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A STUDY OF THE STRUCTURE, DISTRIBUTION

AND FUNCTION OF PHAGOCYTIC CELLS IN

THE IMMUNE SYSTEM OF FISH

NICOLE M. PARISH

Thesis submitted to the Council for National Academic
Awards in partial fulfilment of the requirements for
DOCTOR OF PHLOSOPHY.
September,1981.

Research was carried out at Plymouth Polytechnic in
collaboration with Middlesex Hospital Medical School.

Declaration

This work has not been accepted, and is not concurrently being submitted, for any other degree, and is a record of work carried out by the candidate herself.

CANDIDATE: *Nicole Parish.*

SUPERVISOR: *J E Kaus*

DATE: 12·10·81

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Finally I should like to thank Dr J.E. Harris for his supervision and help with this research project.

Advanced Studies.

In addition to the research described in this thesis, the candidate also followed a third year honours degree option in immunology.

An opportunity to meet others engaged in research in comparative immunology was provided by attending three conferences.
1.On: Lymphocyte development and differentiation-at the University of Durham,1979.
2.On: Phylogeny of immunological memory -at Tampa, Florida December,1979.
3.On: Aspects of developmental and comparative immunology-at University of Aberdeen, August,1980.

Furthermore the candidate visited various establishments engaged in immunology/fish biology research thereby affording further opportunity to discuss the work contained in the present thesis.

Abstract	A Study of the Structure, Distribution and Function of Phagocytic Cells in the Immune System of Fish.
	Nicole M. Parish.

Dogfish leucocytes were characterised by histochemistry, light and electron microscopical methods and qualitative phagocytic studies. This enabled the recognition of the major phagocytic cells and their role in the immune response of the fish was further evaluated.

In addition to _in vitro_ and _in vivo_ studies of phagocytosis, the phagocytic cells were examined for certain surface properties such as the presence of Fc and C_3 receptors. Attempts were also made to demonstrate delayed hypersensitivity by _in vivo_ skin tests and _in vitro_ migration inhibition tests.

All the major leucocytic elements found in mammals were demonstrated in dogfish blood together with some, as yet, unidentified cells. The main phagocytic cells of the blood were the neutrophil and the monocyte. Clearance of antigen and other particulate material from the circulation was effected chiefly by the spleen and to a lesser extent by the gill. In contrast to mammals, the liver showed no phagocytic activity.

Although _in vitro_ studies indicated that the identified phagocytes were efficient at endocytosing antigenic material, _in vivo_ clearance studies showed that low numbers of viable bacteria and yeasts persisted in the circulation for long periods after injection. This suggests a functional deficiency in the phagocytic ability of dogfish compared to higher vertebrates. This may however be augmented by natural elements in their circulation. Delayed hypersensitivity reactions and possession of receptors (which may mediate cellular interactions) were not demonstrated in fish. This may suggest a less sophisticated system is operating in these fish compared with mammals or conversely that these phenomena can not be demonstrated by the methods employed in this study.

CONTENTS.

List of Abbreviations.

A.D.C.C.   –   Antibody dependent cell-mediated cytotoxicity.

B cells.   –   bone marrow-derived lymphocytes.

BCG   –   Bacillus Calmette Guerin.

BGG.   –   bovine gamma globulin.

BSA   –   bovine serum albumin.

C_3 –   3rd. component of complement.

Con A   –   Concanavalin A.

CRP   –   C-reactive protein.

DH   –   Delayed Hypersensitivity.

Fc   –   Fragment crystallizable portion of immunoglobulin.

FCA   –   Freund's Complete Adjuvant.

FIA   –   Freund's Incomplete Adjuvant.

FITC   –   fluorescein isothiocyanate isomer 1.

Ig   –   Immunoglobulin.

i.d.   –   intradermal.

i.p.   –   intraperitoneal.

i.v.   –   intravascular.

KLH   –   Keyhole Limpet Haemocyanin.

LM   –   light microscopy.

LPS   –   lipopolysaccharide.

MIF   –   migration inhibition factor.

MMC   –   melanomacrophage centre.

NBT   –   nitroblue tetrazolium.

O.D.   –   optical density.

P.A.S.   –   periodic acid Schiff.

PAS-GL   –   periodic acid Schiff positive granulocyte.

PBS   –   phosphate buffered saline.

p.f.u.   –   plaque-forming units.

PHA   –   phytohaemagglutinin.

PMN   –   polymorphonuclear leucocyte.

RBC   –   red blood cell.

RDC   –   rapid decalcifier.

SDS   –   sodium dodecyl sulphate.

SEM   –   scanning electron microscopy.

SOD   –   superoxide dismutase.

T cells.   –   thymus-derived lymphocytes.

TCM   –   tissue culture medium.

TEM   –   transmission electron microscopy.

WBC   –   white blood cells.

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INTRODUCTION

Comparative immunology has recently attracted increased
attention and particularly towards two major objectives:-

1. to elucidate mechanisms underlying the functional
   differentiation of immunologically competent cells; and

2. to obtain simplified, experimental models which facilitate
   investigations which would be impossible in the usual
   mammalian systems (Marchalonis, 1977).

This latter aim implies a fundamental similarity of the immune
system in all groups of animals throughout the phylogenetic spectrum.
However, in different organisms evolution often finds independent
answers to the same environmental challenges, such that a defence
mechanism present in an ancestral form may be replaced by different
mechanisms in some of the descendant lines (Mayr, 1966). It has
been observed that, when interpreting data on the phylogeny of
immunity, natural selection will act to defend and adapt the
population in the best way possible for a particular species in its
particular environment (Rosser, 1976).

These observations are especially relevant in the study of the
immune response of fish, which is perhaps one of the most rapidly
expanding areas of comparative immunology. This is due to the
realization that a knowledge of how these mechanisms operate would
assist in disease prevention which is an important economic aspect
of aquaculture - itself a rapidly expanding discipline (Sniezko and
Axelrod, 1971).

The immune response may be divided into cellular and humoral
aspects. The cellular mechanisms are considered more primitive,
although at the level of the higher vertebrates at least, there are

complex interactions of the cellular response with the humoral system
(Roitt, 1971).

Studies on fish immunity have been concerned mainly with humoral
aspects due to the requirement for efficient vaccination procedures in
the aquacultural situation.

Conversely the comparative immunology interest in fish
immunology has been directed towards the role of lymphocytes in
immunity such as:- a) at what level have lymphocytes and plasma cells
emerged phylogenetically, b) the minimal structural requirements of
the lymphoid system which allow cellular and humoral immunity to
function and c) when the distinction between B + T cells arose
(Marchalonis, 1977).

Thus, the importance of phagocytic cells first noted by
Metchnikoff as present in all animal forms (Metchnikoff, 1905), has
received less attention in recent comparative studies than earlier
workers suggested they deserve.

In higher vertebrates phagocytes are organised into a
sophisticated system interacting with other cell types and involved in
processes quite distinct from phagocytosis. Even when research has
been confined to the phagocytic act, the results demonstrate an
intricate mechanism which can be modified by a variety of
intracellular and extracellular conditions (Van Furth, 1970).

The phagocytic cells of mammals are widely distributed and
display certain morphological differences which can be attributed to
the structure, metabolism and blood supply of the various organs of
which they are part (Vernon-Roberts, 1972).

The mononuclear phagocytes originate from the mesoderm during
embryogenesis and form part of all tissues although they are
particularly prominent in bone marrow, liver, spleen, connective

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tissues, serous cavities and blood. Here they are involved in diverse functions related in large measure to their endocytic activities (Cohn, 1968).

In the blood, in addition to monocytes, two other cell types are present which are capable of phagocytosis. These are the polymorphonuclear leucocyte (P.M.N.) and the eosinophil. However the principal phagocyte of the blood and also the bone marrow is the PMN which participates extensively in the eradication of pyogenic bacteria and certain fungi from extravascular sites (Baehner and Boxer, 1979).

The discriminatory capacity of all mammalian phagocytes is very fine and particles which elicit ingestion by phagocytes have surface properties that produce this effect (Stossel, 1975).

The purpose of the immune response is to defend the host against disease and 'non-specific' mechanisms such as phagocytosis are enhanced by the development of adaptive (or specific) immunity (Roitt, 1971). This perhaps suggests that in animals, such as fish, where adaptive immunity is apparently less sophisticated, phagocytosis per se may play a relatively more important role in the defence of the organism.

In fish the phagocytic system has been defined in very few species. In those species so far studied the phagocytic system comprises 'fixed' and 'free' cells which, as with mammals, may be mobilised under appropriate conditions (Mackmull and Michels, 1932; Ellis, Munro and Roberts, 1976). Unlike other vertebrates fish also have pigment containing macrophages - melanomacrophages, which perform unique functions (Agius, 1979).

The present study was undertaken as a consequence of a previous study (Morrow, 1978) which covered many aspects of the immune system of an elasmobranch, the common dogfish (Scyliorhinus

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<u>canicula</u> L.). From the information gained during the project it was apparent that a deeper knowledge of the cells comprising the immune system was required.

The subject of the present study is mainly concerned with the role of phagocytic cells in fish. Initially, it was necessary to identify the phagocytic cells by morphological, histochemical and functional experiments, and subsequently to elucidate their roles in immunological phenomena in which they are known to be involved in higher vertebrates. The principal species used was the dogfish (<u>S</u>. <u>canicula</u>), and also the European eel (<u>Anguilla</u> <u>anguilla</u> L), the plaice (<u>Pleuronectes</u> <u>platessa</u> L.) and the thornback ray (<u>Raja</u> <u>clavata</u> L).

Literature Survey

This literature review is confined to an appraisal of the work
on cellular immunity in fish. However, humoral immunity is also
active in fish defence mechanisms and has been comprehensively
reviewed (Clem and Leslie, 1969; Marchalonis, 1977).

Early work on cellular immunity in fish centred mainly on the
identification of the leucocytes both of the peripheral blood and
of the tissues, and hypothesizing as to their possible precursors
and sites of development (Jordan and Spiedel, 1924; Yoffey, 1929;
Duthie, 1939; Catton, 1951).

1. Peripheral Blood Leucocytes

Not only is there a considerable amount of information on fish
leucocytes as the most recent comprehensive review (Ellis, 1977)·
demonstrates, but there is also controversy over characterization of
leucocytes even within a single species.

i) Lymphocytes

In all studies encountered a lymphocyte has been described.
This cell type is essentially similar to that of higher vertebrates
and has been demonstrated from agnathans to the phylogenetically
more advanced teleosts (Jordan and Spiedel, 1924; Duthie, 1939;
Good and Finstad, 1964; Clawson, Finstad and Good, 1966; Saunders,
1966; Hildemann and Thoenes, 1969; Ellis, 1976). The cells are
variable in size from species to species and most species appear to
have both a smaller and larger type. They are round and have a
nucleus which nearly fills the cell, leaving a thin peripheral rim
of basophilic cytoplasm. Early workers (Jordan and Spiedel, 1924;
Yoffey, 1929; Duthie, 1939) considered the lymphocyte to be the

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common precursor to all other blood cell types including erythrocytes, thrombocytes and granulocytes. In the last twenty five years it has emerged that the lymphocyte is a fully differentiated 'end' cell with specific functions. However, the conclusion that it is a multipotential, stem cell, given the technology available to the earlier workers, was reasonable.

ii) Thrombocytes

In the majority of fish species, thrombocytes have been identified. These are generally described as oval or spindle-shaped, again with a thin rim of variably staining cytoplasm (Saunders, 1966; Karmarkar and Gazdar, 1967; Ellis, 1976). The nucleus is round or oval. A variety of sizes and shapes of thrombocytes are seen even within a single fish (Saunders, 1968) and the cytoplasm may contain granules (Lewis and Shirakawa, 1962). They invariably contain PAS positive material indicative of a significant glycogen content (Lewis and Shirakawa, 1962; Pitombeira, 1970). There are reports of thrombocytes taking up injected carbon (Ferguson, 1976a; Morrow and Pulsford, 1980), however Ellis (1977) considers this not an active process rather a "mechanical entrapment of particles in the cytoplasmic labyrinthine vesicles which communicate with the environment via stomata in the cell membrane". The main function of these cells, as with mammalian platelets, appears to be in the blood clotting mechanism. It may be that in studies in which this cell is reported absent (Catton, 1951) that partial clotting of the blood has occurred depleting the remaining blood of thrombocytes. It could also be that they have been mistakenly identified as lymphocytes.

iii) Granulocytes

Granulocytes are present probably in one or more forms in the peripheral blood of most fish. It seems that the equivalent of the mammalian neutrophil probably exists as a phagocytic granulocyte but may vary in appearance from species to species. A cell considered here to be analogous to the neutrophil has been variously named in the literature as 'heterophil' (Barber and Mills Westermann, 1978), 'special granulocyte' (Fey, 1960) and 'fine granulocyte' (Catton, 1951). [Some authors (Saunders, 1966; Sherburne, 1974) consider the heterophil as distinct from the neutrophil] These cells have been described as phagocytic (Duthie, 1939; Catton, 1951), capable of adherence, spreading (Catton, 1951), amoeboid movement (Jordan and Spiedel, 1924; Duthie, 1939), aggregation (Duthie, 1939) and as having peroxidase activity (Duthie, 1939; Catton, 1951; Blaxhall and Daisley, 1973). It seems probable therefore that these cells constitute an equivalent to the neutrophil. The granules tend to be variable in shape, but are generally smaller than in eosinophils.

The presence of eosinophils is variable in fish blood, but in species where they are not present in peripheral blood they are usually present in the tissues. They have also been described as 'coarse granulocytes' (though in some cases the coarse granulocytes have basophilic granules). They have not been observed to phagocytose but can adhere and spread on glass, and are capable of amoeboid movement (Duthie, 1939). The granules vary in shape from fish to fish but seem most often to be oval to rod-shaped, and stain pink to brick-red by Romanowsky stains. The crystalloid inclusions commonly seen in the granules of mammalian eosinophils are not always present.

The basophil is rarely encountered in fish blood. In

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elasmobranchs only two reports suggest the presence of a basophil in the peripheral blood (Karmarkar and Gazdar, 1967, 1968) and then only in small numbers. Morrow and Pulsford (1980) describe an oval granulocyte with square granules having 'some of the characteristics' of the mammalian basophil, in S. canicula; however, the granules stain eosinophilically and it therefore seems unlikely that they are in fact representatives of this cell type. Basophils have been found in the African lungfish (Protopterus Owen) and Atlantic herring (Clupea harengus harengus L.) (Barber and Mills Westermann, 1978a), in Synodus intermedius (Agassiz), Holocentrus ascensonis (Osbeck), H. rufus (Walbaum), Caranx bartholomaei (Cuvier) and Dactylopterus volitans (L) (Saunders, 1966) and occasionally in Carassius auratus L. (Weinreb, 1963). These eight species are from surveys covering 180 different fish species, thereby demonstrating the relative paucity of this cell type in fish blood.

