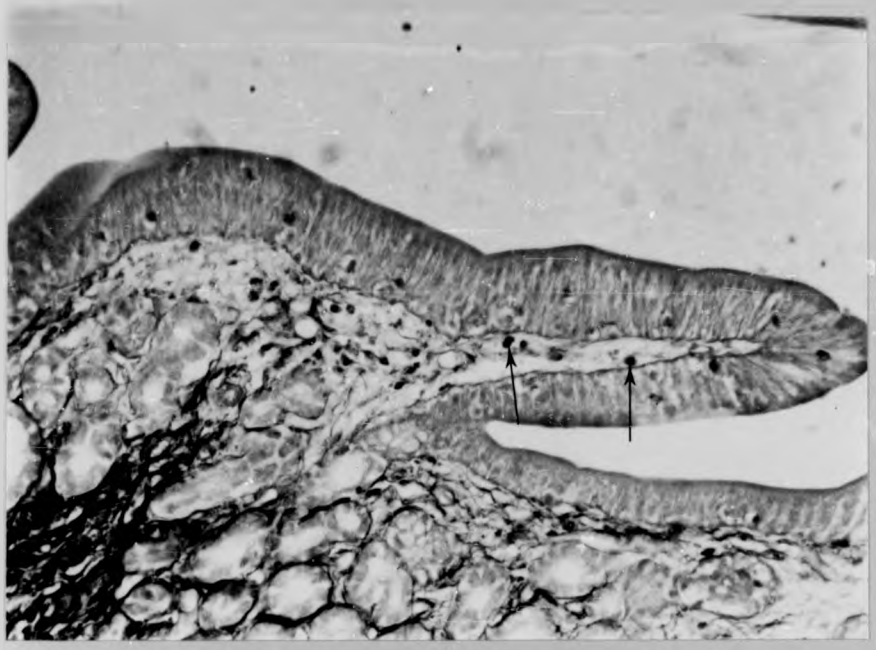

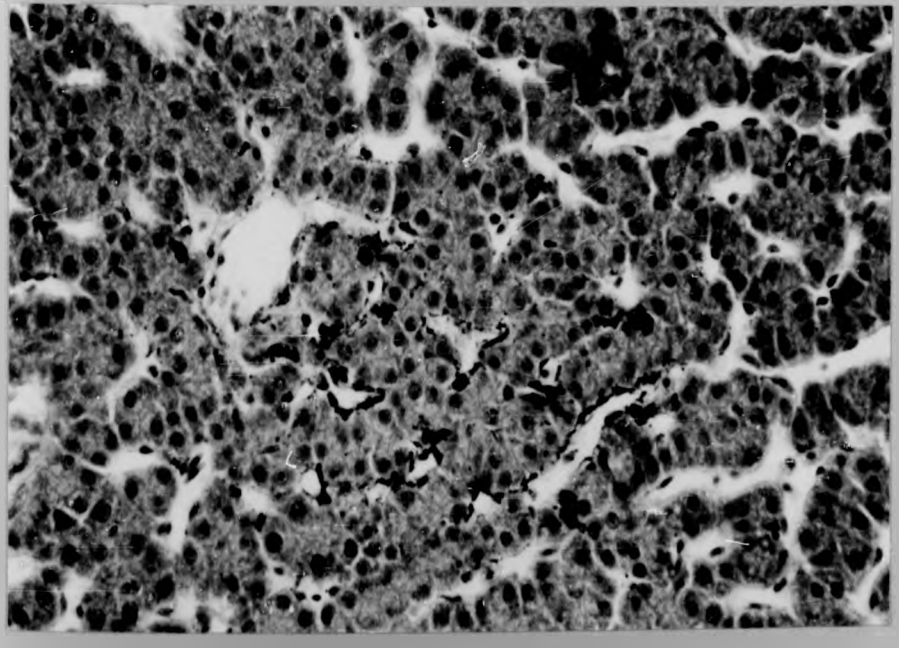

b. secondary lamellae

Cells, centrally placed within the lamellae, contained trypan blue. These were round cells, and not spindle-shaped as in the primary lamellae. It was impossible to determine whether these were circulating leucocytes or pillar cells. Pillar cells were reported by Hughes & Weibel, 1972, to constitute part of the R.E.S. of teleosts.

8. Gut (small and large intestine, and mesentery).

a. small intestine

There was quite a number of large rounded macrophages in the lamina propria. These appeared to be migrating out through the epithelium into a gut lumen, as they were present not only at the base of the brush border, but also at the extreme edge, next to the lumen (Fig. 1). Those cells nearest the lumen were a very diffuse blue and were surrounded by a clear halo, whereas those in the lamina propria contained the trypan blue in discrete vacuoles. The macrophages in the lamina propria were concentrated in what appeared to be small foci of haemato-poietic tissue situated around the longitudinally disposed blood vessels.

b. large intestine

Larger numbers of macrophages, as described above, were present in both the epithelium, and the lamina propria.

c. mesentery

Only those parts attached to the sampled organs were examined. Many fibroblast-like cells were evident, having taken up considerable quantities of trypan blue.

9. Gonads.

Many spindle-shaped fibroblast-like cells were present in the inter-septal connective tissue and these contained quite a lot of trypan blue. Very large rounded cells were present within the stroma of the gonadal tissue. These macrophages may have been responsible for clearing effete cells and tissue during the ovarian reproductive cycle.

10. Eye and brain.

No cells containing trypan blue were observed in these organs.

B Colloidal carbon

Only where a variation in dose level affects the results, is mention made of the fact.

1. Liver (excluding pancreas and biliary system).

Only at the very high dose level (16 mg/100g) did the liver

Fig 3. Section of spleen from a plaice sacrificed 15 minutes after the intra-venous injection of colloidal carbon. Observe the distribution of the carbon within the walls of the ellipsoids (arrows).

H & E x 450

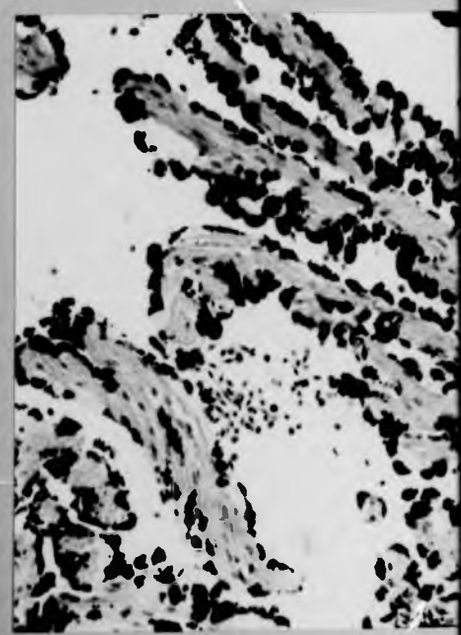

Fig. 4 Section of atrium from a plaice sacrificed 6 hours after the injection of colloidal carbon. Observe that nearly every endocardial lining cell is replete with carbon.

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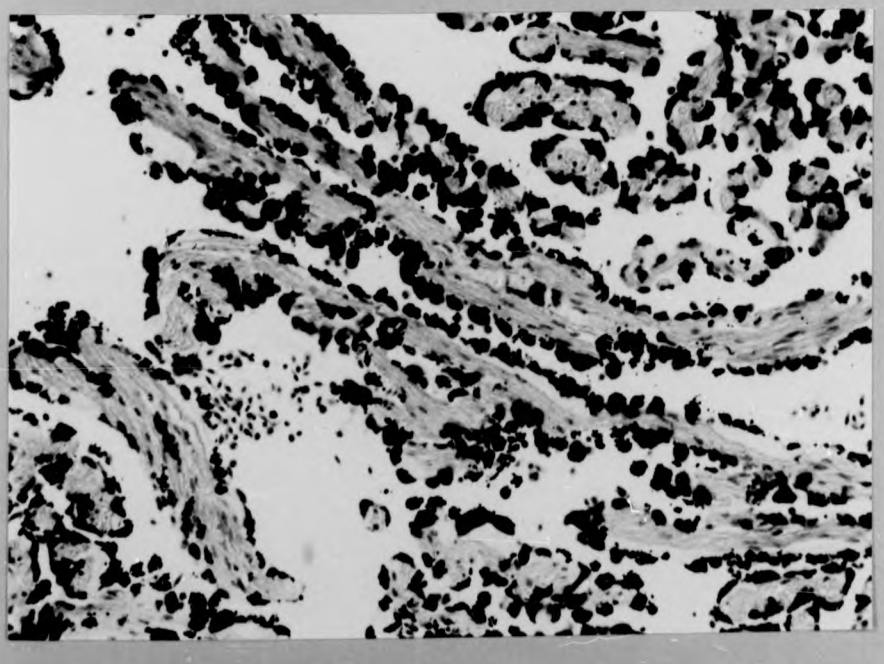


appear black and then, only at the margin.

Microscopically, at the dose rate of 8 mg/100g, small amounts of carbon were present in a peri-sinusoidal position (Fig. 2). Occasional histiocytes, usually associated with bile ducts, were also present.

No evidence for the presence of Kupffer cells was found in plaice, turbot or rainbow trout, i.e. although carbon was seen in a peri-sinusoidal position, no carbon-containing cells which in any way resembled Kupffer cells, were seen.

2. Spleen.

Masses of carbon were seen to be trapped in the ellipsoids almost immediately after its introduction into the bloodstream (Fig. 3). It could not be determined at the LM level however whether the carbon was intra-cellular or not. The marking became maximal at about 12 hours, and the ellipsoids contained carbon for as long as 3 or 4 weeks post injection, after which time, they were clear. After this length of time, the melano-macrophage centres appeared to have accumulated considerable amounts of carbon and were the only areas of the spleen where carbon was to be found.

Following the initial marking of the ellipsoids the cells next seen to contain any carbon were the endothelial cells lining the sinuses. This however was only seen at high dose levels. At all times between 15 minutes, and 4 weeks, carbon-containing macrophages were scattered throughout the stroma of the spleen, unrelated to any recognisable structure.

3. Heart (conus arteriosus, atrium and ventricle).

At all dose levels, the macroscopic appearance was that of a black atrium and ventricle, the latter containing less carbon at the lower dose levels.

a. conus arteriosus

No phagocytic cells were present in the structure. Occasional carbon-containing circulating leucocytes were observed within the lumen.

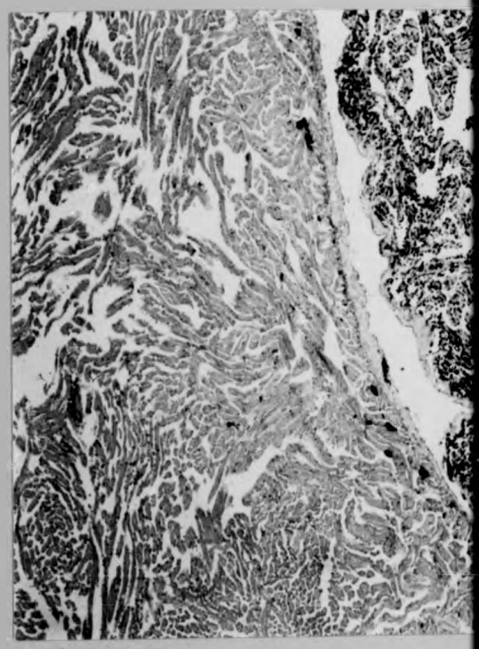
b. atrium

Nearly every endocardial lining cell was extremely prominent, and all were loaded with carbon to such an extent that individual phagosomes could not be distinguished (Fig. 4).

After approximately 4-6 hours, many were in the process of rounding up and floating off.

Fig. 5 Low power photomicrograph of heart from a plaice sacrificed 8 hours after the intra-venous injection of colloidal carbon. Observe that the ventricle (left) contains virtually no carbon whereas the atrium is almost black.

H & E x 450



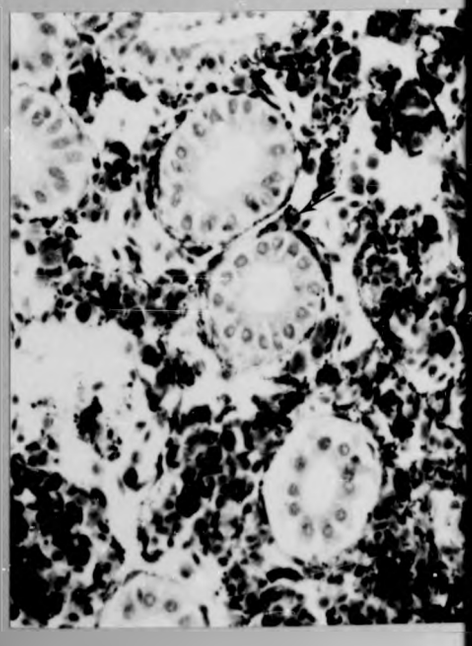


Fig. 6 Posterior kidney from a plaice sacrificed 1 hour after the injection of colloidal carbon. Observe the carbon-containing endothelial cells of the "peri-tubular capillaries" (arrows).

H & E x 1,800

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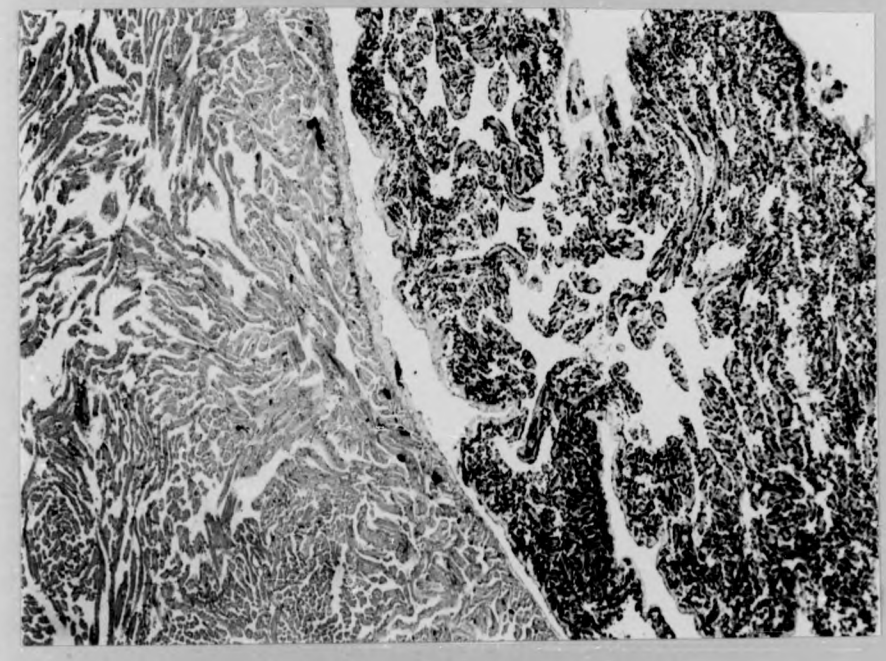

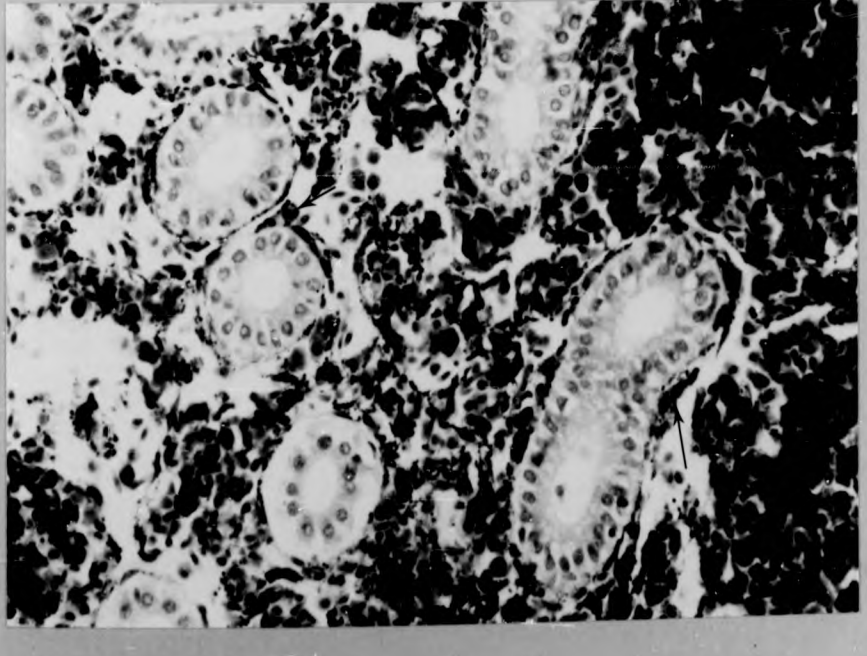

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ries" (arrows).

These macrophages represented the first site at which phagocytosis could be seen to have occurred following the injection of colloidal carbon, and occurred after as short a time as 15 minutes post injection. Maximal marking took approximately 8 hours. After 3 weeks, some cells still contained carbon, but the atrium as a whole, was by this time virtually clear.

The atrial endocardium of both turbot and rainbow trout took up carbon in a similar manner to that of plaice.

c. ventricle

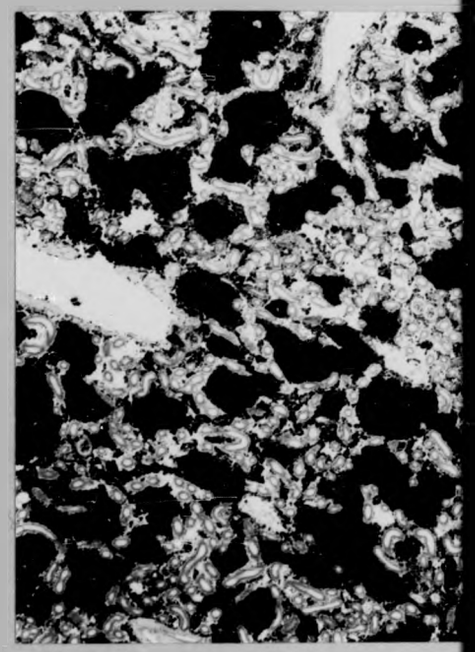
Compared to the atrium (Fig. 5), very few endocardial cells contained carbon, and those that did, did not contain as much, nor were they so prominent. Only at the higher dose levels did any appreciable degree of uptake occur, usually 2-3 hours post injection as opposed to the 15 minutes post injection for the atrium. There was no evidence of any endocardial cells in the process of rounding up and floating off.

4 Kidney (anterior and posterior).

The endothelial lining cells of the "peri-tubular capillaries" became very well marked with carbon after approximately 1 hr. (Fig. 6), and appeared to round up and float off. Following this initial marking, carbon-containing macrophages were seen in the inter-

Fig. 7 Section of posterior kidney from a plaice sacrificed 3 weeks after the intra-venous injection of colloidal carbon. Observe that all the carbon has aggregated within the melano-macrophage centres.

H & E x 180



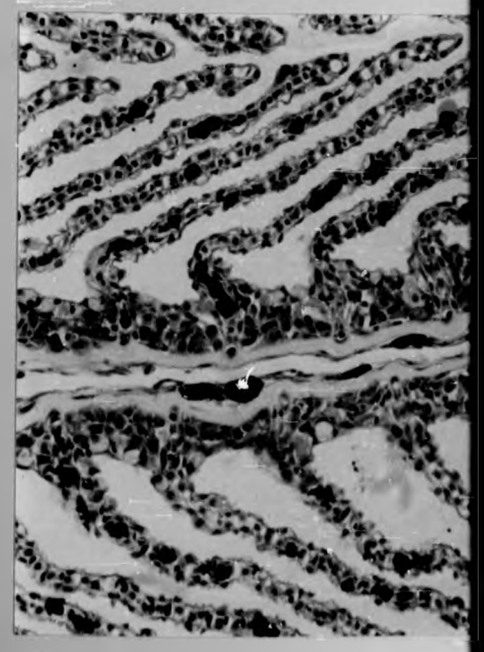


Fig. 8 Section of gill from a plaice sacrificed 4 hours after the injection of colloidal carbon. Observe carbon-containing cells within both the primary and the secondary lamellae.

H & E x 1,250

ificed 3 weeks

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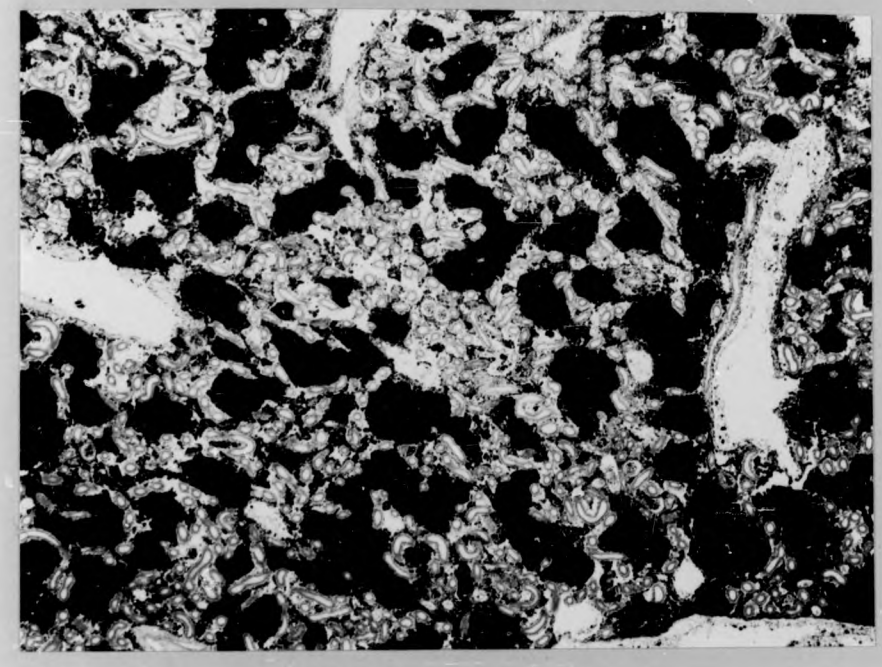

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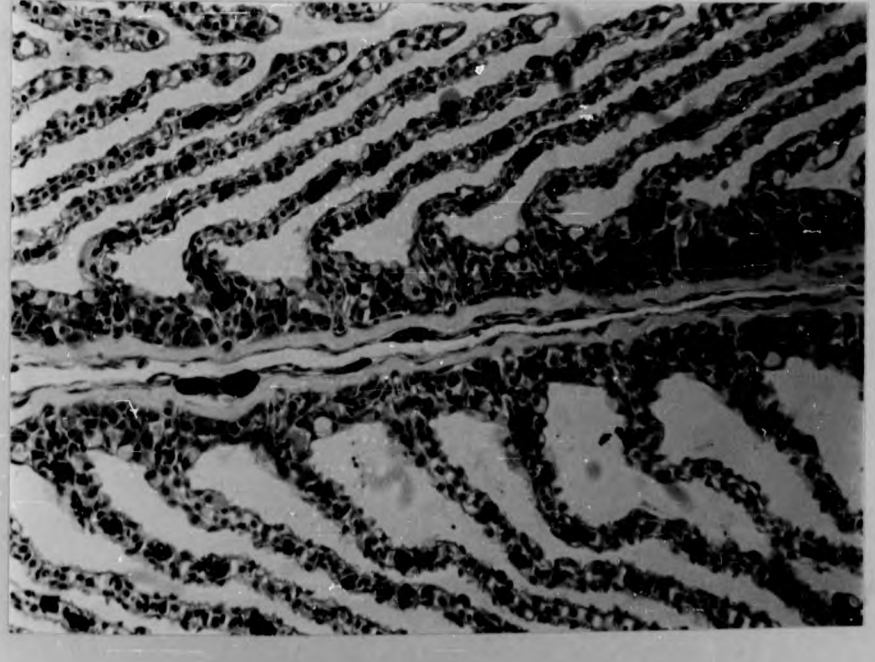

tubular stromal tissue. These were present up to 3 or 4 weeks post injection when both the inter-tubular stroma and the endothelium of the "peri-glandular capillaries" were clear of carbon. All the carbon in the kidney at this time was within the melano-macrophage centres (Fig. 7).

5. Gills.

Carbon-containing cells were seen in the same locations as described for trypan blue (Fig. 8). Although no carbon-containing cells were ever seen to be detaching, the gills were virtually clear of carbon by 3-4 weeks.

6. Blood - leucocytes.

At the LM level, only 3 cell types could be readily distinguished:-

a. thrombocyte

b. lymphocyte

c. monocyte/neutrophil type

Differentiation of the thrombocyte from the lymphocyte was possible only in very well prepared smears, when the spiked poles of the thrombocytes or their cross-hatched nuclei were visible. The third cell type was not easily classified into either monocyte or neutrophil, despite descriptions of these cells by Ellis (1974) and McGregor (1975).

The thrombocyte took up carbon, as did some of the neutrophil/ monocyte types. The lymphocyte did not. The carbon within the thrombocytes was present as a very dense spot just above the nucleus, corresponding to the pore demonstrated by Wardle (1971).

C. Yeast Cells (particles).

1. Liver.

No yeast cells were seen within any liver cells. A few were seen in the peri-glandular area.

2. Spleen.

Very few particles were present within the walls of the ellipsoids, the few that were, being situated right at the periphery of the sheaths, or within the lumen of the axial vessel. More particles were however present within the stroma.

After approximately 4 weeks, there were many particles present within the melano-macrophage centres, many in some stage of degeneration, but this segregation was evident as soon as 5 days post injection.

Fig. 9 Light micrograph of atrium from a plaice sacrificed 24 hours after the intra-venous injection of yeast cells. Observe the close association of many yeast cells with the endocardial lining.

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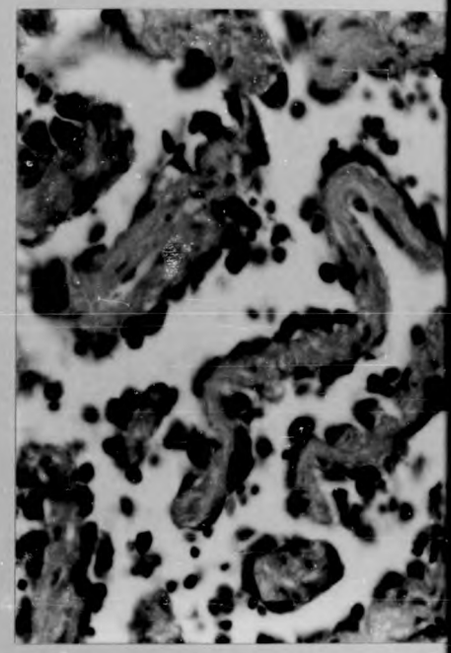

Fig. 10 Light micrograph of epon-bedded atrium from a plaice sacrificed 24 hours after the intra-venous injection of yeast cells. Observe the presence of yeast within the cytoplasm of the endocardial cells (arrows).

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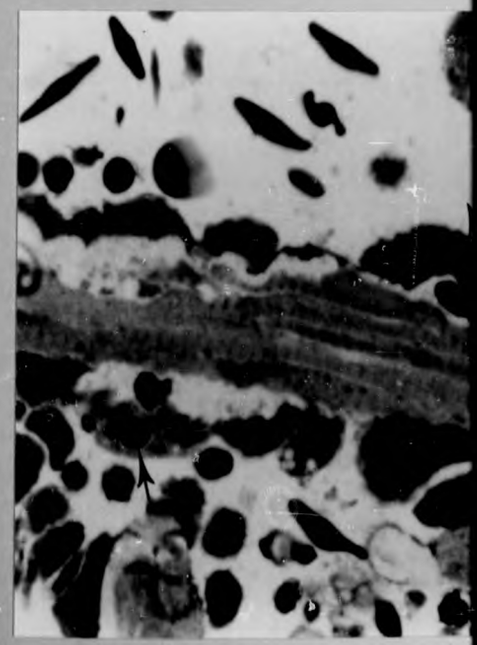

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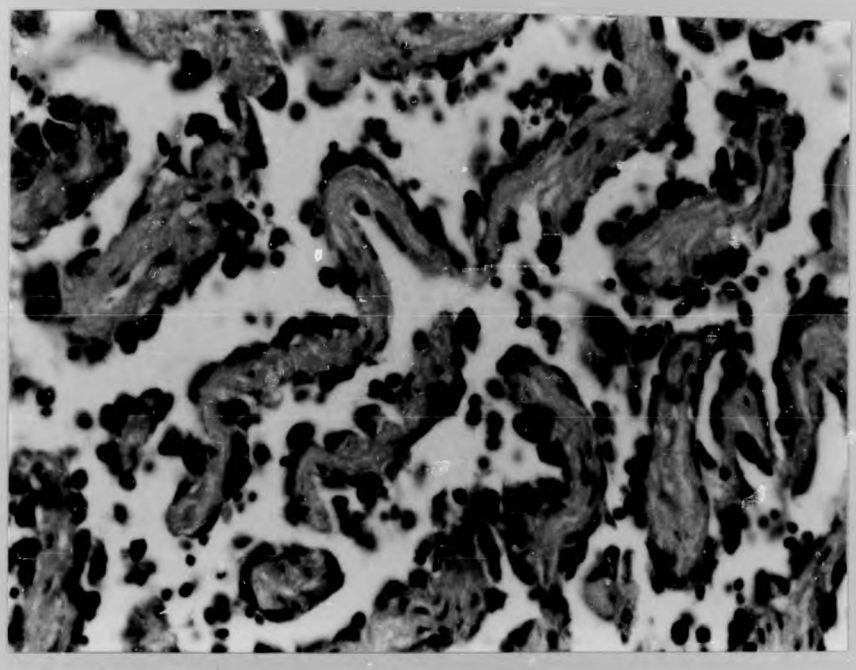

a plaice sacrificed

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3. Heart (atrium and ventricle).

a. atrium

The endocardial cells were loaded with yeast particles (Fig. 9). Some cells appeared to contain as many as 3 or 4 particles (Fig. 10), occupying a total volume far in excess of the original size of the cell. Rounding up and floating off of the endocardial cells was frequently observed and after 4 weeks, the atrium was nearly clear of yeast.

b. ventricle

Very few endocardial cells contained any yeast, and then usually only a single particle. This small degree of yeast uptake had also resolved by 4 weeks.

4. Kidney (anterior and posterior)

a. anterior

A few yeast-containing macrophages were present within the stroma, although these had cleared by 4 weeks. The aggregation of particles within the melano-macrophage centres was very evident after 5 days, and after 4 weeks, many were broken down.

Fig. 11 Posterior kidney from a plaice sacrificed 5 days after the intra-venous injection of yeast cells. Observe the presence of yeast (arrows) within the melano-macrophage centres.

x 1,800

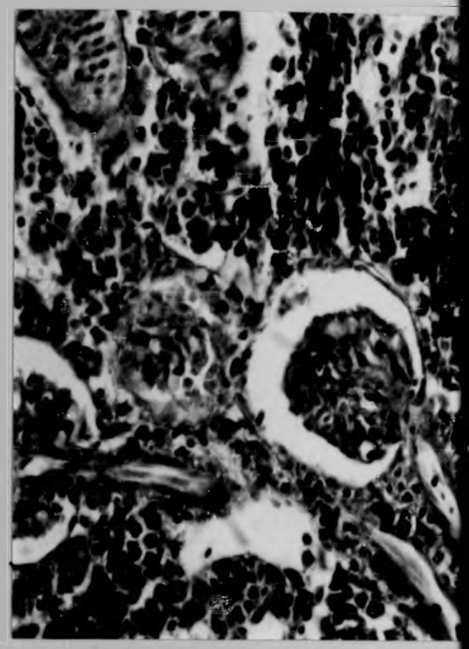
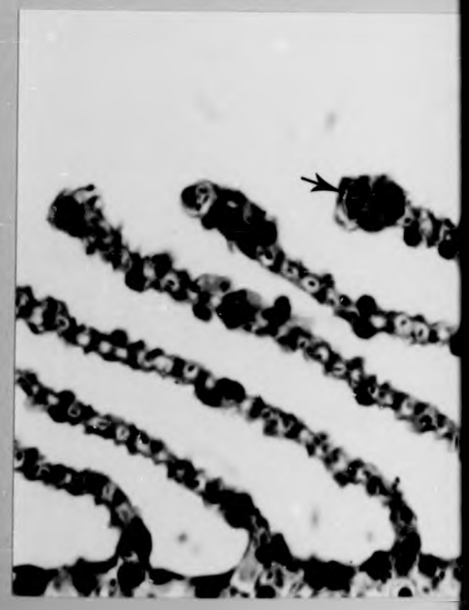


Fig. 12 Gills from a yeast-injected plaice. Observe the small foci of yeast cells (arrows) within the secondary lamellae.

x 1,800



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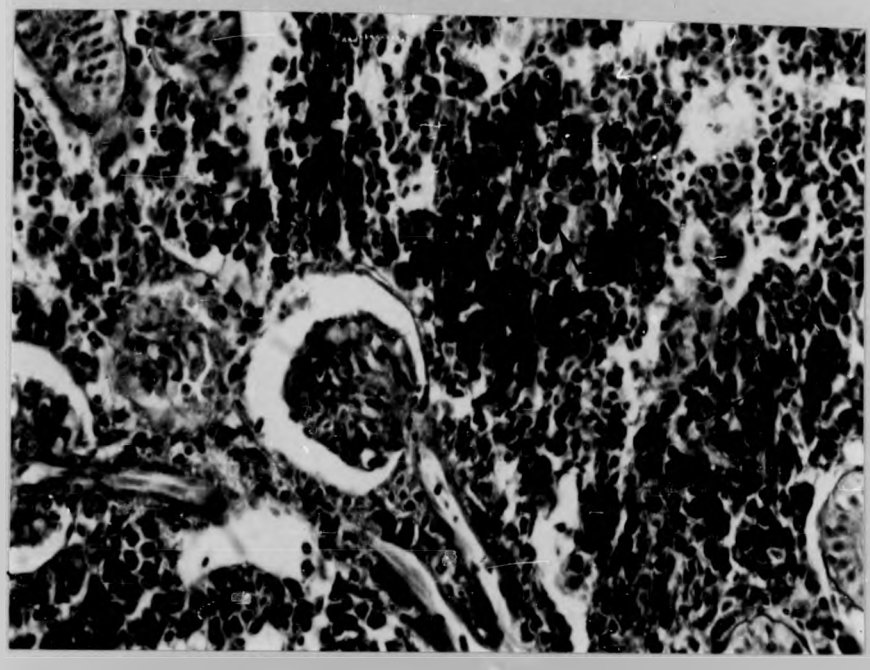

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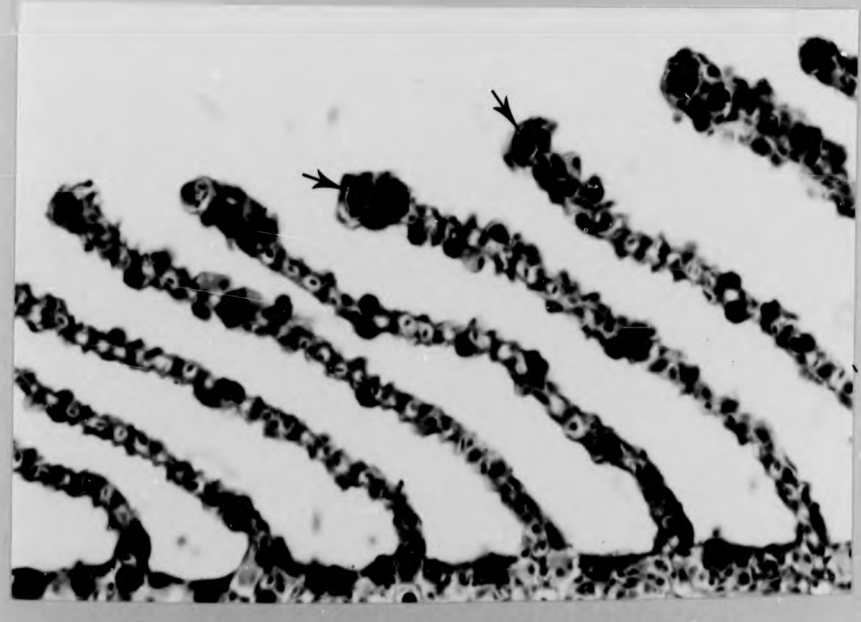

b. posterior

Most yeast was within the inter-tubular stroma, and had cleared by 4 weeks. The accumulation of the yeast within the melano-macrophage centres was the same as in the anterior kidney (Fig. 11).

5. Gills.

Many particles were present within the secondary lamellae (Fig. 12), although, as in the case of the carbon and trypan blue, the cellular association was not determined. The primary lamellae contained few particles.

D. B.C.G. bacilli.

1. Liver.

Acid-fast bacilli were only occasionally encountered, associated with the Space of Disse. Dose level did not affect this result.

2. Spleen.

At low dose levels, only a few bacilli were present within macrophages scattered throughout the stroma. At high dose levels, many were present, mainly associated with the outermost regions of the sheaths. None were ever seen within the walls of the ellipsoids.

3. Heart (atrium and ventricle).

a. atrium

Only a few small clumps of bacilli were present within any cells associated with the endocardial lining cells, but whether these were intra-cellular or not was hard to determine.

b. ventricle

No bacilli were present.

4. Kidney (anterior and posterior).

a. anterior

A few bacilli were present within the stroma, and accumulation within the melano-macrophage centres had commenced 4 days post injection.

b. posterior

Initially, the bacilli were associated with the endothelial lining cells of the peri-tubular capillaries, and, as in the anterior kidney, accumulation within the melano-macrophage centres was prominent only 4 days post injection. The posterior kidney contained more bacilli than any other organ.

