ASPECTS OF THE MELANO-MACROPHAGE CENTRES IN FISH

by

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DECLARATION

I hereby declare that this thesis has been composed by myself and is the result of my own investigations. It has neither been accepted nor is being submitted for any other degrees. All the sources of information have been duly acknowledged.

.....Carmelo Agius.....

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PREFACE

This thesis is concerned with aspects of the melano-macrophage centres of fish and is divided into eight chapters. Following a general introduction to the subject, some phylogenetic and ontogenic aspects are discussed. Chapters 4 and 5 describe how various parameters affect these pigmented structures, both in fry and adult fish. Chapter 6 evaluates the role of melano-macrophage centres in the storage of iron both under normal conditions and in conditions of stress such as starvation and disease. In the next chapter, the possible modes of genesis of the pigment granules of melano-macrophages are investigated; this study is carried to the ultrastructural level. The final chapter presents the general conclusions on the possible nature of these centres in the light of the available evidence.

The body of experimental work is described in Chapters 2 - 7; each of these has its own introduction, materials and methods, results and discussion. Consequently, many of the more specialized references are quoted only in their relevant chapter and not in the general introduction or discussion.

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ABSTRACT

A number of aspects of the melano-macrophage centres of fish were investigated. In teleosts these centres consist of aggregates of pigment-laden macrophages and various leucocytes. They are usually embedded in haemopoietic tissue (mainly within the spleen and kidney) in association with the blood-supplying vessels and in a few species a distinct lymphoid cuff surrounds the entire structure. Salmonids are exceptional in that their melano-macrophages are scattered throughout the haemopoietic tissue and do not form distinct aggregates. At least four types of pigments have been described to occur in these phagocytes, viz. melanin, the lipogenic pigments ceroid and lipofuscin, and the haematogenous pigment haemosiderin. In light microscopy the centres appear in varying hues of yellow, brown and black. It has been suggested that these centres could well represent the primitive analogues of germinal centres of the lymph nodes of birds and mammals.

As all previously available information came exclusively from a restricted number of teleost species it was considered of primary importance to carry out a study of the distribution and cytological organization of these centres in living representatives of Agnatha, Chondrichthyes and Osteichthyes. Seventy-two species of fish had their haemopoietic tissues examined by light microscopy for pigment-containing macrophages. Except for the lamprey Lampetra fluviatilis, all fish species were observed to possess these pigment cells. An evolutionary pattern was evident in both

the distribution and the degree of organization of melano-macrophages; three major evolutionary trends were discernible, viz:

i. a progressive increase in the abundance of pigment cells;

ii. a structural evolution from a random distribution of individual pigmented macrophages observed in Agnatha and Chondrichthyes to organized centres characteristic of all Osteichthyes except the salmonids;

iii. a change in organ location of these pigment cells from the liver in Agnatha, Chondrichthyes and the primitive bony fishes to the spleen and kidney in the advanced bony fishes.

The increasing sophistication in cytological organization of the melano-macrophage centres is concomitant with the increasing levels of complexity of the cytoarchitecture of the lymphoid system. The increasing proclivity of the centres for the main lymphoid organs follows closely upon the evolution of the lymphoid system and represents a major advance in the evolution of lympho-reticular relationships. These analogies provide additional evidence that these centres are of a lymphatic nature and may well represent the primitive analogues of germinal centres of birds and mammals.

Ontogenically pigment-bearing macrophages appear following upon first feeding. Immunological maturity appears to be attained at first feeding and the fact that it is shortly afterwards that melano-macrophages appear within the lymphoid tissues (eventually

leading to melano-macrophage centre formation within them), seems to add weight to the evidence of a structural and functional relationship between melano-macrophage centres and lymphoid tissues.

The very marked changes observed in the melano-macrophage centres during cachexia provided a convenient tool for studying these pigmented macrophages. In adult rainbow trout Salmo gairdneri and plaice Pleuronectes platessa kept at 12°C, the density of melano-macrophages and melano-macrophage centres respectively had increased considerably after 6 weeks of complete deprivation of food and by 10 weeks very high densities were observable. At higher temperatures (25°C), employing Tilapia zillii and swordtails Xiphophorus helleri, a very marked increase in the density of these centres was already evident after 3 weeks of complete starvation. With first feeding rainbow trout fry kept at 12°C, high densities of melano-macrophages were observed within the spleen and kidney after 3 weeks of complete starvation. No other treatment employed in this study was observed to induce any significant changes in the melano-macrophages of either fingerlings or adult fish.

These results suggest that tissue atrophy is a major factor contributing to the formation of the pigments observed within the melano-macrophages. Electron microscopic observations employing normal and cachectic plaice indicated the following possible modes of origin for the pigments within melano-macrophages:

i. melanin granules seem to be derived from their being simply phagocytosed from the classical melanin-containing

cells that have been ruptured or otherwise damaged;

ii. lipogenic pigments appear to derive from damaged cellular components such as effete mitochondria through the process of peroxidation of their unsaturated lipids;

iii. haemosiderin is almost certainly derived from the breakdown of haemoglobin from effete erythrocytes.

Since lipid peroxidation and recycling of iron compounds lead to the formation of free radicals and cations, these potentially toxic entities are bound to arise spontaneously within melano-macrophage centres. In view of this there is raised the possibility that the melanin within the centres could be playing a very important role through its well-recognized ability to absorb free radicals and its strong affinity for cations. While this would account for the presence of all these types of pigments within melano-macrophages, it could also explain why pigment cells are so often observed at sites of infection or tissue injury.

All ingested cellular debris appears to be subjected to lysosomal enzyme activity and it is the indigestible residues (indigestible unsaturated lipids mainly) that give rise to the pigments which gradually accumulate. The absence of pigment in young larval fish, its steady accumulation with age in clinically normal fish and its presence without exception in older fish indicate that the pigments being studied seem to satisfy the criteria set forth for a basic biological aging process.

The role of melano-macrophage centres in iron storage

in normal and diseased fish was studied. The spleen, kidney and liver centres of fourteen species of clinically normal teleost fish were examined histochemically for haemosiderin. This was found to be present in varying amounts within the splenic centres of most specimens, but in contrast it was rarely found in the centres of the kidney and the liver. Under conditions of starvation and in diseased fish, a markedly increased deposition of ferric iron occurred in the splenic centres of nearly all fish examined. By comparison, the iron content in the kidney and liver centres was generally still very low. These results suggest that although the centres in the various haemopoietic organs resemble each other morphologically and in their relation with the associated tissues, there could well be important functional differences between the centres of different organs. When rainbow trout that had been splenectomised were starved, accumulation of haemosiderin was diverted to the kidney melano-macrophages; the liver was still virtually devoid of iron. The possible implications of these findings are discussed.

In conclusion, all the available evidence seem to indicate that the melano-macrophage centres of fish and the germinal centres of birds and mammals are similar in many ways. A major difference is the high levels of pigments in the centres of fish. This seems to be related to the inability of the latter to control their body temperature. Fatty acids of living organisms shift towards greater unsaturation under lower environmental temperatures as a means of maintaining protoplasmic viscosity within the range necessary for normal metabolic processes. Thus

fish, because of their poikilothermic nature, have high levels of unsaturated fatty acids in their bodies and are thus more prone to lipofuscin formation. It has also been suggested that intracellular digestive processes of fish macrophages may not be well developed on the evolutionary scale. This could also lead to increased accumulation of indigestible materials within the melano-macrophage centres.

Finally these results are discussed with special emphasis being placed on the following three points:

i. that the melano-macrophage centres should be regarded as sites where a large variety of materials are aggregated, processed, sifted and disposed of in a variety of ways rather than regarding them as static areas passively accepting and storing any materials that come their way. Of special significance are those materials that are required for recycling such as iron-containing compounds;

ii. that there are important functional differences between the melano-macrophage centres of different organs;

iii. that as more information becomes available the melano-macrophage centres may well become useful as sensitive indicators of the state of health of the fish.

A technique for bleaching the pigments within melano-macrophage centres in ultra-thin sections was developed. Treatment of the sections with permanganate for five minutes followed by fifteen minutes in metabisulphite resulted in complete bleaching of almost all the pigments.

CHAPTER 1

GENERAL INTRODUCTION

1.1 DISTRIBUTION OF PIGMENT CELLS IN FISH

Melanin-containing cells can be found in many sites within the tissues of fish. In addition to the well-recognized cells of the pigmentary system, melanin-containing cells are found in the peritoneum, around blood vessels and lymphatics and within healing wounds. Moreover, melanin-containing cells, which very often form distinct aggregates, are commonly encountered in the haemopoietic tissues. In teleosts, haemopoiesis is located in the stroma of the spleen, the interstitium of the kidney and to a lesser extent the periportal areas of the liver, the intestinal submucosa and the specialized lymphoid organ, the thymus. These aggregations of melanin-containing cells are typically found in the spleen, kidney and liver.

1.2 NOMENCLATURE

The nomenclature of vertebrate melanin-containing cells has for a very long time been a very controversial issue. The generally accepted classification agreed upon at the Sixth International Pigment Conference, Sofia, is that of Fitzpatrick et al. (1967). Unfortunately during this same meeting it was decided to delete the terms melanophage and macrophage as it was widely felt that the macrophage should be regarded only as an incidentally pigmented cell in which the incorporation of

melanin granules is but one expression of its generalized phagocytic activities. Roberts (1975) proposed the name melano-macrophage centres for the aggregates of pigment cells within the haemopoietic tissues because of their high pigment content and their ultrastructural resemblance to macrophages. These structures have not been reported in higher animals.

1.3 BRIEF REVIEW OF THE LITERATURE

Jolly (1923) was the first to mention the centres when he described them as collections of macrophages containing greenish-brown pigment which represents breakdown products of haemoglobin from degenerated red blood cells. Mackmull and Michels (1932) working with the cunner Tautogolabrus adspersus demonstrated that intraperitoneally injected carbon eventually homed on these sites.

Tokumaru and Ferri (1970) combining solubility, bleaching and histochemical tests on the pigments in the spleen, kidney and liver of three fresh-water teleost species (viz. Pimelodus maculatus, Prochilodus scrofa and Cyprinus carpio) suggested that they are ferrigenous lipochromes, comparable to the Gandy-Gamma nodules observed in humans suffering from some infections. The first detailed descriptions of the melano-macrophage centres were published by Ellis (1974) and Roberts (1975); this latter worker also reviewed the available data. Roberts described the centres as periodic acid -Schiff (PAS) -, Schmorl's -, and Ziehl-Neelsen positive and generally yellow-black in colour.

Ferguson (1976a, b) in his ultrastructural studies of the spleen of the turbot Scophthalmus maximus, described the

relationship of the centres to the splenic ellipsoids. These latter are specialized arterioles or capillaries and comprise a flattened endothelium surrounded by a sheath of macrophages which is bounded by a fibrous membrane. The main findings of Ferguson are summarized in section 1.7.

Oguri (1976) investigated some histochemical properties of the pigment cells in the rainbow trout Salmo gairdneri kidney and concluded that the pigment granules are melanins. More recently, analysis for lysosomal enzymes in such pigment cells of the yellow perch, Perca flavescens and the golden shiner, Notemigonus crysoleucas, has provided additional support for the suggestion of Roberts (1975) that the pigmented centres are indeed composed of macrophages (George, personal communication).

1.4 THE PIGMENTS

Edelstein (1971) put forward a definition for melanins to include "all biologic yellow, yellow-brown and black organic substances that are polycyclic polymers of high molecular weight, insoluble in most solvents, and resistant to all but the severest acid or base digestion techniques"; such a definition includes both classical melanins and lipofuscins. From the published data it is clear that the macrophages under study carry different types of pigments frequently even within the same cell. Employing histochemical techniques, at least four types of pigments have to date been shown to be present in the melano-macrophages of fish haemopoietic tissues, viz.: melanin, the lipogenic pigments

lipofuscin and ceroid and the haematogenous pigment haemosiderin (as suggested by the presence of ferric iron). The origin and nature of these pigments are clearly different; it is very likely that their functional significance and fate vary markedly as well. The issue has unfortunately been slightly confused due to the unjustified free interchangeability of the terms ceroid, α-tocopherol deficiency pigment and lipofuscin, by numerous workers. The chemical structure of these pigments is still enigmatic and only their chemical characterization could settle this point; a reappraisal of a large body of literature will then be certainly required.

Within the macrophages under study, lipofuscin appears to be by far the most abundant pigment when viewed across the various fish lineages; melanin is the other major component. Haemosiderin can be present in considerable quantities under certain conditions (vide infra). In this study the terms melano-macrophages and melano-macrophage centres will be retained to denote the pigment-carrying macrophages under study and aggregates of them, respectively. Where relevant the specific pigment is named; otherwise the more general term "pigment" is employed.

For a fuller account of these pigments and a tabulation of their histochemical characteristics the reader is referred to Thompson (1966).

1.4.1 Melanins

True melanin is synthesized from tyrosine in a stepwise process. Edelstein (1971) presented two pathways, one for the

production of integumental melanin and one for visceral or neuromelanin. Pigmentary melanins are polymers of 3, 4 dihydroxyphenylalanine-quinone (DOPA-quinone) synthesized by oxidation of the amino acid tyrosine to DOPA then to DOPA-quinone by the enzyme tyrosinase. Such synthesis takes place on laminar intracellular organelles, the premelanosomes, and the mature pigmented granule, the functional pigmentary organelle, is known as the melanosome. Whereas in higher animals melanogenesis takes place within melanocytes situated in the base of the epidermis which then pass the pigment to the Malpighian cells in the outer epidermal layers, in fish these activities are largely confined to the dermis. Here, the pigmentary melanin-containing cells are the melanocytes and melanophores. Melanocytes are immature melanophores actively producing melanin but capable of becoming melanophores and moving up into the functional melanophore layer (Bullock and Roberts, 1974). Neuromelanin is seen within such visceral structures as the neurones of the brain, myocytes of the heart, and the hepatocytes and Kuppfer cells of the liver. This type melanin may serve as an important clue to the understanding of certain abnormal or disease states, such as the brown atrophy of the heart and liver which is thought to result from abnormal pigment metabolism and is thought to be one of the significant manifestations of visceral aging (Edelstein, 1971). The neuromelanin found in the substantia nigra of the brain is thought to arise from the neurotransmitter, dopamine. According to Lillie (1957: cited in Thompson, 1966), neuromelanin appears to be a distinct substance, perhaps not

even closely related chemically to the other melanins.

1.4.2 Lipofuscins

The lipofuscins constitute a homogenous group of lipogenic pigments. They are derived, in part at least, by the oxidation of unsaturated tissue lipids or lipoproteins, and are observed to be widely distributed in the body of man, fowls, reptiles and lower animals. These pigments are thought to be derived as lipid waste products from the catabolism of various organs and tissues; Bloom and Fawcett (1975) suggested that they represent an end stage of lysosomal activity associated with normal wear and tear accumulate in the cell. They are commonly referred to as "brown atrophy", "wear and tear", or "waste" pigments. A large body of data indicates that destruction of certain portions of cells (i.e. cell membranes) results in deposits of lipid pigments which then may be associated with neuromelanin in the form of a lipofuscin (liponeuromelanin) granule (Edelstein, 1971).

1.4.3 Ceroid

The term ceroid, meaning wax-like, was first used by Lillie, Daft and Sebrell (cited in Hartroft and Porta, 1965) to describe the pigment found in macrophages in cases of nutritional cirrhosis in rats. Since then it has been reported from a variety of animals (see for example Wood and Yasutake, 1956; Hartroft and Porta, 1965; and Thompson, 1966). Ceroid is a yellow to brown, lipogenic pigment which is insoluble in a variety of hydrocarbons that dissolve normal lipids and which reacts with fat stains. It seems probable that this pigment is

derived from highly unsaturated lipid components of cells which have been phagocytosed by macrophages and have subsequently undergone gradual oxidation and polymerization within such cells. The formation of ceroid is apparently inhibited by anti-oxidants (Thompson, 1966) and the pigment has been encountered in a wide variety of experimental conditions in animals and in different human tissues in cases of nutritional or metabolic disorders (Hartroft and Porta, 1965). Whilst much remains to be known as to the mechanism of formation and deposition of ceroid, the available data indicates that dietary choline deficiency is a vital prerequisite for the formation of ceroid *in vivo* (Thompson, 1966). However, such has been the confusion with respect to the exact identity of this pigment that it has been variously termed haemofuscin, lipoprotein, fat pigment, lipochrome, lipofuscin and "wear-tear" or "age" pigment (Hartroft and Porta, 1965). In addition to its spontaneous occurrence, ceroid has been produced *in vivo* and *in vitro* by several investigators, using unsaturated fats as a precursor of the pigment.

1.4.4 Haemosiderins

In higher animals, iron is normally stored in the body in the form of ferritin (a ferric iron-protein substance containing 23% ferric iron); this is seldom observed in routine tissue sections since it is soluble in water and is generally lost with the fixative. When the body as a whole, or a particular organ or tissue, becomes saturated with ferritin, iron continues to be stored intracellularly, but in the form of

haemosiderin rather than ferritin (Herbut, 1959). Haemosiderin is a brown, granular, relatively insoluble pigment which is apparently closely related to ferritin in as much as it contains a protein component (apoferritin) and an iron (ferric) component. One difference between ferritin and haemosiderin is that the former contains approximately 23% ferric hydroxide and haemosiderin contains approximately 35%. According to Dubin (1955), the formation of haemosiderin may be an abnormal stage of iron deposition beyond that of ferritin. When iron continues to pour into a cell that is saturated with ferritin, the ferric hydroxide micelles incorporated in ferritin may form abnormally large polymers that pile up between the protein moieties; these enlarged ferritin molecules could then combine by cross-linkage to form huge granules of haemosiderin. With this in mind, Thompson (1966) discussed the possibility that certain cell types, which are capable of storing iron, may become saturated with ferritin more readily and would tend to store iron as haemosiderin earlier than other types of ferritin containing cells. It would be conceivable then, that such variable abilities to normally store iron as ferritin could account for the more frequent observation of haemosiderin in reticulo-endothelial cells as compared with parenchymal cells.

1.5 THE PIGMENT CARRYING CELLS

The pigment-bearing cells being studied have been shown to be macrophages. According to Carr (1973) a macrophage is an avidly phagocytic metazoan cell with the cellular enzymes required to digest what it has ingested, and the cellular

apparatus necessary to make more enzymes. Cells showing phagocytic capabilities have traditionally been grouped together under the name of "reticulo-endothelial system". This concept has been widely criticized because while these cells had the common ability of phagocytosis, the capacity to do so varied from cell type to cell type, and they were not necessarily morphologically identical. The reticulo-endothelial system of Aschoff (1924) is shown in Table 1.1. It was recently suggested that macrophages should be referred to as a group and called "the mononuclear phagocyte system" (van Furth, 1970). 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 (Rabinovitch, 1968). The components of the mononuclear phagocyte system of mammals are listed in Table 1.2. Workers with fish, however, still regard the system of phagocytic cells within the fish body as more closely allied to the concept of those comprising the reticulo-endothelial system than to those of the mononuclear phagocyte system. The systems are not identical; the relatively avidly phagocytic cells are found in very different locations compared to the mammalian situation. Moreover, dissimilar mechanisms may be operating in regulating the uptake of particulate matter (Ferguson, 1976a); certainly the rate of phagocytosis in a poikilothermic animal is much slower than in a homeothermic one.

In teleosts the precursor cells are thought to reside in the haemopoietic tissue of the kidney (Ellis, 1974). Mature

Table 1.1. The reticulo-endothelial system (RES) of Aschoff

Increase of phagocytic activity ↓	Endothelial cells			
	Fibrocytes			
	Reticular cells of spleen and lymph nodes	RES in a strict sense	RES in a wider sense	
	Reticulo-endothelial cells of lymph and blood sinuses, including Kupffer cells			
	Histiocytes			
	Splenocytes and monocytes			

Table 1.2. The mononuclear phagocyte system of mammals

Cells	Localization
PRECURSOR CELLS	bone marrow
↓	
PROMONOCYTES	bone marrow
↓	
MONOCYTES	bone marrow, 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?)

macrophages are known to exist in the peritoneal lining of the abdominal cavity, lining the ellipsoids of the spleen, in the white and red pulp of the spleen, around the portal tracts of the liver, throughout the haemopoietic tissue of the kidney and in the intermuscular spaces in the atrium of the heart. Mainly within the spleen and kidney, phagocytes including melano-macrophages aggregate in association with lymphoid elements forming the melano-macrophage centres. Thus, these centres can be considered as an integral part of the reticulo-endothelial system.

1.6 MORPHOLOGY OF THE MELANO-MACROPHAGE CENTRES

Melano-macrophage centres are usually nodular with a delicate argyrophilic capsule and generally closely applied to vascular channels. In a few species, such as the turbot, they are frequently seen in close association with clusters of lymphocytes. In salmonids the morphology of the melano-macrophage centres is less well-defined and the cells are distributed at random throughout the interstitial haemopoietic tissue of the kidney, the white pulp of the spleen and the periportal areas of the liver (Roberts, 1975).

In flatfish spleens these aggregates of pigment bearing cells are often found in the axil of an ellipsoid branch and a fine fibrous membrane continuous with that of the ellipsoid usually delimits the centre. A few leucocytes and pyroninophilic cells are dispersed among the pigmented cells. A lymphocytic cuff surrounds the entire arterial system and their associated melano-macrophage centres (Ellis, 1974; Roberts, 1975;

Ferguson, 1976b). In the flatfish kidney too, the centres are bounded by a thin fibrous membrane and surrounded by white pulp devoid of erythrocytes through which run thin-walled, narrow blood vessels (Ellis, 1974).

1.7 ULTRASTRUCTURE OF THE MELANO-MACROPHAGE CENTRES

Ultrastructurally, melano-macrophages are very complex (Roberts, 1975). They have indented nuclei and large numbers of membrane-bounded vacuoles containing a variety of materials. The pigment granules appear to be contained in groups in such vacuoles, suggesting they may be phagocytosed, but the origin of the cells and the pigments they contain has still to be resolved. Ferguson (1976b) claimed that in turbot spleens, the melano-macrophage centres have a definite capsule, composed of both cellular and acellular elements, separating them from the surrounding lymphoid elements. According to Ferguson the centres themselves are composed of cells in varying degrees of degeneration, replete with dense osmiophilic debris. The associated lymphoid tissue comprise lymphocytic cells and typical plasma cells which occasionally show cytoplasmic interdigitations with closely apposed dendritic macrophages.

1.8 POSSIBILITY THAT CENTRES COULD REPRESENT THE PRIMITIVE ANALOGUES OF GERMINAL CENTRES

Ellis (1974), Roberts (1975) and Ferguson (1976a, b) have all suggested that melano-macrophage centres could well represent the primitive analogues of germinal centres of the lymph nodes of birds and mammals. The lymph nodes described

in birds are located in the region of the neck whereas those of mammals are found not only in the neck but also in the thorax, abdomen and extremities. Within lymph nodes, reticular cells and lymphocytes are grouped in nodules or follicles and a nodule that enlarges after antigenic stimulation, termed a germinal centre, also contains plasma cells and macrophages with cytoplasmic extensions or dendritic processes. Thus cytologically, germinal centres and melano-macrophage centres both consist of ordered aggregations of reticular cells, lymphocytes, macrophages and plasma cells. A major difference is the pigment content of fish macrophages. In mammals there appear to be two largely separate types of cells involved in the trapping and processing of antigens to trigger lymphocyte response, namely, macrophages which sequester antigen into inclusions (phagolysosomes) and dendritic cells which retain antigen on their surface, though the distinction between the two is not absolute (White, 1963; Nossal, 1976). Ellis (1974) could detect the presence of immunoglobulin in a dendritic pattern within the melano-macrophage centres in the kidney of normal plaice Pleuronectes platessa suggesting the presence of dendritic cells. This same worker also reported that about 10% of the macrophages in a cell suspension prepared from plaice kidney and spleen carried immunoglobulin on their surface membranes. Usually such cells did not form "caps". Often these macrophages contained melanin granules and may be classified as melano-macrophages. Ferguson (1976a, b) also observed both rounded and dendritic macrophages in the turbot. Stuart (1970) reported that in the lungfish Protopterus

aethiopicus the phagocytic cells are found in two forms: the dendritic macrophage, the chief function of which seems to be phagocytosis of lipid droplets, and the rounded form which takes up pigment released by the breakdown of cells. He reckons that the former type of cells are involved in the lipid metabolism of this fish. It would be interesting to establish whether these two forms of macrophages that are seemingly analogous, at least morphologically, to those found in mammals, occur in all fish. The available evidence suggests that this could well be the case.

By following the fate of intraperitoneally injected carbon particles, Mackmull and Michels (1932) demonstrated that in the cunner circulating macrophages homed specifically on these sites. Later studies on adsorption of carbon from the peritoneal cavity in various species have shown that the process is similar in a wide variety of teleosts (Ellis, 1974; Ferguson, 1976a). It has also been shown that melano-macrophage centres are capable of concentrating circulating Salmonella organisms. In the parasitic infection of roach (Rutilis rutilis) caused by Myxobolus pseudo-dispar, the spores of the organism localize specifically in the centres of the spleen and kidney (Roberts, 1975). Colloidal carbon injected into plaice (Ellis, 1974) and turbot (Ferguson, 1976a) is initially trapped in the ellipsoids but after a period of about three weeks the ellipsoids are clear of carbon, most of which has accumulated within the melano-macrophage centres. This suggests that carbon-laden macrophages migrate from the ellipsoids towards and into the melano-macrophage centres (Ellis et al., 1976;

Ferguson, 1976a; Bogner and Ellis, 1977). It is likewise possible that macrophages from the ellipsoid sheaths, once they are replete with cellular debris of host or foreign origin, migrate to the melano-macrophage centres which themselves originate in the outer part of the sheath. During such a migration, the macrophages come into close contact with the immunologically-competent cells lining their route before segregating within the centres. Cytoplasmic interdigitations of plasma cells with dendritic macrophages can be seen under the electron microscope (Ferguson, 1976a). In some ways this pattern is analogous to the migration in the fowl of either macrophages or dendritic cells from the ellipsoids into the lymphoid malpighian areas, with the consequent formation of a germinal centre should the dendritic cell be carrying antigenic material (Ellis, 1974; Ferguson, 1976a, b).

Through autoradiographic studies Ellis and de Sousa (1974) have shown that circulating lymphocytes in the plaice contain a population of cells which selectively migrate to the lymphoid tissues surrounding the melano-macrophage aggregations in the kidney and spleen and while resident these synthesize RNA. Eventually some labelled lymphocytes could also be seen among the melano-macrophages. Such an observation led him to speculate that aggregations of macrophages in the plaice spleen and kidney could provide sites of concentrated antigen through which circulating lymphocytes percolate and where competent lymphocytes would be immunologically influenced in a similar way as occurs in the formation of germinal centres (White and Gordon, 1970) by the aggregation of antigen-bearing dendritic

macrophages in the spleen of the chicken.

An important role of germinal centre cells is the phagocytosis of pyknotic small lymphocytes. It has been suggested that phagocytosis of small lymphocytes conserves nuclear DNA which is subsequently re-used in lymphocytopoiesis (Stuart, 1970). It would be interesting to establish whether this takes place in fish melano-macrophage centres. Both the pigment centres in fish and the germinal centres in higher animals have frequently been observed to contain cellular debris, much of it within phagocytic cells (Stuart, 1970; Ferguson, 1976a).

Up to a few years ago various workers had stated categorically that lymph nodes are not present in animals which are phylogenetically more primitive than birds. In 1964, Kent and his coworkers demonstrated encapsulated nodes of lymphoid tissue in the upper thorax, neck and axilla of the amphibian Bufo marinus. These nodes, which were observed to phagocytose India ink and form antibody to bovine serum albumin, resembled mammalian lymph nodes in both anatomical and functional characteristics. This marine toad was again studied by Diener and Nossal (1966). They observed that uptake of injected flagellin and carbon was effected by the jugular bodies, kidney and spleen. The localization of the antigen in the jugular bodies was to the surface of dendritic cells which spread throughout the whole organ, there being no organization of the lymphoid tissue into germinal centres similar to the mammalian lymph node. Turner (1969) described the presence of lymphoid

follicles, though without germinal centres, in the spleen of Xenopus laevis, and though lymphoid cells were present in the kidney and liver, the only structurally organized lymphoid tissue was in the spleen. Upon injection of colloidal carbon, phagocytic cells were found in large numbers in all three organs but only in the spleen was there a marked response, the carbon-containing cells migrating from the red pulp into the white pulp to form aggregates in the centre of the lymphoid follicles. Manning and Turner (1972) suggested a similar fate for the soluble antigens human gamma globulin (HGG) and bovine serum albumin (BSA) when given in Freund's complete adjuvant (CFA). It is very significant to note that from 8 weeks onwards after the CFA/antigen injections, melanin-containing cells were also present in the splenic lymphoid follicles accompanying the vacuolated macrophages arising from the injections. Vacuolated macrophages were also observed amongst the groups of lymphoid cells in the kidney and liver. Among reptiles, there has recently been described a lymphoid nodule present in the lymphatic near the anterior limb in a lizard. This nodule resembles in its histological features the jugular bodies of amphibians (Johnston, 1972: cited in Du Pasquier, 1973). Moreover, lympho-epithelial tonsils have been observed in the pharyngeal region of the alligator (Du Pasquier, 1973). It is obvious that much more information is needed especially with regard to poikilotherms before the evolutionary development of germinal centres of higher animals can be traced.

1.9 THE ROLE OF MELANO-MACROPHAGE CENTRES IN DISEASE PROCESSES

Roberts (1975) discussed the involvement of melano-macrophage centres in various fish diseases, mainly in those of parasitic, viral and bacterial aetiology. This same author later (1976) also reported the association of pigment cells with lymphocystis lesions in plaice. Ellis and Wootten (1978) observed that melano-macrophages were common in primary and secondary gill lamellae in Ichtyobodo-infected salmon Salmo salar smolts. They described how in areas of heavy Ichtyobodo infections large amounts of melanin, much apparently present as free granules, were observed external to the blood vessels of the primary lamellae. In the course of the present study some specimens of the deep-sea scabbard fish Aphanopus carbo were found to be heavily infected by an Ichthyophonus-like fungus. Melano-macrophage centres were closely associated with the induced granulomas and individual melanin cells could be observed throughout the granulomatous tissue. These results have been published separately (Agius, 1978) and a reprint is appended. Huizinga et al. (1979) noted an abundance of melano-macrophage centres in the liver, kidney and spleen of red-sore diseased (Aeromonas hydrophila) largemouth bass (Micropterus salmoides). These were especially abundant immediately surrounding encapsulated trematode metacercariae.

CHAPTER 2

PHYLOGENETIC DEVELOPMENT OF MELANO-MACROPHAGE CENTRES

2.1 INTRODUCTION

It is amply clear from the foregoing introduction that the existing literature is confined to studies on a very restricted number of species, all of them teleosts. It was considered of primary importance therefore that, as a starting point to such a comparative study of the melano-macrophage system, a survey of the main histological features of melano-macrophage centres in representatives of the Agnatha, Chondrichthyes and Osteichthyes be carried out. Such a comparative approach was also expected to yield information on the phylogeny of the lympho-reticular system in three major lineages of vertebrates.

2.2 BRIEF REVIEW OF THE MAJOR HAEMOPOIETIC ORGANS OF FISH

Fish have no lymph nodes; they do, however, have abundant masses of lymphoid tissues that are widespread throughout their bodies (Bertin, 1957). The exact distribution of these haemopoietic and lymphoid tissues in fishes is however subject to some disagreement particularly at the level of the cyclostomes.

Hagfishes have no thymus and no circumscribed spleen (Good and Finstad, 1967). They do, however, possess a primitive spleen in the form of an islet-like arrangement of the lymphoid tissue of the sub-mucosal region (Adam, 1963). Papermaster *et al*.

(1963) maintained, however, that this is purely haemopoietic tissue and is entirely lacking in lymphoid structure. Hagfishes also possess a primitive haemopoietic organ in the pronephros; according to Fänge (1963), the pronephros is probably a lymphoid tissue producing lymphocytes and similar cells, but periodically it may also have other functions. The hagfish gill has also been described as having haemopoietic properties (Linna et al., 1966: cited by Good and Finstad, 1967) while the nasopharyngeal duct has a subepithelial haemolymphatic component.

In the lamprey there is no recognizable thymus or spleen (Good et al., 1972). Its lymphoid tissue is located in the gut wall in the ammocoete and in the provertebral arch in the adult. While Papermaster et al. (1963) maintained that these haemopoietic tissues are entirely lacking in lymphoid structure, Good and Finstad (1967) reported that the provertebral arch tissue resembles bone marrow and contains lymphoid elements that proliferate in response to antigenic stimulation.

Thymus and spleen are clearly identifiable in all groups above the Agnatha (Good et al., 1966). Melano-macrophages have apparently never been observed in the thymus although phagocytes are abundant. According to Ellis et al. (1976), this is the only lymphoid organ in fish which appears not to participate in phagocytosis of exogenous material. It is possible that, as in mammals, the thymus of fishes is protected from foreign material in the blood by a specialized vascular endothelium. Ellis (1974, 1979) observed that in plaice the thymus does not participate in the trapping of the soluble antigen bovine serum albumin, further suggesting a central role in the immune system of fishes

similar to the thymus of higher vertebrates.

Elasmobranchs show a major phylogenetic advance on the cyclostomes in that the thymus and spleen are well developed lymphoid organs. Moreover they possess lymphoid and haemopoietic foci along the gastro-intestinal tract especially in the region of the spiral valve (Good and Finstad, 1967). Contrary to earlier reports (for example Bridge, 1904 and Bertin, 1957), Good and Finstad (1967) found no evidence of Peyer's patch-type tissue or other lympho-epithelial organs along the gut tract in either the elasmobranchs or the chondrostean fishes. In some of the higher elasmobranchs, lymphoid and plasma cells have been found in the organ of Leydig surrounding the oesophagus and in the haemopoietic tissue of the genito-urinary apparatus (Good et al., 1966). Chimeroids lack haemopoietic tissue in the kidney but they exhibit an interesting phylogenetic variation in that they have a very well developed myelo-lymphoid organ in the orbit of the eye and the roof of the mouth (Janković and Mitrović, 1967).

In the Dipnoi the spleen is represented by a large compact lymphoid mass which is closely connected with the dorsal and lateral walls of the stomach. Protopterus is remarkable among vertebrates for the extraordinary development of lymphoid tissue which, apart from its distribution in the submucosa, is abundantly present between the longitudinal and circular muscle layers and the peritoneal and muscular coats of the intestine. A thick mass of lymphoid tissue also invests the kidneys and gonads.

In chondrostean fishes there are lymphoid and haemopoietic foci along the gastro-intestinal tract especially in the region of the spiral valve. The paddlefishes, as exemplified by Polyodon spathula, have a highly developed haemopoietic system probably representing a rather special phylogenetic adaptation. Foci containing lymphocytes and plasma cells are found in the anterior kidneys and pericardial haemopoietic organs; plasma cells in these foci as well as in the spleen proliferate in response to antigenic stimulation (Finstad et al., 1964).

The taxonomic position of Amia and Lepisosteus is still very controversial (see for example Patterson, 1973). The term Holostei, as used in this study, includes the two species examined in the course of this work, viz. Amia calva and Lepisosteus platostomus. These have a well-developed thymus, and the spleen, gut-associated peripheral lymphoid tissue and gonadal lymphoid tissue have a relatively complex structure (Good et al., 1966). In Lepisosteus osseus and Lepisosteus platyrhincus the spleen, kidney and meningeal myeloid tissue are haemopoietic (Andrew, 1965; MacLeod et al., 1978). The meningeal myeloid tissue, unique to holosteans, is thought to be primitive bone marrow (Scharrer, 1944).

In teleost fish, haemopoietic tissue is mainly located in the stroma of the spleen and the interstitium of the kidney. In some teleosts, well-developed haemopoiesis is found in the periportal areas of the liver, the gonads, the intestinal submucosa and in the specialized lymphopoietic organ, the thymus.

2.3 MATERIALS AND METHODS

2.3.1 Fish

The species of Agnatha and Chondrichthyes examined are given in Table 2.1; the species of Osteichthyes studied are listed in Table 2.2. The classifications adopted were those of Lagler *et al*. (1977) and Norman and Greenwood (1977). Species were identified using published keys and descriptions. Wherever possible, at least one species from each order was studied and where an order comprises more than two suborders one species was selected from every other suborder. In order to gain a better insight into any possible variations that could occur within an order, a number of species were examined from the Gadiformes and Perciformes.

The fish were obtained through several sources; most were caught in the wild while a very small number of species were obtained from fish farms and pet shops. A small number of species (indicated by an asterisk in Table 2.2) were obtained from museum or private collections; the rest were caught specifically for this study. Wild-caught marine species were obtained from the Caspian Sea, the Mediterranean Sea, and the north-east Atlantic Ocean from depths of up to 2000 m. Fresh-water species were obtained from Africa, Britain, India, Malaysia, North America and Tasmania. Six clinically normal adult specimens were examined from each species.

2.3.2 Histological procedures

The organs examined from the various fish species are listed in Table 2.3. In most cases, the fish were dissected

quickly upon killing; in some instances the specimens were fixed whole, with the necessary incisions being made to allow for proper penetration of the fixative, and the dissections performed later. Samples of marine origin were fixed in 10% formal saline; 10% neutral buffered formalin was otherwise employed. Specimens obtained through museum or private collections had been preserved for some time in alcohol or methylated spirits after fixation in formalin. After routine processing in an automatic tissue processor (Shandon Elliot Ltd), 5 µm thick paraffin sections were cut on a rotary microtome (Leitz Wetzlar). The sections were stained with haematoxylin and eosin (H & E), periodic acid-Schiff (PAS), slow Giemsa and Gomori's silver stain for reticulin (Drury and Wallington, 1967; Pearse, 1968, 1972; Culling, 1974).

2.4 RESULTS

The major evolutionary trends observed in terms of the distribution and cytological organization of the melano-macrophage centres in fish are summarized in Fig. 2.1.

Melano-macrophages were observed within the haemopoietic tissues in all species examined with the exception of the lamprey (Fig. 2.2). While the incidence of these cells in the hagfish and the Chondrichthyes was rather limited, in the Osteichthyean species very high levels of pigmentation were quite common and aggregates of pigment cells reached diameters of 500 µm.

In the hagfish and the Chondrichthyes the pigment-carrying macrophages were randomly distributed either singly

Table 2.1. List of the species of Agnatha and Chondrichthyes which had their major lymphoid organs examined for melano-macrophages

Agnatha	
CLASS Cephalaspidomorphi	
SUBCLASS Cyclostomata	
ORDER Myxiniformes	
Family Myxinidae	Myxine glutinosa Linnaeus
ORDER Petromyzontiformes	
Family Petromyzontidae	Lampetra fluviatilis (Linnaeus)
Gnathostomata	
CLASS Chondrichthyes	
SUBCLASS Holocephali	
ORDER Chimaeriformes	
Family Chimaeridae	Chimaera monstrosa Linnaeus
SUBCLASS Elasmobranchii (Selachii)	
ORDER Squaliformes	
Family Scyliorhinidae	Scyliorhinus canicula (Linnaeus)
Family Squalidae	Squalus acanthias Linnaeus
ORDER Rajiformes (Batoidei)	
Family Rajidae	Raia clavata Linnaeus

Table 2.2. List of the species of Osteichthyes examined for melano-macrophages within their major lymphoid organs. The levels of pigmentation and the pigment colouration within their spleen, kidney and liver are given. The levels of pigmentation were determined empirically; xxx indicates very heavy pigmentation, xx indicates a moderate level of pigmentation and x indicates the presence of only very few melano-macrophages. Concerning pigment colouration, species are classed as yellow (Y), black (B) and yellow/black (Y/B); this latter indicates the presence of both kinds of pigments in substantial proportions. It is worth noting that even in those species classified as strict yellows or blacks slight traces of the other pigment (up to 10% of the total pigment content) are often present. Relative amounts of yellow to black pigments in the species classified as yellow/black are very variable (see text). A dashed line indicates complete absence of pigment cells.

Species marked by an asterisk represent those obtained through museum or private collections, which had perforce been preserved for some time.

			Spl.	Kid.	Liv.	
CLASS Osteichthyes						
SUBCLASS Dipnoi						
ORDER Dipteriformes						
Family Lepidosirenidae	*	Protopterus aethiopicus Heckel	xxx	xx	xx	Y/B
SUBCLASS Actinopterygii						
ORDER Polypteriformes						
Family Polypteridae	*	Polypterus senegalis Cuvier	xxx	x	xxx	Y
ORDER Acipenseriformes						
Family Acipenseridae		Huso huso (Linnaeus)	-	-	xxx	B
		Polyodon spathula (Walbaum)	x	x	xxx	Y/B
ORDER Lepisosteiformes						
Family Lepisosteidae	*	Lepisosteus platostomus Rafinesque	x	-	xxx	Y/B
ORDER Amiiformes						
Family Amiidae	*	Amia calva (Linnaeus)	x	xx	xxx	Y/B
ORDER Anguilliformes						
Suborder Anguilloidei						
Family Anguillidae		Anguilla anguilla (Linnaeus)	xxx	xxx	x	Y
Family Congridae		Conger conger (Linnaeus)	xx	xx	x	Y/B
ORDER Osteoglossiformes						
Suborder Osteoglossoidei						
Family Osteoglossidae	*	Scleropages formosus (Muller = Schlegel)	xx	xx	xx	B
ORDER Salmoniformes						
Suborder Salmonoidei						
Family Salmonidae		Salmo trutta Linnaeus	xx	xxx	x	B
		Salmo gairdneri Richardson	xx	xxx	x	B
		Salmo salar Linnaeus	xx	xxx	x	B

		Spl.	Kid.	Liv.	
Suborder Argentinoidei					
Family Argentinidae	Argentina silus (Ascanius)	xxx	xxx	x	Y/B
Suborder Galaxioidei					
Family Galaxiidae	Galaxias auratus Johnston	x	x	-	Y
Suborder Esocoidei					
Family Esocidae	Esox lucius Linnaeus	xx	xxx	-	Y/B
Suborder Alepocephaloidei					
Family Alepocephalidae	Alepocephalus bairdii Goode& Bean	xx	xxx	xx	Y
ORDER Gonorynchiformes					
Suborder Chanoidei					
Family Chanidae	* Chanos chanos (Fårskal)	xxx	xxx	x	Y/B
ORDER Cypriniformes					
Suborder Cyprinoidei					
Family Cyprinidae	Carassius auratus (Linnaeus)	xxx	xxx	x	Y
Family Cobitidae	Noemacheilus barbatulus (Linnaeus)	xx	xx	x	Y
ORDER Siluriformes					
Family Ictaluridae	Ictalurus punctatus (Rafinesque)	xxx	xxx	xx	Y
ORDER Gadiformes					
Suborder Gadoidei					
Family Moridae	Antimora rostrata Günther	xxx	xx	x	Y
	Lepidion eques (Günther)	xx	x	x	Y
	Mora moro (Risso)	xxx	xxx	xx	Y/B
Family Gadidae	Gadus morhua Linnaeus	xx	xx	-	Y
	Melanogrammus aeglefinus (Linnaeus)	xxx	xxx	x	Y
	Merlangius merlangus (Linnaeus)	xx	xx	-	Y/B
	Trisopterus minutus (Linnaeus)	xxx	xx	x	Y

		Spl.	Kid.	Liv.	
	Trisopterus esmarki (Nilsson)	xxx	xx	x	Y
	Brosme brosme (Ascanius)	xxx	xxx	x	Y
	Phycis blennoides (Brünnich)	xx	xx	-	Y
Family Merlucciidae	Merluccius merluccius (Linnaeus)	x	xx	x	Y/B
Suborder Zoarcoidei					
Family Zoarcidae	Zoarces viviparus (Linnaeus)	xx	x	-	Y
Suborder Macrouroidei					
Family Macrouridae	Trachyrhynchus murrayi Günther	xxx	xxx	x	Y
	Nezumia aequalis (Günther)	xxx	xxx	x	Y/B
	Coelorhynchus occa (Goode & Bean)	x	x	x	Y
	Coryphaenoides rupestris Gunnerus	xxx	xx	x	Y
	Chalinura mediterranea Giglioli	xx	x	x	Y
ORDER Lophiiformes					
Suborder Lophioidei					
Family Lophiidae	Lophius piscatorius Linnaeus	x	x	-	Y
ORDER Atheriniformes					
Suborder Exocoetidae					
Family Belonidae	Belone belone gracilis Lowe	xxx	xxx	x	Y/B
Suborder Cyprinodontoidei					
Family Poecilidae	Poecilia sp.	xxx	xx	xx	Y/B
ORDER Beryciformes					
Suborder Berycoidei					
Family Trachichthyidae	Hoplostethus sp. (Cuvier)	xx	xx	x	Y
ORDER Zeiformes					
Family Zeidae	Zeus faber Linnaeus	xxx	xxx	x	Y/B

		Spl.	Kid.	Liv.	
ORDER Gasterosteiformes					
Suborder Gasterosteoidei					
Family Gasterosteidae	Gasterosteus aculeatus Linnaeus	x	x	x	Y
ORDER Scorpaeniformes					
Suborder Scorpaenoidei					
Family Scorpaenidae	Sebastes sp. (Delaroche)	xxx	xx	x	Y
Family Triglidae	Peristedion cataphractum (Linnaeus)	xxx	xx	-	Y
ORDER Perciformes					
Suborder Percoidei					
Family Serranidae	Serranus scriba (Linnaeus)	xx	xx	x	Y
Family Centrarchidae	Lepomis macrochirus Rafinesque	xx	x	x	Y
	Micropterus salmoides (Lacépède)	xx	xx	xx	Y
Family Apogonidae	Epigonus telescopus (Risso)	xx	xx	x	Y/B
Family Carangidae	Trachurus trachurus (Linnaeus)	xxx	xxx	xx	Y/B
Family Coryphaenidae	Coryphaena hippurus (Linnaeus)	xx	xx	-	B
Family Sparidae	Boops boops (Linnaeus)	xx	xx	xx	Y/B
	Oblada melanura (Linnaeus)	xx	xx	-	B
Family Mullidae	Mullus barbatus Linnaeus	xxx	xxx	x	Y/B
Family Cichlidae	Tilapia mariae Boulenger	xxx	x	x	Y
Family Cepolidae	Cepola rubescens Linnaeus	xx	xx	x	Y
Suborder Mugiloidei					
Family Mugilidae	Mugil sp. Linnaeus	xxx	xx	xx	Y
Suborder Labroidei					
Family Labridae	Coris julis (Linnaeus)	xx	x	x	Y
Suborder Trachinoidei					
Family Trachinidae	Trachinus sp. Linnaeus	x	xx	x	Y
Suborder Blennioidei					
Family Pholididae	Pholis gunnellus (Linnaeus)	xx	x	-	Y

		Spl.	Kid.	Liv.	
Suborder Callionymoidei					
Family Callionymidae	*Callionymus maculatus* Rafinesque	x	x	-	Y
Suborder Scombroidei					
Family Trichiuridae	*Aphanopus carbo* Lowe	xxx	xxx	x	Y/B
ORDER Pleuronectiformes					
Suborder Pleuronectoidei					
Family Scophthalmidae	*Scophthalmus maximus* (Linnaeus)	xxx	xx	-	Y
	Pleuronectes platessa Linnaeus	xxx	xxx	x	Y/B
ORDER Tetraodontiformes					
Suborder Tetraodontoidei					
Family Tetraodontidae	*Tetraodon* sp.	xxx	xxx	x	Y
ORDER Channiformes					
Suborder Channoidei					
Family Channidae	*Channa striata* (Bloch)	xx	xx	x	Y

Table 2.3. List of organs examined from the various fish species

Myxine glutinosa	liver, pronephros, mesonephros, the gut wall from all regions of the alimentary tract, gills, nasopharyngeal duct wall
Lampetra fluviatilis	liver, kidney, the gut wall from all regions of the alimentary tract, all regions of the fat column
Chondrichthyes	liver, kidney, spleen, the gut wall in the oesophageal and spiral valve region
Protopterus aethiopicus	liver, kidney, spleen, the gut wall from all regions of the alimentary tract, gonads
Chondrostei and Holostei	liver, kidney, spleen, the gut wall from all regions of the alimentary tract
Teleostei	liver, kidney, spleen

or as very small groups (less than thirty cells per aggregation). This was observed in the livers of hagfish and all Chondrichthyes examined, as well as in the spleen of all Chondrichthyes (Figs. 2.3, 2.4 and 2.5). In all elasmobranch species examined the liver was more heavily pigmented than the spleen but in Chimaera monstrosa the spleen was the more heavily pigmented (Figs. 2.6 and 2.7). All pigments observed in the hagfish were yellow to yellow-brown; those in the Chondrichthyes were either yellow, yellow-brown or black. All other organs examined were devoid of pigment cells (Figs. 2.8, 2.9 and 2.10).

Pigments in a diffuse form were observed in various other tissues; Lampetra fluviatilis specimens carried varying amounts of yellow to yellow-brown pigments in the intestinal submucosa while considerable amounts of yellow-brown to black pigment were present in the muscular layers of the oesophagus of Chimaera monstrosa. In Myxine glutinosa very significant amounts of yellow to yellow-brown pigments were evident in the epithelial cells of the mesonephric ureters as well as beneath the gill subepithelial connective tissue mainly at the bases of the primary lamellae. Indeed some specimens of a good number of both cartilaginous and bony fish species were also observed to carry small amounts of pigment in the epithelial cells of a very small proportion of their kidney tubules. Virtually nothing is known about these pigments and they will not be given any further consideration in this study.

In salmonids too , the melano-macrophages were more or less randomly distributed; however in the spleen they tended to occur somewhat more in association with the ellipsoids

(Figs. 2.11, 2.12 and 2.13). All other Osteichthyes exhibited well-organized, nodular aggregations of these pigment cells (Figs. 2.14 to 2.18) which were generally embedded in lymphoid tissue (except for primitive bony fishes, vide infra) and generally closely apposed to the vascular supply. Leucocytes were often observed dispersed among the pigment cells. In some species, notably in Lepomis macrochirus and Scophthalmus maximus spleens, a distinct leucocytic cuff, composed mainly of lymphocytic cells, surrounded the melano-macrophage centres and the associated ellipsoids (Fig. 2.19). In the kidney, the centres were often in close association with peritubular capillaries and vascular sinuses in the reticular framework. In the liver the centres were almost invariably in very close association with the portal tracts. The centres were invariably delimited by a fibrous membrane continuous with that of the associated blood vessel. In the majority of species such membranes were strongly argyrophilic (Figs. 2.20 to 2.23). It is in this reticulin-containing fibrous sheath that the leucocytic cuff of the splenic centres was enmeshed.