A P.A.S. +ve granulocyte has been described in several bony fish species (Barber and Mills Westermann, 1978a, 1978b). This cell is insensitive to digestion by salivary amylase and, on the basis of its reaction in vivo to the histamine liberator compound $^{48}/80$, the above authors propose that it may be a precursor to the basophil of higher vertebrates. The general absence of these cells and basophils from the chondrichthyes is perhaps to be expected, as this group of fish are reported to be insensitive to the effects of histamine (Dreyer and King, 1948) and in addition do not appear to have the complement component (C5) (Jensen and Festa, 1980) or lg E which mediate granule release from basophils and mast cells in higher vertebrates.

iv) Monocytes

Monocytes (using the nomenclature of Langevoort, Cohn, Hirsch,

Humphrey, Spector and Van Furth, 1970) have been reported present in the blood of some fish species. They appear to be present in plaice (Ferguson, 1976a) but absent from carp (Weinreb, 1963). Duthie (1939) considered that "mononuclears" mentioned by Drzewina (1911) (and presumably distinct from lymphocytes) were 'not present in fish'. In species in which these cells are reported present they take the same general form as their mammalian counterparts. They are phagocytic and constitute a very small percentage of the peripheral blood leucocytes. Where they are reported as absent it may be that they have been identified as lymphocytes, which they may resemble when unspread (Ellis, 1977).

2. Lymphopoietic and Haematopoietic Tissues

i) Thymus

There is no definitive thymus in the hag fishes but from the lampreys up to the teleosts a thymus exists in one form or another. From the information so far gained from studying elasmobranchs, the thymus is epithelial in embryonic development and lymphoid in adults (Corbel, 1975). However most of the work appears to have been performed on rays which have a larger, more accessible thymus than the dogfish. This very difference in size suggests that differences in structure may be present between rays, dogfish and sharks and that perhaps generalisations covering all elasmobranchs from studies on rays, should not be made.

The thymus is a yellowish, lobulated organ situated near the gills and electric organs in the rays, R. clavata L and Torpedo marmorata Risso (Zapata, 1980). In each lobe there is a cortex and a medulla. The capsule is composed of connective tissue and in the sub-capsular area 'blast' cells predominate. The cortex contains

numerous lymphocytes in a reticular network.

The thymus of the plaice is not a filtration organ but is a primary, lymphopoietic tissue, lymphocytes being formed independently of antigen (Ellis, 1977). There is no process by which the blood circulates through the thymus and this has been demonstrated by a technique which studied the fate of radio-labelled lymphocytes introduced intravascularly into plaice (Ellis and de Sousa, 1974). Furthermore carbon injected intraperitoneally is not phagocytosed by thymic macrophages (Ellis, Munro and Roberts, 1976).

ii) Spleen

The structure of the spleen has been studied in elasmobranchs, dipnoi and teleosts (Yoffey, 1929). An essentially open circulation is present where the blood travels through the pulp and around the lymphoid tissue. The arteries terminate by dividing into three or four thick-walled capillaries - the ellipsoids, which open 'directly into the spaces of the pulp reticulum'. The dipnoi examined did not have ellipsoids.

The most primitive of fish - the hagfish has a splenic equivalent in an accumulation of haematopoietic tissue in the gut (Good and Papermaster, 1964). In elasmobranchs the spleen is the main haematopoietic organ together with the testis (Jordan and Spiedel, 1924).

Zapata (1980) studying the two elasmobranchs R. clavata and T. marmorata reported definite red and white pulps. The white pulp is mainly around the large blood vessels, although in R. clavata there is also white pulp around the ellipsoids. The white pulp is more extensive in T. marmorata than R. clavata. The ellipsoids contain large phagocytic cells.

In the new born guitar fish Rhinobatus productus, the spleen

has only red pulp, the white pulp developing in the adult (Pitchappan, 1980). The nurse shark <u>Ginglymostoma</u> (L) and the snapper <u>Lutjanus</u> (Walbaum) are described by the same author as having 'no germinal centres'.

Plasma cells and their possible lymphocyte precursors have been identified in the spleen and haematopoietic tissue of the paddlefish, <u>Polyodon</u> <u>spathula</u> L. a chondrostean, (Clawson <u>et</u> <u>al</u>., 1966). Eosinophils and neutrophils were also reported as present, and two populations of lymphocytes were identified on the basis of their cytoplasmic ribosomal configuration. Pitchappan (1980) described a well-developed white pulp follicle in the same species.

The spleen has immature red and white cells in Triglidae and Labridae (Duthie, 1939), and so even though the kidney is the principal haematopoietic organ, the spleen of several species of teleost retains considerable activity. Pitchappan (1980) describes the spleen of <u>Tilapia</u> <u>mossambica</u> (Gervais) as having poorly developed white pulp with no well-defined lymphoid centres, but extensive red pulp. It may be that in this species lymphopoiesis and haematopoiesis occurs solely in the kidney.

iii) <u>Kidney</u>

In teleosts the anterior kidney is the main haematopoietic organ (Jordan and Spiedel, 1924) although the spleen possesses some activity. The main centre of blood cell formation in several species of Triglidae and Labridae was reported as the kidney (Duthie, 1939). Jordan and Spiedel (1924) described the main granulocyte of this organ as the 'pseudo-eosinophil' (neutrophil) which is actively amoeboid. In roach (<u>Rutilus</u> <u>rutilus</u> (L)) it has been reported that the only haematopoietic organ is the anterior kidney (Catton, 1951). The kidney of <u>Catostomus</u> <u>commersoni</u> Lacapede was shown to contain

heterophils and PAS-GLS but never eosinophils (Barber and Mills
Westermann, 1978b), whereas in _Cyprinus carpio_ L, _Tinca tinca_ (L)
and _Carassius carassius_ (L) kidneys both neutrophils and eosinophils
have been described (Kelenyi and Nemeth, 1969). Specific antibody-
forming cells have been demonstrated by the immunocytoadherence
technique in the pronephros of rainbow trout, _Salmo gairdneri_
Richardson (Chiller, Hodgins, Chambers and Weiser, 1969) and by the
Jerne plaque assay in the same organ of carp (Rijkers, Frederix-
Wolters and Van Muiswinkel, 1980).

iv) Miscellaneous Haematopoietic Tissues

Fänge (1968) reported the presence of large numbers of
granulocytes in a region of the elasmobranch gut known as Leydig's
organ. This is found ventrally and dorsally in the oesophagus and
contains three types of cells:- a) coarse, eosinophilic granulocytes
with numerous, bright, round to oval granules, b) fine granulocytes
full of minute, rod-shaped granules which stain red and c) non-
granular, lymphocyte-like cells.

The testis was mentioned by Jordan and Spiedel (1924) as being
the organ other than the spleen which is involved in haematopoiesis.
They were probably referring to what is now known as the epigonal
tissue.

The intestinal mucosa of teleosts is characterised by many
amoeboid eosinophils (Jordan and Spiedel, 1924). The submucous
coat and the mesentery of Labridae and Triglidae have lymphoid centres
and apart from the kidney, the largest numbers of granulocytes are
found here (Duthie, 1939). Apart from definitive lymphoid centres,
the granulocytes often occur in large numbers migrating to the
surface of the mucosal membrane. This observation may suggest that
these workers are mistaking developmental stages of mucous cells with

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granulocytes. Large numbers of basophils or mast cells were reported in parts of the alimentary canal of salmonids (Bolton, 1933). This again may suggest confusion with mucous cells and their precursors.

Finally, the livers in the adult elasmobranchs Raja ocellata (L) and Mustelus canis (L) were reported as non-haematopoietic, (Jordan and Spiedel, 1924) and there is no suggestion of any such activity in this organ in teleost literature.

3. The Phagocytic System

The Mononuclear Phagocyte System (M.N.P. system) was the term adopted to describe the mammalian phagocytic system by Langevoort et al. in 1970. Previous to this the most common term was the Reticuloendothelial system, a term which is still occasionally used.

The M.N.P. system encompasses cells of common function and origin. The earliest recognisable precursor in the bone marrow is the promonocyte; as this cell matures it enters the circulation, is moderately phagocytic and is known as the monocyte. The monocyte is the incompletely differentiated precursor to the tissue macrophage, into which it will mature when conditions are suitable for phagocytosis (see fig. 1).

It is not known whether this organisation of the phagocytic system exists in fish. Certainly in some fish at least (Ferguson, 1976a; Ellis, 1976; McKinney, 1977) monocytes have been demonstrated in the peripheral blood, and a variety of tissues contain functional macrophages. Whether the latter are mature forms of the monocyte and where the monocyte originates is unknown. Fish do not have bone marrow and the organs which seem probable sources of phagocyte precursors such as spleen and kidney, also have resident

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Fig. 1. The Mononuclear Phagocyte System

Promonocyte   (BONE MARROW)
        |
        |
        ↓
Monocyte   (BLOOD)
        |
        |
        ↓
Macrophage   (TISSUES)
    highly phagocytic
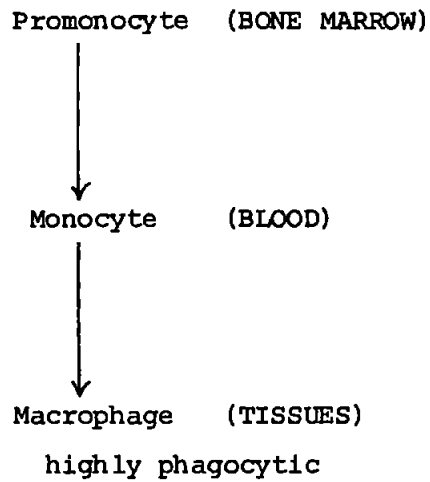
Connective tissue - histiocyte.

Liver - Kupffer cell.

Lung - alveolar macrophage.

Spleen - free and fixed macrophage, sinusoidal lining cell.

Lymph node - free and fixed macrophage.

Bone marrow - macrophages, sinusoidal lining cell.

Serous cavity - peritoneal macrophage.

Bone tissue - osteoclast

Nervous system - (microglia?)

[From 'Mononuclear Phagocytes' ed R. Van Furth, 1970]

mature cells and developmental stages of other cells making
identification of immature forms difficult.

i) Distribution of Phagocytic Cells

Several workers have studied the fish phagocytic system, and
this work has revealed some interesting divergences from the mammalian
system. One of the earliest studies (Mackmull and Michels, 1932) was
on the teleost fish Tautogolabrus adspersus (L). The peritoneal
cavity was injected with colloidal carbon and its fate monitored
histologically. The greatest uptake appeared to be in the spleen
and kidney and by the 'fibroblasts' of the mesentery. These workers
reported the heart as 'appearing very black' but that most of this
could be washed away, any phagocytosed carbon appearing exclusively
in the capillary endothelial cells. There is no resident population
of phagocytes in the gill of this fish species but cells which are
too full of carbon to enter the fine capillaries, lodge in the larger
vessels at the base of the gill. When cells are replete they 'round
up' and become free, wandering macrophages and from one day onwards
migrate towards normally pigmented areas, notably the liver.

Ellis, Munro and Roberts (1976) made a similar study using the
plaice. They noted that only mononuclear phagocytes, never
granulocytes phagocytosed carbon in the peritoneal cavity. Little
carbon appears in the blood although the number of monocytes
increases after injection, some containing carbon granules. The
liver, thymus and gill show no carbon uptake, but a few circulatory
cells containing carbon are seen in the gill. The principal sites
of phagocytosis are the spleen, kidney and to a lesser extent the
heart. As with the previous workers, once the cells are full of
carbon they 'round up' and migrate towards pigment-containing cells
in the spleen and kidney.

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More unusual areas involved in in vivo phagocytosis include the heart of the plaice (Ferguson, 1975; Ellis et al., 1976), where the endocardial lining cells take up carbon, yeasts and to a lesser extent bacteria. The replete cells 'round up' and in time the heart is completely cleared of foreign material.

A recently published report (Chilmonczyk and Monge, 1980) clearly shows gill pillar cells of rainbow trout to be capable of phagocytosing carbon, latex particles and viruses. In some cells melanin granules were present. Ellis et al. (1976) quote Hoskins and Hoskins (1918) as having noted such phagocytic and cytopoietic activity in dogfish gill that it should be considered an important source of free macrophages.

The lungfish (Protopterus Owen) has no resident population of macrophages in its "lung" (Fishman, Daniele and Pietra, 1979), but stimulation with carbon, latex and endotoxin results in a phagocytic exudate and elicits phagocytic potential of the lung lining cells.

From the previous literature it seems that perhaps fish cells have not specialised as completely as in higher vertebrates, and that on appropriate stimulation certain cell types can be induced to phagocytose although this is not their primary function.

ii) Macrophage Morphology

In a descriptive paper on plaice leucocytes, Ellis (1976), the macrophages were described as appearing in three forms. These are: a) as free, rounded cells in the spleen, kidney, thymus, mesentery and peritoneal fluid. These contain a few acid phosphatase positive granules, are PAS positive, and a small number of the splenic cells stain positively for Perl's reaction indicating haemoglobin breakdown. b) Macrophages also appear as fixed cells forming

incomplete linings to the blood sinuses of the spleen, heart and kidney. Once filled with carbon these cells break free. There are also fixed macrophages in the splenic ellipsoids. Finally c) macrophages appear as melanin-containing cells in 'melanomacrophage centres'. These are P.A.S and acid phosphatase positive, and also stain positive with Perl's Prussian Blue for 'ferric' compounds.

iii) Melanomacrophage Centres

The 'melanomacrophage centres' (M.M.C.) deserve special mention as they appear to be unique to fish. Although these cells have been described by earlier workers (Mackmull and Michels, 1932), they were first named by Roberts in 1975 and some preliminary work was performed on them by Ellis (1976); Ellis et al. (1976) and Ferguson (1976b).

However, recently these cells have been studied in much greater depth (Agius, 1979, 1980, 1981; Agius and Roberts, 1981) and their function and distribution is becoming clear.

A survey of the phylogenetic development of M.M.C within fish was performed on 72 species of fish (Agius, 1980) including representatives of agnatha, chondrichthyes and osteichthyes. In the agnathans pigment cells are found only in the hagfish and then only in the liver. In chondrichthyes the melanin-containing cells are more widespread but more are generally found in the liver than the spleen (except in Chimaera monstrosa L where the reverse is true). These pigment cells are not organised into M.M.C in chondrichthyes. In the bony fishes the M.M.C are generally present in spleen, kidney and to a lesser extent liver, and are discrete aggregations of pigment-containing cells with the exception of salmonids in which they retain a more random distribution. From agnathans to the

teleosts there is a progressive shift from liver to spleen and kidney, in the number of M.M.C. found.

Starving dogfish, rainbow trout, plaice, swordtails, Xiphophorus helleri (Heckel) and Tilapia zillii (Gervais) for periods up to ten weeks leads to an increase in M.M.C. in those organs containing them (Agius and Roberts, 1981).

Staining spleen, kidney and liver sections of fourteen species of teleost by Perl's Prussian Blue technique, revealed that spleen M.M.C. have most deposited haemosiderin suggesting erythrocyte breakdown and therefore a functional difference may exist between M.M.C. of different organs even though they are morphologically similar (Agius, 1979).

Splenectomising rainbow trout before starving them results in a shift of deposition of ferric-containing compounds from the spleen to the kidney (Agius, 1981). Elasmobranchs appear to have a different mechanism operating as, although prolonged starvation causes an increase in M.M.C., no haemosiderin can be detected in the body (Agius, personal communication).

Ferguson (1976b) suggested that M.M.C. may act as germinal centres, after observing macrophages migrating to M.M.C. and a concomitant increase in surrounding lymphoid tissue during a coccidial infection of turbot, Scophthalmus maximus (L).

It seems that melanin is produced to assist in the breakdown of antigens, phagocytosed particles and degenerating body tissue. Mawdesley-Thomas and Young (1967) showed an increase in melanophores associated with infection of the skin of the flounder (Platichthys flesus) (L) by metacercariae of a Cryptocotyle sp. Whether it is produced as a by-product of tissue breakdown or directly for its biocidal or biodegradative properties is unknown.

iv) Functional Studies on Phagocytosis

While the cells and tissues of the fish phagocytic system are relatively well-defined, there are fewer reports detailing quantitative studies of either in vivo or in vitro phagocytosis in these animals.