5. Gills.

Many bacilli were present within both the primary and secondary lamellae. They occurred in small clumps and again it was not possible to determine whether they were intra-cellular or not.

The results of these particle distribution experiments are summarised in Table 4.1.

TABLE 4.1 Relative Organ Distribution of Particles

Organ Site	Particles			
	Trypan Blue	Carbon	Yeast	BCG
Liver sinusoids	+	+		+
Liver panenchyma	+	+		
Periglandular area	++++	++	+	+
Spleen ellipsoids	++++	++++•	+	+
Spleen pulp	++	++	++	++
Spleen MMC		++++o	+++o	++o
Heart - atrium	++++	++++•	++++	++
Heart - ventricle	+	++	++	
Kidney - peri-tubular capillaries	+++	++	++	+++•
Kidney - stroma	+++	++	++	++++
Kidney MMC		+++o	+++o	+++o
Gills - 1^{o} lamellae	++	++	++	++
Gills - 2^{o} lamellae	++	++	++	++

Key: • indicates initial marking

o indicates increasing with time

+ indicates the amount. The more ++, the greater the amount.

CHAPTER 4.2 DISCUSSION AND CONCLUSIONS

One of the major problems associated with these definition experiments, was the precise determination of whether the particles were in fact intra-cellular, or merely cell-associated. As Reade (1968) indicated, in relation to mapping out the R.E.S. of the crayfish (Parachaeraps bicarinatus), the colloidal carbon acts as such a dense marker that cellular detail is usually obscured. It is very possible that in the present study a mechanism similar to that described by Spector and Coote (1965) was operating. They showed that mammalian cells were capable of shedding parts of their cytoplasm which thereafter remained in a close extra-cellular location. Another possibility is that, instead of ingestion followed by egestion, the particles merely adhere to the surface of the cells in the same way that antigen is retained on dendritic cells in germinal centres (White, 1963; Nossal et al., 1964; Humphrey et al., 1967), thus providing a framework on which lymphocytes may interact.

The present findings regarding the distribution of the colloidal carbon agree largely with those of Mackmull and Michels (1932) for the cunner (Tautogolabrus adspersus) except for the fact that they described the presence of Kupffer cells, while none were seen in any of the species examined here. The importance of the heart (no distinction was drawn between atrium and ventricle) was emphasised by these authors, as was the accumulation of carbon within the melano-macrophage centres, although no explanation was offered as to the origin or importance of such a process.

The apparent inability of the ellipsoidal sheaths to trap the larger injected particles, i.e. the yeast and B.C.G. bacilli, is in accordance with the findings of White & Gordon (1970) in the chicken. The main route of access of particles from the lumen of the axial vessel into the sheaths was thought by these workers to be via pores in the basement membrane of the endothelial lining, only the smaller particles being capable of passing through, with an upper limit of 2μm. For a full discussion on the significance of the ellipsoids, see later (Chapters 6 and 9).

When salmonid fish are infected with the viral disease infectious pancreatic necrosis (I.P.N.) there is a large increase of eosinophilic cells in the mucosa and lumen of the intestine (Roberts, 1973). This fact was investigated by McKnight (1973) who concluded that these cells were possibly not a pathological finding, specifically associated with this disease. The findings in the plaice i.e. the presence of vitally stained cells in the lamina propria, which showed a migration out through the epithelial brush border and into the lumen, may provide an explanation for the observations in the infected salmonids where possibly a normal process is being accelerated.

In attempting to map out the R.E.S. of plaice, and to generalise the findings by limited repetition in turbot and rainbow trout, it became obvious that there were certain major differences from the mammalian situation. These were:-

1. The liver of plaice did not contain Kupffer cells or any other highly phagocytic cell associated with the sinusoidal lining. This finding in plaice was confirmed in turbot and rainbow trout. In mammals, the liver is the major organ for the uptake of colloidal carbon, and other injected particles. Below a threshold level, the Kupffer cells alone will completely clear the bloodstream (reviewed by Stuart, 1970).

2. The heart, especially the atrium, proved to be highly phagocytic, containing in the endocardial lining cells, possibly the most highly phagocytic cells in the body. This fact was confirmed also in turbot and rainbow trout.

Following the injection of particulate matter into mammals, virtually none locates specifically in the heart (Cappell, 1929).

3. The ellipsoids in plaice were very well developed, and were particularly avid for carbon. Ellipsoids are found throughout the vertebrate phyla (Solnitzky, 1937), and are developed to varying degrees from species to species. Little, however, is known of their ultrastructure.

In addition to the ellipsoids, the other major feature of the spleen was the presence of the melano-macrophage centres, which in the turbot especially, were very well developed. Injected particles accumulated within the centres of both spleen and kidney.

4. Classification of the leucocytes was difficult, even when purely morphological criteria were combined with the information gained from the response to colloidal carbon. The presence of a monocyte was not definitely established and as the precursor of many macrophages in mammals, its existence in plaice needs to be substantiated.

On the basis of these conclusions, it was decided to continue the investigation into the R.E.S. of teleosts, with the following intentions:-

1. To study the histology and ultrastructure of the teleost liver, and compare the findings to the mammalian situation.

2. To study the histology and ultrastructure of the plaice heart with special reference to the endocardial macrophages.

3. To study the histology and ultrastructure of the spleen of plaice and turbot in an attempt to throw some light on:-

   a. the melano-macrophage centres, with regard not only to their structure, but to their relationship to other splenic components.

b. the ellipsoids.

4. To study the ultrastructure of plaice leucocytes in an attempt to define the monocyte and differentiate it from the other cell types.

CHAPTER 5 HISTOLOGY AND ULTRASTRUCTURE I

5.1 Introduction

The ultrastructure of teleost liver has been described with respect to:-

a. the hepatocytes of fasting and feeding fish (Bycykowska-Smyk, 1968),

b. the general hepatic ultrastructure of 2 species of normal and gravid fish (Weis, 1972), and

c. the biliary system of the goldfish (Yamamoto, 1965).

Except for a very brief mention by Weis, however, none of the above authors described the sinusoidal lining or associated cells, including Kupffer cells.

The main work concerning the ultrastructure of teleost cardiac tissue was by Kisch & Philpott for the goldfish (1963), and Santer & Cobb (1972) for plaice. In describing the plaice heart, Santer and Cobb used Kisch and Philpott's work as a model, and only noted differences. No mention was made however of the endocardial cells in either the atrium or ventricle.

The ellipsoids of the spleen were first described by Billroth (1857) in birds. They were first seen in mammals by Key (1861) and by Schweigger-Seidel (1863) in the pig, dog and cat as well as man, and by this latter name i.e. Schweigger-Seidel sheaths, they are often known. Muller (1865) was the first to employ the term "ellipsoids", which he used because of their shape in transverse section in a variety of vertebrates including fish. Yoffey (1928) gave the best comparative account of fish ellipsoids, describing them in 1 species of dipnoid, 3 species of elasmobranch, and 15 species of teleost, including plaice. Solnitzky (1937) gave the best account of mammalian ellipsoids under various experimental conditions. The only work concerning the ultra-structure of ellipsoids however, despite their ubiquitous nature, has been in the fowl (White & Gordon, 1970) and in the dog (Weiss, 1962). Nothing is known of their ultrastructure in fish.

Ultrastructural studies on teleost leucocytes are very few by comparison with the work at the LM level (see review by Ellis, 1975). Weinreb (1963) however stated "The similarity in fine structure noted between the thrombocyte and lymphocyte is striking ...", but no mention was made of the monocyte.

As a result of the initial work on the plaice liver, regarding the possible location of colloidal carbon within the fat-storing cells, or cells of Ito (Ito and Shibasaki, 1968), and the observation that Kupffer cells did not appear to exist, an invitation was extended

from the University of Leiden, The Netherlands, to perform there some of the ultrastructural work, in the Department of Electron Microscopy, directed by Professor Daems.

Accordingly, the results presented in Chapter 6 on the ultrastructure of the livers of bream (Abramis brama) and roach (Rutilis rutilis), were obtained at the above laboratory, and most of the techniques and procedures, especially regarding fixation and embedding of tissues, were adopted for the subsequent ultrastructural work.

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CHAPTER 5.2 MATERIALS AND METHODS

A Fish used

Since some of the work on the ultrastructure of the liver was performed in Holland (see 5.1), wild caught canal fish, i.e. bream and roach of approximately 100g body weight were used. The remainder of the work on the liver was performed on adult plaice as in Chapter 3. The work on the heart and leucocytes was also carried out on plaice. Both plaice and turbot however were used for studying the spleen. During routine histopathological studies, some turbot were found to be infected with an intra-cellular coccidian parasite (Ferguson and Roberts, 1975). Under these conditions, not only was the architecture of the spleen simplified, due mainly to an efflux of cells, but the polysaccharide nature of the parasite meant that it acted as an excellent marker, showing up very well in sections stained with periodic acid-Schiff. Accordingly, both normal and parasitised turbot were studied.

Intra-venous carbon injections, using a dose level of 4 mg/100g body weight, were performed on plaice alone, as in Chapter 3. These fish were sacrificed from 15 minutes to 48 hours post injection.

B Paraffin sections

These were of use only for the spleen, and the fixation and

processing were as described before (Chapter 3). Apart from routine Mayer's haematoxylin and eosin (Culling, 1974), the two stains found to be of particular value were Giemsa's stain (Carleton, 1967) and Martius Scarlet-Blue (M.S.B.) (Carleton, 1967).

C Preparation of ultrathin, epon-embedded sections, for viewing in the electron microscope (E.M.)

1. Fixation.

For all the tissues studied, a 2-stage fixation procedure was employed, using glutaraldehyde, followed by osmium tetroxide, as fixatives.

For perfusion fixation of the liver, a 1.5% glutaraldehyde solution was prepared in a cacodylate buffer (with added sucrose) at pH 7.2, to give a total osmolality of 300 milliosmoles (after Smith and Farquhar, 1966).

For immersion fixation of all other tissues, a 2.5% solution was prepared in a cacodylate buffer (with added sucrose) at pH 7.2, to give a total osmolality of approximately 320 milliosmoles.

Following primary fixation, the tissues were post fixed in a 1% osmium tetroxide solution in a phosphate buffer (Millonig, 1961). The tissues were transferred from the glutaraldehyde into the osmium, without an intervening buffered rinse.

The fixation procedure for each organ was as follows:-

a. Liver.

After exposure of the liver of fish anaesthetised by immersion in a 1 in 12,000 solution of MS 222 (Bell, 1967), a syringe needle ($\frac{1}{2}$ inch 25 gauge Yale Microlance) was inserted into the ventral branch of the hepatic portal vein, and connected to a flexible plastic tube. Fixative was introduced into the vein by means of a peristaltic pump, at the rate of 4 ml/minute. Soon after the start of perfusion, the body of the liver was sliced with a razor blade approximately 1.5 cms behind the anterior margin, to allow the fixative plus blood to escape. The colour of the liver changed from brown to yellow after about 1 minute, and the consistency became that of a hard-boiled egg. After 4 minutes, the perfusion fixative became clear of blood and at this stage, the fixed portion of the liver was removed and cut into $1mm^3$ blocks under the same fixative. These were transferred directly to cold osmium tetroxide (approximately 5 ml) and left for 1 hour at $4^{o}C$, after which they were placed in 70% ethanol.

b. Heart.

The hearts of fish anaesthetised as above were exposed and

a hypodermic needle inserted directly into the ventricle. Cold fixative was introduced as above. After the start of fixation, the conus arteriosus and sinus venosus were cut, to allow fixative to escape. Four minutes later, the heart was removed, and small $1mm^3$ pieces dissected out under fixative. These were allowed to stand for a period of 30 minutes further immersion fixation in 5 ml glutaraldehyde at 4^oC, before transferring them to the osmium tetroxide, as for the liver. After this secondary fixation of 1 hour at 4^oC, the tissue was placed in 70% ethanol at 4^oC.

c. Spleen.

After quickly removing the spleen from a freshly sacrificed fish, it was cut in half, and fixative injected through the capsule into the stroma, at the rate of 4 ml/minute. When the fixative emerging from the cut face was no longer tinged with blood, small ($1mm^3$) pieces were cut under fixative, and treated as for the heart tissue.

d. Leucocytes.

Blood samples were processed according to the method described by Watanabe *et al.* (1967) for human blood, modified here for use in poikilothermic animals. Heparinised blood samples (again taken from the R.P.V.) were centrifuged in an M.S.E. bench centrifuge at

1000 rpm for 30 minutes. After this time, the clear plasma was removed and replaced with the 1.5% glutaraldehyde fixative. After 1 hour at $4^{o}C$, this was replaced with the 1% osmium tetroxide fixative. This was again replaced after 1 hour at $4^{o}C$ with 70% ethanol.

The buffy coat was by now sufficiently hardened to be removed from the siliconised* glass centrifuge tube. It was cut into $1mm^2$ pieces, and again placed in 70% ethanol.

Blood samples were also taken from fish injected intra-venously 24 hours previously with 4 mg colloidal carbon per 100g body weight. These were treated exactly as above.

2. Embedding, cutting, and staining of tissues.

After reaching 70% ethanol, all the tissues were rapidly dehydrated in a graded ethanol series and then embedded in Epon (Luft, 1961) in Number 1 Beem capsules.

The material was cut on an ultrotome** using either a diamond or glass knife. Sections, preferably in the gold region i.e. 90mμ

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*Repelkote, Hopkin and Williams Ltd

**either a Reichert OMU 3, or an LKB 3

(based on the continuous interference colour index - Hayat, 1970), were collected from water onto uncoated diamond or square mesh grids.

The sections were stained by floating the grids on drops of saturated uranyl acetate solution for 20 minutes (Pease, 1964). After washing in distilled water, they were floated for 10 minutes on drops of lead citrate (Reynolds, 1963). They were finally rinsed on 0.02 Molar sodium hydroxide solution and distilled water, and after drying, they were ready for viewing.

The livers of the bream and roach were viewed in a Siemens Elmiskop 1 electron microscope. All other material was viewed in an AEI Corinth electron microscope.

D Preparation of thick Epon-embedded sections, for viewing in the light microscope (LM)

Tissues were fixed and embedded in Epon as described above. Instead of cutting 90mμ sections however, 0.5-1μm sections were cut with a glass knife and after collection on a water bath, were transferred to a hot 1% toluidine blue solution (Hayat, 1970) where they floated for 2-3 minutes before being transferred with a platinum loop, to a water bath. Here, excess stain was removed before the sections were dried onto a glass slide and mounted using synthetic resin and a cover-slip.

This method of preparing tissues provided a very useful link between paraffin sections viewed at the LM level and ultrathin sections viewed at the EM level.

CHAPTER 6 HISTOLOGY AND ULTRASTRUCTURE II

6.1 Results

A Liver

The findings for both normal and carbon injected fish are divided into the following categories:-

i General histology

ii Biliary duct system

iii Sinusoidal lining and associated cells

The findings given are general, species differences being noted where appropriate.

Normal Fish

i. General histology.

The liver tissue of all the fish studied differed from that of mammals in that there was no typical lobulation or arrangement of liver cells into cords. This had previously been noted for the goldfish by Yamamoto (1965). Sinusoids were irregularly

Fig. 13 Electron micrograph of liver of bream, showing 2 adjacent hepatocytes (H) and a bile duct cell. Observe that the centrally placed hepatocyte nucleus contains a very prominent nucleolus. Note also the rough-surfaced endoplasmic reticulum arrayed in rows around the nucleus and just beneath the plasma membrane (arrows). Most of the cytoplasm is comprised of pale-staining glycogen (g).

x 21,000

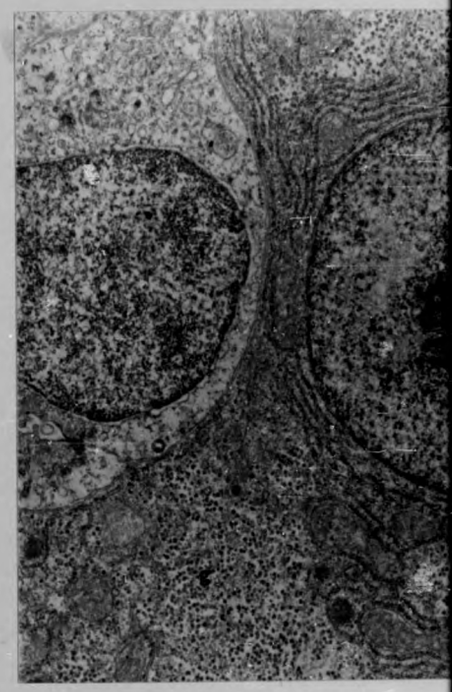
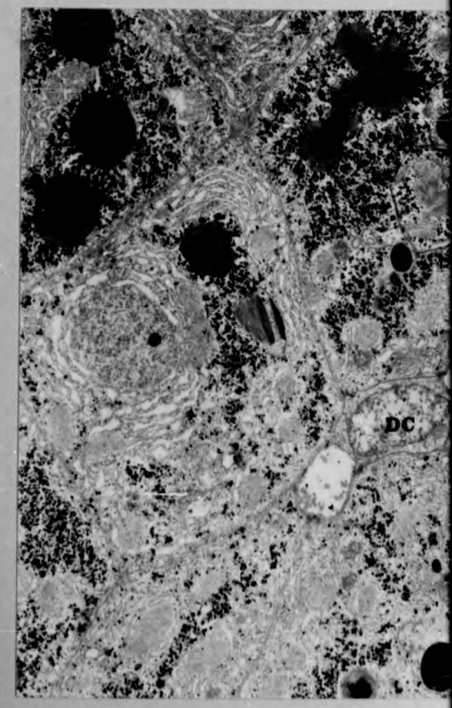


Fig. 14 Electron micrograph of plaice liver showing both an intracellular bile canaliculus (arrow) and the analogue of the mammalian Duct of Hering with a single duct cell (D.C.) contributing to 2 ducts. Observe that there are numerous fat droplets (f.d.) surrounded by darkly staining glycogen granules, within the cytoplasm of the hepatocytes.

x 4,500



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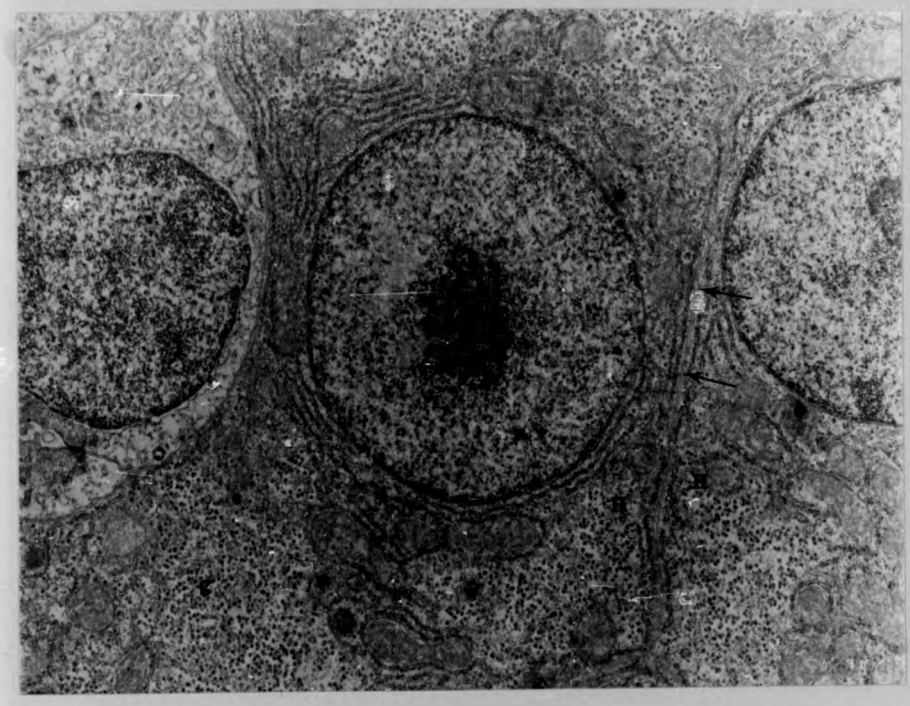

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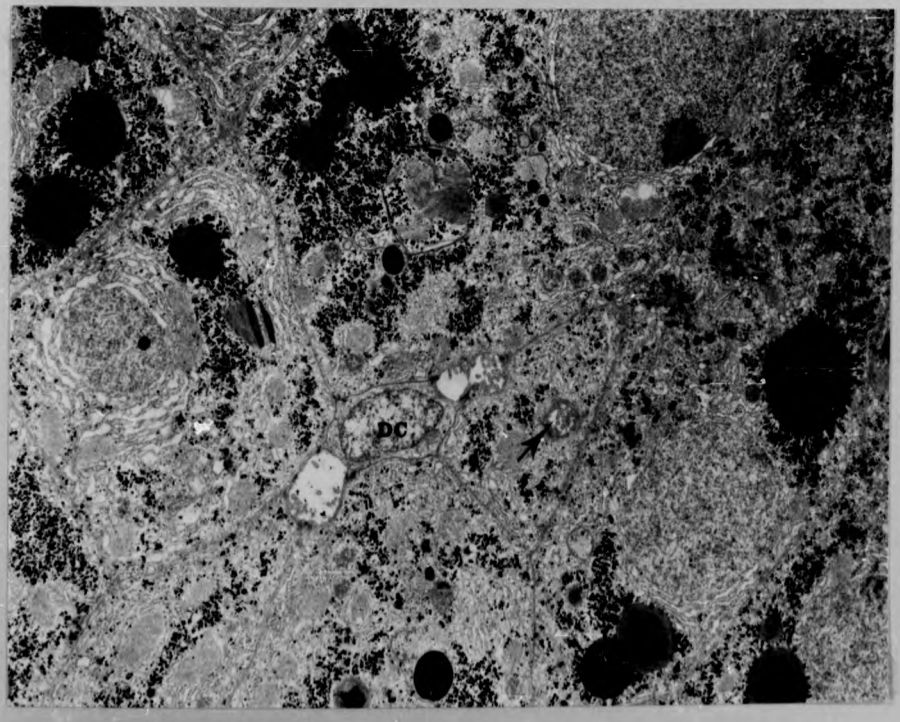

Fig. 15 Intra-cellular bile canaliculus (b.c.) from plaice liver. Observe the desmosomes between the plasma membranes of adjacent hepatocytes and the microvilli from the hepatocytes within the lumen of the canaliculus. Note the condensation of microfibrils at the desmosomes, and the electron-dense substance around the canaliculus.

x 45,000

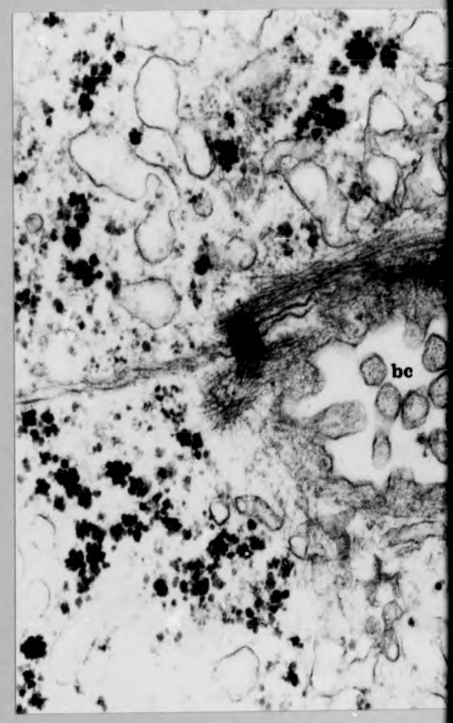

Fig. 16 The analogue of the mammalian Duct of Hering from bream liver. Observe that the microvilli within the duct are contributed to by both hepatocyte and duct cell (D.C.) Note also the electron-lucent vacuoles around the canaliculus.

x 19,500

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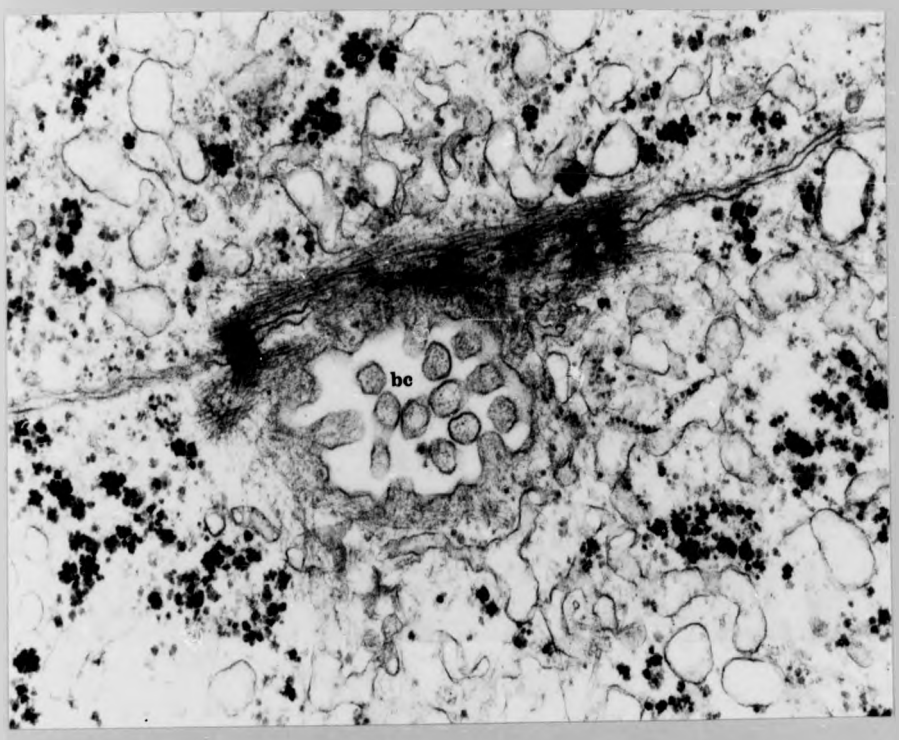

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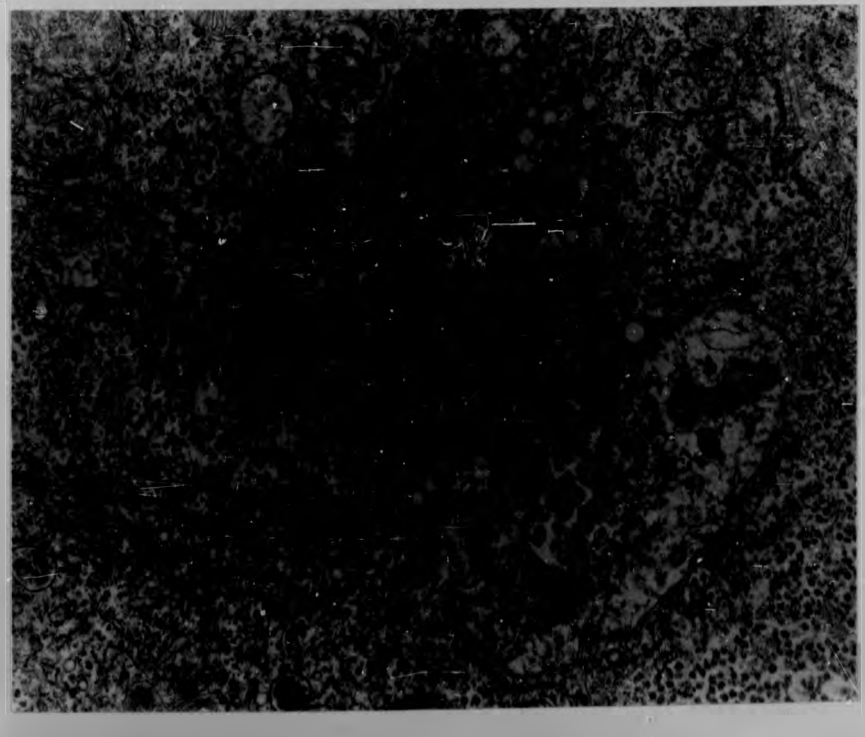

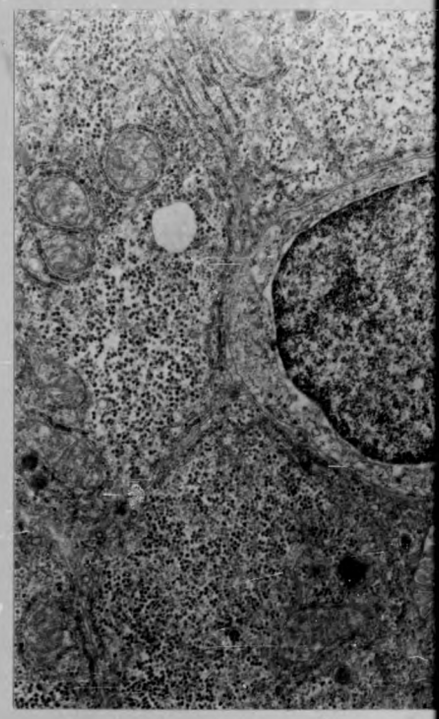

Fig. 17 Electron micrograph of roach liver showing bile duct cell and hepatocyte combining to form a canaliculus (b.c.), the analogue of the mammalian Duct of Hering. Note within the cytoplasm of the bile duct cell, another ductule (arrow) formed by an invagination of the plasma membrane. The apposed plasma membranes are 'sealed' with desmosomes.

x 24,000

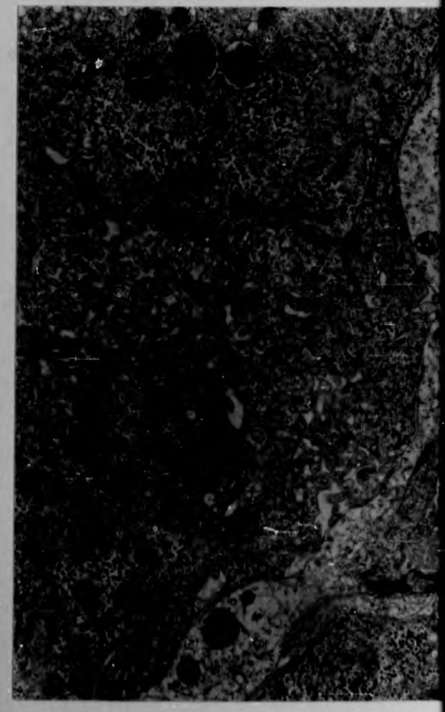

Fig. 18 Electron micrograph of roach liver showing a bile duct cell situated near a sinusoid (S). Observe a ductule (arrows) within the cytoplasm, travelling down the length of the cell and terminating by opening into a bile canaliculus (b.c.) similar to Fig. 17.

x 12,900

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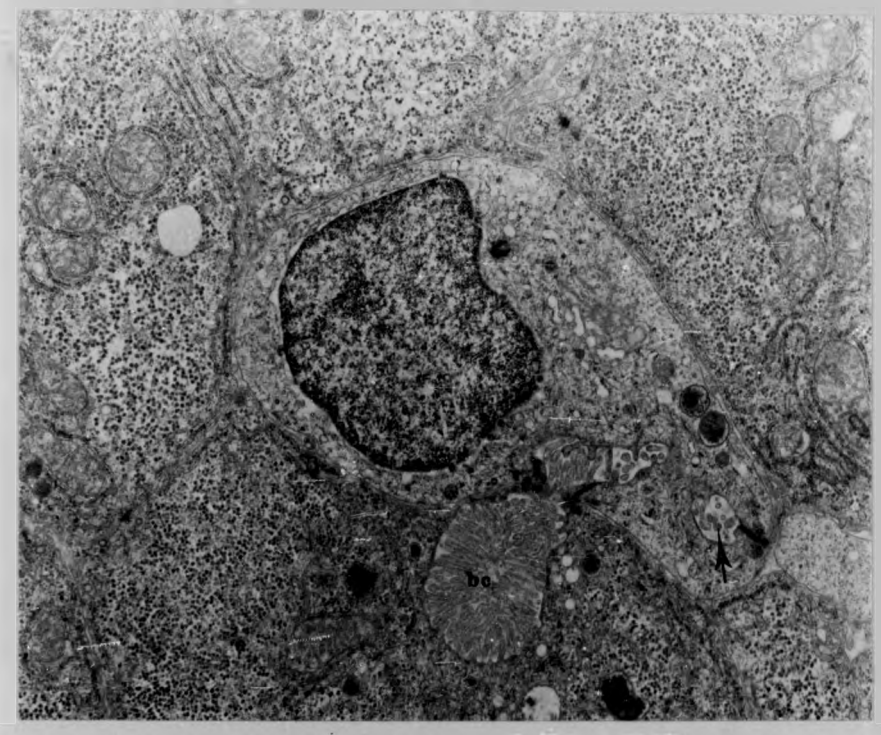

owing a bile duct cell
a ductule (arrows)
the length of the cell
canaliculus (b.c.)

distributed between the polygonal hepatocytes which contained a large prominent nucleolus within an often centrally placed nucleus (Fig. 13). The nuclear chromatin was sparse, and distributed peripherally. Layer upon layer of rough surfaced endoplasmic reticulum (RER) enclosed the nucleus (Fig. 13) and there was also a layer of RER immediately below the plasma membrane. Both of these areas stained darkly with toluidine blue so that they were easily visible, even in the LM. The intervening pale staining area was seen in the EM to be composed mainly of glycogen. The numerous mitochondria were concentrated in the RER areas.

ii Biliary ducts.

The biliary system was also different from mammals, in that intra-cellular bile canaliculi were present (Figs. 14 & 15). These were sometimes situated in the middle of the cytoplasm, but were more often found against the plasma membrane, the gap between the two being filled with an electron-dense substance (Fig. 15). These canaliculi connected up with a single duct cell (Figs. 16 & 17) with microvilli from both hepatocyte and duct cell contributing to the lumen of this duct, the analogue of the mammalian duct of Hering. The next stage of the duct system was a ductule composed of a single duct cell (Fig. 18) (formed by an invagination of the plasma membrane), the duct being "sealed" with desmosomes. These single celled ductules

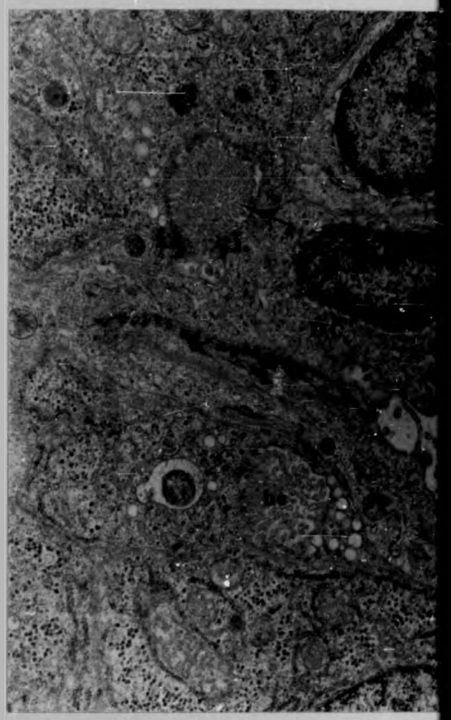

Fig. 19 This electron micrograph of bream liver shows 2 duct cells (D.C.) forming a bile ductule. Observe however a bile canaliculus (b.c.) within the cytoplasm of an adjacent hepatocyte and also another (arrow) connecting with the duct cell.

x 13,5000

Fig. 20 Electron micrograph of roach liver showing bile duct (B.D.) surrounded by connective tissue, and situated near a sinusoid. Observe the endothelial cell (E.C.) projecting into the sinusoid, and the fat-storing cell (F.S.C.), the nucleus of which is indented by a large intra-cytoplasmic fat droplet.

x 6,000

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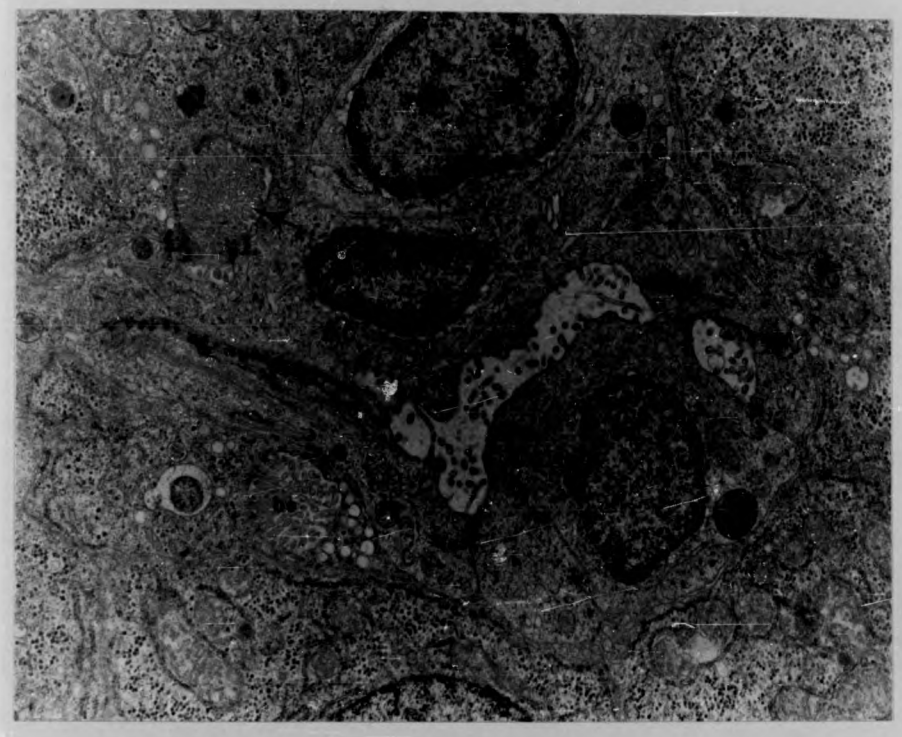

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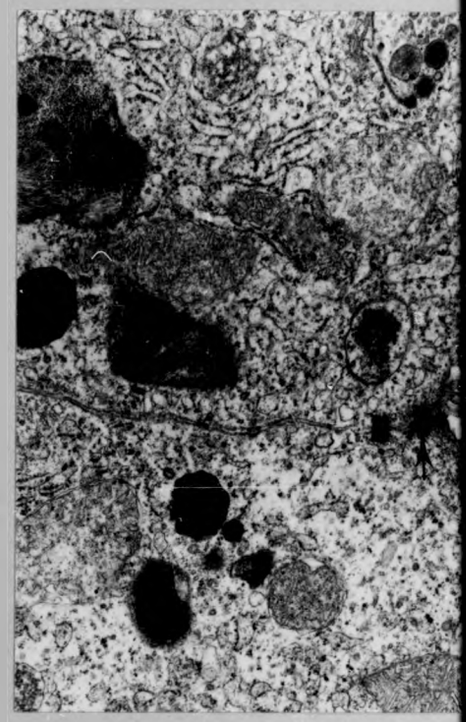


Fig. 21 Plaice liver, showing an inter-cellular bile canaliculus formed by 3 hepatocytes. Observe the desmosomes (arrows) "sealing off" the canaliculus.