Table 2.2 shows the relative degree of pigmentation of the various tissues and the pigment colouration in the species of Osteichthyes examined. Furthermore, in the Dipnoi, chondrosteans and holosteans, melano-macrophage centres were observed in various other organs. Yellow to yellow-brown centres (up to 40 μm in diameter) were observed in the submucosa of the alimentary canal of Polypterus senegalis. While no pigment was observed in the intestinal wall of Huso huso, Polyodon spathula, Amia calva or Lepisosteus platostomus, in

Protopterus aethiopicus all regions of the intestinal wall including the muscular layers carried some randomly distributed brown to black pigments, both cellular and acellular. In this latter species distinct pigment centres were also observed in the haemopoietic tissue investing the gonads. Moreover, the pancreas was heavily pigmented with a dark brown to black pigment; here melano-macrophage centres of up to 100 µm in diameter were common (Fig. 2.24). All these centres were also delimited by an argyrophilic fibrous membrane continuous with that of the associated arterioles and capillaries. At this juncture it is worth pointing out that the pancreas of some teleost fish was also observed to carry melano-macrophage centres eg. Belone belone and Noemacheilus barbatulus (Fig. 2.25). Because this study was not originally designed to include histological examination of the pancreas such observations could, of course, only be made on species having a hepatopancreas.

From the cyclostomes to the teleosts there was observed to be a shift in importance from the liver to the spleen and kidney as the chief organs of eventual deposition of the pigment cells. In fact while in the hagfish the liver was the only organ that carried pigment cells, in all Chondrichthyean species the liver was more heavily pigmented than the spleen (with the exception of Chimaera monstrosa). Moreover, the liver was much more heavily pigmented than either the spleen or the kidney in the primitive bony fishes (Figs. 2.26, 2.27 and 2.28) with the exception of Polypterus senegalis where the spleen was as heavily pigmented as the liver. Indeed, no pigment cells at all could be observed in the kidneys of Huso huso and Lepisosteus

platostomus nor in the spleen of Huso huso while only some scattered pigment cells but no centres could be observed in the kidney of Polyodon spathula and in the spleen of Polyodon spathula, Amia calva and Lepisosteus platostomus. On the other hand, almost all advanced bony fishes carried very similar levels of pigmentation in their spleen and kidney; these levels were normally much higher than those encountered in the liver. In fact, in a good number of teleost fish species the liver totally lacked pigment cells.

While the relative levels of pigmentation of the organs within a species were remarkably constant, substantial variations were evident in the degree of pigmentation of each individual lymphoid organ, not only between species but also between specimens of the same species. Equally variable was the size range of centres observed. It is obvious that any deductions relating to the levels of pigmentation have to be made with extreme care since it is clear that these increase as the fish gets older. This observation was greatly substantiated in this study in the course of which records of fish sizes were coupled with histological observations wherever it proved possible. This was observed, for instance, in Callionymus maculatus, Anguilla anguilla and Ictalurus punctatus. In order to reduce such variations to a minimum adult fish were used throughout this study.

In general, the yellow lipofuscin pigments occurred more commonly in melano-macrophage centres than the black melanin pigments. Actually, the yellow pigments varied in colour from light yellow to dark yellow and sometimes even to yellow-brown.

Fig. 2.1. Synopsis of the major evolutionary trends in the distribution and cytological organization of the melano-macrophage centres in fish.

Subclass Cyclostomata

Holocephali

Elasmobranchii

Actinopterygii

Order Polypteriformes

Acipenseriformes

Amiiformes

Lepisosteiformes

All other Actinopterygii

Subclass Dipnoi

Increase in the abundance of pigment cells

Well-defined morphology (Except salmonids)

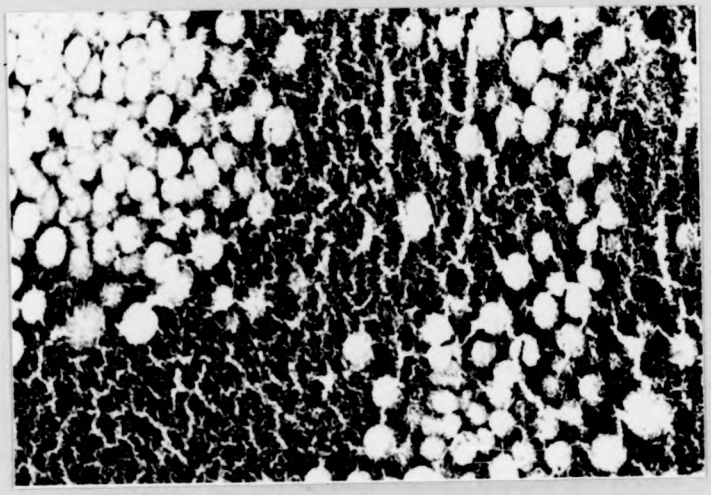
Simple morphology

Pigment cells mainly in spleen and kidney

Pigment cells mainly in liver (Except Polypterus senegalis)

Fig. 2.2. Transverse section through the provertebral arch of an adult lamprey Lampetra fluviatilis. Among the fatty tissue lies haemopoietic tissue (dark areas). While macrophages are a normal component of this haemopoietic tissue no pigment-bearing ones could be observed.
(H & E x 125)

Fig. 2.3. Randomly distributed melanin-containing cells (arrowed) in the liver of the dogfish Scyliorhinus canicula.
(H & E x 125)



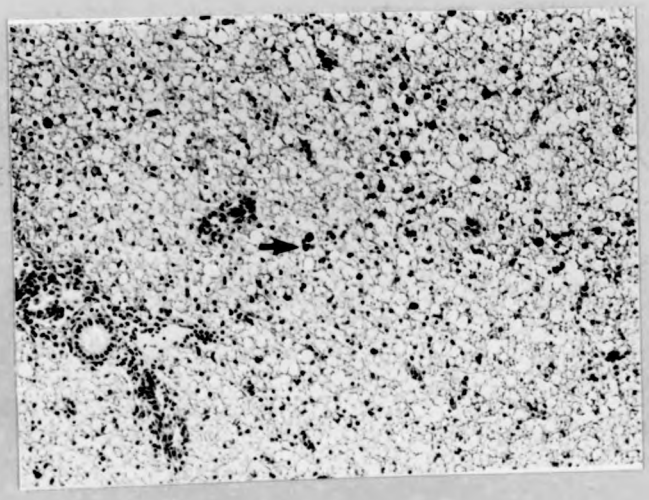


Fig. 2.4. Melanin-containing cells (arrowed) in random distribution in the liver of hagfish Myxine glutinosa. (PAS x 125)

Fig. 2.5. Spleen of dogfish Scyliorhinus canicula. In this one field there is only one pigment cell (arrowed). In all elasmobranch species the spleen was much less heavily pigmented than the liver. (H & E x 125)

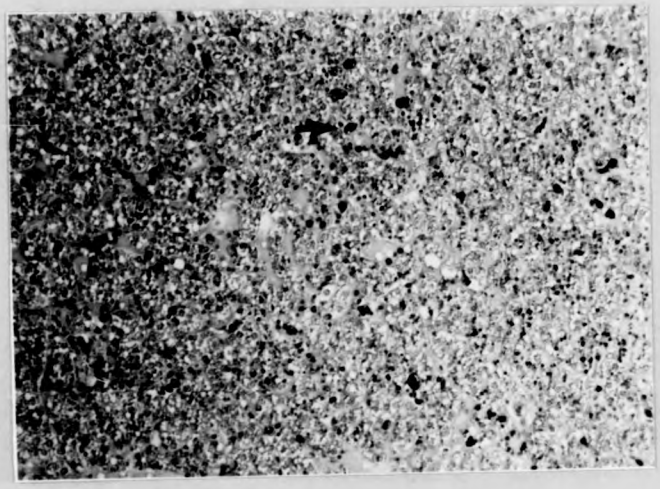
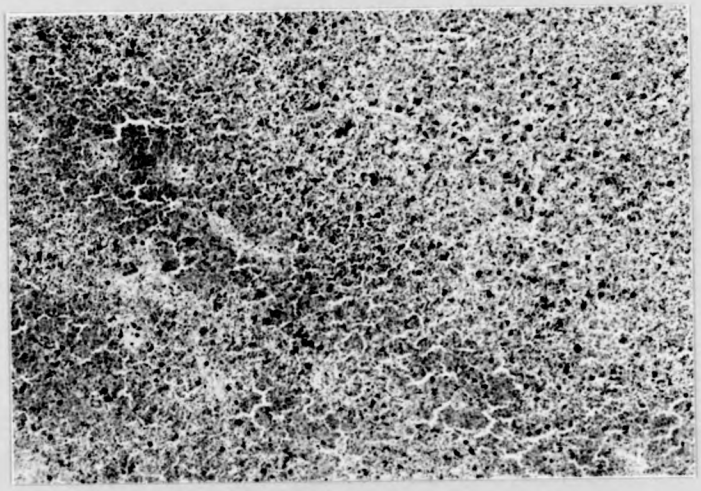


Fig. 2.6. Spleen of Chimaera monstrosa.
(H & E x 125)

Fig. 2.7. Liver of Chimaera monstrosa.
(H & E x 125)

Note the much higher density of pigment cells in the spleen as compared with the liver.



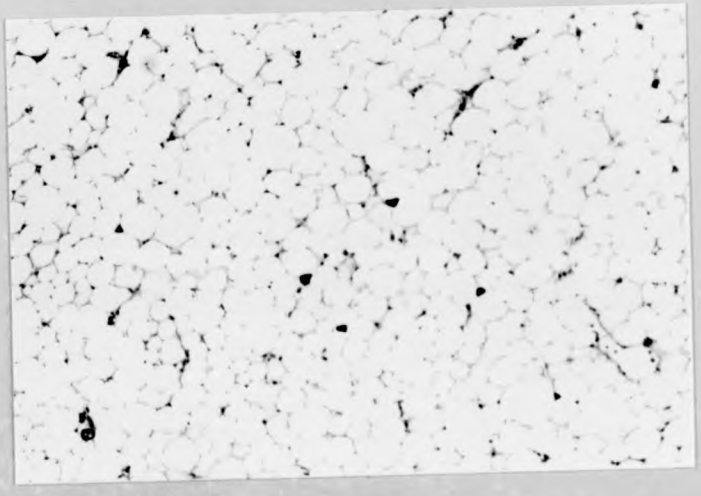


Fig. 2.8. Liver of lamprey Lampetra fluviatilis.
(H & E x 125)

Fig. 2.9. Kidney of dogfish Scyliorhinus canicula.
(H & E x 125)

Both these organs were devoid of pigment cells.

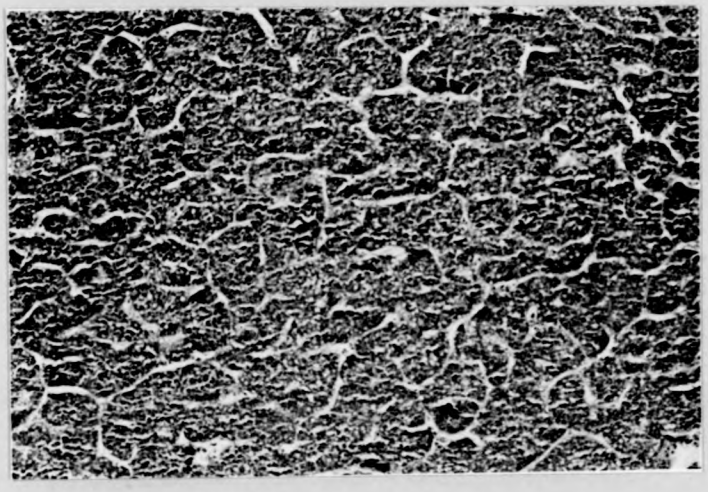
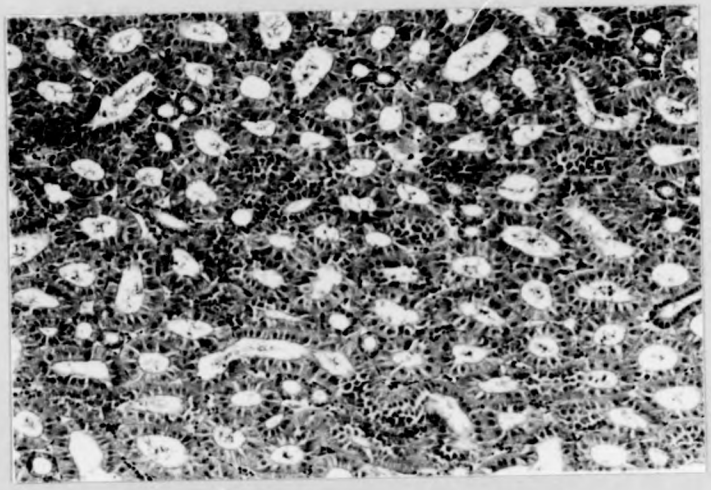


Fig. 2.10. Kidney of Chimaera monstrosa. No haemopoietic tissue and pigment cells were observed in this organ. (H & E x 125)

Fig. 2.11. Trout Salmo gairdneri kidney carrying large numbers of randomly distributed melano-macrophages. (H & E x 125)



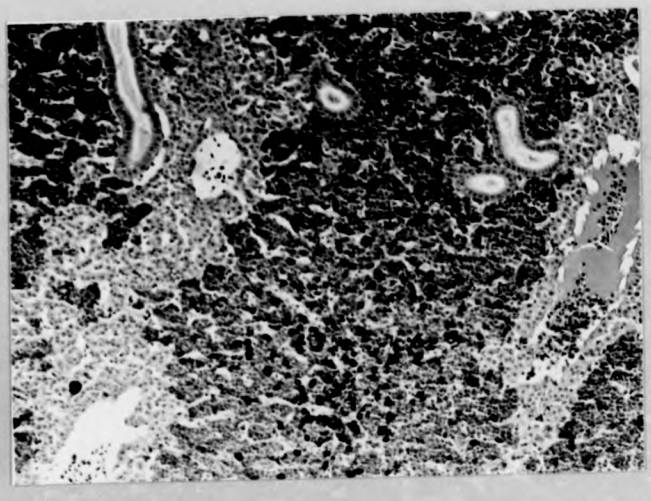


Fig. 2.12. Spleen of trout Salmo gairdneri. Note the somewhat increased concentration of the pigment cells around the ellipsoid (arrowed).
(H & E x 125)

Fig. 2.13. Trout Salmo gairdneri liver. A small number of melano-macrophages can be seen around the portal tracts (p).
(H & E x 125)

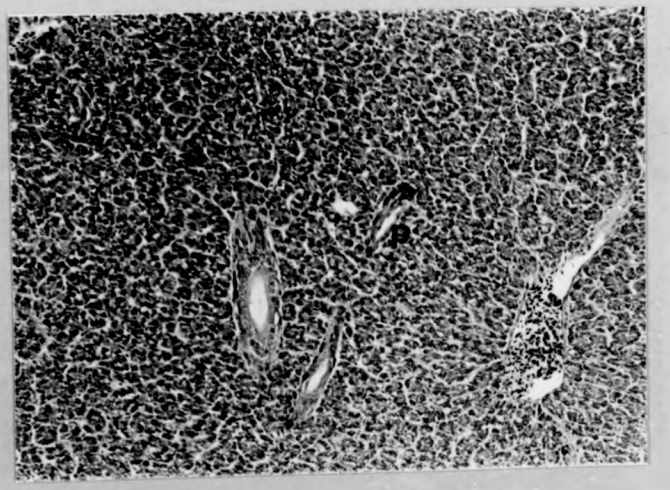
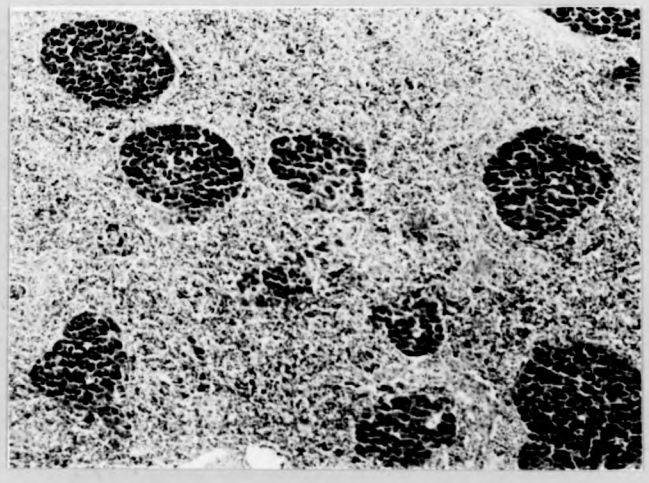

P

Fig. 2.14. Spleen of the teleost Coryphaenoides rupestris showing how heavily pigmented the spleen of this species was observed to be.
(PAS x 125)

Fig. 2.15. High power photomicrograph of one centre from the same organ as that of Fig. 2.14. A membrane (arrowed) continuous with that of the associated ellipsoid (e) delimits the centre.
(PAS x 320)



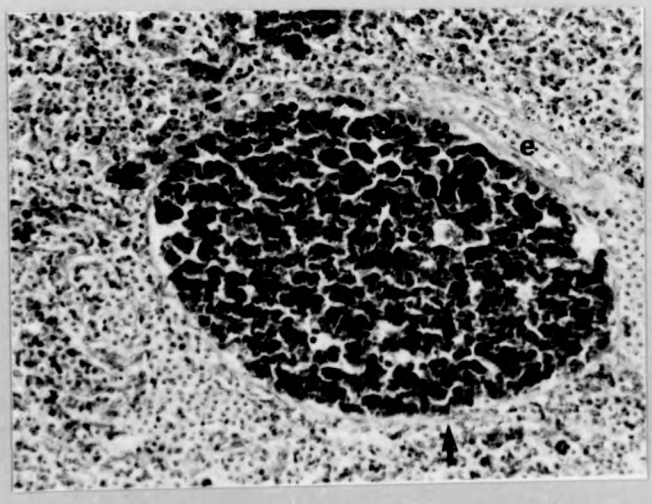
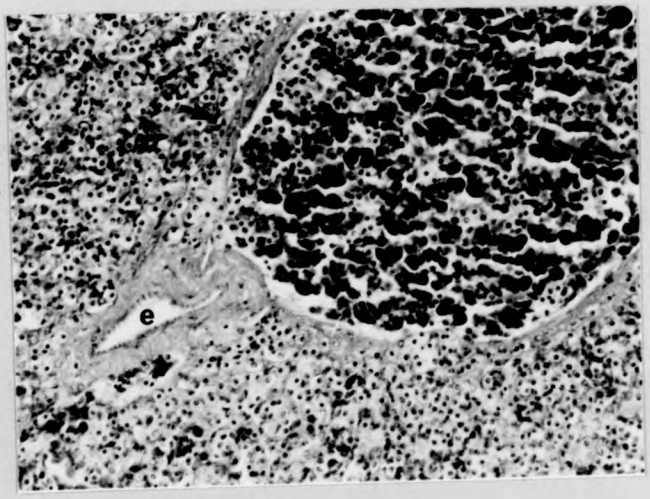

e

Fig. 2.16. Part of a large melano-macrophage centre from the spleen of another Coryphaenoides rupestris specimen. This centre has a very thick connective tissue capsule (arrowed). The wall of the associated ellipsoid (e) is also very thick.
(PAS x 320)

Fig. 2.17. Kidney of the teleost Coryphaenoides rupestris; the melano-macrophage centre present in this field is in intimate association with an arteriole (a).
(PAS x 320)


e

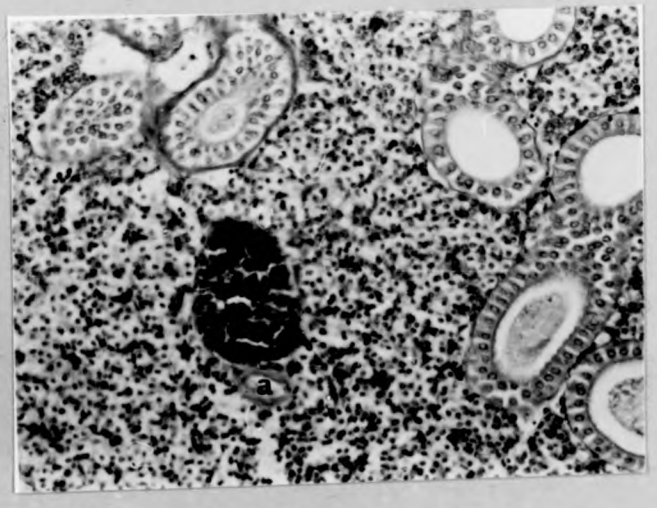
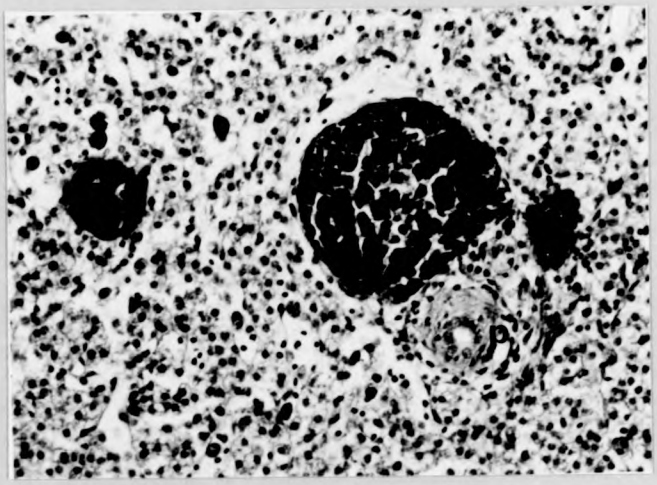
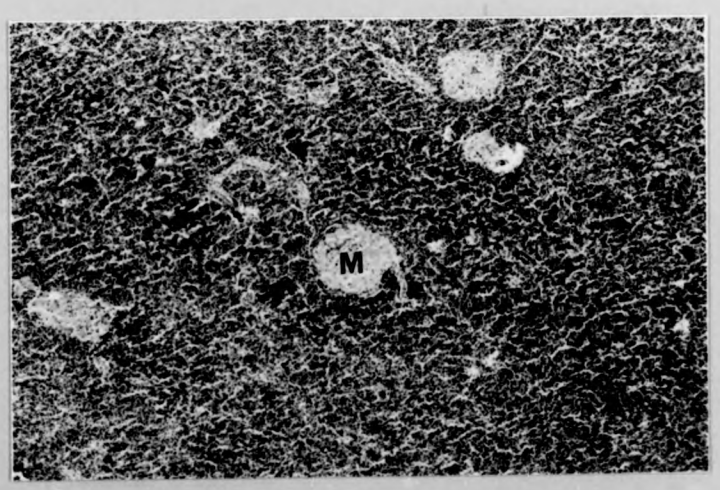

a

Fig. 2.18. Liver of the teleost Alepocephalus bairdii; the larger melano-macrophage centre is closely apposed to a portal tract (p).
(PAS x 320)

Fig. 2.19. Spleen of the teleost Lepomis macrochirus. Most of the melano-macrophage centres (M) in this organ had a distinct lymphoid cuff (arrowed) bordering them. (e) is the associated ellipsoid.
(H & E x 125)

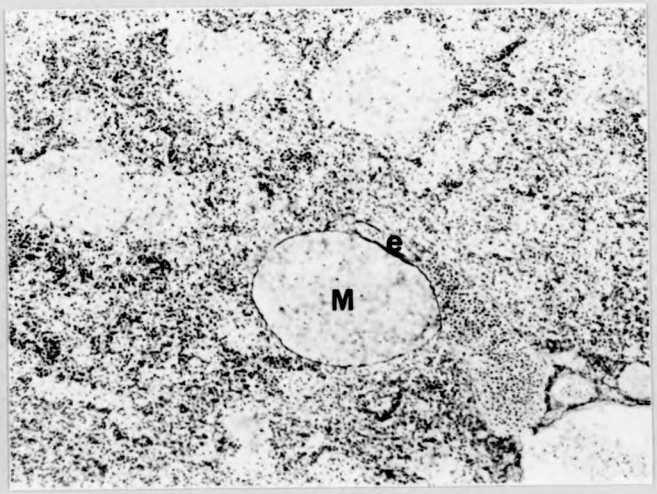


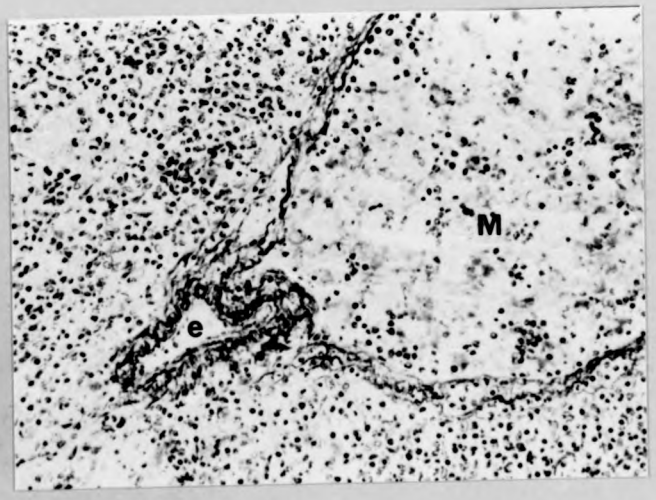

M

Gomori staining to show the reticulin content of the limiting membranes of melano-macrophage centres (M). The pigments in all sections were bleached prior to staining.

Fig. 2.20. Spleen of the teleost Coryphaenoides rupestris. The limiting membrane of the centre is continuous with that of the associated ellipsoid (e).
(x 125)

Fig. 2.21. Part of a large melano-macrophage centre in the same organ as that of Fig. 2.20. (e) is the associated ellipsoid.
(x 320)



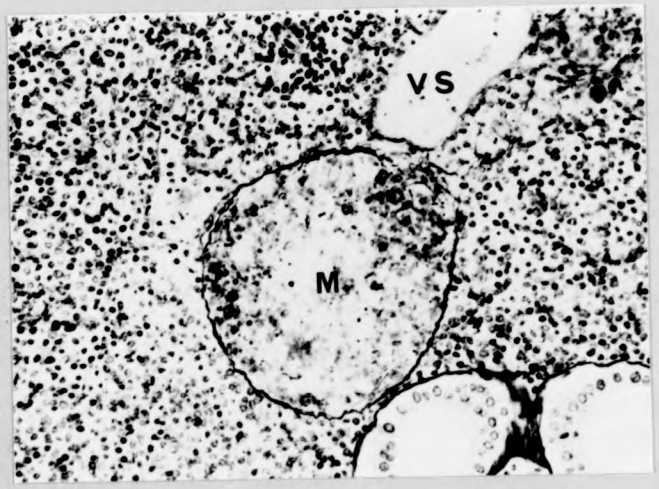


psoid.

Gomori staining to show the reticulin content of the limiting membranes of melano-macrophage centres (M). The pigments in all sections were bleached prior to staining.

Fig. 2.22. Melano-macrophage centre in close association with a vascular space (VS) in the kidney of Coryphaenoides rupestris.
(x 320)

Fig. 2.23. Liver of the teleost Alepocephalus bairdii. The argyrophilic membrane of the centre is continuous with that of the portal tract (p).
(x 320)


VS
M

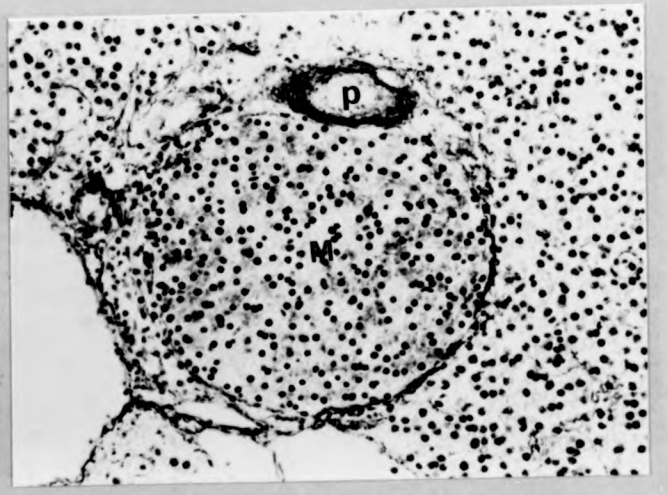
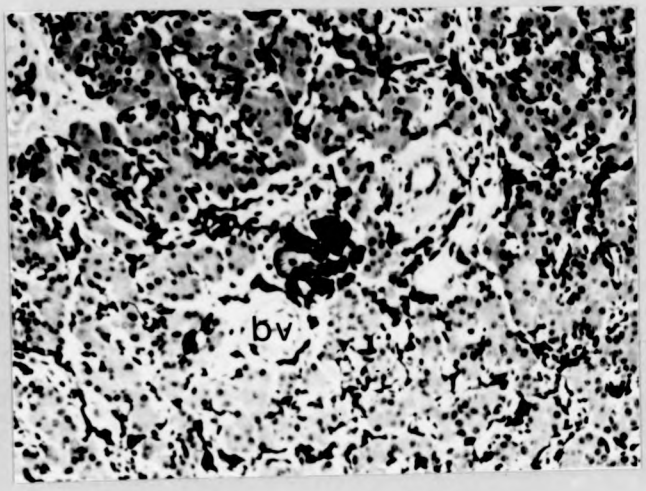

p
M

Fig. 2.24. Pancreas of the lungfish Protopterus aethiopicus showing large amounts of melanin and a compact melano-macrophage centre in close association with a blood vessel (bv).
(H & E x 125)

Fig. 2.25. Liver of the teleost Noemacheilus barbatulus with pancreatic tissue (pt) embedded in it; a melano-macrophage centre (M) and a portal tract (p) are located in the hepato-pancreas.
(H & E x 320)


bv

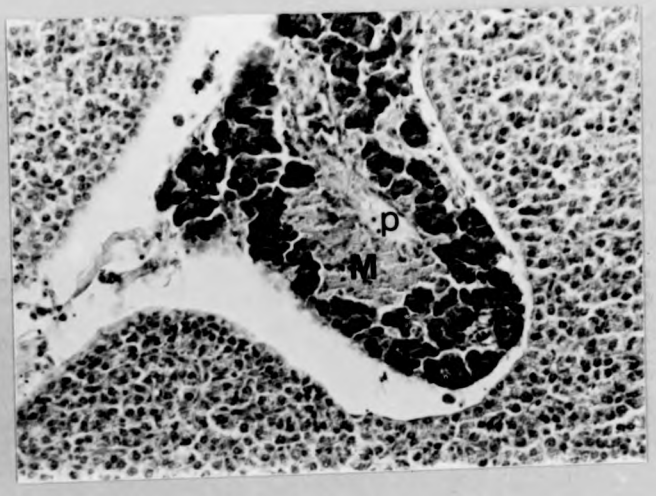
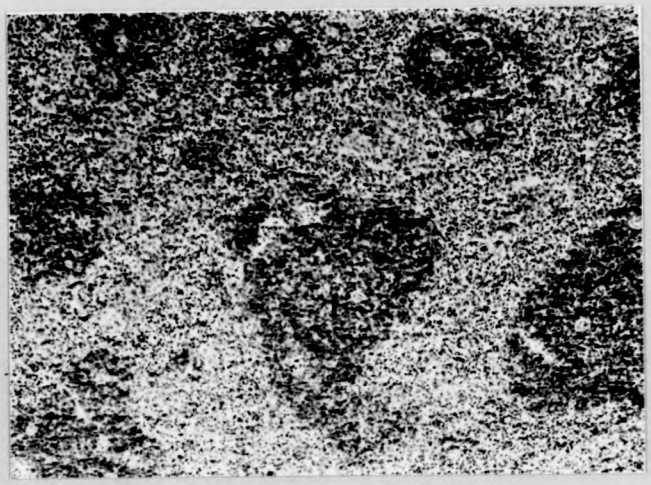

p
M

Fig. 2.26. Spleen of sturgeon Huso huso.
(H & E x 125)

Fig. 2.27. Kidney of sturgeon Huso huso.
(H & E x 125)

Both these organs were observed to be totally devoid of pigment cells. The darker areas (l) are all lymphoid tissue.



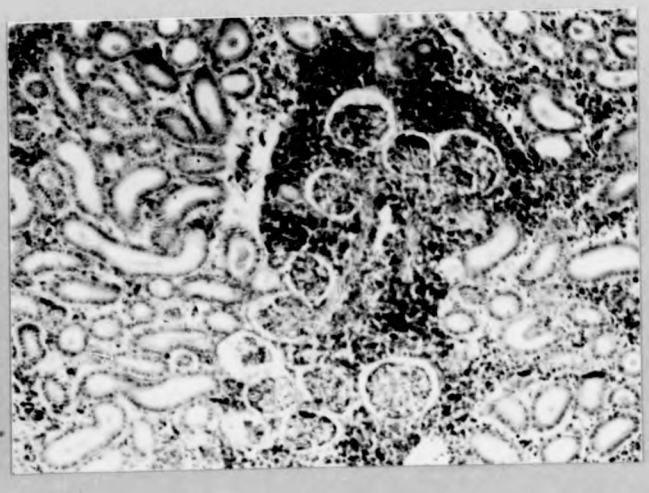
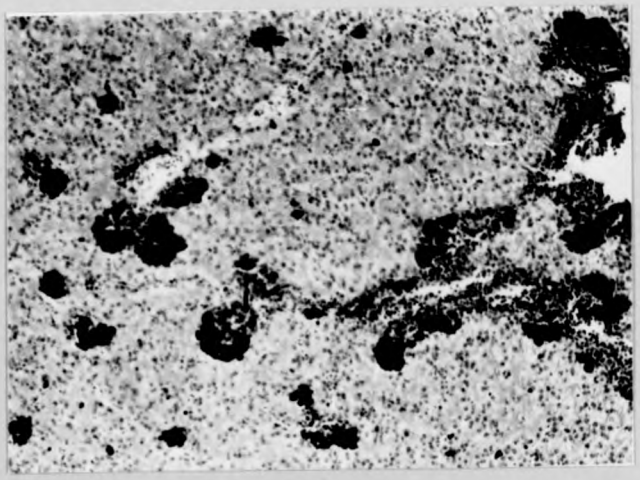


Fig. 2.28. Liver of sturgeon Huso huso. This organ was observed to be heavily pigmented with discrete melano-macrophage centres clearly embedded in the lymphoid tissue (l).
(H & E x 125)



Occasionally macrophages of an orangeish-red appearance were evident in some centres eg. Poecilia sp. The relative amounts of yellow to black pigments in those species classified as yellow/black were very variable. Though no discernible pattern could be made out in this respect it was observed that in most of these species the melano-macrophage centres in the kidney carried a larger proportion of black pigment than those of the spleen. Such examples included Argentina silus, Esox lucius and Conger conger. The extreme example was furnished by Nezumia aequalis whose melano-macrophage centres were all yellow in the spleen but all black in the kidney. Moreover, in Mora moro, the centres in the pronephros were all black while those in the opisthonephros were all yellow. Differences between specimens of the same species also occurred. Thus in Belone belone, for instance, the melano-macrophage centres in the spleen of all six specimens examined carried about equal amounts of yellow and black pigments, while those in the liver were all yellow. The melano-macrophage centres in the kidneys of two out of the six specimens examined carried about equal amounts of yellow and black pigments, in two others they were 80% black while in the other two specimens they were all black.

2.5 DISCUSSION

The cytoarchitecture of the lymphoid system and the molecular diversity of antibodies produced attain progressively higher levels of complexity in the evolution from fishes to mammals (Tam et al., 1976). Indeed, a phylogenetic pathway is apparent in all aspects of the specific adaptive immune response

(Bogner and Ellis, 1977). In birds and mammals germinal centres are considered to be the primary location of the central immune mechanism within the organized lymphoid tissue (Congdon and Hanna, 1967). Evidence that the melano-macrophage centres of fish could represent the primitive analogues of germinal centres is accumulating (section 1.8). The study described in the present chapter has revealed the major phylogenetic steps that led to the attainment of this, apparently highly organized, melano-macrophage centre system of advanced bony fishes. Primarily, there is a progressive increase in the abundance of pigment cells and an increasing tendency for these cells to home in the main lymphoid organs. The end result is that at the level of the advanced bony fishes the pigment cells are largely concentrated in the two major lymphopoietic organs, the spleen and the kidney where they are in intimate association with the immunocompetent cells. Secondarily, but no less important, there is evident an evolution of organization from a random distribution of these cells to the highly ordered centres of advanced bony fishes. The increasing complexity of this system is concomitant with the increasing levels of complexity in structure and immunocompetency of the lymphoid tissues.

Comparisons of immunocompetence at successive phylogenetic levels should take many variables into account. The increasing levels of complexity of lymphoid tissue organization have already been reviewed. Though cyclostomes possess both B- and T-cell competence (Hildemann and Thoenes, 1969), they appear to have a limited repertoire of responsivity associated with limited organization of lymphoid tissue (Tam et al., 1976). They can produce, even

if in low quantities, antibody of a structure similar to that observed in other vertebrate immunoglobulins (Marchalonis and Edelman, 1968). The Chondrichthyes, possessing a well-organized thymus, are capable of both 19S and 7S IgM responses. The plasma cell makes its appearance at the recent elasmobranch level of phylogeny (Good et al., 1966). Integrated cell- and humoral antibody immunity may have first evolved in advanced bony fishes which are admirably responsive to diverse antigens. There is a well developed spleen and thymus, T-cell and B-cell functions are evident and two or more molecular "size" classes of antibodies are demonstrable (Tam et al., 1976).

It is pertinent to mention here that Von Eschen and Rudbach (1974) have also reported an evolutionary development in the activity of non-specific serum factors. They observed that cyclostome and elasmobranch sera were less active in detoxifying and inactivating bacterial endotoxin than sera from teleosts. The factors responsible for inactivation were possibly related to serum complement. Attempts to demonstrate true complement activity in the cyclostomes have so far been unsuccessful and it is possible, although not certain, that the complement system is of more recent phylogenetic origin.

There is also evident a transition from slow to rapid elicitation of transplantation immunity. Agnatha and Chondrichthyes all reject initial allografts by discriminating but slowly mobilised immune reactions (Hildemann, 1970). Among primitive bony fishes as exemplified by the paddlefish Polyodon spathula (Perey et al., 1968) and arrowana Osteoglossum bicirrhosum (Borysenko and Hildemann, 1969), a transition from

slow to rapid elicitation of transplantation immunity is evident. Advanced bony fishes all show acute rejection of first-set integumentary allografts and accelerated rejection of second-set grafts with a vigour of cellular immunity at least equal to that of any laboratory mammal (Hildemann, 1972). Higher fishes also respond vigorously to diverse xenogeneic antigens by abundant antibody production. Very little information is available on the evolution of other aspects of cellular immunity in fish.

This phylogenetic pathway in structure and function of the lymphoid system is closely paralleled by a similar pathway of melano-macrophage centre development. In Agnatha, pigment cells are restricted to the liver and then only in the hagfish; in Chondrichthyes pigment cells are more abundant and in all Osteichthyes the levels of pigmentation are much higher than in Chondrichthyes. Secondly, it is only in the Osteichthyes that the pigment cells become cytologically organized to form discrete centres. Salmonids are exceptional in that, as in all Agnatha and Chondrichthyes, their melano-macrophages are randomly distributed in the haemopoietic tissues with no distinct pattern of organization at all. Thirdly, from cyclostomes to teleosts, there is a shift in importance from the liver to the spleen and kidney as principal areas of pigment cell aggregation.

This tendency of pigment cells to aggregate in areas of very active lymphopoiesis is possibly very significant and could well throw some light on the origin and evolution of function of the melano-macrophages. Phagocytic cells are phylogenetically much older than lymphocytes (Stuart, 1970). It is speculated

that at the primitive level, indigestible residues of host origin are engulfed by macrophages in the liver, an important site for metabolic activities, and are deposited there in the absence of adequately developed reticular and lymphoid systems. With the appearance of organized lymphoid tissues mainly within the spleen and kidney, the improved efficacy of the two systems in association with each other led to the progressive attainment of higher levels of complexity of this association in the spleen and kidney. Gradually the macrophage may have acquired additional immunological functions of trapping, processing and storing antigen and triggering the immune response. It seems therefore that in phylogenetic history, while the phagocytic cell is much older than the immunocompetent cell, the evolution of the lymphoid system resulted in the phagocytic system evolving new functions of association which led to organizational changes. In line with these speculations, Tam et al. (1976) identified the possibility that macrophage functions may have become more numerous and complex with the concomitant development of lymphoid organs and diversification of lymphoid cell types. Before this can be proved, however, there is need for more information on the immune response, both cell- and antibody-mediated; the functions, immunological and otherwise, of the macrophage and melano-macrophage; and the interactions of these within the various fish lineages.

The exception of Polypterus among the primitive bony fishes, in that it has a very heavily pigmented spleen, perhaps reflects its still very controversial phylogenetic affinities. In fact, while many taxonomists treat the Polypteridae as

chondrosteans, even as living Palaeonisciformes, current information suggest that they are more closely related to the Crossopterygians than to the Actinopterygians (Norman and Greenwood, 1977).

Unfortunately, studies with specific phyletic objectives other than morphological are scanty. Freihofer's (1963) work on a single nerve complex and Olsson's (1968) systematic study of the prolactin cells are two such examples. Hildemann (1974) illustrated the major sequential steps in the phylogeny of immunologic reactivity. According to his classification the fishes fall into two main groups, the primitive and the advanced fishes. Primitive fishes (Agnatha and Chondrichthyes) exhibit primordial cell-mediated immunity with memory. This is associated with cooperation of macrophages and T-like lymphocytes in allograft-type reactions. Advanced bony fishes show integrated cell- and humoral-antibody immunity. The primitive bony fishes are not shown in the chart although it is quite clear that they are considered to be intermediate between the primitive fishes and advanced bony fishes since they exhibit a whole range of responses from slow to rapid elicitation with respect to various aspects of the specific immune process like transplantation immunity. Based on the observations of the present study on the distribution and cytological organization of the melano-macrophage centres, fishes are likewise divided into two groups, the primitive fishes (Agnatha and Chondrichthyes) which have the individual melano-macrophages randomly distributed throughout the tissue with their being most abundant in the liver (except for Chimaera monstrosa) and the advanced bony fishes which have

highly organized melano-macrophage centres in close association with the immunocompetent tissues. The only exception to this subdivision are the salmonids, which exhibit random distribution of their pigment cells in their lymphoid tissues. Primitive bony fishes can also here be considered as intermediates since they resemble higher bony fish in the morphological status of their centres but are like Agnatha and Chondrichthyes in having their pigmented cells mainly concentrated in the liver. Polypterus senegalis is exceptional in that its spleen too is heavily pigmented.

In the course of this work it was observed that in most species the melano-macrophage centres stained PAS positive; in a few others only a proportion of the melano-macrophages in each centre proved to be PAS positive (eg. Coryphaenoides rupestris and Zeus faber). This and other reported histochemical properties such as their sometimes being Perls' Prussian blue positive (see Chapter 6) and Ziehl-Neelsen positive reflect the diversity of material that segregate in the centres. The response to the PAS reaction was observed to be remarkably similar for all specimens of each species examined. This most likely reflects the fact that the samples examined from each species originate from the same population which would thus have been subjected to similar environmental experiences, rather than it being a universal characteristic of the species. The response to such staining reactions as PAS, Perls' and Ziehl-Neelsen most probably depends on the environmental stimuli experienced by the fish so that they could well vary between samples of the same species obtaining from different sources.

It needs hardly be emphasized that the full implications of many of the observations made during this study remain obscure for the moment, the more so since the phylogenetic affinities of the different species at this level of phylogeny are very inadequately known (see for example Greenwood et al., 1973; and Norman and Greenwood, 1977). It is hoped that this study will generate more interest in the investigation of the various aspects of the lympho-reticular system from a phylogenetic point of view. Moreover, it would be interesting to establish what additional functions (immunological or otherwise), if any, the pigment gives to the macrophage. Edelstein (1971), in his review of the role of melanin in defence mechanisms in plants and animals, advanced many speculative ideas as to the possible functions the pigments could have. Certainly, the presence of pigmented macrophages in all fishes bar the lampreys, very often in very substantial quantities, strongly suggests a significant role. There is a dire need for a more experimental approach to try and dismember the seemingly complex interaction of functions that are carried out by the melano-macrophages and to characterize chemically the pigments and other components of these pigmented cells.

CHAPTER 3

ONTOGENY OF MELANO-MACROPHAGES AND AGE-RELATED CHANGES

3.1 INTRODUCTION

The integumentary melanin-producing cells of teleosts are derived from the neural crest ectoderm, although it is believed that they can also arise from other cells of the embryonic brain (Oppenheimer, 1949; Newth, 1951). The embryonic origin of the melanin-bearing macrophages within haemopoietic tissues has still to be resolved.

For a long time there has existed controversy as to whether the first haemopoietic sites in the developing teleost body are the intra-embryonic intermediate cell mass of Oellacher located between the somites and the lateral plates or the extra-embryonic blood islands of the yolk sac; indeed, it has been claimed that in some species both sites can be haemopoietic (for review see Al-Adhami and Kunz, 1976). Whatever may be the true answer, these sites only give rise to erythroblasts. Only two papers could be traced that give some information as to the first leucopoietic site in developing teleosts.

Al-Adhami and Kunz (1976) studied the haemopoietic centres of developing angelfish Pterophyllum scalare from the embryo till adulthood, thus providing valuable information as to which haemopoietic centres operate sequentially in the embryonic, post-embryonic and larval phase and at what stage the final switch over to adult centres takes place. At 27°C, the

embryonic phase of angelfish eggs lasts 2 days. The post-embryonic phase lasts 8 days and ends with the complete absorption of the yolk sac (Kunz, 1964). The subsequent larval phase lasts approximately two months at the end of which metamorphosis to the laterally compressed adult begins. The first haemopoietic centres in the embryo, producing only proerythroblasts which on being released into the circulation differentiate into erythrocytes, are found in the blood islands of the yolk sac. In the post-embryo haemopoiesis is carried out by the pronephros, an organ that remains haemopoietic to the adult stage. On the 4th post-embryonic day the pronephros is very active haemopoietically and its cellular composition is indicative of granulocyte, lymphocyte and erythrocyte formation; leucocytes and thrombocytes are seen in circulation only after the yolk has been absorbed. Thus leucocytes and thrombocytes appear much later in development than the red blood cells. Stem cells are not observed to develop within the early pronephros and the number of circulating stem cells decreases drastically after haemopoiesis has started here (early post-embryonic phase). This suggests that circulating stem cells, originating from the blood islands, get trapped or home onto the pronephric reticulum and undergo further development there. The functions of the two haemopoietic centres would, therefore, be complementary: the blood islands (primary centres) produce the stem cells; the reticular tissue in the early pronephros (secondary centre) traps and harbours them.

Ellis (1977) in his study on the histogenesis of the lymphoid organs in Salmo salar noted the presence of haemopoietic foci in the kidney at an early stage of development - earlier than

23 days before hatching. These foci were composed of erythroblasts, granuloblasts, erythrocytes and granulocytes. The thymus is the first organ to become lymphoid (22 days prehatch) followed by the kidney and the appearance of lymphocytes in the vasculature (14 days prehatch). The eggs were here incubated in running tap water at 4°C rising to 7°C in May when hatching occurred, 111 days after fertilization.

In this chapter are recorded and discussed the beginnings of an experimental approach to the problem of elucidating the embryonic origin of the melano-macrophages observed in the haemopoietic tissues of fish. The objectives of this section are to establish when melano-macrophages appear during normal development in a number of teleost fish species and how their numbers vary as fish grow older. Such information is a prerequisite for any surgical procedures, such as defect or transplantation experiments,or indeed for any other investigational approach.

The egg of teleosts has been the object of a considerable amount of embryological work dealing mostly with the three genera Fundulus, Oryzias and Salmo (and other Salmonidae). Interesting investigations have also been carried out on the eggs of Carassius and Brachydanio and to a lesser extent on the eggs of Clupea and Perca (Devillers, 1961). In this study it was attempted to study two cold-water/temperate species viz. rainbow trout Salmo gairdneri and turbot Scophthalmus maximus and one warm-water species Tilapia zillii (Gervais).

3.2 MATERIALS AND METHODS

3.2.1 Salmo gairdneri

Origin and incubation of eggs

In this experiment, carried out in the winter of 1977/78, the eggs were the progeny of two pairs of rainbow trout (four years old) obtained from Howietoun Northern Fisheries, Stirlingshire, Scotland. The mature fish were stripped and fertilization was carried out on the farm by leaving the eggs mixed with milt for ten minutes. The eggs were then washed thoroughly and transported to a constant temperature room in the laboratory aquarium. Two hours after fertilization, 4,000 eggs were laid out on a hatching tray placed in a rectangular hatching tank through which a slow but continuous flow of well-aerated, copper-free tap water was maintained. The temperature of the water was kept at 12°C throughout the experimental period. Dead eggs were removed daily; on average only one or two were removed each day.

First feeding

On the 27th day of incubation hatching commenced and in 48 hours about 90% of the eggs had hatched; the rest were discarded. 300 newly-hatched alevins were transferred to a rectangular tank measuring 1 m x 0.5 m x 0.5 m through which well-aerated, copper-free tap water was allowed to flow at the rate of 5 l/min. The rest of the alevins were reared separately for use in experiments to be described later (see Chapter 5). The fish were subjected to a 12-hour photoperiod. Forty-two days after fertilization, the fry started to feed voraciously even though the yolk had not been entirely consumed. They were

hand fed on a commercial diet; on average they were fed six times daily. As growth proceeded, the fish were thinned down to appropriate densities.

Sampling

Sampling was started with hatching. Six fish were examined each week until 20 weeks post-hatching. Alevins and small fry were fixed in 10% neutral buffered formalin. The egg yolk was then dissected away before cutting serial sections at 5 µm. For the bigger fry, the spleen, kidney and liver were dissected out wherever possible.

Adult fish

One-, two- and three-year old rainbow trout from the same farm were also examined. Five fish of each age class were dissected and portions of their anterior kidney, liver and spleen were fixed in 10% neutral buffered formalin.

3.2.2 Scophthalmus maximus

All attempts to obtain fertilized eggs of this species failed.

Adult fish

One-, two-, three- and four-year old turbot (five of each) were obtained from the Flatfish Farming Unit of the White Fish Authority at Ardtoe, Argyllshire, Scotland. Portions of the anterior kidney, liver and spleen were fixed in 10% formal saline.

3.2.3 Tilapia zillii

Source and maintenance of fry

A batch of 80 newly-hatched tilapias were obtained from

the Tropical Aquaculture Laboratory at Stirling University. These were placed in a tank measuring 0.5 m x 0.3 m x 0.3 m which was fitted with an E-heim filter. The eggs had been incubated at 25°C (incubation lasted 3 days) and the fry were kept at that temperature throughout this experiment under a 12-hour photoperiod. Two days after hatching feeding commenced. The fish were hand fed, about six times daily, on flakes commercially produced for pet fish.

Sampling

Fish were sampled soon after hatching; thereafter fry were examined each week until 20 weeks post-hatching. Samples were also taken 2 days after hatching i.e. when feeding commenced. On each occasion, three specimens were fixed in 10% neutral buffered formalin and after routine processing, serial sections were cut at 5 µm.