Nelstrop, Taylor and Collard (1968) used lampreys (Petromyzon fluviatilis (L)), goldfish (C. auratus) and dogfish to investigate whether fish showed an enhanced clearance of antigen on secondary compared to primary exposure. Using T_1 phage as the test antigen, these workers showed that in all lampreys and half the goldfish a faster clearance rate occurred on secondary exposure (in the remaining goldfish the clearance rate was equal to the primary clearance rate). By comparison the dogfish showed a slower secondary clearance rate, even when the dose was reduced, and it was suggested that a 'blockade' of the phagocytic system may have occurred.

In vitro work has been performed to a greater extent. The clearance of Staphylococcus aureus by goldfish whole blood was monitored (Avtalion and Shahrabani, 1975) using previously immunised and non-immunised fish. There appeared to be little difference in the rate of clearance, implying that there was no opsonic effect of specific antibody present in the immunised fish, but the cells from the immune fish were more efficient at killing intracellular bacteria suggesting that 'activation' of the phagocytes occurs when immunised with bacteria.

Little information was gained in this work as to the relative contributions to clearance by phagocytes, natural antibody, complement and lysozyme.

Macrophages from the pronephros of rainbow trout and Atlantic

salmon (Salmo salar L) phagocytosed sheep erythrocytes, latex, carbon, yeasts and Vibrio anguillarum under in vitro conditions (Braun-Nesje, Berthenssen, Kaplan and Seljelid, 1981).

The gar Lepisosteus (L), a holostean, has been shown by electron microscopy studies to have phagocytes which take up particles by a unique mechanism, termed by the authors "enfoldment" (McKinney, Smith, Haines and Sigel, 1977). This occurs, in addition to the usual engulfment process, by long, narrow pseudopodia 'wrapping' the particle in one or more coils, and the membrane then fusing with the rest of the cell at various points along the 'coil'.

4. Leucocyte Surface Markers and Interactions with Immunoglobulin

i) Leucocyte Surface Markers

In mammals the presence or absence of surface receptors on leucocytes is used to identify the various populations of cells (Roitt, 1971). In fish, most work has been carried out on lymphocytes, in order to demonstrate T and B cell analogues. Thus, many workers are attempting to show differential responses to mitogens such as Concanavalin A (con A), Phytohaemagglutinin (PHA) and bacterial Lipopolysaccharide (LPS), and so identify subpopulations of lymphocytes. Etlinger, Hodgins and Chiller (1976) showed differential mitogen responses by lymphocytes from rainbow trout and proposed that B and T cell equivalents are present in these fish. Cuchens and Clem (1977) showed differential mitogen responses in two lymphocyte populations of the bluegill (Lepomis (L)). Interestingly, these two populations also differ in the optimum temperature for stimulation by the appropriate mitogens.

In addition to surface receptors lymphocytes of some fish have been shown to have surface immunoglobulin. For example, Ellis and

Parkhouse (1975) demonstrated surface immunoglobulin on the surface of skate (Raja naevas Müller & Henle) lymphocytes, by immunofluorescence.

Using a similar technique, Ellis (1976) showed that approximately ten percent of macrophages in suspensions of spleen and pronephros of plaice, have surface immunoglobulin and a large proportion of these cells contain melanin.

ii) Opsonisation

The main function of receptors for immunoglobulin on the surface of mammalian macrophages is in enhancing phagocytosis by a process known as opsonisation (Wright and Douglas, 1903).

Variable results have been gained with fish phagocytes. Coating sheep erythrocytes with specific antibody and complement (from mammals) does not enhance phagocytosis by macrophages from the pronephros of rainbow trout or Atlantic salmon (Braun-Nesje et al., 1981). Similarly, no promotion of phagocytosis is gained, by sensitising sheep erythrocytes with specific antibody from rabbits, by peritoneal macrophages from the marine teleosts Pimelometopon pulchrum and Girella nigricans (Holmquist, Stohlman, Tronsdale, Ganges and Meyer, 1980).

These results would perhaps be expected as it seems unlikely that fish cells would have receptors for mammalian lg G since the only immunoglobulin present in fish is lg M (Marchalonis, 1977).

By comparison Ruediger and Davis (1907) found that serum is necessary for the uptake of a number of bacterial species by fish blood leucocytes in vitro. Mammalian sera were as efficient in this respect as homologous sera.

The disagreement concerning the role of serum may reflect fundamental variations between antigens indicating that several

mechanisms exist to deal with various antigens; or reflect a difference in culture conditions. For example, in this study, if the control cells were maintained in a medium deficient in certain nutrients, the addition of serum may supply these nutrients necessary for phagocytosis to occur while not acting opsonically.

Phagocytic cells from the smooth dogfish Mustelus canis, take up liposomes coated with heat-aggregated (10 min. at 62°C) Ig M at a greater rate than they phagocytose uncoated liposomes or liposomes coated with native Ig M (Weissmann, Bloomgarden, Kaplan, Cohen, Hoffstein, Collins, Gotlieb and Nagle, 1975).

It is difficult to imagine what use a receptor to an 'artificial' form of the immunoglobulin is to the fish in nature, unless there exists a mechanism by which the immunoglobulin may become naturally aggregated.

iii)   Antibody Dependent Cell-Mediated Cytotoxicity

Macrophages may be cytotoxic to some extracellular antigens by a process in which they interact with antibody - a phenomenon known as 'antibody-dependent cell-mediated cytotoxicity' (A.D.C.C.). Cellular antigen coated with small amounts of antibody can cause macrophages to release lysosomal enzymes which mediate lysis of antigen (Roitt, 1971).

Few reports exist on this reaction in fish. Phagocytes of Mustelus canis degranulate and secrete lysosomal enzymes, when presented with immune complexes composed of the oocytes of the sea urchin Arbacia punctatus and heat-aggregated Ig M (Weissmann, Finkelstein, Csermansky, Quigley, Quinn, Techner, Troll and Dunham, 1978). These enzymes cause lysis of the Arbacia eggs. This result relies on the assumption that the dogfish phagocytes have surface receptors for Ig M (which Weissmann et al., (1975) had previously

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demonstrated).

Pettey and McKinney (1980) reported A.D.C.C. in the nurse shark, using natural antibody. They had not investigated the cells in question for receptors but their results suggest their presence.

Peritoneal macrophages from P. pulchrum and G. nigricans did not lyse sheep erythrocytes coated with rabbit antibody (Holmquist et al., 1980) but again there is no reason why fish cells should have receptors for mammalian antibody.

5. Hypersensitivity Reactions

i) Delayed Hypersensitivity

The macrophage is involved in delayed-hypersensitivity reactions in mammals and higher vertebrates (David, 1970). Here, a specific, inflammatory reaction occurs on secondary exposure to an antigen after primary sensitisation. Macrophages are prevented from leaving the site of antigen challenge by a lymphokine released by T-cells - Macrophage Migration Inhibition Factor.

Although fish undoubtedly show inflammatory reactions (Finn and Nielsen, 1971; Timur and Roberts, 1977) there is less evidence for the more specific delayed-hypersensitivity (D.H.) reactions of the kind seen in mammals.

Bullheads (Cottus sp. L) were claimed to show D.H. after tuberculin sensitisation (Papermaster, Condie, Finstad, Good and Gabrielsen, 1963). A delayed corneal reaction to purified protein derivative (P.P.D.) in rainbow trout one month after immunization with Freund's complete adjuvant has been reported (Ridgway, Hodgins and Klontz, 1966).

Cell-mediated immunity is active in the later stages of granulomatous disease of fish (Timur and Roberts, 1977) and in

tuberculous and foreign body granulomas and in lymphocystis, the late stages of the lesion are characterised by invasion of the tissues by lymphocytes. This coincides with a positive 'M.I.F.' test in blood samples (see below).

The Migration Inhibition Factor (M.I.F.) test is an _in vitro_ test of delayed hypersensitivity. Timur (1975) showed that M.I.F. is produced in chronic inflammatory diseases and implicated a lymphocytic cell as being responsible for conversion of macrophages to epithelioid cells and for 'ᴄaseous necrosis'.

The dogfish S. canicula shows a positive M.I.F. reaction against an extract of Proleptus obtusus Dujardin (a common, intestinal parasite) (Morrow, 1978). However, since no uninfected fish could be obtained, there was no negative control in this study.

Rainbow trout have been screened for resistance to furunculosis, using the M.I.F. test after immunization by various methods (Smith, McCarthy and Paterson, 1980). The percentage of fish producing a positive cellular response against the natural challenge strain of Aeromonas salmonicida, corresponded very closely with the percentage found to be resistant to furunculosis in vaccine field trials.

There does therefore appear to be sufficient evidence to show that D.H. occurs in at least some species of fish.

ii)   Immediate Hypersensitivity

Anaphylaxis is the other main hypersensitivity reaction to have been investigated. There are no reports of anaphylaxis in cyclostomes and chondrichthyes although Dreyer and King (1948) noted that elasmobranchs are insensitive to histamine - which mediates this reaction in higher vertebrates.

Fletcher and Baldo (1973) reported positive skin reactions to

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several fungal extracts, in four of five flatfish species tested. The flounder (<u>Platichthys flesus</u> (L)) was the only species tested which did not show immediate hypersensitivity. Furthermore the flounder was also the only species which did not exhibit C reactive protein (CRP) precipitins to the allergens, which led the authors to implicate CRP in mediating type 1 hypersensitivity or anaphylaxis.

Further positive results in teleosts have been reported by Dreyer and King (1948) and also recently by Goven, Dawe and Gratzek (1980). However Lukyanenko (1967) could not repeat the work of Dreyer and King, as the 'anaphylactic-type' reactions such as changes in respiration rate and increased motor activity occurred in control as well as immunized fish. In addition, the same author could not confirm the high reactivity of fish to histamine.

Passive cutaneous anaphylaxis could not be demonstrated in trout using guinea-pig or trout anti-BSA/BSA or trout anti KLH/KLH' systems (Hodgins, Weiser and Ridgway, 1967).

Clem and Leslie (1969) could not demonstrate anaphylaxis in margates (marine teleosts) which had received BSA or margate anti BSA serum intravascularly.

The same workers have similarly been unable to demonstrate 'localised Arthus-type lesions' in teleosts even after monthly immunizations for up to a year. They suggested that the low concentrations of serum antibody present throughout the experiment may have been too low to allow the reaction to occur.

Although the available evidence suggests that hypersensitivity reactions, and immediate-type reactions in particular, are difficult to demonstrate, there are very few reports on these reactions in the literature. It therefore appears to be an area requiring further

investigation, especially in the cases of the lower fish such as agnatha and chondrichthyes.

Chapter 2

Materials and Methods

1. Materials

Fish

i) Dogfish (<u>Scyliorhinus canicula</u> L.) (600 - 1000g), plaice (<u>Pleuronectes platessa</u> L.) and rays (<u>Raja clavata</u>) were obtained from the Marine Biological Association, Citadel Hill, Plymouth as required, and maintained in large polythene tanks in cooled, filtered, recirculating sea-water at 10-12°C with supplementary aeration. They were fed chopped coley on alternate days.

ii) Eels (<u>Anguilla anguilla</u> L.) were obtained from Pudleigh Mill Fish Farm, Chard, Somerset, and maintained in recirculating, 50% sea-water with supplementary aeration. They remained unfed until used for experimental work.

Mammals

i) Closed colony, outbred Wistar rats (250-350g) were obtained from Plymouth Polytechnic Animal House.

ii) New Zealand white rabbits (3 kg) were obtained from Ranch Rabbits Ltd, Crawley Down, Sussex.

Chemicals

Unless otherwise stated all chemicals were obtained from Sigma (Chemicals) Ltd or BDH Ltd (Poole) Dorset. All reagents for electron-microscopy were supplied by E M Scope.

Tissue Culture

All tissue culture media were obtained from Wellcome Ltd and tissue culture plates from Nunc Ltd or Corning Ltd. Media (i.e. Tissue Culture Medium 199 or Hank's Balanced Salts Solution) were

supplemented as outlined in Table 1.

Table 1

Tissue Culture Medium Supplementation.

Fish	Urea	Na Cl	Na HCO$_3$
Elasmobranchs	2.1%	to 0.2 M	0.2%
Marine teleosts	-	to 0.2 M	0.2%
Eels	-	-	0.2%

Where tissue culture media could not be used (e.g. in the investigation
of the effect of divalent ions on phagocytosis) Young's selachian
saline was used as recommended by Lockwood (1961) and supplemented
with glucose (1g 100 cm^{-3}).

Bacteria

Bacterial species used in this project were obtained from
existing laboratory stocks kept at -198°C. All microbiological media
were obtained from Oxoid Ltd or Difco Ltd.

Table 2

Culture media and temperatures employed for micro-organisms used in this study

Bacterial Species	Broth Culture	Differential or Selective Media	Incubation Temp.
1. Escherichia coli	Nutrient Broth	McConkey Agar	37°C
2. Salmonella typhimurium	Nutrient Broth	Bismuth Sulphite Agar	37°C
3. Streptococcus faecalis	Glucose Broth	Blood Agar Base + 5% Horse Blood	37°C
4. Staphylococcus albus	Tryptone Soya Broth + 1.5% NaCl	Staphylococcus 110 Medium	37°C
5. Vibrio anguillarum i) Strain - UNH 569) ii) Strain - COB 408) iii) Strain - A20/76) also	Tryptone Soya Broth + 1.5% NaCl	Tryptone Soya Agar + 1.5% NaCl	25°C
6. Saccharomyces cerevisiae	Sucrose solution	Malt Extract Agar	25°C

2. General Methods

Immunization

i) Dogfish and plaice were immunized by either the intraperitoneal or intravascular route. Injections comprised normally of between 0.25 cm^3 and 0.5 cm^3 of suspensions of Salmonella typhi H antigen, sheep or bovine erythrocytes or KLH; the precise details will be described in the relevant section of the results.

ii) All immunization procedures for fish used in the delayed hypersensitivity studies are given in a later section.

iii) Rabbits (New Zealand white) were immunized by three subcutaneous injections at multiple sites given at 10 day intervals. Antigen suspensions (such as KLH, BGG and Dogfish Ig) were emulsified in an equal volume of Freund's Incomplete Adjuvant (FIA) prior to injection. Cellular antigens (such as sheep or bovine erythrocytes) were injected as a 5% $^v/v$ suspension without FIA. Ten days after the final injection the rabbits were bled from the marginal ear vein. The blood was allowed to clot at room temperature, placed at 4^oC overnight for the clot to contract and the serum harvested and stored at -70^oC.

iv) Rats (outbred Wistar) were immunized in the same way as rabbits but were bled by direct cardiac puncture after anaesthesia with Halothane (May and Baker).

Blood sampling

For all routine and experimental blood sampling dogfish and eels were bled ventrally from the posterior caudal sinus behind the cloaca by means of a 23g hypodermic needle with a heparin-coated syringe.

Rays could only be sampled once directly from the heart again using a 23g hypodermic needle and heparinised syringe.

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Plaice were sampled from the renal portal vein ventral to the vertebral column using a similar needle size and syringe as for the previous fish.

Light microscopy

i)   Small blocks (approximately 1 cm^3) of tissue were fixed overnight in Formol saline at 4°C. Where necessary (e.g. with skin) they were decalcified for an appropriate period in RDC (Bethlehem Instruments). The tissues were subsequently dehydrated in graded alcohols, cleared in xylene and embedded in Fibrowax (Raymond Lamb) (melting point 56°C).