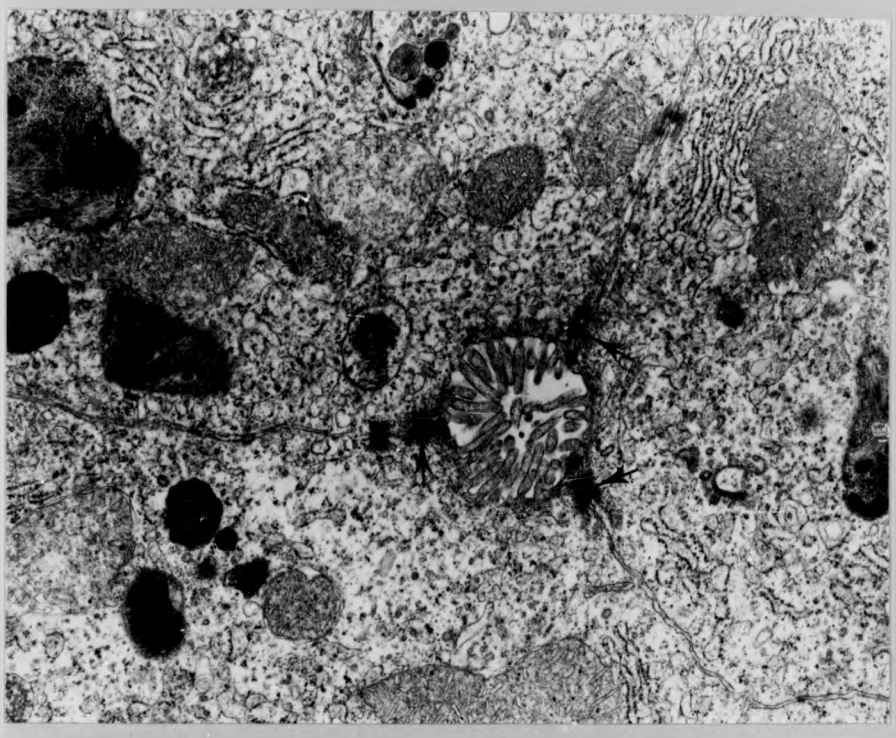
x 18,000

Fig. 22 Plaice liver showing an inter-cellular bile canaliculus formed by 4 hepatocytes. Again, observe the desmosomes.

x 18,000

bile canaliculus

smosomes (arrows)



bile canaliculus formed

desmosomes.

Fig. 23 Electron micrograph of roach liver showing a sinusoid (S) surrounded by the Space of Disse (S.D.). Two nuclei of the endothelial cells (E.C.N.) which comprise the lining can be seen, bulging into the lumen of the sinusoids. Observe microvilli from the surrounding hepatocytes, projecting into the Space of Disse. The sinusoidal lining in this micrograph is complete.

x 15,000

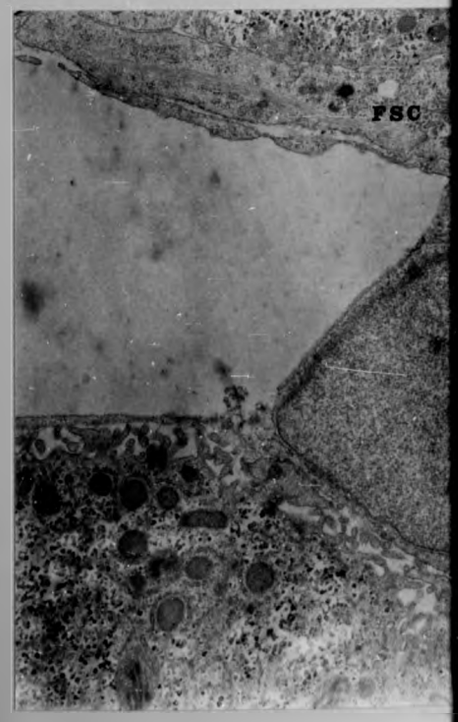


Fig. 24 Electron micrograph of roach liver showing an endothelial cell spanning a sinusoid. Note that the cytoplasmic projections of this cell comprise the sinusoidal lining which shows fenestrations (arrows). Situated in the Space of Disse can be seen a fat-storing cell (F.S.C.), its cytoplasmic projections extending just under the endothelial lining.

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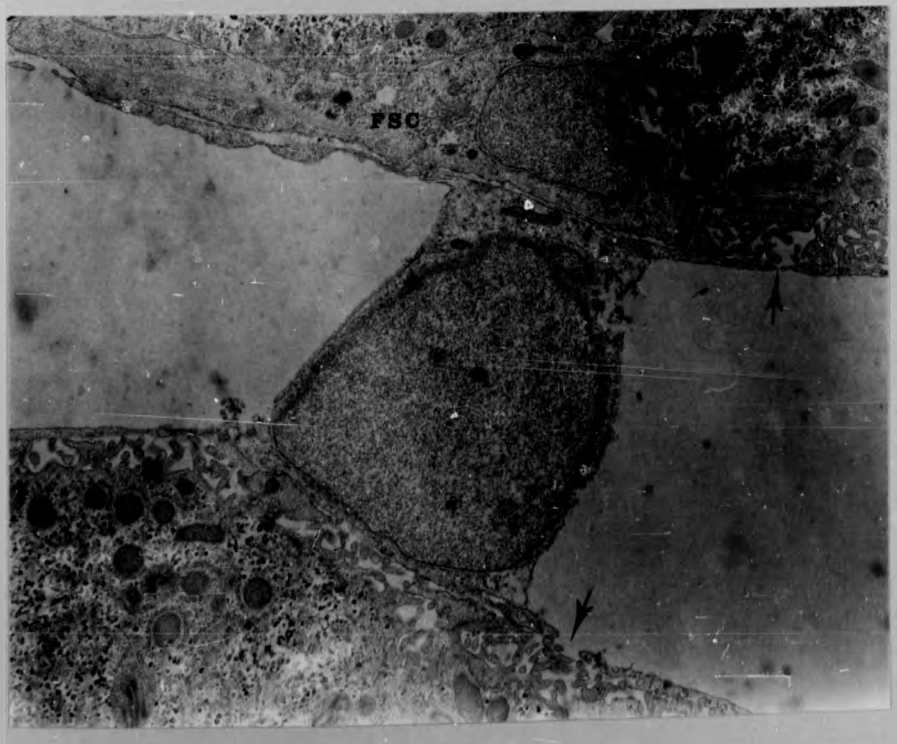

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connected up with ductules comprised of 2 duct cells (Fig. 19) which eventually anastomosed to form typical bile ducts (Fig. 20), situated in connective tissue, and surrounded by periduct cells.

In plaice, intra-cellular bile canaliculi were not as frequent an observation as in either bream or roach, although intra-cellular canaliculi, contributed to by up to 4 hepatocytes, were more frequently seen (Figs. 21 & 22).

Histiocytic cells, usually containing cellular breakdown products, were often found in close association with those ductules composed of 2 or more cells. They were easily recognised by virtue of their comparative electron-lucency and their nuclear chromatin, which was denser, and had a far more patchy distribution. Some cells were small and rounded, while others had long processes inserting either between the adjacent hepatocytes, or around the duct cells.

iii Sinusoidal lining and associated cells.

As described above, the sinusoids were fewer in number than in mammals. They were lined by endothelial cells (Fig. 23) whose nuclei were often very prominent, and in favourable

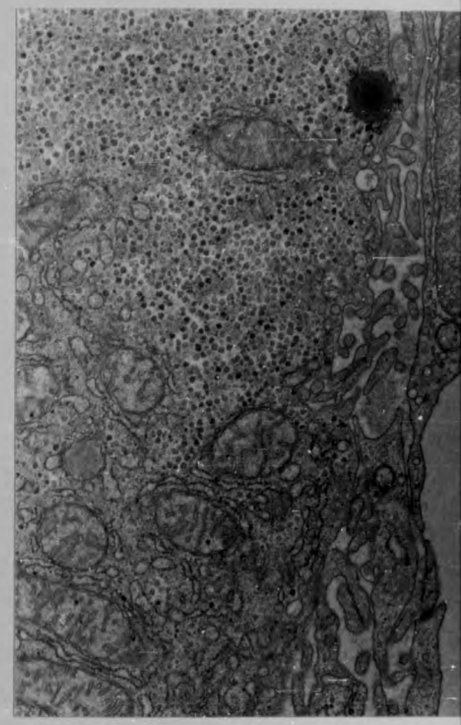

Fig. 25 Electron micrograph of roach liver showing a sinusoid lined by a fenestrated endothelium (arrows) and surrounded by the Space of Disse. Within this Space, observe the numerous projections from surrounding hepatocytes, and material of a buffy nature.

x 21,000

Fig. 26 Note the fenestrations in the sinusoidal lining. Observe within the Space of Disse a cytoplasmic projection from a fat-storing cell (F.S.C.) and material resembling collagen.

x 12,000

a sinusoid lined

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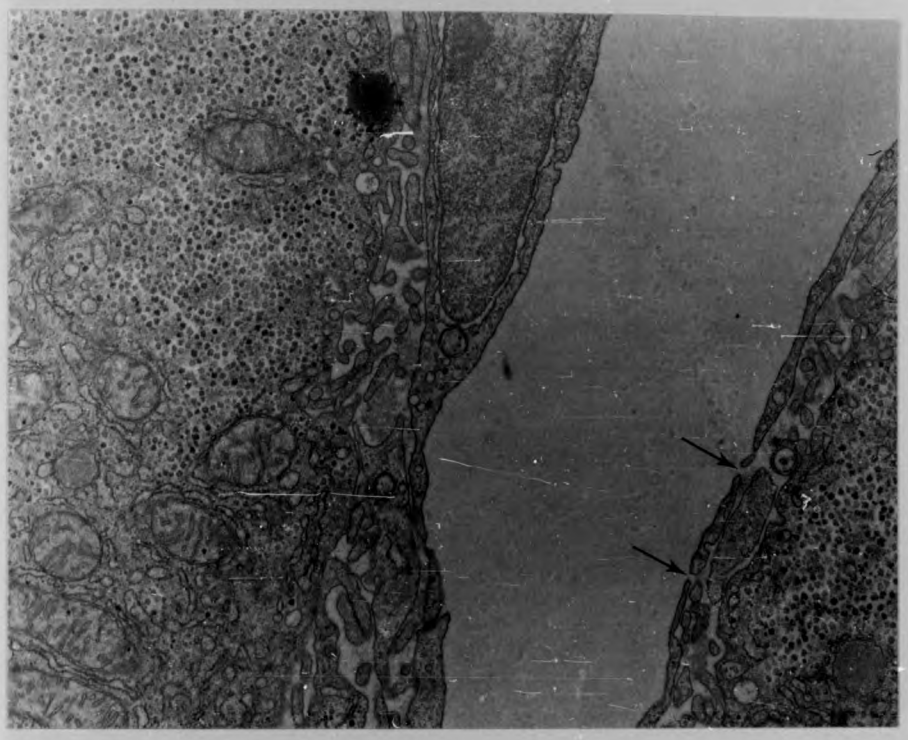

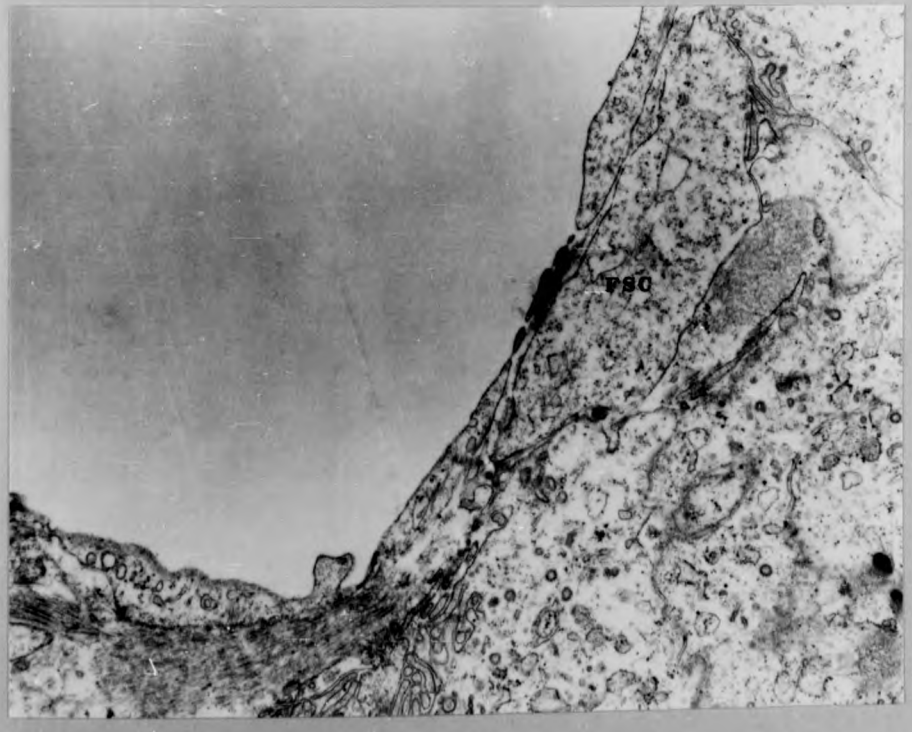

lining. Observe

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Fig. 27 Electron micrograph of plaice liver showing the presence of fat droplets within both hepatocytes (arrows) and fat-storing cells neighbouring a sinusoid.

x 7,500

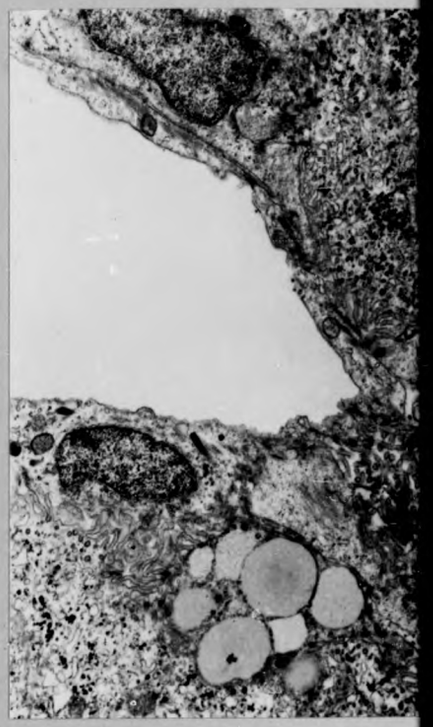

Fig. 28 Observe the cytoplasmic projections from this fat-storing cell extending round the sinusoid, beneath the endothelial lining. Note the pinocytotic activity (arrows) in the F.S.C. and the large fat droplet indenting the nucleus.

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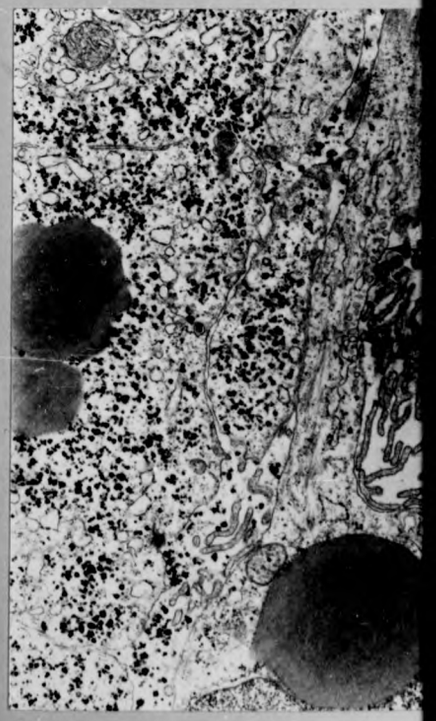

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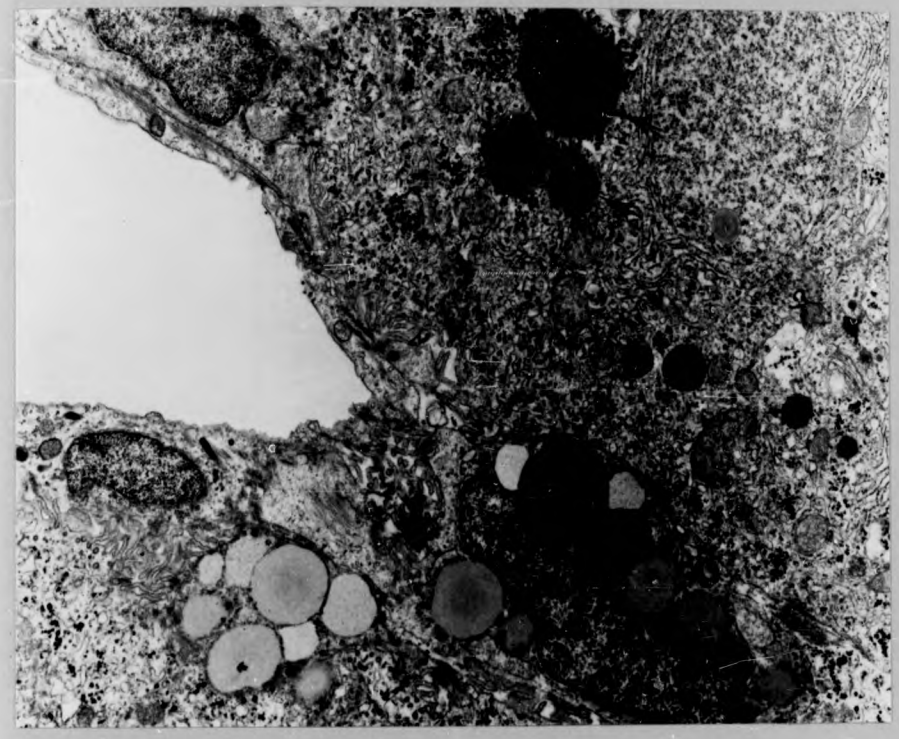

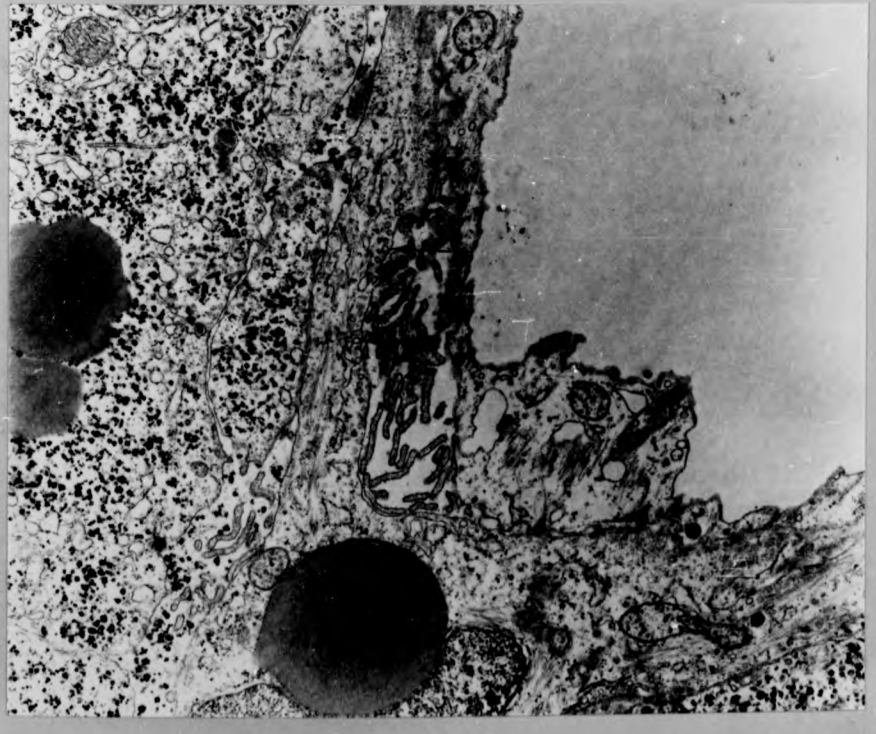

this fat-storing cell

endothelial lining.

the F.S.C. and the

sections, these cells could sometimes be seen to span a sinusoid (Fig. 24). The cytoplasmic processes which comprised the majority of the sinusoidal lining showed fenestrations (Figs. 25 & 26) but these were not as prominent or as extensive as those described in the rat (Wisse, 1974).

Underlying this relatively poorly fenestrated lining was the Space of Disse (Fig. 23) which contained cytoplasmic processes from 3 sources:-

a. Fat-storing cells (F.S.C.) (Figs. 24, 27, 28). These were very numerous and extensive, often running up to half way round the sinusoid.

b. Microvilli from the surrounding hepatocytes, constituting the majority of the cell processes (Fig. 25).

c. Occasional small projections from the endothelial cells themselves.

Apart from these cellular inclusions, the Space of Disse also

Fig. 29 Electron micrograph of plaice liver showing 2 F.S.C. within the Space of Disse, and an overlying endothelial cell nucleus. Observe the collagen (arrow) within the Space of Disse and the considerable amounts of fibrillar material within the cytoplasm of the F.S.C.

x 12,000

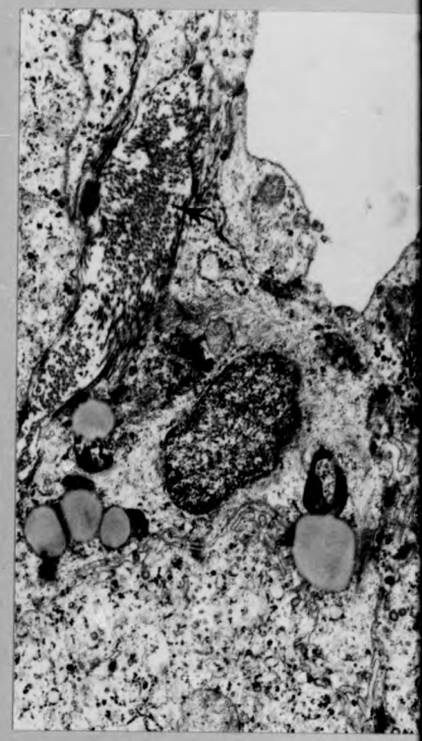

Fig. 30 Electron micrograph of liver from a plaice sacrificed 12 hours after the intra-venous injection of colloidal carbon. Observe the presence of carbon (arrows) within vacuoles in the cytoplasm of the F.S.C.

x 18,000

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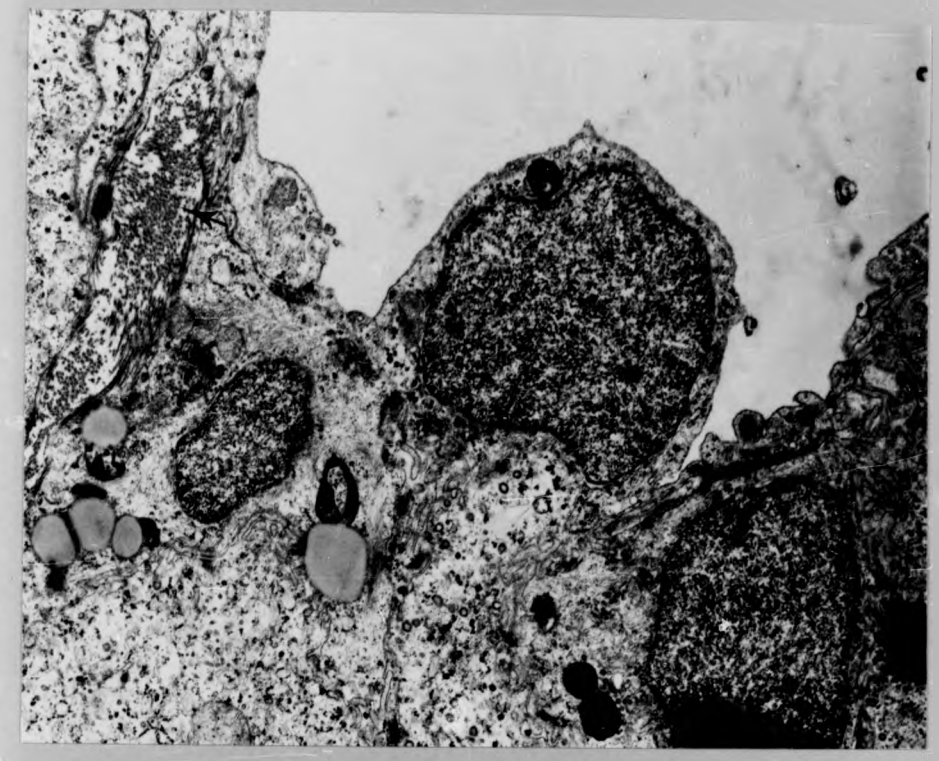

e sacrificed 12 hours

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contained material of a buffy consistency (Fig. 25), microtubules and filaments, some possibly associated with the FSC. In poorly fed fish, there were extensive amounts of collagen associated with the Space of Disse, often running up between hepatocytes. The following desmosomal attachments were seen:-

a. Between FSC and FSC
b. Between FSC and hepatocyte
c. Between FSC and endothelial cell
d. Between endothelial cell and endothelial cell

The FSC were particularly numerous, their cytoplasm containing amounts of collagen and large lipid inclusions which often indented the nucleus (Figs. 27 & 29). Some of their cell processes were in very close association with the adjacent hepatocytes, the respective plasma membranes being separated by an extremely narrow gap. FSC were seen not only in the Space of Disse, but extending up between the hepatocytes and were sometimes situated some distance from a sinusoid.

Carbon Injected Fish

As stated previously, only at high dose levels (greater than 16 mg/100g) was there any macroscopic blackening of the liver.

The carbon in this case was found in the Space of Disse, mainly within the FSC (Fig. 30), or to a lesser extent within the endothelial cells. Some of the carbon had been endocytosed by the hepatocytes, although in toluidine blue stained sections, this did not appear as black, but brown, due to its very finely scattered distribution i.e. it was not within phagosomes.

Even in fish which had received only a moderate dose of carbon so that none was to be seen in the hepatocytes, and very little in the endothelial cells,at an ultrastructural level, the FSC were consistently observed to contain appreciable amounts. Here it was seen within vacuoles containing in addition, dense osmiophilic material with the appearance of cell breakdown products.

B The Heart

The following description applies to both atrium and ventricle.

Normal Fish

i. General histology.

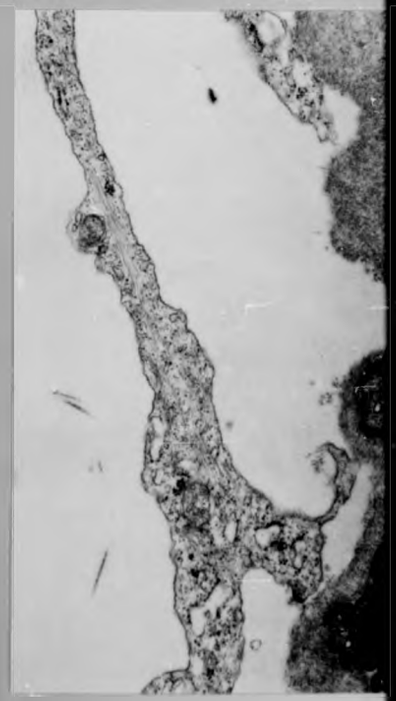
The basic arrangement was that of myocardial cells sandwiched

Fig. 31 Section through plaice atrium to show large sub-endocardial space (S.E.S.) containing a lot of collagen. The endocardial cell (E.C.) and the myocardial cell nucleus (N) with its prominent nucleolus are both well illustrated.

x 7,500

Fig. 32 Plaice ventricle to show sub-epicardial space. Observe that the buffy material within the space has condensed onto the sarcolemma.

x 18,000



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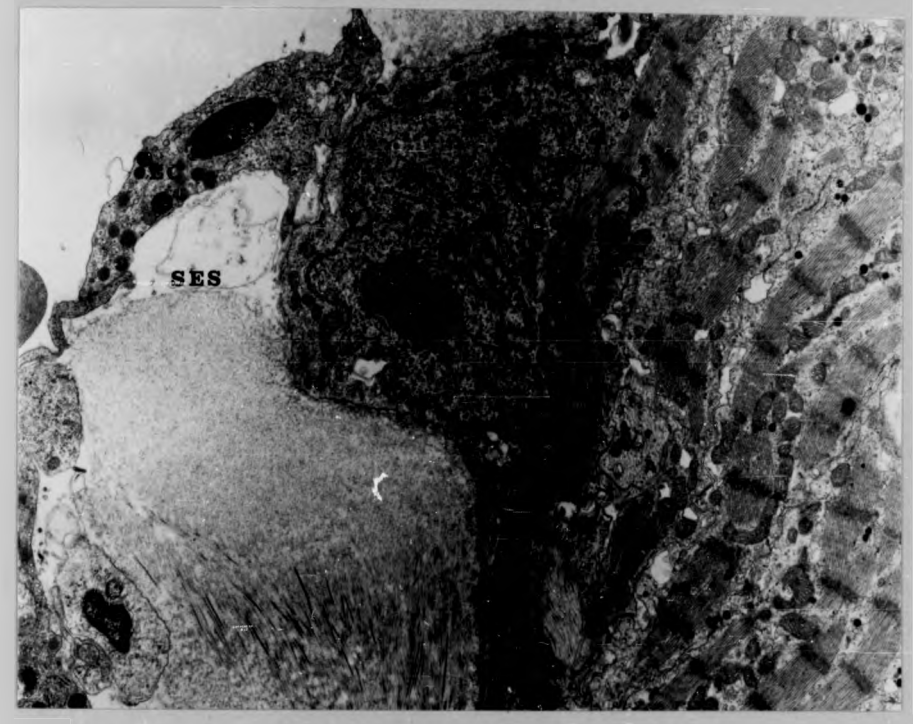
with its prominent



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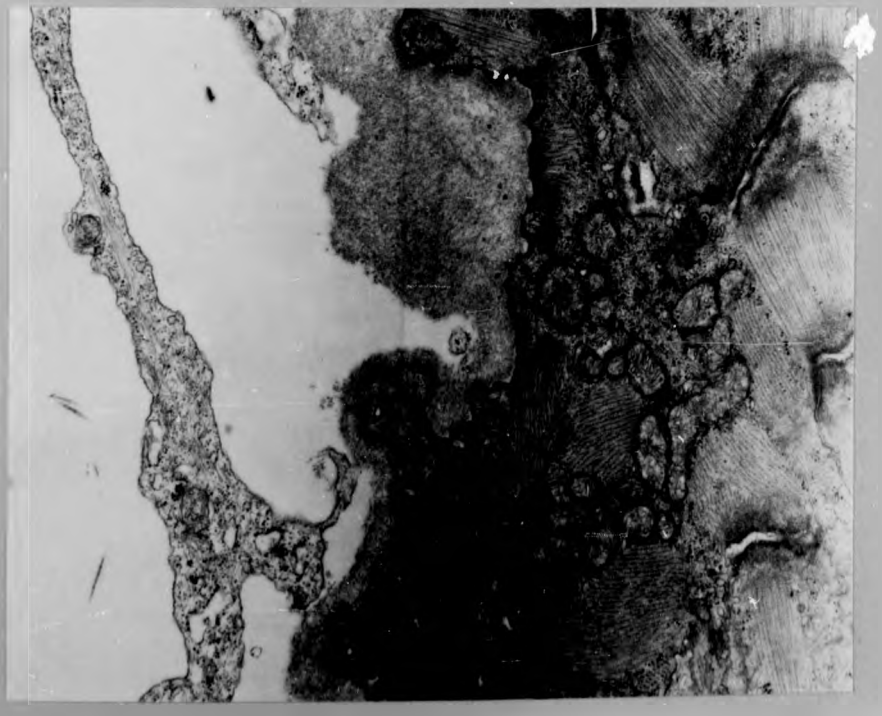

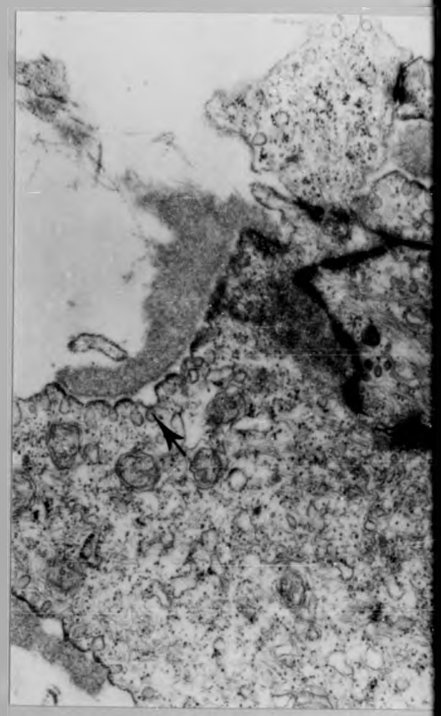

Fig. 33 Electron micrograph of plaice ventricle showing the desmosomal attachments between epicardial cells. Observe the buffy material condensed onto the inner aspect of the plasma membrane. (Compare with Fig. 32 where it is condensed onto the sarcolemma). Note also the extensive pinocytotic activity at this site (arrows).

x 18,000

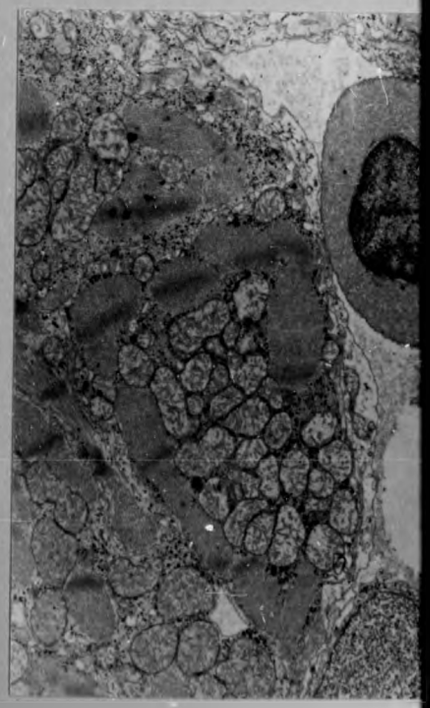

Fig. 34 Electron micrograph of plaice ventricle showing an erythrocyte (E) within one of the rarely encountered coronary vessels, possibly existing merely as a gap between opposing endocardial-lined muscle fibres.

x 7,500

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e the buffy

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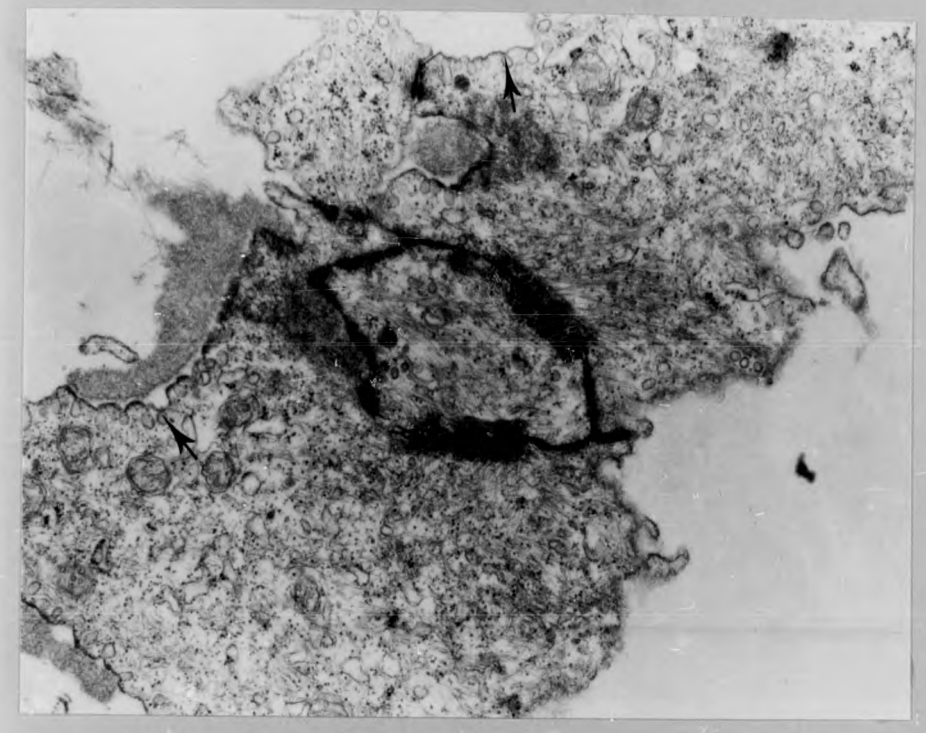

ding an erythrocyte

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Fig. 35 Plaice ventricle showing tightly packed mitochondria between the myofibrils. Observe the appearance of the Z bands (arrows) and the presence of an M band (M).

x 18,000

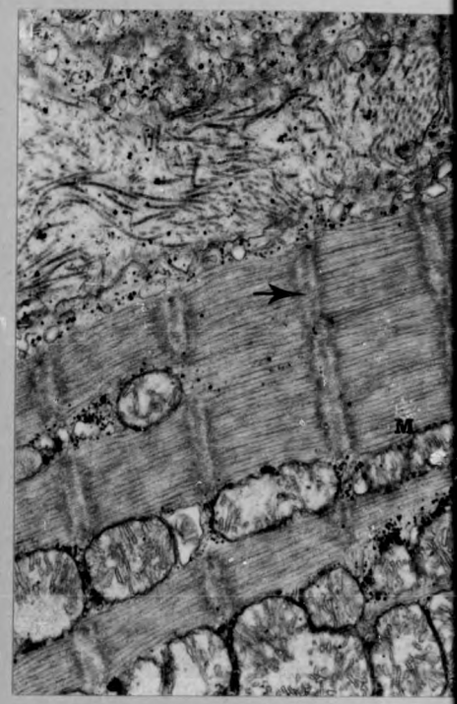

Fig. 36 Plaice ventricle showing an intercolated disc. Observe the mass of fine fibres, the fascia adhaerentes on either side of the disc.