Adult fish

Attempts to obtain enough Tilapia specimens of different age groups proved impossible during the timescale of the study.

3.2.4 Histopathological procedures

Processing, wax embedding and section cutting were performed as described in the previous chapter. All sections were stained with H & E.

3.2.5 Technique for counting melano-macrophages and melano-macrophage centres

As described in the previous chapter, the melano-macrophages of salmonids are randomly distributed throughout the

haemopoietic tissues whereas in flatfish they tend to form discrete centres. This necessitated developing different techniques for counting the pigment cells in the two species. Serial sectioning revealed that in any one fish specimen there are no substantial differences in the distribution of the pigment cells throughout the liver, the spleen and the anterior kidney. Thus, relatively large areas of sections were first scanned microscopically and a field considered to be representative of the whole section in terms of its content of pigment cells was chosen. For sections of trout origin, the individual pigment cells in that field were counted at x 225; this procedure was repeated three times, an average taken and the results expressed as number of melano-macrophages per mm^2 using an eyepiece graticule and a stage micrometer. For sections from flatfish it was observed that centres with a diameter of between 40 and 120 μm represented about 90% of the total pigment cell content and thus counting centres in this size range was considered to be adequate for the purposes of the present study. The same counting procedure as for sections from salmonids was adopted except that in this case counting at x 100 was found to be more practical and the results were expressed as number of centres per mm^2 of tissue section.

3.3 RESULTS

3.3.1 Salmo gairdneri

Ontogenic appearance of melano-macrophages

In rainbow trout, melano-macrophages were first observed in sections of fry that had been feeding for two weeks. They

seemed to appear simultaneously in the spleen and the kidney. Thereafter they increased steadily in numbers in both organs. In comparison, very few melano-macrophages could be seen in the liver even at 20 weeks post-hatching.

Variations with age (Figs. 3.1, 3.3, 3.4 and 3.5)

The density of splenic and renal melano-macrophages was observed to increase steadily with age. The rate of increase was highest during the first year of life but then decreased. The pattern of such an increase was similar for the spleen and anterior kidney but the density of melano-macrophages in the spleen was consistently lower than that in the kidney. At all ages, very low numbers of melano-macrophages could be observed in the liver and three-year old trout only carried at most about 15 such cells per mm^2 of liver section.

3.3.2 Scophthalmus maximus

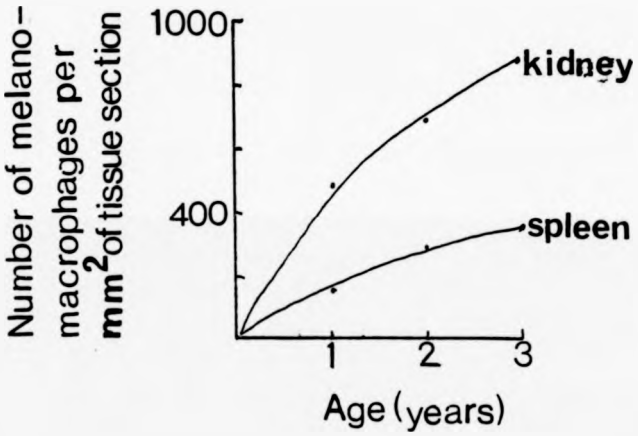
Variations with age (Figs. 3.2, 3.6 and 3.7)

As for trout, the density of splenic and renal melano-macrophages, here forming discrete centres, increased steadily with age. The rate of increase was highest during the first two years after which it decreased. In turbot the density of the splenic melano-macrophage centres was consistently of the same order of magnitude as that of the kidney centres. As for trout, the liver carried a much smaller population of melano-macrophages. In fact, even in four-year old specimens, only a few randomly distributed melano-macrophages, but no centres, could be observed.

An interesting observation was the presence in the older turbot of large amounts of yellow pigments, much apparently

Fig. 3.1. Graph representing the accumulation of melano-macrophages with age within haemopoietic tissue of the spleen and anterior kidney of rainbow trout. At all ages the liver contained very few melano-macrophages.

Fig. 3.2. Accumulation of melano-macrophage centres with age within the haemopoietic tissue of the spleen and anterior kidney of turbot. At all ages the liver carried very few melano-macrophages. Only centres in the size range 40 - 120 μm were counted as these were observed to comprise by far the majority of the total melano-macrophage population.


Number of melano-macrophages per mm² of tissue section
1000
400
kidney
spleen
1
2
3
Age (years)

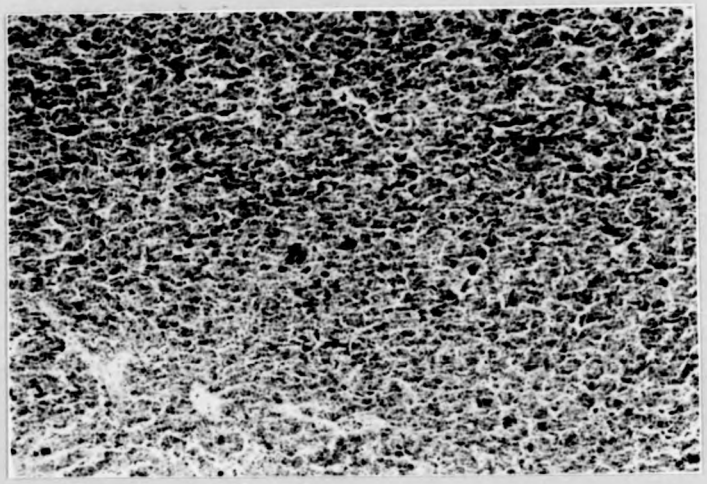

Number of melano-macrophage centres with a diameter 40-120µm per mm² of tissue section
16
8
spleen
kidney
1
2
3
4
Age(years)

Anterior kidney of rainbow trout Salmo gairdneri.

Fig. 3.3. Section taken from four-month old specimen.
(H & E x 125)

Fig. 3.4. Section taken from one-year old specimen.
(H & E x 125)

Note the higher density of melano-macrophages in the older fish.



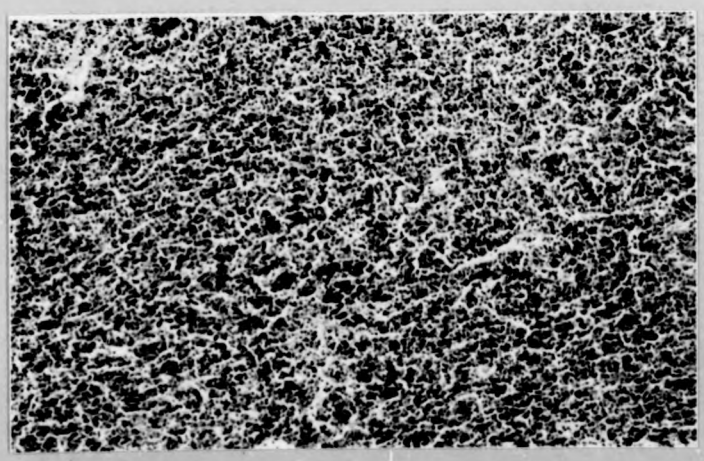
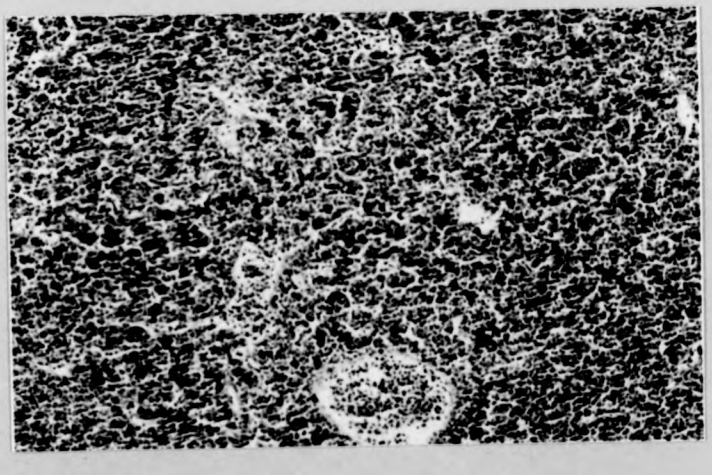


Fig. 3.5. Anterior kidney of a two-year old rainbow trout. Note how dense the melano-macrophages are in older fish.
(H & E x 125)

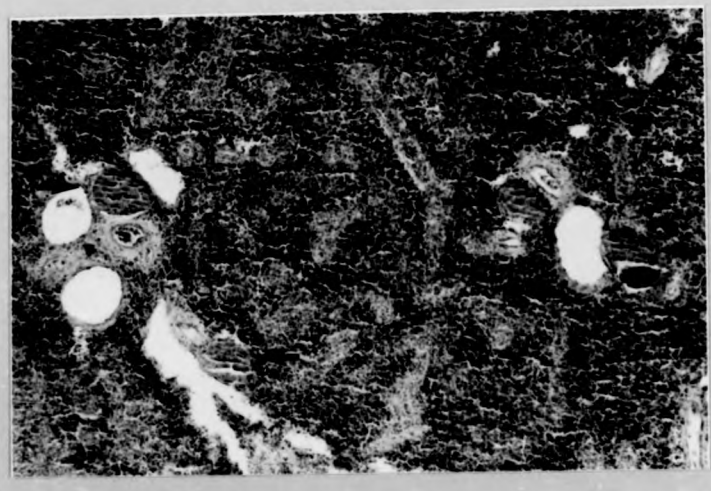


Sections from spleen of turbot Scophthalmus maximus.

Fig. 3.6. One-year old turbot.
(H & E x 125)

Fig. 3.7. Three-year old turbot.
(H & E x 125)

Note that while in the younger fish only one or two small melano-macrophage centres (arrowed) are present, a number of fairly large centres (arrowed) can be observed in the section from the older fish.



present as free granules, trapped within and just external to the fibrous membrane of the ellipsoid walls.

3.3.3 Tilapia zillii

Ontogenic appearance of melano-macrophages

In this species the presence of melano-macrophages could be detected in the kidney one week post-hatching i.e. 5 days after commencement of feeding. However, it was only three weeks after hatching that their presence in the spleen could be established with certainty. The number of melano-macrophages increased steadily in both spleen and kidney and by 20 weeks post-hatching aggregates of about 30 - 40 such cells could be seen starting to form discrete melano-macrophage centres. As with the other species, very few such cells were present in the liver even at 20 weeks post-hatching.

3.4 DISCUSSION

Salmonid eggs can tolerate a temperature of 0 - 16°C (Peterson et al., 1977); temperatures that are either lower or higher than the optimum are known to induce anomalies. In fact, a direct relationship between temperature and abnormal development has often been demonstrated in fish hatcheries and laboratory experiments (Koo and Johnston, 1978). Furthermore, various workers have claimed that newly-hatched alevins of various families of fishes are smaller at higher incubation temperature (see for example Alderdice and Forrester, 1971 for Pacific cod Gadus macrocephalus, Alderdice and Velsen, 1971 for Pacific herring Clupea pallasi, and Peterson et al., 1977 for Atlantic salmon). In this work rainbow trout eggs were incubated at 12°C;

this was considered a high enough temperature to accelerate development considerably, thus reducing to a minimum the risk of losses in future experiments at the same time not inducing any serious abnormalities.

Knight (1963) incubated rainbow trout eggs at the same temperature as that used in the present study and obtained hatching on the 23rd day of incubation. Such a range of a few days in the hatching dates of different groups of eggs appears to be normal. Embody (1934) noted a 3- to 4- day range in the emergence of alevins from the eggs of a single female at high water temperatures. At this temperature, the neural keel becomes very prominent on the dorsal lip of the blastopore at 5 days post-fertilization. This then would be the appropriate time to perform any extirpation experiments designed to establish whether the neural crest ectoderm is in any way responsible for giving rise to melano-macrophages in the same way as it gives rise to the integumentary melanin-producing cells. After 12 - 13 days of incubation the eyes become pigmented, thus becoming visible through the chorion and a week later the body appears much darker because it is covered with many melanophores. By comparison the melano-macrophages of the haemopoietic tissues only appear about two weeks after first feeding. This means that if the embryos are subjected to any treatment, surgical or otherwise, at the stage when the neural keel becomes prominent, they then have to be cultured, frequently under very strict sterile conditions in specially prepared media for a minimum of eight weeks at 12°C before one can hope to obtain results. This is assuming that any treatment administered does not in any way

impair development.

Using a warm water species seems to offer many advantages. At 25°C T. zillii hatch three days after fertilization. Melano-macrophages could be detected (at least in the kidney) one week post-hatching. This opens the possibility that the desired results could be achieved within two weeks. However there are two major drawbacks here, viz., the risks of infection are much greater at the higher temperature and more importantly the Tilapia eggs are very small.

Of the fish species that are readily available and about which adequate knowledge of their embryology is obtainable, salmonids possess by far the largest-sized eggs. The mean yolk diameter of the group of eggs employed in this study was approximately 5 millimetres. The eggs of the Atlantic salmon are even bigger, ranging from 5.5 to 6.0 mm in diameter (Battle, 1944). Eggs in this size range are more amenable to any treatment, surgical manipulations in particular, than for instance Tilapia eggs with a diameter less than 2 millimetres.

The appearance of the melano-macrophages following upon first feeding is of specific interest. Many important changes have been reported as coinciding with first feeding and there is no doubt that this represents a very delicate phase in fish larval development. Ellis (1977) investigated the ontogenic development of the lymphoid system in Atlantic salmon by assaying the appearance of membrane immunoglobulins (m Ig M) on lymphocytes (as a measure of "B-cell" function) and the development of mixed

leucocyte reactivity (MLR) (as a measure of "T-cell" function). Both phenomena appear coincidentally with the onset of feeding. Ellis commented on the likelihood that the prefeeding salmon larva is immunologically immature, possessing neither humoral nor cell-mediated immunity. At first feeding it appears as if immunological maturity is attained. The fact that it is shortly afterwards that melano-macrophages appear within the lymphoid tissues eventually leading to melano-macrophage centre formation within them seems to add weight to the evidence of a structural and functional relationship between melano-macrophages and lymphoid tissues. The spleen in salmon also develops at first feeding; from this study it appears as if the same holds true for rainbow trout. This correlation is significant with respect to melano-macrophage centre development. The present work has shown that melano-macrophages appear simultaneously in the spleen and the kidney. Moreover, the spleen in this species can be fairly heavily populated with melano-macrophages. Obviously further work is needed to discover whether the coincidental occurrence of so many developmental processes with feeding are due to the larvae being subjected to a new diet that may, among so many possibilities, be deficient in some requirements (such as antioxidants leading to lipofuscin pigment formation), or present a large antigenic challenge, or whether this is the normal developmental sequence of events in fish larvae.

Finally, the observations made in the course of this study seem to lend support to the hypothesis that the pigment within the centres represents age pigment. Its absence in young larval fish, its steady accumulation with age in clinically

normal fish and its presence without exception in older fish seem to meet the criteria set forth for a basic biological aging process.

CHAPTER 4

EFFECTS OF STARVATION, HIGH FATTY DIETS AND AN EXPERIMENTALLY INDUCED CHRONIC INFLAMMATORY LESION ON MELANO-MACROPHAGE CENTRES IN ADULT FISH

4.1 INTRODUCTION

In this chapter are described a number of experiments that were designed to identify, under well-defined conditions, factors that induce a measurable effect on melano-macrophage centres in a relatively short space of time. Currently available information comes exclusively from observations on wild-caught or commercially farmed fish (often suffering from some disease) and the environmental conditions to which the fish had previously been subjected are often very poorly recorded. In the experiments to be described fish were starved, fed very oily diets and injected intramuscularly with Freund's complete adjuvant and the melano-macrophages were monitored histologically.

Enlargement of the melano-macrophage centres under starvation conditions has been reported by Roberts (1978).

Numerous investigators have observed a yellow-brown pigment, possessing marked tinctorial and histochemical similarities to ceroid or lipofuscin within various tissues (including the reticulo-endothelial system) of numerous animal species fed diets rich in unsaturated lipids particularly cod liver oil and deficient in α-tocopherol (for review see Thompson, 1966). Cod liver oil contains polyunsaturated fatty

oils of the elasmobranchs (Lowry et al., 1976). In fact, in this oil the fatty acids are approximately 65% saturated and 44% unsaturated (Wood and Tsuchiya, 1956). Hass (1938a, b; 1939) observed that cod liver oil provokes a pronounced and giant-cell response when injected into subcutaneous tissue in rats. Subsequently the injected material was converted into a tuberculosis-like substance. Bullock (1944) reported that prolonged in vitro oxidation of cod liver oil or linseed oil by potassium dichromate produced acid-fast material similar to smegma. He also noted that these oils when injected subcutaneously into rats gradually acquired acid fastness.

The involvement of melano-macrophages in various fish diseases has already been reviewed (section 1.3). Freund's complete adjuvant, consisting of mineral oil, an emulsifying agent and killed mycobacteria, is known to induce inflammatory lesions in fish (Finn and Nielsen, 1971; Lamas, 1975). In this study an inflammatory lesion was elicited by injecting this adjuvant intramuscularly and the melano-macrophages of the spleen, kidney and liver were monitored histologically as the inflammatory response progressed.

4.2 MATERIALS AND METHODS

4.2.1 Experimental design

All experiments were carried out in tanks measuring 1.2 m x 0.3 m x 0.3 m, each fitted with an in-tank filter. Aeration was supplied to each tank and the water was changed every other day. All experiments were carried out at 12°C and the fish were subjected to a 12-hour photoperiod.

acids of the ω3 series (Cowey et al., 1976); in fact, in this oil the fatty acids are approximately 16% saturated and 84% unsaturated (Wood and Yasutake, 1956). Hass (1938a, b; 1939) observed that cod liver oil provokes an eosinophilic and giant-cell response when injected into subcutaneous tissue in rats. Subsequently the injected material was converted into a lipofuscin-like substance. Endicott (1944) reported that prolonged in vitro oxidation of cod liver oil or linseed oil by potassium dichromate produced acid-fast material similar to ceroid. He also noted that these oils when injected subcutaneously into rats gradually acquired acid fastness.

The involvement of melano-macrophages in various fish diseases has already been reviewed (section 1.9). Freund's complete adjuvant, consisting of mineral oil, an emulsifying agent and killed mycobacteria, is known to induce inflammatory lesions in fish (Finn and Nielsen, 1971; Timur, 1975). In this study an inflammatory lesion was elicited by injecting this adjuvant intramuscularly and the melano-macrophages of the spleen, kidney and liver were monitored histologically as the inflammatory response progressed.

4.2 MATERIALS AND METHODS

4.2.1 Experimental design

All experiments were carried out in tanks measuring 0.5 m x 0.3 m x 0.3 m, each fitted with an E-heim filter. Aeration was supplied to each tank and the water was changed every other day. All experiments were carried out at 12°C and the fish were subjected to a 12-hour photoperiod.

4.2.2 Fish

In all three experiments about to be described, rainbow trout were used. A batch of 0+ trout (7 to 10 cm in length) were obtained from a hatchery in Scotland. Before introducing them into the experimental set-up the fish were treated for external parasites by placing them in a 1:5000 concentration of formalin in well-aerated water for one hour. Thirty fish were placed in each experimental tank and these were left to acclimatize for two weeks. During this period they were fed on a commercial diet they had been reared on since first feeding and which will henceforth be referred to as the control diet. Irrespective of the diet employed, all fed fish were given 3% of their body weight/day as food spread over three to five times during the day. All treatments were run in duplicates.

4.2.3 Starvation experiment

After the two-week acclimatization period, the trout were completely deprived of food for a period of ten weeks. The control fish continued to be fed on the control diet.

4.2.4 Feeding of oily diets

Three treatments were carried out; one feeding the control diet, one a 25% oily diet and another feeding a 35% oily diet.

The control diet consisted of:

40% herring meal
25% white fish meal
10% dried distillers soluble
20% vitamins and minerals
5% marine fish oil

This carries a total of about 10% oil. The oil content of this diet was enhanced in two ways viz. by replacing the herring meal which contains about 10% oil by a French fish meal containing 19% oil (commercially called Special G) and by stepping up the percentage of oil employing cod liver oil. Two diets were prepared, viz:

i. 25% oily diet consisting of:

40% Special G
15% white fish meal
10% dried distillers soluble
20% vitamins and minerals
15% cod liver oil

Pellets identical in size to those of the control diet were prepared and fed to the fish.

ii. 35% oily diet consisting of:

34% Special G
13% white fish meal
8% dried distillers soluble
17% vitamins and minerals
28% cod liver oil

This mixture was too oily to be pelleted and was fed as a paste.

With the rate of feeding employed in these experiments the fish quickly swallowed the food as soon as it touched the water and the loss of oil through seepage out of the pellets or paste was considered negligible. The experiments lasted fourteen weeks.

4.2.5 Inoculation with Freund's complete adjuvant

A 1:1.7 emulsion of Freund's complete adjuvant* and sterile physiological saline was prepared. 0.15 ml of this emulsion was injected into the myotomal muscles just below the dorsal fin. Control fish were injected with an equivalent volume of sterile physiological saline. Injections were performed under anaesthesia. All fish were fed on the control diet. These experiments lasted eight weeks.

4.2.6 Sampling and histological procedures

Two fish were sampled from each experimental tank weekly. After pithing, the anterior kidney, spleen and liver were dissected out and fixed in 10% neutral buffered formalin. Following routine processing, 5 μm thick paraffin sections were stained with haematoxylin and eosin (H & E) and Schmorl's ferricyanide method for lipofuscins; in this way counting of lipofuscin-containing macrophages was greatly facilitated.

For the inoculated fish the site of injection was also examined histologically; sections of the muscle tissue from this region were stained with haematoxylin and eosin (H & E).

4.2.7 Plaice starvation experiment

Because of the results obtained (see results section) the starvation experiment was repeated using plaice. These were caught off the Aberdeen coast and measured from 10 to 15 cm in length. In the laboratory fifteen plaice were placed in each

* Bacto Adjuvant, Complete Freund's, Difco, Detroit

of four tanks in water of salinity around 30 ‰. After weaning them onto chopped squid the fish in two of the tanks were completely starved for a period of sixteen weeks whilst the control fish continued to be fed daily on chopped squid in amounts that satisfied their appetite.

Two fish were sacrificed from each tank at the following intervals: 2 weeks, 4 weeks, 6 weeks, 9 weeks, 12 weeks and 16 weeks. After pithing, portions of the anterior kidney, spleen and liver were fixed in formal saline and 5 µm thick paraffin sections were stained with H & E and Schmorl's ferricyanide method for lipofuscins.

4.2.8 Counting of melano-macrophages and melano-macrophage centres

This was performed as described in section 3.2.5.

4.3 RESULTS

The changes in the density of melano-macrophages in the spleen and anterior kidney of the rainbow trout subjected to the various treatments are illustrated in Figs. 4.1, 4.6 and 4.7. Because of the close similarity of the results obtained with the two highly oily diets the results are pooled in Fig. 4.6.

Starvation resulted in a very large increase in the density of melano-macrophages both in the spleen and the anterior kidney (Figs. 4.1 to 4.5). Melano-macrophages were almost entirely absent from the liver except in the final two or three weeks of the experiment when densities as high as 50 pigment

cells/mm^2 were recorded from cachectic specimens. In both spleen and kidney the increase in the density of melano-macrophages followed a sigmoid pattern. However, the density of these cells in the kidney was consistently much higher than that in the spleen. The most marked increase in both organs occurred between week 5 and week 8, after which the rate of increase levelled off. This coincided with the onset of mortalities, the fish having become very debilitated and lost considerably in weight. One very important difference was that while the pigment-laden macrophages laid down in the kidney during the starvation period were nearly all black, a large proportion of those laid down in the spleen were yellow; these were Schmorl's positive and acid-fast indicating that they contained lipofuscin. As will be seen in Chapter 6 these splenic melano-macrophages also carried substantial amounts of haemosiderin.

In the fish fed very oily diets there was only observed a slight decrease in pigment cell density in the anterior kidney especially between week 4 and week 7 (Fig. 4.6). Within the period of study the fish fed such diets showed markedly better growth and post-mortem examination showed that extensive fat depots had been laid down in the liver and around the pyloric caeca; some specimens also developed very marked kidney pallour. Within hepatocytes lipid droplets varied greatly in size and localization but the lipid accumulation was large enough to visibly increase the volume of individual cells and of the whole organ. Pigment cells were almost entirely absent from the liver throughout the experimental period.

No changes in melano-macrophage density were observed in the fish injected with the complete adjuvant (Fig. 4.7) although the inflammatory reaction progressed normally (Fig. 4.8). Once again the densities of pigment cells in the liver are not plotted as these remained very low indeed throughout the experiment. The fish were observed to feed normally on the control diet at all times and the experimental inoculations did not affect the fish clinically in any way.

In view of the results obtained the starvation experiment was repeated with plaice. The changes in the number of melano-macrophage centres per mm^2 occurring in plaice during starvation are plotted in Fig. 4.9. The density of melano-macrophage centres increased considerably in both spleen and kidney during starvation (Figs. 4.10 to 4.13). Two important differences are immediately apparent when the plots are compared with those obtained using rainbow trout. In plaice the numbers of melano-macrophage centres per unit area of section were similar in the spleen and anterior kidney both in normal and cachectic specimens in contrast to trout where the density of the melano-macrophages in the anterior kidney was consistently higher than that in the spleen. Also, while the density of melano-macrophages in trout increased sigmoidally during starvation the number of melano-macrophage centres in plaice increased in a linear fashion.

As no mortalities were recorded the experiments were continued for a longer period of time than those with trout. After ten weeks of starvation melano-macrophage centres started to form in the liver and by sixteen weeks some specimens carried considerable numbers of them in this organ.

Fig. 4.1. Changes in the density of melano-macrophages in the spleen and anterior kidney of 0+ rainbow trout that were completely deprived of food for a period of ten weeks at a temperature of 12°C. Each point represents an average of four determinations.

- ● spleen of control trout
- ⊙ spleen of cachectic trout
- ■ anterior kidney of control trout
- ⊗ anterior kidney of cachectic trout

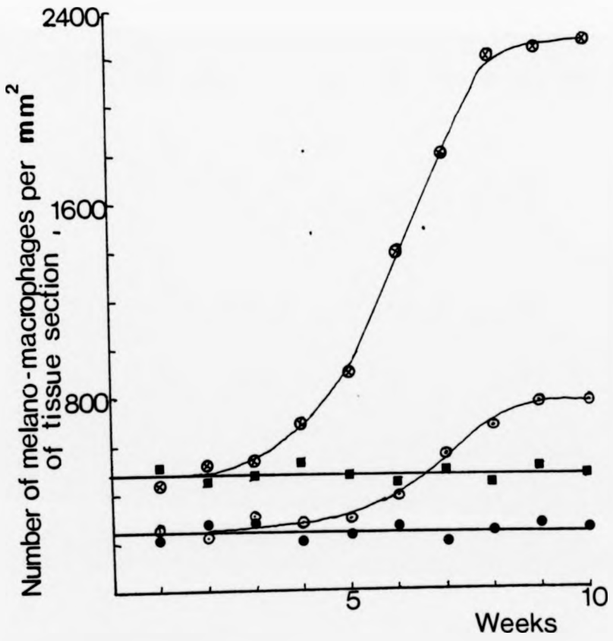


Fig. 4.2. Spleen of a normal 0+ rainbow trout. This section was taken from a control specimen eight weeks after commencement of the starvation experiment. Note the small number of randomly scattered melano-macrophages.
(H & E x 125)

Fig. 4.3. Spleen of a cachectic trout. This section was taken from a 0+ specimen that had been starved for eight weeks at 12°C. Note the much greater density of melano-macrophages as compared to the control (Fig. 4.2).
(H & E x 125)

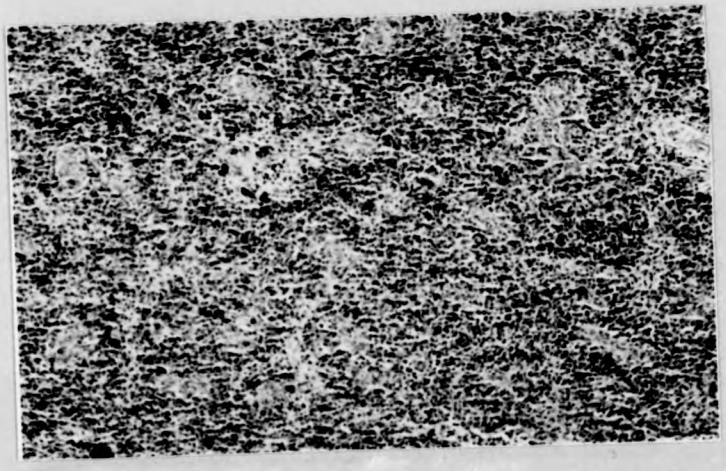
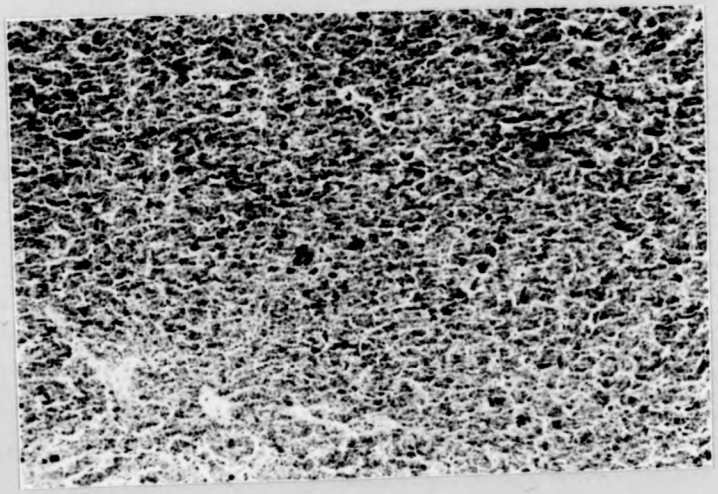


Fig. 4.4. Anterior kidney of a control trout specimen at eight weeks of the starvation experiment.
(H & E x 125)

Fig. 4.5. Anterior kidney of a cachectic trout after eight weeks of total starvation at 12°C. The density of melano-macrophages has increased enormously during the starvation period.
(H & E x 125)



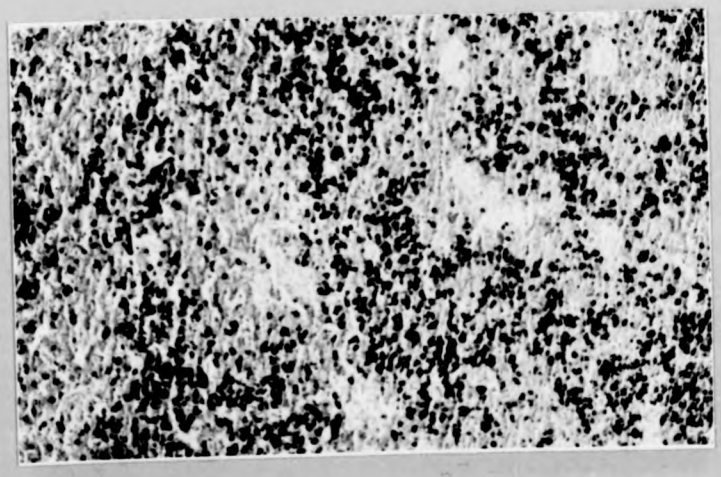


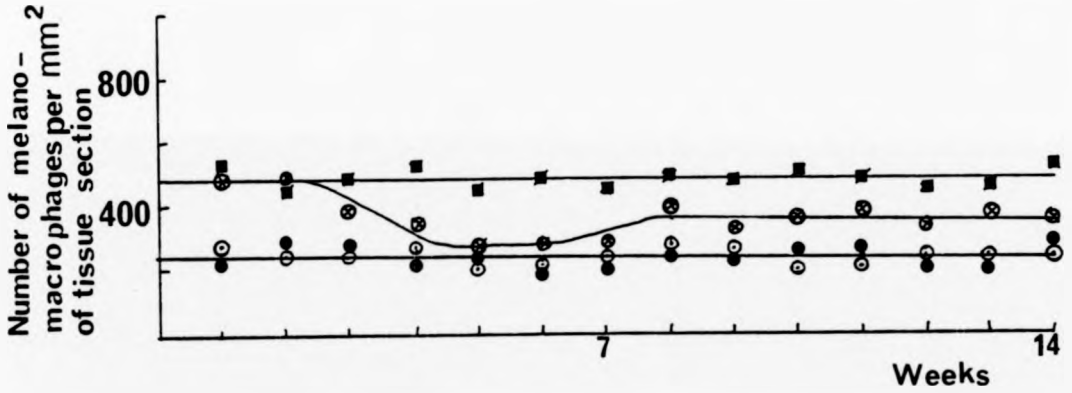
Fig. 4.6. Plots depicting the changes in the density of melano-macrophages in the spleen and anterior kidney of 0+ rainbow trout that were reared on a very oily diet for a period of fourteen weeks at 12°C. Each point represents an average of four determinations.

- ● spleen of control trout
- ⊙ spleen of trout reared on very oily diet
- ■ anterior kidney of control trout
- ⊗ anterior kidney of trout reared on very oily diet

Number of melano-macrophages per mm^2

Fig. 4.7. Graph showing the density of melano-macrophages in the spleen and anterior kidney of 0+ rainbow trout that had received an intramuscular injection of emulsified Freund's complete adjuvant. Monitoring of the haemopoietic organs for these pigmented macrophages was continued for eight weeks after performing the inoculations during which time the fish were kept at 12°C. Each point represents an average of four determinations.

- ● spleen of control trout
- ⊙ spleen of inoculated trout
- ■ anterior kidney of control trout
- ⊗ anterior kidney of inoculated trout


Number of melano-macrophages per mm2 of tissue section
800
400
7
14
Weeks

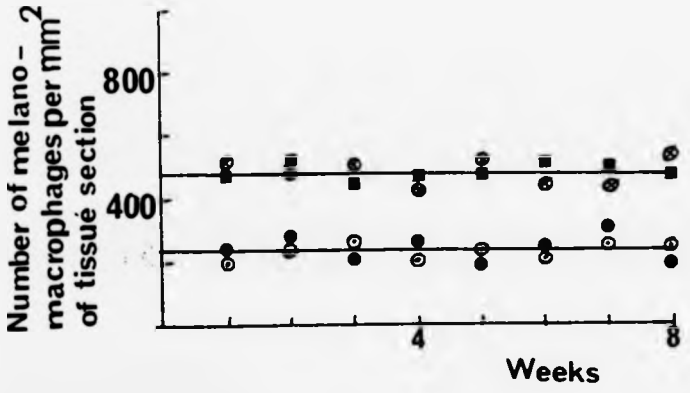

Number of melano-macrophages per mm2 of tissue section
800
400
4
8
Weeks

Fig. 4.8. Inflammatory lesion induced in rainbow trout skeletal muscle by means of an inoculum of emulsified Freund's complete adjuvant. General view of the lesion at 12°C after eight weeks. Note the presence of pigment cells amid the very marked cellular infiltrate.
(H & E x 125)

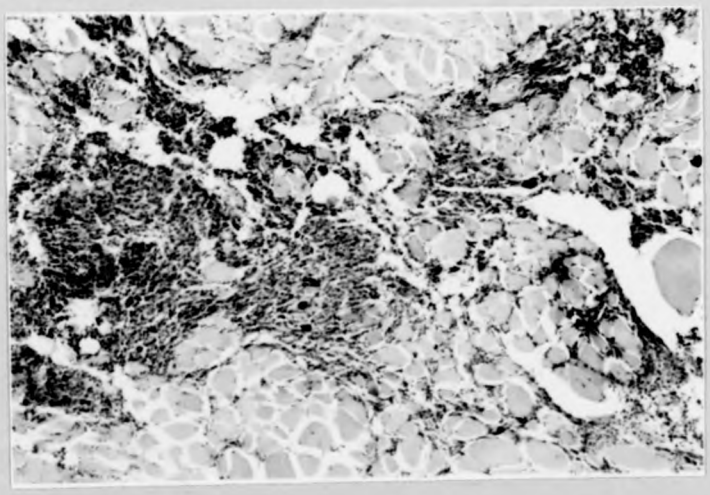


Fig. 4.9. Graph illustrating the changes in the density of melano-macrophage centres in the spleen and anterior kidney of plaice that were starved for a period of sixteen weeks at 12°C. Each point represents an average of four determinations.

- ● spleen of control plaice
- ⊙ spleen of cachectic plaice
- ■ anterior kidney of control plaice
- ◉ anterior kidney of cachectic plaice

Number of melano-macrophage centres with a diameter
40-120μm per mm² of tissue section
40
30
20
10
4
8
12
16
Weeks

Fig. 4.10. Spleen of a control plaice. Only one or two melano-macrophage centres and a few randomly distributed melano-macrophages can be seen in this one field.
(H & E x 125)

Fig. 4.11. Spleen of a cachectic plaice. Note the much higher number of centres as compared to the section from the control specimen (Fig. 4.10). This specimen had been completely deprived of food for sixteen weeks at 12°C.
(H & E x 125)

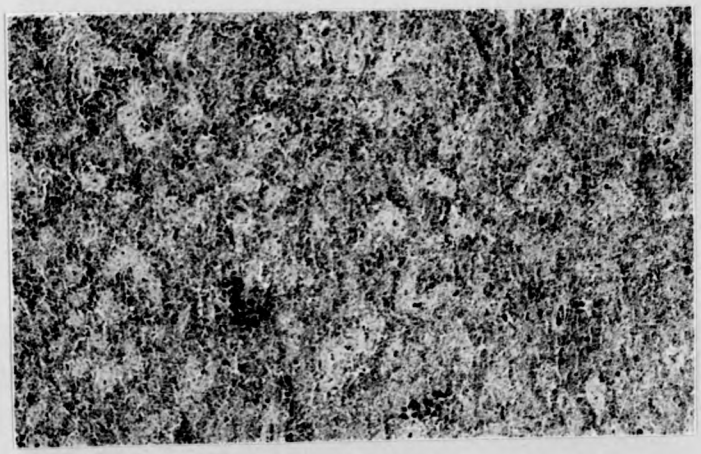
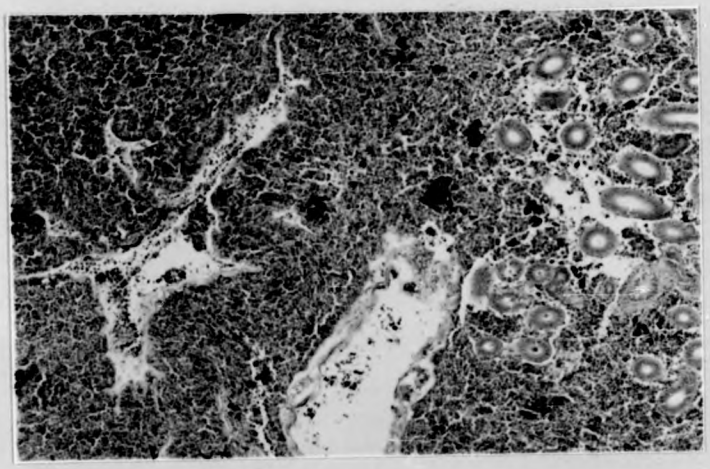


Fig. 4.12. Kidney of a control plaice specimen. A few small melano-macrophage centres can be seen in this one field.
(H & E x 125)

Fig. 4.13. Kidney of a plaice that was starved for sixteen weeks at 12°C. During cachexia the number of centres increased both in size and number.
(H & E x 125)



Starvation experiments employing small numbers of swordtails Xiphophorus helleri (Heckel) and Tilapia zillii showed that in these species too cachexia led to a considerable increase in the numbers of melano-macrophage centres. These experiments however were of shorter duration because of mortalities; it is very likely that this is a consequence of the higher temperatures (25°C) at which these experiments were conducted as compared with those using trout and plaice (12°C).

4.4 DISCUSSION

The very marked changes observed in the melano-macrophage centres during cachexia provide a convenient tool for studying these pigmented macrophages. At higher temperatures a very marked increase of these cells could be induced in the relatively short time interval of about three weeks.

It is interesting that the mode of increase in the density of melano-macrophages in trout and of melano-macrophage centres in plaice was similar in both the spleen and the kidney but at present no explanation can be offered as to why this pattern differed from one species to the other; it is possible that the different techniques adopted in counting the cells partially accounted for this.

The results obtained indicate that tissue atrophy is a major factor (and possibly the sole one) contributing to the formation of the pigments observed within the melano-macrophages. A small number of reports have documented the extensive tissue degeneration occurring in various organs during starvation in a

variety of fish species. Thus, for instance, atrophy of white muscle fibres has been reported in starved cod Gadus morhua (Greer Walker, 1971). Unfortunately, however, most previous works on the histology of starvation in the salmonids (which furnish the main body of data) have dealt with fishes which were not only starving but were also either undergoing a spawning migration or on the point of spawning; this obviously complicates any conclusions deriving from such studies (for review of the literature on this subject see MacLeod, 1978). A detailed account of the biochemical changes accompanying starvation in various fish species has been given by Love (1970). Only Roberts (1978) has referred to the possible effects of starving fish on their melano-macrophage centres. Increased deposition of lipofuscin during cachexia has been observed in various organs of several other animal species including humans (Dubin, 1955; Smith and Jones, 1961). Thus for instance Garnett et al. (1969) noted severe fragmentation of myofibrils and an increased accumulation of lipofuscin at necropsy in the myocardium of a woman who had been subjected to 30 weeks of therapeutic starvation for obesity.

It can be argued that the increase in the density of melano-macrophages is only a relative increase due to atrophy of the rest of the splenic and renal tissues during starvation. Weight determinations on a large number of starved and control trout confirmed that the spleen and kidney do shrink in weight during starvation but the magnitude by which they do so is small when compared to the manifold increase in melano-macrophage density

and can for practical purposes be neglected. In a later chapter of this thesis will be described a histological and ultra-structural study of the possible events linking tissue degenerative changes and melano-macrophage centre enlargement in plaice.

Little is known about the requirements of fish for vitamin E and other biologic antioxidants. Various workers in the past have fed fish on diets rich in cod liver oil and/or deficient in vitamin E. Unfortunately however such experiments were always conducted with other objectives in mind and rarely referred to any pigment formation. To mention a few examples, Cumings (1942) investigated the effects of vitamin E deficiency in the guppy Lebistes reticulatus but did not mention the formation of pigments in his report. Atherton (1975) added two types of fat viz. cod liver oil and lard, at various levels to the diet of rainbow trout. Once again, this author did not mention the formation of any pigments resulting from increased intake of unsaturated fatty acids. On the other hand, Cowey et al. (1976) who fed turbot diets rich in cod liver oil, corn oil and hydrogenated coconut oil observed ceroid deposition in the livers of those turbot given hydrogenated coconut oil.

There are two explanations that could possibly account for the failure of the high oily diets to exert a measurable effect on melano-macrophages. Firstly, the levels of anti-oxidants such as vitamin E could have been adequate to protect the fats from oxidation irrespective of the diet employed in this study. Thus, the well documented accumulation of lipofuscin

pigment in various tissues of vitamin E-deficient animals fed polyunsaturated fats (Wolman, 1964; Porta and Hartroft, 1969) could have been avoided in the present circumstances. Cod liver oil is particularly well known for its high content of polyunsaturated fats and its pigmentogenic characteristics (Tappel, 1972). It seems reasonable to assume, therefore, that significant changes in the melano-macrophages would have been obtained had antioxidants been entirely omitted from the diet or at least represented only in trace amounts. It has been repeatedly demonstrated that lipogenic pigments are not formed, even when diets which are rich in unsaturated lipids are fed, if the diets are supplemented with adequate amounts of α-tocopherol (Trautwein, 1962). Secondly, it is possible that the experiments with feeding oily diets were not extended over a long enough period; on a long term basis the ratio of antioxidants to polyunsaturated fatty acids could have become unsuitable. This need for long-term experimentation in investigations of this nature has been emphasized for mammals (see for example Green, 1972).

Perhaps more useful results would have been obtained had the cod liver oil been injected into the fish rather than it being administered in the diet but this, then, would not have been necessarily relevant to lipid metabolism in the intact animal. Lipogenic pigments have been produced in vivo by direct injection of cod liver oil or linseed oil into the spleen of guinea pigs, the peritoneal cavity of rats, and the subcutaneous tissue of guinea pigs and rats (Hass, 1938a, b, 1939; Endicott,

1944). According to Carr (1973), after the injection of lipids into the peritoneal cavity of rats, lipid can be demonstrated by histochemical techniques in the draining (diaphragmatic) lymph nodes; presumably this could eventually lead to pigment formation.

The slight decrease in pigment cell density in the anterior kidney of trout fed the high fatty diets could possibly have been a consequence of the slightly increased size of the organ due to lipid accumulation.

Atherton (1975) reviewed the literature dealing with the effects of supplementing the fat levels in the diet of rainbow trout. There is general agreement that the inclusion of fat promotes growth. Atherton's own experimental work led him to suggest that levels of 15% and more of oil added to the diet have an adverse effect on the growth of the fish when kept at 12°C. Earlier Ono et al. (1960) had shown that as much as 15% of the diet could be fat without the fish showing any ill effects, provided that the fat was protected from oxidation. Although the fish exhibited markedly increased growth in the course of the present experiments, they seemed to have lost their appetite in the last week or so of the experimental period and had the trials been pursued further there would have almost certainly been observed a trend towards a decrease in the rate of growth.

The failure of the inflammatory reaction elicited by the adjuvant to exert any significant changes in the melano-macrophages seems to indicate that the degree and duration of the insult was not of a large enough magnitude. In fact, the degeneration of muscle fibres and other tissue damage elicited were practically

restricted to the site of injection. It seems likely that significant results would have been obtained had a more extensive reaction been induced by multiple injections and by regularly repeating the inoculations. It would also have been interesting to investigate whether inoculating the adjuvant intravenously would have affected the melano-macrophages. Intravenous injection of the adjuvant in mammals induces specific cellular reactions containing the usual components of the chronic granuloma to develop in a variety of organs such as spleen, liver, kidney and lymph nodes (White et al., 1955; Freund, 1956; Rupp et al., 1960; Steiner et al., 1960; Spector and Willoughby, 1968). Willoughby et al. (1967) have also shown that when Freund's complete adjuvant is injected into lymph nodes there is observed an increase in the number of circulating monocytes. This led them to suggest that there may be a monocytogenic hormone produced in lymphoid tissue which stimulates monocyte production.

Finn and Nielsen (1971) carried out the first extensive experimental histopathological study of the teleost inflammatory response in rainbow trout. They studied the various exudative and cellular changes occurring after lesions were induced in a variety of ways, one of these being the intramuscular injection of Freund's complete adjuvant. They concluded that the response to adjuvant consisted of polymorphonuclear leucocytes, macrophages, lymphocytes, fibroblasts, giant cells and it was remarkably similar to that of the higher animals though the onset of cellular changes was delayed and they were probably quantitatively less severe than in mammals. These workers did not continue their

study for a sufficiently long period for serious observations of the chronic lesion and did not mention the presence of epithelioid cells or plasma cells.

Edelstein (1971) in his review of the role of melanin in defence mechanisms suggested that melanin may have a bactericidal effect as a source of free radicals or, in association with peroxidase, as a source of H_2O_2 from oxidation of NADH; a similar process occurs in mammalian polymorphonuclear leucocytes. It would be expected then that if such a system operates in melano-macrophages, the injected mycobacteria would be phagocytosed and transported to the melano-macrophage centres; subsequently this increased influx of bacteria could lead to increased accumulation of melanin-bearing macrophages. Unfortunately, the results obtained in this study leave this issue unanswered. Indeed the whole question of the possible role of melanin in disease processes is still under very intensive investigation.

CHAPTER 5

EFFECTS OF STARVATION, HIGH FATTY DIETS, HIGH DIETARY TYROSINE LEVELS, INTRODUCING A SOLUBLE ANTIGEN AND A COMBINATION OF THE LATTER TWO FACTORS ON THE MELANO-MACROPHAGES OF RAINBOW TROUT FRY

5.1 INTRODUCTION

In this chapter are described a series of experiments carried out on rainbow trout alevins. These experiments were designed with three main objectives in mind, viz:

i. to attempt to find means of inducing an earlier than normal ontogenic appearance of melano-macrophages and possibly a faster build-up than normal of these cells within haemopoietic tissues;

ii. to consolidate the observations made with adult rainbow trout in the previous chapter; and

iii. to examine other factors which might directly influence melano-macrophages.

It was never intended to subject the results to any rigorous mathematical analysis because of the qualitative nature of the work and when, for technical reasons, the choice had to be made between having a small number of treatments with replicates and a larger number of treatments with no replicates, the latter option was considered to serve better the present purposes.

It is extremely difficult to introduce a particulate antigen into fish as small as those being used in this study. A soluble antigen, bovine serum albumin (BSA), was therefore employed. The method employed to introduce this antigen into the fish was the hyperosmotic infiltration technique for soluble antigens developed by Amend and Fender (1976). This involved placing the fish in a hyperosmotic solution of sodium chloride followed by immersion in a solution of bovine serum albumin. The serum albumin thus enters the fish's body to replace the water lost while in the hyperosmotic solution.

The physical state of a protein antigen can affect its antigenicity. Thus bovine serum albumin has been observed to show wide divergences in its ability to stimulate immunity in mammals, depending to some extent on the physical state in which it is administered. Tyrosine has been found to be of value in rendering a polypeptide immunogenic. In fact, aromatic amino acids consistently enhance the immunogenicity of a polypeptide in mammals (Weir, 1973). It is possible then that tyrosine may act as an immunogen in fish. Furthermore, it is well established that excessive levels of tyrosine stimulate formation of melanin. The dependency of melanin formation upon the availability of three substrates, viz. tyrosine, tyrosinase and molecular oxygen, is well documented (Lerner, 1955; Herbut, 1959). The absence of any one of these results in decreased melanin formation and an overabundance of all three substrates results in increased melanin production. Although it has not as yet been clearly established whether tyrosine per se is the precursor of melanin

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in the animal body or if tyrosine is but a component of the precursor, it seems most likely that the tyrosine which is oxidized in the presence of tyrosinase (at least within the cytoplasm of mammalian melanocytes) is free tyrosine and not incorporated in a protein chain (Thompson, 1966). In this study, a batch of rainbow trout fry were fed a tyrosine-enriched diet and a separate batch that were similarly reared on this diet were also challenged with bovine serum albumin. The spleen and kidney were regularly monitored histologically to determine whether any of these treatments resulted in an increased melanin accumulation within the melano-macrophages of haemopoietic tissues. It was realised from the onset that this was highly unlikely to occur in view of the fact that melano-macrophages have not been found to be DOPA-positive (Ellis, 1974).