Sections (5-10μ) were cut using a Leitz rotary microtome. For general use wax sections were dewaxed in xylene, brought to water in decreasing graded alcohols and stained with Mallory's Triple Stain (Putt, 1972). More specific histochemical staining procedures were also performed (see Table 3).

ii)   Whole blood smears, adherent leucocytes and organ imprints were air-dried, fixed in methanol for 5 min. and then stained in Giemsa stain for 5 min. They were washed and allowed to air-dry. The most suitable Giemsa procedure was using the following:-

Giemsa buffer (BDH)	2 parts
Methanol (BDH)	1 part
Distilled water	8 parts

Three volumes of the above were mixed with 1 volume of Giemsa concentrated stain (BDH) (Wrathmell, personal communication). In addition, blood smears and tissue imprints were stained histochemically as outlined in Table 3.

Electron Microscopy

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Transmission Electron Microscopy (TEM). Small blocks of dogfish tissues (approximately 1 mm^3) and leucocyte suspensions were fixed in 2% glutaraldehyde in Young's selachian saline (Lockwood, 1961) for 24h at $4^{o}C$. They were washed three times in dogfish saline and post-fixed in 1% osmium tetroxide in the same diluent, for at least 2h. The samples were again washed three times, dehydrated in graded alcohols, transferred to propylene oxide and impregnated with Spurr resin (Spurr, 1969).

Gold sections (60-100 nm) were cut using glass knives on Porter Blum MT2B or Reichert-Jung Ultracut ultramicrotomes, and mounted on uncoated copper grids. They were stained with saturated aqueous uranyl acetate and Reynold's lead citrate and examined on a Philips EM 300 transmission electron microscope.

Scanning Electron Microscopy (SEM). Samples for SEM were fixed and dehydrated as for TEM. They were critical point dried using CO_2 astransition fluid (Samdrie PBT3 critical point dryer). The samples were then mounted on stubs, gold-coated and observed using a Jeol 35S scanning electron microscope.

Bacterial Counts

Enumeration of bacteria was performed by ten-fold serial dilution in peptone water and plating 0.02 cm^3 of suitable dilutions in duplicate by the method of Miles and Mizra (Miles, Mizra and Irwin, 1938). Alternatively, in vitro clearance was monitored by using a spiral plater (Spiral Systems) to enumerate residual bacteria in suitably diluted samples.

Fluoresceination of bacteria (after Gelfand, Fauci, Green and Frank, 1976)

Bacteria were grown in appropriate broth media for 48h with

aeration to produce a dense culture. They were heat-killed at 60°C

for 30 min, washed three times in PBS and resuspended to give an

OD reading of 2.0 at 540 nm.

One volume of this culture was diluted with 5 volumes of 0.5M

carbonate/bicarbonate buffer, pH 9.5. Two volumes of a solution of

fluorescein isothiocyanate isomer 1 (FITC) (0.03g 100 cm^{-3}) in the

same buffer were added and incubated for two hours at room

temperature. The fluoresceinated bacteria were washed three times in

Veronal buffered saline with 0.15 mM Ca, 1mM Mg and 1 mg cm^{-3} gelatin,

and resuspended in the same buffer. They were divided into suitable

aliquots and stored at -20°C.

Fluoresceination of soluble antigen (after Hudson and Hay, 1980) (i.e.

of Keyhole Limpet Haemocyanin (KLH) (Calbiochem) and Bovine Gamma

Globulin (BGG))

A protein solution was prepared (20 mg cm^{-3}) in 0.25M carbonate/

bicarbonate buffer, pH9, and 0.05 mg of FITC was added per mg. total

protein. The solution was allowed to mix overnight at 4°C and the

free fluorochrome separated from the conjugated protein by passing

the mixture down a G-25 Sephadex column (Pharmacia) equilibrated with

PBS.

Trypan-blue staining of yeasts (after Rivero, Abaca, Valles,

Vannucci, Diumenjo and Moravenik, 1979)

Yeast (Saccharomyces cerevisiae) was suspended in PBS to give a

suspension of 1.2 x 10^8 cells cm^{-3}. These were then heat-killed in a

boiling water bath for 10 min. and stained by adding 0.1 cm^3 of a

trypan blue solution (20 mg cm^{-3}) per cm^3 of yeast suspension. The

yeasts were then washed three times in PBS and resuspended to the

original cell concentration.

Preparation of monomeric IgM from dogfish plasma

Dogfish plasma containing natural antibody activity was taken and separated by column chromatography using Sephadex G200 (Pharmacia). The appropriate fractions were tested against rabbit anti dogfish IgM, by agar-gel diffusion. Those fractions giving precipitin bands were pooled, concentrated and subjected to mild reduction by dithiothreitol (Rhodes, 1973). The resulting solution was run via polyacrylamide gel electrophoresis with SDS, with appropriate molecular weight standards to identify any monomer which may have formed.

Rocket immunoelectrophoresis (Weeke, 1975)

Fifty cm^3 of agarose (1g 100 cm^{-3}), (made up in 0.02 M barbital buffer pH 8.6) containing antiserum were poured on to a level pre-warmed, glass plate (26.15 x 12.45 cm) to give a gel approximately 1.5 mm thick.

Wells of 2.5 mm diameter were cut in the gel and the residual agarose gently removed by suction. The plate was placed on a LKB multiphor system and the samples applied to the wells as quickly as possible to prevent any diffusion from the wells. The buffer baths were each filled with 1 dm^3 of 0.02M barbital buffer, pH 8.6 and the wicks positioned. The samples were run for 2 h at 8-10 v cm^{-1}. The gels were subsequently washed in PBS and transferred to stiff plastic. They were pressed overnight under a heavy weight, stained with Coomassie Brilliant Blue R, destained by the method of Weber and Osborn, (1975), and were finally pressed and dried. Any rockets were measured by noting their heights and widths at the mid-point.

Migration inhibition factor (MIF) tests

Blood and spleen leucocyte suspensions were taken from fish which had been appropriately sensitised (see details under delayed hypersensitivity). They were adjusted to approximately 10^7 leucocytes cm^{-3} of tissue culture medium. Uniformly mixed suspensions of cells were taken up in sealed heparinized capillary tubes. The cells were centrifuged at 750g for 5 min. The capillary tubes were broken at the interface between leucocytes and medium and the cell-containing segment positioned at the edge of the wells in MIF plates (Sterilin) by means of silicone grease.

Various concentrations of test antigen were placed in the wells, including control medium containing no antigen and each well was covered by means of a glass cover slip. Each test concentration was performed using at least four replicates.

After over-night incubation at room temperature the projected migration patterns were outlined on paper. The resulting paper discs were cut out and weighed and each test antigen concentration was expressed as a percentage of the migration of control suspensions.

3. Identification, Characterisation and Morphology of Dogfish Blood and Tissue Leucocytes

Treatment of fish

Dogfish were bled and subsequently killed by a sharp blow to the head and the spleen, liver, heart, gills and oesophagus removed.

In order to locate phagocytic cells, 0.1 cm^3 of colloidal carbon (150 mg cm^{-3}) was injected intravascularly via the caudal sinus two days prior to blood sampling and sacrifice.

Blood

i) Whole blood was diluted to $1/10$ in Young's selachian saline and total blood counts were performed by haemocytometry. In addition blood smears were made on glass slides and stained by the method of Giemsa or by other histochemical methods outlined in Table 3. Differential leucocyte counts were performed on Giemsa-stained smears.

ii) Whole blood was centrifuged at 750g for 5 min (MSE bench centrifuge) and the buffy coat was removed and suspended in tissue culture medium before being dispensed into tissue culture plates. These were left for at least 1 h at room temperature to allow cells to adhere to the plate after which time non-adherent cells were washed off with tissue culture medium. Some of these cell cultures were then provided with yeasts or bacteria and incubated for 30 min. All cultures were then washed and fixed for Giemsa staining, histochemistry or SEM. In addition unfixed cells were observed by phase contrast on a Zeiss photomicroscope.

iii) Whole blood was centrifuged and the buffy coat removed and suspended in 2% glutaraldehyde in Young's selachian saline for TEM.

Spleen and liver

i) Pieces (approximately 1 cm^3) of freshly dissected spleen and liver were cut and the cut surface blotted on paper tissue to remove excess moisture. This surface was then used to make imprints on glass microscope slides which were subjected to a variety of histochemical procedures (see Table 3).

ii) Small pieces of spleen and liver (1 cm^3) were processed for wax histology. In addition smaller blocks (1 mm^3) were processed for TEM and SEM.

iii) Liver and spleen tissues were teased apart in a petri dish containing heparinised tissue culture medium. The cell suspension was

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then centrifuged at 750g for 5 min and the white cells suspended in tissue culture medium before being dispensed into tissue culture plates and treated in the same way as adherent blood cells.

Gill, heart and oesophagus

These tissues were taken from control and carbon-treated dogfish, processed for wax histology and stained by Mallory's triple stain.

Histochemistry

Table 3 shows the histochemical procedures used in this study.

Lysozyme

Attempts to detect lysozyme (N-acetylmuramoyl hydrolase) in blood and tissues of fish involved three separate assays:-

i) The method of Ghoos and Vantrappen (1970). Chitin was coated on glass slides and buffy coat preparations were smeared on these coated slides and incubated in a moist chamber for 1-2 h at room temperature. The slides were then allowed to dry, fixed in methanol for 2 min and stained as follows:-

Alcian Blue 8GS (400 mg 100 cm^{-3}, pH4)   –   8 min

Nuclear Fast Red (100 mg 100 cm^{-3} of 5%

Aluminium Sulphate pH 3.5)   –   2 min

A blue colour concentrated especially in the cytoplasm of neutrophils signifies lysozyme activity, the nuclei staining brick red.

ii) The method of Briggs, Perillie and Finch (1966). Blood was mixed with an equal volume of a suspension of Micrococcus

Table 3

Histochemical procedures performed on dogfish cells and tissues

Stain	Fixative	Authority/Ref.	Wax section, imprint or smear
1. Non-specific esterase	$Na_2 HPO_4$ - 20 mg KH_2PO_4 - 100 mg DW - 30 mls Acetone - 45 mls formaldehyde (30%) - 25 ml pH 6.6	Pearse, 1968	Imprint/smear
2. Acid phosphatase	Formalin vapour	Hayhoe & Flemens 1969	Imprint/smear
3. Alkaline phosphatase	Buffered acetone	Cartwright, 1968	Imprint/smear
4. Sulphatase	Formalin vapour	Austin & Bischel, 1961	Imprint/smear
5. β glucuronidase	Methanol : Formalin 3 : 7	Lorbacher, Yam & Mitus, 1967	Imprint/smear
6. Lipase	Formalin vapour	Gomori, 1945	Imprint/smear
7. Peroxidase	2% glutaraldehyde	Pearse, 1968	Imprint/smear
8. Periodic-Acid Schiff (PAS)	40% Formalin : Ethanol 10 : 90	Hayhoe & Flemens, 1969	Wax, imprint or smear
9. Sudan Black B.	Formalin vapour	Hayhoe & Flemens, 1969	Wax, imprint or smear

Table 3 Continued

Stain	Fixative	Authority/Ref.	Wax section, imprint or smear
10. Perl's Prussian Blue	– Wax sections	Drury & Wallington, 1967	Wax
11. Whitby & Hynes Method for leucocytes	– Live cell suspensions	Culling, 1969	Live cell suspensions
12. Csaba's mast cell stain	– Wax sections	Culling, 1969	Wax/smear
13. Acridine orange -	– Live cell suspensions	Allison, 1976	Live cell suspensions
14. Qualitative nitroblue tetrazolium reduction	– Adherent cells	Sugimoto, Ando, Senba & Tokuomi, 1980	Adherent blood cells

lysodeikticus (40 mg cm^{-3}) in dogfish saline, mixed thoroughly and

smears made on glass slides. These slides were then placed in a

moist chamber and incubated at room temperature for 10-15 min.

The slides were subsequently air-dried, fixed in methanol and stained

by the method of Giemsa.

Evidence of cellular lysozyme activity consists of capsular

swelling and diminution in the protoplast size of micrococci

immediately surrounding an active blood cell.

iii)· The plate method of Osserman and Lawlor,(1966). An agar

gel (Oxoid Agar No. 1) was prepared (10g dm^{-3}) in PBS. When the

molten agar cooled below 55°C, dried Micrococcus lysodeikticus were

added (0.25 mg cm^{-3}) and mixed thoroughly with the agar. The agar-

bacteria suspension was poured into sterile disposable petri dishes

(Sterilin) and allowed to gel.

Wells were cut in the agar using a sterile cork borer and

constant volumes of varying dilutions of egg white lysozyme were

added to the wells. In addition dogfish plasma, blood and spleen

leucocyte suspensions, ultrasonicated leucocyte suspensions, and

supernatants from phagocytosing cells were added to the wells.

After 24 h incubation at room temperature the diameters of any

zones of lysis were recorded.

Quantitative nitroblue tetrazolium reduction (after Sugimoto, Ando,

Senba and Tokuomi, 1980)

Nitroblue tetrazolium (NBT) is reduced to a blue formazan

product by reducing agents. In human phagocytes this reduction is

largely due to the superoxide anion (O_2^-), the activity of which is

demonstrated by the inhibition of NBT reduction by the addition of

superoxide dismutase.

Standard aliquots of a uniform suspension of dogfish blood leucocytes were placed in tissue culture plates and allowed to adhere for at least 1 h. The non-adherent leucocytes were washed away and the medium replaced with 2 cm^3 of tissue culture medium 199 containing NBT (0.2g 100 cm^{-3}). The cells were incubated in this medium for 90 min. The effects of the presence of latex and bacteria were tested (i.e. whether phagocytosis affected NBT reduction) and also the effects of superoxide dismutase (30 units cm^{-3}) and catalase (0.1 mg cm^{-3}).

After incubation the reaction was stopped by means of 1 cm^3 of 1M HCl, and the cells scraped off the plates into glass tubes. These were centrifuged at 750g for 5 min and the overlying supernatant discarded. The precipitated cells were solubilised in 5 cm^3 pyridine and the optical density of each tube monitored at 515 nm against a 5 cm^3 pyridine blank. Each test mixture was assayed in replicate.

4. Endocytic Studies

a) A qualitative study of in vitro endocytosis

i) Blood and spleen leucocyte suspensions in tissue culture medium were allowed to adhere to tissue culture plates for 1 h before non-adherent cells were washed off and tissue culture medium containing the test antigen or particle (see Table 11) were placed on the plates. After 1-2 h the overlying medium was removed, the cells washed in medium and fixed and stained by the method of Giemsa or prepared for SEM.

ii) In some cases blood leucocyte suspensions were incubated in siliconised glass vessels with the test antigen or particle for 1-2 h

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before smears were made on glass slides and similarly stained by the method of Giemsa.

b) A qualitative study of in vivo phagocytosis

Bacteria, yeasts and carbon were injected intravascularly into dogfish for quantitative monitoring of clearance. After these experiments were terminated the fish were killed, their blood, spleens, livers and gills processed for wax histology and TEM and examined for evidence of phagocytosis. In addition, BCG (Glaxo) was injected intravascularly into three dogfish (i.e. 0.1 cm^3 of a 1 cm^3 vial) and the blood and tissues processed for light and electron microscopy as above. Cells from these fish were also used for quantitative, in vitro phagocytic studies in order to see if they were in any way 'activated' (see section 4c, vi)).

c) A quantitative study of in vitro phagocytosis and the effects of various factors on this process

Phagocytosis was monitored in vitro by using adherent monolayers of dogfish blood leucocytes and trypan blue-stained yeasts as the phagocytic particle.