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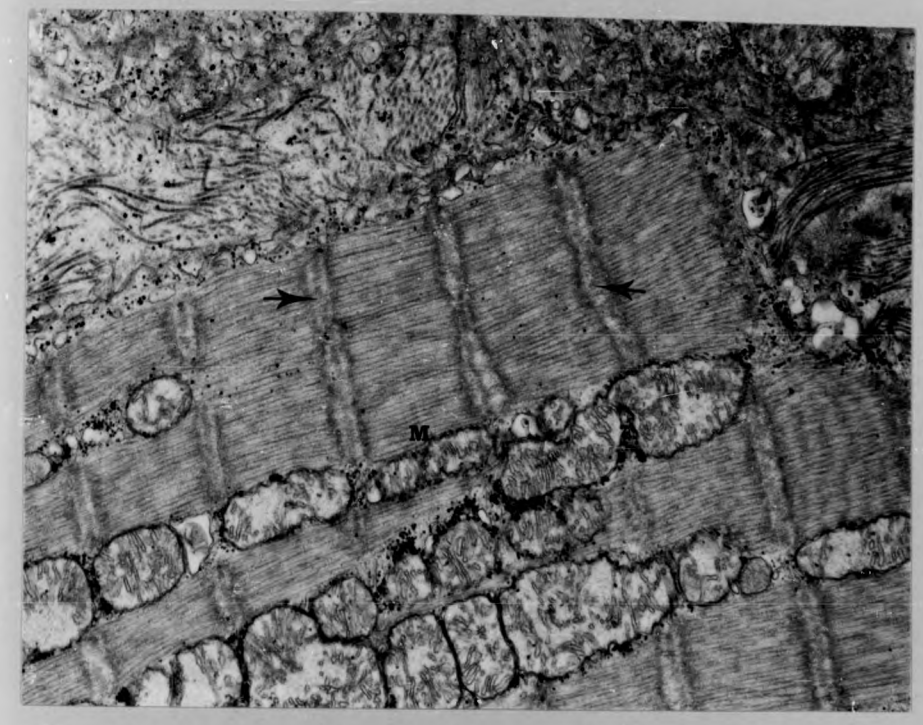

c. Observe the

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between endocardium and epicardium, with both a sub-endocardial and a sub-epicardial space (Figs. 31 & 32). The endocardium was thrown into deep folds or lamellae, so that in transverse section the cardiac tissue was a meshwork of endocardial-lined myocardial trabeculae. (This basic arrangement holds true for plaice, trout and turbot. The following description however at an ultrastructural level, applies to plaice only).

The ventricular sub-epicardial space was more extensive than the atrial, and contained considerable amounts of collagen, many macrophages, and a buffy material which was condensed so as to form a thick layer on the sarcolemma (Figs. 32 & 33). This latter showed very extensive pinocytotic activity, probably directed towards endocytosing the buffy material because the pinocytotic vesicles contained material of an identical density and organisation.

No coronary blood supply was present in the atrium, and only a few vessels were present in the ventricle (Fig. 34), but these were probably merely gaps between apposing muscle fibres. In the myocardial cell itself, the following striations were visible:-

1. Z bands. These consisted of 2 dark lines with a clear band in between, which was itself bissected by a dark line (Fig. 35).

Fig. 37 Plaice ventricle showing intercalated disc. Observe the connections with the sarcoplasmic reticulum (S.R.)

x 45,000

Fig. 38 High power electron micrograph of an intercalated disc from plaice ventricle. Observe the desmosomes (d) and gap junctions (arrows) along the length of the disc.

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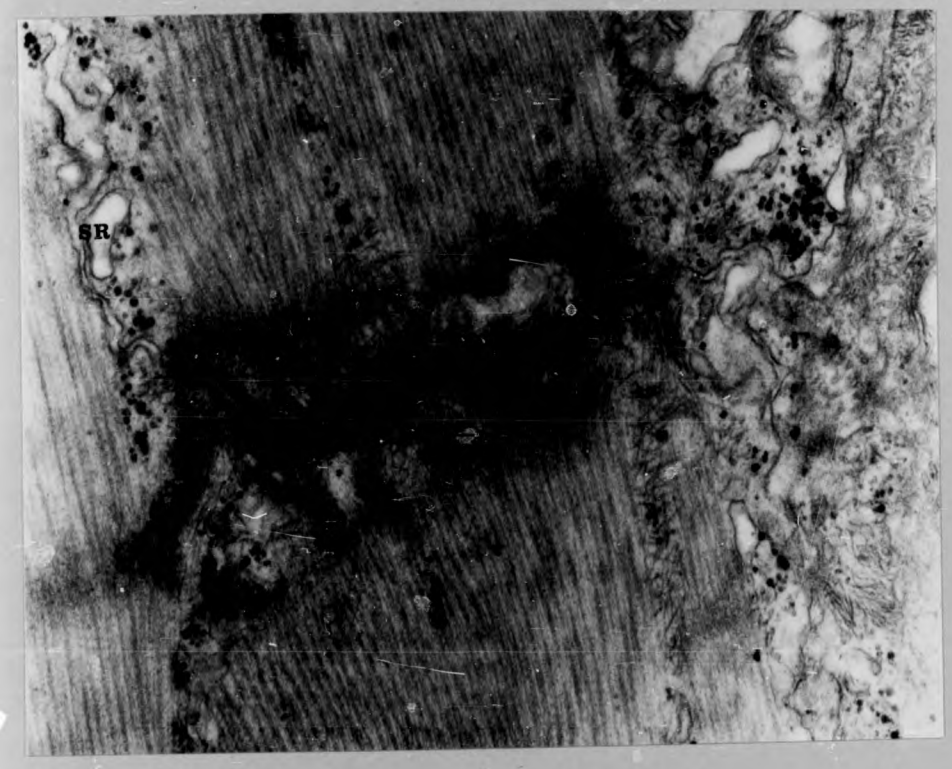

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This appearance was probably related to the state of contraction of the muscle at fixation.

2. A bands. Although visible, they were not very prominent.

3. H and M bands. This latter M band was very prominent (Fig. 35).

The regions of intercellular connection, the intercalated discs (Fig. 36) were very prominent slightly wavy lines composed of the following structures:-

a. fascia adhaerentes (cardiac adhesion, plaques, intermediate junctions). This was a dense meshwork of fine fibres extending on either side of the disc (Figs. 36 & 37), intermingling with the contractile fibres of the cell on one side, and inserting into the apposed plasma membranes on the other.

b. desmosomes

c. gap junctions (nexuses)

Both of these were present within the fascia adhaerentes (Fig. 38) and appeared almost to alternate with each other along the length of the disc. Desmosomes were also present between the plasma membranes of the longitudinal axes of adjacent cells.

Fig. 39 Plaice ventricle showing an irregularly-shaped myocardial nucleus (N) surrounded by mitochondria; a small cluster of dense-cored vesicles (V) surrounded by glycogen granules; and extensive amounts of collagen throughout.

x 12,000

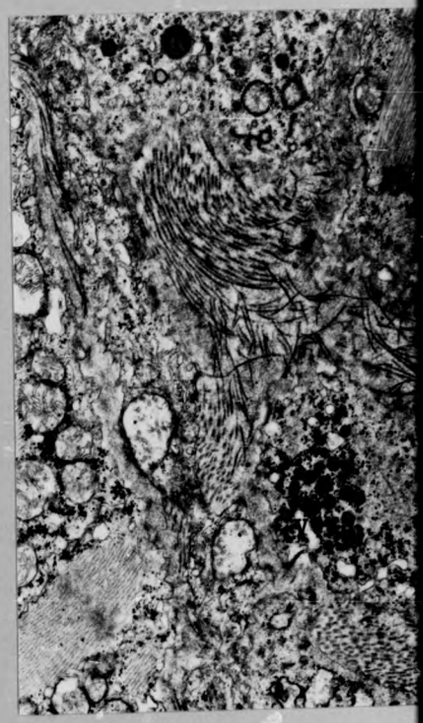
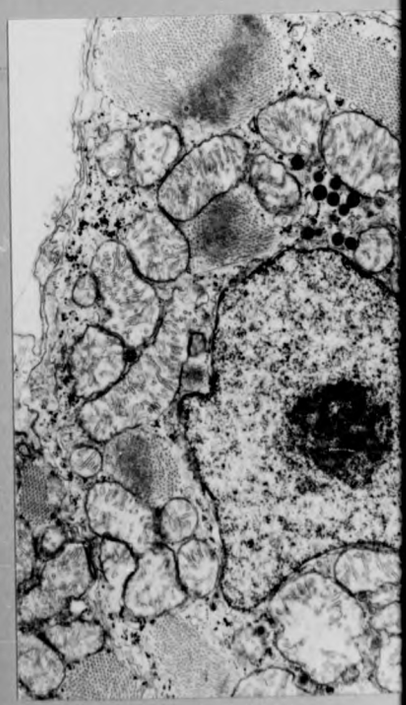


Fig. 40 A single myocardial cell from plaice ventricle. Observe the centrally-placed nucleus with its prominent nucleolus, and the large mitochondria distributed amongst the myofibrils, most of which are cut in T/S.

x 12,000



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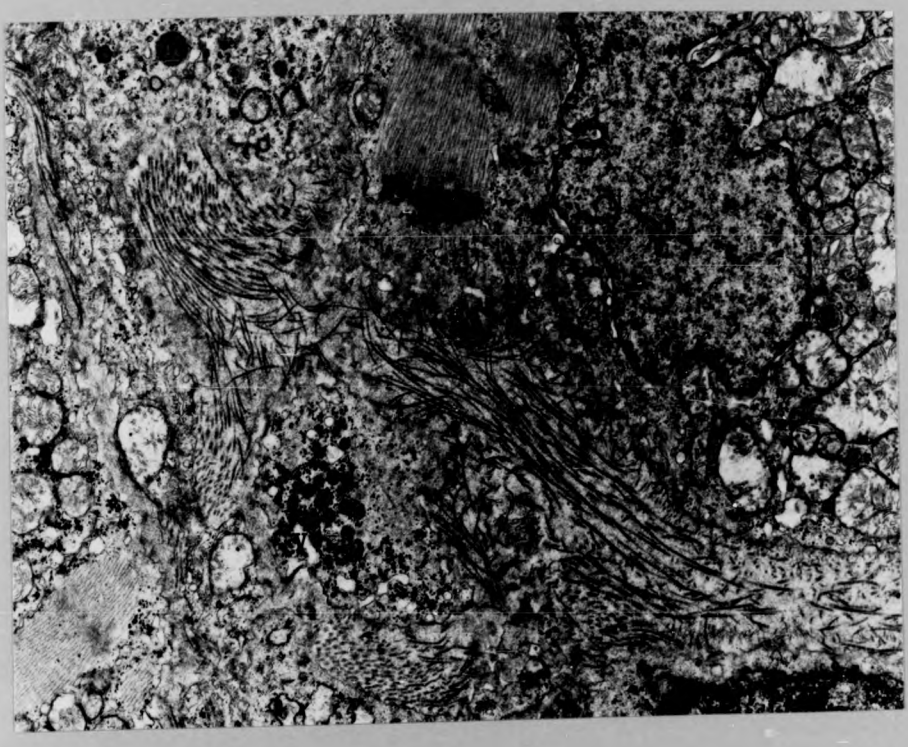

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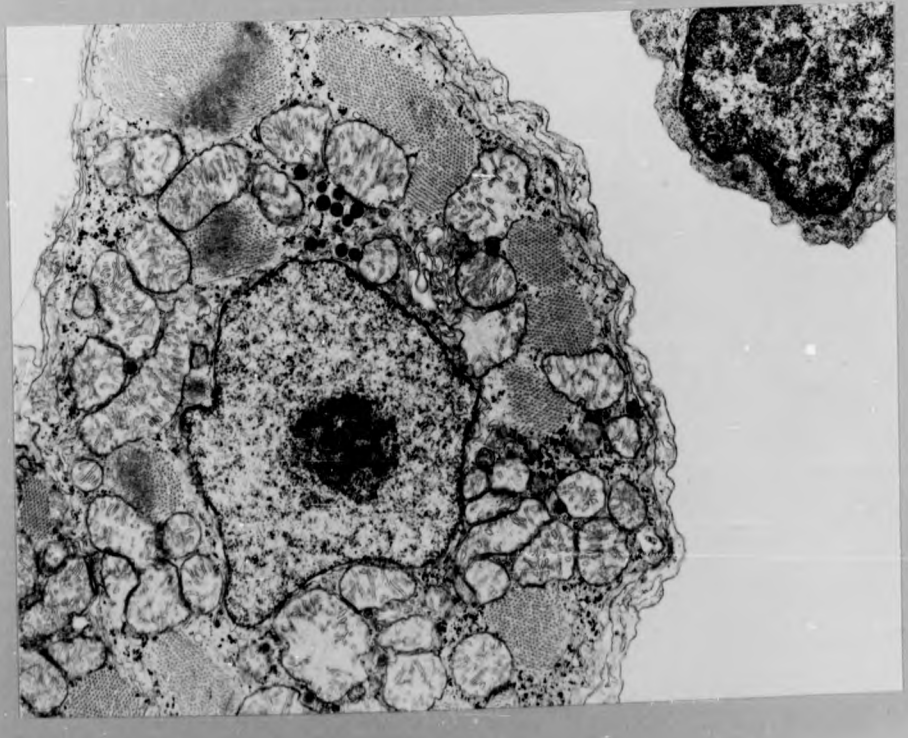

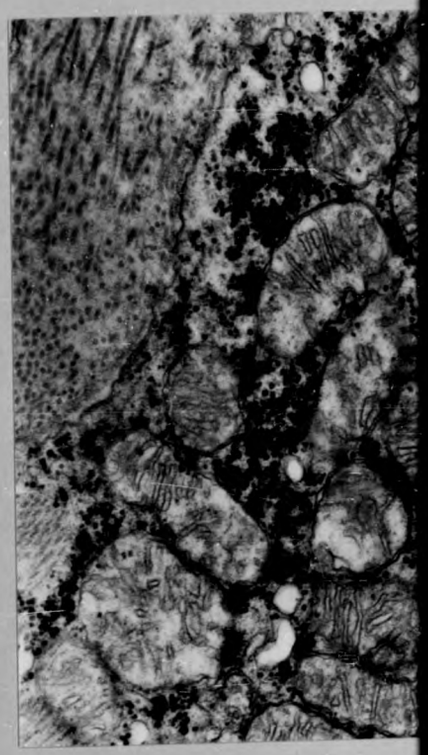

Figs. 41 & 42 Plaice ventricle to show groups of mitochondria between the myofibrils. The top micrograph also shows the presence of densely-staining glycogen granules.

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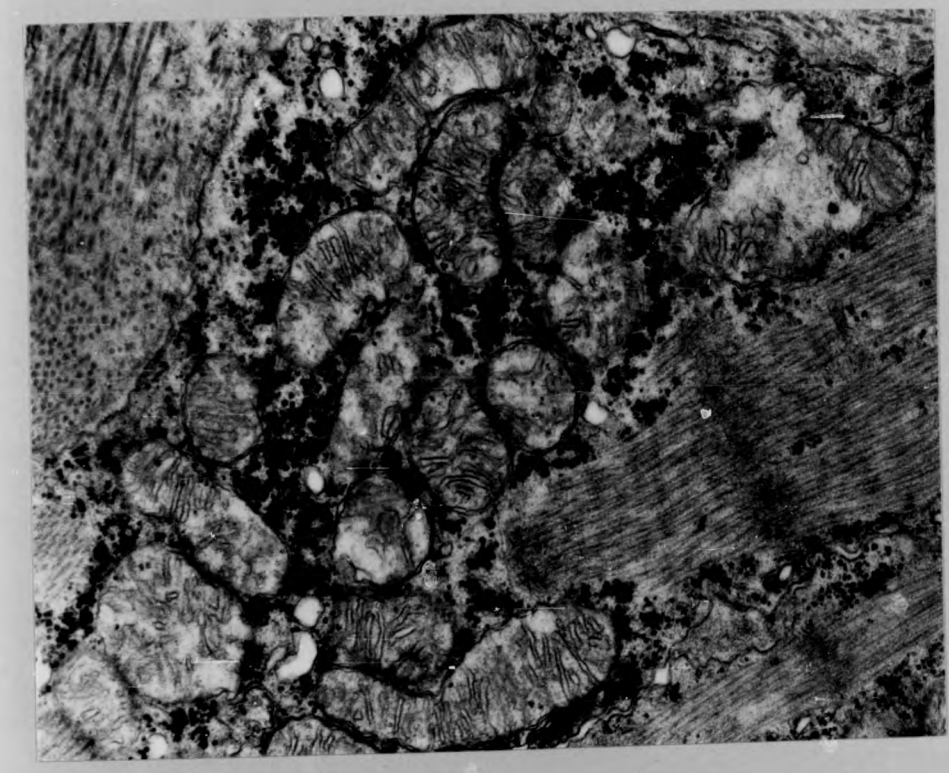
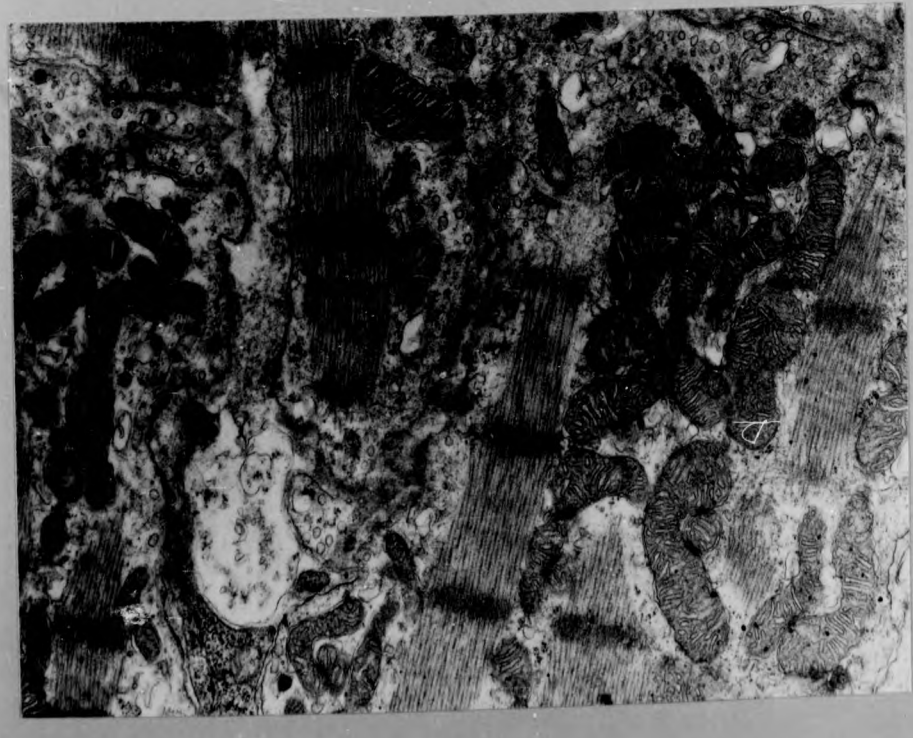
x 18,000





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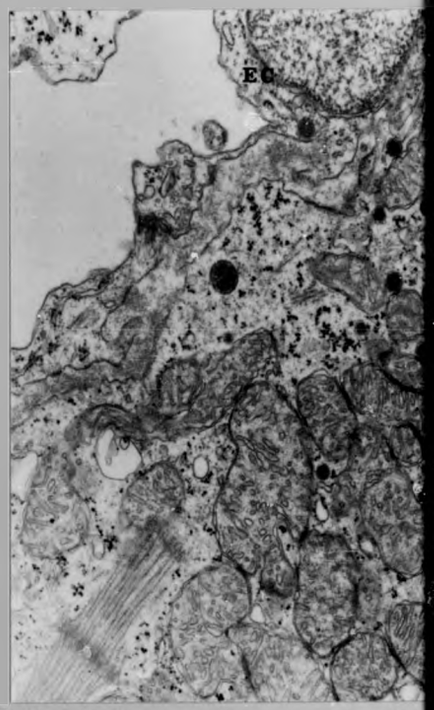

Fig. 43 Plaice atrium showing mitochondria packed between the few myofibrils. Observe the endocardial cell (E.C.) and the sub-endocardial space.

x 18,000

Fig. 44 Plaice ventricle showing 2 adjacent myocardial cells. Note the group of dense-cored vesicles in the lower (V) and the endocardial cell (E.C.) with limited cytoplasm covering the other.

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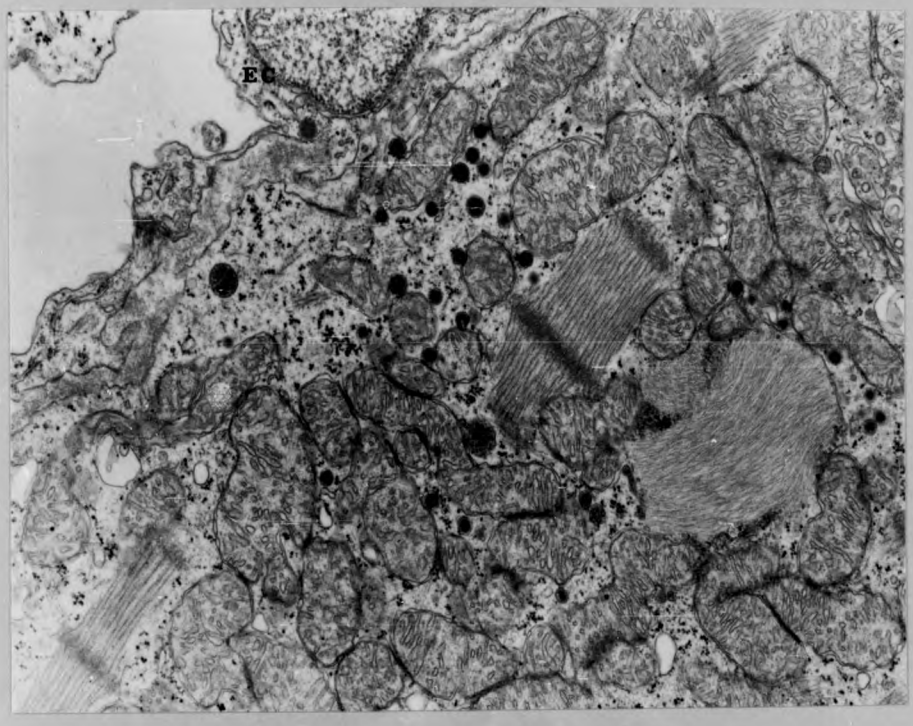

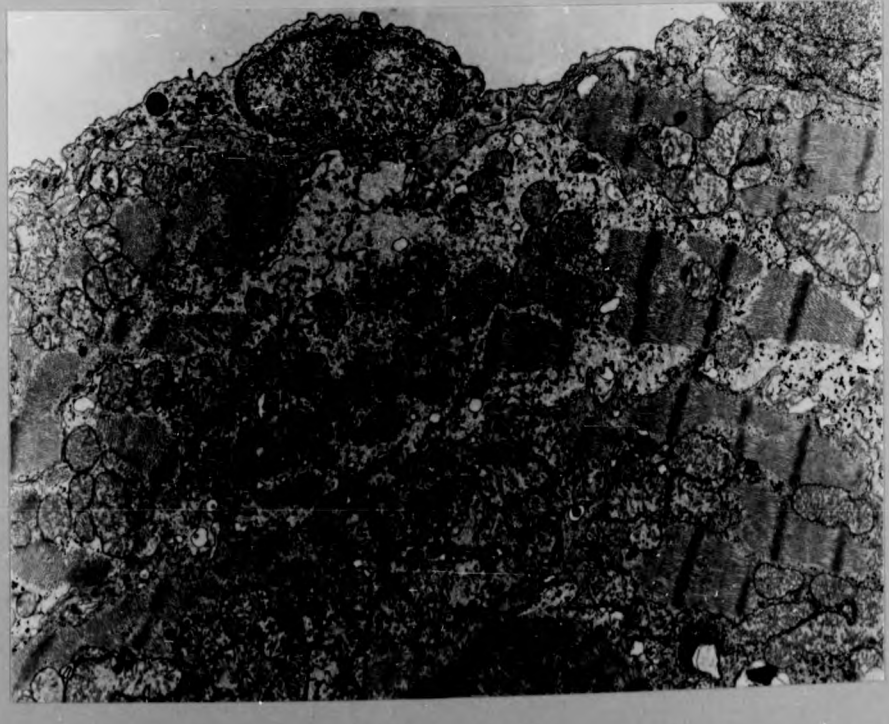

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No transverse tubular system was present as is obvious in skeletal muscle, and the sarcoplasmic reticulum showed only moderate development (Figs. 36 & 37). The often centrally placed nuclei were usually irregularly shaped (Figs. 39 & 40) and had a condensation of chromatin not only round their periphery, but also round the prominent nucleolus.

Vast numbers of tightly packed mitochondria were dispersed between the myofibrils (Figs. 41-43). This was the most prominent cytoplasmic feature, although other components were present:-

1. Small clusters of dense cored vesicles (Figs. 39 & 44). (These were thought by Santer & Cobb (1972), to contain biogenic amines).

2. Masses of glycogen granules, usually surrounding either the dense cored vesicles (Fig. 39), or the mitochondria (Fig. 41).

3. Varying amounts of collagen (Fig. 39).

4. Dense bodies (lysosomes).

Fig. 45 Plaice ventricle showing a typical endocardial cell with limited cytoplasm containing a few dense bodies (arrows). The myofibrils have been cut in T/S.

x 13,000

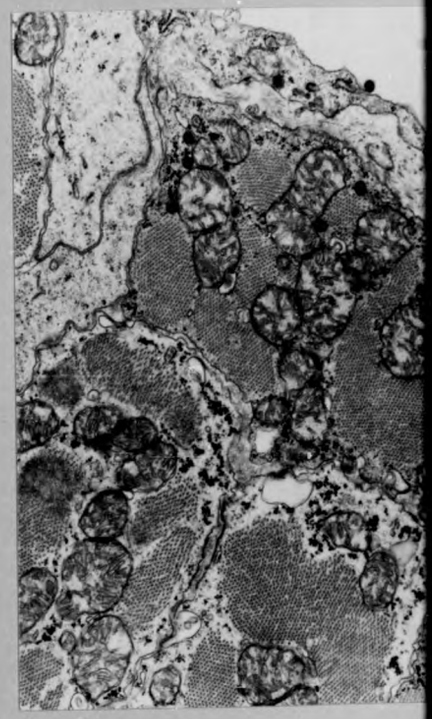
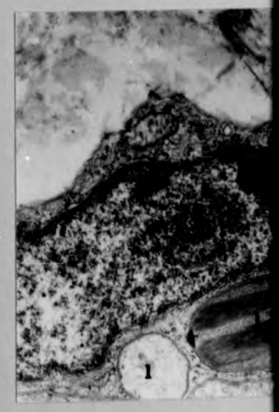


Fig. 46 Endocardial cell from plaice atrium. The cytoplasm contains 3 large lysosomes (l) in various stages of development. The central one has inclusions of a fibrillar nature.

x 13,000



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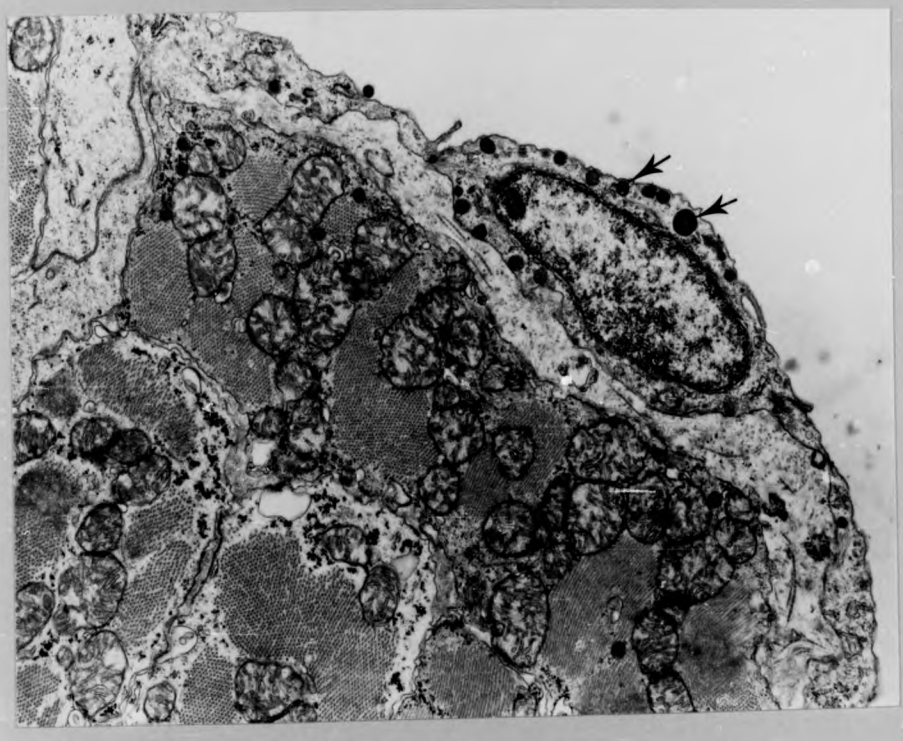

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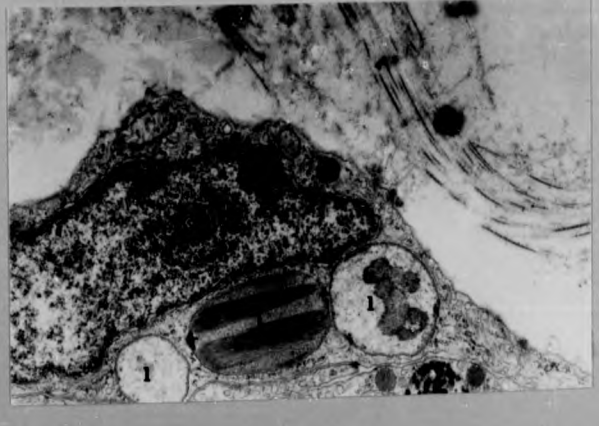

Histiocytes, situated between the myocardial cells were occasionally encountered.

A comparison of atrial and ventricular tissue:

Ventricular cells had larger numbers of tightly packed mitochondria but atrial cells contained more of the dense cored vesicles.

The major difference between atrium and ventricle however lay in the endocardial lining, the cells of which in the atrium, were much larger with a more "active" looking cytoplasm. (Compare Figs. 45 and 46) By this term is meant that larger numbers of both primary and secondary lysosomes were present, plus a better developed golgi apparatus. Some of the larger secondary lysosomes had a fibrillar appearance (Fig. 46), similar to that of the granules of the neutrophils which were often encountered closely associated with the endocardium.

Atrium of Carbon Injected Fish

The plasma membrane of the endocardium was not "sticky" for the colloidal carbon particles, as is the case with mammalian Kupffer cells. This fact was confirmed by employing different

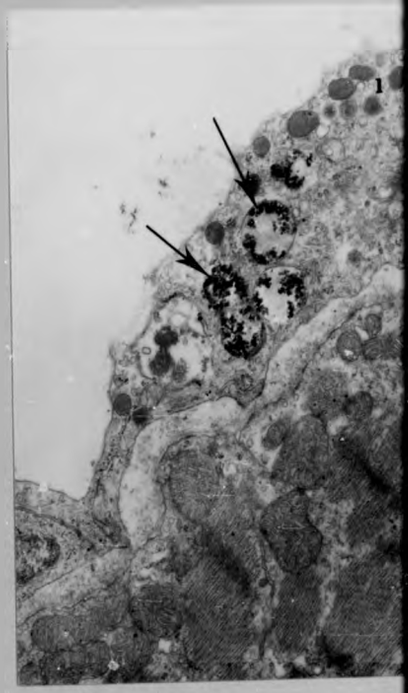

Fig. 47 Electron micrograph of atrium from a plaice sacrificed 15 minutes after the intra-venous injection of colloidal carbon. Observe the presence of carbon-containing vacuoles within the cytoplasm of the endocardial cell (arrows) and the numerous secondary lysosomes (l) lying just beneath the plasma membrane.

x 12,000

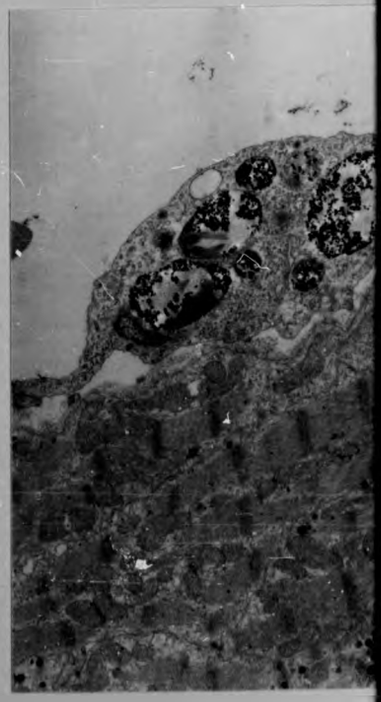

Fig. 48 Atrium from a plaice sacrificed 1 hour after the intra-venous injection of colloidal carbon. Observe the large amounts of carbon in the cytoplasm of the endocardial cell.