5.2 MATERIALS AND METHODS

5.2.1 Fish

Rainbow trout alevins were obtained as described in section 3.2.1. About 200 newly-hatched alevins were placed in each of 9 tanks. Each tank, which measured 0.4 m x 0.3 m x 0.3 m, was fitted with an E-heim filter. Adequate aeration was supplied and the water was changed regularly. All experiments were carried out at 12°C and the fish were subjected to a 12-hour photoperiod. Two weeks after hatching the fry started to feed voraciously even though the yolk had not been entirely consumed. Irrespective of the diet employed the fry were hand fed about six times daily. As growth proceeded, the fish were thinned down to the appropriate densities.

5.2.2 Preparation of fatty diets

Diets containing 25% and 35% oil were prepared as described in section 4.2.4. Pellets of the 25% oily diet were ground as finely as possible and the 35% oily paste was cut into very small pieces prior to feeding.

5.2.3 Preparation of tyrosine-rich diet

The composition of the commercial diet used throughout this work as the control diet has been given in section 4.2.4. A batch of this feed was prepared with 1 gm of L-tyrosine* being added to each kilogram of the mixture of raw materials before pelleting. Pellets of this 0.1% tyrosine-enriched diet were finely ground before feeding.

5.2.4 Administration of bovine serum albumin*

Bovine serum albumin was administered to the fry by hyperosmotic infiltration after the method of Amend and Fender (1976). A two-step procedure was adopted. The fry were first placed in a 5.32% sodium chloride solution for about 2 minutes, they were then immersed in a 2% solution of the albumin for 3 minutes. About 2% of the fry were lost during such treatment. At all stages of the treatment a constant temperature of 12°C was maintained.

5.2.5 Treatments

The nine tanks were set up as follows:

* Sigma Chemical Company

Tank 1 : Control fry

The fry were fed the normal diet

Tank 2 : Low fatty diet

The fry were reared on the 25% oily diet

Tank 3 : High fatty diet

The fry were fed the 35% oily diet

Tank 4 : BSA-treated fry

The fry were treated with bovine serum albumin 5 days after commencement of feeding. They were reared on the normal diet throughout

Tank 5 : Fry treated repeatedly with BSA

The fry were treated three times with bovine serum albumin, viz. 5 days, 3 weeks and 5 weeks post first feeding. These were fed on the normal diet

Tank 6 : Tyrosine-rich diet

The fry were reared on the tyrosine-rich diet

Tank 7 : BSA-treated fry fed a tyrosine-rich diet

The fry were treated with bovine serum albumin 5 days after commencement of feeding. They were reared on the diet to which 0.1% tyrosine had been added

Tank 8 : Starved fry

The fry were fed on the normal diet for two weeks post first feeding and then were completely starved

Tank 9 : Starved fry

The fry were reared on the normal diet until four weeks after first feeding and then entirely deprived of food

Very few mortalities occurred throughout these experiments.

5.2.6 Sampling and histological procedures

The experiments lasted 12 weeks i.e. 10 weeks after commencement of feeding. The experiments in which the fry were starved were terminated earlier. Each week six fry from each experimental tank were fixed in 10% neutral buffered formalin. The egg yolk was dissected away before cutting serial paraffin sections at 5 μm. All sections were stained with H & E.

5.3 RESULTS

A summary of the results is presented in Table 5.1. As observed earlier on in this work, melano-macrophages appeared about two weeks after commencement of feeding. In the control fish their numbers increased steadily and by the tenth week post first feeding somewhat large numbers could be observed. Whilst this held true for both the kidney and the spleen the density of such cells in the spleen was consistently lower than that in the kidney. None of the treatments to which the fry were subjected, apart from starvation, appeared to have induced any significant changes in the melano-macrophages as compared to the controls.

In starved fish a rapid build up of melano-macrophages occurred within the spleen and the kidney. Within three weeks

Table 5.1. Incidence of melano-macrophages in the spleen and kidney of rainbow trout fry subjected to various treatments. No melano-macrophages were observed prior to the onset of first feeding.

Abbreviations used: C, the fry used as controls; LFD and HFD, those fed on the low and high fatty diets respectively; BSA, the fry treated once with bovine serum albumin and reared on the control diet; BSA (rep), the fry treated repeatedly with bovine serum albumin and fed the control diet; Ty, the fry fed the tyrosine-rich diet; BSA + Ty, the fry treated once with bovine serum albumin and reared on the tyrosine-rich diet; ST 1, the fry starved as from the second week post first feeding; and ST 2, the fry starved as from the fourth week post first feeding.

Explanation of symbols

-	indicates that no melano-macrophages could be observed
d	indicates that the presence of melano-macrophages was doubtful
x	indicates their presence in very low numbers
xx	indicates their presence in somewhat larger numbers
xxx	indicates their presence in moderately large numbers
xxxx	indicates their presence in very large numbers

Melano-macrophages were very rarely encountered in the liver.

The starvation experiments were terminated earlier than the rest.

Weeks after first feeding	C		LFD		HFD		BSA		BSA (rep)		Ty		BSA + Ty		ST 1		ST 2	
	kid.	spl.	kid.	spl.	kid.	spl.	kid.	spl.	kid.	spl.	kid.	spl.	kid.	spl.	kid.	spl.	kid.	spl.
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	x	d	x	-	x	x	-	-	x	-	-	-	d	d	x	d	x	x
3	x	x	x	x	x	x	x	x	x	x	x	x	x	x	xx	x	x	x
4	xx	x	xx	x	x	x	x	x	xx	x	xx	x	xx	x	xxx	xx	xx	x
5	xx	x	xx	x	xx	x	xx	x	xx	x	xx	x	xx	x	xxxx	xxx	xxx	xx
6	xx	x	xx	x	xx	x	xx	x	xx	x	xxx	x	xxx	x	xxxx	xxx	xxx	xx
7	xxx	x	xxx	x	xx	x	xx	x	xxx	xx	xxx	xx	xxx	xx			xxxx	xxx
8	xxx	x	xxx	xx	xxx	xx	xxx	xx	xxx	xx	xxx	xx	xxx	xx			xxxx	xxx
9	xxx	xx	xxx	xx	xxx	xx	xxx	xx	xxx	xx	xxx	xx	xxx	xx				
10	xxx	xx	xxx	xx	xxx	xx	xxx	xx	xxx	xx	xxx	xx	xxx	xx				

of complete starvation, very high densities of melano-macrophages could be observed within the haemopoietic tissues of both batches of starved fish. Having obtained satisfactory results these experiments were terminated earlier to avoid unnecessary sufferings by the fish.

As observed with adult rainbow trout in the previous chapter, a large proportion of the melano-macrophages accumulated in the spleen of cachectic fry were yellow whereas those in the kidney were almost all black.

Melano-macrophages were almost entirely absent from the liver but in a few cachectic specimens they were to be observed, although in very low numbers.

5.4 DISCUSSION

Of the parameters tested, only starvation induced any significant changes in the melano-macrophages of first-feeding fry. Within three weeks of their being completely deprived of food both batches of fry carried very high densities of melano-macrophages in their haemopoietic tissues. The possible reasons underlying this increased accumulation of pigment cells during starvation have already been reviewed. It seems reasonable to assume that those same processes that occur in adult fish also occur in fry. Reference has already been made to the fact that melano-macrophages appear within haemopoietic tissues following shortly upon first feeding (Chapter 3). It would be interesting to investigate the appearance and subsequent build-up of melano-macrophages, if there will be any, in fry that have never been

fed. It might be possible then that the ontogenic appearance of these cells will be induced at an earlier stage of development.

As with adult fish, feeding fry on very oily diets failed to exert any measurable effects on the melano-macrophages. Nor did the feeding of a tyrosine-rich diet affect in any way the melano-macrophages.

It is generally believed that lower vertebrates show poor response to soluble protein antigens unless administered in complete Freund's adjuvant. Manning and Turner (1972) postulated that this may be due to a limited capacity of lower vertebrates to trap antigen unless the reticulo-endothelial system is stimulated by adjuvant. The results of Ellis (1974 and 1979) however failed to support this hypothesis, though it must be noted that this latter worker administered the antigen to plaice separately from the adjuvant and not in an emulsion with it. Whatever the true answer may be, the failure of any of the treatments with the bovine serum albumin to elicit any significant changes certainly emphasizes the dire need for more experimental work designed to establish the precise immunological role of melano-macrophage centres.

No bioassay for bovine serum albumin in fry was performed in this study. However, Amend and Fender (1976) subjected juvenile rainbow trout (weighing on average 4 gm) to exactly the same hyperosmotic infiltration procedure as that employed here and recorded a high concentration of about 160 µg of BSA per millilitre of fish serum within 60 minutes of applying the treatment; this concentration remained high for at least 48 hours. This uptake of

BSA into the fish appeared to take place primarily through the lateral line system and secondarily through the gills. It is true that these workers employed a higher temperature throughout viz. 20°C as compared to 12°C as used in this study but even at 12°C substantial uptake of the serum albumin would certainly have taken place, especially so in those fry that were subjected to the antigen on a regular basis.

Ellis (1974, 1979) in his studies of antigen trapping in the plaice investigated the process of the localization of bovine serum albumin within the lymphoid tissues. He concluded that antigen is held, probably as an immune complex, scattered throughout melano-macrophage centres as well as in other sites. It can be surmised that in a similar fashion, the albumin administered in the course of the present experiments, or at least some of it, must have eventually reached the melano-macrophages within the spleen and kidney of the fry. This increased uptake of antigen does not seem to have affected in any way the pigmented macrophages. It is very clear that more work needs be done to establish what the fate of the antigen is and what might be the role of the pigment, if it has any at all, in the participation of the melano-macrophage in the sequestration of the antigen and in the triggering of the immune response.

CHAPTER 6

THE ROLE OF MELANO-MACROPHAGE CENTRES IN IRON STORAGE IN NORMAL AND DISEASED FISH

6.1 INTRODUCTION

Melano-macrophage centres frequently stain positive by Perls' Prussian blue staining method (Drury and Wallington, 1967) indicating that they can contain ferric iron which they probably derive from the breakdown of haemoglobin from phagocytosed effete red blood cells. Iron is an essential element involved in the physiological functions of oxygen transport and cellular respiration. A large amount of literature has resulted from measurements made on human subjects and rats which deals with iron absorption, transport, utilization and conservation. There is, however, relatively little information concerning iron metabolism in lower vertebrates.

Recently there has been an increasing interest in the role of iron and iron binding proteins in the immune system of mammals particularly in relation to the role of iron in lymphocyte traffic through lymph nodes. This, and the suggested possibility that melano-macrophage centres could represent the primitive analogues of germinal centres of higher animals (Roberts, 1975), prompted the present study of the incidence of iron in the centres of a variety of teleost fish under normal and pathological conditions.

6.2 MATERIALS AND METHODS

6.2.1 Fish

Normal specimens

Five adult fish from each of fourteen species of teleosts of marine, freshwater, temperate and tropical origin (Table 6.1) were obtained as described in section 2.3.1 and sections were taken from the spleen, kidney and liver.

Clinical specimens

The spleen, kidney and liver of specimens of rainbow trout and turbot suffering from disease conditions in which haemolytic anaemia plays a recognized role as well as fish suffering from a condition where haemolytic anaemia is not a major factor were examined for comparison with the normal fish (Table 6.2).

Cachectic specimens

Because of the recognized role which starvation plays in modifying the structure of melano-macrophage centres, material from the haemopoietic organs of three species of fish, viz. rainbow trout, plaice and swordtails, from various experimental cachexia studies were also examined for comparison with the normal teleost. The experimental procedures employed in these cachexia studies have already been described in section 4.2.

Splenectomised specimens

Twenty-five one-year old rainbow trout had their spleen removed. Fish were anaesthetised in Benzocaine. When all reflex activity had stopped but opercular movements were still continuous

the fish were placed ventral surface uppermost in a V-shaped cradle. A ventral midline incision was made 3 cm long anterior to the pelvic muscles. The incised skin was lifted up and the spleen located. Using a pair of 4" forceps the spleen was grasped from underneath and gently removed. This involved removing some fat from the area where the spleen was resting. Any attempts to grasp the spleen itself resulted in fragmentation of this organ because of its friability. Occasionally the spleen was present as two discrete bodies and these were both removed. No bleeding was seen to occur after removal so that haemostasis was not considered necessary. The skin was closed with horizontal mattress sutures of catgut (00 medium chronic). This meant that the fish did not have to be handled at a later date to have the sutures removed.

The splenectomised fish were then put back in a tank at 12°C and completely deprived of food for 12 weeks when the experiments were terminated. Two more experimental tanks, one with 25 trout that were only starved but not splenectomised and one with 25 trout that were not operated on and fed normally thus serving as controls were set up. Other details of experimental design have been described in section 4.2. No mortalities were recorded either during or post- operation. Every three weeks three fish were killed from each of the three tanks.

6.2.2 Histopathological techniques

Portions of the spleen, kidney and liver of freshly killed fish were dissected out and fixed in 10% neutral buffered formalin

or formal saline. After routine processing, 5 µm thick paraffin sections were cut and stained by Perls' Prussian blue method for the demonstration of ferric iron (Drury and Wallington, 1967).

6.3 RESULTS

The results of the survey of the normal material are summarized in Table 6.1. In most species, the splenic centres of the majority of specimens carried iron, very often in high amounts. By comparison, the centres in the kidney and liver contained insignificant amounts (Figs. 6.1a, b). Staining by the specific method for iron in the ferrous (Fe++) form employing potassium ferricyanide instead of potassium ferrocyanide gave negative results indicating that melano-macrophage centres store iron-containing compounds only in the ferric (Fe+++) form.

The levels of iron accumulation in rainbow trout and turbot suffering from various diseases are shown in Table 6.2. Haemogregariniasis, pansteatitis and vibriosis resulted in considerable accumulation of iron in the splenic centres, at the same time little or none could be detected in either the kidney or liver (Figs. 6.1c, d, e, f). Proliferative kidney disease did not result in any increased iron deposition in any of the organs.

During starvation of trout both the number of iron-carrying cells and the iron content within the cells increased progressively within the spleen till eventually a very heavy deposition of iron was observable. In contrast, comparatively little accumulation had occurred in either the kidney or liver

Table 6.1. Response of the melano-macrophage centres found in the spleen, kidney and liver of five specimens of the various fish species examined to the Perls' Prussian blue test for the presence of free ferric iron. + signifies a strong positive reaction implying that the majority of cells within all the centres stained positively, $\overset{+}{-}$ indicates that only a small to a moderate proportion of the pigment cells stained positively, t signifies the presence of iron only in trace amounts and - indicates a negative reaction denoting complete absence of ferric iron. Pigment centres are often absent in the liver and this is indicated by an asterisk.

ORGAN	SPLEEN					KIDNEY					LIVER				
SPECIES	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Anguilla anguilla (L.)	-	t	t	-	+	-	-	-	-	-	-	t	t	*	*
Aphanopus carbo Lowe	-	t	-	-	-	-	-	-	-	-	*	*	*	-	*
Boops boops (L.)	t	+	+	+	+	-	$\overset{+}{-}$	t	t	-	-	-	-	-	-
Brosme brosme (Ascanius)	+	$\overset{+}{-}$	$\overset{+}{-}$	$\overset{+}{-}$	$\overset{+}{-}$	$\overset{+}{-}$	t	-	-	$\overset{+}{-}$	t	*	*	*	*
Coryphaenoides rupestris Gunnerus	+	+	+	+	+	-	-	-	-	-	t	*	-	-	t
Mora moro (Risso)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mugil sp.	+	-	-	-	t	t	-	-	-	-	t	-	-	-	-
Peristedion cataphractum (L.)	$\overset{+}{-}$	-	$\overset{+}{-}$	$\overset{+}{-}$	$\overset{+}{-}$	-	-	-	-	-	*	*	*	*	*
Pleuronectes platessa L.	+	$\overset{+}{-}$	t	+	$\overset{+}{-}$	-	-	-	-	-	*	*	*	-	*
Salmo gairdneri Richardson	-	t	-	$\overset{+}{-}$	t	-	-	-	-	t	-	*	*	*	*
Sarotherodon mossambicus (Peters)	+	+	+	+	+	-	-	-	-	-	*	*	*	*	*
Scophthalmus maximus (L.)	-	t	-	$\overset{+}{-}$	-	t	-	-	-	-	*	*	*	*	*
Xiphophorus helleri (Heckel)	$\overset{+}{-}$	-	$\overset{+}{-}$	t	-	-	-	-	-	-	*	*	*	*	*
Zeus faber L.	$\overset{+}{-}$	-	+	$\overset{+}{-}$	-	-	-	-	-	-	-	-	$\overset{+}{-}$	*	*

Table 6.2. Response of the melano-macrophage centres found in the spleen, kidney and liver of rainbow trout Salmo gairdneri and turbot Scophthalmus maximus suffering from various diseases. Explanation of symbols same as for Table 6.1.

Disease Condition	SPLEEN					KIDNEY					LIVER				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Pansteatitis (Rainbow trout)	+	t	t	+/-	+/-	-	-	-	-	-	*	-	-	-	*
Proliferative Kidney Disease (Rainbow trout)	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*
Vibriosis (Rainbow trout in salt water)	t	t	+	+/-	+/-	-	-	t	-	t	-	*	-	*	*
Haemogregariniasis (Turbot)	+	t	t	+/-	-	-	-	-	-	-	*	*	-	*	*

Table 6.2. Response of the melano-macrophage centres found in the spleen, kidney and liver of rainbow trout Salmo gairdneri and turbot Scophthalmus maximus suffering from various diseases. Explanation of symbols same as for Table 6.1.

Disease Condition	SPLEEN					KIDNEY					LIVER				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Pansteatitis (Rainbow trout)	+	t	t	±	±	-	-	-	-	-	*	-	-	-	*
Proliferative Kidney Disease (Rainbow trout)	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*
Vibriosis (Rainbow trout in salt water)	t	t	+	±	±	-	-	t	-	t	-	*	-	*	*
Haemogregariniasis (Turbot)	+	t	t	±	-	-	-	-	-	-	*	*	-	*	*

even after this prolonged period of stress which resulted in substantial mortalities.

In plaice, both the size and number of iron-carrying centres as well as their iron content increased progressively within the spleen and eventually a heavy deposition of iron was evident in all centres. As in the case of trout, very little iron deposition was observable in either the kidney or the liver centres (Figs. 6.2 and 6.3). As no mortalities were recorded the experiments were continued for a longer period of time during which the accumulation of iron in the splenic centres reached very high levels while considerable levels were also observed in the liver centres (Fig. 6.4). The kidney centres still carried comparatively low levels of iron-containing compounds.

The experiments with swordtails were of shorter duration (see section 4.3). During the three-week period of starvation massive accumulation of ferric iron had occurred but once again this was restricted almost exclusively to the splenic centres.

The results of the splenectomy experiment are summarized in Table 6.3. While in the unoperated fish accumulation of ferric iron resulting from starvation was observed to take place almost exclusively in the spleen, in fish that had been splenectomised prior to starvation, such a process was diverted to the kidney (Fig. 6.5). An important observation, already pointed out in Chapter 4, is that during starvation of trout a large proportion of the melano-macrophages accumulating in the spleen were yellow in contrast to those of the kidney which were

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Table 6.3. Response of the melano-macrophages in the haemopoietic organs of rainbow trout Salmo gairdneri from an experiment in which some were splenectomised prior to being starved. Explanation of symbols same as for Table 6.1. Three specimens were examined in each case and the results are pooled together.

Weeks	Controls			Starved			Splenectomised and starved	
	kid.	spl.	liv.	kid.	spl.	liv.	kid.	liv.
0	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-
6	-	-	-	-	t	-	+ -	-
9	-	-	-	t	+	-	+ -	-
12	-	-	-	t	+	-	+	-

nearly all dark brown or black. The former cells were also observed to carry substantial amounts of ferric iron. When the spleen was removed, these cells accumulated in the kidney. The liver in all trout, splenectomised and non-splenectomised, remained almost entirely devoid of any iron-carrying cells throughout the experiments (Fig. 6.6).

6.4 DISCUSSION

Any evaluation of the foregoing results must be made bearing in mind that the histochemical method employed measures almost exclusively the iron bound as haemosiderin (this has already been reviewed in section 1.4). However, since reticulo-endothelial cells seem to store excess iron mainly in this form, ferritin, the other form in which iron is normally stored in the body, is likely to occur in much smaller quantities within melano-macrophage centres.

In mammals the reticulo-endothelial system is intimately involved in the metabolism of iron; reserves of this metal are normally present in the reticulo-endothelial cells of the liver, the spleen, the bone marrow and also in the parenchymal cells of the liver (Stuart, 1970). The melano-macrophage centres can be considered as an integral part of the reticulo-endothelial system of teleosts, acting as repository centres for effete materials which cannot be metabolised further or that are required for recycling (Roberts, 1975). The present study has shown that storage of haemosiderin takes place almost exclusively in the splenic centres implying that although the centres in the

Fig. 6.1. Light micrographs (x 125) of sections stained by Perls' Prussian blue method for iron-containing compounds and counterstained with neutral red.

(a) Splenic melano-macrophage centres of clinically normal Sarotherodon mossambicus showing large quantities of haemosiderin

(b) Kidney centres (arrowed) from same specimen as (a) showing the complete absence of Perls' positive material

(c) Spleen from rainbow trout suffering from pansteatitis, with greatly increased deposition of iron-containing compounds (arrowed)

(d) Pigmented macrophages from the kidney of the same trout as (c) showing complete absence of iron

(e) Turbot spleen filled with considerable amounts of iron-containing compounds; this specimen suffered from haemogregariniasis

(f) Melano-macrophage centres (arrowed) from anterior kidney of same turbot specimen as (e) entirely devoid of Perls' positive material

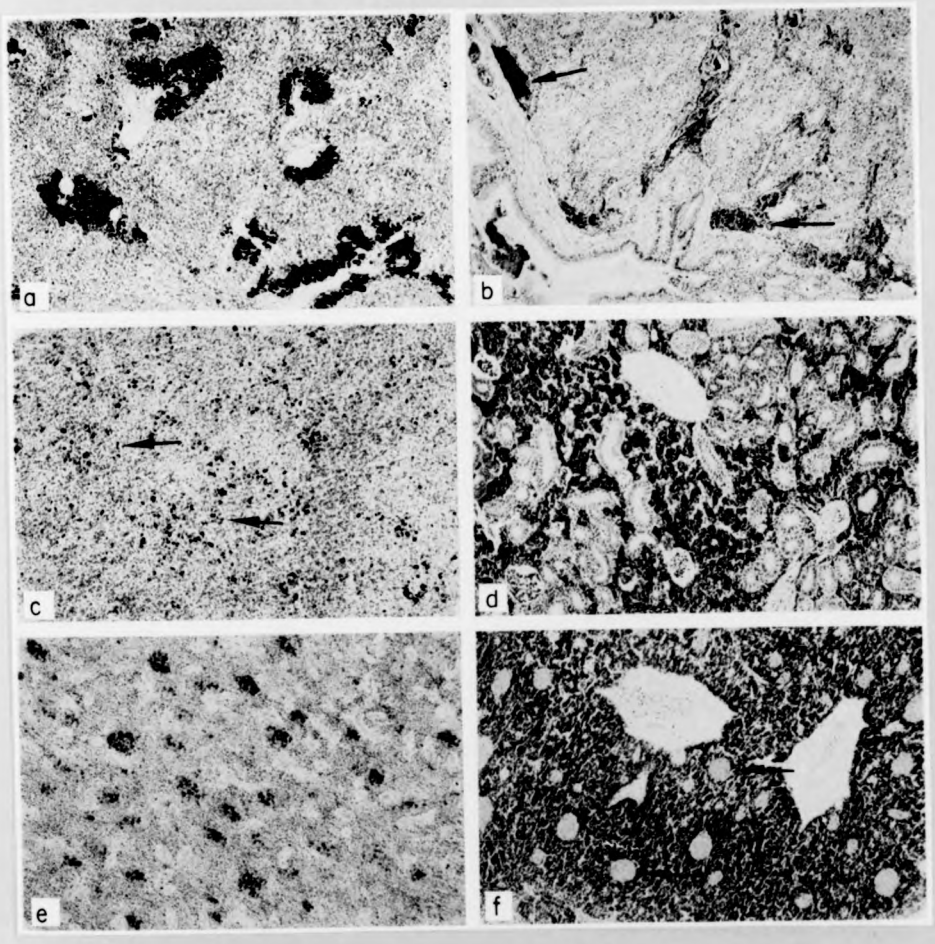

a
b
c
d
e
f

Fig. 6.2. Splenic centres with massive deposition of ferric iron-containing compounds from plaice subjected to prolonged starvation.

Fig. 6.3. Melano-macrophage centres within the kidney of the same plaice specimen containing comparatively very little or no iron.

Perls' Prussian blue method and counterstained with neutral red.

(x 125)

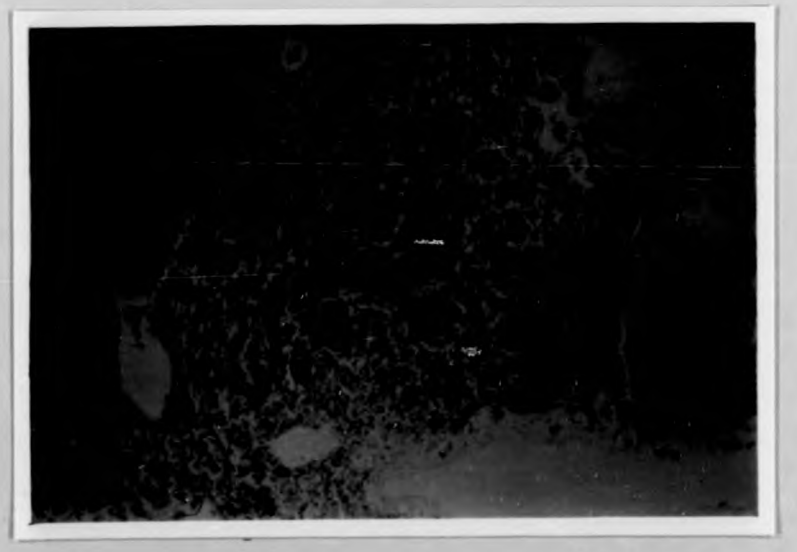


Fig. 6.4. Liver of plaice that was subjected to sixteen weeks of starvation. After this prolonged starvation considerable levels of iron-containing compounds were observed in the liver centres.

Perls' Prussian blue method and counterstained with neutral red.

(x 125)

Fig. 6.5. Perls' positive cells within the anterior kidney of a rainbow trout that had been splenectomised and then starved for ten weeks.

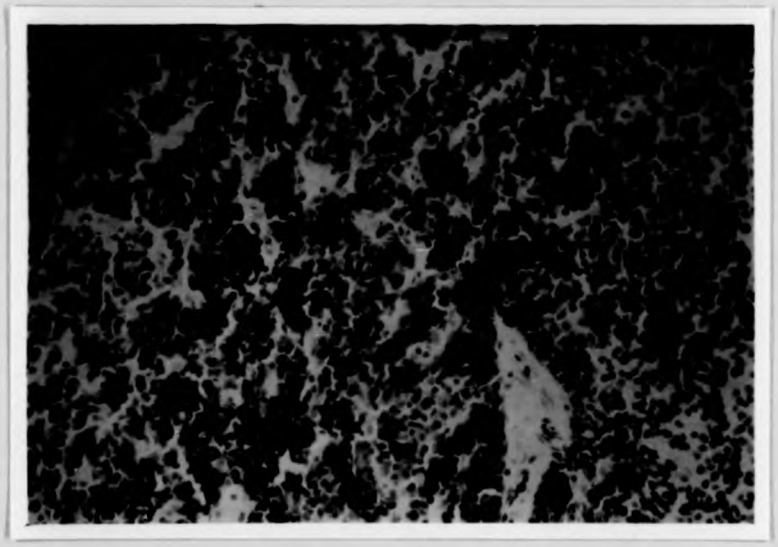
Perls' Prussian blue method and counterstained with neutral red

(x 320)

Fig. 6.6. Liver from splenectomised cachectic trout showing complete absence of haemosiderin.

Perls' Prussian blue method and counterstained with neutral red.

(x 125)



three organs resemble each other morphologically and in their relation with the associated tissues, there could well be important functional differences.

Tokumaru and Ferri (1970) applied Perls' staining method for the presence of iron in the centres of the liver, spleen and kidney in Pimelodus maculatus Lacépède, Prochilodus scrofa L. and Cyprinus carpio (L.). Although they failed to point it out, their results clearly show that in all three species the iron content in the splenic melano-macrophages is considerably higher than that of the kidney counterparts; in all three species the liver pigment cells of most specimens also showed a positive reaction. From their mode of presentation of the results, however, it is difficult to deduce whether the differences they obtained were of the same order of magnitude as those observed in the present study. Yu et al. (1971) demonstrated the presence of haemosiderin bodies in the spleen, liver and kidney of the blue gourami Trichogaster trichopterus with the spleen being by far the predominant site of accumulation. In another report, Oguri (1976) came to the conclusion that there was no iron present in the kidney melano-macrophages of the fifteen rainbow trout specimens he studied. The results of previous workers thus corroborate to a large extent the observations made in the present study.

Walker and Fromm (1976), employing chemical analytical methods, determined the total iron content of various tissues of a batch of 100g hatchery-reared rainbow trout. They concluded that the spleen had the highest iron concentration but the liver,

due to its mass, represented the prime storage area for iron in trout. The head kidney, pyloric caeca and intestine contained very low levels of iron. In iron-deficient trout, the liver was found to contain the most important labile iron storage pool which can be utilised for the accelerated erythropoietic process. This iron was observed to be stored mainly as easily mobilized ferritin in the parenchymal cells and no haemosiderin could be detected histologically using Perls' reaction. This points to the need to investigate further the importance of the centres as sites for iron storage in relation to the total iron stored in the body. Ideally such an analysis should give due cognizance to the different forms in which iron could be bound, viz. ferritin and haemosiderin.

Hevesy et al. (1964) analysed chemically for the distribution of iron in the various organs of the tench Tinca vulgaris as part of their study on iron metabolism and erythrocyte formation in fish. They found that the iron content per gram weight was by far highest in the spleen, while the iron content per gram liver and kidney was about the same (about one-sixth that in the spleen). The total iron content of the blood, liver and spleen were the highest and between them comprised about 65% of the total iron pool of the body. Unfortunately, however, their results again do not yield any insight relating to the form in which the iron was bound.

There seems to be general agreement that iron deficiency due to bleeding significantly reduces liver-iron content. However, attempts to determine the responsiveness of splenic

iron stores to such acute induced iron deficiency have so far yielded contradictory results. Thus for instance while Yu et al. (1971) reported a decrease in splenic haemosiderin bodies in blue gourami within 24 - 48 hr. following bleeding indicating the mobilization of splenic iron for erythropoiesis, Walker and Fromm (1976) did not observe any decrease in splenic total iron content as a result of bleeding rainbow trout. While the possibility that this could be due to species differences should not be excluded, there is an obvious need to extend these studies before any general conclusions can be drawn.

The very limited amount of iron traceable in the kidney centres tempts one to speculate that the kidney plays a very limited role, if any, in iron storage and perhaps also in its metabolism. This is surprising when it is recalled that the teleost kidney is known to be very active haemopoietically. Thus, in trout, for example, it is well established that the head kidney is the major site of erythropoiesis (Catton, 1951; Topf, 1953; Klontz et al., 1969). In plaice too, erythropoiesis is largely confined to the kidney (Ellis, 1974). It could well be that the destruction of effete red blood cells is restricted to the splenic centres where the excess iron is stored. That the teleost spleen is haemoclastic rather than haemopoietic has been suggested by several workers (Topf, 1953; Zwillenberg, 1964; Haider, 1966). More recently, Al-Adhami and Kunz (1976) also observed that in the angelfish the spleen seems to be the site where blood cells are destroyed and the only haemopoietic organs are the pronephros and, to a lesser extent, the mesonephores.

It is possible then that iron will be mobilized in the required amounts from the spleen to the areas where it is needed for erythropoiesis or other purposes. Alternatively, newly-produced blood cells could well have to circulate through the spleen to receive the full complement of iron. In this context it is very significant that de Sousa and Bognaki (unpublished data) recently observed that in mice there is a significant increase in the number of labelled lymph node cells from a donor migrating through the spleen of a syngeneic recipient that had been repeatedly injected subcutaneously with ferric citrate. Thus the iron seems to be acting in some way as a stimulus for lymph node cells to migrate through the spleen of the recipient. Whether a similar mechanism operates in fish remains to be ascertained. In many specimens, irrespective of the organ, a number of Perls'-positive cells were observed outside the centres; these very often were in close association with the vascular channels. Whether these iron-laden cells were on their way towards the centres or whether they were moving out into the circulation is unknown.

Increased storage of iron is known to occur if the degradation of the subject's own erythrocytes is accelerated (Thompson, 1966) such as occurs during starvation. Kawatsu (1966) described some of the changes occurring in the haematological parameters of rainbow trout during starvation. He noted that the erythrocyte count and haemoglobin levels increased in the early stages of starvation but decreased thereafter. The anaemia resulting from starvation was characterized by the

erythrocytes becoming smaller and the disappearance of immature erythrocytes reflecting a decrease of haemopoietic activity. The severe haemolytic anaemia that can be caused by vibriosis and haematoprotozoan parasites is well documented (Richards and Roberts, 1978; Kirmse, 1978). Roberts et al. (1979) report that rainbow trout suffering from pansteatitis had reduced packed cell volume (PCV) levels and a buffy coat (white cell) layer that was 25 - 50% greater than normal. During starvation, the number of melano-macrophages increases enormously in both the spleen and the kidney. No concomitant increase in the number of such cells occurs in the liver although in plaice liver an appreciable number of centres were observable after prolonged starvation (section 4.3). The fact that under these conditions as well as in disease the marked increase in iron deposition was restricted almost exclusively to the splenic centres confirms that there are well-defined pathways of iron storage and metabolism that could well be of survival value to fish.

It would be interesting to establish whether under any of the stressful conditions studied, the fish loses any iron from its body. Walker and Fromm (1976) in their study on the metabolism of iron by rainbow trout concluded that there was essentially no detectable loss of ^{59}Fe (which they administered intraperitoneally) in the urine or faeces of either normal control or iron-deficient fish (the latter they obtained by bleeding prior to intraperitoneal injection of ^{59}Fe). They concluded that the iron cycle in fish is a closed recycling system with very little iron input or excretion and in this respect is similar to the iron cycle in humans.

The absence of iron from any of the melano-macrophages of some clinically normal specimens e.g. Mora moro and of the control trout used in the splenectomy experiments as well as of trout suffering from proliferative kidney disease needs further consideration. While in the former two cases this could well be a sign of good health, the latter case could perhaps partly be due to the fact that the anaemia associated with this disease is of a hypoplastic rather than a haemolytic nature (Ferguson and Needham, 1978).

The fact that in splenectomised trout iron-laden melano-macrophages find their way into the kidney suggests that this latter organ can become more involved in the recycling of iron even if the conditions under which it was observed to do so were very abnormal. It would be interesting to resolve why in splenectomised trout iron-carrying melano-macrophages accumulate principally in the kidney whereas in plaice subjected to prolonged starvation it seems as if it is the liver rather than the kidney that acts as a second store of haemosiderin once the spleen has become replete. Perhaps one approach that could throw some light on this problem would be to administer iron in various concentrations into different fish species. In mammals, administration of iron-containing solutions, either therapeutically or experimentally by intravenous, intraperitoneal, or subcutaneous routes may result in the deposition of haemosiderin in body tissues in the absence of haemoglobin degradation (Golberg et al., 1960). Initially the deposits are limited to cells of the reticulo-endothelial system (Best and Taylor, 1961) but if high

quantities are administered, the animal can become "overloaded" and haemosiderin will be deposited within parenchymatous cells (Golberg et al., 1960). A few reports describing experiments of this nature in fish have been published so far (see for example Yu et al., 1971, and Walker and Fromm, 1976) but results have been inconsistent.

CHAPTER 7

A HISTOLOGICAL AND ULTRASTRUCTURAL INVESTIGATION OF THE POSSIBLE MODES OF ORIGIN OF THE PIGMENT GRANULES OF THE MELANO-MACROPHAGE CENTRES IN NORMAL AND CACHECTIC PLAICE

7.1 INTRODUCTION

A large body of work has been published on electron microscopical studies of pigment cells in mammals (see for example Montagna and Hu, 1967). In fish, a fair amount of attention has been given to the pigmentary system but very little data is available on the ultrastructural features of other pigment-carrying cells.

According to Roberts (1975), melano-macrophage centre cells have indented nuclei and large numbers of membrane-bounded vacuoles containing a variety of materials. The pigment granules appear to be contained in groups in such vacuoles, suggesting that they may be phagocytosed, but the question of whether melano-macrophages are melanogenic or merely phagocytose melanin has still to be resolved. Ferguson (1976b) provided evidence that the splenic melano-macrophage centres of coccidia-infected turbot have a definite capsule separating them from the surrounding lymphoid elements. He described the centres as being composed of cells in varying degrees of degeneration, replete with dense osmiophilic debris.

The marked increase in melano-macrophage centre cells in

cachectic specimens has already been remarked upon. In an attempt to gain some insight into the possible mechanisms leading to this build-up of pigmented macrophages during cachexia, a light and electron microscopic study of the centres in the spleen and anterior kidney of clinically normal and cachectic plaice was coupled with a similar study of the skin, peritoneal lining (with special reference to the pigment cells) and skeletal muscle. A method for bleaching the melanin pigments in ultra-thin sections was also developed.

7.2 MATERIALS AND METHODS

7.2.1 Experimental design

The experimental design employed in the cachexia studies has been described in Chapter 4.

7.2.2 Preparation of tissues for light and electron microscopy

Tissues were taken from clinically normal plaice and from plaice which had been completely starved for sixteen weeks at a temperature of 12°C. Portions of the following tissues were examined:

spleen

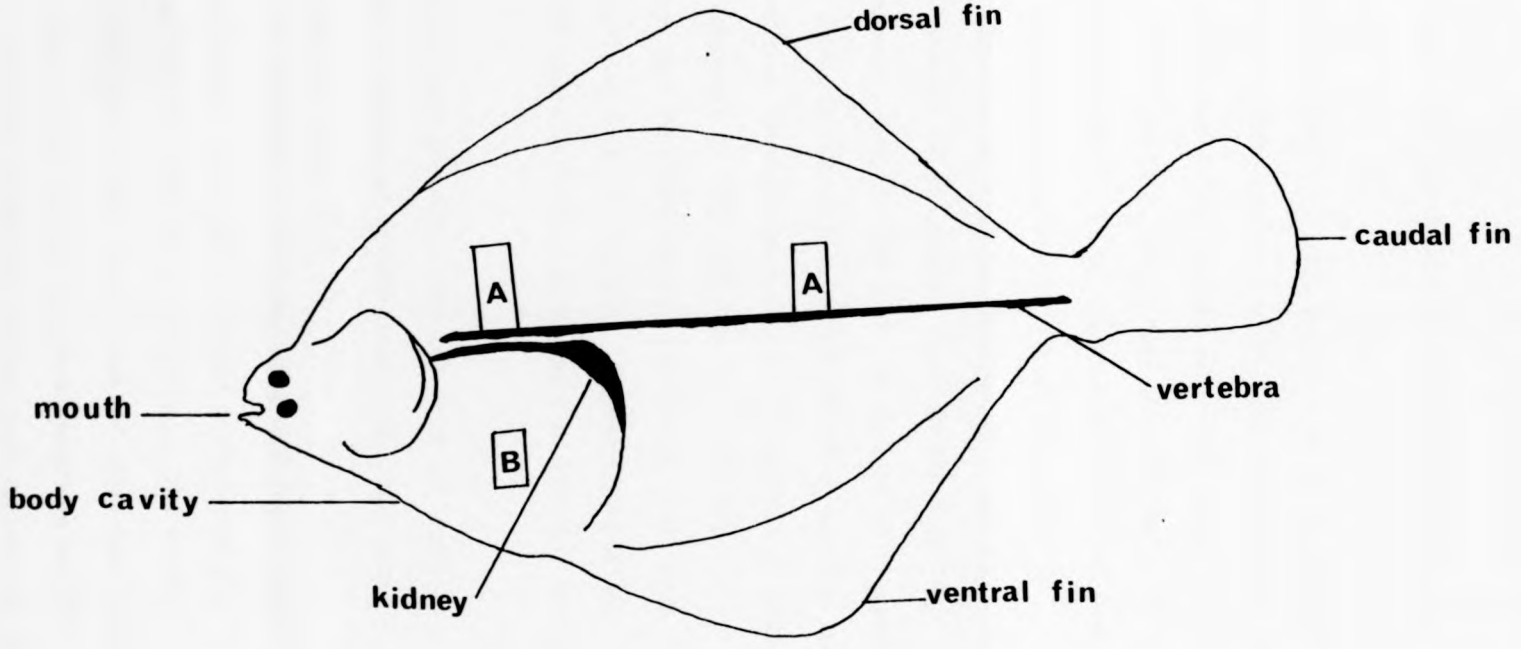
anterior kidney

skin and skeletal muscle from the areas of the oral side marked A in Fig. 7.1

body cavity wall from the area of the oral side marked B in Fig. 7.1

0.5 cm^3 blocks were prepared for light microscopy as described in Chapter 2 and stained by H & E.

Fig. 7.1. Diagram of a plaice. Skin and skeletal muscle from regions A as well as body cavity wall from region B (to include peritoneum) were examined by light and electron microscopy.


dorsal fin
caudal fin
A
A
vertebra
mouth
B
body cavity
kidney
ventral fin

1 mm^3 blocks were prepared for electron microscopy as follows:

Tissues were fixed in a 2.5% solution of glutaraldehyde in 0.2 M sodium cacodylate buffer of pH 7.2 for a minimum of two hours at room temperature. The tissues were then rinsed in 4 - 6 changes of the buffer solution, drip-dried on blotting paper and fixed for another hour at 4°C in 1% osmium tetroxide. Following osmication the tissues were dehydrated in graded series of alcohols, viz. first in 50% with two changes of 15 minutes, next in 70% with two changes of 15 minutes, then in 80% with two changes of 15 minutes, then in 90% with two changes of 15 minutes and finally in 100% alcohol with four changes of 15 minutes each. The tissues were then immersed in a mixture of propylene oxide and resin (see Appendix I) 75% - 25% for one hour, then in a 50% - 50% mixture for another hour and finally in 100% resin overnight. Finally tissues were placed in plastic capsules, covered with 100% resin and cured in an oven at 60°C for twenty-four hours.

2 µm thick sections were first cut from the resin embedded blocks of tissues on an LKB pyramitome 11800 using glass knives prepared on an LKB knife maker. These thick sections were stained with 1% toluidine blue and viewed under a binocular microscope. This enabled rapid location of the desired area for further ultra-thin sectioning. The blocks were trimmed accordingly with a razor blade and ultra-thin sections were then cut using either diamond or glass knives on an LKB ultrotome III. Sections, preferably in the gold region i.e. 90 nm (based on the continuous interference colour index) were collected

from water onto formvar-coated diamond or square mesh grids.

The sections were stained by floating the grids on drops of saturated uranyl acetate solution for 20 minutes. After washing in distilled water, they were floated for 20 minutes on drops of lead citrate. They were finally rinsed in 0.02 M sodium hydroxide solution and distilled water, and after drying, they were ready for viewing. All material was viewed in a Jeol JEM-100C electron microscope.

7.2.3 A procedure for bleaching pigments in ultra-thin sections

After a number of preliminary trials a two-step procedure was adopted. Prior to staining, the grids were first floated on drops of a 1% potassium permanganate solution. After thorough washing with distilled water, the grids were then floated on drops of a 3% potassium metabisulphite solution. They were then washed again with distilled water. For comparison, the grids were subjected to four different time regimes. The melano-macrophages of the spleen and kidney as well as the pigment cells of the skin and peritoneal lining from both normal and cachectic plaice were subjected to this treatment. The time regimes employed were as follows (all times are in minutes):

Potassium permanganate	Potassium metabisulphite
2	10
2	20
5	15
10	30

After treatment with the bleaching agents the sections were double stained with uranyl acetate and lead citrate.

7.3 RESULTS

7.3.1 Light microscopy

In the cachectic specimens, degenerative changes were readily evident in all tissues examined. In the spleen and anterior kidney degeneration of haemopoietic cells and a very marked enlargement of the melano-macrophage centres (see Chapter 4) were observed. The splenic ellipsoids became very prominent (Figs. 7.2 and 7.13). In the skin, the thickness of the epidermis was markedly reduced (Fig. 7.3). In both skin and peritoneum, ruptured pigment cells were occasionally observed with the release of their pigment granules. Degenerative changes in the muscle were very extensive leading to prominent vacuole formation in the muscle bundles (Figs. 7.4 and 7.5). Such changes originated in the centre of the bundles and spread peripherally; muscle bundles gradually attained a granular appearance and eventually seemed to be completely resorbed thus forming spaces between fascicules. There was observed a general loss of staining affinity resulting in marked differences in eosinophilicity of muscle bundles. Some pyknotic nuclei and a few areas of marked erythrocytic and leucocytic infiltration were also seen.

7.3.2 Electron microscopy

Melano-macrophage centres are generally closely applied to vascular channels. In the spleen, the arteries branch into the pulp as thick-walled arterioles called ellipsoids. The ellipsoidal lumens are very narrow and the blood cells pass in single file. Sometimes, a lymphocytic cuff surrounds the entire

arterial system and the associated melano-macrophage centres. Within the centres themselves, leucocytes and pyroninophilic cells are dispersed among the pigmented cells. Pigmented and non-pigmented macrophages are sometimes seen closely apposed to lymphocytic cells with cytoplasmic extensions that greatly increase the area of contact between the two types of cells. Some of these features of melano-macrophage centres are illustrated in Figs. 7.6 to 7.12.

In cachectic specimens, the splenic ellipsoids became somewhat denuded of cells and the outer limiting membrane was very prominent (Fig. 7.13). A considerable degree of degeneration of haemopoietic cells during cachexia resulted in large intercellular spaces becoming evident in the pulp areas of both spleen and kidney. The number of macrophages increased enormously, more so within the melano-macrophage centres; most macrophages had numerous and very extensive dendritic processes (Figs. 7.14 and 7.15). Moreover, in the spleen, a type of cell with a small cell body but very long dendritic processes was frequently observed. The dendrites from the different cells, including melano-macrophages, ramified and came into close apposition with each other thus forming a kind of network in which blood cells were to be found (Fig. 7.16). Moreover, lymphocytes were often observed to possess dendritic processes in close contact with those from cells of the monocyte series.

In cachectic specimens, the macrophages were very heavily laden with lysosomes or lysosome-like bodies which varied enormously in size (Figs. 7.14 and 7.15). In many instances,

especially so within the melano-macrophage centres themselves, the macrophages comprised little more than a nucleus and masses of such lysosomes. These single-membrane bounded inclusions had, in general, an amorphous or granular internal structure. Fibrillar processes were at times present. The term lysosome, used hereafter instead of the term lysosome-like body, is used on purely morphological grounds, although it is accepted that identification at the ultrastructural level of acid phosphatase within them, which was not carried out, might be considered a significant further proof of their identity.

Although pigment granules were observed in very small numbers within macrophages throughout the spleen and kidney, the macrophages containing significant amounts of pigments were largely confined to the melano-macrophage centres. Under the electron microscope, the vast majority of the pigment granules of melano-macrophages resembled, to a large extent, the melanin-bearing granules of the skin and peritoneal melanophores. They were of the same size order, but the shapes of the melano-macrophage granules were rather more variable; most of them could however still be defined as being spherical, oval or rod-shaped. The pigment granules of the melano-macrophages were also less frequently recognizable as being surrounded by a limiting membrane. Most of the granules observed within the plaice melano-macrophages were uniformly very electron dense. Sometimes granules with an electron dense shell bounding a less electron dense core were also observed (Fig. 7.17). Less frequently, very electron dense bodies with electron transparent spaces within them were to be observed (Figs.

7.18 to 7.20). All three types of pigment granules were also to be seen in the pigment cells of the skin and peritoneum. Granules with less regular outlines and irregularly shaped patches of different electron densities were sometimes observed in melano-macrophages (Figs. 7.48 and 7.49).

The disposition of the melanin granules within melano-macrophages fell into two main categories. A considerable proportion of the granules were grouped in rather large numbers in large vacuole-type clear bodies henceforth referred to as large vacuoles. These structures were clearly membrane delimited and apparently carried no other inclusions apart from the pigment granules (Fig. 7.21). A fairly large proportion of the melanin granules were observed to be localized within lysosomes (Fig. 7.22). Generally a lysosome carried a small number of granules although on occasions large lysosomes containing much greater numbers of pigment granules were seen. Very often a melano-macrophage contained melanin granules both in the large vacuole-type bodies and in lysosomes (Figs. 7.23 to 7.25). This mode of distribution of the melanin granules contrasts with that of the melanin granules in the integumental and peritoneal melanophores in which the granules are randomly distributed throughout the cell cytoplasm (Figs. 7.26 to 7.28). Figs. 7.29 to 7.38 are high power electron micrographs of pigment granule-bearing lysosomes from splenic and kidney melano-macrophage centre cells.

Lysosomes with a fine granular appearance were frequently seen within splenic melano-macrophages from cachectic specimens (Figs. 7.35 and 7.39). The fine granular material most probably

represented iron-containing compounds such as haemosiderin. At times this could also be observed in various other regions of the cytoplasm not in association with any specific intracellular structure.

Close examination of melano-macrophages from both spleen and kidney sections revealed structures which may be various degeneration stages of mitochondria, suggesting that a possible mode of origin, even if only for a proportion of the pigment granules, is by a degenerative transformation of effete mitochondria to lipofuscin granules (Figs. 7.40 to 7.49). Without exception, such degenerating bodies were observed to lie free in the cytoplasm and never enclosed in lysosomes or the large vacuole-type bodies. Some of them still contained what appeared to be remnants of disorientated mitochondrial cristae.

Very occasionally myelin-like whorls were seen among the cellular debris of melano-macrophage centres (Figs. 7.50 and 7.51).

Limited evidence of macrophages actively phagocytosing cellular debris was obtained within the kidney of cachectic specimens. Figs. 7.52 and 7.53 show a macrophage, apparently as yet containing no pigment granules, about to phagocytose some cellular debris possibly including a mitochondrion-like body. This activity is taking place well outside a melano-macrophage centre and the macrophage which already carries a considerable number of large lysosomes will presumably migrate to a melano-macrophage centre once it is replete with indigestible residues.

The slightly increased rupturing of the skin and peritoneal pigment cells of cachectic specimens seen under light microscopy was confirmed by electron microscopy. Their contents were observed to be released both into the underlying muscles and into the abdominal cavity (Figs. 7.54 to 7.56). Melanin is known to be potentially toxic to living cells so that upon release the melanized organelles could well be phagocytosed and eventually transported to the melano-macrophage centres.