Heparinised, dogfish blood was centrifuged at 750g for 5 min and the buffy coat removed and suspended to approximately 2 x 10^7 leucocytes cm^{-3}.

Equal aliquots of the uniformly suspended leucocytes were dispensed into an appropriate number of tissue culture plates and the cells allowed to adhere for at least 1 h at room temperature before non-adherent cells were washed off. The overlying medium was replaced with tissue culture medium containing heat-killed, trypan blue stained yeasts in appropriate numbers (see below). At various time intervals,

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or after a fixed incubation period the overlying medium and residual yeasts were washed off and the adherent phagocytes fixed and stained by the method of Giemsa. The number of cells containing yeasts were counted and expressed as a percentage of total adherent cells in at least six microscope fields (x 400). In addition the number of yeasts per positive phagocytic cell was counted.

The above procedure was used to investigate the effect of altering the physical and chemical parameters described below.

i) Effect of pH

Equal numbers of yeasts were added to 10 cm^3 volumes of tissue culture medium 199 adjusted to a pH range of 5.5 to 8.5 (with 0.5 pH unit intervals), and phagocytic indices calculated after a fixed incubation period (The optimal pH was used for all subsequent experiments).

ii) Effect of temperature

Duplicate plates were incubated for 1 h at the following temperatures - 4°C, 10°C, 20°C, 25°C, 37°C, 45°C.

All subsequent assays were performed at room temperature (18-21°C).

iii) Effects of plasma

a) Any opsonic effect of autologous plasma was assayed by either adding

      1) heat-inactivated (45°C for 25 min) plasma

      2) whole plasma

      3) no plasma

to the incubation medium or by previously incubating yeasts with the above.

Rates of uptake were assayed by fixing plates with methanol at

10 min intervals up to 1 h.

b) To remove any opsonic plasma elements which may be permanently attached to phagocytic cells, the adherent leucocytes were subjected to trypsin (2 mg cm^{-3}) or rabbit anti dogfish IgM (heat-inactivated 56°C for 30 min) for 30 min prior to the addition of yeasts $\pm$ plasma, and counts made after incubation for 1 h.

iv) **Effects of metabolic inhibitors**

The effects of three metabolic inhibitors were examined by supplementing the incubation medium as indicated below.

a) Iodoacetate (glycolytic inhibitor) - 0, 0.1, 1.0 and 10 mM.

b) Sodium azide (cytochrome inhibitor) - 0, 0.1, 1.0 and 10 mM.

c) Puromycin dihydrochloride (protein synthesis inhibitor) - 0, 0.1, 1.0 and 10 μg cm^{-3}.

v) **Effects of divalent ions**

This assay was performed using Young's selachian saline (Lockwood, 1961) supplemented with glucose (1g 100 cm^{-3}) as:-

1. the complete ringer

2. without calcium salts

3. without magnesium salts

4. without calcium or magnesium salts

in place of tissue culture medium.

vi) **Effects of 'activation'**

Blood leucocyte suspensions were prepared from an untreated fish and a fish injected 2 weeks previously with BCG intravascularly.

They were adjusted to similar leucocyte concentrations and their phagocytic activity compared by the method previously described at the beginning of section 4c.

In vitro uptake of KLH

Equal volumes of dogfish leucocyte suspensions were allowed to adhere to tissue culture plates as previously described. The adherent cells were overlaid with 2 cm^3 of tissue culture medium 199 containing KLH (a range of concentrations between 40 and 250 $\mu g\ cm^{-3}$ were tested). At various times up to 24 h 50 μl samples were taken and residual KLH was assayed by Rocket Immunoelectrophoresis. A control plate containing only tissue culture medium and KLH was sampled at the same time. Assays were also performed using cells from KLH immunized fish and cells from control fish.

In vitro bacterial clearance

Leucocyte suspensions were allowed to adhere as before. Adherent cells were overlaid with medium containing bacteria at a concentration of approximately $10^5\ cm^{-3}$. Samples of this medium were taken at various intervals and the numbers enumerated by the spiral plater technique. The effect of 1.5 mM Iodoacetate was assayed and control plates containing bacteria only or bacteria and Iodoacetate only were sampled at the same time.

The following bacterial species were assayed in this way:-

Vibrio anguillarum strain	UNH 569
+ strain	COB 408
Salmonella typhimurium	
Escherichia coli	
Streptococcus faecalis	
Staphylococcus albus	

The plates were incubated for 24-48 h and counted.

d) **A quantitative study of in vivo antigen clearance**

i) **Bacteria and yeasts**

Bacteria were cultured overnight in broth. They were centrifuged at 3,000g for 20 min and resuspended in PBS. They were then serially diluted in peptone water and enumerated by the Miles and Mizra technique. By using OD readings it was possible to inject 10^8 - 10^9 bacteria intravascularly into dogfish. The volume injected was never greater than 1 cm^3.

Bacteria were injected via the caudal sinus and blood samples taken from the same site at various intervals afterwards. The samples were serially diluted in peptone water and enumerated by the Miles and Mizra technique. The following microorganisms were assayed in this way:-

1) S. typhimurium - a) 'short' and 'long-term' clearance

b) clearance after immunization with

S. typhi H. antigen

c) clearance 1 day after injection of

0.1 cm^3 of colloidal carbon

(150 mg cm^{-3}) i.v.

2) E. coli - 'short term' clearance.

3) Strep. faecalis - 'short term' clearance.

4) Staph. albus - 'short term' clearance.

5) V. anguillarum - 'short term' clearance of strains A20/76 and COB 408.

6) Saccharomyces cerevisiae - 'short term' clearance.

Precise numbers injected and sampling times are detailed in the results.

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At the termination of the experiments the fish were killed and the blood and organs processed for light microscopy and TEM.

ii) <u>In vivo clearance of KLH</u>

Dogfish were immunized by injecting 50 mg KLH intravascularly via the caudal sinus. Four to six weeks later these fish and untreated control fish were each injected intravascularly with 75 mg of KLH. They were bled at various intervals up to 24-48 h and the blood plasma assayed for residual KLH by Rocket . Immunoelectrophoresis.

5. <u>Cell Surface Properties</u>

a) <u>Fc receptors</u>

Blood and spleen leucocytes of four fish species were monitored for the presence of Fc receptors. Rat spleen cells were used as positive controls. The following sera and antigens were employed.

<u>Sera</u>   i)   Rat anti sheep or bovine erythrocyte serum

 ii)   Plaice·anti sheep erythrocyte serum

 iii)   Natural antibody from dogfish, rays, plaice and eels

 iv)   Dogfish monomeric Ig

 v)   Dogfish 'anti KLH' serum

 vi)   Rabbit anti KLH serum

 vii)   Rabbit anti BGG serum

<u>Antigens</u>   i)   Sheep or bovine erythrocytes

 ii)   Fluoresceinated KLH or BGG

<u>Methods</u>

i)   Sheep or bovine erythrocytes in Alsevers were washed three times in PBS. They were resuspended to 5% ^{v}/v and one aliquot was

incubated for 30 min with an aliquot of a sub-agglutinating dilution of heat-inactivated serum. (For each fish species autologous and heterologous sera were tested). In the case of rat antiserum the incubation with erythrocytes was performed at $37^{o}C$, whereas fish sera were incubated at room temperature.

After incubation the erythrocytes were again washed three times in PBS and resuspended to 5% $^{v}/v$.

Aliquots of coated and uncoated erythrocytes were added to adherent blood and spleen cells in tissue culture plates and also to blood and spleen leucocyte suspensions. The cells were examined at various times between 10 min and 24 h of incubation.

ii)   Fluoresceinated KLH was titrated against heat-inactivated rabbit anti-KLH serum, dogfish anti-KLH serum and dogfish natural antibody. The highest 'sub precipitating' concentration was added to adherent blood and spleen leucocytes from dogfish. Control cell monolayers received KLH only or fluorescein only. The cells were examined after 1 - 2 h for surface fluorescence.

iii)   Adherent leucocytes from dogfish were treated with trypsin (20 mg cm^{-3}) for 30 min before being washed and tested for Fc receptors using dogfish, plaice and rat antibodies.

b) C_3 receptors

i)   The method used to test for C_3 receptors was identical to the method for Fc receptors except that the sera used to coat erythrocytes were not heat-inactivated and so sub-lytic concentrations were used to coat erythrocytes.

ii)   A second method used the observations of Gelfand et al.

(1976) and Rivero et al. (1979) that Gram negative bacteria and yeasts can cause C_3 to be deposited on their surfaces by directly activating the properdin system (alternate complement pathway) in the absence of antibody.

FITC-conjugated E. coli and trypan blue stained yeasts were incubated in sublytic dilutions of normal fish sera. After 30 min the yeasts and bacteria were washed three times in PBS and added to adherent blood and spleen leucocytes and also suspensions of these cells. The cells were examined for rosettes of yeasts or bacteria from 10 min to 24 h. The serum was tested for its lytic properties before and after incubation with yeasts or bacteria.

iii) Adherent leucocytes from dogfish were tested after trypsin treatment and 'anti-Ig' treatment using erythrocytes coated with sublytic concentrations of whole serum.

c)   Surface immunoglobulin

Dogfish adherent spleen and blood leucocytes were tested for surface immunoglobulin by incubating first in rabbit anti-dogfish Ig serum, and then, after washing with sheep anti-rabbit Ig, conjugated with FITC (Wellcome Ltd). Appropriate controls were included such as rabbit-anti KLH and then sheep anti rabbit Ig (to ensure the cells were not pinocytosing the foreign protein) and also sheep anti-rabbit Ig only.

The plates were examined using a Vickers fluorescent microscope.

6.   Delayed Hypersensitivity

The experimental work performed in an attempt to demonstrate delayed hypersensitivity in the three fish species used is presented in Table 4.

Table 4

Details of Procedures employed to demonstrate Delayed Hypersensitivity.

Expt.	Fish	No. of fish	Antigen	Sensitising Dose	Time Interval Between Injections	Challenge Antigen	Time After Last Sensitising Dose	MIF Test Performed ±
1	Dogfish	2	KLH	1 x 25mg i.v.	-	KLH, BGG, PBS 1mg i.d.	10 days	+
	Dogfish	2	-	-	-	"	-	+
	Dogfish	1	KLH	1 x 25mg i.v.	-	"	10 weeks	+
2	Dogfish	1	BSA in FCA	3 x 25mg i.p. in 0.5cm^3 FCA	7 days	BSA 1mg i.d.	7 days	+
	Dogfish	1						-
3	Dogfish	1	BSA in FCA	2 x 25mg i.p. in 0.5cm^3 FCA	7 days	(i.d.) BSA in FCA	5 days	+
	Ray	1	"	"	"	Ray Died	-	-
4	Dogfish	2	BCG	3 x 0.15cm^3 i.p.	7 days	BSA (i.d.) BCG	7 days	+
	Dogfish	2	BSA	3 x 37.5mg i.p.	"	" "	"	+
	Dogfish	1	-	-	-	" "	-	+
5	Dogfish	1	BCG	2 x 0.15cm^3 i.p.	7 days	PPD i.d.	7 days	+

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Table 4 Continued

Expt.	Fish	No. of fish	Antigen	Sensitising Dose	Time Interval Between Injections	Challenge Antigen	Time After Last Sensitising Dose	MIF Test Performed $\pm$
6	Dogfish	2	-	-	-	Proleptus Ext. (i.d.) PPD + PBS	-	-
7	Dogfish	2	KLH in FIA	2 x 10mg i.p.	7 days	KLH	7 days	+
	Dogfish	1	-	-	-	KLH	-	+
8	Eel	2	BCG	$3 \times 0.15 cm^3$ i.p.	7 days	BCG $0.1cm^3$i.d. BSA $0.1cm^3$i.d.	7 days	+
	Eel	2	BSA	3 x 20mg i.p.	7 days	" "	7 days	+
	Eel	2	-	-	-	" "	-	+
9	Eel	2	SRBC	$1 \times 0.25cm^3$ of 10% ^{v}/v i.p.	-	SRBC	14 days	-
	Eel	2	SRBC in FCA	$1 \times 0.25cm^3$ of 10% ^{v}/v + $25cm^3$ FCA i.p.	-	SRBC	14 days	-
	Eel	2	BCG	$1 \times 0.25cm^3$ i.p.	-	BCG	14 days	-
	Eel	2	FCA	$1 \times 0.25cm^3$ i.p.	-	BCG	14 days	-
10	Plaice	2	BCG	$1 \times 0.15cm^3$ i.p.	-	PPD, BSA, BCG	14 days	-
	Plaice	1	-	-	-	"	-	-

Chapter 3

Identification, Characterisation and Morphology of Dogfish Blood and

Tissue Leucocytes

Results

Blood Cell Identification

A comprehensive study of the blood leucocytes of the dogfish was

made using both Giemsa-stained blood smears and electron microscopy.

The object of this study was to identify major blood cell types which

could be further characterised by histochemical and functional

experiments and to identify the phagocytic cells.

Morphological observations on the major blood leucocytes are

described below.

Lymphocyte. A lymphocyte-type cell was observed (see Plate 1) which

measured approximately 8.25 x 10.4μ (mean of 10 cells). These cells

possessed a large nucleus which almost filled the cell surrounded by

a thin rim of basophilic cytoplasm. A prominent nucleolus was

evident under the electron microscope (Plate 1b). The cells were

generally round, but at times formed a single large pseudopodium at

one end of the cell or otherwise numerous minute pseudopodia over the

whole surface of the cell. The cytoplasm contained few mitochondria

or other organelles.

Thrombocytes. Cells designated as thrombocytes existed in several

forms (see Plate 2). The two most obvious types were:- a) an oval

cell often with the cytoplasm elongated at each pole to form a

spindle-shape and a few vacuoles contained within the cytoplasm

(Plate 2b). This form was able to trap carbon in these vacuoles

(see following chapter). The cytoplasm stained variably by

Romanowsky stains. The nucleus was oval. b) an oval cell, but in

this case the cytoplasm was full of eosinophilic granules which, by electron microscopy (Plate 2c), were seen as square, crystalloid granules. These cells also formed spindle or spiked cells by elongation of the cytoplasm at one or both poles. The nucleus was again oval.

Both cell types were of similar size (16.25 x 7μ) and under the light microscope forms could be seen which appeared to be intermediate between the two types. Thrombocytes were capable of adhering to glass and tissue culture plates (particularly the agranular type) and accumulations of adherent cells often trapped antigens such as yeasts and foreign erythrocytes while not actively endocytosing them*.

Monocytes. Cells with the morphological characteristics of mammalian monocytes were in evidence and were apparently actively endocytic (see Plates 3 & 4). They possessed abundant cytoplasm which was slightly basophilic and was often vacuolated. The nucleus was eccentric and frequently 'kidney-shaped'. These were the main blood leucocyte to phagocytose injected colloidal carbon (Plate 3a). Monocytes from the blood of untreated fish were regularly observed containing other cells and nuclei (Plate 3b) and were presumably active in clearing effete cells from the circulation. Monocytes would adhere and spread on glass or tissue culture plates (Plates 4a & b) and were not removed by washing or trypsinisation. When unspread they were often indistinguishable from large lymphocytes but when positively identified measured 15 x 15μ. When spread they were up to 31.25 x 31.25μ.