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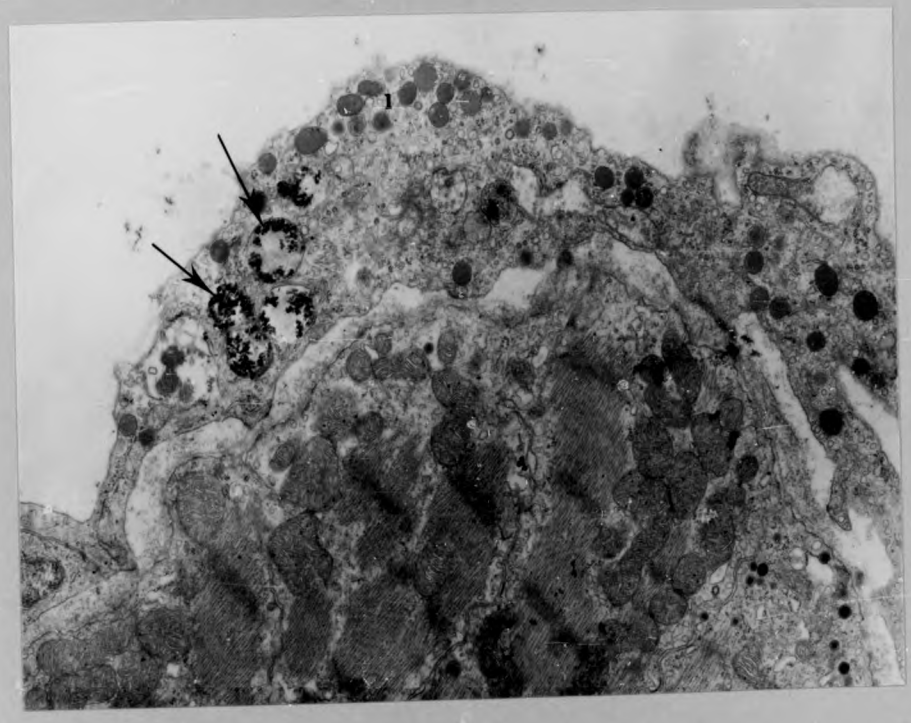

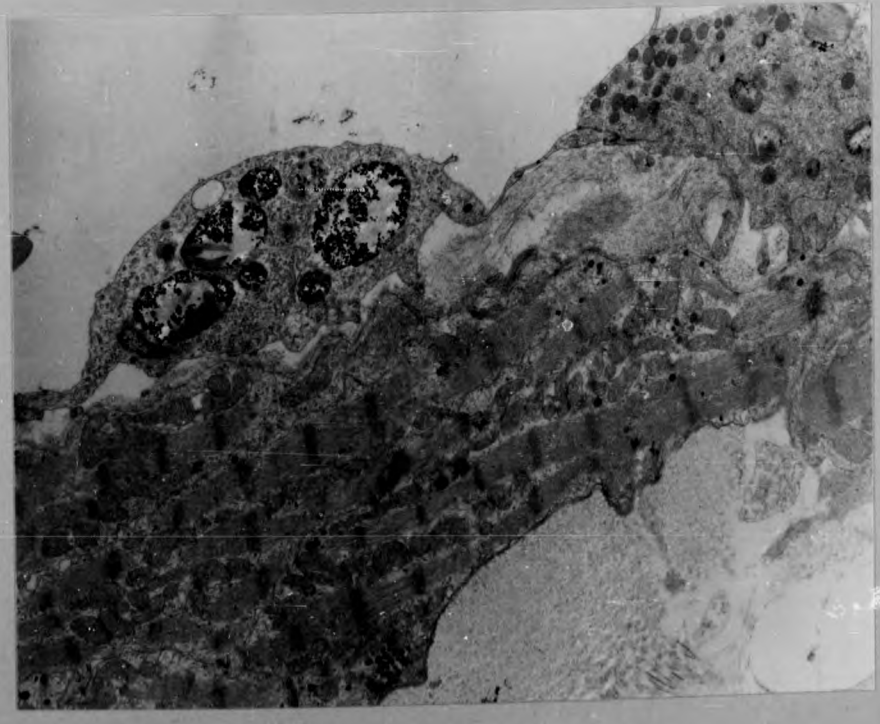

Fig. 49 Atrial endocardial cell from a plaice sacrificed 1 hour after the injection of carbon. Observe the golgi apparatus (G) actively budding off primary lysosomes, and the extensive pinocytotic activity at the plasma membrane (arrows). One of these half-formed vesicles contains carbon particles.

x 30,000

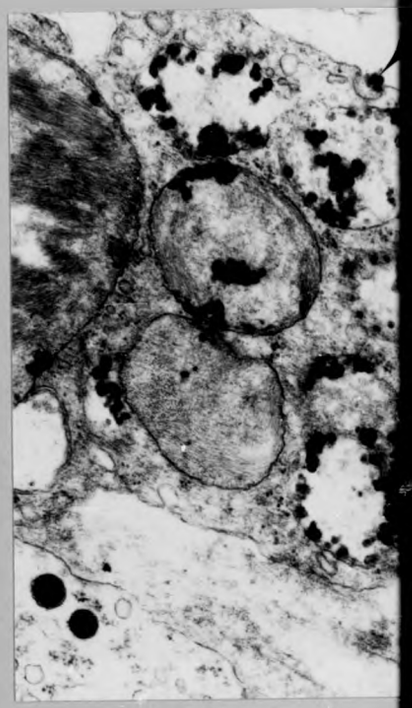

Fig. 50 Atrial endocardial cell showing large carbon-containing phagolysosomes, (Ph).

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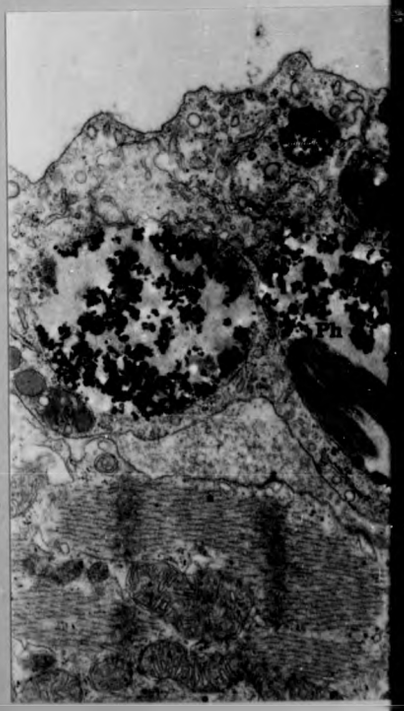

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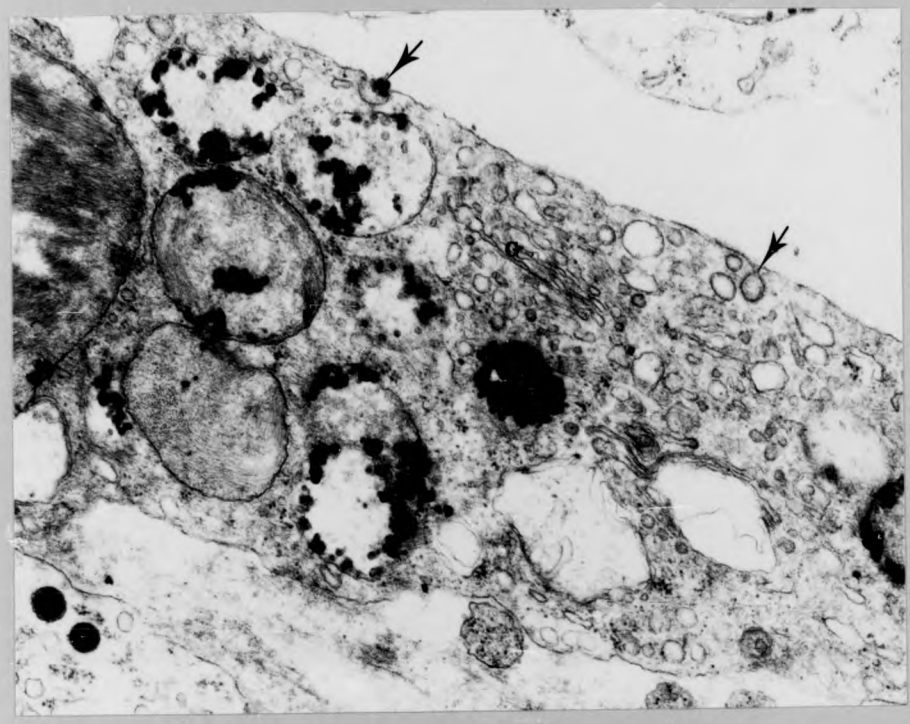

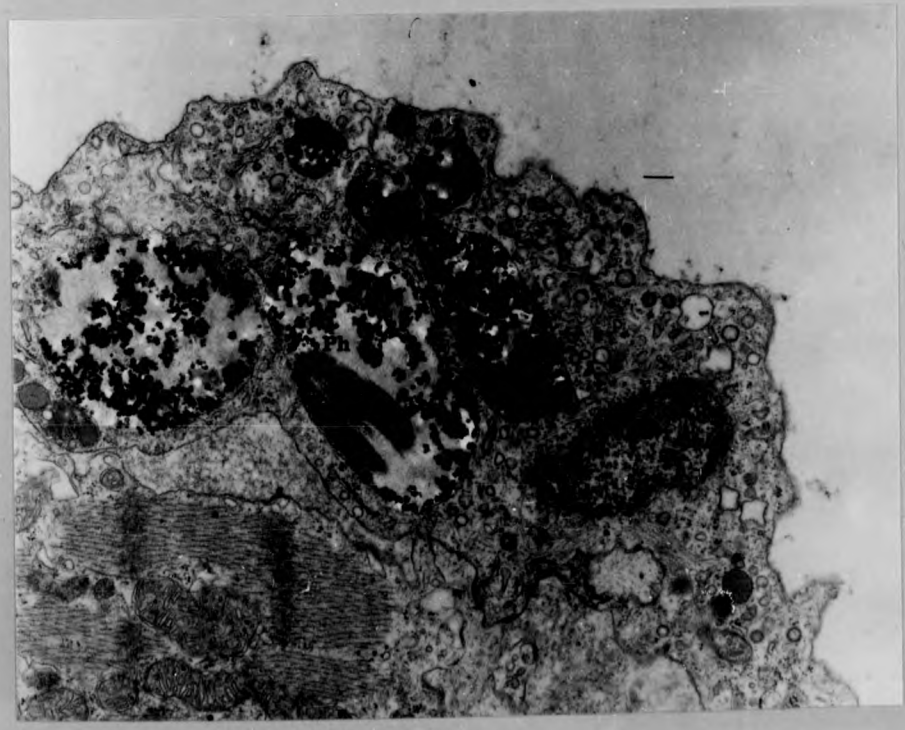

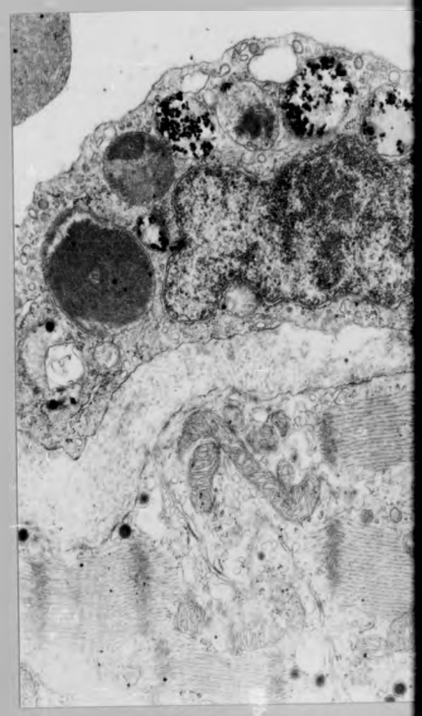

Fig. 51 Atrial endocardial cell from a carbon-injected plaice and adjacent erythrocyte (E). Note the presence of macro-pinocytotic vesicles at the plasma membrane (arrows). Some contain carbon particles while others are endocytosing a buffy material, possibly plasma elements.

x 18,000

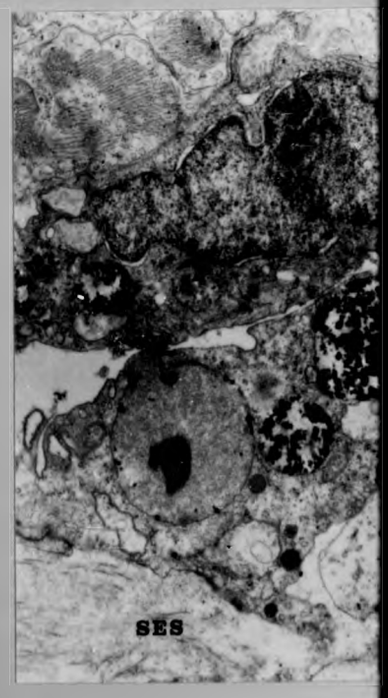

Fig. 52 Atrium from a carbon injected plaice showing 2 apposed carbon-containing endocardial cells. Beneath the lower, in the sub-endocardial space (S.E.S.) note the presence of a sub-endocardial cell (S.E.C.) which has an electron-dense nucleus, an electron-lucent cytoplasm containing a few mitochondria, and a small golgi apparatus.

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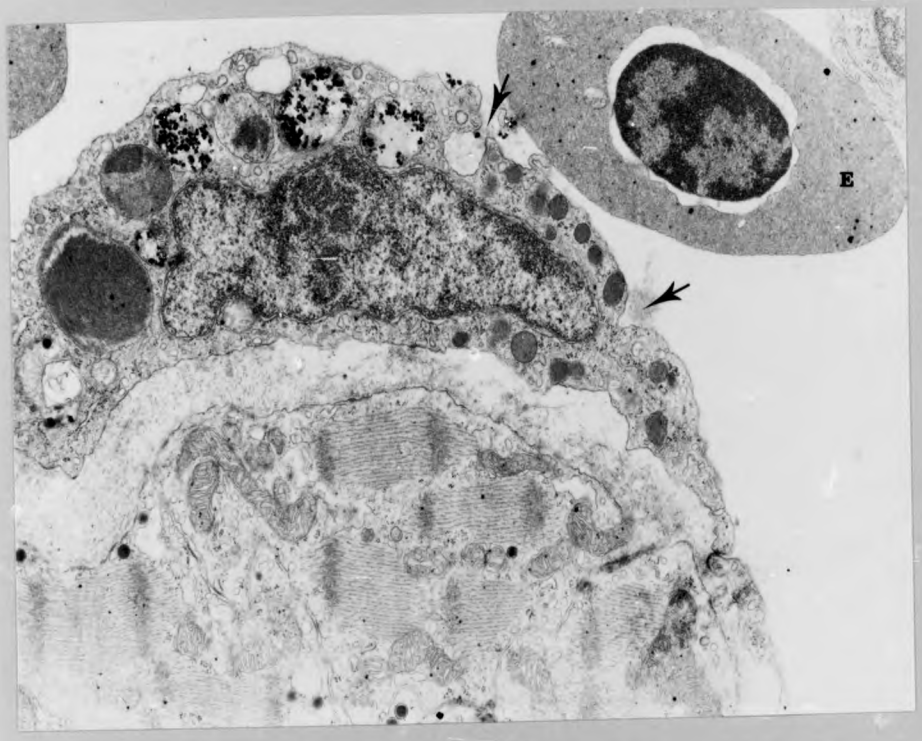

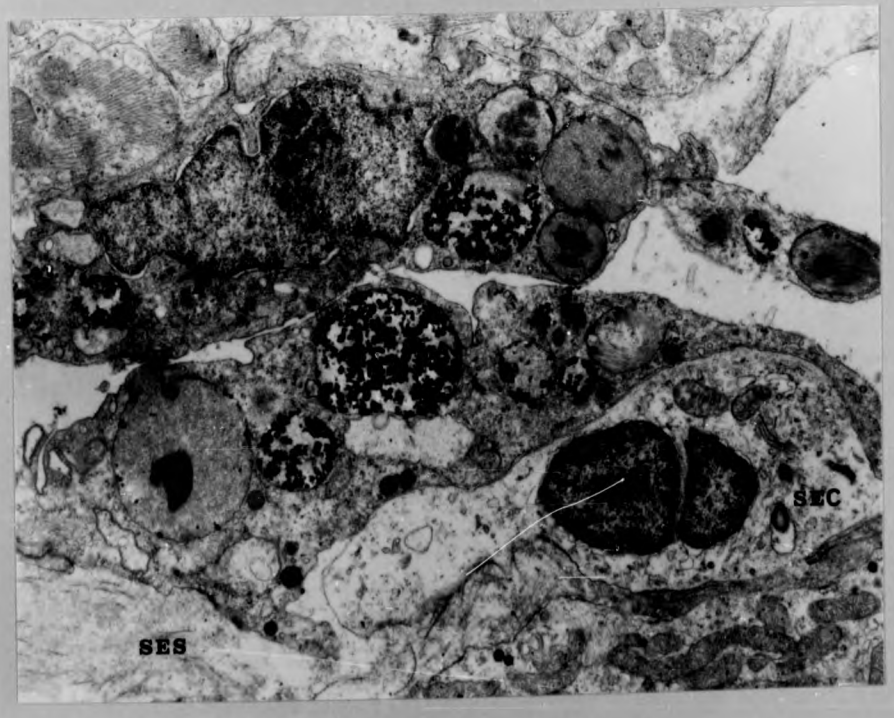

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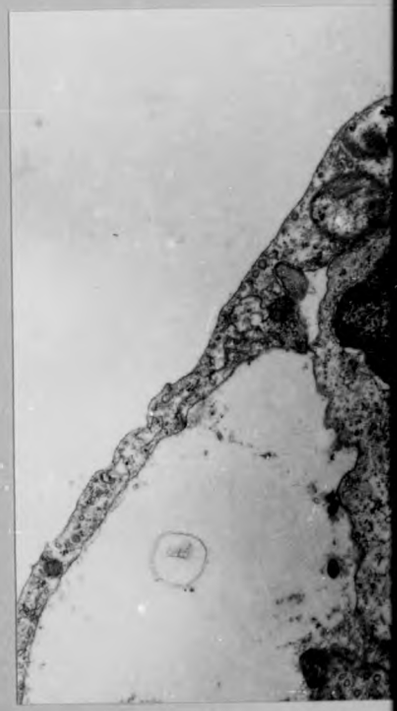

Fig. 53 Atrium from a carbon-injected fish, showing the presence of a sub-endocardial cell (S.E.C.) in an extensive sub-endocardial space.

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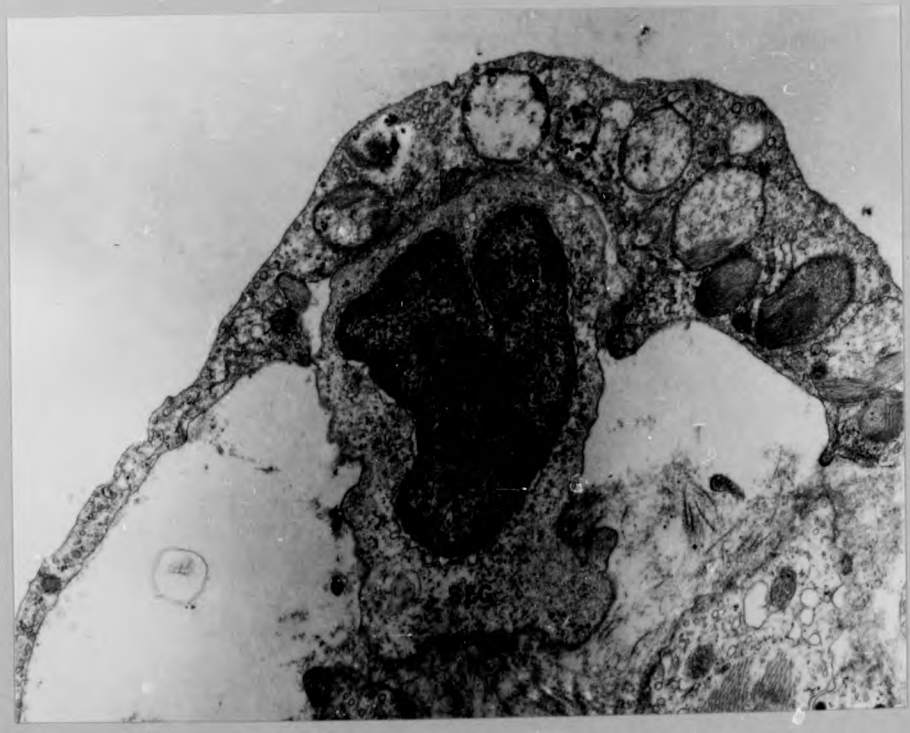

fixation methods e.g. osmium tetroxide alone, which showed that no fuzzy coat existed, to which the carbon particles could have adhered.

Despite this however, the carbon content of the endocardial cells increased during the period 15 minutes up to 8 hours post injection. Most of this carbon was present within phagolysosomes (Figs. 47 & 48) and fusion of phagosomes with secondary lysosomes was frequently observed (Figs. 49 & 50). Micropinocytotic and macropinocytotic vesicles containing a few carbon particles were occasionally seen near the plasma membrane (Figs. 49 & 51).

Sub-endocardial cells were occasionally observed in a sub-endocardial space (Figs. 52 & 53) which appeared to be enlarged to accommodate them. They did not contain any carbon.

C Spleen

Normal Fish (plaice and turbot)

General histology

The splenic capsule was devoid of muscle, and a trabecular

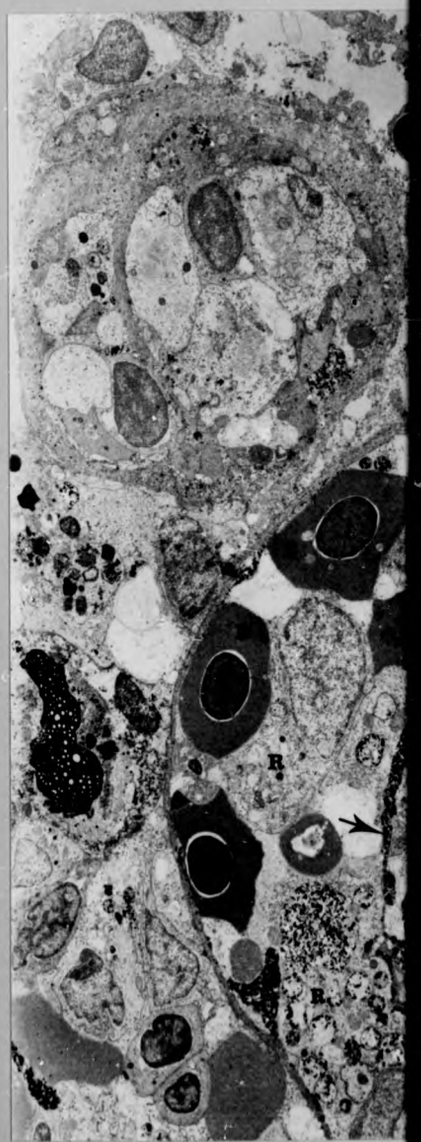

Fig. 54 Electron micrograph of spleen from a plaice sacrificed 1 hour after the intra-venous injection of colloidal carbon, showing 2 closely situated ellipsoids. Observe in the lower and larger of the two, the centrally placed axial vessel (A). A lot of carbon has located to the basement membrane of this vessel (arrows). Within the outer part of the ellipsoid, observe the numerous erythrocytes (E) and the large electron-lucent reticular cells (R) which are in the the process of endocytosing the carbon. Note also the presence of a small cell with long processes (triangle) situated on the basement membrane of the axial vessel.

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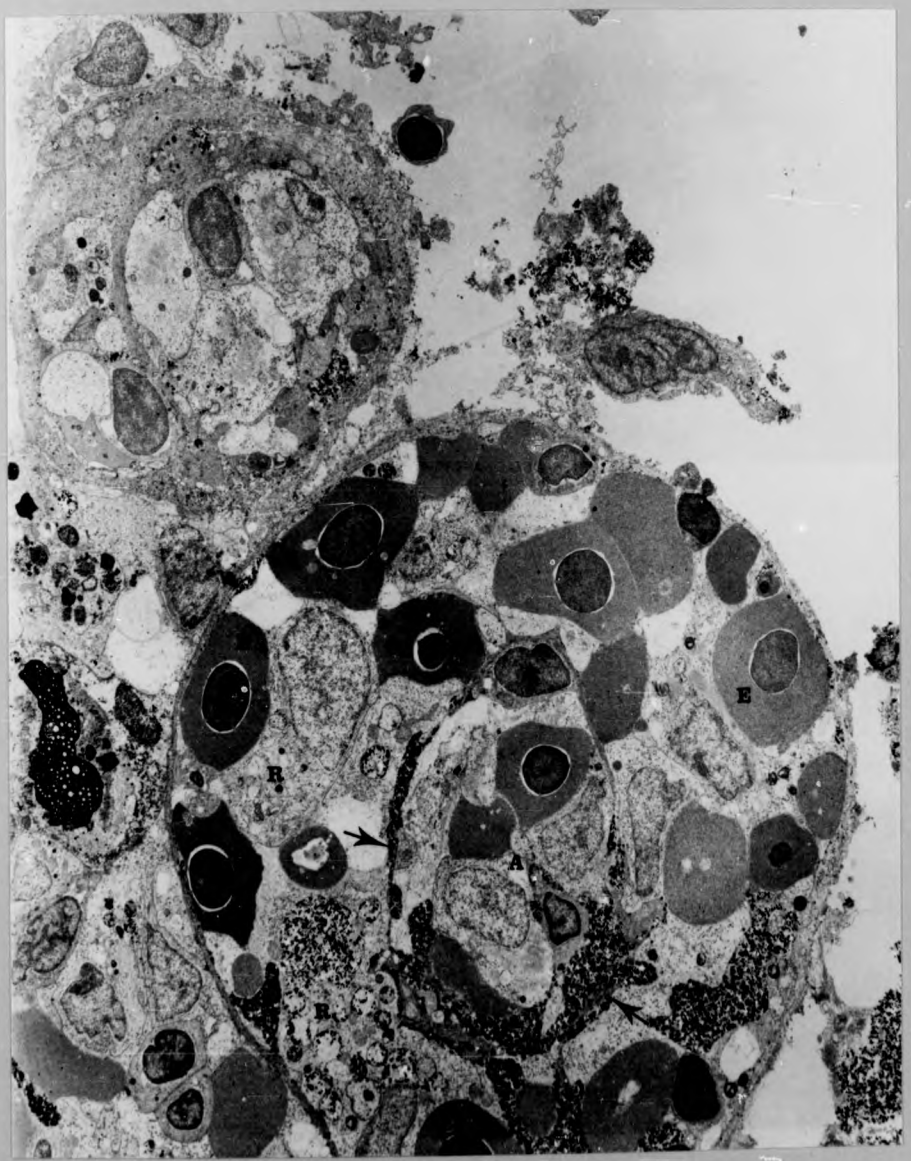

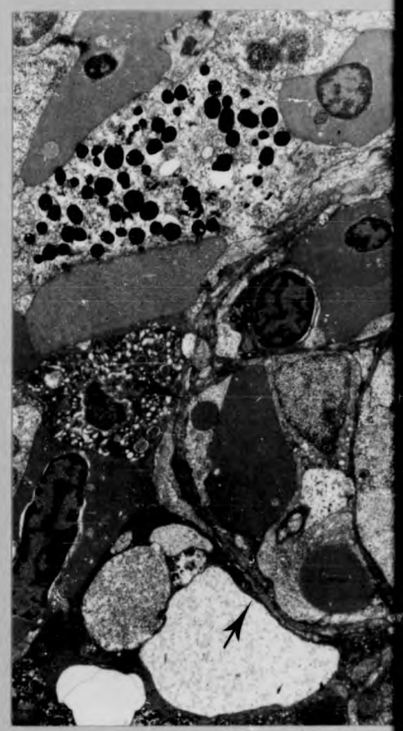

Fig. 55 Spleen from a normal turbot showing one ellipsoid, the boundaries of which are shown by arrows. Observe however that the ellipsoid is highly compartmentalised within the outer limiting membrane. The lumen of tha axial vessel is inapparent.

x 4,500

Fig. 56 Spleen from a normal plaice showing the most frequently encountered type of endothelial cell of the ellipsoidal axial vessel. Observe the intercellular desmosomal linkages (arrows); the pseudopodial projections at the base of the cells, into the basement membrane (bm); and the extensive micropinocytotic activity over the whole surface of the cell. Note also the fact that these are relatively high endothelial cells, and that the cytoplasm contains an active golgi apparatus and a multitude of microfibrils.

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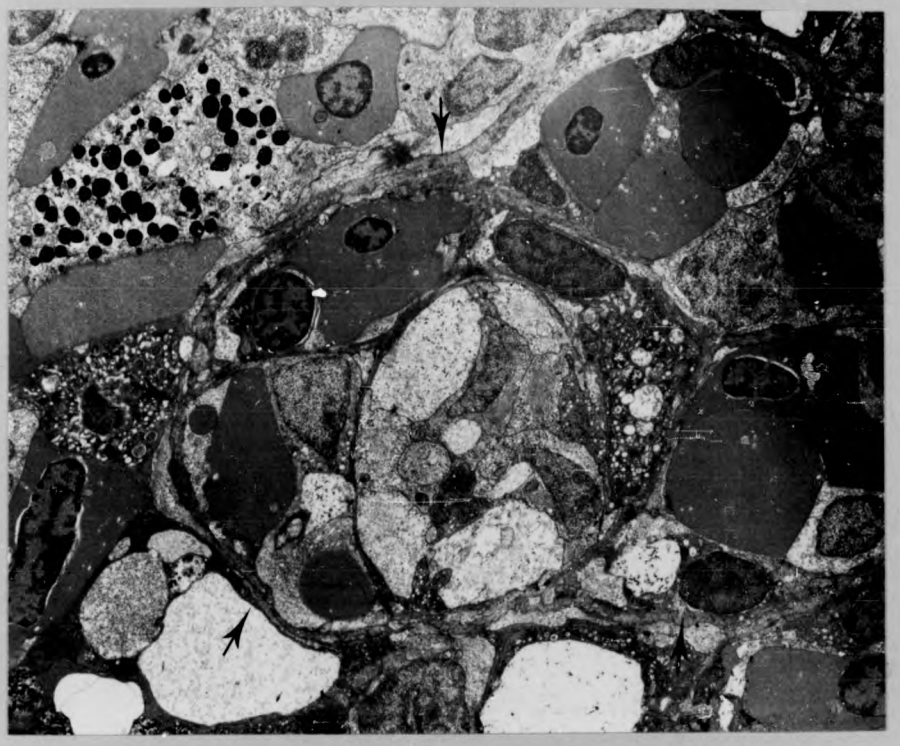

system was absent. The main elements were ellipsoids, pulp, lymphoid tissue and melano-macrophage centres.

a. Ellipsoids.

The centrally placed arterioles terminated by dividing into 3 or 4 thick-walled ellipsoids which in transverse section comprised small groups (Fig. 54). They were extensively distributed throughout the splenic stroma and in reality represented the capillaries of the spleen.

They were composed of one large 'tube' of thick basement membrane containing an eccentrically placed endothelial-lined vessel, surrounded or 'sheathed' by compartments, walled off from one another by basement membrane (Fig. 55). This latter was continuous with both the outer limiting wall of the 'tube' and the inner basement membrane of the axial vessel on which the endothelial cells were situated. The cells comprising the endothelial-lined axial vessel were of 2 types:-

1. In some ellipsoids, one cell type only was present. This was very high and they were sometimes conjoined at their bases by desmosomes and pseudopodial interdigitations (Fig. 56).

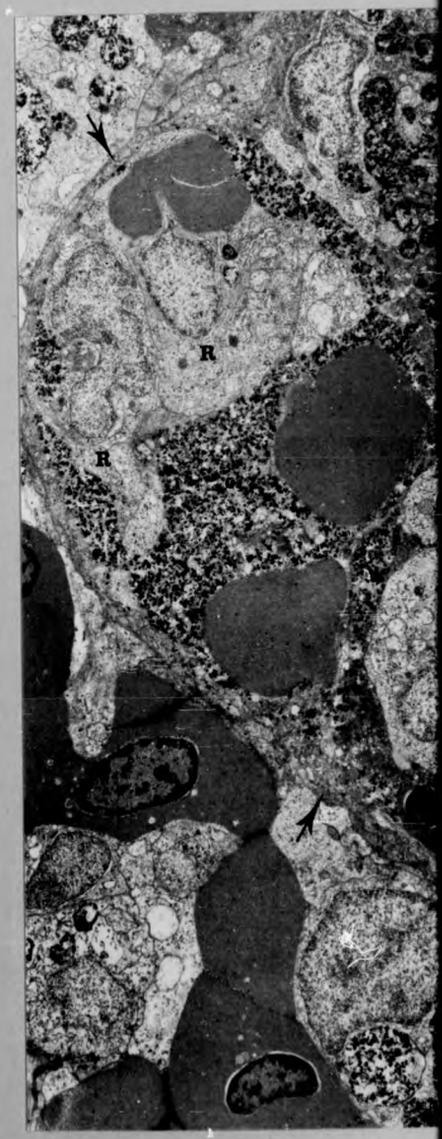

Fig. 57 Electron micrograph of spleen from a plaice sacrificed 1 hour after the intra-venous injection of colloidal carbon, showing a single ellipsoid, the outer membrane of which is shown by arrows. Observe that the endothelial lining of the eccentrically placed axial vessel is incomplete and that erythrocytes (E) are situated between adjacent endothelial cells. Observe also the large pale reticular cells (R) in the outer parts of the ellipsoid. Some of these cells are in the process of endocytosing some of the carbon (C) trapped within the wall. Note the large pale carbon-containing cells surrounding the ellipsoid and attached to the limiting membrane.

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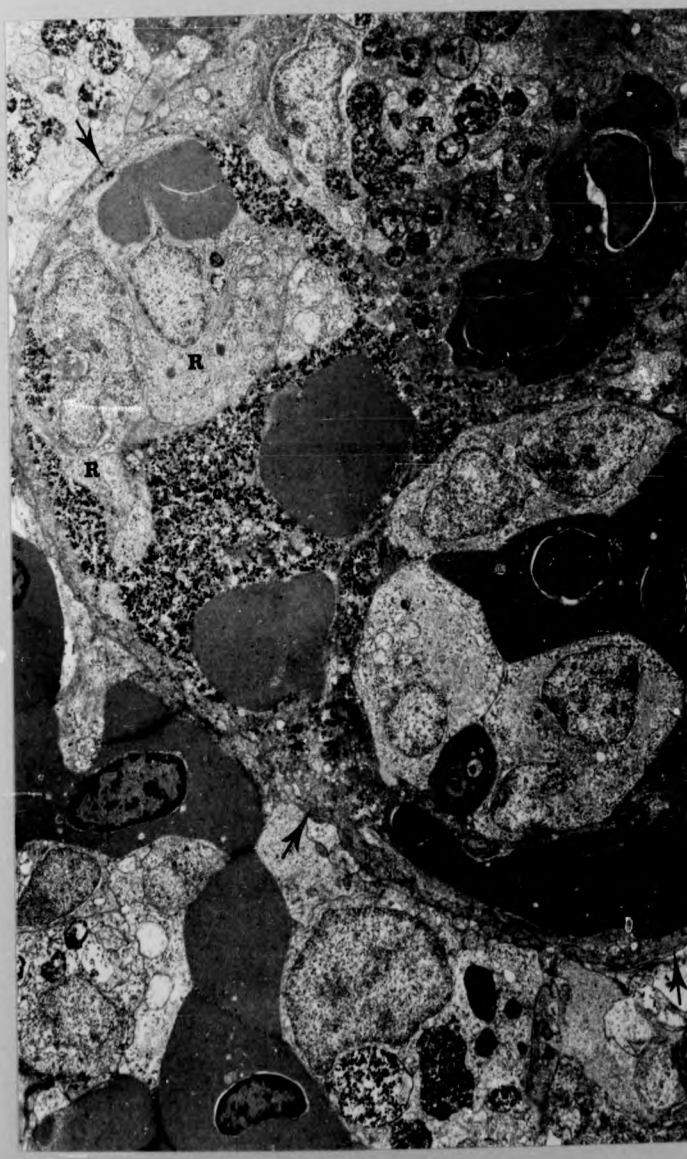

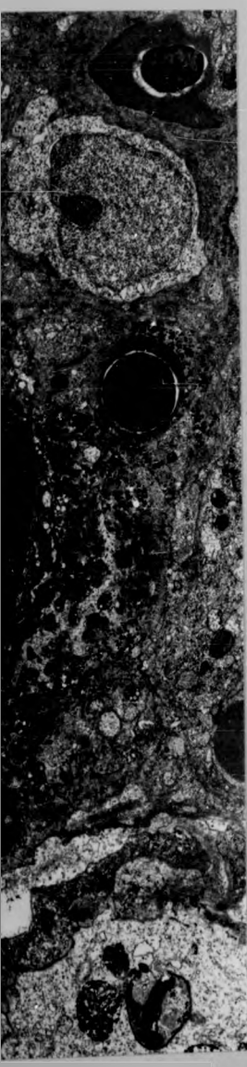

Fig. 58 Spleen from a carbon-injected plaice showing a gap (arrow) in the basement membrane of the axial vessel. Observe an erythrocyte (E) in the gap.

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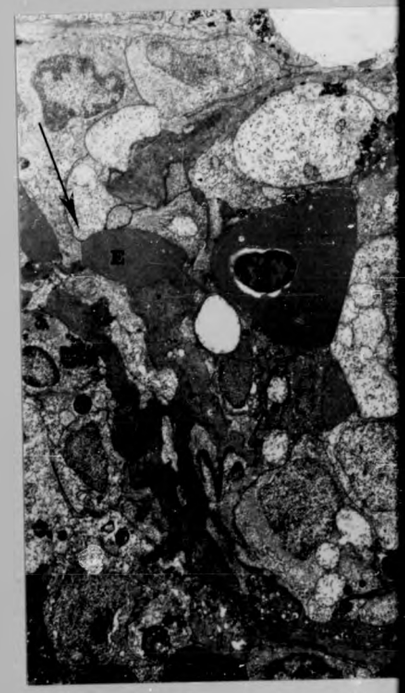

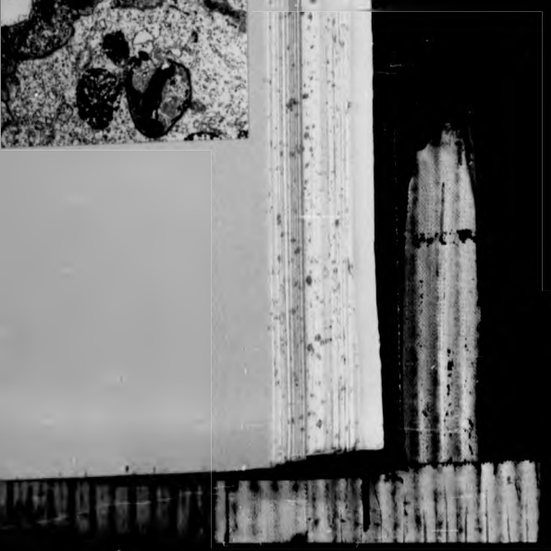

Fig. 59 Spleen from a carbon-injected plaice showing the endothelial-lined axial vessel. Observe the relatively electron-lucent cells (1) situated on the basement membrane, and the electron-dense cells (2), mostly overlying them, but also inserted onto the basement membrane (arrow). Note that the electron-density of cell type 2 is mainly due to whorls of microfibrils and glycogen granules.