The degenerative changes that took place in the muscle during cachexia left little recognizable structure at the level of the electron microscope. The fibrils were severed in several places and a great deal of resorption had taken place. The resulting vacuoles contained nothing more than very small amounts of cellular debris (Figs. 7.57 to 7.59). Occasionally a macrophage laden with lysosomes and cellular debris could be observed among the almost totally degenerated myotomes. In one or two cases some lysosomes contained pigment granules. Moreover, among the phagocytosed cellular debris, large numbers of degenerating bodies, morphologically suggesting a mitochondrial origin, were observed (Figs. 7.60 to 7.62).

7.3.3 Bleaching

Treatment of the sections with permanganate for five minutes followed by fifteen minutes in metabisulphite resulted in complete bleaching of almost all the pigment granules i.e. those in melano-macrophages as well as those in skin and peritoneal pigment cells. The electron denseness was removed and the granules were transformed into electron transparent granules

apparently lacking any characteristic internal structure. With the pigment granules located within lysosomal bodies this resulted in the complete bleaching of the pigment-containing entities but leaving the rest of the lysosomes unmodified. This is amply demonstrated in Figs. 7.63 to 7.70.

The full results of the bleaching procedures adopted are shown in Table 7.1. Treatment of the sections from normal plaice with permanganate for 2 minutes and subsequently with metabisulphite for 20 minutes resulted in only very slight or no bleaching irrespective of the melanized tissue being examined. Subjecting the sections from cachectic specimens to identical treatment, however, resulted in a considerable proportion of the pigment granules within the spleen and kidney melano-macrophages being almost totally bleached. All other pigment granules including those of the pigment cells of the skin and peritoneum were only very slightly bleached or not bleached at all (Figs. 7.71 to 7.75).

In ultra-thin sections, melanin granules sometimes appear to comprise an electron dense shell enclosing a less electron dense core (vide infra). The bleaching technique developed in the course of this study has shown that this electron dense outer rim is more resistant to bleaching than the inner core (Fig. 7.76).

Treatment of the sections with permanganate for a minimum of 5 minutes followed by a minimum of 15 minutes in metabisulphite resulted in complete bleaching of the pigments in all tissues examined; the electron dense pigment granules were transformed into transparent bodies lacking any obvious

Table 7.1. Results of the treatment of ultra-thin sections from normal and cachectic plaice with the bleaching reagents potassium permanganate and potassium metabisulphite

Treatment (in minutes)	Spleen and kidney melano-macrophages		Pigment cells of peritoneal lining and skin	
	Control Specimens	Cachectic Specimens	Control Specimens	Cachectic Specimens
2/10	very slightly bleached	very slightly bleached	very slightly bleached	very slightly bleached
2/20	very slightly bleached	moderately bleached	very slightly bleached	very slightly bleached
5/15	totally bleached	totally bleached	totally bleached	totally bleached
10/30	totally bleached	totally bleached	totally bleached	totally bleached

internal structures. In many cases, however, trace amounts of the electron dense material were still evident on the membrane delimiting the granule further confirming its resistance to digestion by bleaching agents (Figs. 7.77 to 7.81).

As a technical note it is worth pointing out that the longer the sections are left in permanganate the longer they need to be washed with distilled water in order to eliminate deposits of electron dense droplets.

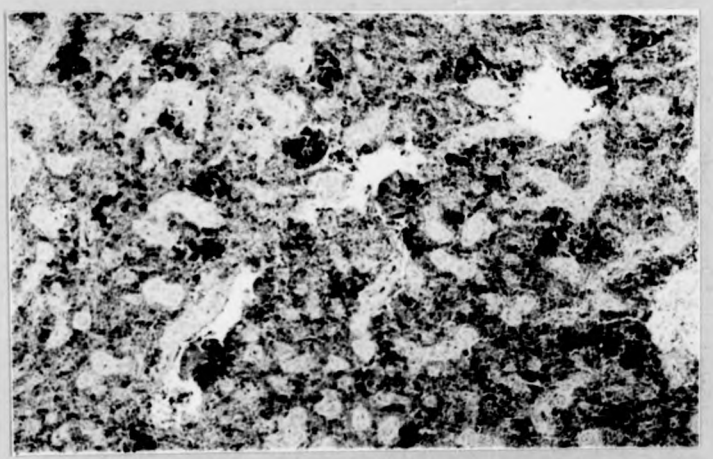
7.4 DISCUSSION

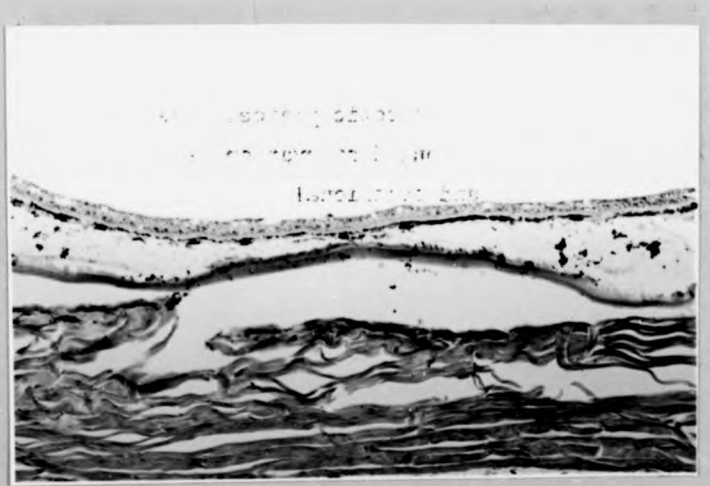
Any conclusions drawn from the foregoing results must be evaluated with due cognizance of the potential hazard involved in interpreting electron micrographs without supporting data from other modes of investigation.

The heterogeneity of pigments within the melano-macrophage centres poses the first serious problem to any attempts at elucidating the origin of the pigments within these cells. Lipofuscin appears to be by far the most abundant pigment when viewed across the various fish lineages. Melanin is the other major component but haemosiderin can also be abundant under certain conditions (vide infra). A number of theories regarding the genesis of lipofuscin granules have been advanced (see for example the review by Toth, 1968). The mode of origin of melanin has been well documented (see for example Montagna and Hu, 1967). The present study suggests that lipofuscin is a metabolic product inherently related to degeneration of cellular components such as mitochondria through the process of lipid

Fig. 7.2. Light micrograph of a spleen from a cachectic plaice. Melano-macrophage centres and ellipsoids are very prominent.
(H & E x 125)

Fig. 7.3. Skin of a cachectic plaice. The most marked changes that resulted from prolonged starvation were a reduction in epidermal thickness and occasional rupturing of melanophores.
(H & E x 125)

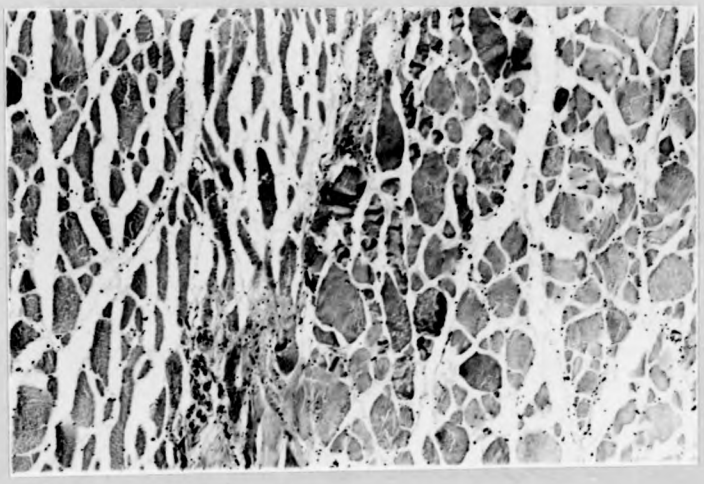




Light micrographs showing the very extensive degenerative changes brought about in plaice skeletal muscle through prolonged fasting. For full description see text.

Fig. 7.4. H & E x 125

Fig. 7.5. H & E x 320



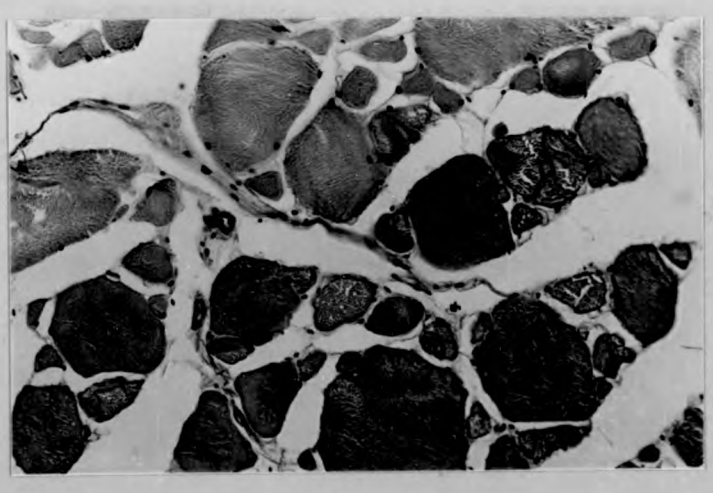
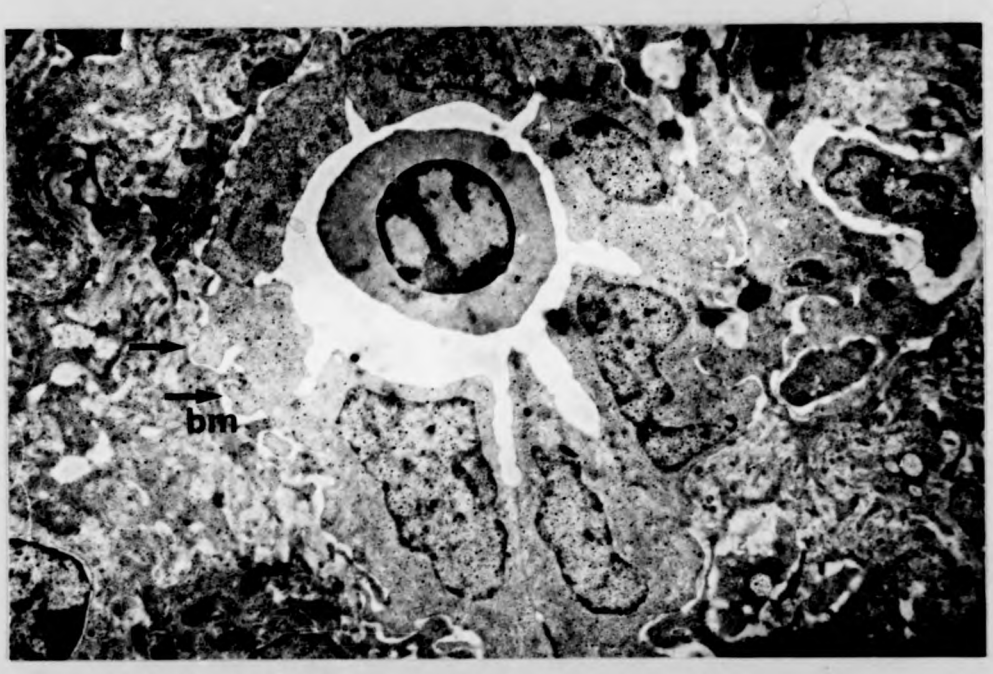


Fig. 7.6. Spleen from a normal plaice showing an ellipsoidal axial vessel. The lumen of ellipsoids is very narrow causing the blood cells to pass in single file; in this micrograph an erythrocyte is going through. Note the pseudopodial projections at the base of the endothelial cells (arrows) into the basement membrane (bm).
(x 7,000)

Fig. 7.7. Electron micrograph of a melano-macrophage from a splenic centre of a normal plaice. Note how electron dense the pigment granules (m) are. The pigment cell is in intimate contact with two lymphocytic cells (L). Cytoplasmic extensions (arrows) increase the area of contact between the two cell types.
(x 10,500)


bm

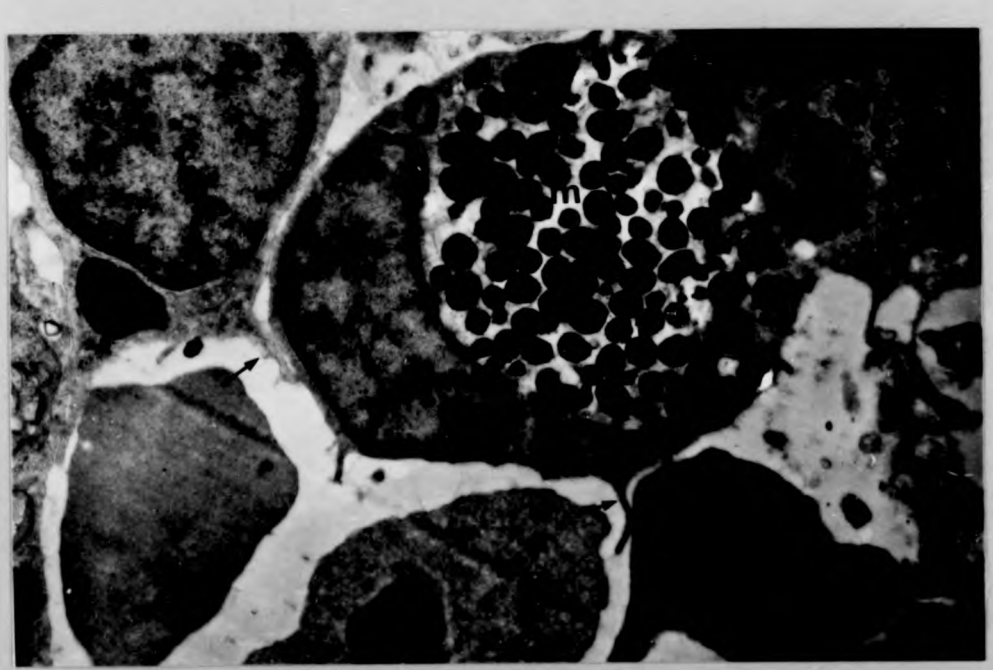
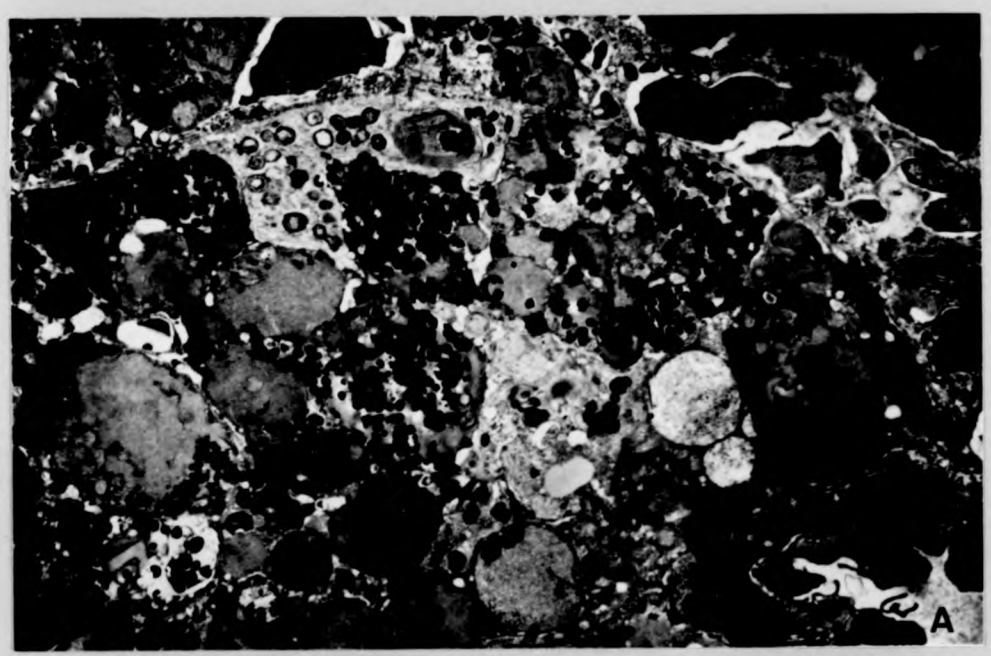

m

Fig. 7.8. Anterior kidney of normal plaice. Low power micrograph of a melano-macrophage centre (MMC) adjacent to an arteriole (A). Note that the MMC seems to be walled off from the surrounding haemopoietic tissue (arrows). Besides the melanin granules note also the presence of considerable amounts of cell debris and the large number of large lysosomal bodies.
(x 3,500)

Fig. 7.9. Enmeshed in the limiting membrane (arrows) of the melano-macrophage centre (MMC) shown in Fig. 7.8 is a lymphoid cuff (LC) in which are normally found plasma cells, primitive lymphocytic cells, lymphocytes and dendritic macrophages.
(x 3,500)


A

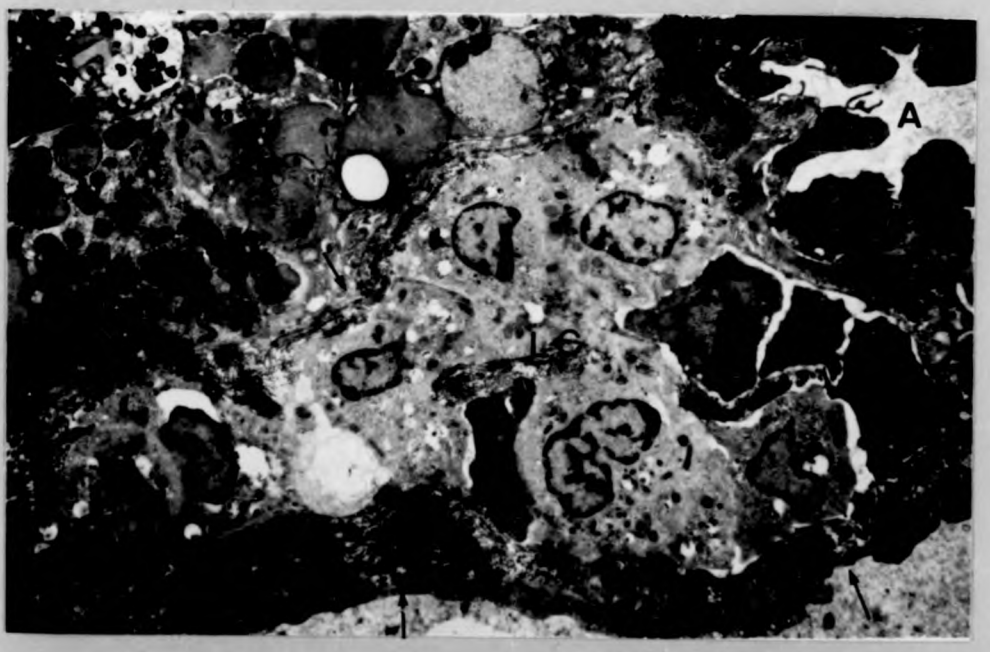

A

Fig. 7.10. Anterior kidney from a normal plaice showing a lymphocytic cell (L) embedded in the wall of an arteriole (A). Next to the arteriole is a melano-macrophage centre; the electron dense bodies are melanin granules. Antigenic material being transferred from the arteriole lumen to the centre possibly influences such lymphoid cells to produce an immune reaction.
(x 17,500)

Fig. 7.11. Electron micrograph of the anterior kidney from a normal plaice showing a thrombocyte-like cell among melano-macrophages within a melano-macrophage centre.
(x 17,500)


A

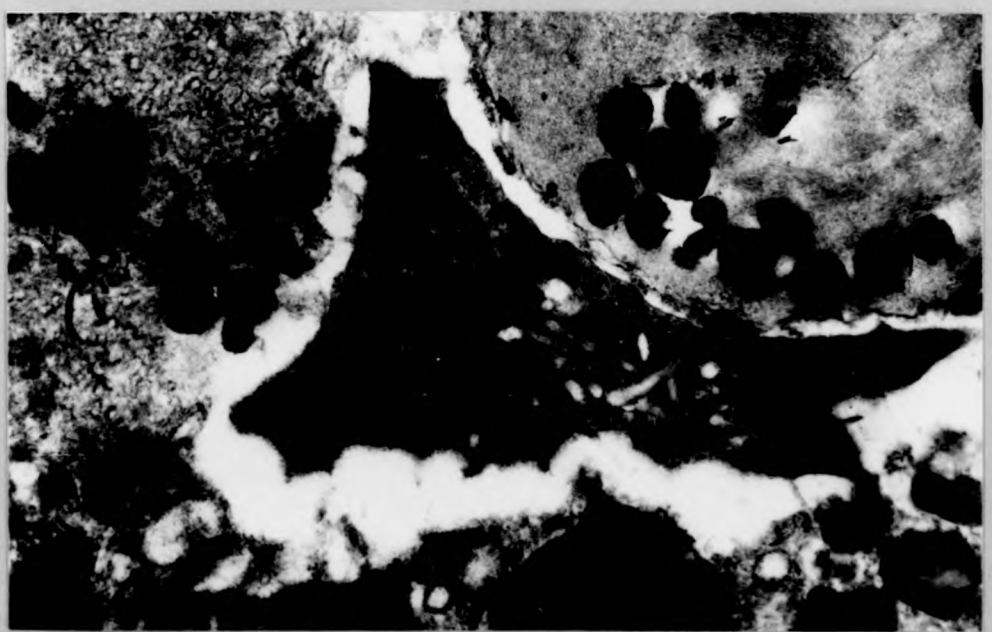


Fig. 7.12. Spleen from normal plaice. This electron micrograph shows two melano-macrophages in close apposition; one carries only a few pigment granules (none of them shown in this photomicrograph) while the other is replete with such granules. A long process (arrowed) extends from each cell to embrace its counterpart for the greater part of its length. The significance of this phenomenon remains to be determined.
(x 8,750)

Fig. 7.13. Electron micrograph of an ellipsoid from the spleen of a cachectic plaice. A is the axial vessel and the outer limiting membrane of the ellipsoid is arrowed. Note that the ellipsoid is relatively denuded of cells and in the surrounding haemopoietic tissue there are extensive intercellular spaces (S).
(x 3,500)

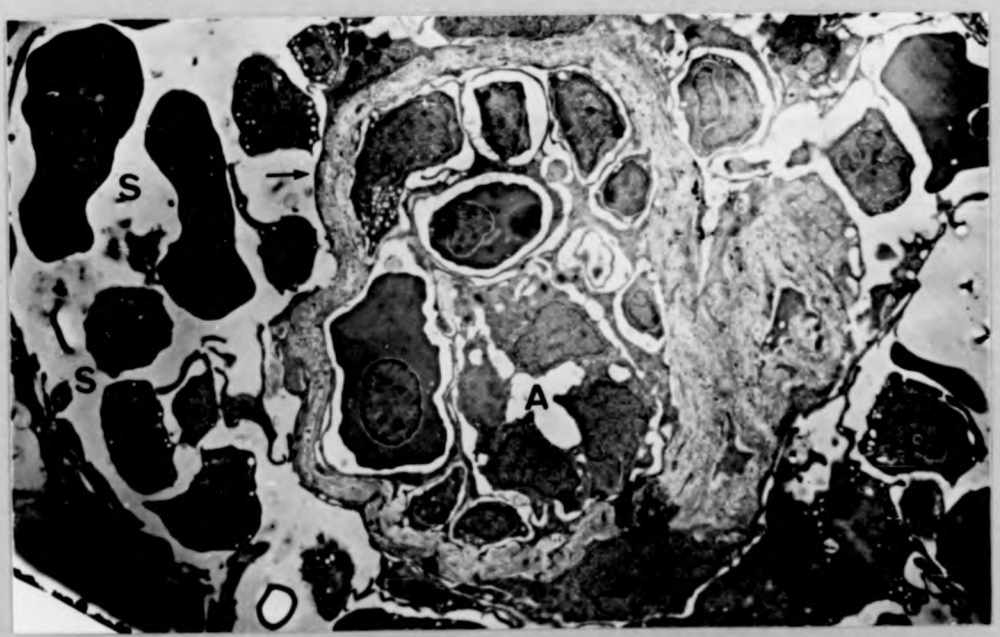

S
S
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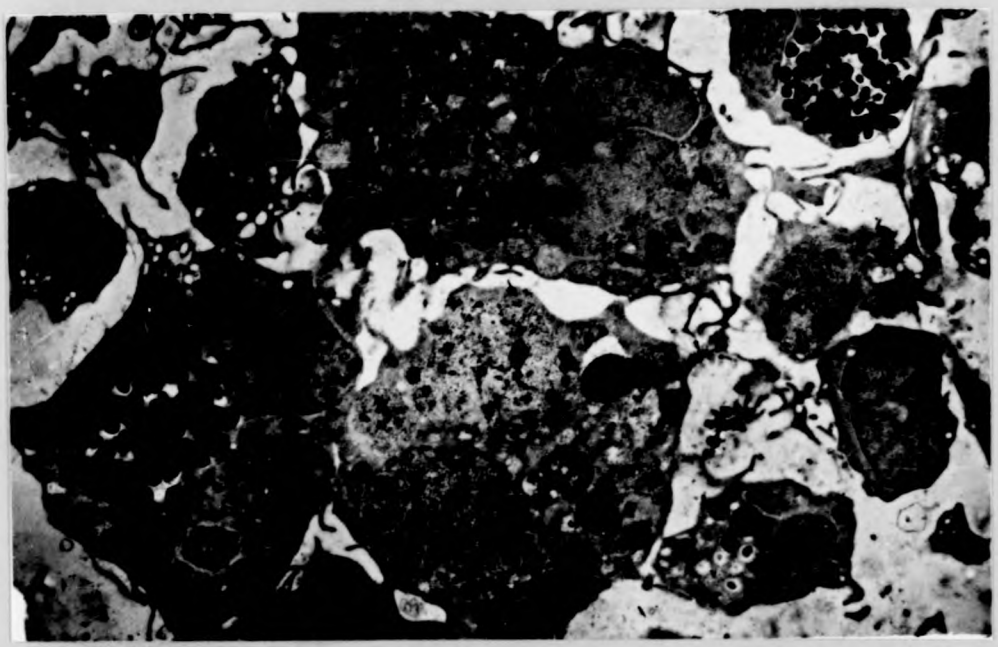
Fig. 7.14. Spleen of a cachectic plaice. This electron micrograph shows a number of macrophages (two carrying pigment granules) with extensive dendrition. Note also the way in which the macrophages are replete with lysosomes (L) which are very variable in size.
(x 5,250)

Fig. 7.15. Electron micrograph of a spleen from another cachectic plaice specimen to show the large number of macrophages with extensive dendrition and bearing very high numbers of lysosomes. Cell A for example is particularly densely packed with lysosomal bodies.
(x 7,000)



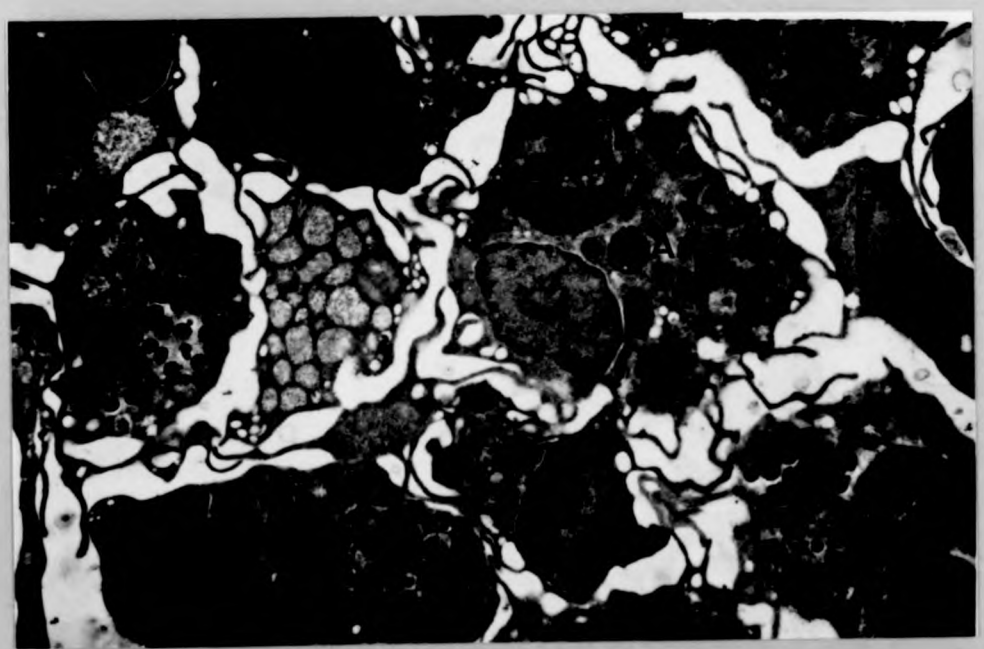


Fig. 7.16. Spleen of a cachectic plaice. This electron micrograph shows a cell-type commonly encountered in the spleen of starving plaice. This type of cell had a small cell body (CB) and long ramifying dendritic processes(arrowed) that came into close apposition with cytoplasmic extensions of monocytes, macrophages, melano-macrophages as well as lymphocytes.
(x 10,500)

Fig. 7.17. Electron micrograph of some melanin granules within a melano-macrophage of a normal plaice spleen representing a fair range of the observed variations in ultrastructural appearance of pigment granules in melano-macrophages. Note that in the majority of granules the entire structure is very electron dense whereas in a small number of granules there is an electron dense shell bounding an electron transparent core.
(x 14,000)

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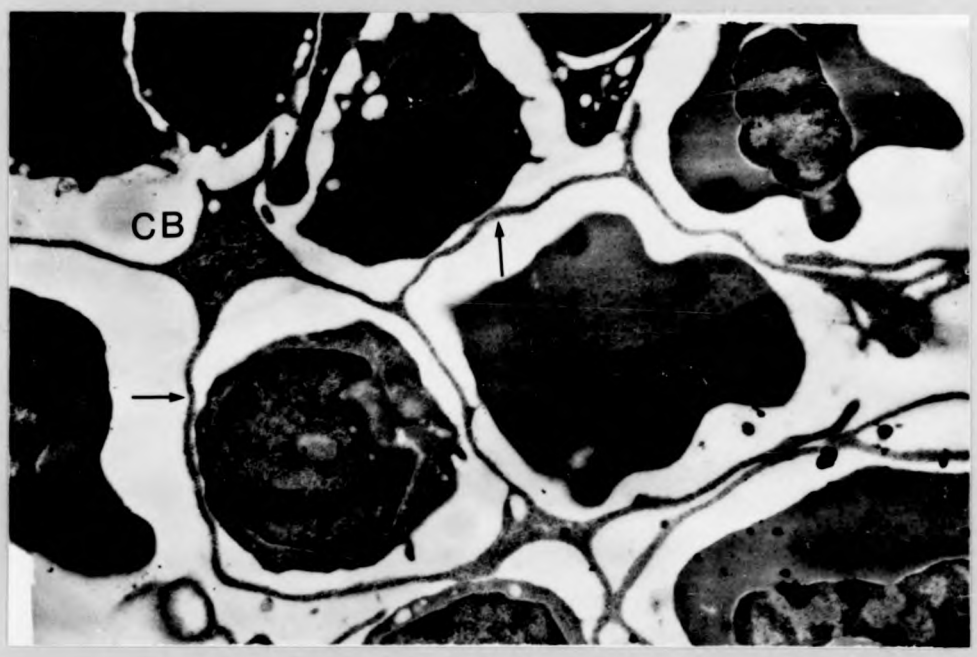

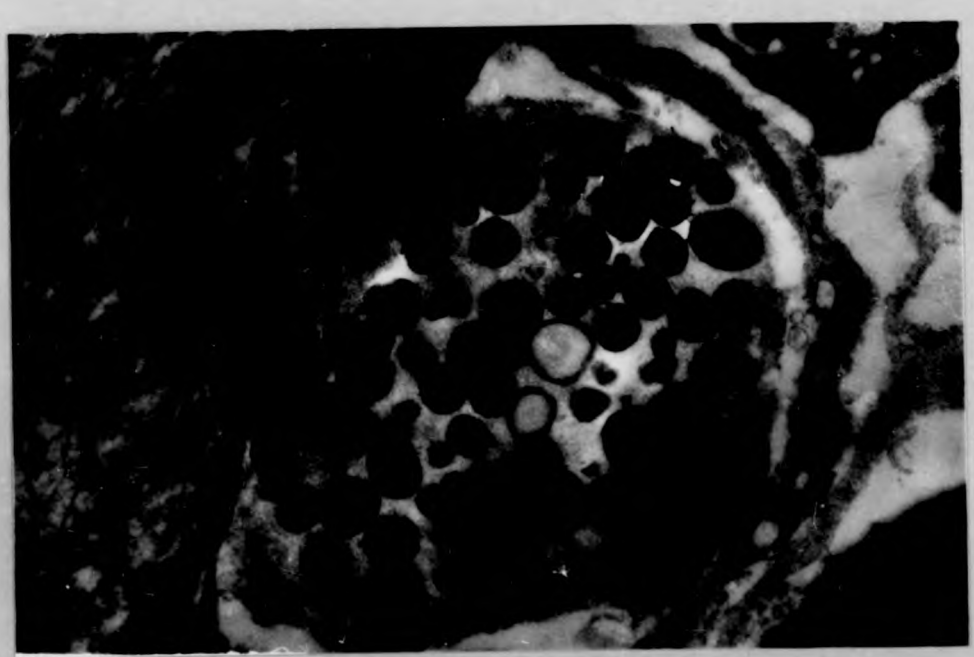

Fig. 7.18. Electron micrograph of a group of pigment granules from a kidney melano-macrophage of a cachectic plaice. Note how electron dense all the granules are, but some clearly exhibit electron transparent spaces within them. These presumably represent multivesicular bodies (premelanosomes) wherein the lumens of the internal vesicles appear as electron transparent spaces. Upon maturation, the whole structure becomes uniformly electron dense as the internal vesicles become filled in by melanin.
(x 35,000)

Fig. 7.19. High power electron micrograph of one of the multivesicular bodies shown in Fig. 7.18. Note the number of internal vesicles, some of which have little or no melanin within them as yet.
(x 175,000)

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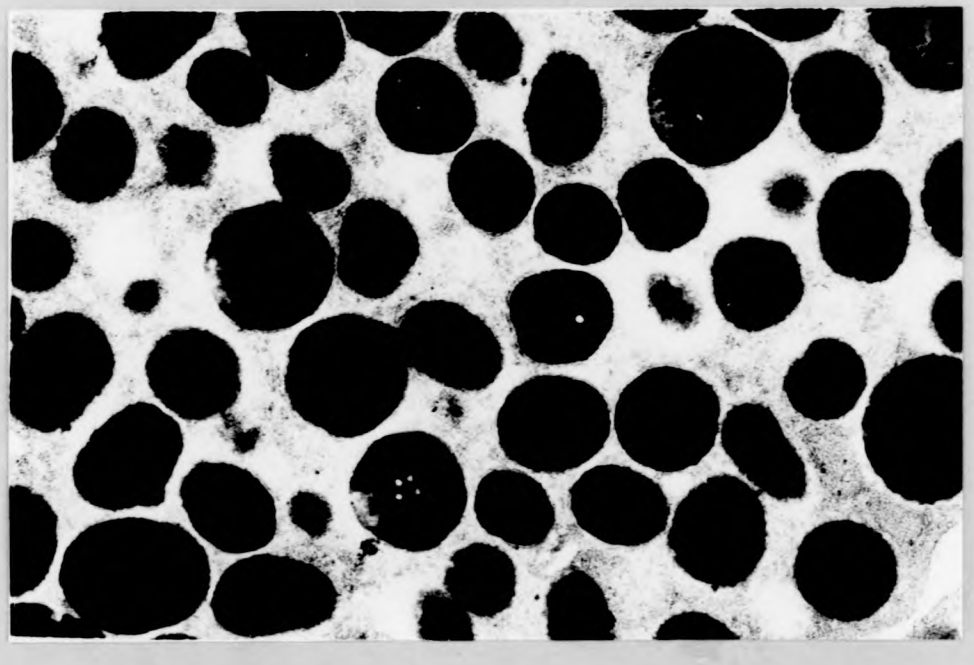


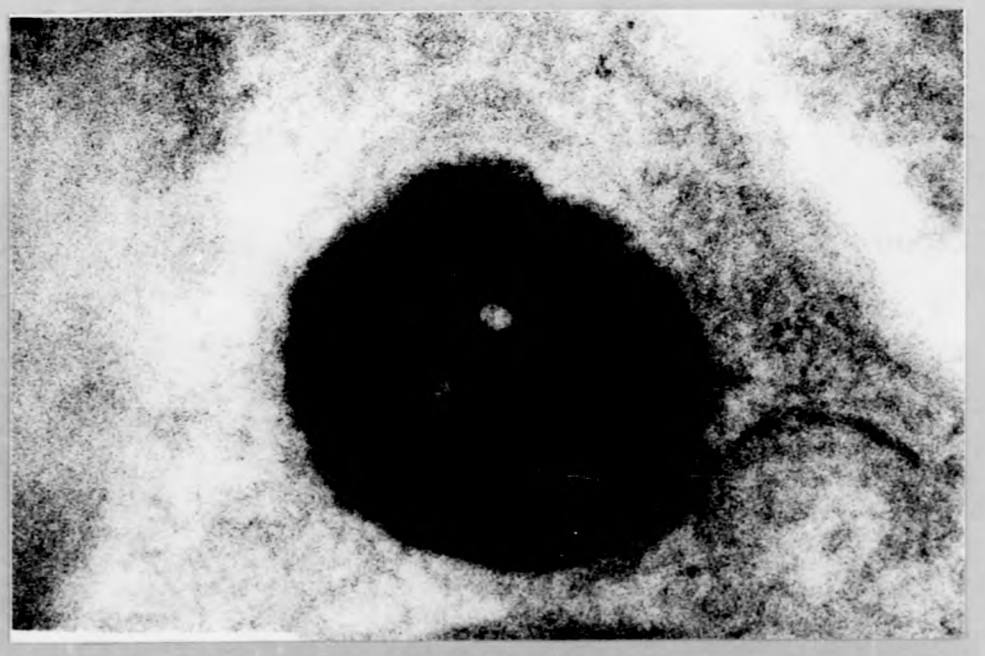
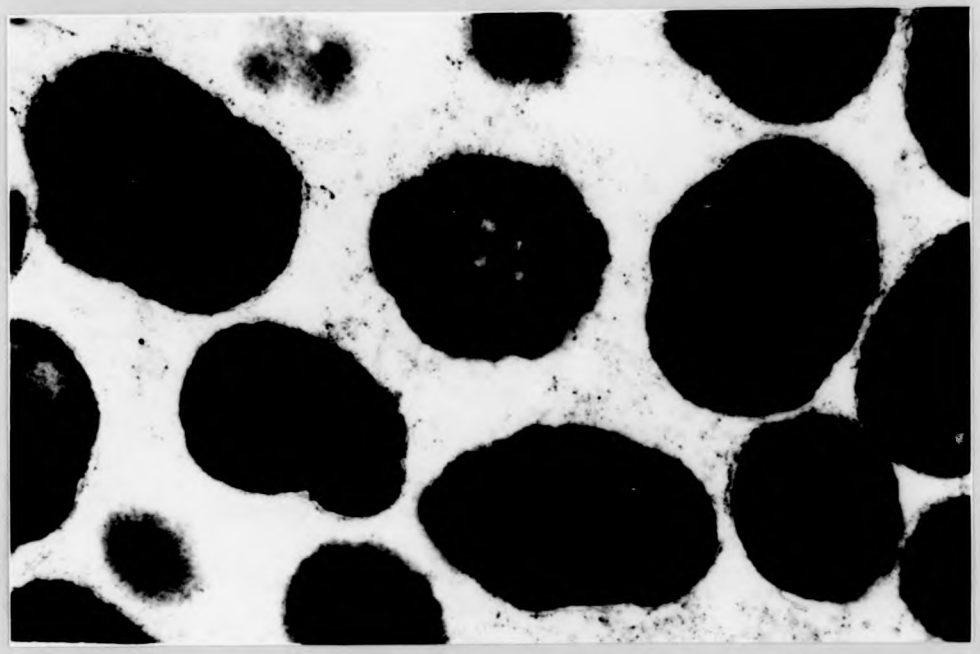


Fig. 7.20. A multivesicular body within a peritoneal melanophore of a cachectic plaice specimen. (x 70,000)

Fig. 7.21. Electron micrograph of a melano-macrophage within the spleen of a cachectic plaice. Note the disposition of the melanin granules in two large vacuole-type bodies. Note also the large number of lysosomes within the cell. (x 10,500)



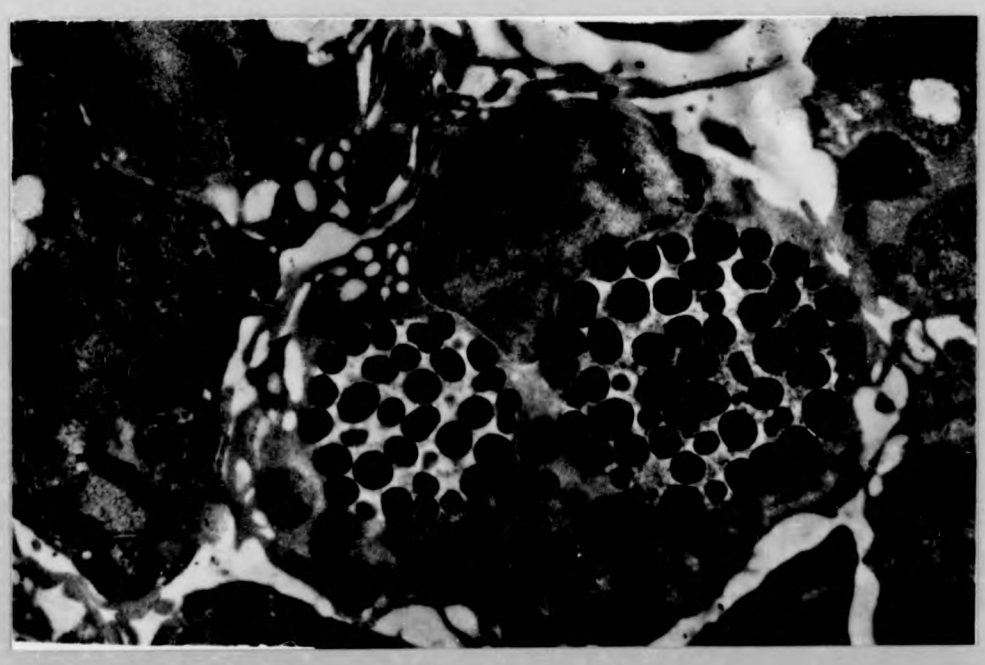


Fig. 7.22. Electron micrograph of a melano-macrophage within the spleen of a normal plaice. Note the disposition of the melanin granules within lysosomes (L).
(x 17,500)

Fig. 7.23. Electron micrograph of a melano-macrophage within the spleen of a normal plaice. Note the presence in this one cell of melanin granules in large vacuole-type body (V) as well as in lysosomes (L).
(x 10,500)

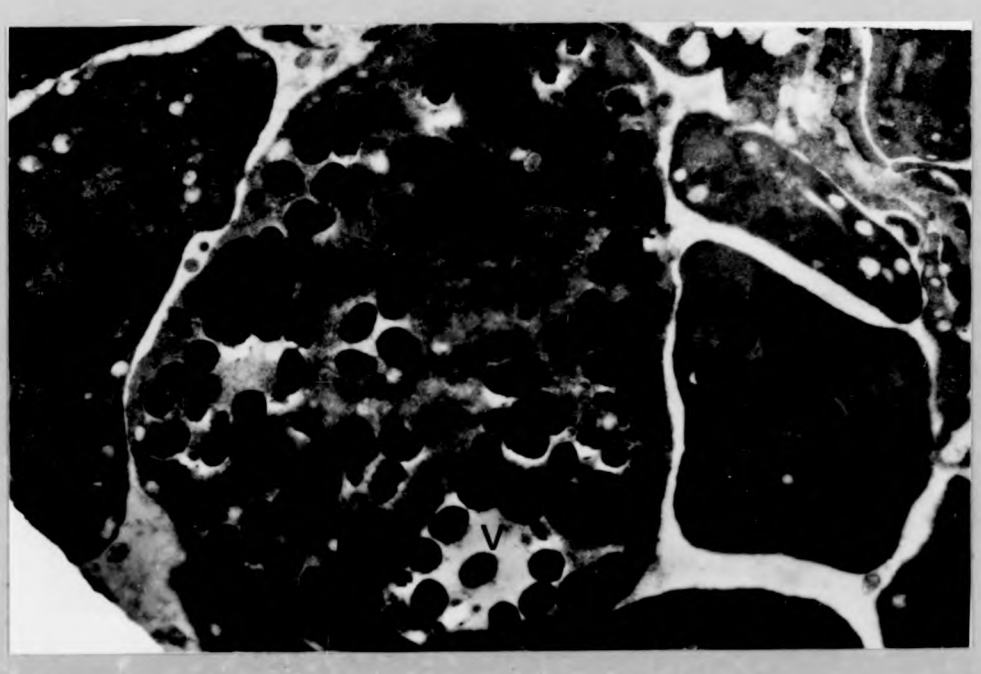
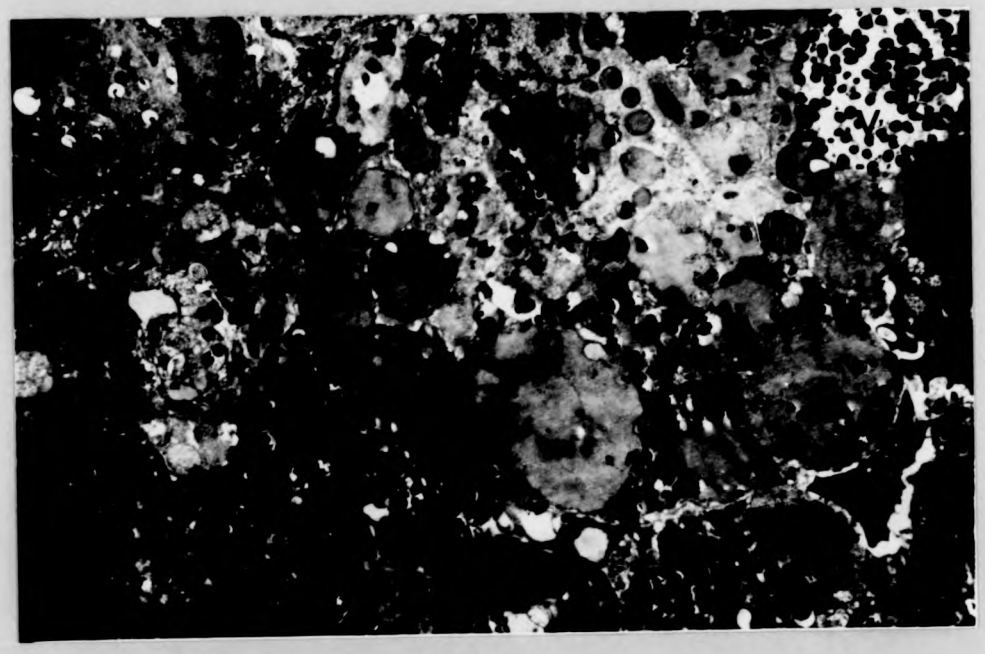

V

Fig. 7.24. Low power electron micrograph within a melano-macrophage centre of the kidney of a normal plaice. As in the spleen, melanin granules are generally held either in large vacuole-type bodies (V) or in lysosomes (L).
(x 3,500)

Fig. 7.25. High power electron micrograph within a splenic melano-macrophage of a normal plaice showing melanin granules in a large vacuole-type body (V) and in lysosomes (L). Note how the vacuole contains no other detectable inclusions.
(x 35,000)


V


V

Fig. 7.26. Electron micrograph of a contracted skin melanophore from normal plaice. The very electron dense melanin granules are randomly distributed throughout the cytoplasm with no inclusion of granules in any intracytoplasmic organelles.
(x 7,000)

Fig. 7.27. Low power micrograph of melanophores in the peritoneum of a normal plaice showing how the melanin granules are randomly distributed throughout the cell cytoplasm with no inclusion of granules within any intracellular organelles.
(x 5,250)

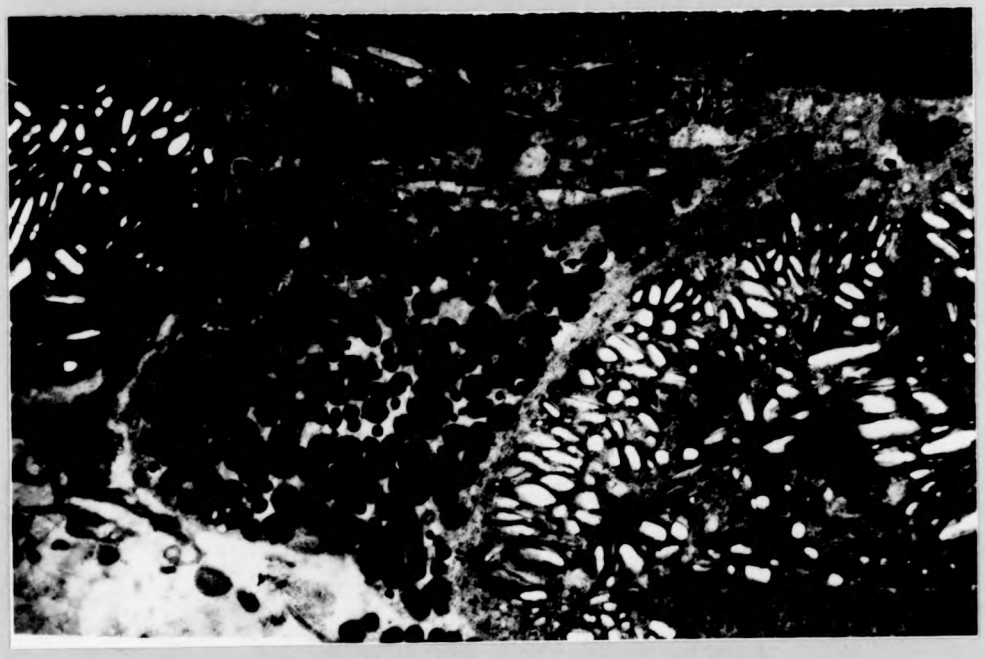
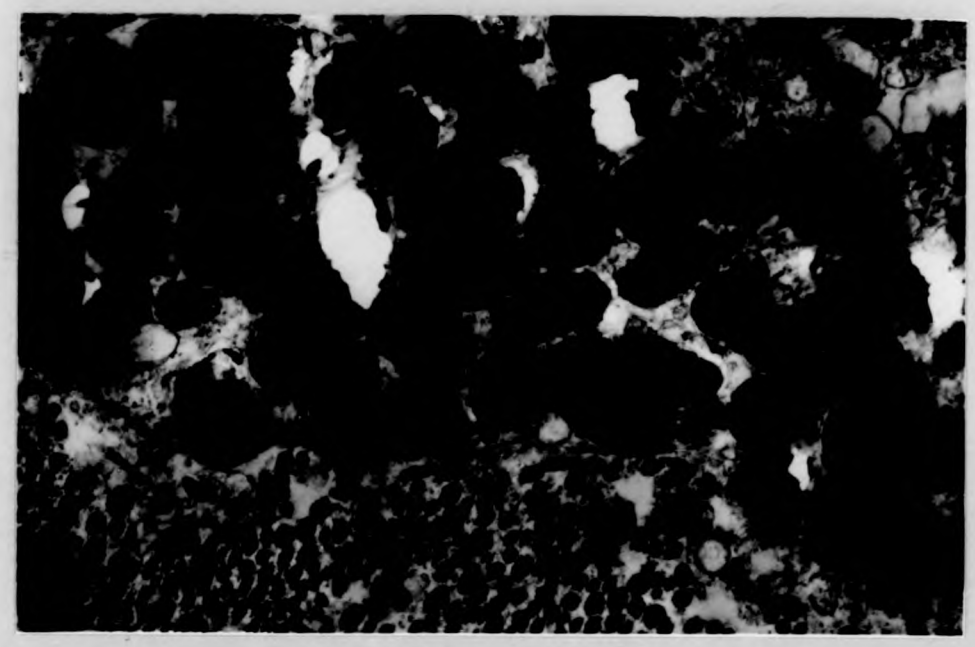