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*Functional studies of phagocytosis are described and discussed in the next chapter.

<u>Granulocytes</u>. Three main types of granulocyte were evident (see Plates 5, 6 & 7).

i) <u>Type 1 granulocyte</u>. This cell was considered to be analogous to the mammalian neutrophil due to its phagocytic properties.

Within type 1 granulocytes were two forms which may represent developmental forms of one another as both were phagocytic. Both types were of similar size, i.e. 12.5 x 13.75μ when unspread and 37.5 x 22.5μ when spread.

a) The most commonly encountered form (Plate 5) possessed large round eosinophilic granules (Plate 5b, d & e) in a light grey-blue cytoplasm. The nucleus was irregular in shape but rarely polymorphic. They were capable of adhering and spreading and were often seen in blood smears in large aggregations with monocytes (Plate 5a). Spreading tended to be less centrifugal than in monocytes such that they were longer along one axis than the other (Plate 5c). Although these cells could adhere to tissue culture plates they were easily removed by thorough washing, in contrast to monocytes. Amoeboid movement was exhibited by the extension of the membrane, before the cytoplasm and its granules flowed into the newly formed pseudopodia (Plate 5b). The granules measured approximately, 0.75μ in diameter.

b) The second form of type 1 (Plate 6) contained much smaller granules (approx. 0.25μ in diameter) and the granules stained more faintly by Giemsa (Plate 6b) than those with large granules. The nucleus was not generally polymorphic. There appeared to be a greater amount of endoplasmic reticulum than in the latter form (Plate 6a). This was the only granulocyte type ever seen with engulfed carbon (see next chapter), and as with the other form was capable of adherence and spreading on glass and plastic.

ii)   Type 2 granulocyte (see Plate 7a,b & c).   These granulocytes had
slightly elongated, rod-shaped granules.   The granules generally
stained brighter and pinker than Type 1 granulocytes.   The granules,
measuring 0.25 x 0.58µ, tended to be radially arranged around a
prominent centrosome (Plate 7a & c).   The nucleus was invariably
polymorphic (Plate 7c) and eccentric.   These granulocytes were
capable of adherence and spreading (Plate 7b) on glass and plastic
and of amoeboid movement, but were never observed phagocytosing any
material.   Unspread type 2 granulocytes measured 14.25 x 14.25µ.

iii)   Type 3 granulocytes.   The remaining granulocyte possessed
granules which did not stain at all by Giemsa, but which appeared in
blood smears as a very bright round cell with an eccentric nucleus
which was invariably 'dumb-bell' shaped (Plate 7d).   They measured
approximately 15 x 16.25µ and were never observed to spread or
adhere to glass.   Under the electron microscope the cell appeared
highly vacuolated, the round vacuoles containing small granules which
never completely filled the vacuole (Plate 7e).   The vacuoles had an
average diameter of 0.53µ and the contained granules a diameter of
0.276µ.

        These cells formed the main blood leucocytes observed.   In
addition to these there were many cells which appeared to be
precursors of leucocytes or erythrocytes.   Indeed, it was difficult to
ascertain, particularly with regard to the granulocytes and type b)
thrombocytes which were developmental stages of one cell and which
were distinct cell types.

        Plate 8 shows an electron micrograph of a granulocyte observed
on only one occasion, but which had definite crystalloid type inclusions

in the granules reminiscent of eosinophils of mammals.

Spleen. The spleen of the dogfish possessed red and white pulp though not as clearly delineated as in mammals (Plate 9a). The white pulp consisted entirely of developmental stages of lymphoid type cells. The red pulp contained red cells in all stages of development, thrombocytes and their precursors (Plate 9b & c), ellipsoids containing the phagocytic elements of the spleen and other leucocytes.

The ellipsoids were shown by carbon injection to contain the phagocytic cells (see next chapter for details of carbon uptake) which are shown by light and electron microscopy (Plates 10 & 11). Scanning electron microscopy of a cut surface of the spleen showed the ellipsoids very clearly (Plates 11a & b), with macrophages suitably placed to take up foreign material as it arrived in the spleen via the arterioles which the ellipsoids themselves surround. The cells could be seen to possess large quantities of cytoplasm characteristic of phagocytic cells.

The spleen contained very few pigment-containing cells, but those which were present were isolated cells with dark-granules in the cytoplasm (see Plate 11c).

Liver. The liver of the dogfish appeared to function mainly in lipid storage. Light microscopic examination revealed very loose vacuolated tissue where the normal lipid content had been broken down by fixation and dehydration processes (Plate 12a). Among the lipid storage cells, there were small groups of pigment-containing cells or melanomacrophages (Plate 12b). There was no evidence indicating that the liver was directly involved in phagocytosis of circulating foreign matter, as in mammals. Pigment-containing cells contained numerous granules of the pigment (melanin?), often arranged around

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vacuoles which sometimes appeared to contain cellular debris (Plate 12c & d).

<u>Gill</u>. The gill was evidently involved in carbon clearance (see Plate 22c and Fig. 6 in following chapter). However there did not appear to be any specially modified phagocytic cells, rather the normal structural cells which comprised the gill occasionally took up material - presumably by phagocytosis.

<u>Heart</u>. Examination of the dogfish heart revealed no evidence of leucocytic activity, phagocytic or otherwise.

<u>Oesophagus</u>. Within the wall of the oesophagus is a granulopoietic organ known as Leydig's organ (see Fig. 2). This consisted of large sac-like structures within a space in the connective tissue. It appeared to be involved only in granulopoietic development, as no injected carbon could be detected within the organ and there was no evidence of its involvement in phagocytosis.

<u>Blood Cell Numbers</u>. The results of counts of blood samples taken from ten dogfish of similar size (750g) are given in the following Tables 5 and 6 and Figure 3. The counts recorded were highly variable as indicated by the standard deviations.

Fig. 2

Diagrammatic representation of a transverse section of Leydig's organ
in oesophagus of dogfish.

"Sacs" containing granulocytes which comprise Leydig's organ.

Epithelium.

Connective tissue.
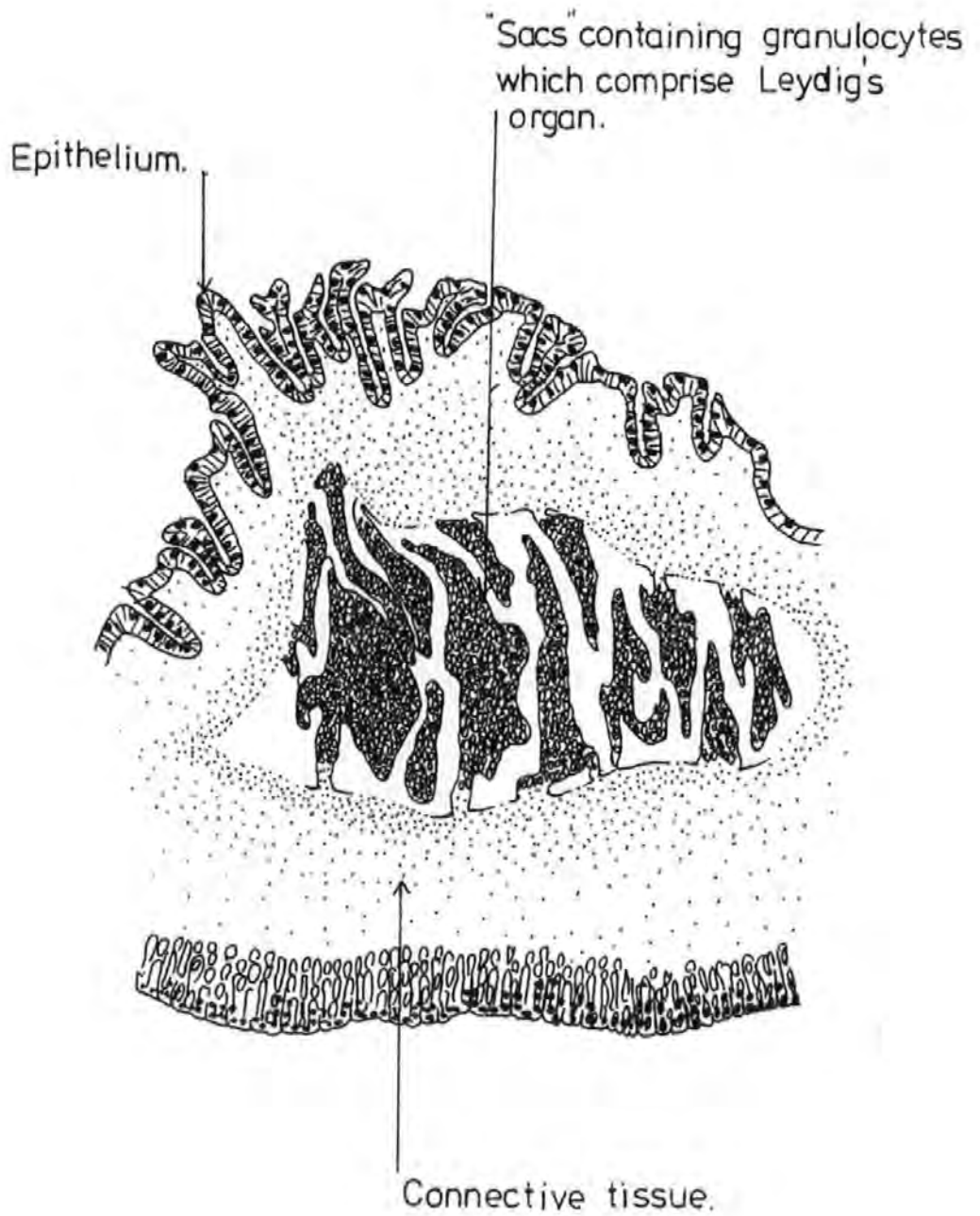
Table 5

Blood cell numbers in ten dogfish

Fish	RBCs/cm^3	% of total blood cells	WBCs/cm^3	% of total blood cells	% of total leucocytes						
					T(a)	T(b)	L	M	G(1)	G(2)	G(3)
1	1.12×10^8	89	2×10^7	11	48		23.5	6.7	-	16.8	5.0
2	1.85×10^8	92	2.6×10^7	8	41		22	4.4	23.5	2.9	5.8
3	1.39×10^8	91	1.95×10^7	9	48		31	2.1	10.6	7.4	1.1
4	1.26×10^8	84	2.75×10^7	16	58.5		24.4	1.2	13.4	2.4	-
5	1.99×10^8	87	3×10^7	13	48.7	16.6	30.5	1.6	1.6	1.06	-
6	1.3×10^8	84	2.45×10^7	16	47.3	9.3	31.3	4	6.0	1.4	0.7
7	2.13×10^8	82	4.65×10^7	18	29	18	33	5.5	0.85	13.6	-
8	1.08×10^8	85	1.85×10^7	15	62	7.8	15.6	1.3	7.8	2.5	2.5
9	1.36×10^8	80.3	1.35×10^7	19.7	53	6.3	23	0.8	3.2	11.9	2.4
10	1.1×10^8	88.7	1.4×10^7	11.3	57.1	11.6	22.4	1.3	0.7	4.1	2.7

RBC = Red blood cell
WBC = White blood cell
Ta = Type a thrombocyte
Tb = Type b thrombocyte

L = Lymphocyte
M = Monocyte
G(1) = Type 1 granulocyte
G(2) = Type 2 granulocyte
G(3) = Type 3 granulocyte

Table 6

Relative blood cell numbers in dogfish blood

	RBC	WBC	Ta	Tb	L	M	G(1)	G(2)	G(3)
mean nos. cells/cm^3	1.46×10^8	2.4×10^7	1.1×10^7*	3.3×10^6*	6.4×10^6	7.8×10^5	1.6×10^6	1.58×10^6	4.1×10^5
Standard deviation of cells/cm^3	3.9×10^7	9.6×10^6	2.95×10^6*	2.9×10^6*	3.7×10^6	7.6×10^5	1.94×10^6	1.9×10^6	4.9×10^5
Percentage of total blood cells (mean of 10 fish)	86.3	13.7	49.5*	11.6*	25.7	2.89	6.77	6.41	2.02
Standard deviation of percentage cells	3.9	3.9	11.4*	4.8*	5.5	2.09	7.43	5.71	2.07
Range of % values of cell types	80.3-92	8-19.7	29*-62	63*-18	15.6-33	0.8-6.7	0.7-23.5	1.06-16.8	0-5.8
Range of Nos/cm^3 values of cell types	1.08×10^8 $\rightarrow 2.13 \times 10^8$	1.35×10^7 $\rightarrow 4.65 \times 10^7$	7.2×10^6* $\rightarrow 1.5 \times 10^7$	8.5×10^5* $\rightarrow 4.3 \times 10^6$	2.89×10^6 $\rightarrow 1.54 \times 10^7$	1.1×10^5 $\rightarrow 2.6 \times 10^6$	0 $\rightarrow 6.1 \times 10^6$	3.2×10^5 $\rightarrow 6.3 \times 10^6$	0 $\rightarrow 1.7 \times 10^6$

*based on 6 values not 10 as rest of results

For explanation of abbreviations refer to Table 5.