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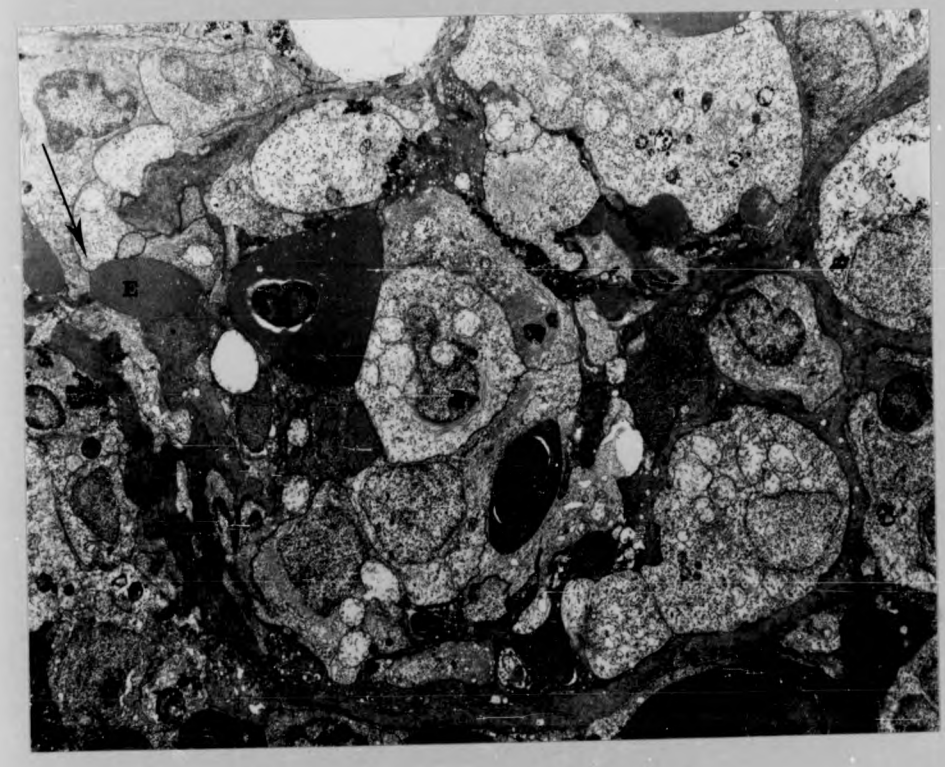

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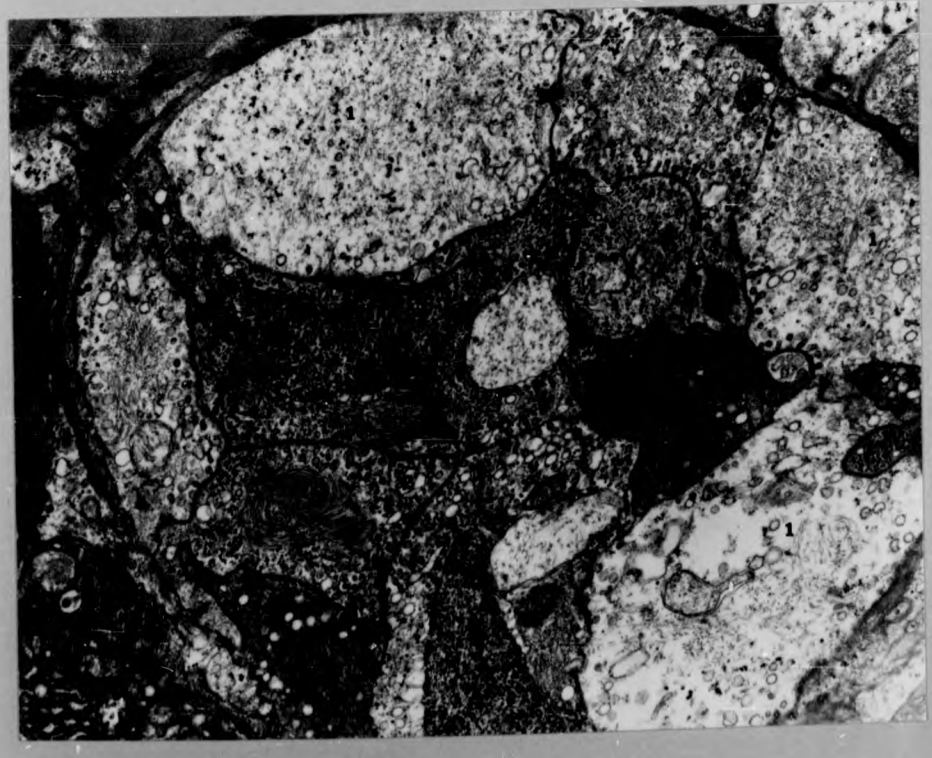

Fig. 60 Spleen from carbon-injected plaice showing an ellipsoid. Observe the very numerous erythrocytes round the axial vessel, the lumen of which is inapparent.

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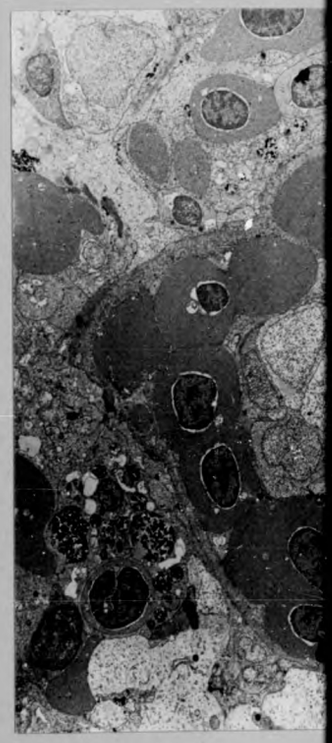

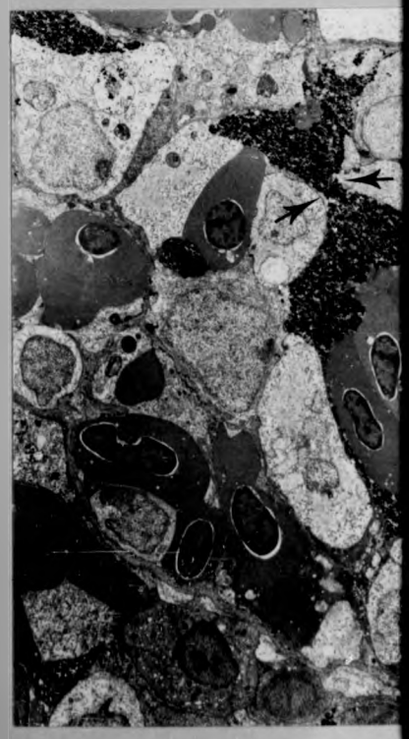

Fig. 61 Spleen from a carbon-injected plaice showing an ellipsoid with masses of carbon particles and erythrocytes in the lumen of the axial vessel. Observe the carbon particles migrating out of the lumen of the axial vessel between opposing endothelial cells (arrows).

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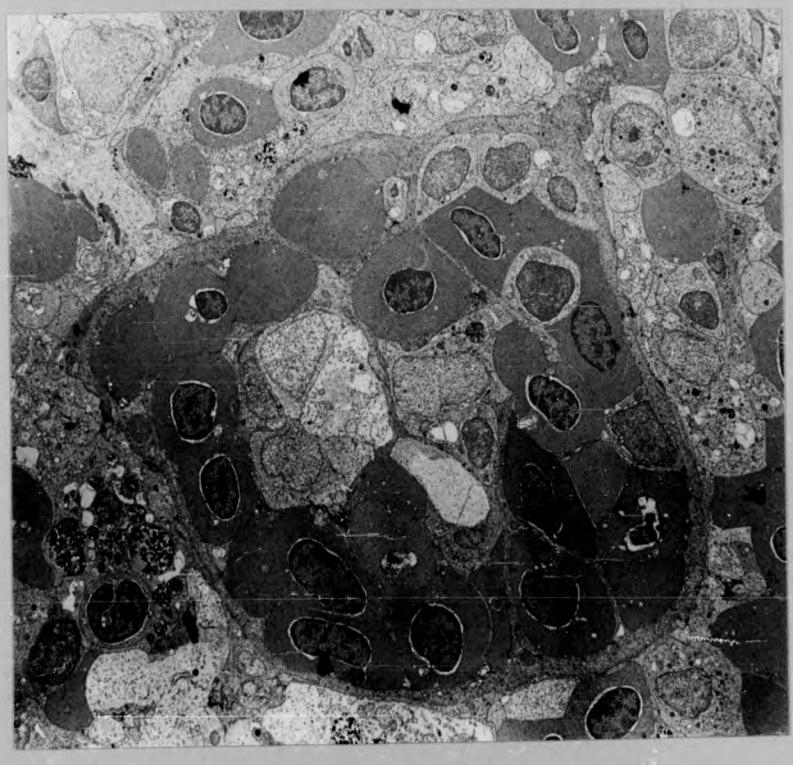

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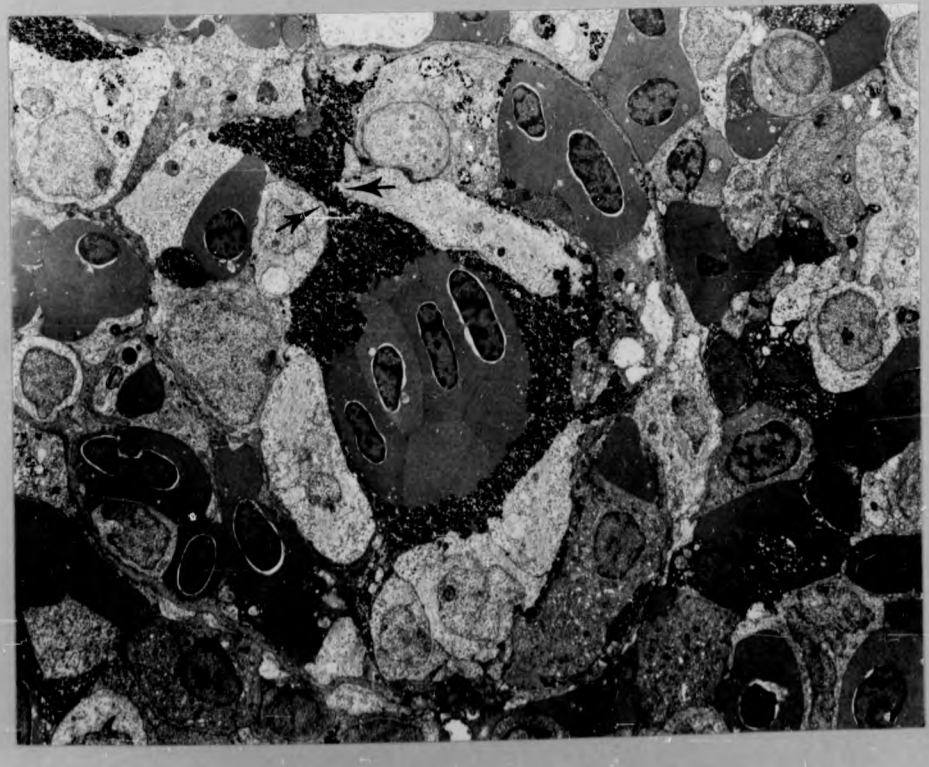

The cytoplasm contained very numerous whorls of fine fibres and usually an active golgi apparatus (Fig. 56). The nucleus was large and often irregular and the plasma membrane was the site of intense micropinocytotic vesicular activity. The endothelial lining was often incomplete and erythrocytes were present, situated so as to give the appearance of passing between the cells into the outer regions of the sheath (Fig. 57), via occasionally observed apposed gaps in the basement membrane (Fig. 58). Axial vessels composed of low endothelial cells were also observed, although these were comparatively rare.

2. In other ellipsoids a second cell type was encountered, situated between, and usually overlying the normal endothelial cells (Fig. 59). These were more osmiophilic due to the presence of enormous masses of intra-cytoplasmic fibres. At the points where these cells were encountered, the lumen of the axial vessel was either inapparent, or so narrow, that an erythrocyte would pass only with very considerable distortion.

The compartmentalised sheath surrounding the axial vessel contained numerous erythrocytes (Fig. 60) and very large electron-lucent cells often closely associated with both the supporting walls of the sheath and with each other (Figs. 54 & 57). The cytoplasm of

these cells often contained degenerating erythrocytes and other phagocytic vacuoles. Other cell types invariably present in the sheaths were firstly, a small darkly staining cell with long processes (Fig. 54), closely associated with the connective tissue of the sheath, and secondly a large darkly staining cell with extremely numerous cytoplasmic vacuoles. This latter cell type was very similar to a thrombocyte.

The ellipsoids were situated either in close apposition to, or were surrounded by endothelial-lined sinuses. Cytoplasmic projections from the endothelial cells crossed the sinus to insert into the outer connective tissue wall of the sheath.

b. Pulp and lymphoid tissue.

These were almost inextricably intermingled. The lymphoid tissue was finely condensed around the melano-macrophage centres and ellipsoids, this feature being especially prominent in the turbot. It was composed of macrophages, some containing melanin, and lymphocyte-type cells. The pulp consisted of argyrophilic fibres supporting lympho-reticular cells and intermingled erthrocytes.

c. Melano-macrophage centres (MMC).

These were prominent in both plaice and turbot but were more numerous in the latter. Those of turbot contained less melanin, the pigment being pinky-yellow and staining with the characteristics of ceroid (Roberts, 1974a). The discrete nature of these ceroid centres was one of their most striking characteristics. Ultrastructurally, the dense osmiophilic contents were separated into discrete clumps by what appeared to be cell membrane remnants. Degenerate nuclei were occasionally seen within these clumps but no viable cells were present.

Carbon Injected Fish (Plaice)

General histology

As already stated under the 'mapping out' experiments, the carbon initially located in the ellipsoidal walls, but after a period of 3 or 4 weeks, virtually all of it was within the MMC.

At the LM level, the toluidine blue stained epon sections showed that initially, the carbon trapped within the sheaths was for the most part not in a phagocytosed form i.e. it was not concentrated or aggregated in any way, but just diffusely

Fig. 62 Electron micrograph of a "melano-macrophage" from splenic sinus of a carbon-injected plaice. Observe the extremely electron-dense melanin granules (m) plus the carbon-containing phagolysosomes (arrows).

x 18,000

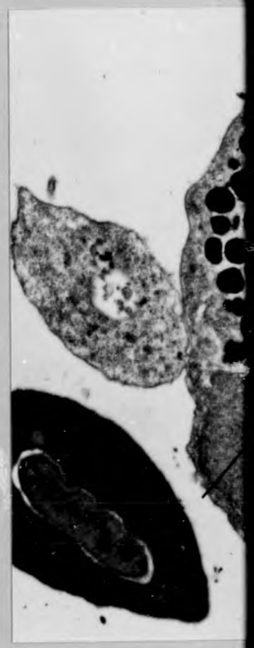

Fig. 63 Light micrograph of spleen from a protozoan-infected turbot. Observe 2 ellipsoids (e) almost totally surrounded by an endothelial-lined sinusoid (S). Note the small groups of darkly-staining macrophages at the periphery of the ellipsoid (arrows) still, however, within the outer limiting membrane of the sheath. Note also the prominent meshwork appearance of the thick ellipsoidal walls.

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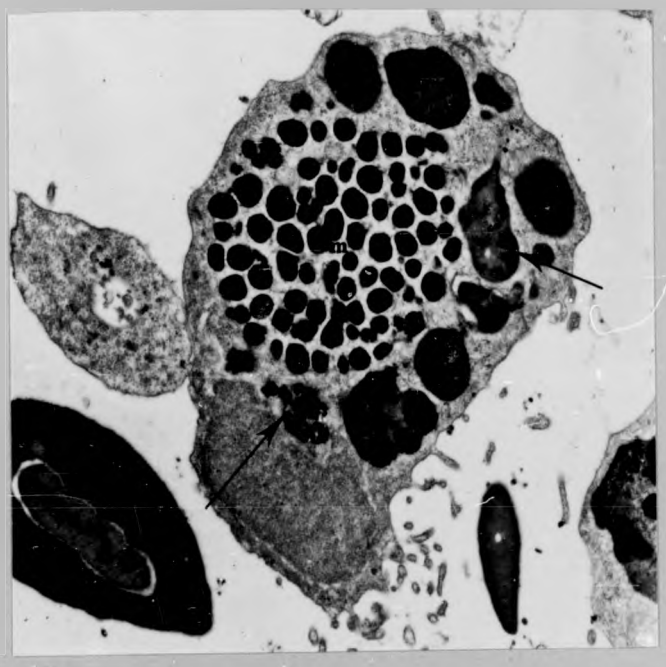

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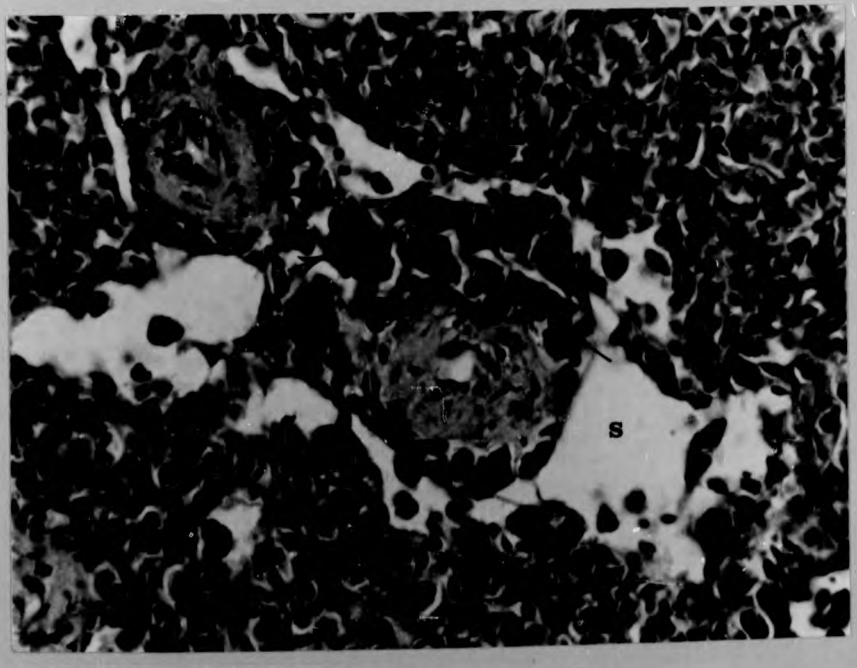

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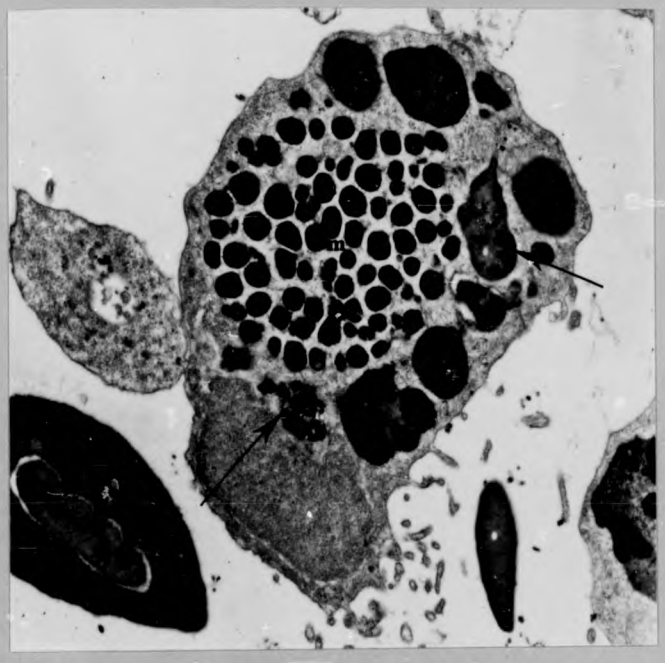

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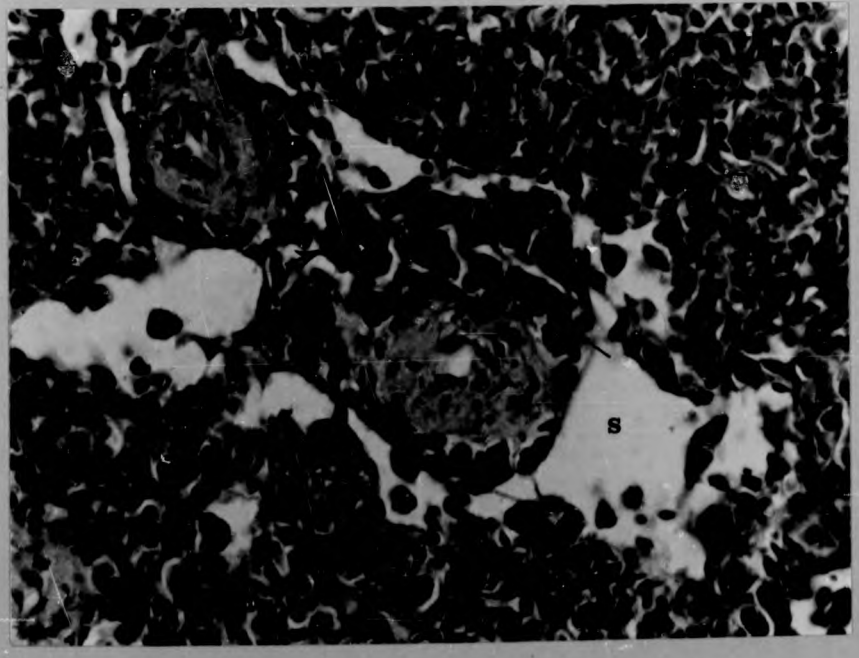

scattered throughout the sheath. At the periphery of the sheath however were a few macrophages which had phagocytosed some carbon. The number of these increased with time as the carbon content of the sheaths became less and less.

The presence of carbon within the lumen of the axial vessel was confirmed at the ultrastructural level (Fig. 61). In addition it could be seen to be scattered throughout the reticulin meshwork of the sheath and from here and from the basement membrane of the axial vessel to which it had specifically located, it was in the process of being endocytosed by the large electron-lucent cells of the sheath (Figs. 54 & 57). Macropinocytotic and micropinocytotic carbon-containing vesicles were very much in evidence at the surface of these cells. No carbon was present within the endothelial cells themselves, although it was present between them, in the process of gaining access to the sheath, from the lumen of the axial vessel (Fig. 61).

Some carbon-containing melano-macrophages were present within the pulp and sinuses (Fig. 62).

Parasitised turbot

The ellipsoids of these fish were especially prominent (Fig. 63) as were the MMC, and there was a good lymphoid

Fig. 64 Light micrograph of spleen from a protozoan-infected turbot. Observe the proliferation of lymphoid tissue virtually enclosing both the ellipsoid (e) and the immature melano-macrophage centre (MMC). Note that the lumen of the axial vessel of this ellipsoid is comparatively wide.

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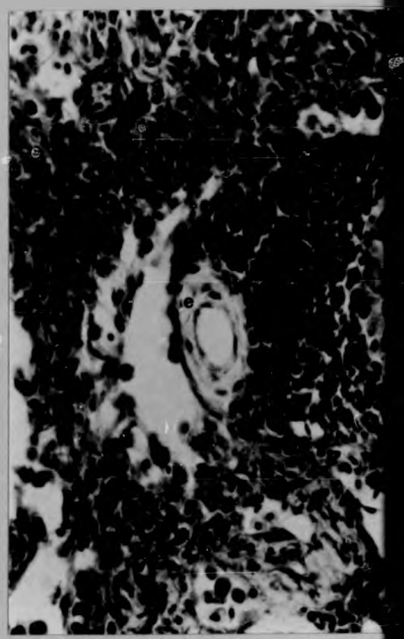

Fig. 65 Light micrograph of spleen from a protozoan-infected turbot. Observe the PAS-positive macrophages around the periphery of the ellipsoid (arrows)

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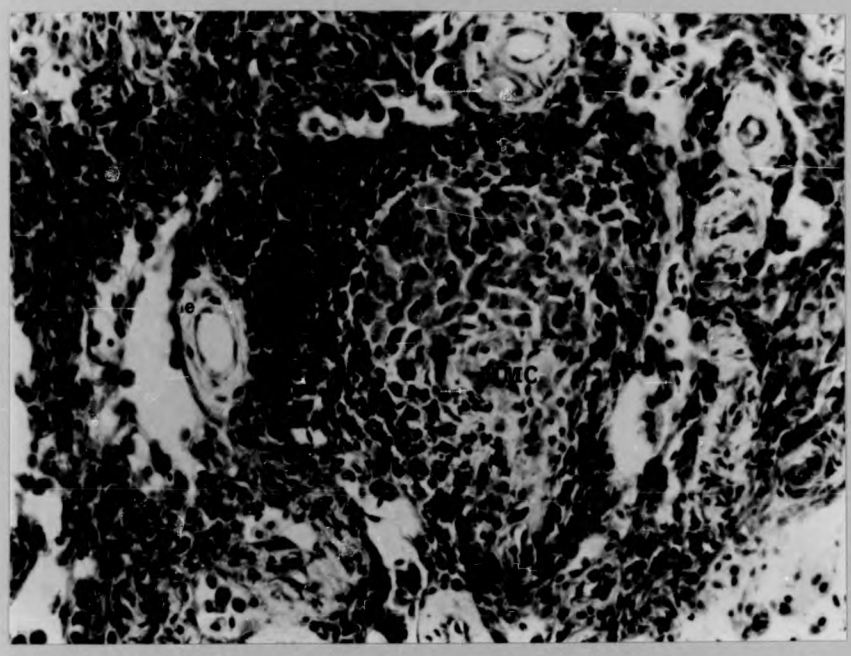

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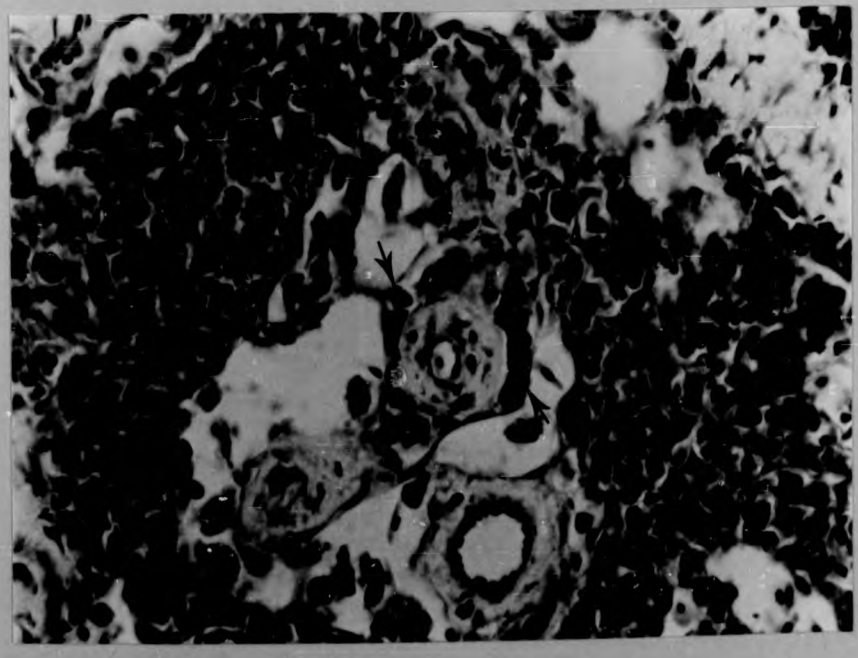

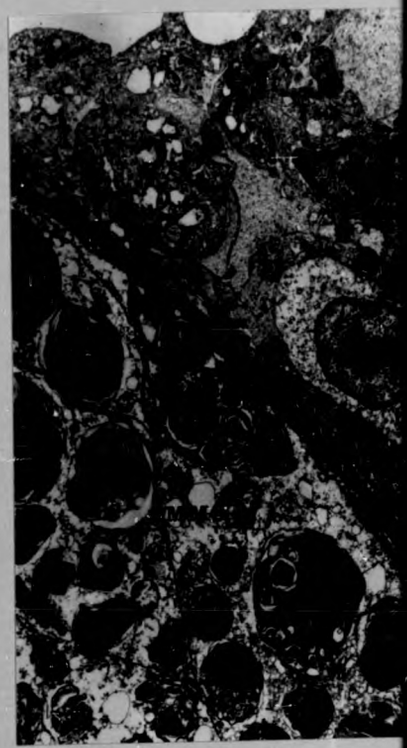

Fig. 66 Electron micrograph of spleen from a protozoa-infected turbot showing a melano-macrophage centre (MMC) invested by a lymphoid cuff in which can be seen typical plasma cells (PC), primitive lymphocytic cells (LC) and dendritic macrophages (arrow). Note that the MMC is walled off from the lymphoid cuff and that it contains osmiophilic material, possibly of protozoal origin.

x 5,000

Fig. 67 High power electron micrograph of the lymphoid cuff investing a melano-macrophage centre from a protozoan infected turbot, showing an immature plasma cell (PC) almost totally invested by a dendritic macrophage. Observe the cytoplasmic interdigitations between the two cells (arrows), and the RER nearby.

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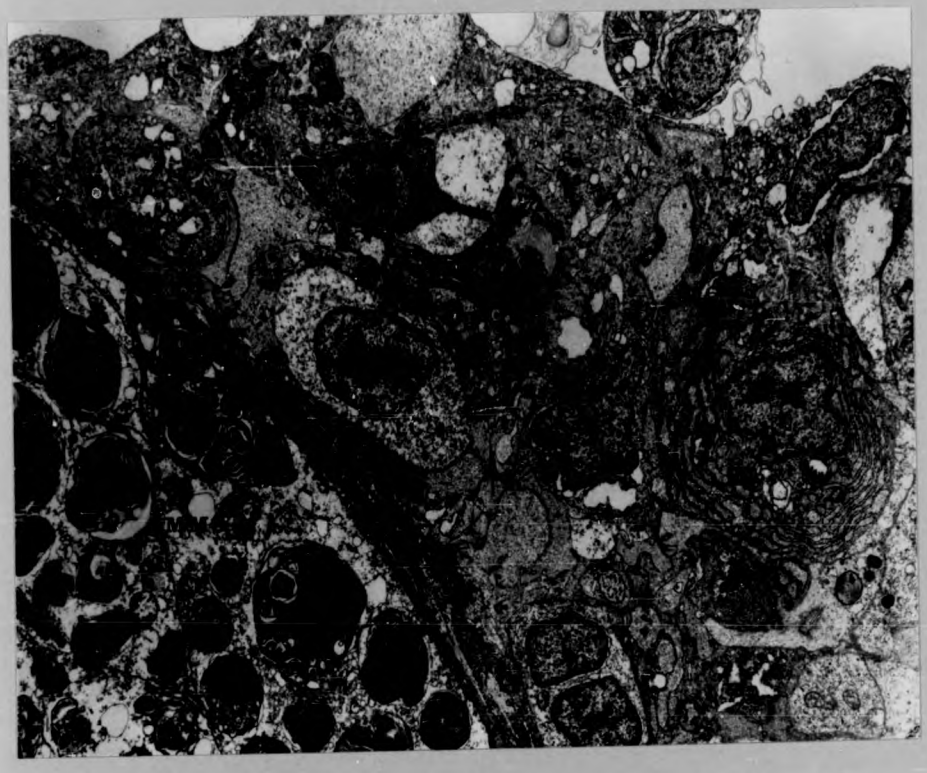

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proliferation around both (Fig. 64). The prominence of the ellipsoids was due to the fact that most of the cellular content was distributed around the periphery of the sheaths as macrophages containing PAS positive material (Fig. 65). The reticulin meshwork of the sheaths therefore was almost denuded of cells.

The peripherally distributed macrophages were in clusters of varying sizes, the largest being accumulations of a greater diameter than the ellipsoids themselves, and frequently projecting into the surrounding vascular sinus. Even the largest however still retained an outer limiting membrane continuous with the outer connective tissue of the sheath, thus resulting in typically discrete MMC.

The normally fine lymphoid cuff was highly developed and surrounded both MMC and ellipsoids, joining the two together. Pyroninophilic cells (possibly plasma cells) and PAS positive macrophages were other cell types found within the lymphoid tissue.

Ultrastructurally, the MMC had a definite capsule separating them from the surrounding lymphoid tissue (Fig. 66) and this was composed of both cellular and acellular elements. The

centres themselves were composed of cells, probably macrophages, in varying degrees of degeneration (Fig. 66). These were replete with dense osmiophilic cell debris, possibly of protozoal origin. The lymphoid cuff comprised lymphocytic cells and typical plasma cells (Fig. 66) which occasionally showed deep invaginations of their plasma membranes at the point of contact with dendritic macrophages (Fig. 67). The lymphocytic cells had a virtually clear cytoplasm except for a few scattered ribosomes, but they had a very chromatin dense nucleus.

D Leucocytes

Four different white blood cell types could be readily distinguished.

a. Lymphocyte - no attempt has been made to classify them into small, medium or large

b. Thrombocyte

c. Monocyte

d. Neutrophil type

a) Lymphocyte

This was the smallest of the 4 leukocytes and was characterised

Fig. 68 Electron micrograph of 2 plaice lymphocytes. Observe the prominent mitochondria and the plicated plasma membrane.

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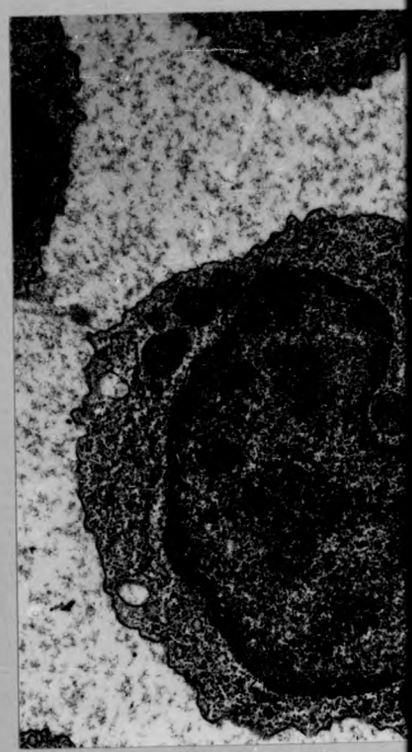

Fig. 69 Electron micrograph of 3 lymphocytes and 1 thrombocyte from plaice blood. Note that the diameter of the thrombocyte is only slightly greater than that of the lymphocytes. Observe that the plane of section excludes the nucleus of the thrombocyte but that within the cytoplasm can be seen centrioles (arrow), many vesicles (v), a small golgi apparatus (Ga) and mitochondria.

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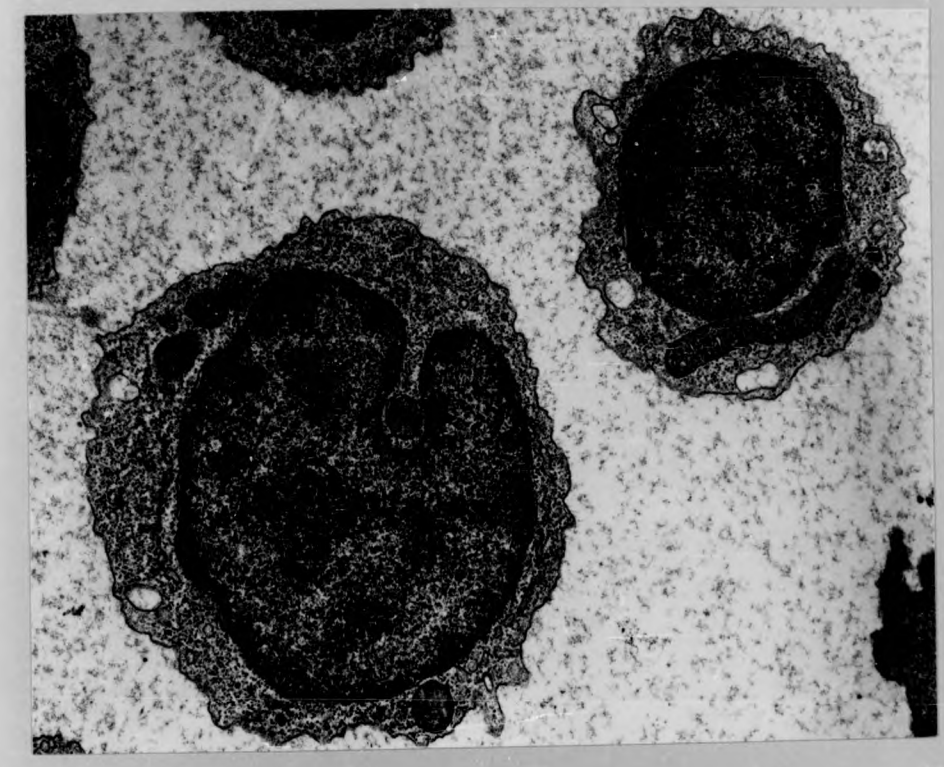

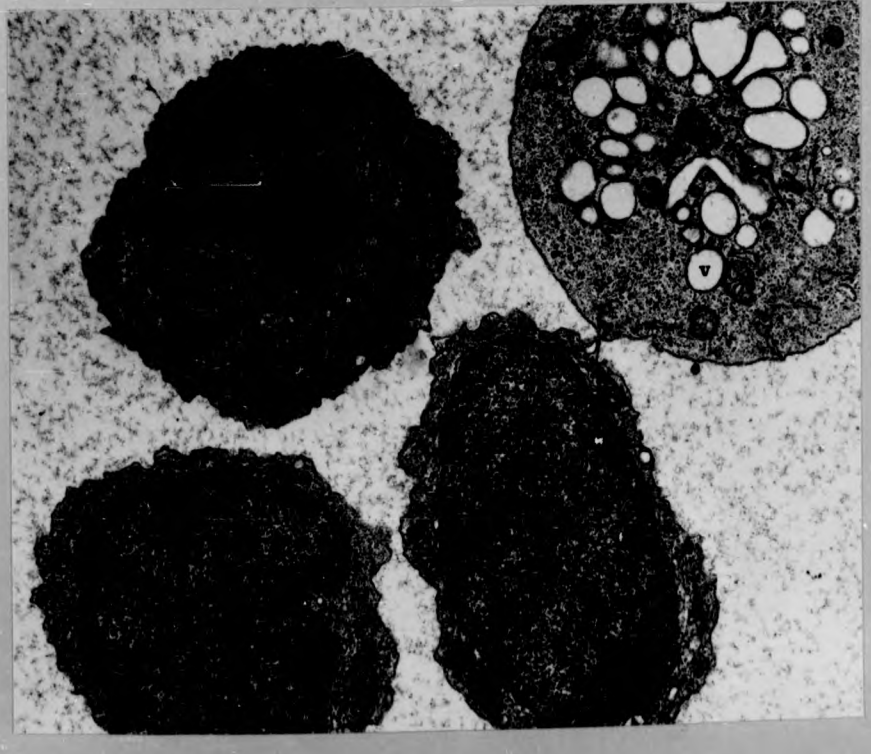

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by its large nucleus surrounded by only a thin rim of cytoplasm (Fig. 68). The plasma membrane was plicated and occasional small pseudopodia were evident.