Fig. 7.28. High power micrograph of one of the melanophores shown in Fig. 7.27. Note the melanin granules freely dispersed throughout the cytoplasm.
(x 26,250)

Fig. 7.29. High power electron micrograph of melanin granules enclosed within a lysosome. This is from a splenic melano-macrophage of a normal plaice.
(x 105,000)



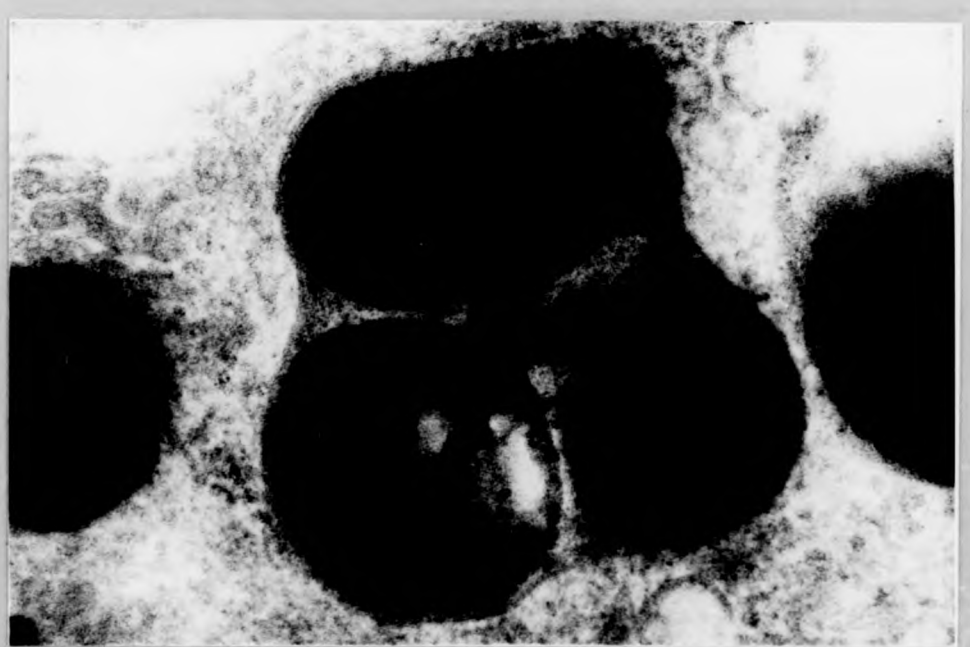


Fig. 7.30. This electron micrograph of a splenic melano-macrophage from a normal plaice shows several lysosomes bearing pigment granules. One of these bodies also contains what look like membrane remnants (arrow).
(x 26,250)

Fig. 7.31. High power electron micrograph of a splenic melano-macrophage of a normal plaice. The lysosome with a fine fibrillar appearance (L) bears numerous pigment granules mainly near the periphery. One or two also seem to be bulging out raising the possibility that they are being engulfed by or extruded from the lysosome.
(x 52,500)

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Fig. 7.32. A large lysosome bearing two pigment granules in this one plane. Splenic melano-macrophage of a normal plaice. (x 43,750)

Fig. 7.33. Another lysosomal inclusion in the cytoplasm of a splenic melano-macrophage from a normal plaice. The pigment granule to the left of the lysosome has an electron dense coat enclosing a less electron dense core.
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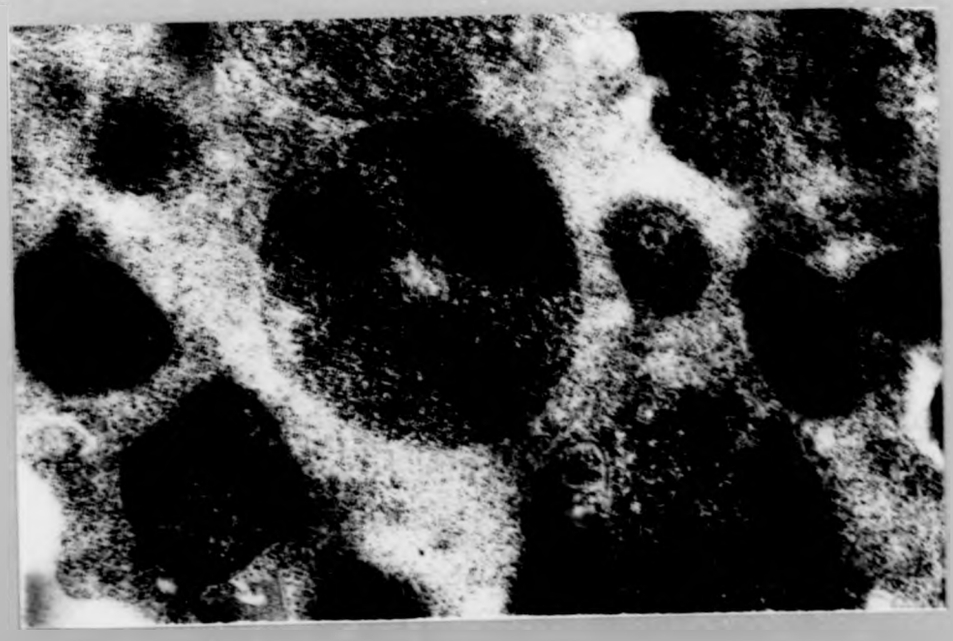
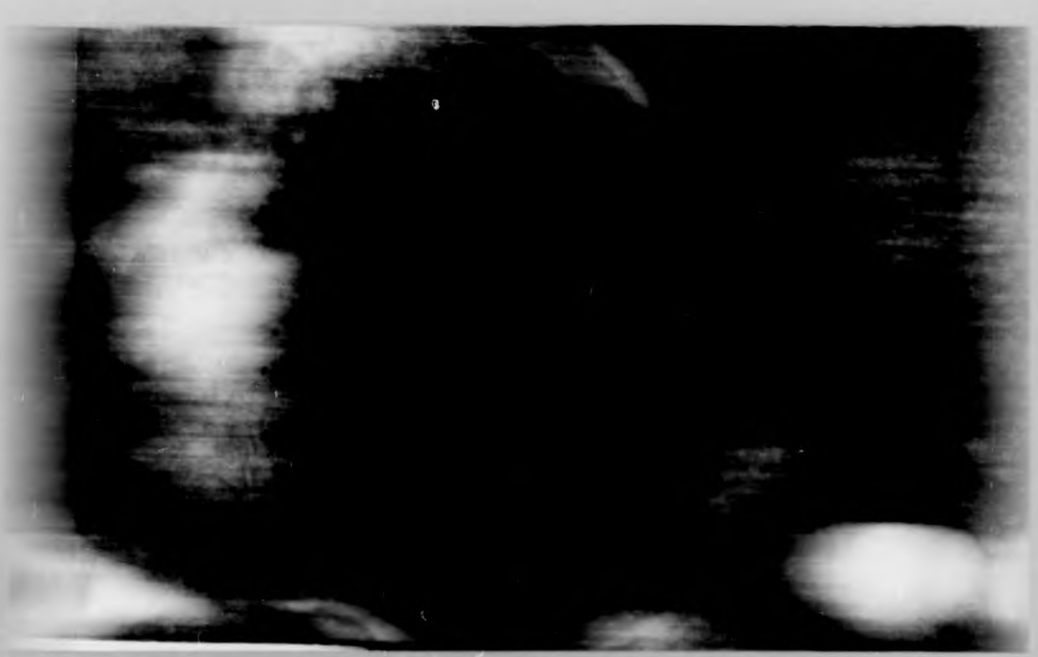


Fig. 7.36. Lysosomes bearing melanin granules within a kidney melano-macrophage of a normal plaice.
(x 26,250)

Fig. 7.37. High power electron micrograph of a lysosome containing pigment granules. This was observed in a kidney melano-macrophage of a cachectic plaice.
(x 87,500)



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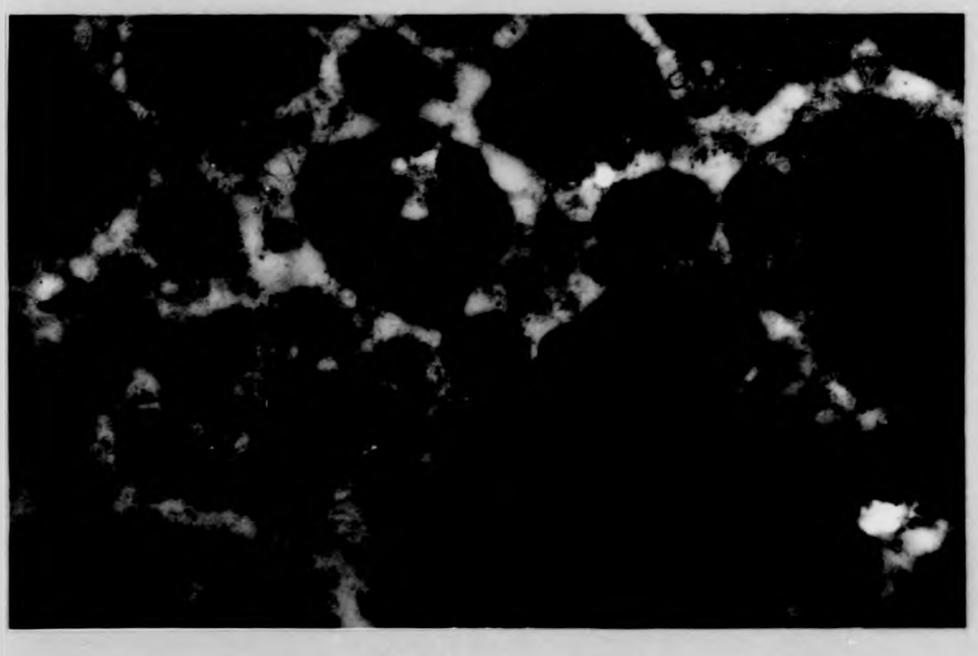

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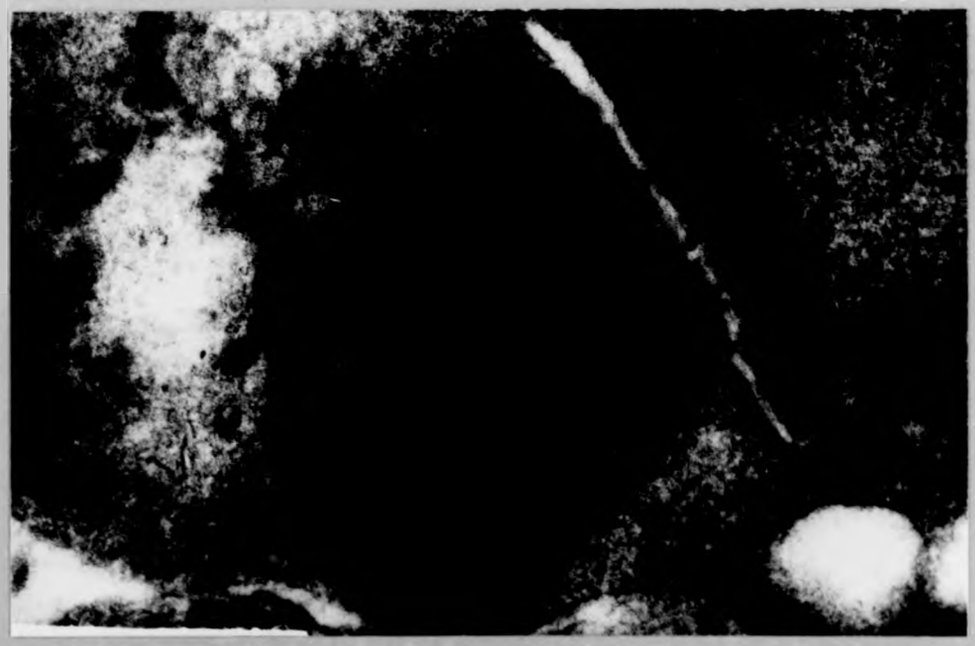

Fig. 7.38. A melano-macrophage from the anterior kidney of a cachectic specimen. The appearance of the granules in this lysosome tempts one to speculate that some of them are being formed within this body through oxidation of indigestible lipid residues to pigment.
(x 105,000)

Fig. 7.39. Electron micrograph of a splenic melano-macrophage from a cachectic plaice. The lysosomes (L) appear to be heavily laden with iron compounds. Considerable quantities of granules suggestive of iron compounds can also be seen throughout the rest of the cytoplasm.
(x 43,750)

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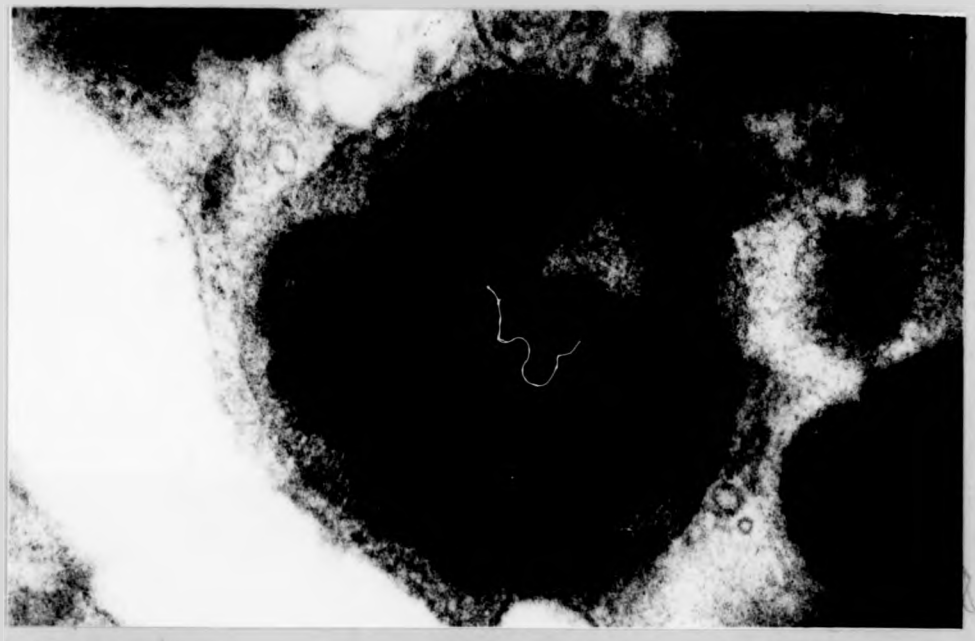

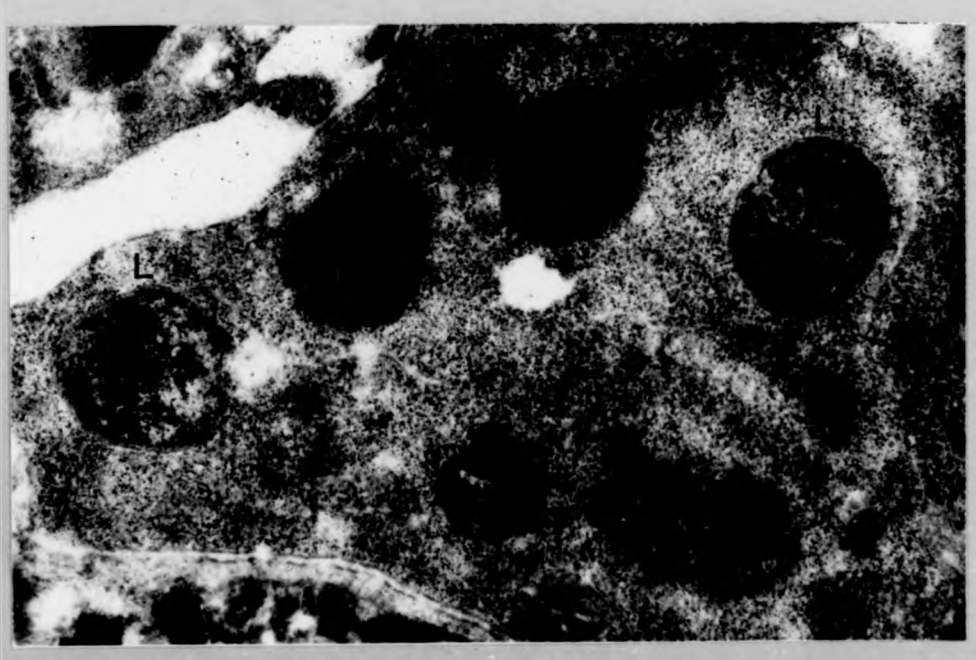

Fig. 7.40. Anterior kidney melano-macrophage from a normal plaice containing numerous mitochondria (arrows). The mitochondria lie free throughout the cell cytoplasm and most of them look still viable in this cell.
(x 10,500)

Fig. 7.41. Electron micrograph showing what looks like a still viable mitochondrion adjacent to several melanin granules. This is a high power picture within the same cell shown in Fig. 7.40.
(x 52,500)

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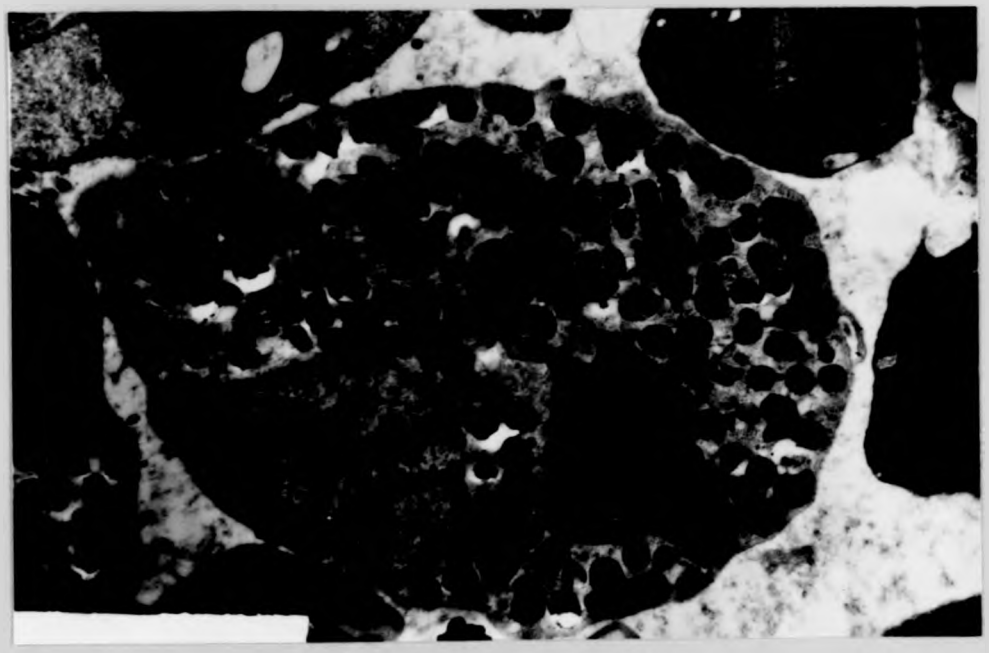

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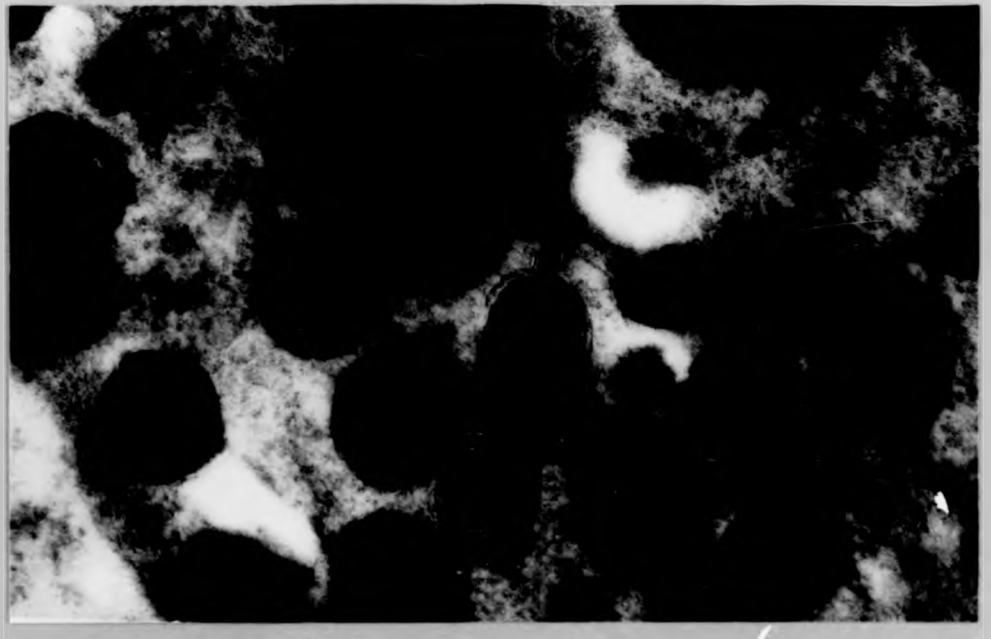
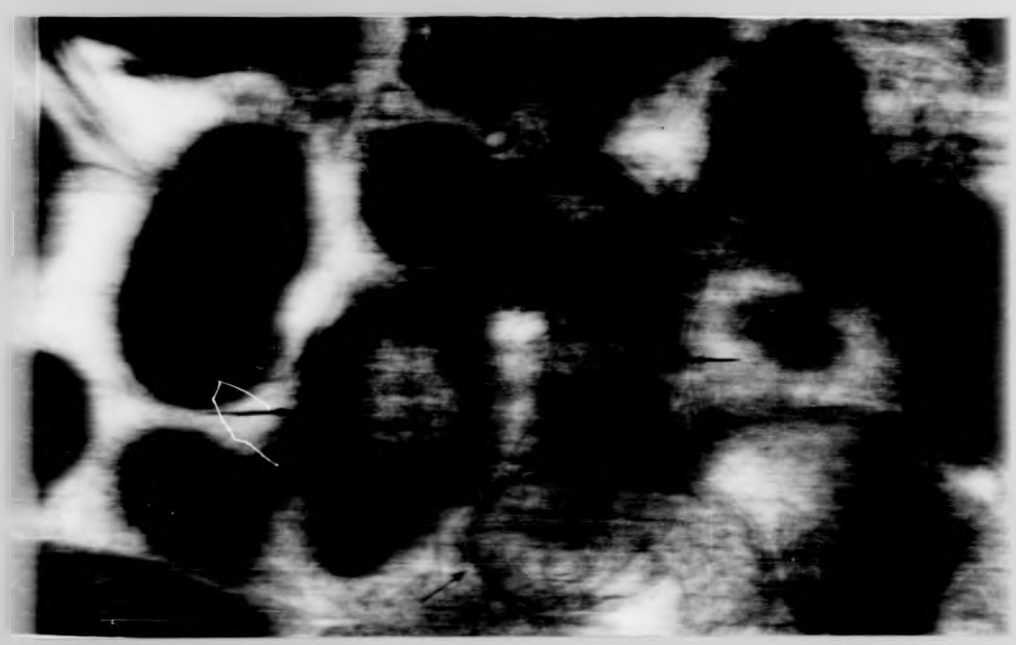
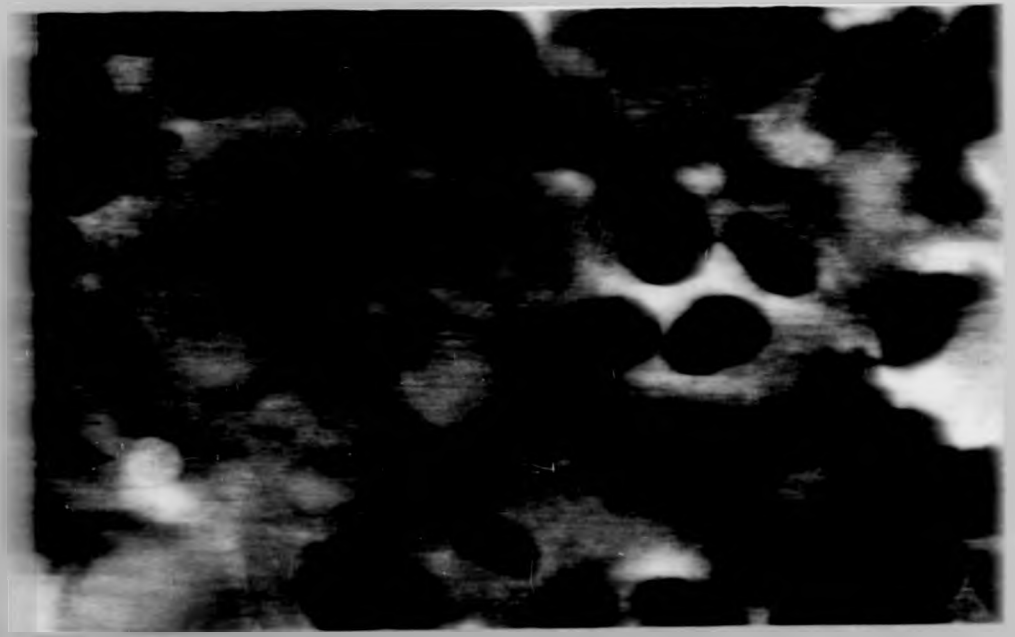


Fig. 7.42. Electron micrograph within the cytoplasm of a kidney melano-macrophage of a cachectic plaice. Observe what appear to be different stages (arrowed) of a degenerative transformation of mitochondria into pigment granules.
(x 70,000)

Fig. 7.43. Splenic melano-macrophage from a normal plaice. Observe the three arrowed bodies that appear like mitochondria in an advanced stage of degeneration.
(x 26,250)





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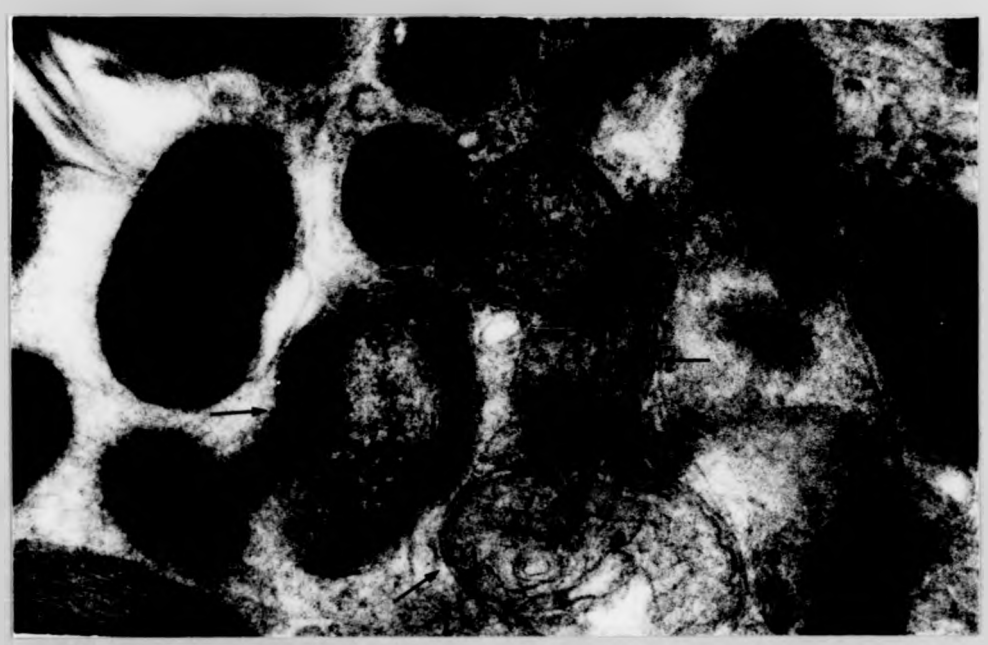

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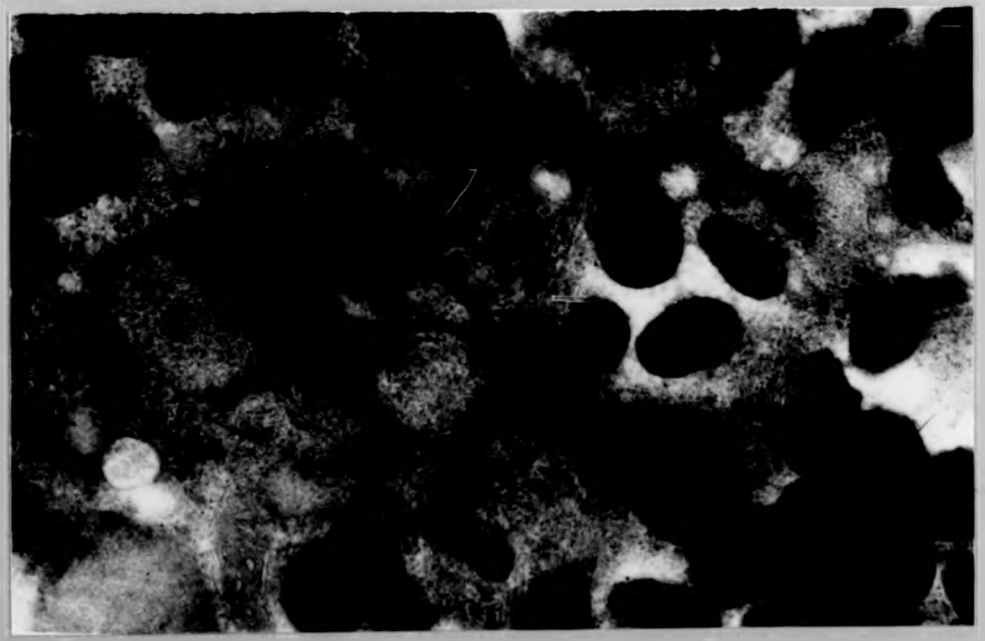

Fig. 7.44. Splenic melano-macrophage of another normal plaice. Four bodies suggestive of mitochondrial origin appear to be undergoing transformation into pigment granules. These and numerous similar bodies were evident in the spleen and kidney of this specimen; most of them lay free in the cytoplasm of melano-macrophages among mature pigment granules.
(x 70,000)

Fig. 7.45. Kidney melano-macrophage of a normal plaice. A number of what appear to be mitochondria degenerating into pigment granules were observed among the melanin granules. The one shown in this high power electron micrograph still has what appear to be remnants of mitochondrial cristae (arrowed).
(x 87,500)

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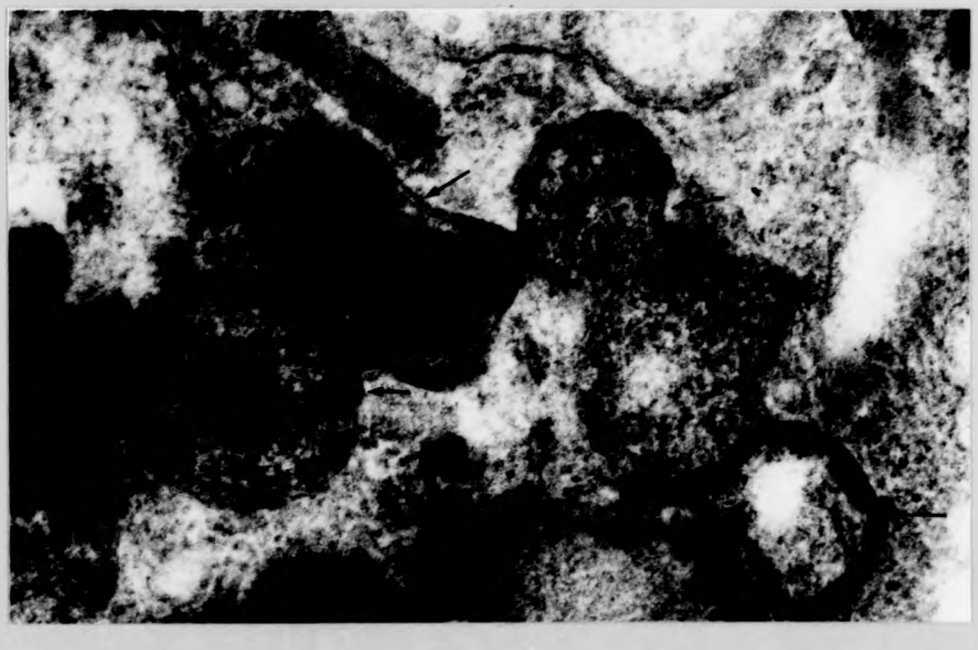

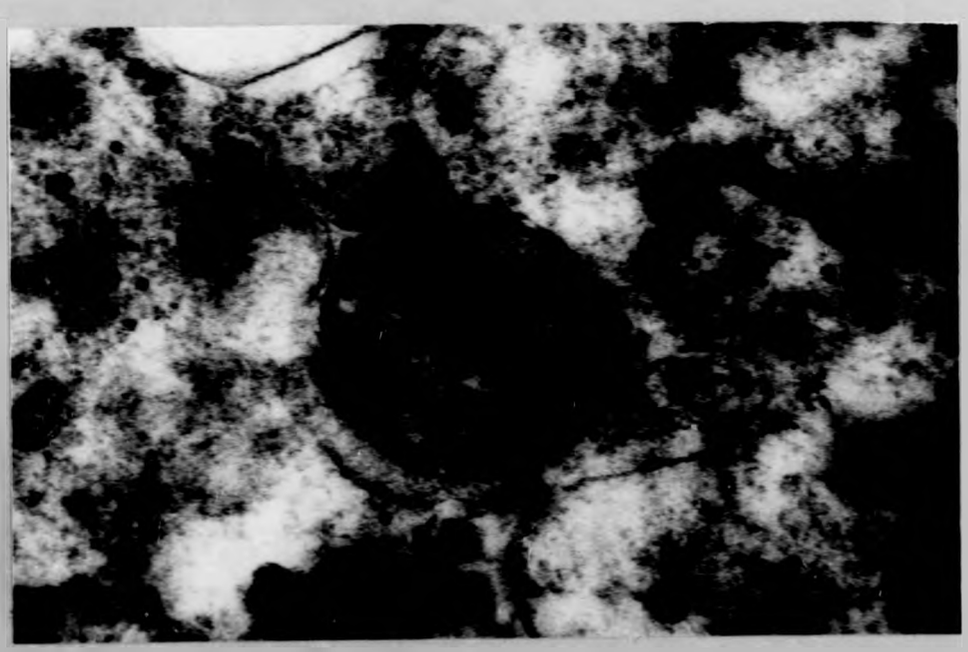

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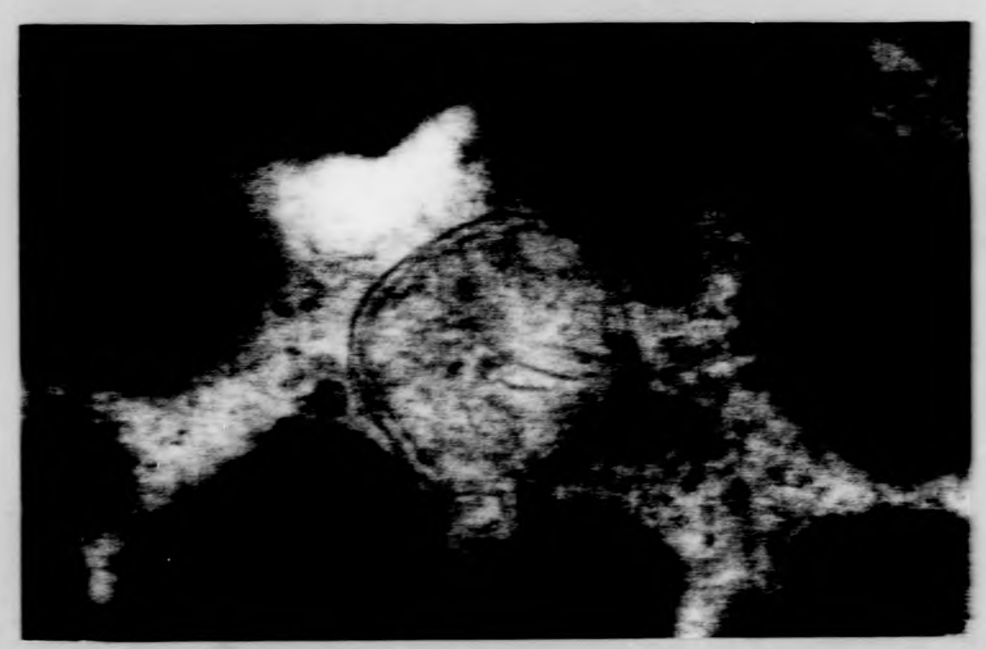
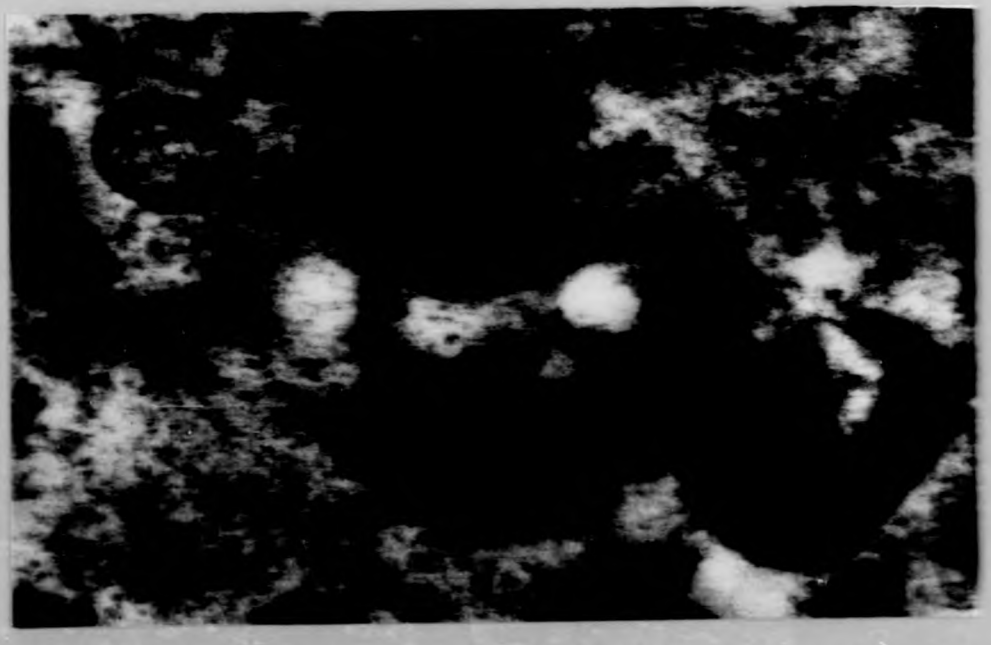
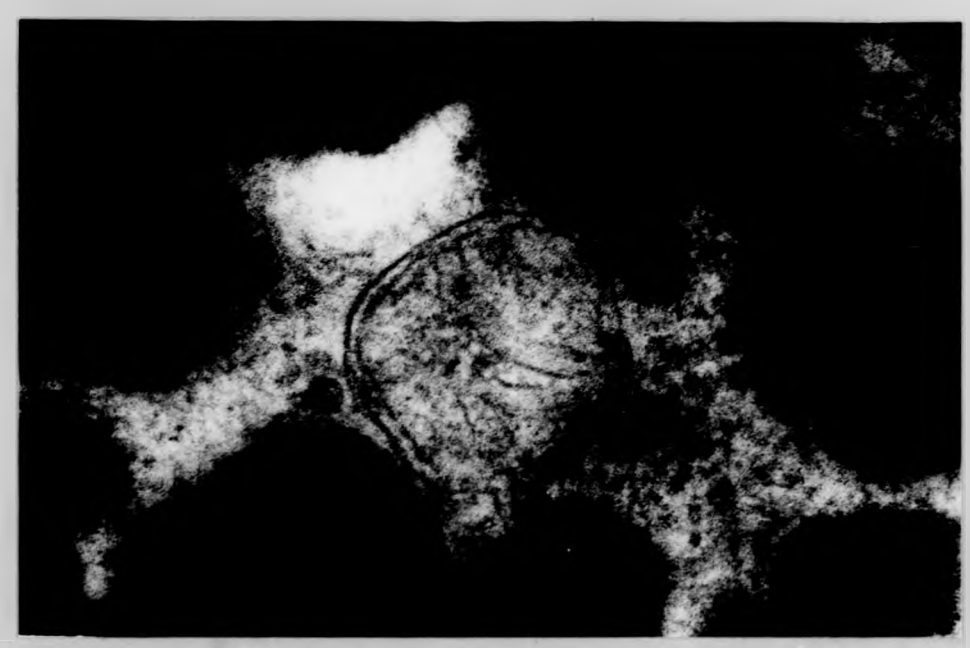
Fig. 7.46. High power electron micrograph of another mitochondrion-like degenerating body lying among mature pigment granules in the cytoplasm of a splenic melano-macrophage of a normal plaice.
(x 105,000)

Fig. 7.47. Some more bodies suggestive of mitochondrial origin seemingly undergoing transformation into pigment granules. Close-by is a fully mature pigment granule. These bodies were not observed to be within a macrophage but lay free among masses of cellular debris within a melano-macrophage centre in the anterior kidney of a normal plaice.
(x 70,000)







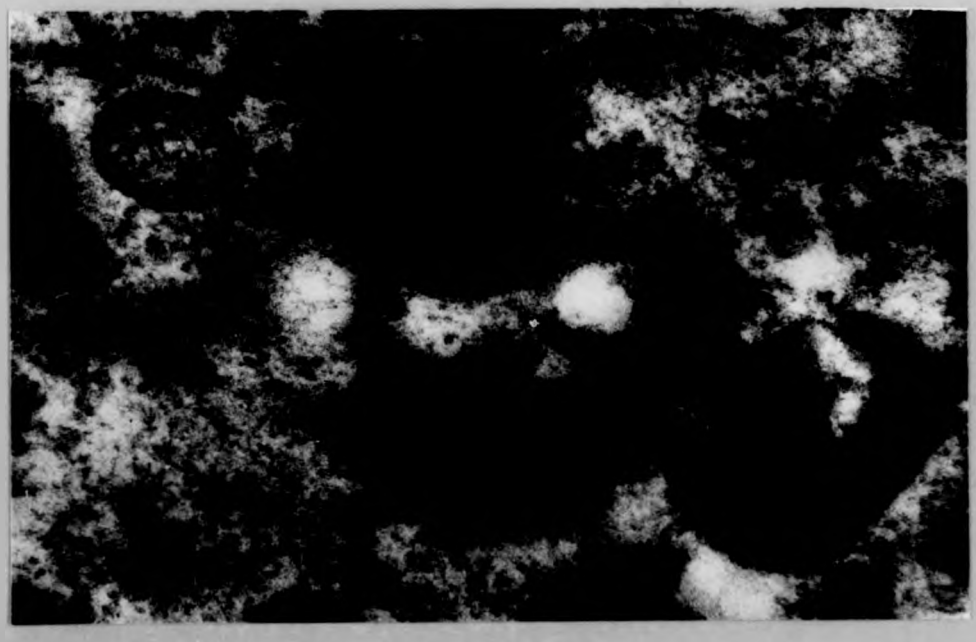
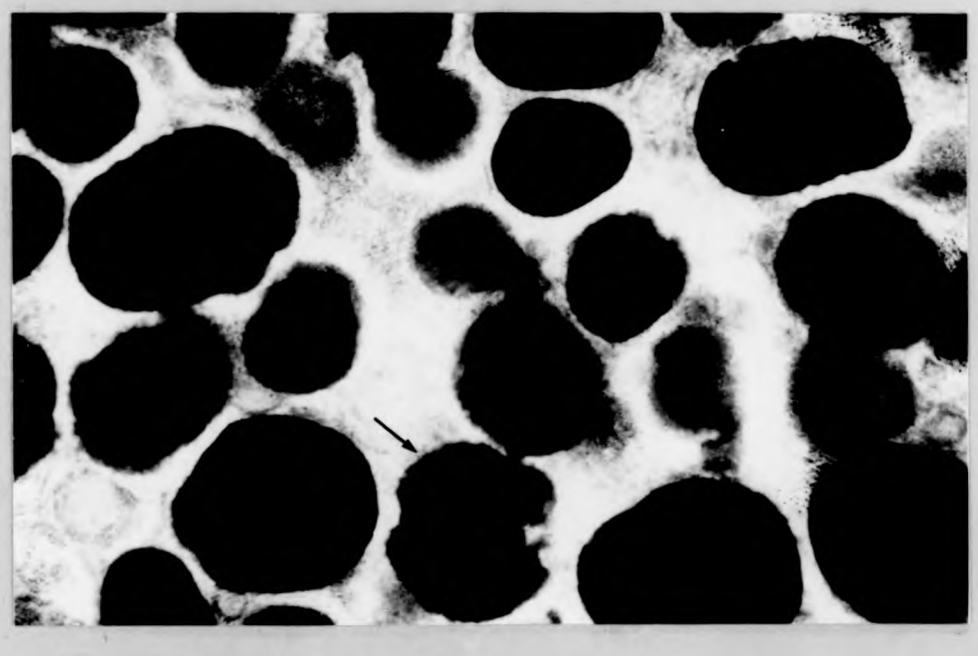


Fig. 7.48. Splenic melano-macrophage from a cachectic plaice.
(x 52,500)

Fig. 7.49. Kidney melano-macrophage from a normal plaice.
(x 70,000)

Bodies like the ones arrowed could well represent nearly fully oxidized pigment granules possibly derived from some effete cellular components such as mitochondria.