Fig. 3

a) Erythrocyte and leucocyte cell numbers and percentages in dogfish peripheral blood. (arrow represents standard deviation)
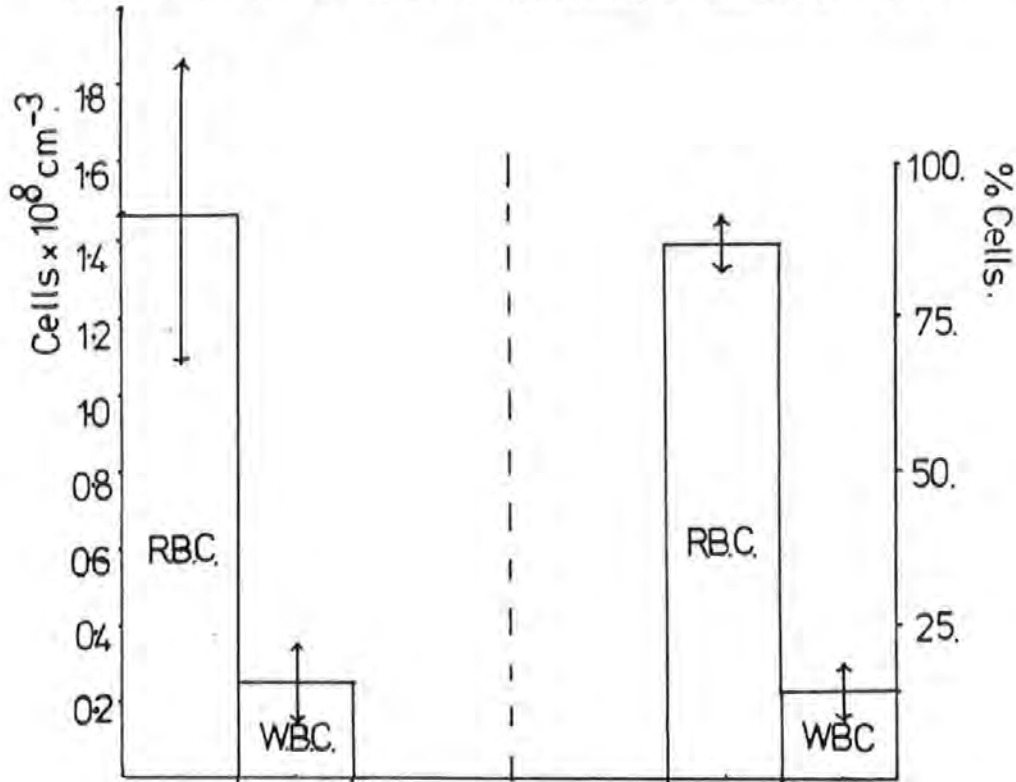
b) Differential leucocyte counts in dogfish peripheral blood. (arrow represents standard deviation)
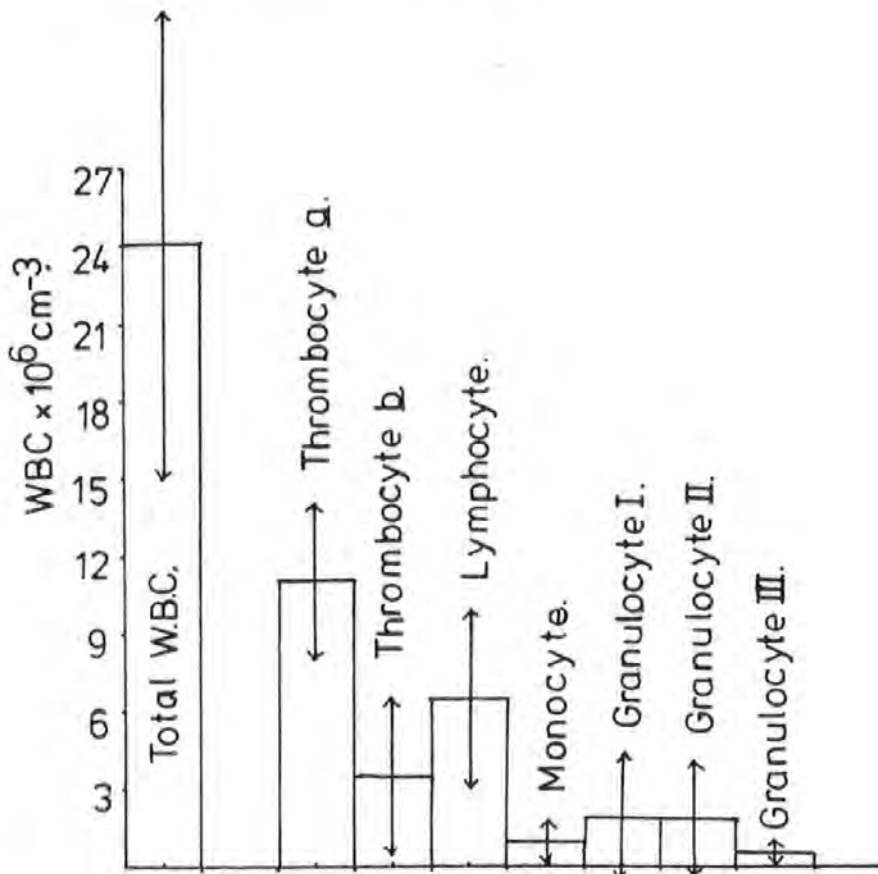
Histochemistry

The results of the histochemical methods performed on blood leucocytes are summarised in Table 7 and some of these are shown in Plates 13-17.

The results of histochemistry on spleen and liver imprints are summarised in Table 8 and shown in Plate 18.

Lysozyme

In all cases lysozyme was not detected in serum, cell suspensions or supernatants from cultured cells with or without latex. It is possible however that the cells produce too small an amount to be detected by the three methods described.

The plate method gave a diameter of lysis of 18 mm with 50 μls of 0.5 mg cm^{-3} egg white lysozyme which corresponded to a total lysozyme content of 25 μg in the sample, thereby demonstrating that quite small amounts of enzyme could be detected by this technique.

NBT reduction

In both sets of results presented in Fig. 4 the cells tested were monocytes. If a means of obtaining firmly adherent type 1 granulocytes had been found the results may have been more meaningful as, from qualitative data these cells were most active in reducing NBT.

Addition of superoxide dismutase (SOD) to resting adherent monocytes decreased the amount of NBT reduced to 60-67% of non-inhibited cells. Cells provided with latex showed only 72% of the reducing activity of non-phagocytosing cells. Addition of SOD further decreased the reducing activity to 60%.

The provision of bacteria (V. anguillarum) as the phagocytic

Table 7

Histochemical reactions of dogfish peripheral blood leucocytes

CELL ENZYME	LYMPHOCYTE	THROMBOCYTES	MONOCYTE	GRANULOCYTE TYPE 1	GRANULOCYTE TYPE 2	GRANULOCYTE TYPE 3	PLATES
Peroxidase	-ve	-ve	occasional +ve granule in cytoplasm	+ve	-ve	-ve	Plate 13a & b
Acid Phosphatase	-ve	-ve	-ve	slightly +ve cytoplasm	very positive cytoplasm	+ve nuclei -ve cytoplasm	Plate 14a, b & c
Alkaline Phosphatase	-ve	-ve	-ve	-ve	-ve	-ve	-
Non-specific Esterase	-ve	Slightly pink	Dark red +ve	Slightly pink	Slightly pink	-	Plate 15a & b
Sulphatase	+ve activity associated with membrane	type a) +ve vacuoles type b) +ve granules	Clear cytoplasm a few dark red granules	Pink cytoplasm and a few dark red granules	Dark pink cytoplasm, no darker granules	-ve	Plate 16a, b & c
β-glucuroni-dase	-ve	-ve	-ve	-ve	-ve	-ve	-
Lipase	-ve	-ve	-ve	-ve	a few granules stain slightly brown	-ve	-
PAS	-ve	Very +ve vacuoles	+ve	+ve particularly round membrane	Very +ve	Slightly +ve particularly around the edge	Plate 17a & b

Table 7 Continued

ENZYME \ CELL	LYMPHOCYTE	THROMBOCYTES	MONOCYTE	GRANULOCYTE TYPE 1	GRANULOCYTE TYPE 2	GRANULOCYTE TYPE 3	PLATES
Sudan Black B	-ve	-ve	-ve	-ve	-ve	-ve	-
Acridine Orange	Green fluorescent nuclei	Green fluorescent nuclei. No orange fluorescence	Bright orange	Bright orange	Bright orange	Bright yellow	-
Csaba's mast cell stain	-ve	-ve	-ve	-ve	-ve	-ve	-
Whitby & Hynes	-ve	-ve	Bright red-orange granules	Bright orange granules	Dull orange granules/ cytoplasm	Maroon granules/ cytoplasm	Plate 17c, d & e

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Table 8

Histochemical reactions of dogfish spleen and liver imprints

Enzyme	Spleen	Liver	Plate
β-Glucuronidase	Two macrophages in one imprint, contain one red 'globule'.	Few positive areas in some cells. Some positive reaction associated with MMC	18a
Lipase	No activity	Some generalised activity not associated with any particular cell	-
Sulphatase	General activity not associated with particular cells. Granulocytes and macrophages react as granulocytes and monocytes in blood	General very positive activity; also positive reaction in granulocytes and macrophages	18b,c & d
Acid Phosphatase	Type 1 and type 2 granulocytes positive. Generally negative.	Type 2 granulocytes positive. Generally negative	-
Non Specific Esterase	Macrophages are dark maroon, granulocytes pink. Otherwise negative	Melanomacrophages positive, but other cells also slightly positive	-
Peroxidase	Some type 1 granulocytes positive, macrophages contain the odd positive granule	NOT DONE	-
Haemosiderin	Negative in all fish, even those which had been injected with yeasts and bacteria	Negative	-

Fig. 4

Nitroblue Tetrazolium reduction by adherent blood monocytes under a variety of conditions. (Results expressed as a percentage of control (untreated) cells)

Experiment 1.

Experiment 2.

66
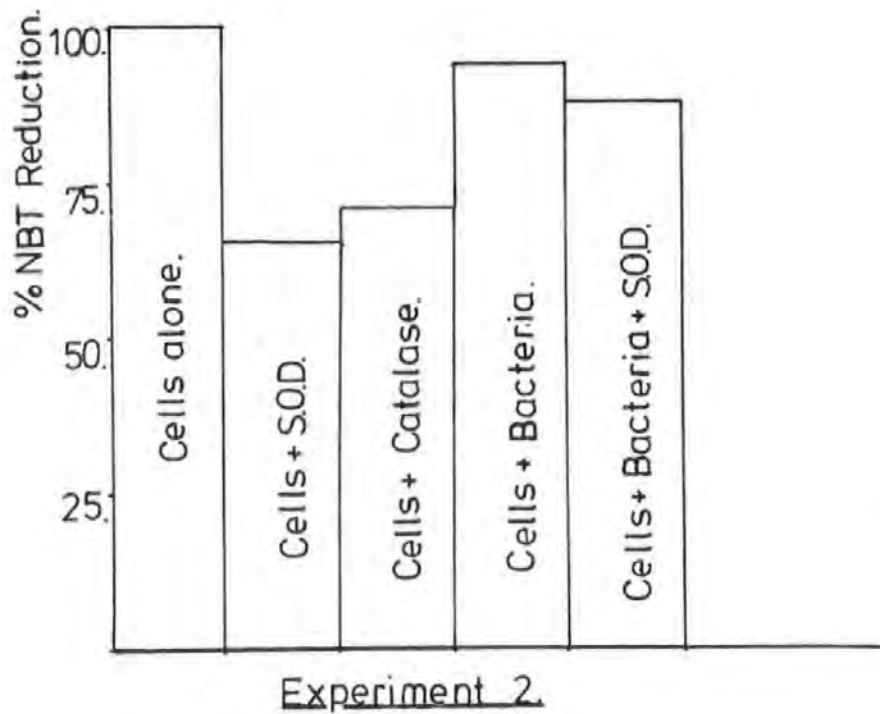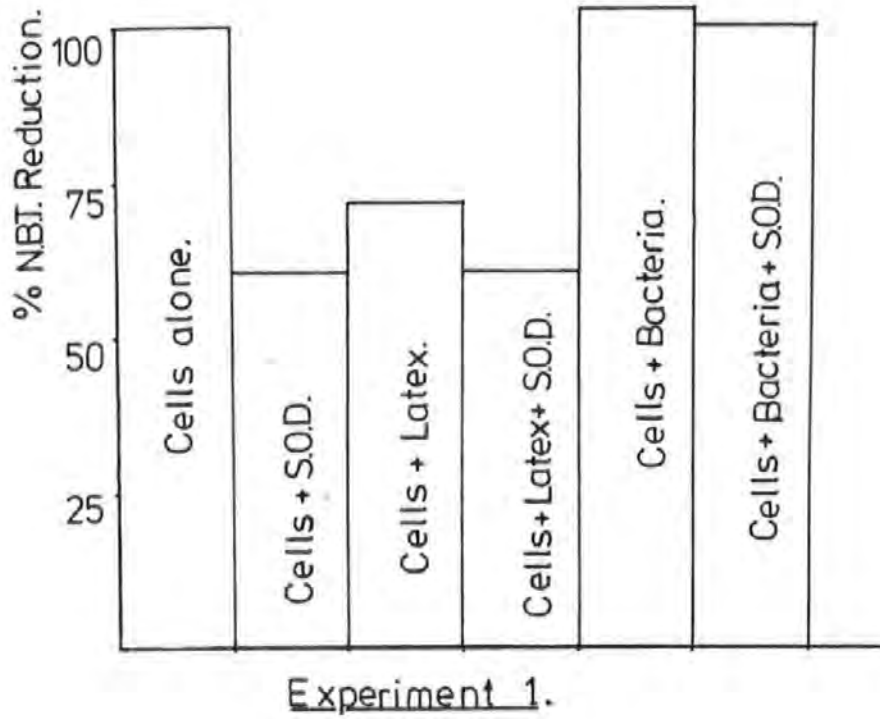
particle had variable results. On one occasion the reduction of NBT was increased by 2-3% and on the other occasion the activity was decreased by 6-7%. Addition of SOD very slightly inhibited NBT reduction as compared to cells and bacteria without SOD.

Plate 1

a) Dogfish blood smear showing various leucocytes. LM (x 573)

Giemsa. Bar ≡ 10μ.

$$L \quad = \quad \text{lymphocyte}$$

$$T_a \quad = \quad \text{thrombocyte type a}$$

$$T_b \quad = \quad \text{thrombocyte type b}$$

$$G_1 \quad = \quad \text{granulocyte type 1b}$$

$$G_2 \quad = \quad \text{granulocyte type 2}$$

b) Dogfish peripheral blood lymphocyte. TEM (x 13,655) Bar ≡ 1μ.

$$C \quad = \quad \text{cytoplasm}$$

$$N \quad = \quad \text{nucleus}$$

$$n \quad = \quad \text{nucleolus}$$

a

b
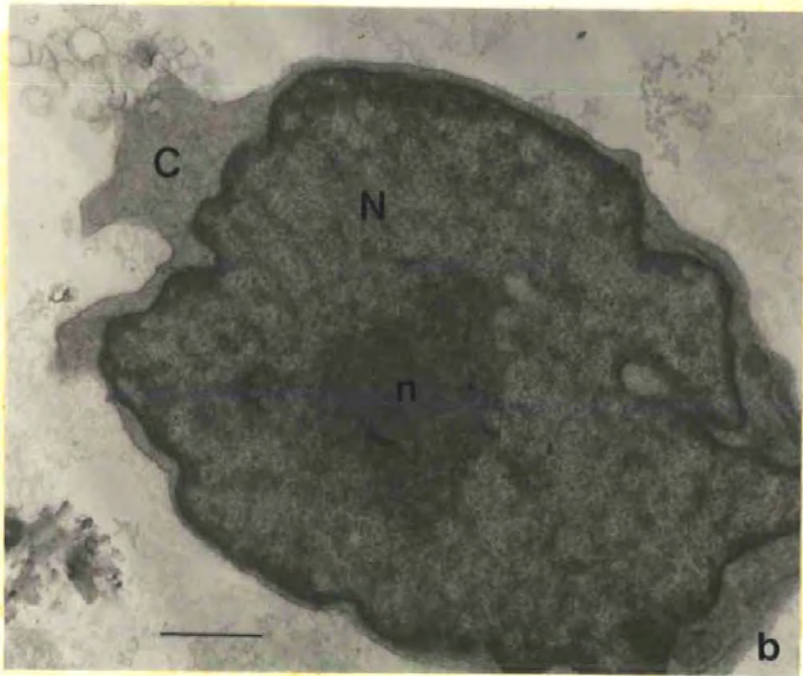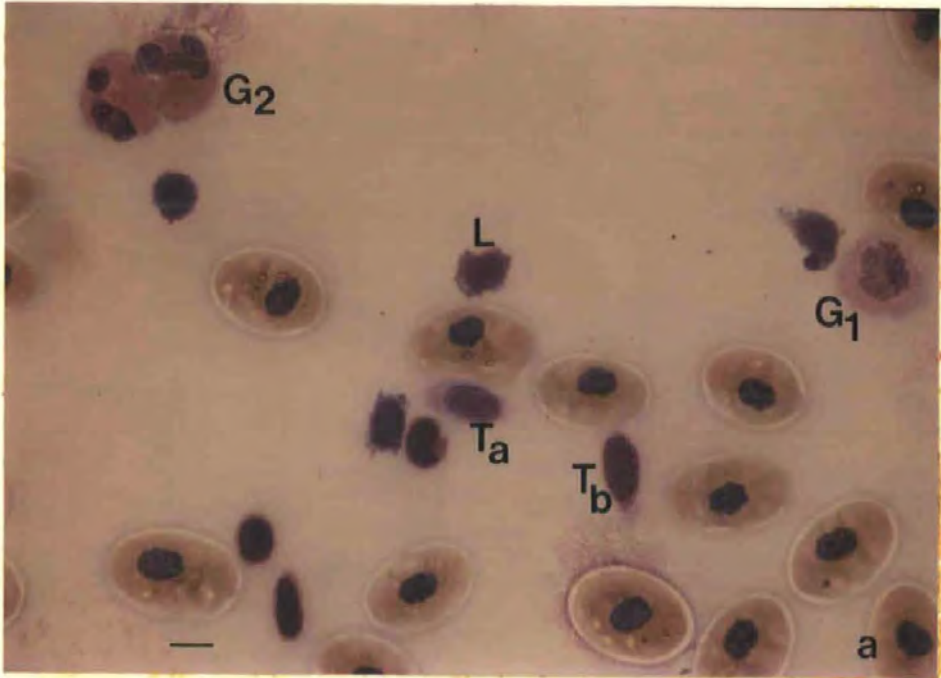
Plate 2

a) Dogfish peripheral blood smear showing the two types of
thrombocyte observed in this study. LM (x 1,431) Giemsa.
Bar ≡ 10μ.