The nuclear chromatin was dense, but was mainly peripheral in distribution. The nucleus was often indented or clefted, so that in suitable planes of section, two nuclei were apparent.

Small vesicles, limited amounts of rough endoplasmic reticulum (RER) and scattered ribosomes comprised the bulk of the cytoplasm. The most prominent cytoplasmic features however, were the extremely large and often elongated mitochondria (Fig. 68).

b) Thrombocytes

As described in phase contrast microscopy (Wardle, 1971) the thrombocyte was an elongated fusiform cell. There was usually an indentation in the plasma membrane just above the nucleus, and in injected fish, this was invariably 'marked' with the colloidal carbon. Ultrastructurally, the plane of section rarely included both poles, and in transverse section, the thrombocyte was usually slightly larger than the lymphocyte (Fig. 69). In this plane, the nucleus was usually clefted and

Figs 70 & 71 Thrombocytes from plaice blood. Observe the deeply indented nuclei, and the prominent cross-hatching of the nuclear chromatin.

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Fig. 72 Transverse section of plaice thrombocyte to include nucleus. Microtubules (arrow) radiate from the centrioles. Observe the prominent vesicles lined with a dintict fuzzy coat.

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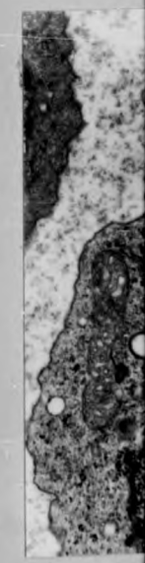

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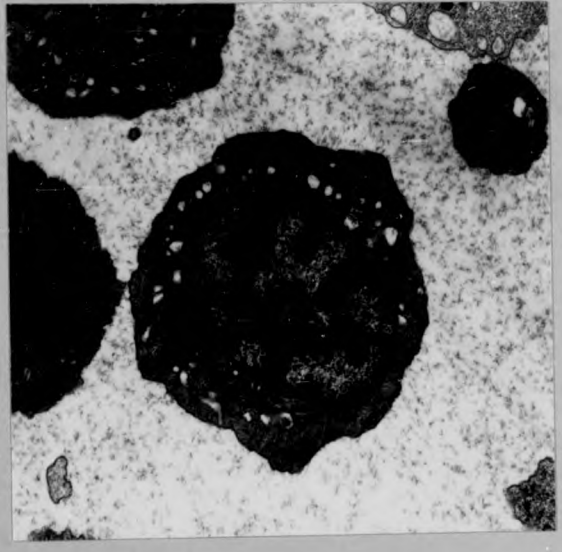
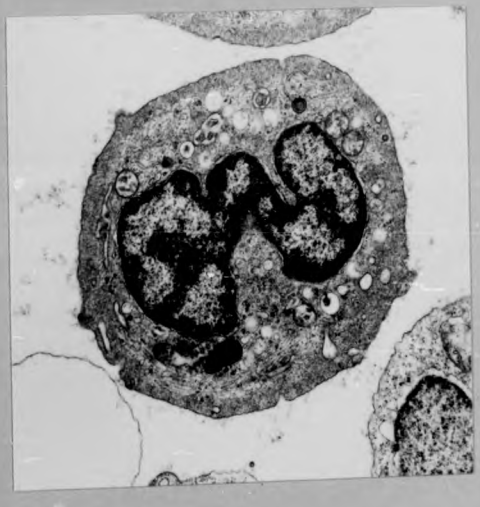




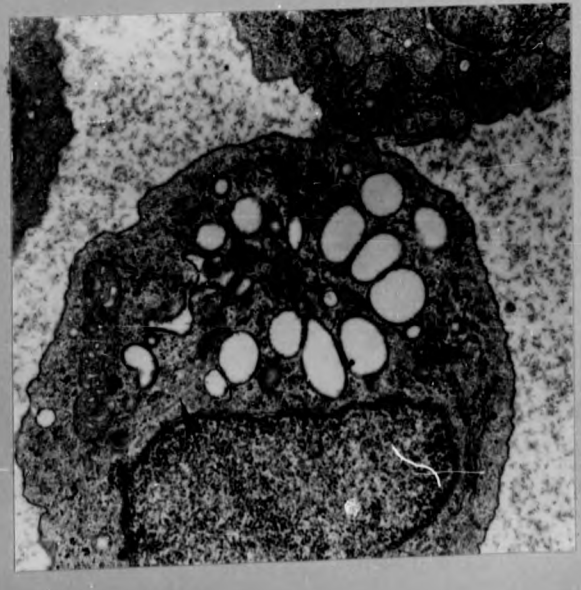


Fig. 73 Tangentially sectioned thrombocyte showing the microtubules.

x 30,000

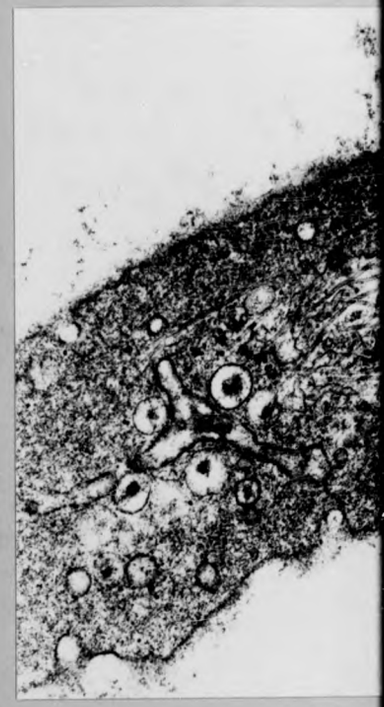
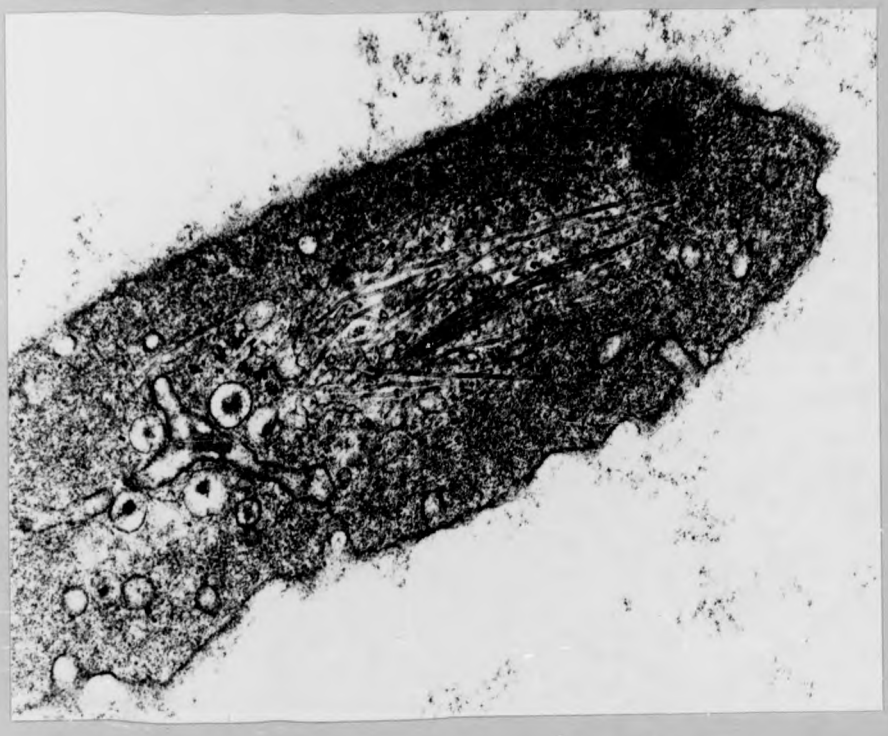
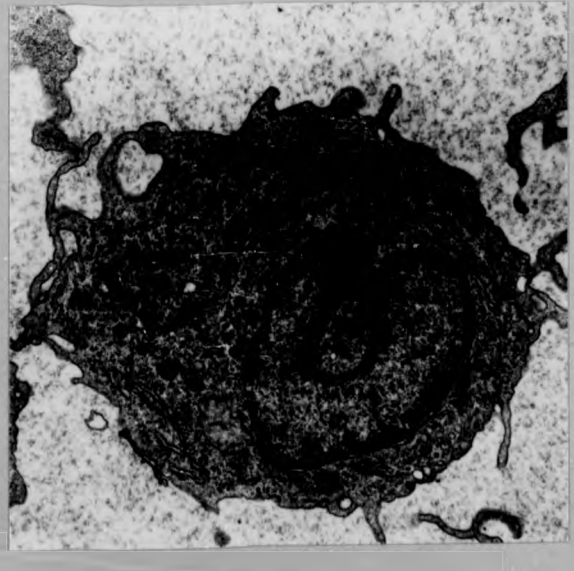


Fig. 74 Electron micrograph of a plaice monocyte. Observe the pseudopodia, the prominent golgi apparatus (arrow), and reniform nucleus.

x 12,000





again surrounded by only a thin rim of cytoplasm and at a very superficial level therefore, the thrombocyte resembled the lymphocyte.

On closer examination, however, it could be seen that the nuclear chromatin had a very marked 'cross-hatched' appearance, with very clear separation between the light and dark phases (Figs. 70 & 71). (This was visible even in the LM). The most prominent feature of the cytoplasm was the number of vesicles (Figs. 69 & 72). These were lined with the same 'fuzzy' coat as could be seen on the plasma membrane of cells fixed with osmium alone. These vesicles were considered analogous to the surface-connecting system of the mammalian platelet.

Favourable sections included the centrioles, from which radiated micro-tubules. Other microtubules could be seen just beneath the plasma membrane and concentrated at the poles, similar again to the mammalian platelet. These were best illustrated by a tangential section (Fig. 73). Sparse RER scattered ribosomes, a small golgi apparatus, and a few mitochondria comprised the rest of the cytoplasmic organelles.

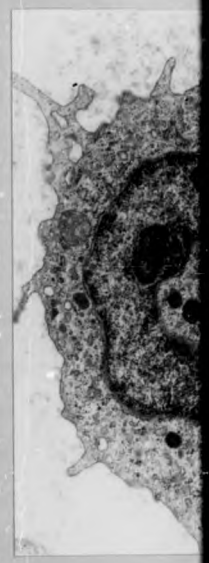
Many thrombocytes demonstrated their ability to take up appreciable amounts of carbon. Uptake of carbon by platelets has been

Fig. 75 Plaice monocyte. Observe the nucleolus within the reniform nucleus, pseudopodia, and the electron-dense secondary lysosomes (arrows).

x 12,000

Fig. 76 High power electron micrograph of centrosomal area from a plaice monocyte. Observe the microtubules radiating from the centrioles (c), the prominent golgi apparatus (Ga) giving rise to primary lysosomes, and the secondary lysosomes or dense bodies (arrows) showing a gradation in density.

x 45,000



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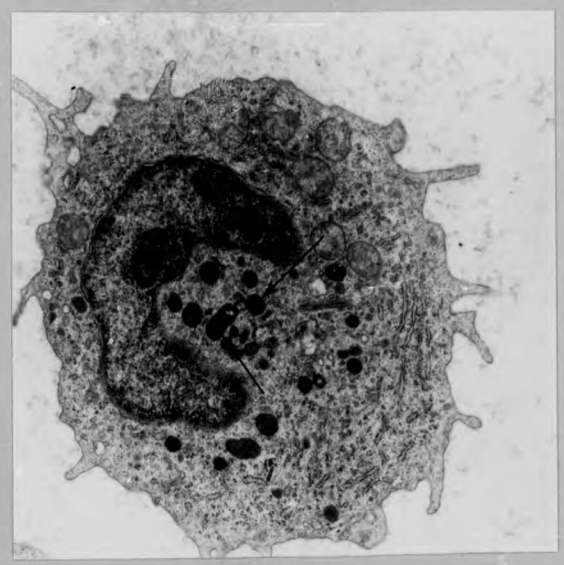


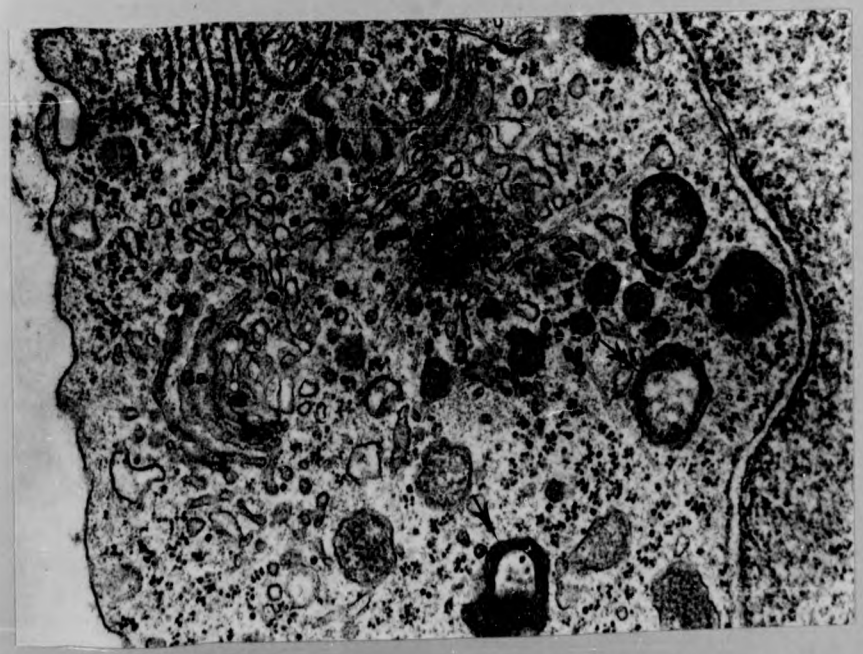


Fig. 77 Monocyte from a plaice sacrificed 4 hours after the intra-venous injection of colloidal carbon. Observe the presence of carbon within the monocyte cytoplasm. Note also the large golgi apparatus.

x 30,000

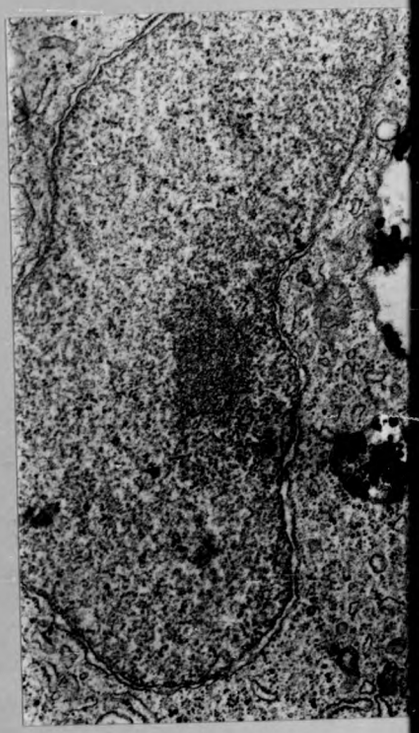
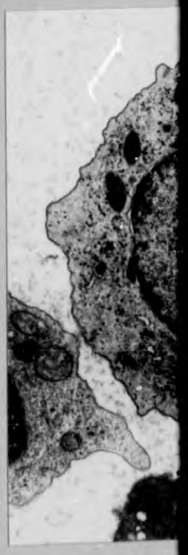


Fig. 78 Plaice neutrophil. Observe the numerous prominent cytoplasmic granules. Although irregular in outline, the cell lacks pseudopodia.

x 12,000



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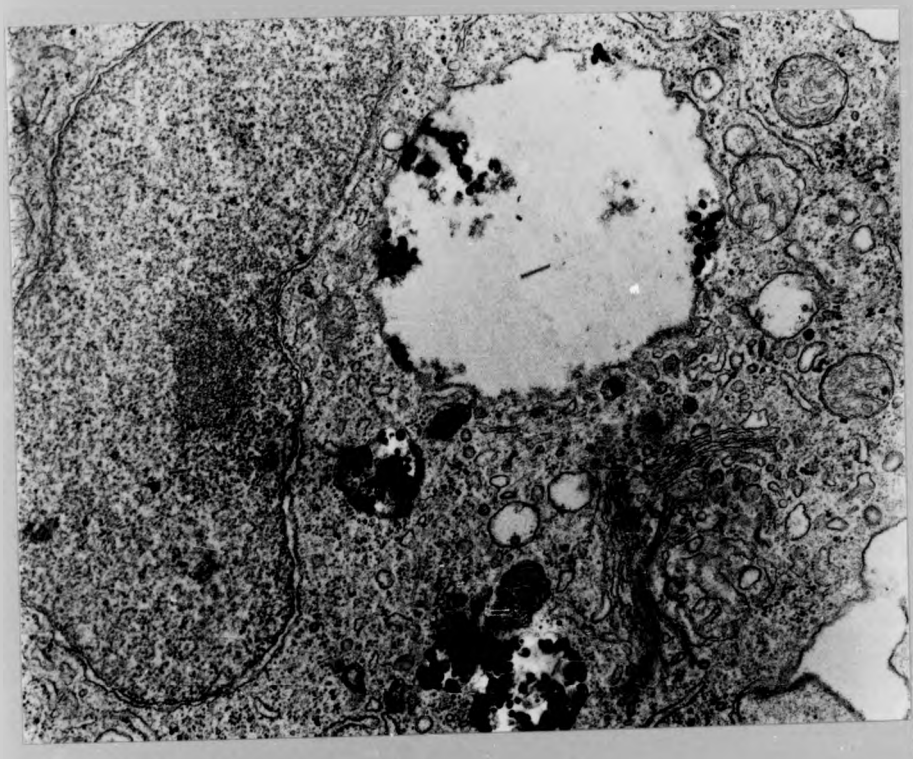

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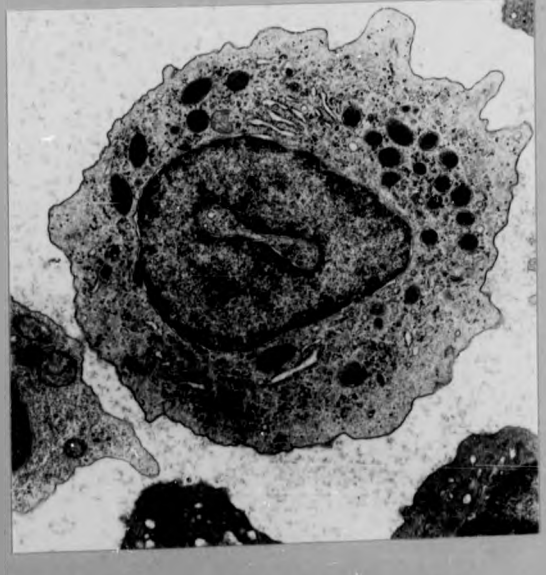

described for mammals (Van Aken and Vreeken, 1970).

c) Monocytes

The plaice monocyte (Figs. 74 & 75) was only slightly larger than the neutrophil type, due mainly to the more irregular outline and pseudopodia. The nucleus occupied approximately one quarter of the cell and was usually reniform. It was relatively electron-lucent apart from a narrow peripheral band of chromatin and its large nucleolus was often very apparent even in the LM. The cytoplasm had quite large ovoid or round mitochondria, some RER and micro-tubules, radiating from the centrosomal area (Fig. 76). The most prominent cytoplasmic features were the golgi stacks from which small primary vesicles could be seen to be forming (Fig. 76) and the extensive vesiculation and vacuolation throughout the entire cytoplasm. The vesicles were of varying size and electron density, the smaller ones being the more electron-lucent. The content of the larger vesicles did not appear to possess much determinable fine structure apart from a distinct granularity and although some had a rough outline, most were smooth.

In injected fish the colloidal carbon was taken up by these cells and its presence within phagolysosomes was obvious (Fig. 77).

Fig. 79 Plaice neutrophil showing the fine structure of the cytoplasmic granules. Note that they have a fine fibrillar nature.

x 20,000

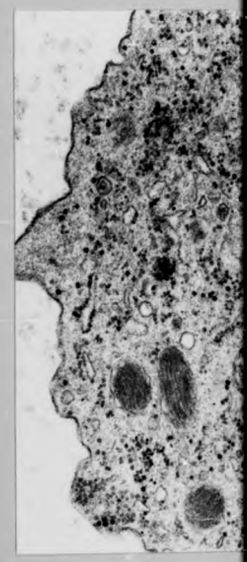

Fig. 80 Electron micrograph of white muscle from a plaice, to show the presence of M-bands (M).

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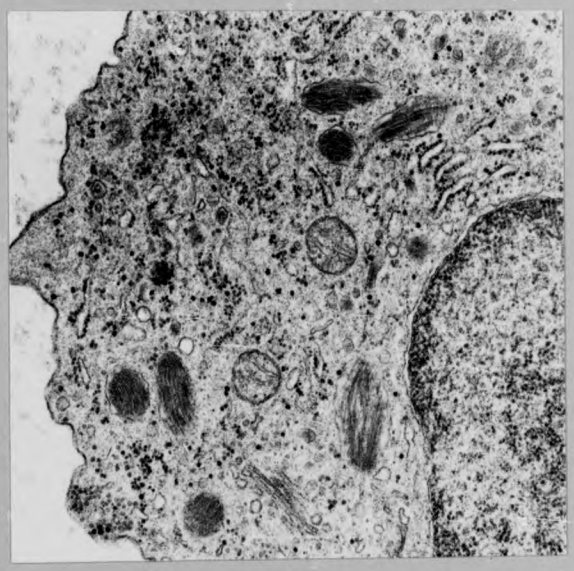

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d) Neutrophil type

The neutrophil (Fig. 78) was the most numerous leukocyte. Its outline was often irregular, but pseudopodia were absent. The nucleus was more irregular in outline that that of the monocyte but was not seen to be multilobed. The nuclear chromatin was denser and more patchy in its distribution than in the monocyte.

The cytoplasm was distinguished by its numerous large, round to elongate, granules (Fig. 79). The content of these granules showed varying degrees of organisation but they were nearly all "fibrillar" with the fibrils running either parallel or irregularly throughout the granule. Some had a banded appearance.

The golgi apparatus was not so prominent as in the monocyte nor was the degree of vesiculation so great, and these cells only very rarely showed any carbon uptake. However, the cytoplasm of the neutrophil, in comparison to that of the monocyte, did show an overall greater granularity, due to the presence of more ribosomal material.

CHAPTER 6.2 DISCUSSION AND CONCLUSIONS

A The Liver

One cell type only formed the sinusoidal lining, and using the criteria established in rats (Wisse, 1974), this cell type was classified as an endothelial cell rather than a Kupffer cell. The criteria identifying it as an endothelial cell were:-

i it formed the poorly fenestrated sinusoidal lining situated between the bloodstream and the parenchymal cells.

ii it was streamlined, probably in a fixed position and was not amoeboid. No cells in the process of detaching were ever seen.

iii the plasma membrane was not "sticky" to circulating carbon particles, and the endocytotic capacity was quite small.

Kupffer cells, therefore, were not seen in plaice, bream or roach. LM observations suggest that they do not exist in rainbow trout or other species of fish, but more work is required to establish this fact.

By contrast, fat-storing cells (FSC) were very numerous. They have been reported by Ito and his co-workers to exist in a variety of animals, including fish, but have never been described in fish at an ultrastructural level. Various functions have been ascribed to the F.S.C.:-

i The storage of vitamin A. This has been demonstrated by Wake (1971).

ii The formation of collagen fibrils (Wood, 1963).

iii The storage of fat, which has gained access to the Space of Disse.

iv The support of the sinusoid by means of the cytoplasmic processes projecting into the Space of Disse.

All of these above functions are discussed by Ito and Shibasaki (1968). The present findings would tend to support the idea that FSC are responsible for the formation of some of the fibres within the Space of Disse, although the significance of the large amounts of intra-cellular fibres is unknown. Whether because of the fibres, or the long cytoplasmic processes however, the sinusoids were well supported, as

evidenced by the fact that good patency (in contrast to the mammalian situation) was maintained even in the absence of perfusion fixation.

The consistent finding of carbon within the FSC raises the question as to how it got there. Several possibilities exist:-

i The particles of carbon were small enough to pass through the fenestrations of the endothelial lining, into the Space of Disse from where they were endocytosed by the FSC.

ii Having gained access to the Space of Disse through the endothelial fenestrations, they were endocytosed by the parenchymal cells and then passed to the FSC along with other cellular products.

The carbon was present within the FSC in phagosomes containing either carbon alone, or carbon plus cellular products, some resembling debris, and it is therefore possible that both mechanisms exist, unless sufficient lipoidal products gained access to the Space of Disse via the fenestrations to account for all of the lipid products within the FSC. The

consistent finding of carbon within the FSC also raises the question as to whether they should be included within the RES of the plaice. The fact that this was only really prominent at high dose levels would tend to suggest that it was an artificial finding, and under more natural conditions, the FSC would probably not participate in blood clearance. Accordingly, they are included within the RES, but are not considered to be one of the major components.

The biliary system of roach, bream and plaice differed little from Yamamoto's description of the biliary passages of goldfish. This however is the only such description of any detail, and it is interesting to note that in the species used in the present investigation, the biliary passages also extended to an intra-cellular level, as opposed to the inter-cellular level found in all mammals. It is additionally interesting that a single bile duct was capable of forming a patent passage, merely by an invagination of its cytoplasm. The significance of the association of histiocytes with the bile ducts is unknown, although it is possibly related to the fact that macrophages are thought to be responsible for the formation of bile pigments (Dumont et al. 1962).

B The Heart

The lack of an outer compact layer in the myocardium is

in accordance with the fact that the plaice is neither a very large nor a fast moving fish. Ostadal and Schiebler (1971) stated that fish with low body weights do not have an outer compact layer.

The present ultrastructural findings largely agree with those of Santer and Cobb (1972) for the plaice and Kisch (1963) for the goldfish. In neither of these works however was a proper description of the endocardial cells or the intercalated discs included. A good comparative account of intercalated discs was given by Martinez-Palomo and Mendez (1971) for the hearts of non-mammalian species, with special reference to gap junctions which are thought to be responsible for the spread of electrical activity from cell to cell. Within the fascia adhaerentes the gap junction was the only kind of junction described for all species examined. No mention was made of desmosome-like junctions within the fascia adhaerentes although they were noted as existing between adjacent cells of their longitudinal axes. The precise significance of the presence of the desmosome-like junctions within the intercalated discs of plaice is unknown, although certainly, inter-cellular adhesion would be improved.

Adjustments in cardiac output in fish are characterised by large changes in stroke volume, and small changes in heart

rate (Randall, 1968), and high cardiac outputs could place considerable strain on the cardiac tissue. Johansen (1965) stated that the meshwork-like structure of the cardiac tissue of both amphibia and fish possibly facilitates ventricular function, by reducing the strain on individual fibres (see review by Priede, 1975). Additional protection could possibly be provided by the numerous desmosomes in the intercalated discs.

Santer and Cobb mentioned the presence of A and I bands, and an indistinct M band. Kisch however stated that, although not seen in goldfish, M bands had been seen in other species, but were never seen in skeletal muscle. A generalisation was then proposed for all poikilotherms in which it was stated that M bands are present in cardiac muscle, but not in skeletal muscle. Reference to Fig. 80, however, shows that this statement is inaccurate.

Unfortunately, even examination of carbon-injected fish at an ultrastructural level could not determine precisely whether the uptake of carbon by the endocardium was regulated by pinocytosis or phagocytosis, although the former is thought to be the more important, as extensive pseudopodia, or flaps of cytoplasm projecting from the plasma membrane were not seen. The metabolic inhibitor cycloheximide prevents pinocytosis, but

not phagocytosis (Cohn, 1970) and this could possibly be used to determine precisely which is the rate limiting process.

C The Spleen

In both plaice and turbot it was concluded that the melano-macrophage centres did not contain viable cells although it is reasonably certain that material is brought to the centres within macrophages. The method of entry into the centres is unknown, although the relationship to the ellipsoids suggests that for a while at least, the encapsulation of the MMC is incomplete, and that direct connections with the pulp exist, so that at some stage viable cells may exist within the centres.

The existence of gaps in the basement membrane of the ellipsoidal endothelium whereby cells can pass out of the axial vessel, has always been in contention. Various workers have reported that they do exist and that cells e.g. erythrocytes and neutrophils, can be seen migrating out of the central vessel into the sheath. Yoffey (1928) claimed that gaps do exist in fish, whereas Solnitzky (1937) denied their presence in all those animals he studied, the few cells seen to be migrating out being very distorted, and that the situation

was therefore no different from cellular migration out of any other capillary i.e. diapedesis.

Ultrastructurally, White & Gordon (1970) reported 1-2µm gaps in the basement membrane with pseudopodia from the overlying endothelial cells projecting down through this gap. The desmosomal linkages of the endothelial cells at this point were absent, and they maintained this to be the access route, though often tortuous, of particulate matter into the sheath. The use of different sized particles confirmed that only those smaller than the pore size of the basement membrane could get out of the axial vessel into the sheath.

In contrast to Solnitzky's observations, the present work confirms the existence of large gaps not only in the basement membrane of the axial vessel, but in the continuity of the endothelium itself, these therefore being the routes of access of erythrocytes and carbon into the sheath, and of carbon to the basement membrane. It is not known why the yeast or bacteria used to map out the RES did not locate in the sheaths, because the gaps would have appeared to have been big enough to have allowed them to pass through. One possible explanation may lie in the fact that these gaps were seen only when cells e.g. erythrocytes, were migrating out, and thus possibly they

were actively created by the cells. The yeast or bacteria used would be incapable of any active migration. The carbon however would have been filtered out of the blood in accordance with the proposed physiological 'plasma skimming' function of the ellipsoids (Schweigger-Seidel, 1862; Mills, 1926; Weiss, 1962), gaining access to the sheath via small discontinuities in the endothelial lining unassociated with migrating cells.

The ellipsoids have been described as having numerous physiological functions, most of which relate to regulating the blood supply to the spleen, by means of nervous control (Muller, 1865), a contractile response in the absence of any muscle (Knisely, 1936) or by means of a one-way valve action. Solnitzky reviewed the literature, and said that no real evidence existed for any of these mechanisms nor indeed very little to suggest that any form of lumen regulation occurs.

Weiss (1962) however, noted the presence of fibres in the cytoplasm of the endothelial cells of the ellipsoids in the spleen of dogs, and used this as evidence to support Knisely's proposal of a contractile capacity for the ellipsoids which followed his observation, using quartz rod illuminators, that an abrupt cessation of flow in the axial vessel was possible. The present work supports Weiss's ultrastructural observations and extends the evidence in support of an axial vessel capable of a contractile

response by observing the presence of a second type of endothelial cell with a high content of cytoplasmic fibrils.

This second cell type was an inconstant finding possibly due to the fact that only some ellipsoids possessed them or more likely, due to the fact that they were situated only at intervals along each ellipsoid. Bearing in mind this contractile capacity, blood flow through the ellipsoids could occur in one of the following ways:-

1. There is sufficient contraction of the axial vessel to completely block the flow, and due to a build up of back pressure, blood may be forced through alternative pathways or "shunts" (analogous in this respect to Knisely's observations) which possibly lack such a capacity for contraction. The very high micropinocytotic activity of the endothelial cells may indicate some form of passive swelling rather than an active contraction resulting in a reduced blood flow. This in itself possibly represents some form of regulatory mechanism concerning haemoconcentration.

2. The lumen of the axial vessel is reduced only enough to slow the blood flow, thus forcing erythrocytes through small gaps into the sheaths, and in the process, exposing them to considerable trauma. This mechanism may represent

therefore the normal physiological means for the removal of effete blood cells. A reduced blood flow would apply only to cellular elements and in this way, the plasma would be separated or "skimmed".

The relationship of the ellipsoids to the MMC, derived from the observations on the parasitised turbot, will be discussed in Chapter 9.

D Leukocytes

At the ultrastructural level, the thrombocyte and the lymphocyte were readily differentiated, but could possibly be confused (Weinreb, 1963), especially if the thrombocyte were cut in transverse section sc as to exclude its poles; both cells then appearing as dark nuclei surrounded by a thin rim of cytoplasm.

The significance of the high degree of carbon uptake demonstrated by thrombocytes, with regard to their possible role in the uptake of normally encountered pathogens, is unknown, but further study on this would be considered worthwhile.

Unlike the situation reported in the goldfish by Weinreb &

Weinreb (1969), where a cell corresponding to the mammalian monocyte was reported to be lacking, the morphological findings described here for plaice, are considered sufficient evidence, based on the criteria established for mammals (Carr, 1973), to separately identify these cells as monocytes.

In plaice blood therefore, there are four morphologically distinct types of cells, three of which can be recognised by use of mammalian criteria (lymphocyte, thrombocyte and monocyte). There is, however, a fourth type which cannot be easily recognised on morphological grounds as being the equivalent of the mammalian neutrophilic, basophilic or eosinophilic granulocyte. Firstly, the nucleus is not multilobed i.e. it is not polymorphonuclear. Secondly, in mammalian neutrophils, 2-5 granule types have been described and only one granule type was seen in the mature plaice neutrophil.

The ultrastructure of this granule, with its close packed fibrillar arrangement bears no resemblance to the azurophil or specific granules of mammalian neutrophils or to the granules of the basophil or eosinophil granulocytes.

It does, however, greatly resemble the third granule type of the human neutrophil, which under the same fixation procedures has a very similar fibrillar arrangement (Daems, 1968).

Kelenyi (1972) reported very similar findings in several teleost species. He believed this granule type to be common to all vertebrate leukocytes and termed the cell 'azurophil leukocyte'. The present author prefers to retain the commonly used term 'neutrophil' since this is sufficient to distinguish the cell from the basophil and eosinophil, which although not occurring in plaice, are commonly found in other fish, especially cyprinids, while still allowing the descriptive terminology to remain within the accepted nomenclature of comparative pathology. It must be appreciated when using the term 'neutrophil' however, just how different this cell is from its mammalian counterpart and possibly from the neutrophils of other fish such as the salmonids (Conroy, 1971) where the nucleus has been shown to be lobed.

CHAPTER 7 QUANTITATIVE CLEARANCE STUDIES I

7.1 Introduction

Since the original finding by Wyssokowitch (1886) that the reticulo-endothelial cells of the liver and spleen could phagocytose circulating bacteria, many workers have emphasised the efficiency of this system in clearing the bloodstream (see review by Rogers, 1960).

Clearance in mammals can be divided into 3 phases:-

i Phase 1 is very rapid and lasts from 10-90 minutes

ii During Phase 2, the bacteria may disappear or persist at low levels

iii Phase 3 is the ultimate stage, during which the low levels of Phase 2 may disappear completely or flare up, depending on the virulence of the organism.

The kinetics of blood clearance of isotopically labelled Salmonellae were compared by Biozzi et al. (1960) with their previous work concerning the clearance of stable colloids in mice. Reade (1968) performed colloid clearance experiments on the Australian freshwater crayfish or yabbie (Parachaeraps bicarinatus) and the common garden snail (Helix pomatia) using colloidal carbon. These experiments represent one of the few examples of kinetic studies using colloids.

performed on poikilotherms.

The effect of temperature on the rate of phagocytosis in carp, using ^{131}I labelled bovine serum albumin, was examined by Avtalion *et al.* (1974) as part of an important study on the influence of environmental temperature on the immune response in fish. Avtalion's group re-examined the original work of Bisset (1946-9) who had also studied the effect of environmental temperature on the immune response in poikilotherms, and had concluded that antibody production could be divided into two stages; induction and manufacture. This latter stage, i.e. the manufacture of antibodies, Bisset considered to be temperature dependent, so that at low temperatures, production was inhibited. In contrast however, the Israeli workers showed that it is the induction stage which is the temperature sensitive event.

In the present work therefore, it was hoped to establish to what extent the pattern of clearance of bacteria from the bloodstream of plaice and the subsequent organ distribution, resembled that already established in mammals. In addition, by studying the effect of temperature on the observed clearance rate, it was hoped to substantiate Avtalion's findings.

CHAPTER 7.2 MATERIALS AND METHODS

A Fish used.

Adult plaice were used as described previously.

B Experimental conditions.