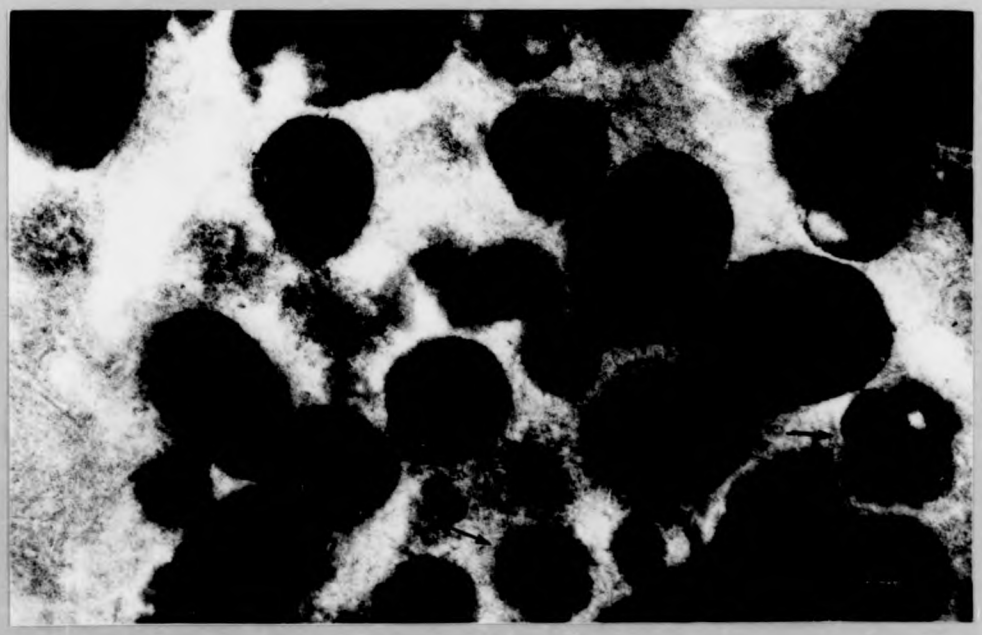


Fig. 7.50. Myelin-like whorls within a melano-macrophage centre of the spleen of a normal plaice. Such whorls are frequently encountered when degenerating protoplasm is present but their exact significance is not understood. They were encountered only very occasionally in the course of the present study. Note also the remnants of degenerating rough endoplasmic reticulum (arrowed).
(x 26,250)

Fig. 7.51. Higher power electron micrograph of a portion of another melano-macrophage in the same section as that from which Fig. 7.50 was taken. Observe again the myelin-like whorls.
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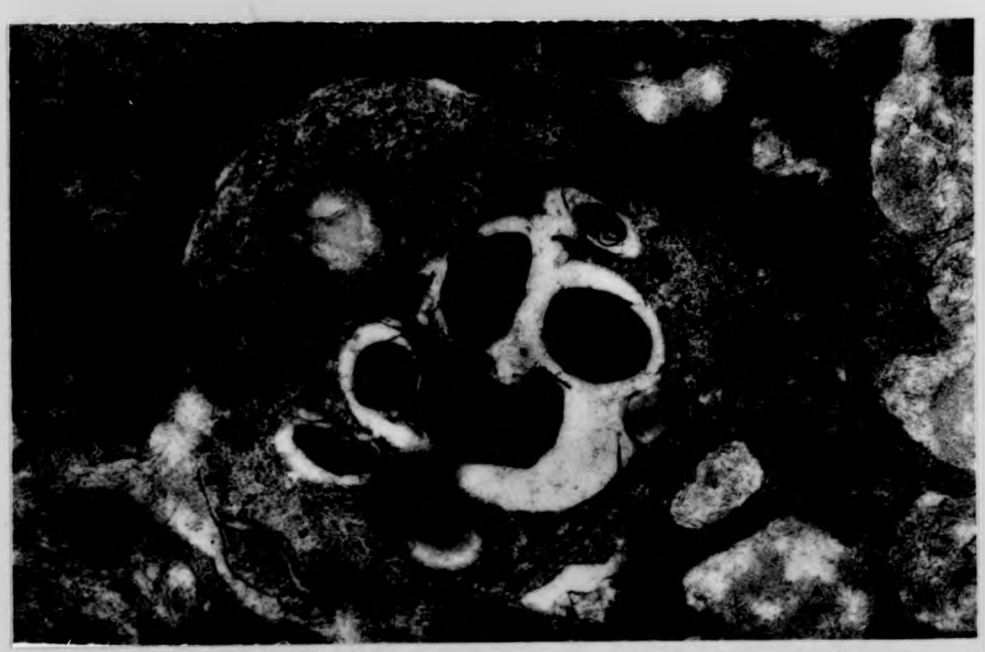

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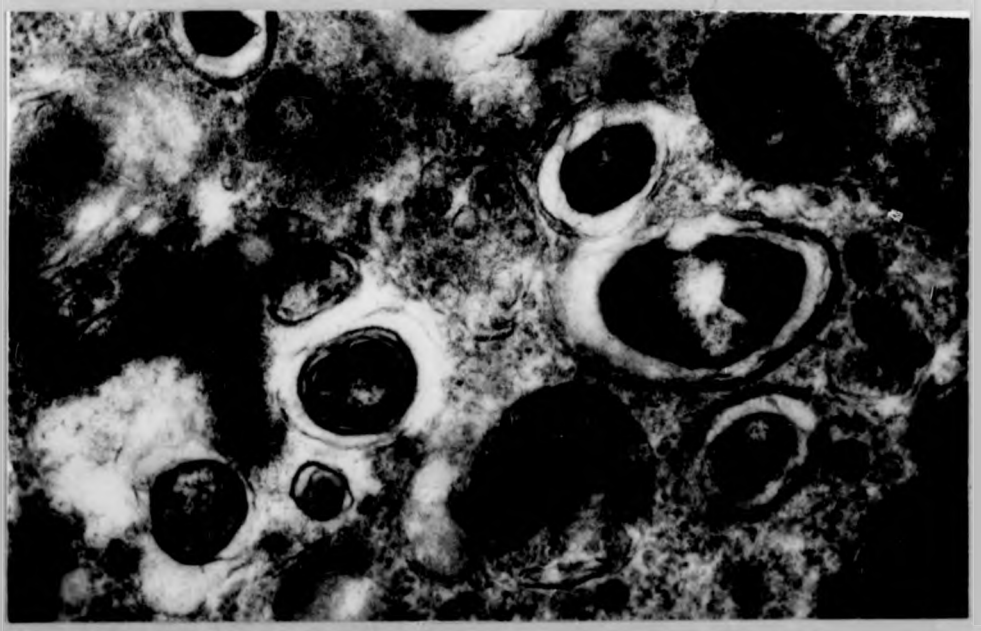

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(x 26,250)

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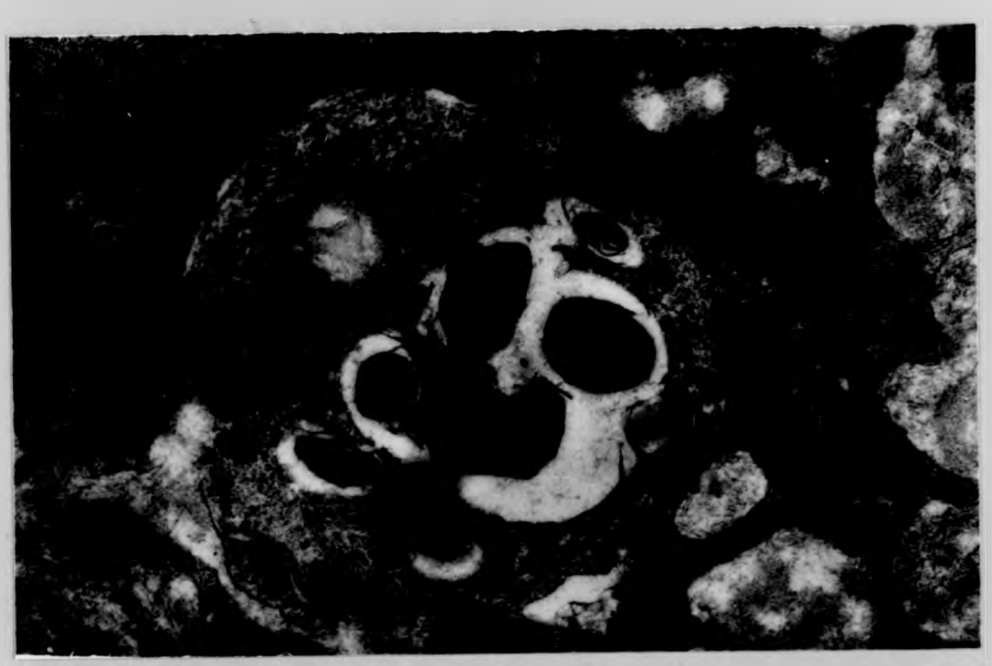
(x 52,500)



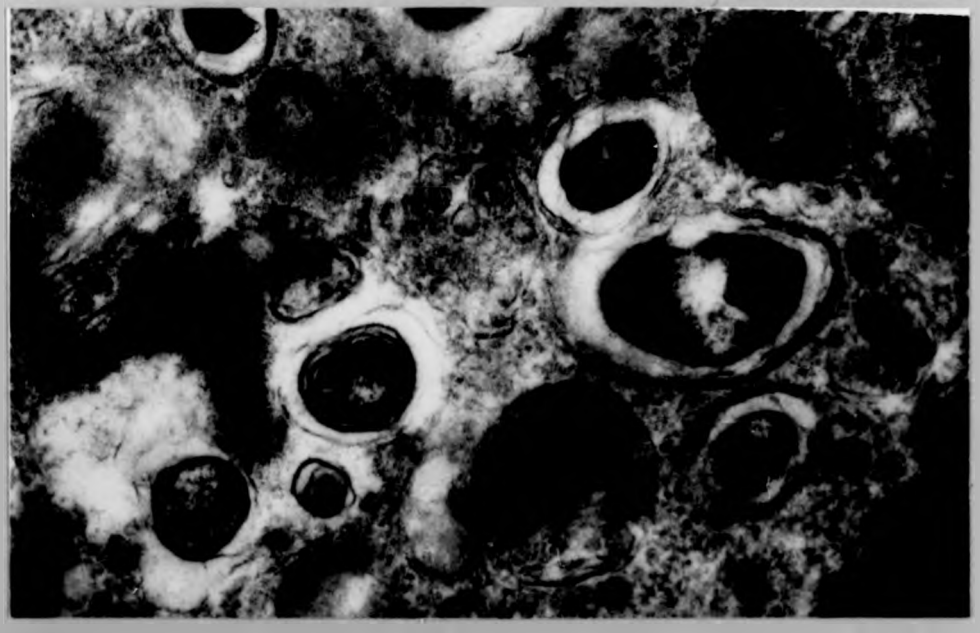


Fig. 7.52. Electron micrograph of a macrophage actively phagocytosing cellular debris (arrowed) from among the haemopoietic cells of the anterior kidney of a cachectic plaice. The material being engulfed possibly includes a mitochondrion-like body (indicated by top arrow). Observe how the macrophage which is well away from any melano-macrophage centre is nearly fully laden with lysosomes of various sizes but in this particular cell no pigment granules can be seen as yet.
(x 5,250)

Fig. 7.53. High power electron micrograph of the macrophage shown in Fig. 7.52 showing the process of phagocytosis in greater detail. The mitochondrion-like body being engulfed is almost totally surrounded by the pseudopodial extensions. Note also the very long mitochondrion (M).
(x 17,500)

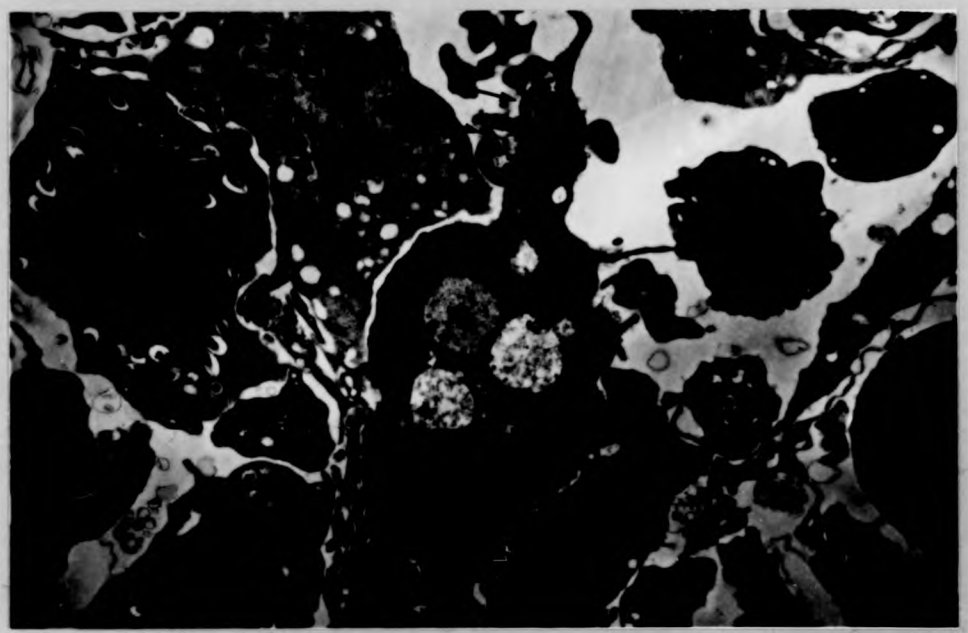
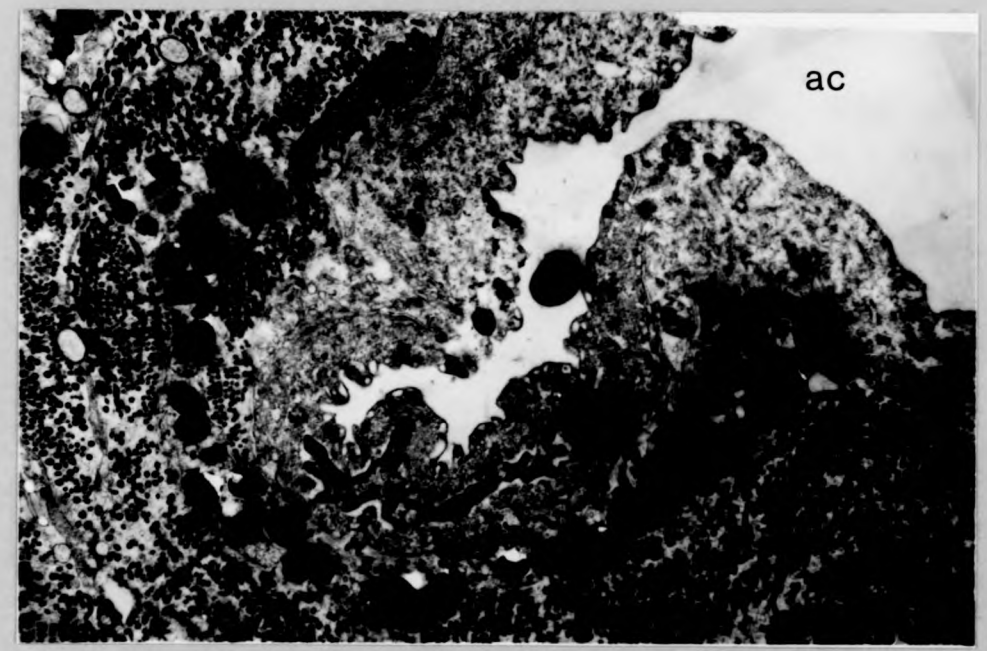


Fig. 7.54. Electron micrograph of a melanophore in the lining of the peritoneum. Note the melanin granule being extruded into the abdominal cavity (ac). Possibly this will be phagocytosed and carried to the melano-macrophage centres. Such a phenomenon seen here taking place in a normal plaice presumably occurs to a great extent during cachexia.
(x 10,500)

Fig. 7.55. A ruptured peritoneal melanophore of a cachectic plaice. Here the melanosomes are being released into the direction of the underlying muscles. These will possibly be phagocytosed and transported to the melano-macrophage centres.
(x 8,750)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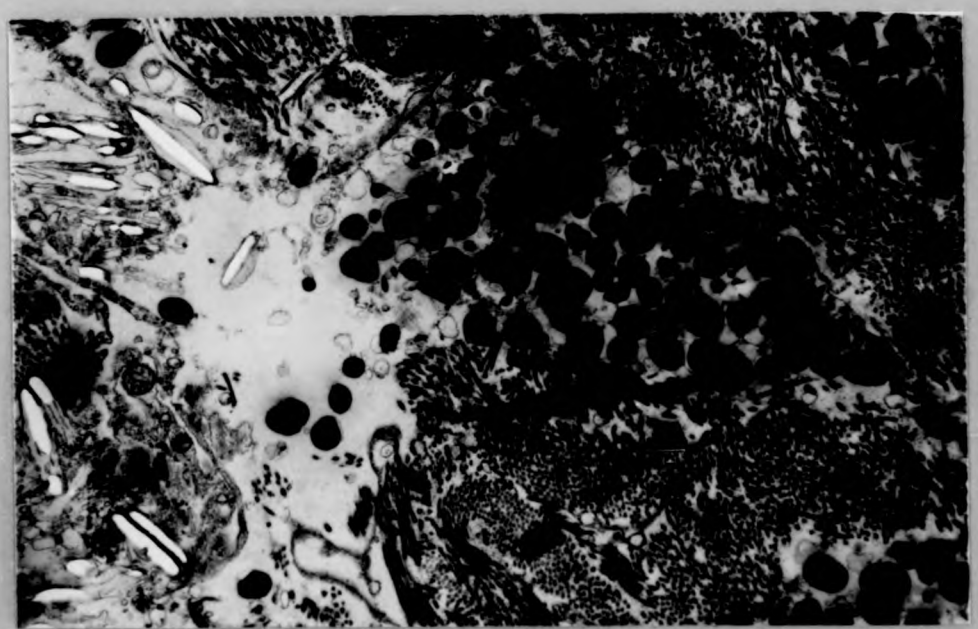
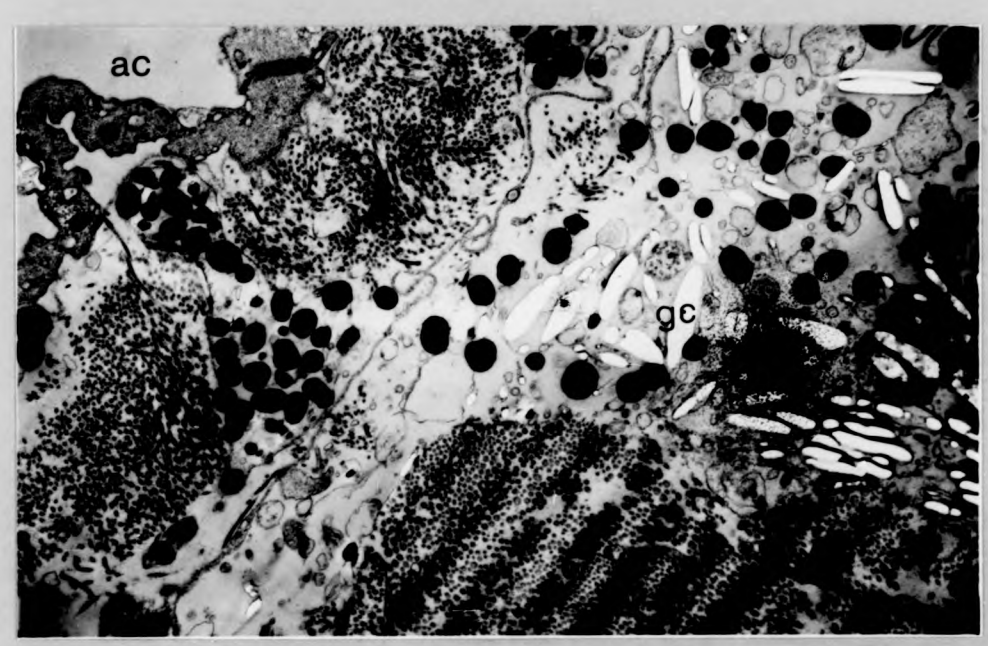


Fig. 7.56. Electron micrograph of the peritoneum of a cachectic plaice. Cachexia has led to increasing numbers of rupturing pigment cells and numerous free-lying melanin granules can be observed among the guanine crystals (gc). Melanin is potentially toxic to living cells, so presumably these granules will be quickly phagocytosed and possibly transported to the melano-macrophage centres. ac is the abdominal cavity.
(x 7,000)

Fig. 7.57. Electron micrograph of skeletal muscle from normal plaice. This is a longitudinal section through the myotomes; fibrils run obliquely and their bands are marked (Z, I, A, M). Interfibrillar spaces are occupied by membrane-limited sacs (arrows); these form part of the transverse tubular system.
(x 26,250)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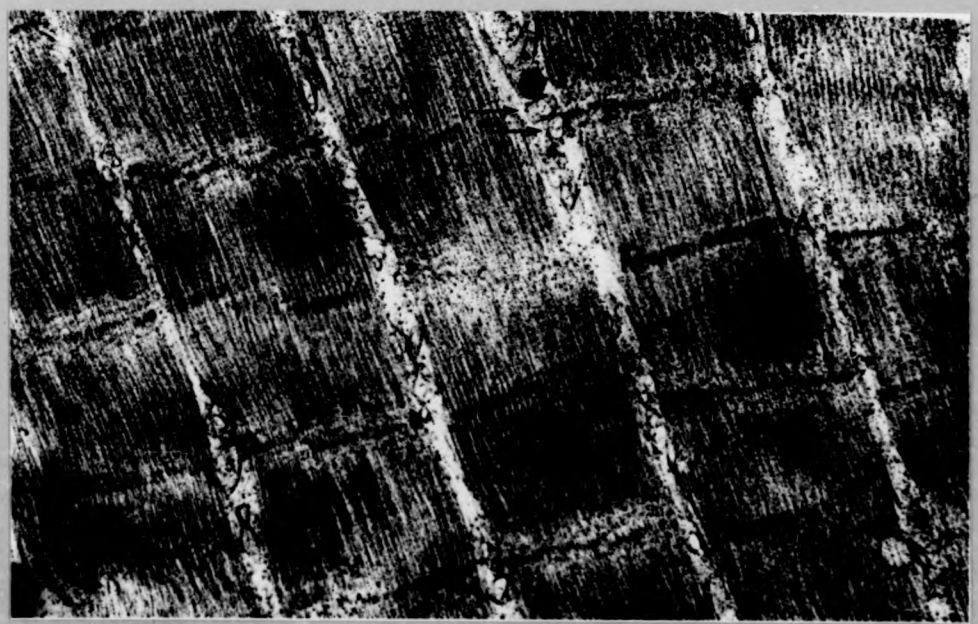


Fig. 7.58. Electron micrograph of skeletal muscle from a cachectic plaice. Degenerative changes have left little by way of recognizable structures; the fibrils have lost their continuity. The mitochondrion in this micrograph also seems to be degenerating.
(x 35,000)

Fig. 7.59. Electron micrograph of degenerated skeletal muscle from another cachectic plaice specimen. Note the almost complete destruction of the muscle.
(x 17,500)

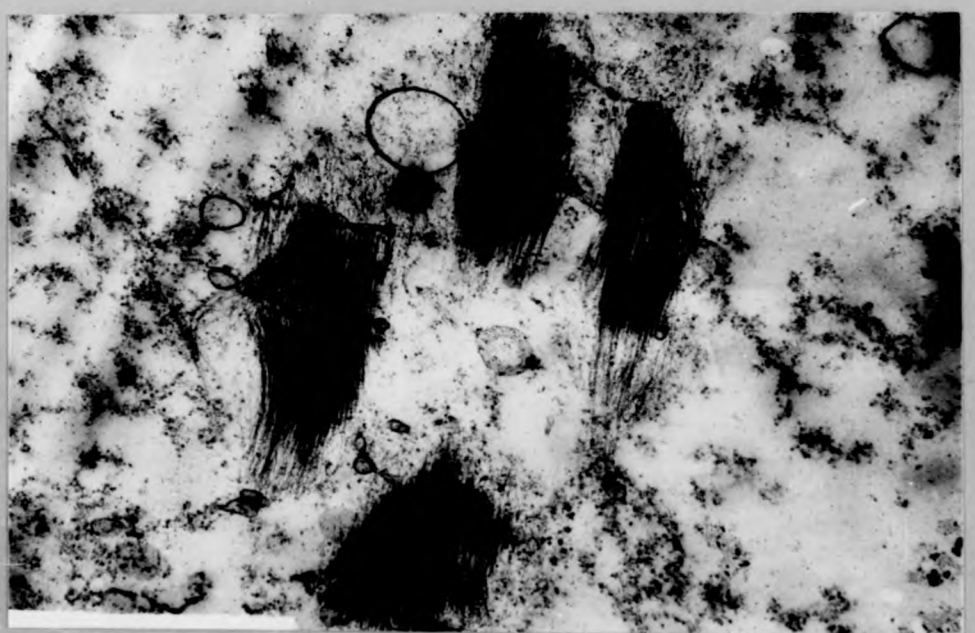
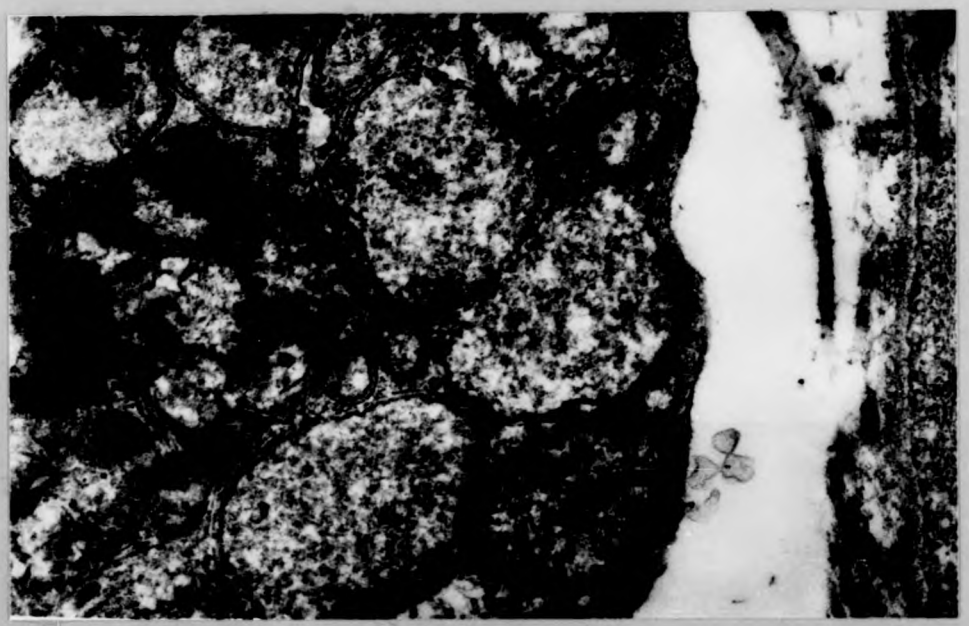


Fig. 7.60. Part of a macrophage observed amongst atrophied muscle of a cachectic plaice. This macrophage was heavily laden with bodies suggestive of mitochondrial origin (M). Lysosomes were also present, some of which contained a few melanin granules (arrow); a few others had inclusions of a fibrillar nature (double arrows). This macrophage was probably responsible for extensive phagocytosis of indigestible residues resulting from tissue atrophy.
(x 14,000)

Fig. 7.61. High power electron micrograph of a lysosome bearing pigment granules from the same macrophage shown in Fig. 7.60. Close by is a degenerating body suggestive of mitochondrial origin (M). Note also what appear to be remnants of degenerating rough endoplasmic reticulum (arrowed). Once replete with cell debris this macrophage will possibly find its way to a melano-macrophage centre.
(x 52,500)

Fig. 7.62. Another high power electron micrograph within the macrophage shown in Fig. 7.60. Several bodies suggestive of mitochondrial origin are present within the cytoplasm. Some still have what appear to be disorientated remnants of mitochondrial cristae (arrow).
(x 35,000)

Fig. 7.63. Splenic melano-macrophage from a cachectic plaice specimen. This electron micrograph shows a lysosome containing four pigment granules, three of which have been totally bleached by treatment with permanganate (5 minutes) and metabisulphite (15 minutes) prior to double staining. One granule has resisted bleaching. Note also how the rest of the lysosomal contents remain unaffected.
(x 26,250)



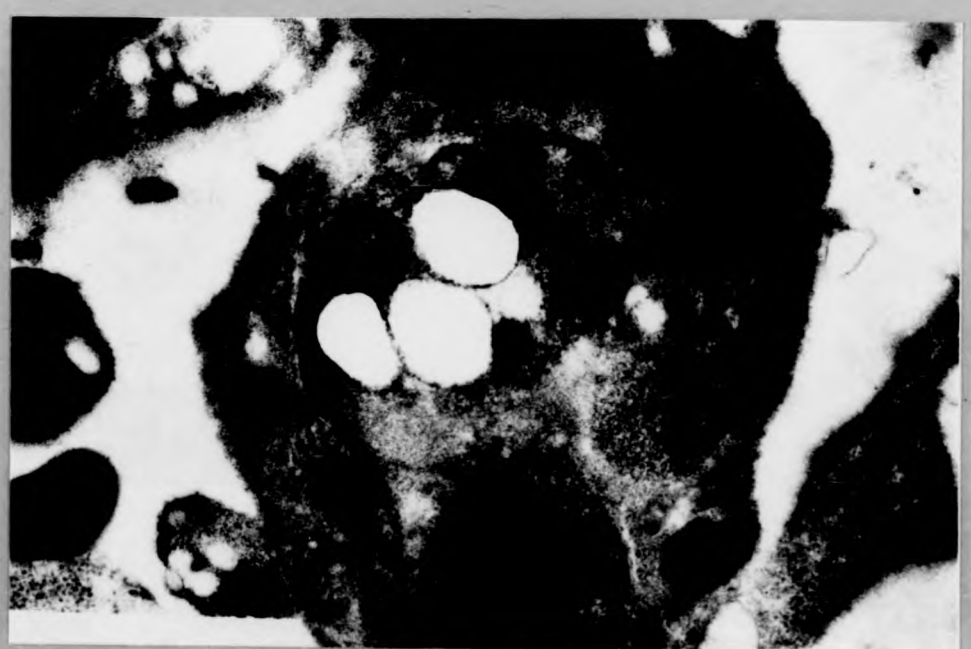
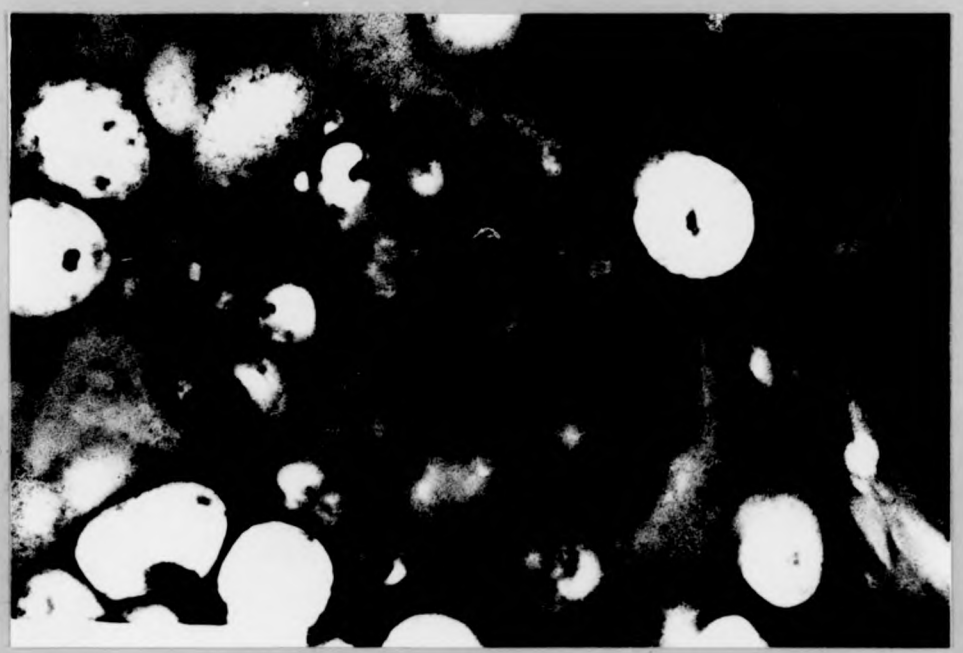


Fig. 7.64. Another lysosome within a splenic melano-macrophage of a cachectic specimen. This contains several pigment granules which have been totally bleached with permanganate (5 minutes) and metabisulphite (15 minutes) prior to double staining. The rest of the lysosomal contents are unaffected.
(x 35,000)

Fig. 7.65. Three fully bleached melanin granules within a lysosome of a splenic macrophage (arrowed). One of them (A) gives the impression of it being on the point of entering or leaving the lysosome. Note also the shape of granule (B); quite a few granules with that shape were observed. Section bleached with permanganate (5 minutes) and metabisulphite (15 minutes) prior to double staining.
(x 43,750)



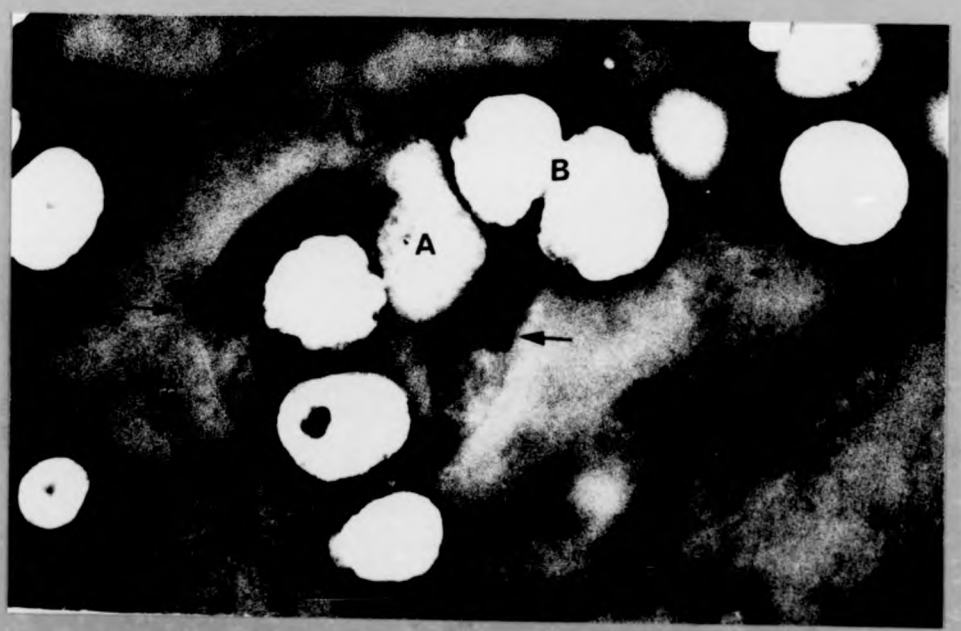

B
A

Fig. 7.66. Another example of a lysosome bearing pigment granules that have been fully bleached. This was observed within a splenic melano-macrophage of a cachectic specimen (5 minutes in permanganate; 15 minutes in metabisulphite; double stained).
(x 26,250)

Fig. 7.67. Splenic melano-macrophage of a cachectic specimen. Note the large lysosome with some fully bleached melanin granules in it. Most of the granules outside the lysosome have remained unaffected by the bleaching agents. Some melanin granules proved to be resistant to bleaching even after treatment for 5 minutes with permanganate and for 15 minutes with metabisulphite. Note also the large amount of debris in this lysosome.
(x 14,000)

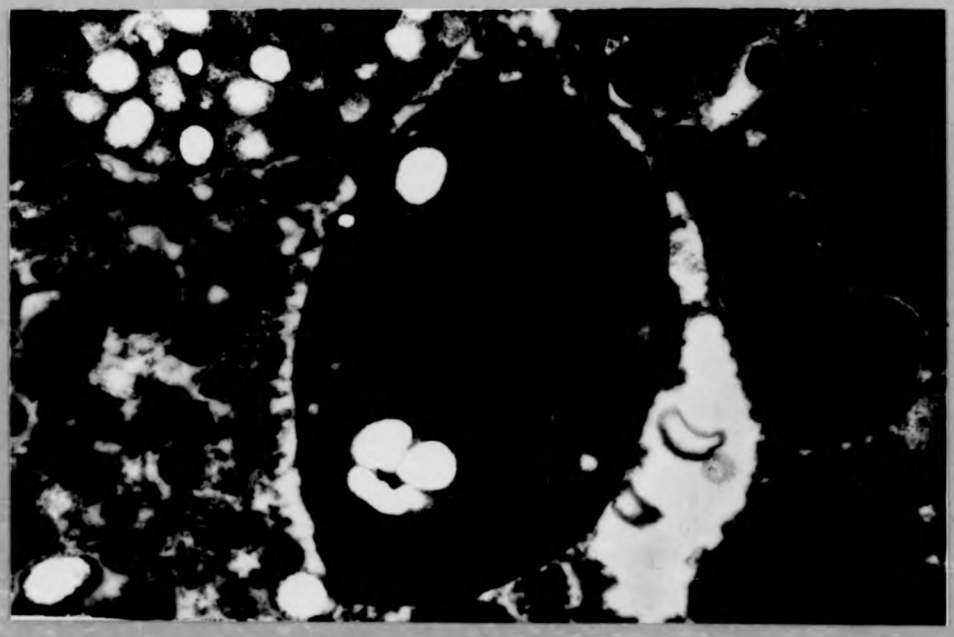


Fig. 7.68. High power electron micrograph of a pigment granule-bearing lysosome within a melano-macrophage in the anterior kidney of a cachectic specimen. The granule has been fully bleached by treatment with permanganate (2 minutes) and metabisulphite (20 minutes) prior to double staining .
(x 70,000)

Fig. 7.69. Fully bleached melanin granules within a lysosome of a kidney melano-macrophage from a normal plaice. This section was bleached by treatment with permanganate (5 minutes) and metabisulphite (15 minutes) and then was double stained. Also observe what appear like fibrillar processes within the lysosome.
(x 17,500)

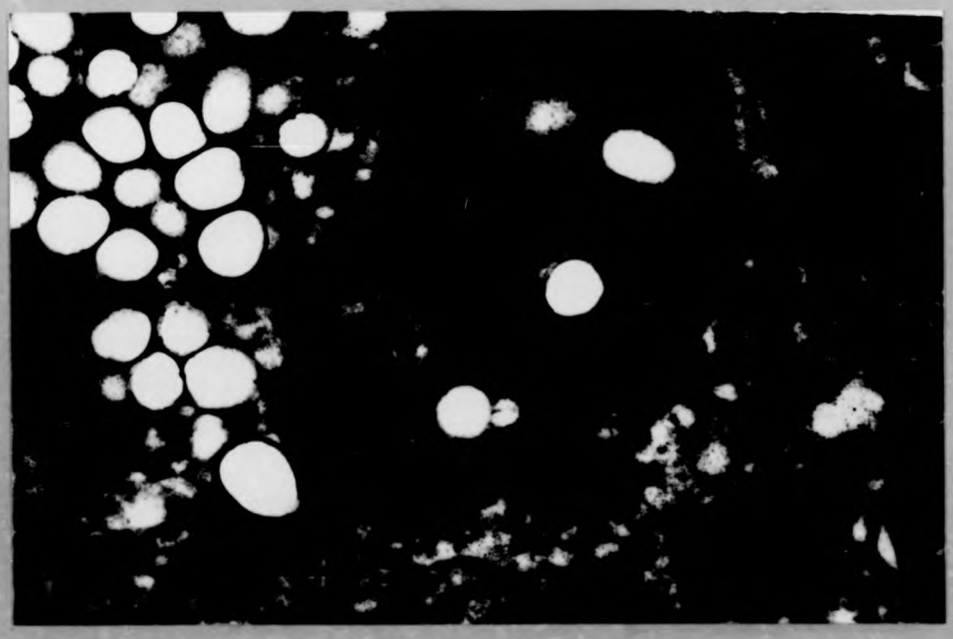


Fig. 7.70. A lysosome containing a fully bleached pigment granule from a kidney melano-macrophage of a control plaice. (10 minutes in permanganate, 30 minutes in metabisulphite, and double stained).
(x 52,500)

Fig. 7.71. Splenic melano-macrophage from a cachectic plaice. This section was treated with permanganate for 2 minutes and then with metabisulphite for 20 minutes prior to being double stained. Note that melanin granules in all stages of bleaching are present in this one cell.
(x 8,750)

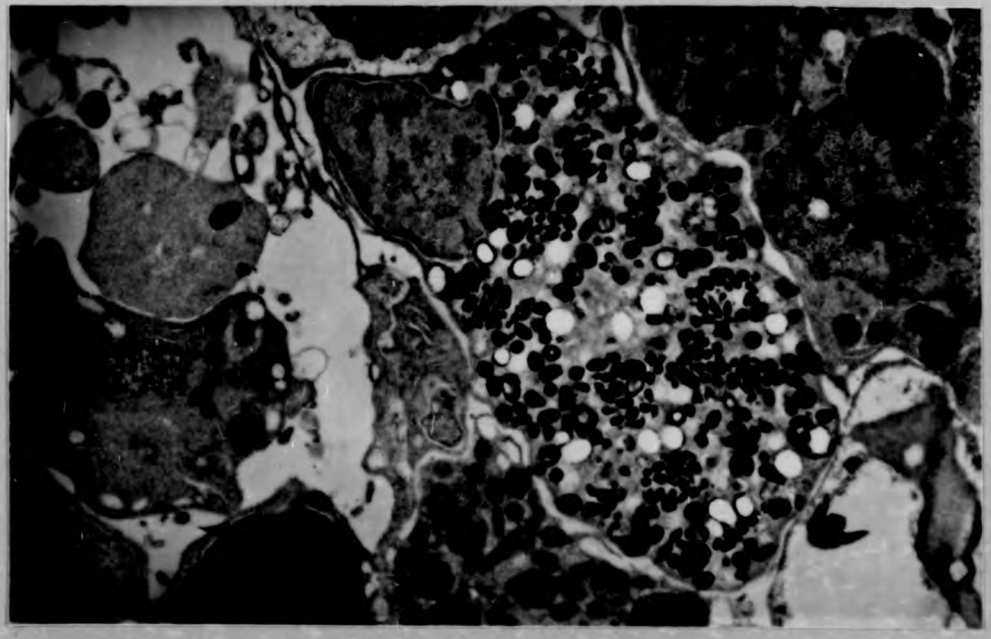
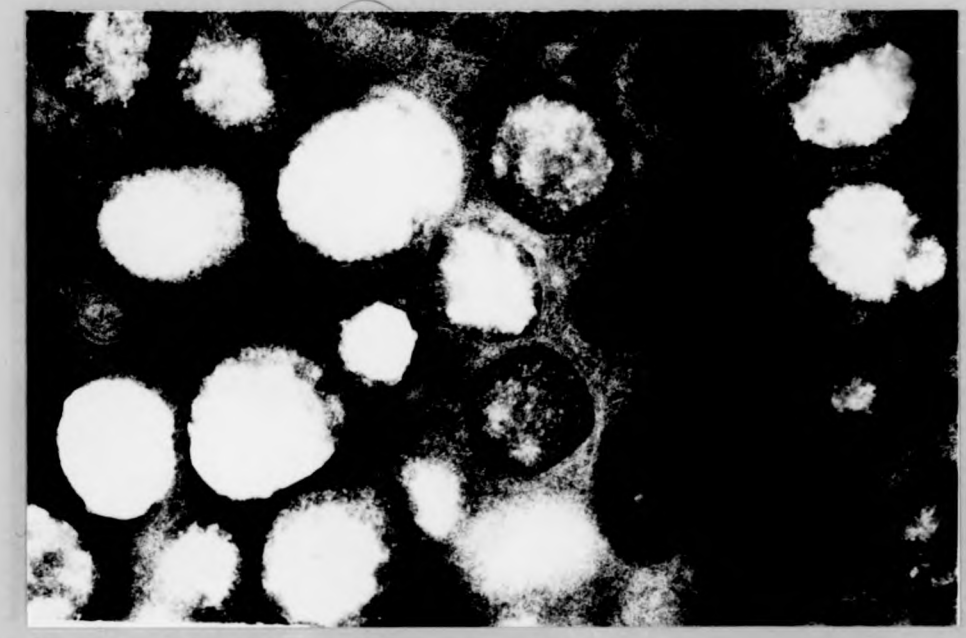


Fig. 7.72. High power electron micrograph of melanin granules within a splenic melano-macrophage of a cachectic plaice. Note the granules in different stages of bleaching. This section was treated with permanganate (2 minutes), metabisulphite (20 minutes) and then double stained.
(x 52,500)

Fig. 7.73. Low power electron micrograph of the anterior kidney of a cachectic plaice specimen. Several melano-macrophages are shown in this one field; this section was treated with permanganate for 2 minutes and with metabisulphite for 20 minutes. This resulted in about 50% of the melanin granules being bleached. After bleaching the section was double stained.
(x 3,500)



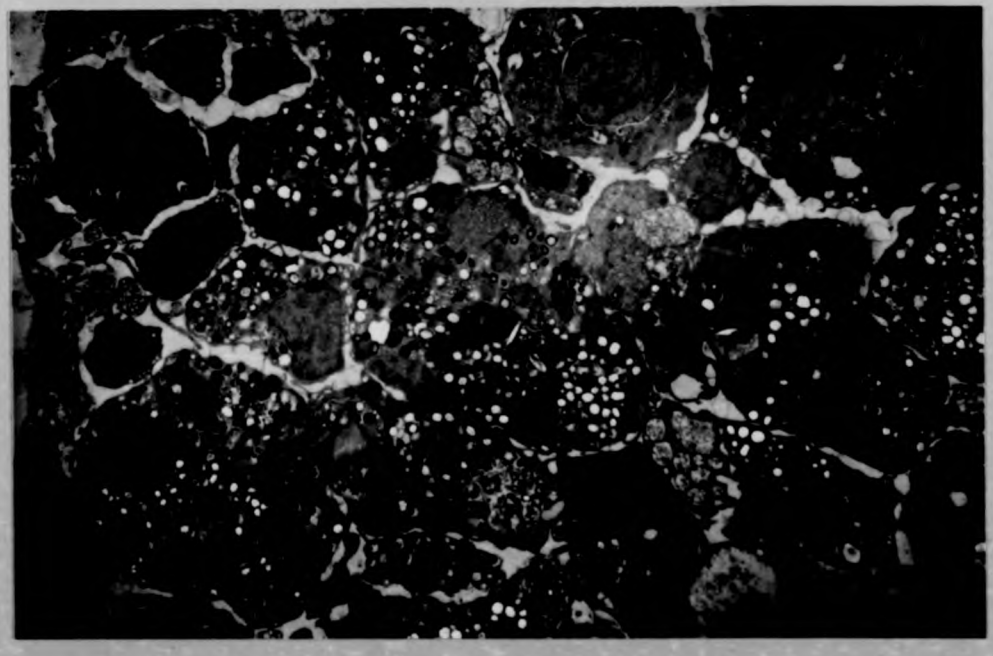
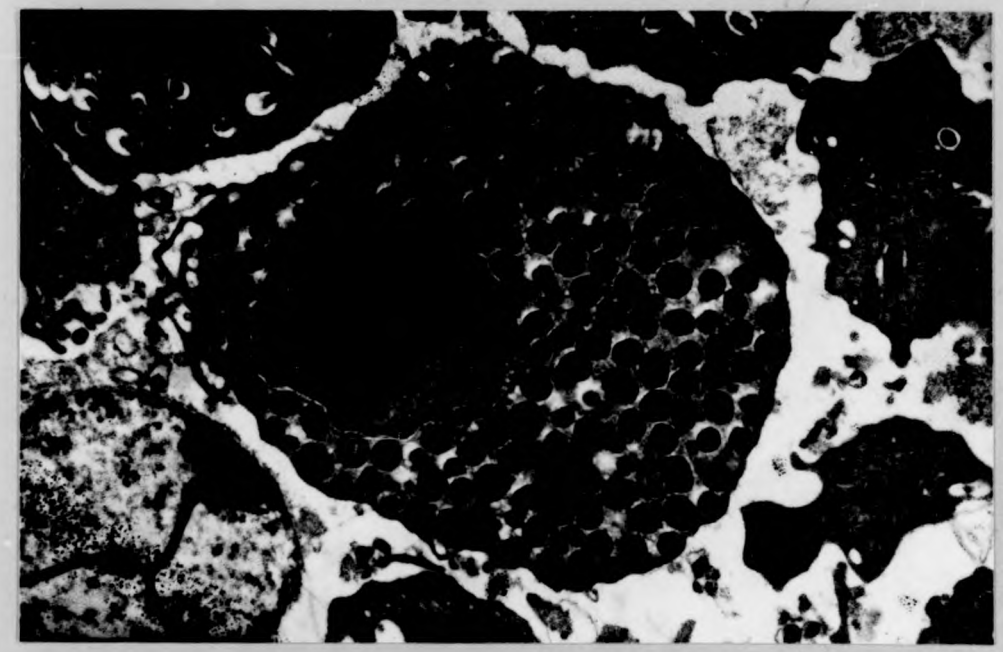


Fig. 7.74. Higher power electron micrograph of a melano-macrophage from same section from which Fig. 7.73 was taken showing that in this one very little of the pigment has been removed by the bleaching agents. This differential behaviour is perhaps indicative of slight differences in the nature of the contents of the pigment granules or in the way in which the pigments are bound.
(x 5,250)

Fig. 7.75. Some almost fully bleached melanin granules from a melano-macrophage of kidney from a cachectic plaice. This section was subjected to permanganate for 2 minutes and subsequently to metabisulphite for 20 minutes prior to staining. This one section contained fully bleached melanin granules as shown in this figure and others that had experienced no bleaching at all.
(x 43,750)



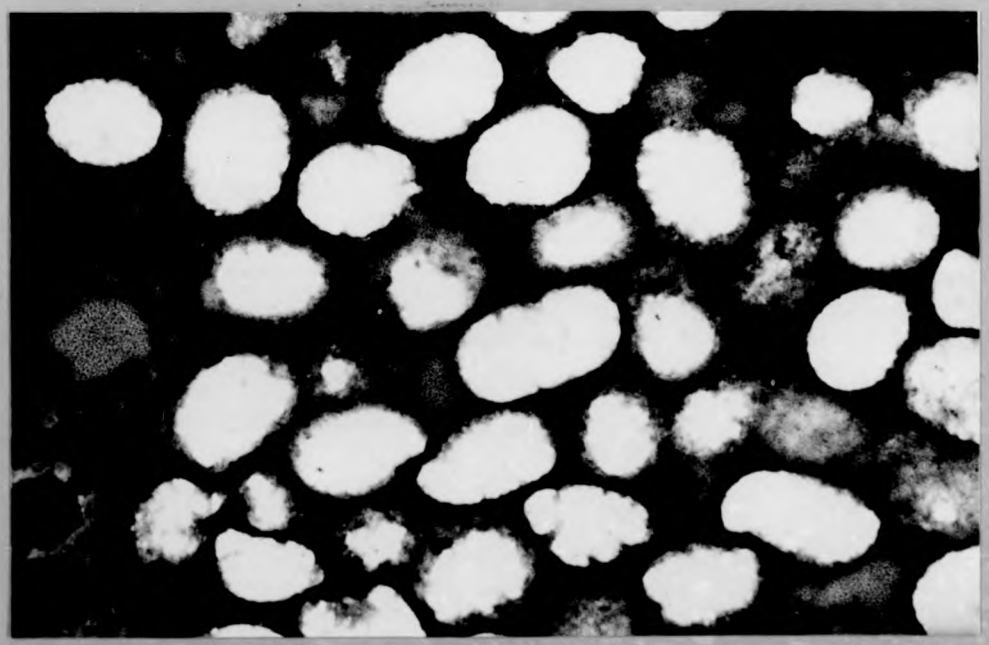
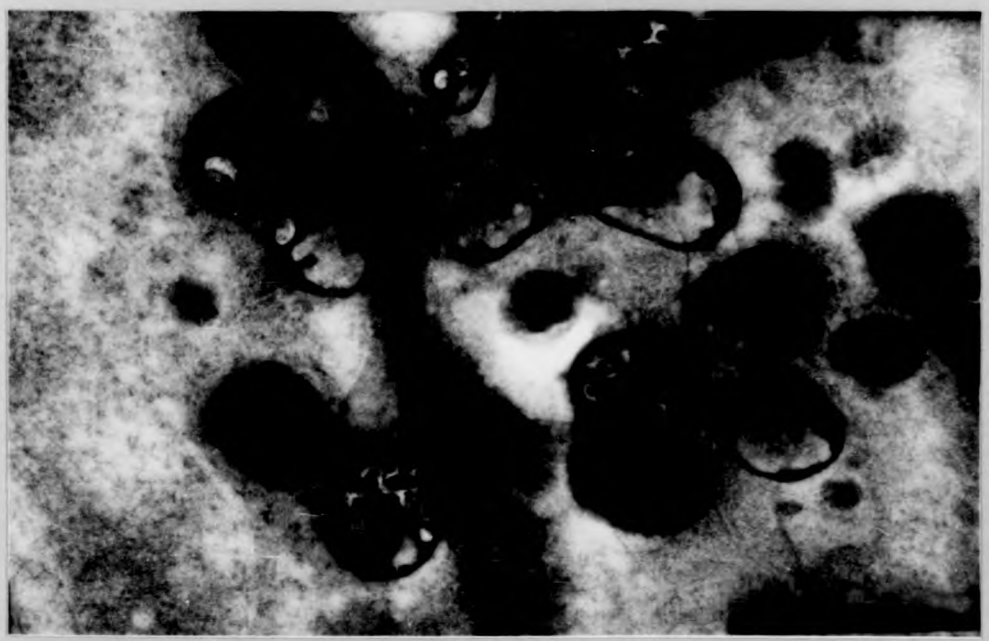


Fig. 7.76. Melanin granules within a melano-macrophage of a cachectic plaice. The section was first treated with permanganate for 5 minutes. A thin film of metabisulphite was then placed on it and was viewed under the electron microscope while the pigment was being bleached. This picture shows the granules in a semi-bleached stage. Note the variable and irregular shapes of the granules resulting in some of the granules being cut in several places. Observe also that the internal core is apparently easier to bleach than the outer shell.
(x 43,750)

Fig. 7.77. Low power electron micrograph of two splenic melano-macrophages from a cachectic plaice. All the pigment granules have experienced total bleaching following treatment with permanganate (10 minutes) and metabisulphite (30 minutes).
(x 5,250)



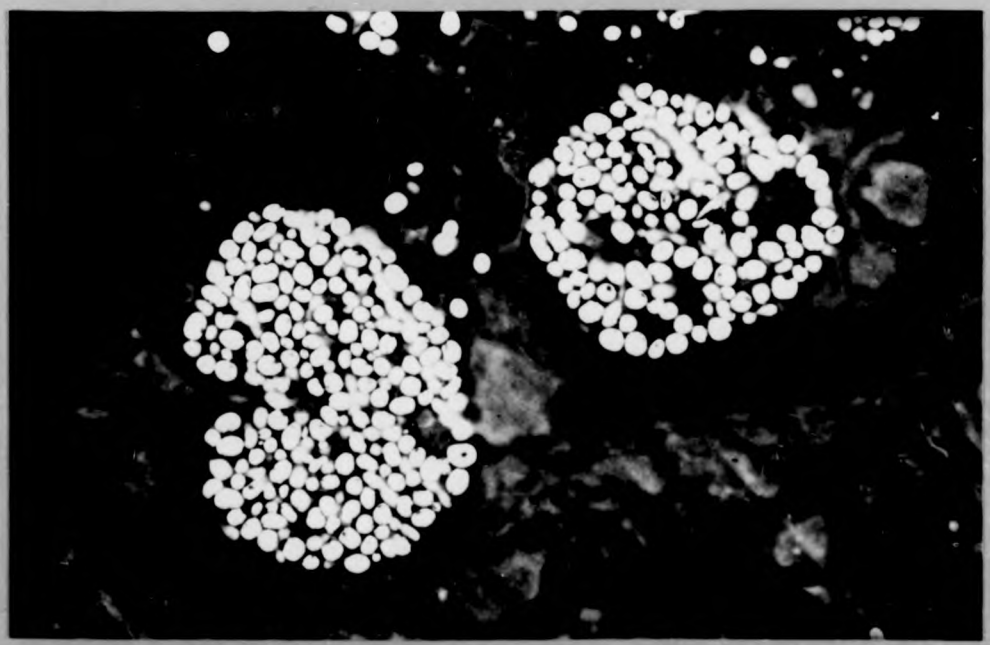
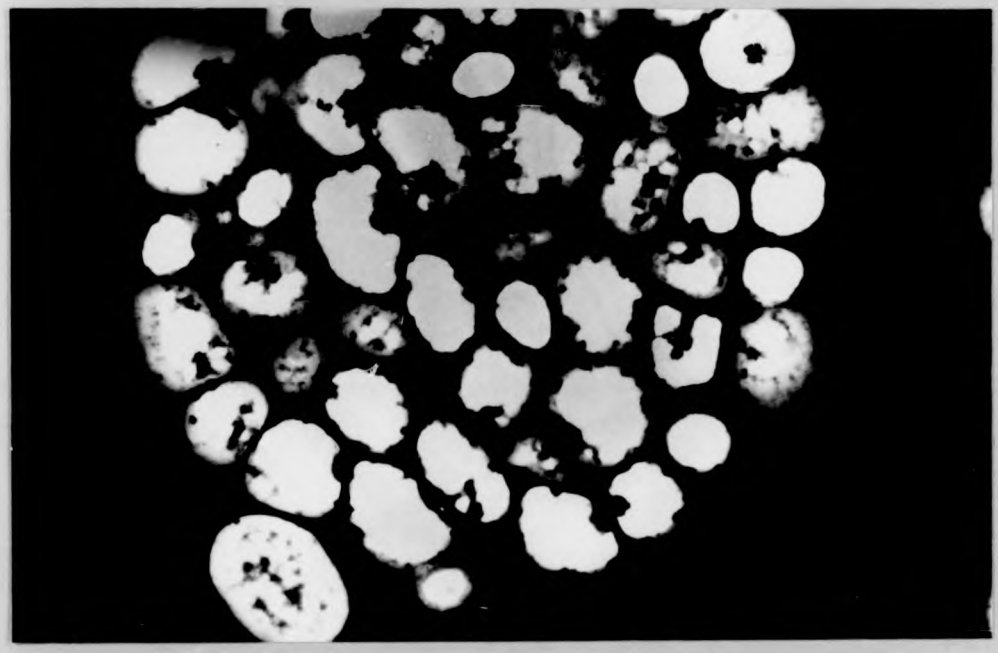
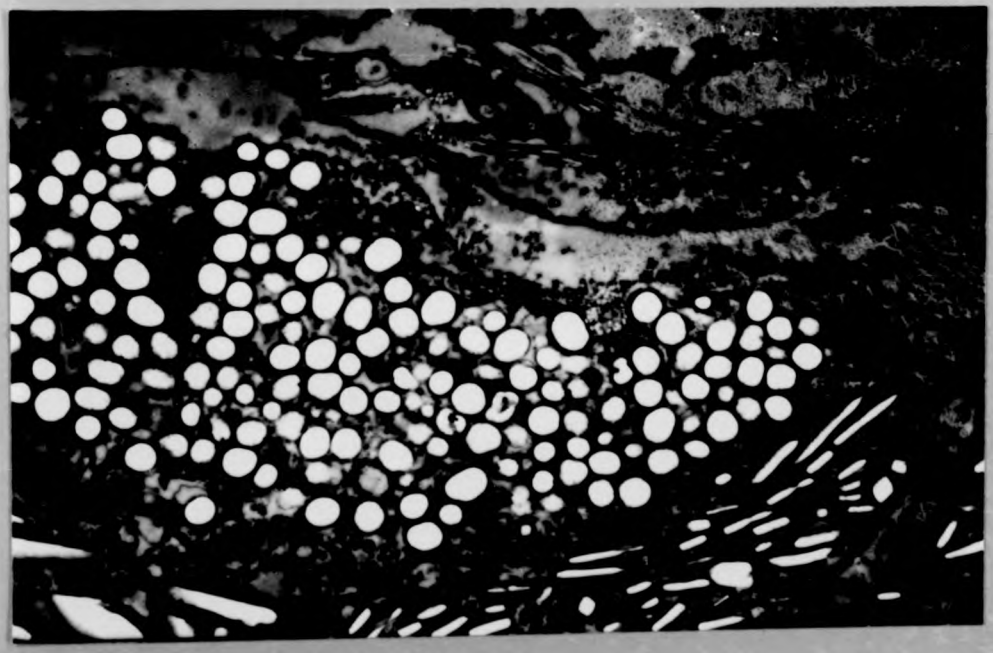


Fig. 7.78. A mass of pigment granules in a large vacuole-type body of a splenic melano-macrophage from a cachectic plaice. Observe that while the central core has been completely bleached, small amounts of electron dense material are still evident on the outside of the granules.
(x 26,250)

Fig. 7.79. Part of a skin melanophore in which all melanin granules have been fully bleached by treating the section with permanganate for 5 minutes and metabisulphite for 15 minutes prior to double staining. This section was taken from a normal plaice specimen.
(x 8,750)





peroxidation. Melanin, or at least some of it, is seemingly derived more simply from phagocytosis of melanin granules and/or their precursor organelles from normally occurring melanin-containing cells that have ruptured or otherwise been damaged by some noxious agent. Haemosiderin is almost certainly derived through breakdown of haemoglobin from effete erythrocytes. Such degenerative processes are seemingly occurring all the time in normally functioning tissue, being in fact an essential component of wear and tear processes. Under cachectic conditions however the catabolic processes outweigh anabolic ones leading to the observed accelerated accumulation of these pigments.

A large body of literature has been published on the fine structure and biochemical composition of mammalian pigment granules especially those of human origin (see for example Montagna and Hu, 1967). The normal melanosomes of mammals are composed of a system of coiled helical filaments and of folded sheets upon which melanin is deposited. As melanin gradually accumulates, the melanosome is eventually transformed into a uniformly dense and structureless particle called the melanin granule. In contrast, very few similar investigations have been carried out on fishes. Turner et al. (1975) were the first to investigate the ultrastructure of melanosome formation during melanocytogenesis in fishes. They noted that in goldfish the internal lamellar matrix and the system of coiled helical filaments characteristic of mammalian melanosomes were absent. Instead they observed a previously undescribed premelanosome, a multivesicular body derived by fusion of large vesicles blebbed

from rough endoplasmic reticulum and Golgi-derived small vesicles. Each large vesicle fuses with several small ones; the latter, containing the enzyme tyrosinase, fuse with the membrane of the large vesicle, invert and reform within the large vesicle exposing the tyrosinase. Melanin synthesis begins around the periphery of the inverted vesicles and eventually fills the intervesicular spaces and finally the inverted vesicles. Thus the electron transparent spaces of the multivesiclar bodies represent the lumens of the internal vesicles. The failure in the present study to detect within plaice melanosomes any internal structure characteristic of mammalian melanosomes together with the finding of multivesicular bodies very similar to those reported in goldfish raise the possibility that the process of melanocytogenesis in fish may differ slightly from that in higher animals. The fact that all three types of granules observed in the melanophores of plaice were also present within melano-macrophages perhaps provides added proof to the hypothesis of their having been phagocytosed after release from the classical pigment cells of the body unless melano-macrophages possess a similar capacity for melanocytogenesis for which there is absolutely no evidence as yet.

The multivesicular bodies reported in goldfish by Turner and his coworkers as well as those observed in the present study are quite similar in appearance to multivesicular bodies in other cell types. Many investigations have attempted to elucidate the function and origin of these cellular organelles found in rat vas deferens, anterior pituitary, renal glomerulus, epididymis,

neurones and liver (Friend, 1969). Moreover, various workers have proposed theories to account for the mode of assembly of these bodies. (See for example Friend, 1969; Gordon et al., 1965; and Novikoff et al., 1968).

The granules with less regular outlines and irregularly shaped patches of different electron densities possibly represent intermediate stages in the transformation of damaged cellular components to lipofuscin granules. However, interpretation regarding the exact nature of these and other granule types are rather complex especially since it has not as yet been determined whether the degree of melanization is the only factor that determines the electron density of the melanin-containing organelle (Fitzpatrick et al., 1967). Thus for example Mishima (1966) noted that the electron density observed in human melanosomes might be due in part to the accumulation of zinc and copper within these organelles. It seems as though melanin is a type of chelating agent and can bind metallic ions from the surroundings (Lane Brown, 1966). Biochemical analyses of the melanin granules of melano-macrophages would of course provide the answer but these have as yet to be performed.

Formation of lipofuscin pigment appears to involve peroxidation of polyunsaturated lipids of subcellular membranes (Chio et al., 1969). Its formation has also been inversely correlated in experimental animals with the vitamin E content of the diet (Tappel, 1972). Fish, with their high content of unsaturated fatty acids (Sargent, 1976) and relatively low levels of vitamin E (Dicks,1965: cited in Tappel, 1975) are particularly

prone to the formation of lipofuscin.

How cellular organelles and constituents become indigestible can be explained by the process of lipid peroxidation, which produces malonaldehyde - a good tissue fixative. Malonaldehyde reacts with primary amino groups of proteins, phospholipids and nucleic acids to form a Schiff-base product which has fluorescent and excitation maxima very similar to those of lipofuscin (Chio et al., 1969). The rate at which lipid peroxidation occurs is directly dependent on the oxygen concentration. It is perhaps ironic that oxygen, the fuel of electron transport, can be the destroyer of unsaturated lipid membranes if these are left unprotected. In a way incompletely understood, α-tocopherol enters into the structure of highly unsaturated phospholipid membranes and reduces their vulnerability to free-radical attack (Lucy, 1972; Tappel, 1972). According to Gruger (cited by Tappel, 1972) α-tocopherol inhibits peroxidations by furnishing hydrogen atoms to free radicals. However, it needs hardly be emphasized that the reactions of and the damage caused by free radical peroxidation of lipids are complex topics, and in a relative sense the mechanisms of damage are not well understood, although they appear to have counterparts in radiation chemistry (Tappel, 1972). Moreover, although vitamin E has been under continuous investigation for a long time, there is still some disagreement as to its biological role; for instance its role as an antioxidant has been strongly debated (see for example Green, 1972 and Schwarz, 1972).

A large body of data dealing with the fatty acid composition

of subcellular organelles has been reviewed by Fleischer and Rouser (1965). They concluded that mitochondria always have a relatively high content of polyunsaturated fatty acids when compared with other subcellular organelles. Moreover, while there are encountered wide variations in the specific polyunsaturated fatty acids, in fish the acids of the linolenate family predominate while in higher animals, linoleic, arachidonic and docosahexaenoic acids are the most commonly encountered. The higher double bond character of fish mitochondrial lipids is in keeping with the fact that fish body temperatures are generally lower than those of warm-blooded animals since fluidity is presumably more readily maintained with increase in unsaturation. This will be discussed more fully in the final chapter.

Evidence that in higher animals mitochondria can be transformed into granules of lipofuscin has been accumulating (Toth, 1968; Glees, 1975; Ahmed and Glees, 1977; Koobs et al., 1978). The pigment has been shown to arise from peroxidative destruction of mitochondrial polyunsaturated lipid membranes. Chio et al.(1969) described the in vitro formation of lipofuscin from isolated rat liver mitochondria as a function of lipid peroxidation; this, he found, could be prevented by an anti-oxidant in the incubation medium. In mammals the chemical characteristics of lipofuscin are consistent with a mitochondrial origin in view of the highly unsaturated nature of the phospho-lipid in these membranes, the richness of enzymatic protein, and the presence of nucleic acids in these organelles (Koobs et al., 1978)

Moreover, various studies of the oxidation rates of isolated subcellular organelles have shown that mitochondria peroxidize faster than most other organelles. These differences were found to correlate both with differences in lipid composition and with the proximity of catalysts of peroxidative reactions such as iron compounds (see for example Tappel, 1975 and references therein).

It is very interesting to mention here that Woods et al. (1963) expressed the view that the melanin granules of mammalian melanocytes represent modified mitochondria that have acquired melanin in the course of a specialized ontogeny. According to this view the divergence between differentiation as a melanin granule or as an ordinary mitochondrion could begin at a premitochondrial stage. Any connection between these various ideas, if there is any, remains highly speculative at this stage.

The author believes that the electron micrographs presented in this study support the concept that lipofuscin can be derived from mitochondria. Numerous mitochondria-like bodies were observed within melano-macrophages; these ranged from the still viable to the highly degenerate ones that resembled to a considerable extent lipofuscin granules. These were observed to be dispersed among pigment granules and phagocytosed cellular debris; their presence in lysosomes or in the large vacuole-type bodies could not be ascertained. At times these degenerating mitochondria-like bodies still contained membrane remnants reminiscent of cristae.

In this study a substantial proportion of the pigment

granules within melano-macrophages were observed to be held within lysosomes. It seems as if all pigment granules, irrespective of whether they arise from degenerating cellular organelles, from phagocytosis of melanin granules (and their precursor organelles) or from other as yet unrecognized sources, come into lysosomal contact at some stage or other. How such bodies would be modified within the lysosomes and whether any useful substrates are extracted remains to be determined. Evidence for such an association of pigment granules with lysosomes has been growing since the advent of the electron microscope. Thus for instance Ehrlich et al. (1960) and Essner and Novikoff (1960) have shown that hepatic lipofuscin granules may be deposited in lysosomes within hepatocytes. Moreover, Brandes (1966), in his account of the various stages by which lipofuscin bodies may evolve from lysosomes in mouse macrophages, published electron micrographs of mitochondria undergoing degradation within autophagic vacuoles. Alterations of normal metabolic pathways had been induced in the mice under study by the alkylating agent Cytoxan. Toth (1968) in reviewing the possible modes of origin of lipofuscin pigments arrived at the conclusion that lysosomes represent the ultimate source of lipofuscin and that lysosomal enzyme activity can account for the apparent formation of lipofuscin from all other cell organelles. A number of investigators consider lipofuscin granules to be a special type of lysosome and believe that they represent residual bodies (Bloom and Fawcett, 1970; Brunk and Ericcson, 1972); others have failed to demonstrate lysosomal enzymes in these structures (Brunk and Ericcson, 1972).

The concept of lipofuscin being regarded as the indigestible residue of lysosomal enzymatic activity does not indicate whether the quality of indigestibility is present before or after lysosomal contact. Because lysosomes have only hydrolytic enzymes, which degrade stepwise the macromolecules of organelles, indigestibility is likely to be acquired prior to lysosomal encounter (Robbins, 1974); as has already been discussed, this indigestibility is acquired through lipid peroxidation. The association of lysosomes with lipofuscin granules therefore can be interpreted as a frustrated attempt to recycle the components of organelles that are damaged by peroxidation (Koobs et al., 1978).

The present observations seem to substantiate those reported for higher animals. The finding of melanosomes concentrated within lysosomes has been reported to occur in dermal melanophages of humans suffering from a type of melanoma known as Dubreuilh's melanosis (Mishima, 1967). These melanophages, while rich in acid phosphatase-positive lysosomes, possess no tyrosinase activity indicating that the melanin has been phagocytosed. According to Mishima, the melanosomes phagocytosed within the lysosomes undergo degradation by the action of the contained hydrolytic enzymes. Furthermore, this same worker provides evidence of an actively melanosome-synthesizing cell of malignant melanocytoma which is concentrating and degrading melanosomes with the formation of lysosomes. Thus, under these abnormal conditions, lysosomes also occur in melanin synthesizing cells.

Drochmans (1967) provides evidence that in human pigmented tissues, melanin granules within lysosomes of dermal melanophages are broken down with release of melanin particles from the melanin granules. It is suggested that the particles are then reduced into smaller units and eliminated in the extracellular medium. As with the present study, Drochmans did not verify the presence of acid phosphatase to prove the lysosomal nature of the inclusions bearing melanin granules. Whether a similar process occurs in melano-macrophages remains to be ascertained.

Lysosomes whose entire structure seemed to be transforming into a lipofuscin granule were also observed in this study. The possible transformation of lysosomes to lipofuscin granules was inferred by Brunk and Ericsson (1972) who worked on the lipofuscin bodies in the neurones of the rat cerebral cortex and pontine ganglia. They observed that these bodies contained acid phosphatase and noted the presence of apparently transitional forms between autophagic vacuoles and lipofuscin-like bodies indicating that the lipofuscin bodies represented the residual body type of lysosomes resulting mainly from cellular autophagy. They attributed the accumulation of these bodies to the inability of the cells to exocytose residues accumulating in lysosomes.

The nature of the large vacuole-type bodies laden apparently exclusively with pigment granules has still to be worked on. The most likely explanation seems to be that they correspond to what de Duve and Wattiaux suggested be called telolysosomes or post-lysosomes. These develop from secondary

lysosomes which are replete with residues of digestion. Such bodies do not show the characteristically high enzymic activities expected on the basis of their lysosomal origin. (De Duve and Wattiaux, 1966). Moreover, they are commonly much larger than the lysosomes normally present in the same type of cell. There are three possible explanations here: that lysosomal digestive residues accumulate progressively in a secondary lysosome, that residues separated from lysosomes can aggregate, or that fusion of several residue-laden lysosomes occurs (Daems et al., 1972). Only further research will resolve whether any of these mechanisms operate in fish melano-macrophages.

The suggested possibility that the pigment granules within melano-macrophages originated from various different sources and the fact that they (or at least the vast majority of them) appeared rather similar in gross morphology are perhaps difficult to reconcile. Published electron micrographs of lipofuscin granules presumed to be derived from mitochondria in higher animals show that these granules differ slightly in appearance from any of those observed in the present study. Those described by Koobs and his coworkers (1978) who worked with material of human origin, were irregular in outline and irregular clumps of electron dense material were dispersed throughout the fine granular matrix material. The lipofuscin bodies of Brunk and Ericcson (1972), thought to have been derived from lysosomes, were in general irregular in shape and contained lipid droplets and clumps of electron dense material in the fine granular matrix material; their appearance however varied slightly depending on the age

of the rat and the method employed for processing the tissues. A variety of reasons could possibly account for these apparent discrepancies. Primarily cells from different tissues and from very different animal groups are being compared. Moreover, the sequence of events that leads to the formation of the fully mature lipofuscin granule is largely unknown so that we may well be looking at different stages of one and the same process of degenerative transformation from damaged cell components to lipofuscin granules. The possibility that slightly different techniques employed by the various workers in the preparation of their material for electron microscopy could account for some of the differences observed must not be overlooked. With regard to this latter point, Mishima (1966) holds that in human tissues variations in the gross morphology of melanosomes following various fixation methods are minimal although the appearance of the internal structure may be affected. It is interesting to note that Mishima also concludes that the majority of melanosomes and melanin granules in various diseases are distinctly different and can be identified with a high degree of certainty in most cases.

While the increased dendriticity of the macrophages within the centres of starving plaice is highly indicative of intense phagocytic activity, this study has produced little direct evidence of phagocytic activity of effete organelles in the skin, peritoneum and muscle during cachexia. This could be partly due to the fact that such processes like ingestion and fusion between phagosomes and lysosomes are not very often caught by electron

micrographs (Daems et al., 1972). Possibly more information could have been obtained had material for ultrastructural study been taken at more regular intervals. Perhaps as occurs in amphibian tadpole tail metamorphic atrophy cell injury was so overwhelming that autophagy could not be employed to eliminate the damaged cells (Helminen, 1975). There is in the literature, however, much evidence of the scavenging function of fish macrophages. Such examples include the phagocytosis of debris in trout tissue infected with infectious pancreatic necrosis virus (Wolf and Quimby, 1969); phagocytosis in situations of experimentally induced inflammation provoked by various agents such as bacteria and adjuvants in rainbow trout (Finn and Nielsen, 1971) as well as adjuvants and carrageenin in plaice (Timur, G. et al., 1977; Timur, M. et al., 1977); in the clearance of cellular debris caused by parasitic Iernaeid copepods in the white bass, Morone chrysops (Joy and Jones, 1973); in muscle damaged by tagging Atlantic salmon parr (Roberts et al., 1973) and in the phagocytosis of carbon particles in cunner, plaice, turbot and other teleosts (Mackmull and Michels, 1932; Ellis, 1974; Ferguson, 1976a).

In the course of the present study, macrophages laden with lysosomes and what looked like degenerating mitochondria were occasionally seen among degenerating muscle cells; occasional pigment granules were sometimes contained within these macrophages. After sixteen weeks of complete starvation the spleen and kidney carried much higher numbers of macrophages than those of the control specimens. The majority of these were

replete with cellular debris, lysosomes of varying sizes and some, especially those of the melano-macrophage centre region, carried considerable numbers of pigment granules. Very occasionally, macrophages with pseudopodial extensions in the process of phagocytosing cell debris were observed within the pulp areas of the spleen and the kidney. Before the whole sequence of events linking tissue atrophy with enlargement of the centres can be worked out, however, there is a dire need to resolve whether damaged cell components are phagocytosed immediately and transported to the lymphoid organs within the macrophages or whether they are transported by the blood stream and engulfed by such components of the endothelial system as the macrophage sheath of the splenic ellipsoids. Obviously, the sooner damaged organelles are disposed of the better, since malonaldehyde, a by-product of lipid peroxidation, can react with nuclear DNA blocking template activity. Nuclear damage of this kind could reduce the capacity for protein synthesis and limit mitochondrial and contractile protein replacement (Koobs et al., 1978). The eventual aggregation of melanin-bearing macrophages into centres that can possibly be completely separated from the surrounding lymphoid tissues may well be of importance in protecting tissues from the potential toxicity melanin pigments are known to possess.

Very few records of attempts to bleach pigment granules could be traced. Laxer et al. (1954) described the appearance of melanin granules isolated from wool by digestion with phenolthioglycollic acid mixtures and subsequently treated with H_2O_2. The granules were reduced in size and had an irregular

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surface suggesting that the peroxide had removed a superficial shell of melanin. Birbeck and Barnicot (1959) examined thin sections of the shaft of a mature black hair after bleaching in H_2O_2. They found no evidence that the surface of the granule was preferentially attacked. Drochmans (1967) employed potassium permanganate to bleach melanosomes of human origin. In favourable conditions he could distinguish a fine structure within the cortical region. This fine structure consisted of small granules arranged in a regular pattern and more or less aggregated in units 30 - 50 nm in diameter; these units he called melanin particles. Drochmans proposed that this regular assembly of such particles may be related to the structure of the melanin polymer.

No such fine structure of the cortical region or indeed any details of internal structure could be observed in any of the pigment granules examined in the present study. The bleaching treatment did demonstrate however that the outer osmiophilic cortex is harder to bleach than the core. Partial bleaching also showed that the granules particularly those within melano-macrophages could be of very diverse shapes. Moreover, in the splenic and renal melano-macrophages of cachectic specimens a proportion of the pigment granules were more readily bleached than others. During cachexia the formation of pigment granules was greatly accelerated and those pigment granules that proved easier to bleach could well represent the newly-formed ones where oxidation had not progressed to any large extent. It is possible that the degree of oxidation of the pigment precursors

dictates the ease with which they can be removed by bleaching. In fact numerous studies have shown that the fine structure of age pigment becomes increasingly complex as the result of progressive and prolonged autoxidation of unsaturated lipid precursors, which accounts for alterations in various histochemical tests and increasing insolubility (Toth, 1968).

CHAPTER 8

GENERAL DISCUSSION, CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

Macrophages participate in the defence of the organism in a number of ways. They remove debris during tissue involution and repair, they destroy micro-organisms by phagocytosis, they may take up and process antigens, they may kill antigenically deviant cells notably neoplastic cells and they may actively secrete various substances such as lysozyme and interferon. While only further research can establish whether fish melano-macrophages are capable of carrying out all these functions, the present study has shown that melano-macrophage centres do indeed execute a multiplicity of functions. At the two extremes, they seem to be of a lympho-reticular nature as well as act as dumps for a wide range of materials. The term "dump" however needs much qualification in the light of the probable recycling of various substances such as iron compounds.

The phylogenetic and ontogenic studies described in this thesis provide additional evidence that melano-macrophage centres are of a lymphatic nature possibly representing the primitive analogues of germinal centres of birds and mammals. One property attributable to germinal centres is that they contain areas of degeneration and accumulation of noxious substances (Congdon and Hanna, 1967). In fact, large phagocytic cells containing cellular debris are often observed within these

structures (Congdon, 1969; Chen et al., 1978). The present study has amply demonstrated that cellular degeneration is a major feature of fish melano-macrophage centres. These results undoubtedly invite further intensive investigation of the immune processes occurring within the centres and the associated lymphoid tissue. Most of the available evidence at present is based on purely anatomical grounds and only Ellis (1974, 1979) and Ellis and de Sousa (1974) have examined such immune phenomena as the role of the centres in antigen-trapping and the recirculation of lymphocytes through them. The major findings of these workers have already been reviewed.

From the available evidence it is clear that considerable amounts of materials such as iron compounds are recycled and are only stored in the centres temporarily, presumably more so under stressful conditions. Thus the centres should be regarded as sites where a variety of materials are aggregated, processed, sifted and disposed of in a number of ways rather than regarding them as static areas passively accepting and storing indefinitely any material that comes their way. Carbohydrate, protein and lipid moieties as well as iron can all be re-utilised. It would seem then that only those compounds that are highly insoluble and which the animal otherwise has difficulty in handling (such as melanin and lipogenic pigments) accumulate gradually in the centres. It is probable that such pigments or at least some of them if formed elsewhere in the body eventually also accumulate in the centres. In cirrhotic livers of rats much of the ceroid produced by the liver

eventually ends up in lymph nodes (Hartroft and Porta, 1965). In humans, lung tissue may be cleansed of inhaled matter very rapidly and eventually ends up in lymph nodes (Stuart, 1970).

On the basis of such observations one would expect similar distinct aggregates of pigmented cells to be found in the lymph nodes of higher animals; the available evidence indicates that this is not the case. The reasons for this could well lie in the differing abilities of animals to control their body temperature. As they occur in animal tissues, saturated fats are solid at ordinary temperatures and unsaturated fats are liquid and therefore are oils (Lowy and Harrow, 1940). It seems therefore that it is imperative for poikilotherms to have high levels of unsaturated fats in their body tissues. In fact it has long been known that the fatty acids of living organisms shift towards greater unsaturation under lower environmental temperatures as a means of keeping protoplasmic viscosity within the range necessary for normal metabolic processes (see for example Lewis, 1962; Cowey and Sargent, 1972; Hazel and Prosser, 1974; Swan, 1975; Aloia, 1978). Cowey and Sargent (1977) presented evidence that fish reared at low temperatures require more highly unsaturated fatty acids than do fish reared at higher temperatures. Membrane fluidity depends to a large extent on the degree of unsaturation of the fatty acids esterified to the polar lipids. This fluidity permits movement of enzymic protein molecules within the membrane, and on this many of the functions of the membrane are dependent. Knipprath and Mead (1968) working with goldfish demonstrated that low

environmental temperatures resulted in increased unsaturation of tissue fatty acids. Kemp and Smith (1970) also working with the lipids from goldfish, found that if the rearing temperature of the fish was increased by 20°C then the percentage of polyunsaturated fatty acids in the membrane lipids from the intestinal submucosa was halved while that of the more saturated acids was nearly doubled.

Studies of this type have been extended to the organelle level. Richardson et al. (1961) analysed for the fatty acid contents of mitochondria from chicken liver, beef heart, rat liver, catfish liver, carp liver, salmon liver and salmon heart, and found that the degree of unsaturation of the fatty acids increased in the above order. Comparison of the fatty acid composition of rat liver and catfish liver mitochondria revealed a 15% greater degree of unsaturation in the mitochondrial fatty acids from fish (Richardson and Tappel, 1962). Leslie and Buckley (1976) demonstrated that the fatty acids incorporated into the phospholipids of goldfish liver microsomes were more highly unsaturated at incubation temperatures of 10°C than at 30°C; this indicates that temperature is the main factor determining which fatty acids are inserted into membrane phospholipids. Moreover, Caldwell and Vernberg (1970) reported that the lipids of mitochondria isolated from the gills of cold-acclimatized (10°C) goldfish were more unsaturated than the lipids of warm-acclimatized (30°C) specimens. These results are consistent with those of Irving and Watson (1976) who found the fatty acid composition of tropical fish mitochondrial membranes

to be more saturated than in previously reported cold-water fish mitochondrial membranes. However, while the necessity of enhancing membrane unsaturated fatty acids at low temperatures is obvious, the reverse, that higher temperatures induce formation of fats of higher melting point is not always true.

Lipofuscin, the hallmark of aging, appears to indicate inadequate protection from ubiquitous oxygen and other free-radicals that are induced metabolically. In plaice, mitochondria appear to be the major contributors to the lipofuscin component of melano-macrophage centres. Lipid peroxidation is a free radical chain reaction and once set in motion can give rise to a widening zone of pathology within the cell. The formation of lipid peroxides in biological membranes is accompanied by an extensive disturbance of their organized structure and loss of many specialized functions. Thus, there is loss of mitochondrial function, liberation of lysosomal enzymes and loss of integrity of the cell membrane (Slater, 1972).

Lipofuscin formation has been reported in practically every organ in homeotherms (for review see Thompson, 1966) and some of it is eventually transported to and deposited in the lymph nodes. Thus it seems as if a higher rate of formation of lipofuscin pigment from peroxidation of unsaturated lipids and its eventual accumulation in what are thought to be primitive germinal centre analogues is to be expected in fish. It would be interesting to examine pigment formation from a phylogenetic point of view especially in relation to the ability of different animal species to regulate their body temperature.

Another important suggestion that could give some insight as to why fish have such prominent areas of phagocytic cells heavily laden with indigestible materials is that intracellular digestive processes of fish macrophages may not be well developed on the evolutionary scale. The hypothesis of a poorly developed digestive enzyme system in fish phagocytes has been proposed by Corbel (1975) to explain the slow digestion of phagocytosed bacteria by phagocytes in the rainbow trout and by Bach et al. (1978) to explain the resistance of the bacterium Aeromonas hydrophila to intracellular enzymatic digestion by the macrophages in channel catfish fingerlings.

The electron microscope studies suggest that the melanin granules observed in melano-macrophage centres are phagocytosed from normally occurring melanin-containing cells after the latter rupture or are otherwise damaged. Such a process has been observed to occur in higher animals. Thus, for instance, Thompson (1966) observed that in normal human skin the melanocytes are continually being renewed and melanin is continually being eliminated by passing through the skin and being shed with the stratum corneum. Some melanin however is also normally eliminated by being absorbed by the lymphatics. In this respect, phagocytic macrophages within the dermis and subcutis may phagocytose melanin eliminated by the melanocytes and transport this melanin to the lymphatics. Such macrophages are known as melanophages and like the fish melano-macrophages under study, they do not give a positive DOPA reaction but they may be PAS positive (normal skin melanocytes are DOPA positive and PAS negative

unless they come from albinos). However, the possibility that in both circumstances such granules could be transferred from the classical melanin-synthesizing cells, the melanocytes, to the macrophages for some specific purpose, must not be overlooked.

In fact it seems very possible that the melanin within the centres could have a very important role through its well-known property of absorbing free radicals and cations thereby rendering them inactive and thus harmless (Edelstein, 1971; Gan et al., 1977). According to Mason et al. (1960) melanin can act as a biological electron exchange polymer and thus protect cells against reducing or oxidizing conditions which might release highly reactive free radicals which in turn can possibly cause alterations in cell metabolism and in cell membrane structure and function. The present study has shown that the centres represent areas of extensive lipid peroxidation reactions and, at least in the spleen, of a great deal of recycling of iron-containing compounds. To further complicate matters, iron compounds are known to actively catalyse the oxidation of lipids leading to increased production of malonaldehyde in animal (including fish) tissues (Castell and Bishop, 1969). The possible mobilization of melanin in order to neutralize the activity of these potentially reactive molecular entities could then be of immense value. While this would account for the presence of so many pigment types within melano-macrophages it could also explain why melanin is so commonly observed at sites of infection or tissue injury. It seems possible that the melanin at such sites absorbs any free radicals

that are formed through lipid peroxidation of damaged subcellular organelles. The sequence of events in this suggested interaction between the melanin-synthesizing cell, damaged cell components, free-lying radicals and cations, the freely wandering melano-macrophage and the melano-macrophage centres provides a most interesting challenge to future research.

If, as has been concluded in the present study, the melanin granules within melano-macrophages are acquired from the classical melanin-containing cells of the body, one would expect that no such granules would be observed in the melano-macrophage centres of albino fish species. True albino fish proved difficult to obtain but histological examination of one or two clinically normal albino catfish (Clarias sp.) showed that all the pigment within the melano-macrophage centres of the spleen, kidney and liver was yellow, presumably lipofuscin-type.

It is well established that in higher animals haemosiderin is frequently present at the same sites at which lipogenic pigments are deposited indicating that deposition of haematogenous and lipogenic pigments may occur concomitantly. The same appears to hold for fish since macrophages laden with iron compounds, melanin and lipogenic pigments abound, particularly within the splenic centres.

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mention that in the rat spleen some macrophages are observed to be surrounded by clusters of mature red blood cells, presumably old erythrocytes, undergoing phagocytosis while others are surrounded by or actually contain erythroblasts. Pictet et al. (1969: cited in Carr, 1973) postulated that the macrophages may be acting as nurse cells for the erythroblasts, somehow facilitating their maturation.

How effete red blood cells are phagocytosed and broken down as well as the fate of the various components are still largely unknown. In higher animals, the breakdown of effete erythrocytes takes place in lysosomes; proteins, iron and bile pigment precursors derived from the haemoglobin of these cells are re-utilised in metabolic processes elsewhere in the body (Muir and Niven, 1935; Essner and Novikoff, 1960; Rifkind, 1966). Although from this study it appears that a very similar sequence of events occurs within fish melano-macrophages, no insight whatsoever could be gained as to how any of these processes are carried out. The initial rupture of the red blood cell membrane is probably a consequence of peroxidation of membrane lipids resulting in the loss of integrity of red cell membranes and ultimate osmotic lysis. This process, which has been described to occur in mice (Mengel, 1972), is apparently enhanced in stressful environmental conditions such as starvation. Of particular interest, though still largely obscure, is the mechanism whereby material digested in lysosomes and earmarked for re-utilization is safely transported to the cytoplasm and further. Edwards and Simon (1970) who carried out a detailed

study of the process of ingestion of effete red blood corpuscles in the rat spleen, described how the matrix of the red blood cell in phagocytic vacuoles became heterogenous and granular; the phagosome membrane then apparently became inverted into the red blood cell so that a series of tunnels of cytoplasm finally invaginated it. Ferritin appeared first within the red cell cytoplasm and seemed to be able to penetrate the cytoplasmic membrane readily to reach the adjacent cytoplasm. In the rabbit, once again whole red blood cells were ingested but prominent tunnels of cytoplasm were not formed (Simon and Burke, 1970). Daems et al. (1972) thought that probably iron leaves the erythrocyte-containing lysosomes by the budding-off of small iron-containing, acid phosphatase-positive vesicles.

This study has identified only one stressful factor that directly influences melano-macrophage centres, viz. starvation. The need to determine what other factors could stimulate or depress the function of these pigment cells is obvious. Bern (1958) has studied the effect of a variety of hormonal, dietary and operative procedures on the formation of lipofuscin pigment in the adrenal glands of mice. He observed that both oestrogen administration and hypophysectomy induced the formation of the pigment. It would be interesting to investigate whether similar treatments affect in any way lipofuscin deposition in the melano-macrophage centres of fish. What is certain is that these centres do not fit into any one discipline so that a multi-disciplinary approach must be adopted in studying them.

The formation of autophagic vacuoles enclosing portions

of host cells during starvation has been observed in a variety of animal species and has been interpreted as constituting a mechanism which enables the cells to feed on their own substance without irreparably damaging the entire cell. Thus to mention two extreme examples, autophagic vacuoles have been observed in the liver of starved rats (De Duve, 1963) and in the unicellular organism Euglena (Brandes et al., 1964). During starvation of this latter organism, portions of the cytoplasm that become encapsulated within autophagic vacuoles have also been observed to include mitochondria. It seems that lysosomal enzymes are deficient in lipolytic enzymes and that lipid digestion remains incomplete within these bodies, giving rise to residual bodies or aging pigment (De Duve, 1964). It also appears that lower species can eliminate this aging pigment through cellular defaecation but higher organisms appear to have lost their ability to do so due to cellular "constipation" acquired in the course of evolution and thus aging pigment accumulates (De Duve, 1964).

It is commonly accepted that melanin, being a final product of metabolism, is metabolically inert. However, it has yet to be ascertained that the melanin and lipogenic pigments accumulated in melano-macrophage centres, or at least some of them, are not disposed of in some way. It is true that their steady accumulation with age and during stressful conditions such as starvation, seems to argue against the presence of an efficient cellular defaecation mechanism but the observation of Drochmans (1967) that in humans melanin can apparently be destroyed by

dermal melanophages shows that this question is still open to investigation and should stimulate biochemists to search for a melanin depolymerase.

The present study has shown that despite the morphological similarity of the melano-macrophage centres of the kidney, spleen and liver, there are important functional differences between the centres of the three organs. Both in normal and disease conditions, only the splenic centres are involved in the handling of haemosiderin to any large extent; this appears to hold true for all teleost species. Only when rainbow trout were splenectomised was that role taken over by the kidney. Important differences have also been reported with respect to the involvement of the centres in the immune response. Ellis (1974, 1979) who studied the process of antigen trapping within the lymphoid tissues of the plaice observed important differences in the pattern of distribution of the soluble antigen bovine serum albumin and in the associated immunoglobulin production as well as in the persistence of the immune-complexes in the centres of the spleen as compared with those of the kidney. Thus, for instance, while in the spleen the antigen appeared to be present extracellularly and could be so detected within the centres, in the kidney uptake of BSA appeared to be a predominantly intracellular process and antigen could be so detected in aggregates of lymphocyte-type cells adjacent to melanin centres but not actually within centres.

When more differences of this nature are unravelled, we may be able to establish correlations between various factors

such as melano-macrophage centre distribution within the various haemopoietic organs (as well as their composition and funotions) and the relative erythropoietic and leucopoietic (particularly lymphopoietic) activity of these different organs within a particular species. The available evidence indicates that some very important processes are confined largely, if not exclusively, to the splenic centres. Indeed, Moltenius (1970) asserts that the main task of the spleen is to monitor the bloodstream in the defence of the organism against bacteria and toxins. The longer-term persistence of extracellularly trapped antigen on dendritic cells within the splenic centres has been described in plaice and the possible implications of this observation in fish immune mechanisms have been fully discussed by Ellis (1979). The almost exclusive restriction of the breakdown of effete erythrocytes and the subsequent storage of excess quantities of haemosiderin to the splenic centres must certainly involve other differences in the functioning of the melano-macrophage centre systems of the different organs. In the light of these important differences it would be extremely valuable to investigate whether there exist any direct interactions between the centres of different organs and in particular whether there occurs any exchange of information or material between them.

Ellis *et al.* (1976) who studied the fate of intra-peritoneally injected carbon in plaice observed that the main route of uptake by the spleen is via the ellipsoid sheaths which appear to be a source or a residence of large macrophages which phagocytose free carbon particles present in the ellipsoid lumen

and which ultimately form aggregates in the splenic pulp. Bach et al. (1978) who carried out experimental infections of channel catfish fingerlings with Aeromonas hydrophila also commented on the marked ability of the ellipsoids to sequester massive numbers of bacteria which are somehow recognized and filtered out from the blood as this flows through their lumens. If during cachexia effete organelles and other cellular debris from degenerating tissues are similarly released into circulation to be eventually engulfed by ellipsoidal macrophages, that could explain why so little phagocytic activity was traceable within the skin, peritoneum and skeletal muscle. The kidney was the other major site of phagocytosis and storage of carbon particles; once again, the carbon appeared to be carried to the kidney as free particles (Ellis et al., 1976). A similar type of cytophilic attraction or "cellular togetherness" is demonstrated in the melano-macrophage centres of the kidney. It is not known, however, 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 association of melanin with sites of infection in fish and other organisms suffering from various diseases has been well documented (Edelstein, 1971; MacQueen et al., 1973; Roberts, 1975). The involvement of melano-macrophage centres in various fish diseases has already been remarked upon in the introductory chapter. From the foregoing discussion, it appears that this

involvement could be of three types, viz:

i. direct involvement; such as for example the pigment could exert a bactericidal effect as suggested by Edelstein (1971);

ii. due to tissue damage at the sites of infection; peroxidation of unsaturated lipid cellular components to lipofuscin (possibly in association with melanin, vide infra) and their possible eventual transport to the melano-macrophage centres could result in enlargement of the latter (anaemia also leads to haemosiderin deposition); and

iii. due to tissue damage through debilitation; by this is implied a more widespread form of tissue damage caused for instance by fish going off food; starvation results in marked enlargement of the centres.

It needs hardly be emphasized that more work needs be done to enable us determine how these pigment cells fit in the pathogenesis of the various diseases. Equally important is the question of whether this gradual accumulation of pigment within haemopoietic tissues impairs their functioning in any way. Moreover, the potential danger of having large amounts of pigments, that are highly toxic to living cells, released into the surrounding tissues should the centres rupture for some reason or other, must be given due consideration.

Strehler et al. (1959) reported the results of a quantitative study of the age-wise occurrence of cardiac lipofuscin pigment in the human myocardium. They observed that the

pigment concentration increased linearly at a rate of approximately $\frac{1}{3}$% heart volume/decade. The fraction of the myocardium occupied by pigment and total cardiac pigment also showed linear rates of increase with time ($\frac{2}{3}$%/decade and 100 mg/heart X year, respectively). It would be interesting to establish the equivalent figures for the pigment within fish haemopoietic tissues based on large samples. Apart from its theoretical interest such an exercise could prove to be of great practical significance.

With the increasing threat of pollution in recent years, fisheries biologists have been searching for histological - anatomical indicators in fish of environmental quality and physiological stress (see for example Smith and Ramos, 1976). The involvement of melano-macrophage centres in various disease conditions and the changes brought about in them by such factors as starvation strongly indicate that once a reliable baseline is established such analysis should provide sensitive indicators of stressful conditions in the aquatic environment with which the fish live in such an intimate contact. In particular, the accumulation of iron within the splenic centres could be extremely useful. Blois (1965) observed that melanin binds to a large number of compounds including para-aminobenzoic acid-C14, chlorpromazine and a host of others. The possible role of melanin in protecting living cells against various pollutants has been reviewed in detail by Edelstein (1971). It seems possible then that chemical pollutants might be preferentially concentrated within melano-macrophage centres.

Much effort has been directed at attempting to utilize haematological parameters in assessing sublethal concentrations of environmental pollution (Blaxhall, 1972). Results, however, have been inconsistent partly because of the lack of species normal values and partly because such values could alter very rapidly depending on such simple phenomena as methods of capturing and transporting the fish (see for example Hattingh and Van Pletzen, 1974). In view of this, attempts to develop melano-macrophage centres as indicators of the state of health of the fish should be given even greater priority.

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APPENDIX I

Mixtures A and B were first prepared as follows:

Mixture A:	Epon 812	26 gm
	DDSA	41 gm
Mixture B:	Epon 812	18 gm
	MNA	15 gm

A and B were then mixed in a 7 : 3 ratio for blocks of medium hardness.

Immediately before use, the reaction accelerator DMP-30 was added to a final concentration of 1.5 - 2%. All mixtures were well-stirred at all stages. Mixtures A and B can be stored in a refrigerator for about two months.

APPENDIX II

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

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SHORT COMMUNICATION

Infection by an *Ichthyophonus*-like fungus in the deep-sea scabbard fish *Aphanopus carbo* (Lowe) (Trichiuridae) in the North East Atlantic

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Ichthyophonus disease, a systemic granulomatous infection, is one of the most serious mycoses of both marine and freshwater teleosts. Sindermann (1970) and Hendricks (1972) reviewed the literature on those fish species that have so far been reported to act as hosts for this parasitic fungus. No record of it occurring in deep-sea species so far exists.

The livers, spleens and kidneys (anterior and posterior) of six specimens of *Aphanopus carbo* (Lowe) from the N.E. Atlantic were dissected out and fixed in 10% neutral buffered formalin. Four of the specimens were randomly selected from a total catch of 83 trawled from 735m at 56° 38.9′N 09° 10.8′W to 56° 35.8′N 09° 13.6′W. The other two specimens represented the total catch of a trawl from 56° 37.1′N 09° 15.2′W to 56° 33.8′N 09° 17.4′W at a depth of 980 m. The length range of all fish examined was 90–116 cm. The distance between the two trawls was approximately 5 km so that they may well have belonged to the same aggregation. After routine processing, 5 μm thick paraffin sections were stained with haematoxylin and eosin (HE), periodic acid-Schiff (PAS), Martius scarlet-blue (MSB), Ziehl-Nielsen (ZN) and Gram Humberstone.

Five out of the six specimens examined contained multiple granulomas; two of them were very severely affected. No gross lesions were visible on any of the fish examined but all of the organs examined microscopically carried granulomas. In extreme cases the inflammatory lesions had replaced much of the normal tissues. The granulomatous reaction type observed was indistinguishable from that found in ichthyophoniasis in gadoids and clupeids (Roberts, 1977, personal communication). All stages, ranging from early to fully mature, were evident in the development of granulomata (Fig. 1). The centres of the lesions considered to be fully mature were caseous but there was no evidence of major accumulations of lymphocytes suggestive of a cell-mediated immune caseation. There was a marked host response in the form of connective tissue proliferation resulting in encapsulation of the spores and obliteration of the normal parenchymatous and vascular tissues of the organs. Accumulation of melanin bearing cells was also observed in some lesions. Staining by Ziehl Nielsen or modified Gram methods failed to reveal the presence of any bacterial pathogens.

Sproston (1944) described several stages of the life history of *Ichthyosporidium*

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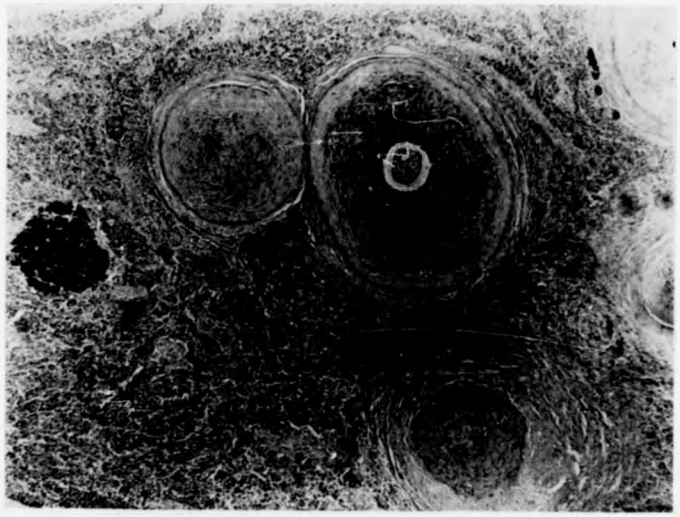

Figure 1. Granulomas at different stages of maturation elicited by *Ichthyophonus*-like fungus in the spleen of *Aphanopus carbo*. A resting cyst containing a multinucleate spore (arrowed) can be seen in the centre of one of the foci (H & E, ×100).

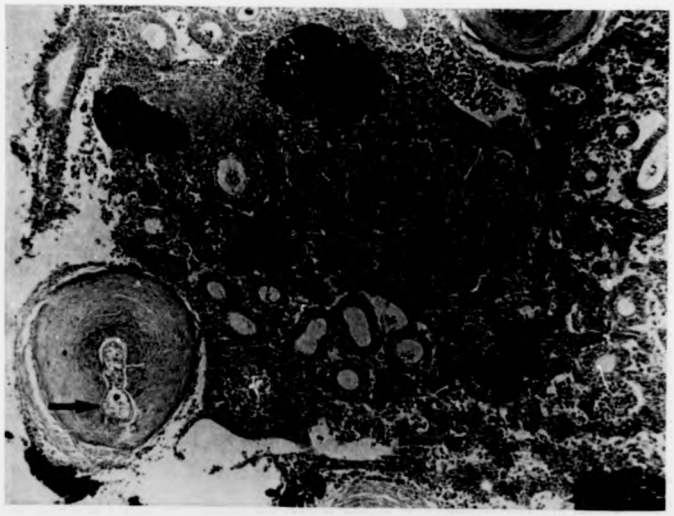

Figure 2. Germinating spore (arrowed) in the posterior kidney of the same specimen as for Fig. early granuloma (g) can also be seen in this figure (H & E, ×100).

hoferi. However, Sprague (1965) maintains that, like so many others in the past, Sproston was using *Ichthyosporidium* as the name of a genus of fungi. Only two distinct forms could be identified in the present study (Figs 1 & 2). Most prominent were the large spores (35–100 μm in diameter) surrounded by a thick, double wall; in many cases the contents had somehow been shed and only the deeply PAS-positive staining spore wall or cyst could be identified. Some spores in the early phases of germination could also be seen; no well-developed hyphal stages could be detected.

These observations indicate the possibility of a much wider host range for the distribution of this disease. How fish at such depths become infected with this parasitic fungus, till now reported mainly in pelagic species, is a matter for speculation although, as far as is known, transmission normally occurs *per os*. Further studies on the prevalence and distribution are needed before any conclusions can be drawn as to the role this parasite could be playing in controlling deep sea populations. It is already recognized as one of the limiting factors controlling N.W. Atlantic clupeid fishes (Sindermann 1963). Of particular significance may be the fact that scabbard fish smaller than 90 cm have been noted to be absent from catches in recent years (Gordon, 1977, personal communication). Although this observation cannot as yet be directly linked with disease, and there are always a number of interacting factors affecting any fish population structure, the very high prevalence of the disease in the fish examined suggests the need for further studies, especially in relation to epizootics in wild populations.

Acknowledgments

The scabbard fish were caught on S.M.B.A. 'Challenger' cruise 7/77, one of a series undertaken by Dr J.D.M. Gordon to study the biology of deep-sea demersal fish on the continental slope. The author wishes to acknowledge the help of Dr Gordon in providing information on the scabbard fish populations and Mr I.H. MacRae for collecting the material.

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