Groups of 3 fish were kept under a variety of experimental regimes, as follows:-

i Fish were kept at room temperature i.e. 17°C

ii Fish were kept at a high temperature i.e. 24°C

iii Fish were kept at a low temperature i.e. 4°C

iv Fish had prior exposure to the antigen at 17°C

v Fish had prior injections with colloidal carbon at 17°C

Under the above experimental conditions, two parameters were studied:-

i The rate of clearance of Salmonella gallinarum from the bloodstream.

ii The organ distribution of the bacteria, after their injection.

The fish were maintained in fresh recirculating aerated sea-water (recirculated by a filtration unit*), and allowed an acclimitisation

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*Eheim Ltd.

period of at least 48 hours before experimentation.

The high and low water temperatures were achieved by means of a thermostatically controlled water heater, and a cold room, respectively.

The fish which had been previously exposed to the antigen were kept at 17°C and over a period of 9 weeks, received 2 intramuscular injections of 50/50 complete Freund's adjuvant and sonicated Salmonellae culture. The size of each inoculum was 0.5 ml and contained 2.0×10^9 bacteria. The second injection was made 6 weeks after the first. The fish were used 3 weeks later when antibody levels from the secondary response would be expected to be high (Avtalion et al., 1974). These levels were not quantitatively measured, but qualitative serum agglutination tests showed good specific agglutination against Salmonellae, whereas control serum did not agglutinate.

The fish to be previously injected with colloidal carbon received their injections of 16 mg colloidal carbon in a 0.5 ml inoculum, 12 hours before the clearance experiments.

C Bacterial labelling.

Blood agar plates were inoculated from a type culture of

Salmonella gallinarum on Dorset Egg medium, in order to confirm the purity of culture. The bacteria were shown to be in the smooth form (S) by their failure to autoagglutinate in 0.85% saline, even when acriflavine was added (Wilson & Miles, 1964).

After 24 hours incubation at 40°C, single colonies were transferred to nutrient broth in 5 ml bijou bottles. After a further 24 hours incubation, one of these was used to inoculate 1 litre of nutrient broth in 5 lots of 200 ml. Half a millilitre of culture was used to inoculate each 200 ml bottle.

After 18 hours incubation, a small aliquot of this culture was diluted to 10^{-5} and 10^{-6}, and 0.1 ml of each dilution used for the inoculation of blood agar plates. These were incubated for 48 hours at 40°C, after which time, the number of colonies was counted, thus providing both a viable bacterial count, and providing a check on the purity of the culture. Their failure to auto-agglutinate was again shown, indicating that no smooth to rough (S→R) variation had occurred.

The remainder of the culture, meanwhile, was centrifuged at 12,000 rpm (R.C.F. value at this speed equals 23,000 g) for 15 minutes, on an M.S.E. High Speed 18 centrifuge, at room temperature. The supernatant was discarded, and the bacteria resuspended in 0.85%

saline. By this means, the culture was washed 3 times, before being finally suspended in 10 ml saline.

From the blood plates, at 10^{-5} dilution, there were 60 bacterial colonies, while at 10^{-6} dilution there were 8 colonies. Thus in the 10 ml saline, there were approximately 8.0×10^{9} bacteria/ml. They were killed by heating for 1 hour at $60^{o}C$ and then labelled with the sodium chromate (^{51}Cr) solution B.P. as described by Howard et al. (1959). This process involved incubation of the culture, suspended in 10 ml saline, with 2.5 millicuries of sodium chromate (^{51}Cr) at $37^{o}C$ for 48 hours. After this, 0.5 ml of the culture was removed and made up to 100 ml in a volumetric flask with saline - (1).

By washing the culture with saline and then centrifuging, Howard et al., were able to remove unbound ^{51}Cr. This was tried, but it was not found possible to obtain a radioactive-free wash, even after 5 or 6 successive centrifugations. Accordingly, the culture was placed in dialysis tubing and dialysed firstly overnight against running water, and secondly, for 2 hours against 3 changes of 1 litre or normal saline. The culture was then removed from the dialysis tubing, and placed in a glass screw-top bottle. Another 0.5 ml was again taken and made up to 100 ml with saline - (2).

This solution represented the standard, and whenever the radio-

activity of any material was counted, an aliquot of the standard was also counted so that an estimate could be made of the degree of radioactive decay, in order that adjustment of the resulting counts could be made.

By comparing the radioactivity of equal aliquots of (1) and (2) i.e. before and after dialysis, a measure of the efficiency of labelling was obtained. This was found to be approximately 80%.

D Injection, sampling and counting procedures.

Each fish received an intra-venous injection of 0.5 ml of the labelled bacterial culture into the R.P.V. Anaesthetics were unnecessary for this painless inoculation.

Immediately after the last fish in each experiment had been injected with the labelled bacteria, a blood sample was taken i.e. usually approximately 15 minutes after the start of the injections.

Blood samples were taken from the R.P.V. The first was taken immediately anterior to the point of injection of the labelled bacteria, each subsequent sample being taken just anterior to the preceding one. By this means, it was hoped to avoid sampling

difficulty due to reaction or clotting around the site of injection. A 25 gauge 5/8" disposable needle was inserted into the vein, the blood pressure alone being sufficient to force blood up into the plastic base of the needle without the need for suction. Once the blood had half-filled the base of the needle, it was removed, and a wet finger placed over the sampling site, to reduce blood loss as much as possible. A heparinised micro-haematocrit tube of standard bore was then inserted into the base of the needle, and blood was drawn up to a level of 4.2 cms. The needle was then discarded. By this means, very small (approximately 0.02 ml) blood samples were removed from the fish without unduly stressing them, or greatly altering the total blood volume. Samples were removed from the fish at the following times post injection:-

15 minutes	4 hours
30 minutes	6 hours
1 hour	8 hours
2 hours	24 hours

The samples were placed in a small numbered rack after blocking one end of the tubes with plasticine. For the preliminary experiments, haematocrit values were obtained from the tubes as a check on the efficiency of the technique as it could be readily determined from an abberrant haematocrit whether any lymph vessels had been punctured, or whether any water had been inadvertently taken into the tubes.

All samples were retained until the last had been taken i.e. after 24 hours, and then, together with the organs, they were all assayed at the same time. After the last sample had been taken, various organs were removed and placed in glass counting bottles of a known weight which were weighed again in order to obtain by subtraction, the weight of the organ. The organs taken were:-

Liver

Spleen

Heart (atrium and ventricle)

Kidney (divided into anterior and posterior)

As each batch of organs and blood samples were assayed, 2 additional samples were assayed:-

1. An empty glass bottle, in order to obtain an estimate of the background count.

2. A 1 ml sample of the standard.

All samples were assayed for 2 minutes in an automatic scintillation counter. Constant geometry of the microhaematocrit tubes was obtained by placing them in the glass counting bottles, as for the organs, where they stood virtually upright.

TABLE 8.1

MICROHAEMATOCRIT CLEARANCE VALUES

Time	Normal Temperature			Low Temperature			High Temperature		
	1	2	3	1	2	3	1	2	3
15 mins	40,907	44,527	35,860	13,216	12,891	14,739	12,145	7,755	10,012
30 mins	35,869	32,368	32,802	12,616	10,403	18,429	5,116	6,745	5,115
1 hour	27,002	26,597	25,727	8,126	9,288	10,480	3,568	2,305	3,192
2 hours	25,649	17,799	19,035	9,338	8,768	11,434	2,483	1,929	2,923
4 hours	11,278	6,855	10,126	6,147	5,693	7,840	2,904	1,158	1,816
6 hours	3,384	3,654	7,432	4,523	3,664	4,789	1,505	915	1,691
8 hours	2,443	3,080	-	3,486	2,866	3,139			
24 hours	1,463	846	996	1,242	957	1,293	2,362	723	2,221

Time	Immune			Control			Carbon			Control		
	1	2	3	1	2	3	1	2	3	1	2	3
15 mins	2,290	1,496	3,348	6,095	7,086	5,713	12,240	12,720	7,822	11,640	15,555	11,220
30 mins	700	359	996	2,352	3,218	3,445	12,625	14,020	6,012	6,440	6,725	7,665
1 hour	594	346	366	1,980	2,527	2,657	9,990	3,600	2,908	8,400	4,170	8,510
2 hours	285	215	137	1,005	2,039	1,468	5,675	3,320	2,940	3,800	5,100	2,820
4 hours	149	509	86	538	1,815	1,096	5,570	3,850	4,065	4,550	3,300	2,550
6 hours	137		100	896	951	695	2,912	4,060	2,200	3,740	2,280	2,255
24 hours	145		112	302	301	360						

Table 8.2 Initial and Secondary rates of clearance for each fish in each experiment (except carbon) plus their means

	Initial rate	Mean	Secondary rate	Mean
Normal	0.445 0.475 0.350 1.270	0.423	0.445 0.475 0.350 1.270	0.423
Low	0.450 0.450 0.375 1.275	0.425	0.150 0.167 0.218 0.535	0.178
High	1.400 1.650 2.500 5.550	1.850	0.118 0.217 0.333 0.668	0.223
Immune	6.600 5.500 5.300 17.400	5.800	0.250 0.383 0.167 0.800	0.267
Control	2.800 3.000 1.900 7.700	2.567	0.283 0.157 0.183 0.616	0.205

Lease significant difference (LSD) for means of 3 = 0.591

TABLE 8.3

ORGAN DISTRIBUTION

Organ	Normal Temperature			Low Temperature			High Temperature		
	1	2	3	1	2	3	1	2	3
Spleen	18,143	11,980	9,000	3,657	11,111	9,333	4,000	7,085	5,130
Heart	150	129	132	283	160	309	63	350	177
Liver	278	147	117	117	112	85	637	154	142
Ant. Kidney	2,333	1,975	2,117	539	557	1,945	1,550	1,708	2,064
Post. Kidney	2,747	1,930	2,016	1,467	1,367	2,405	1,185	1,216	1,368

The above figures represent the 10^3 counts per g. of tissue, measured over a period of 2 minutes.

Fig. 8.1 Comparison of Mean Clearance Values.

Each point represents the mean of 3 fish.

X = Normal

L = Low

H = High

O = Control

i = Immune

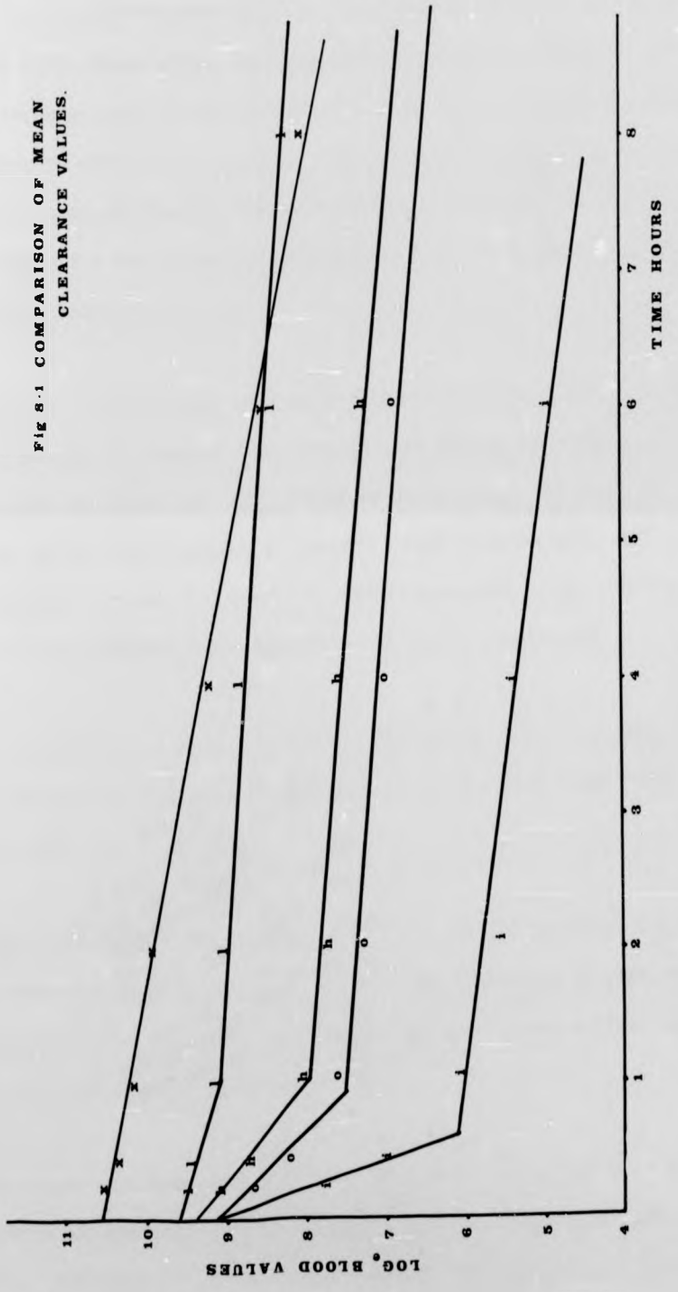

Fig 8·1 COMPARISON OF MEAN CLEARANCE VALUES.

CHAPTER 8.1 RESULTS AND INTERPRETATION

A. Results

The results obtained for the microhaematocrit tubes are shown in Table 8.1. These values were expressed as Natural Logarithms, and the means at each sample time for each experiment, (except for carbon clearance) were plotted against time, as shown in Graph 8.1. It can be seen that in general, the clearances are two phase, and the rate of clearance for each phase can be obtained from the slope of the line for that phase.

Graphs of clearance were plotted for individual fish, and the rates of clearance for each of the 2 phases were determined. These values are shown in Table 8.2 An analysis of variance was performed on these values (see Appendix I) which showed that overall, the differences between the first and second clearance rates, and the interaction between these two, were all highly significant.

By calculating the least significant difference (LSD) (= 0.591), it was possible to pinpoint the differences between individual treatments and rates.

Table 8.3 represents the organ distribution of the bacterial label, 24 hours after its injection. The relative importance of each organ was obtained by expressing the radioactive counts over a fixed period of time, per gram of tissue.

The values were expressed as Log_e rather than Log_{10} (which previous workers had used) as the former were derived from considerations of growth and decay factors and are therefore mathematically more correct.

Due probably to peri-vascular injection, the second sample value sometimes exceeded the first. Where this occurred, an average of the 2 figures was used for both the 1st and 2nd sample values, when plotting the graphs or for the analysis of variance.

B. Interpretation

From Graph 8.1 it can be seen that when the dose of bacteria exceeds a critical initial level i.e. injected dose, the clearance is exponential. As the injected dose is reduced, the clearance becomes biphasic, this being more obvious, the lower the injected dose.

The size of the inoculum remained constant throughout the study and corrections for radioactive decay were incorporated into the assay methods. Despite this, however, the first sample readings became progressively lower. Various explanations are offered to account for this:-

1. The ^{51}Cr label separated from the bacteria and thus when injected, were immediately excreted through the kidneys before the first sample could be taken. Dialysis of some old bacterial preparation however demonstrated conclusively that practically no label did separate, the radioactivity of equal aliquots taken before and after dialysis, remaining the same.

2. The bacterial preparation decomposed in some way so that label was still attached to fragments of the bacteria, but these were easily and rapidly cleared by the R.E.S., again before the first sample could be taken.

3. There were variations in the size of the fish used so that the relative dose levels differed. The weights of the fish did vary, and this probably accounts for the majority of the differences between

the initial dose levels.

From Graph 8.1, it can be seen that the first phase rate of clearance of the low temperature fish was approximately the same as for the normal fish. If however, one takes into account the fact that the average weights of the fish were 190g and 127g respectively, then the rate of clearance for 100g was appreciably slower in the cold fish than in the normals, e.g. using the formula:-

$$\text{rate} = \frac{\log_e \text{ value at 15 mins} - \log_e \text{ value at 1 hour}}{\text{t}}$$

	Mean Rate	Rate/100 grams
Normal Temperature	0.56	0.44
Low Temperature	0.56	0.295
High Temperature	1.59	1.1

From this it can also be seen that the 1st phase clearance rate of the high temperature fish exceeds that of the normal fish.

The injected dose levels in the immune and their control fish were the same, having been sampled at the same time. This is supported by Graph 8.1 where it can be seen that extrapolation of the respective lines back to time zero, brings them to the same point. The 1st phase clearance rate in the immune fish however was very much greater than in the control fish and reached the 2nd phase much faster.

The data from the carbon clearance experiment was not plotted on the graph or included in the analysis of variance as it appeared obvious that there was no real difference between the 2 groups of fish, i.e. the prior injection of a large dose of colloidal carbon had no effect

on the subsequent clearance of a dose of bacteria from the blood stream.

From Table 8.3 it can be seen that the relative importance of each organ did not vary with temperature, and in descending order of importance, these were as follows:-

spleen

kidney (anterior and posterior combined)

Liver/Heart

The heart comprises both atrium and ventricle, but in the experiments mapping out the R.E.S. it was demonstrated that the ventricle played only a minor role in cleaning the blood stream. Accordingly, the figures for the heart should be much greater, and the atrium is therefore considerably more significant in clearing the blood stream than the liver.

The major difference between treatments, therefore, lies in the first phase, the second phase remaining approximately the same for each treatment. The first phase lasted for approximately 1 hour except for the immune fish ($\frac{1}{2}$ hour). The second phase of clearance lasts up to approximately 8 hours, by which time, most of the bacteria have been cleared from the blood stream. By 24 hours, a small residual level remains in the blood stream.

CHAPTER 8.2 DISCUSSION AND CONCLUSIONS

A Normal, immune and carbon injected fish

In comparison to the mammalian situation (Biozzi et al, 1960), the clearance of Salmonellae from the blood stream of the plaice is much slower; approximately 8 hours as opposed to 20 minutes. This finding however is in accordance with those of Nelstrop et al (1968), who studied the clearance of bacteriophage in several poikilotherms including the goldfish. The latter workers also noted that, as in the present work, clearance appeared to be more rapid over the first 2 hours. Biozzi et al. (1960) found that in mice, approximately 60% of the injected bacteria were cleared exponentially, the rate of clearance then slowing down until a residual radioactivity remained after 20-30 minutes. These orders of clearance apply also to the present work.

The same explanations as to why clearance becomes progressively slower in mice are valid for plaice. Biozzi suggests that there is a residual population of bacteria which resists phagocytosis, possibly related to their electric charge (Choucroun, 1936), and quotes the work of other authors to support this contention. Rogers (1956) explained the ability of certain bacteria to evade the R.E.S. by their location within circulating granulocytes. No attempt was made in the present work to establish the involvement of the neutrophil in the phagocytosis of the bacteria.

In his review article on host mechanisms which remove bacteria from the blood stream of mammals, Rogers (1960) noted that immunisation specifically increases the trapping ability of the splanchnic organs (liver and spleen) for bacteria. Increased trapping within other

organs could not be demonstrated. The findings of the present work in the immune fish, that the presence of specific antibody greatly increased not only the initial rate of phagocytosis, but also reduced the length of time of 1st phase clearance from approximately 1 hour to ½ hour support the work of Benacerraf et al.(pers. comm. in Biozzi et al., 1960) who considered that the limiting factor for the maximum clearance of bacteria in mice, was the availability of circulating antibody due to its opsonic effect.

It was unfortunate that the organ distribution for the immune fish was not determined as this would possibly have shown an increased efficiency of trapping, not of both liver and spleen as in mammals, but of spleen alone.

As in mammals, the prior injection of colloidal carbon did not significantly alter the efficiency of uptake of the Salmonellae in the plaice, although the possibility of other colloids such as heat denatured serum albumin successfully competing with the bacterial clearance should perhaps be investigated. Another aspect requiring investigation is whether the bacterial species used would affect the pattern of clearance i.e. would the pattern of clearance of a bacterial species to which the plaice is liable to come into contact e.g. Vibrio anguillarum, be very different from that of the heat killed Salmonella gallinarum to which it is very unlikely to come into contact? In mammals, the efficiency of extraction varies greatly (from 36-81%) with the species of bacterium employed (Wardlaw and Howard, 1959).

Can the clearance of the Salmonellae from the blood stream be explained wholly on the basis of phagocytosis? Rogers (1960) suggests that other factors do affect the clearance because even after complete

blockade of the R.E.S., the blood stream is still cleared and mechanisms such as intra-vascular clumping of bacteria, trapping within the lungs, or uptake by granulocytes which subsequently disappear from the blood stream, are suggested as being responsible. Although in fish, pulmonary trapping of bacteria is not possible, filtration by the gills may play some part, as indicated by the B.C.G. bacilli and Salmonellae trapped there during the mapping out studies (Chapter 4).

Despite the fact that the trapping of bacteria within the splenic ellipsoids during the mapping out experiments was not a conspicuous occurrence, the trapping of colloidal carbon was very marked, and this fact together with the organ distribution figures for the ^{51}C labelled Salmonellae (in every case the spleen was absolutely and relatively the most important organ) indicate that the spleen does act as a filter bed for the bacteria. Thus the clearance of the Salmonellae from the blood stream could possibly be explained as due not only to phagocytosis, but also to a sequestration partly within the capillary beds of the gills and possibly kidney, but mainly within the Schweigger-Seidel sheaths of the spleen i.e. a 'passive' clearance effect.

First phase clearance therefore is variable, and probably reflects alterations in phagocytic activity. Second phase clearance however is independent of environmental conditions, having remained nearly constant for every experiment. This finding suggests that second phase clearance is due mainly to mechanisms which are independent of both metabolic activities and of circulating antibody levels. These mechanisms are probably not accounted for by a purely trapping effect e.g. within the Schweigger-Seidel sheaths, because prior injection with colloidal carbon (even large amounts) does not significantly alter subsequent

bacterial clearance, unless of course their capacity were large and therefore not exceeded by the amount of carbon injected.

How can the greatly increased efficiency and rate of removal of Salmonellae from the blood stream of the immune fish be explained? Is this solely due to the opsonic effect of specific antibody?

As reviewed by Stuart (1970) it has been suggested that prior exposure to an antigen may lead to the appearance of specifically sensitised macrophages. Nelstrop et al.(1968) demonstrated that an increased clearance of a secondary dose of T_1 bacteriophage in the absence of any detectable neutralising antibody, may occur in goldfish, lamprey and shore crab. This was also demonstrated in the rabbit by the same authors. Papermaster et al.(1964) in their work on the evolution of the immune response, demonstrated that in teleost and elasmobranch fish, there was an increased clearance of a secondary dose of antigen, but that this time, antibody was associated.

There is however no real evidence for the existence of an "immune macrophage", i.e. "a cell which has the ability to phagocytose and destroy one specific organism for reasons other than the presence of antibody absorbed on its surface" (Carr, 1973). The probable explanation for this increased secondary clearance is that, following the primary exposure to an antigen, macrophages, both free and fixed cells, are induced to mature. As shown by Hard (1969, 1970), peritoneal macrophages from mice immune to Corynebacterium ovis, contained more hydrolytic enzymes, and although they used less oxygen, were more active in glycolysis than normal macrophages. They also contained many more lysosomal granules than normal cells i.e. they were more mature.

The weight of evidence from the mammalian viewpoint therefore, tends to suggest that increased RE clearance is due to a non-specific maturation of cells and that the increased avidity demonstrated by macrophages in immune lesions is due to their possessing a layer of cytophilic antibody on their surface.

It is debatable whether or not there is in addition, any proliferation of the non-specific trapping mechanisms to augment clearance i.e. do the ellipsoidal walls become thicker following a primary exposure to an antigen?

It is highly probable that the antibodies produced by the plaice in response to the Salmonellae were IgM type antibodies, and these have been shown in mammals, (Robbins et al., 1965) to be 22 times more active than IgG in inducing agglutination and 500 to 1000 times more efficient as an opsonin, although they do have a shorter intra-vascular half-life.

B The influence of temperature on the clearance

Neither the low nor the high temperature inhibited the clearance of the Salmonellae. Taking into account the relative sizes of the fish, the rate of clearance at the low temperature was approximately half that at 17°C, and although clearance is not solely the result of phagocytosis, phagocytic activity would be slowed due to a purely metabolic effect, and this probably accounts for the majority of the reduced clearance rate. For a drop of 13°C, there has been an approximate halving in the clearance rate i.e. Q_{10} is approximately equal to 2 (see Prosser and Brown, 1961), and this therefore is in accordance with the supposition that the reduced clearance rate is due to a metabolic effect.

Similarly, the raising of the temperature to 24°C increased the metabolic rate, and hence clearance was faster. After 24 hours at the high temperature however the levels of activity in the blood were still quite high. The following mechanisms may be operating to account for this:-

1. Following rapid phagocytosis of bacteria by fixed macrophages, they round up and float off, as was shown to occur with colloidal carbon, and are therefore counted along with free bacteria in the circulation.

2. Due to a faster metabolic rate, the ^{51}C label becomes detached from the bacteria which are being rapidly digested. This label could then enter the blood stream associated possibly with metabolites or plasma proteins.

3. An increase in uptake by the monocytes or neutrophils.

The amount of radioactivity in the blood stream at any one time must be a balance between the rate at which the bacteria are being phagocytosed, and the rate either at which macrophages are being released into the circulation, or at which the bacteria are being digested with the subsequent release of label. In this context, it is interesting to note that the clearance at the high temperature may show an upwards tendency after 6 hours indicating possible release of label in one form or other. A more complete sampling procedure could possibly establish this point.

For the low temperature experiments, the blood stream was still not cleared, at 24 hours, to the same degree as that of the normal 17°C fish. Continuation of the sampling procedure beyond 24 hours would possibly have shown this figure dropping to as low a level as for the normal fish.

Fish populations appear to be particularly susceptible to bacterial disease at high environmental temperatures e.g. vibriosis of flatfish caused by Vibrio anguillarum. Bisset (1947) stated that this was due to the fact that although antibody production increases at higher temperatures, so too does the replicative power of the bacteria, and the outcome must be determined by the balance achieved between these two opposing factors. Although many other factors operate such as water quality, and over-crowding, the survival of the fish is more likely to be determined by whether or not they can acquire sufficient antibody protection in time, before the massive increase in bacterial numbers (Avtalion et al., 1974). Although therefore the rate of phagocytosis of the R.E.S., increases with a rise in temperature and the trapping capacity increases due to a non-specific maturation of cells, in the absence of specific antibody, this alone would be insufficient to cope with the numbers of bacteria in the blood stream. In this context, it should be noted that in the immune fish, the clearance of the blood stream was faster than at the higher temperature.

At low environmental temperatures on the other hand, many authors (reviewed by Avtalion et al., 1974) have shown that the immune response of fish is greatly reduced, or completely inhibited. Avtalion however has shown that it is the priming of the immune response which is the temperature sensitive stage and not the production or release of antibodies as stated by Bisset (1948). These findings mean that as long as a fish is allowed a sufficient length of time at normal (or high) temperatures during which the antigen can be processed, the production and release of antibodies will occur at low temperatures, although at a reduced rate due to a purely metabolic effect.

Avtalion (unpublished, 1974) has in fact demonstrated that carp can clear ^{131}I labelled bovine serum albumin from their blood streams, at

low temperatures, and uses this, plus *in vitro* tests on leucocytic destruction of *Staphylococcus aureus*, as evidence to state that phagocytosis is not the temperature sensitive stage of the priming event. He postulates that the temperature sensitive stage is possibly the interaction of macrophages with immunocompetent cells. The present findings support those of Avtalion i.e. that the clearance of bacteria from the blood stream is uninhibited at low temperatures.

From the study, it is felt that the following main conclusions can safely be drawn:-

1. Plaice are able to clear their blood stream of bacteria at high and low environmental temperatures, although the rate of this clearance varies, due probably to a metabolic effect.

2. Previous exposure to an antigen enhances the initial rate of clearance.

3. Prior injection with colloidal carbon does not affect the subsequent clearance of *Salmonellae*.

TABLE 9.1 RES of Plaice, and probably other species of Higher Teleosts

Organ	Site
Atrium	Endocardial cells
Spleen	The sinus lining cells and the stromal macrophages, especially those closely associated with the ellipsoids. The large, pale, reticular cells of the Schweigger-Seidel sheaths are included, but they have a lower phagocytic capacity
Kidney	The endothelial cells of the 'peri-tubular capillaries', plus the many stromal macrophages.
Blood	Monocytes, and to a lesser degree, thrombocytes
Gills	Macrophages possibly associated with pillar cells
Gut	Macrophages in the lamina propria
Liver	Bile duct macrophages. Fat storing cells
Gonads	Stromal macrophages

Approximate order of activity (descending)

CHAPTER 9 GENERAL DISCUSSION

The results of the present study would suggest that the reticulo-endothelial system of plaice, and probably other species of higher teleost fish, can be defined as shown in Table 9.1.

By complete contrast with the mammalian situation, it is surprising to find that the liver plays virtually no part in the clearance of particulate matter from the blood. The number of sinusoids is comparatively small and hence the surface area of sinusoidal lining in direct contact with the blood is small. The subsequent reduction in filtering capacity is augmented by the lack of actively phagocytic cells in the sinusoids i.e. Kupffer cells are not present.

The reduction in sinusoidal development, means that instead of hepatic cords composed of double rows of cells, the cords in fish are composed of 4, 5, 6 or 7 cells. Probably as a result of this, in the initial stages of the ducting of bile into the major bile ducts, the pathways extend to an intra-cellular level i.e. due to the less regular orientation of hepatocytes, the ducting has to be more specific, to enable efficient transport of bile to take place. Where the cords are sufficiently small, inter-cellular canaliculi similar to those found in the mammalian liver are found.

The extensive distribution of fat-storing cells within the Space of Disse accounts for the often considerable amounts of lipofuscin/ ceroid-type material found here in many fish and visible in the light microscope. They endocytose material which gains access to the Space of Disse, but due to the reduced fenestrations of the endothelial lining, it is thought unlikely that any virulent particle other than viruses would be capable of this. The large numbers of fat-storing cells are possibly closely associated with the often extensive fat content (in the form of droplets) of the hepatocytes.

Fish liver therefore functions as a digestive organ in that it stores and metabolises fat and manufactures bile, but it does not serve as a filtration organ for the removal of particles from the blood stream. This role is fulfilled by the kidney, spleen and atrium.

The spleen and kidney are responsible for the clearance of most of the injected matter but the fact that the atrium is active at all means that it is in a vulnerable position. This is particularly evident, when one considers that the teleost fish has no lymph nodes, and thus in comparison to the mammal, many more routes exist whereby pathogens can reach the heart or any other major organ, without first coming into contact with reticular tissue. Clinical evidence of this vulnerability has been

regularly observed in the routine diagnostic work of this laboratory. Young cultured fishes, both salmonids and flatfish, are extremely susceptible to acute septicaemias, resulting from infection by aeromonad or vibrionic bacteria, and they succumb very rapidly. When the hearts of such fish are examined, most of the atrial endocardium has usually sloughed, and there is evidence of atrial myocardial necrosis. Ascites and loss of pigmentation control are often the only outward manifestations of disease in such fish, the oedema possibly reflecting cardiac failure, or at least impaired function.

It is suggested that the acute diseases clinically observed to affect the atrium are directly attributable to the capacity of the endocardium for phagocytosis. As originally suggested by Bisset (1946), fish may tolerate a bacteraemia at low temperatures and only when the temperature is raised does the production of antibodies occur in amounts great enough to clear the bloodstream, despite similarly enhanced bacterial replication. If however, the rise in temperature is rapid, antibody production may lag behind bacterial replication. Under these circumstances, the hearts of the fish are clinically affected as are the other organs.

The kinetic studies, using radio-labelled bacteria, show that the rate of clearance of bacteria from the bloodstream in the absence

of specific antibody is enhanced at higher temperatures, due probably to a purely metabolic effect. The atrial macrophages are thus capable to endocytosing at an increased rate, the larger numbers of bacteria present.

Chronic bacterial endocarditis however is a poorly recognised entity in fish. This may be explained in one of two ways. Firstly, if an antigen of a less virulent nature is endocytosed, then the macrophages have time to round up and detach, thus clearing the heart, in a similar manner to the way in which the carbon was cleared, approximately three weeks after injection. Secondly, it may be that few bacteria are taken up, because of some discriminatory mechanism at the cellular level. This latter explanation i.e. a discriminatory mechanism for bacteria, may account for the relatively low numbers of B.C.G. bacilli and Salmonellae bacteria seen to localise in the atrium, in comparison to the high numbers of yeast or colloidal carbon particles.

The precise mechanism responsible for internalising most of the carbon was not immediately apparent although pinocytotic activity was very evident. This means of uptake however could not account for the endocytosis of the yeast particles, unless more than one macrophage were involved, similar to the situation reported in mammals by Shirahama et al. (1971) who noted that co-operation by

several macrophages was necessary in order to interiorise large particles. In the case of the injected yeast cells, the atrium is possibly acting as a framework or scaffolding with a very large surface area, onto which circulating phagocytes can attach, in order to co-operate with the existing macrophages in engulfing the particle. The close association of monocytes and neutrophils with the atrial endocardium was a constant finding although initially, the possible implications were overlooked. An ultrastructural investigation of the mechanism whereby the endocardial macrophages can endocytose such large particles, could possibly provide valuable information relating to the involvement of the atrium in disease processes of viral, bacterial, mycotic or protozoal etiology.

A replacement mechanism for those endocardial cells which rounded up and detached was not evident, although a possible explanation may be found either in division of the endocardial cells themselves (mitotic figures were occasionally observed) or by maturation of the sub-endocardial cells. The latter cells were reported by Kisch (1962) to be also phagocytic.

The meshwork structure of the heart helps to reduce the strain on individual fibres during periods of high output (stroke volume and not heart rate, increases). A further safeguard is possibly provided by the presence of desmosomes in the inter-

calated disc (see Chapter 6), the apparently large alterations in sarcomere length possibly being accommodated by the very well developed sub-endocardial space.

Regarding atrial involvement in disease processes in mammals, Jarplid (1964) indicated that in his experience, the right auricle was the primary site of leukotic changes in the bovine heart. He associated this with the atrium's property of embryonal haematopoiesis, and also reviewed various disease conditions affecting the atrium in other mammalian species, including man. It would appear that such a contentious association can be supported teleologically by reference to the fish atrium, which not only has a capacity for embryological haematopoiesis (Faure-Fremiet, 1931) but also retains the property, in the adult, of active phagocytosis.

The observations on the spleen of the protozoan infected turbot strongly suggest that there is a pattern of cellular migration from the ellipsoids to the melano-macrophage centres. Macrophages from the sheaths, replete with cellular debris, probably of protozoal origin but possibly of host origin, migrate to the melano-macrophage centres which themselves originate in the outer parts of the sheath. During this migration, the macrophages come into contact with the immunologically competent cells lining their route

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and cytoplasmic interdigitations of plasma cells with dendritic macrophages are encountered. Subsequently, the macrophages are segregated within the melano-macrophage centres which would therefore appear to be acting as a focus, or a kind of germinal centre for the immune reaction.

It is tentatively suggested that this migration pattern is analogous to the well recognised pattern in the fowl (White and Gordon, 1970) where either macrophages or dendritic cells migrate from the ellipsoids into the lymphoid malpighian areas, with the consequent possible formation of a germinal centre, should the dendritic cell be carrying antigenic material.

A migration pattern such as this would explain the accumulation of virtually all types of injected particle within the melano-macrophage centres of the spleen. A similar example of cytophilic attraction or 'cellular togetherness' is demonstrated in the melano-macrophage centres of the kidney. It is not known whether the macrophages comprising these centres all originate in the endothelial lining of the 'peri-tubular capillaries' or whether some other, as yet unidentified, source exists, e.g. capillaries sheathed in a similar though much reduced manner, to those of the spleen.

The melano-macrophage centres however are also the site where, under normal conditions, metabolically stable products of host catabolism e.g. phospholipid and haemosiderin, are deposited. These are probably derived from host cells such as effete blood cells, and this material is again transported by macrophages closely associated with the ellipsoids. Thus at the two extremes, melano-macrophage centres can act as 'metabolic dumps' for material of host origin, and 'germinal centres' for antigenic material.

The fact that particles larger than the colloidal carbon were not seen to locate in large numbers within the Schweigger-Seidel sheaths may mean that as previously suggested, the gaps seen in the endothelial lining of the axial vessel were present due only to active cellular migration, and that large particles such as yeast or bacteria may first require to be phagocytosed by either fixed or circulating macrophages before they can be transported intra-cellularly across the endothelial lining into the sheath.

Viruses, as well as carbon particles, could probably gain direct and immediate access to the sheaths because of their small size. An investigation into the effects of viruses on splenic pathology could possibly elucidate this point which may provide one explanation for the affinity of fish viruses for haemopoietic tissue.

Appendix 1

Analysis of Variance for rates of clearance of Salmonellae

	Initial rate	Secondary rate	
Normal	0.445 0.475 0.350	0.445 0.475 0.350	
	1.270	1.270	2.540
Low	0.450 0.450 0.375	0.150 0.167 0.218	
	1.275	0.535	1.810
High	1.400 1.650 2.500	0.118 0.217 0.333	
	5.550	0.668	6.218
Immune	6.600 5.500 5.300	0.250 0.383 0.167	
	17.400	0.800	18.200
Control	2.800 3.000 1.900	0.283 0.150 0.183	
	7.700	0.616	8.316
	33.195	3.889	37.084

Source of variation	Sums squares	degrees freedom
Total	89.722627	29
Treatments	28.957078	4
Rates	28.628054	1
Interaction	29.726042	4
Residual	2.411453	20

	Mean Square	Variance ratio
Treat.	7.239269	60.04***
Rate	28.628054	137.43***
Inter.	7.431510	61.63***
Resid.	0.120573	

*** = Highly significant

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