T_a = thrombocyte type a

T_b = thrombocyte type b

b) Type a. thrombocyte. Note spindle-shape and characteristic
vacuoles (v) in the cytoplasm. TEM (x 11,337) Bar ≡ 1μ.

c) Type b. thrombocyte. Note the large numbers of granules (g)
in the cytoplasm. TEM (x 5,349) Bar ≡ 1μ.
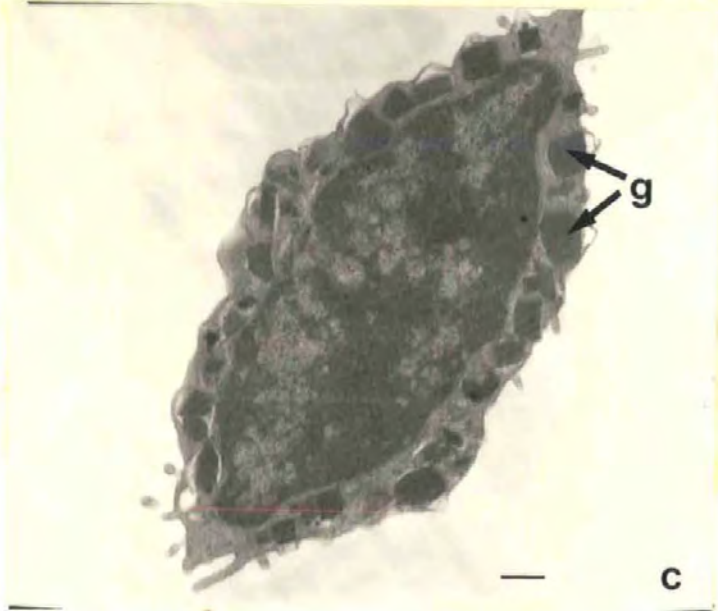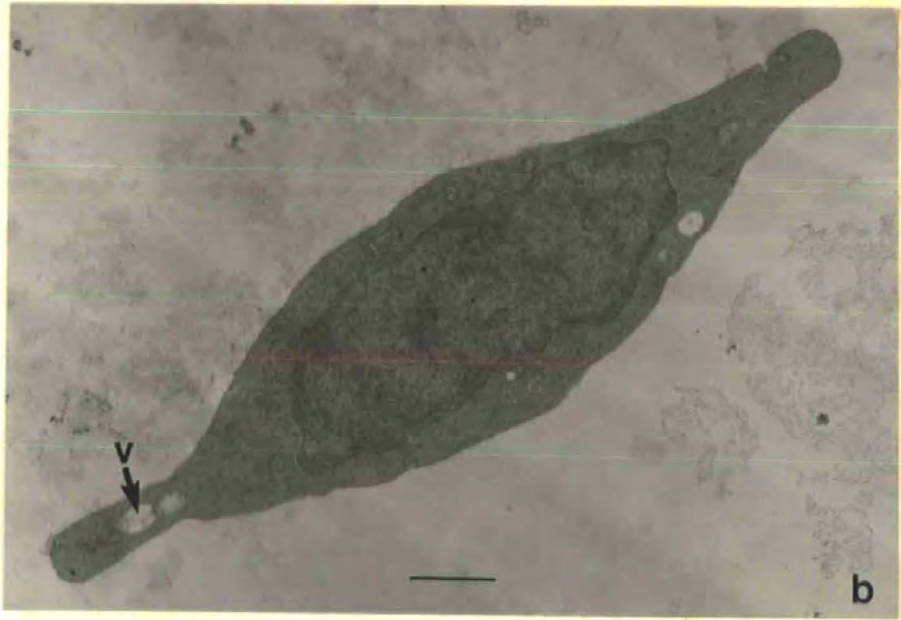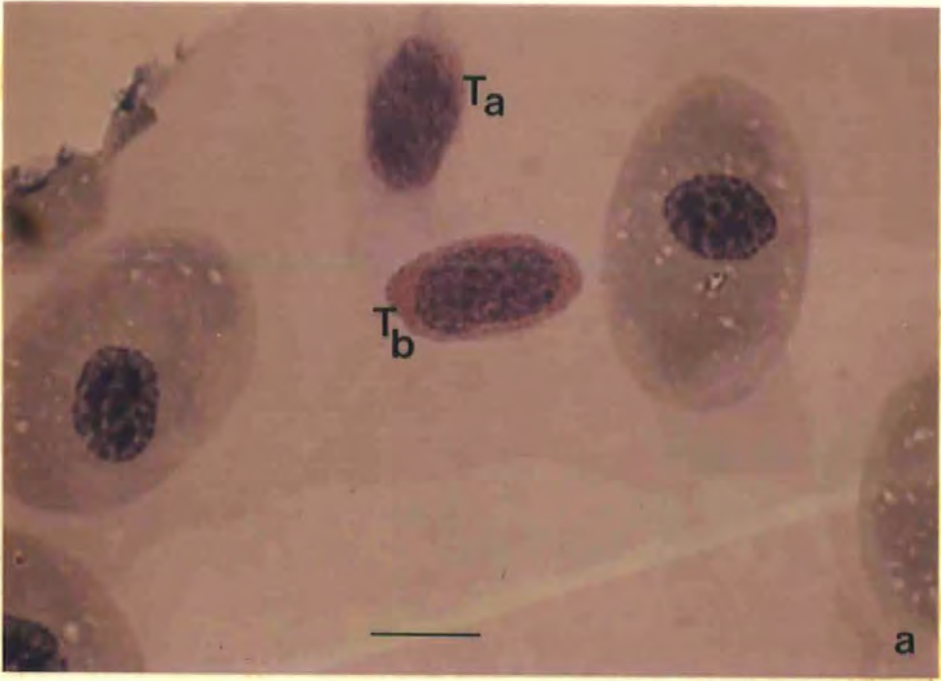
Plate 3

a)   Dogfish peripheral blood monocyte containing carbon. Note the

loose, vacuolated cytoplasm. LM (x 1,431) Giemsa. Bar ≡ 10μ.

M   =   monocyte

C   =   carbon granules

(T$_a$ =   thrombocyte type a)

b)   Peripheral blood monocyte with vacuolated cytoplasm and vacuole

containing effete cell or nucleus of dogfish origin. TEM

(x 8,817). Bar ≡ 1μ.

V   =   vacuole

dv =   digestive vacuole

N   =   nucleus

c)   Surface of monocyte demonstrating pseudopodial formation and

evidence of pinocytosis. TEM (x 101,277). Bar ≡ 1μ.

p   =   pseudopodium

pv =   pinocytic vacuole

a
b
c
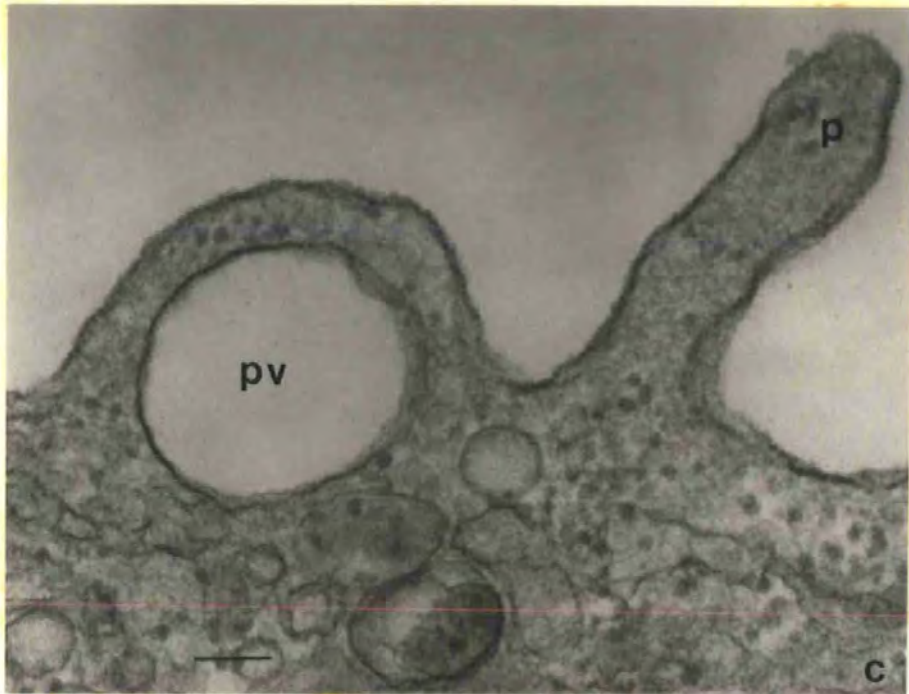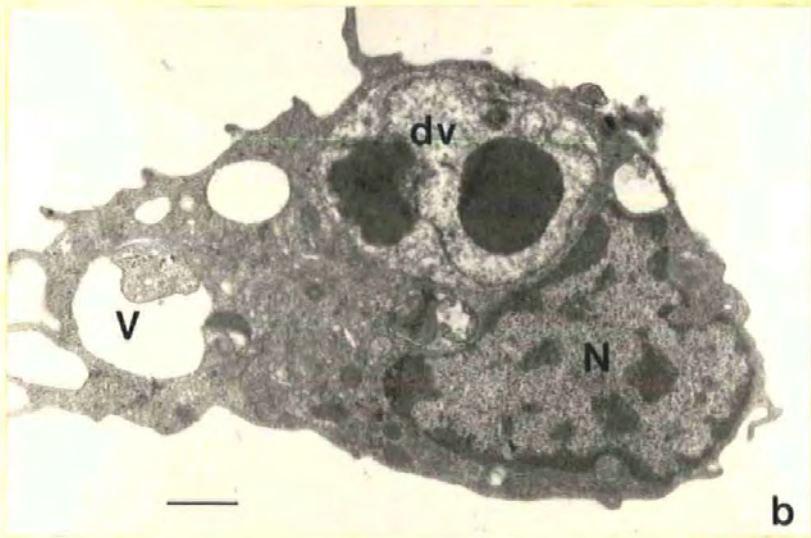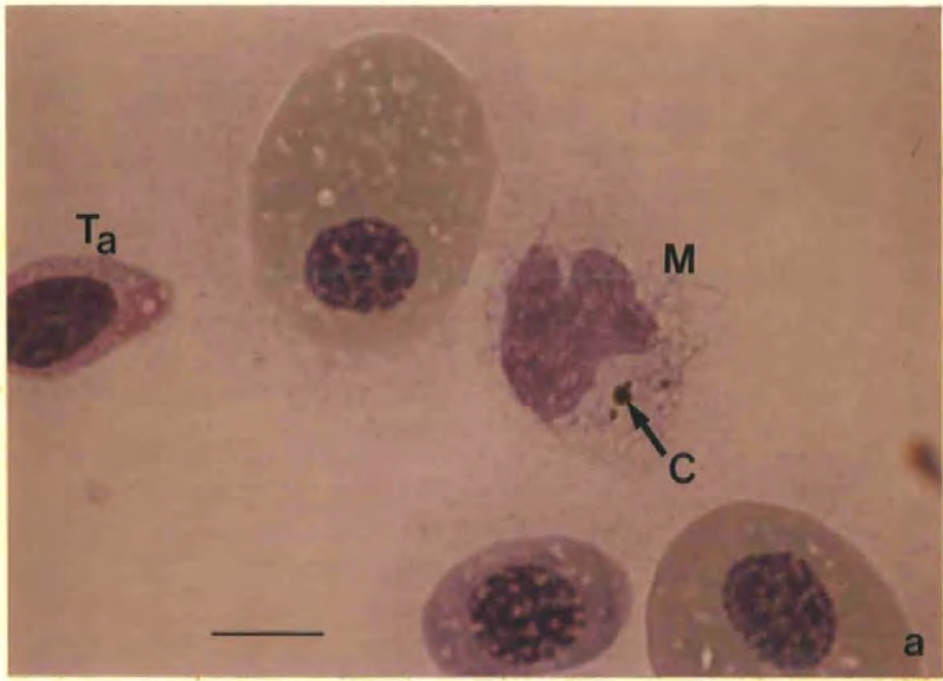
Plate 4

a)   Dogfish adherent monocyte in process of engulfing yeast cell.

SEM (x 3,040). Bar ≡ 10μ.

Y = yeast cell

m = monocyte

N = nucleus

b)   Live preparation of adherent leucocytes in dogfish peripheral

blood. LM (x 527) Phase Contrast. Bar ≡ 10μ.

m =   monocyte

G_1 = granulocyte type 1a

G_2 = granulocyte type 2

71
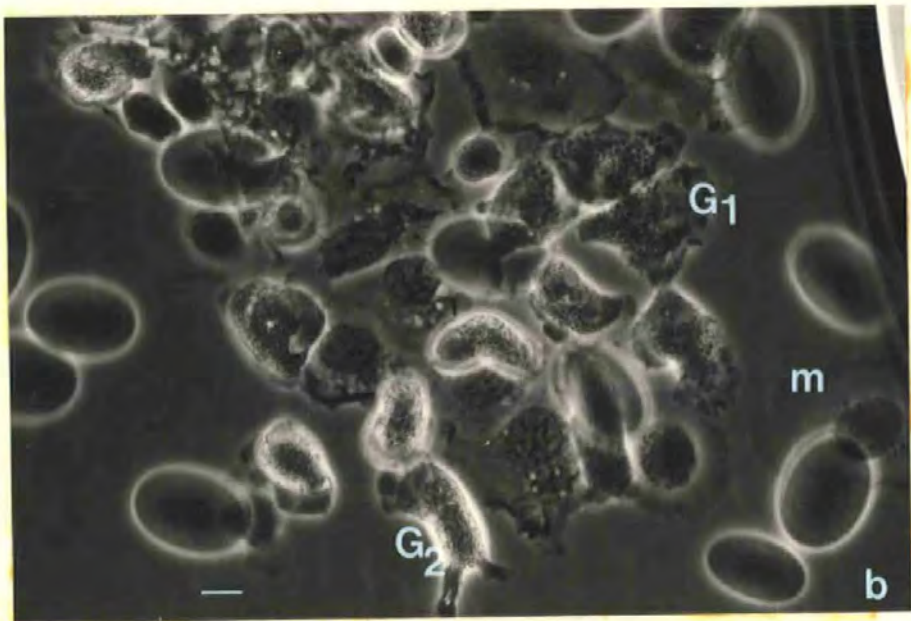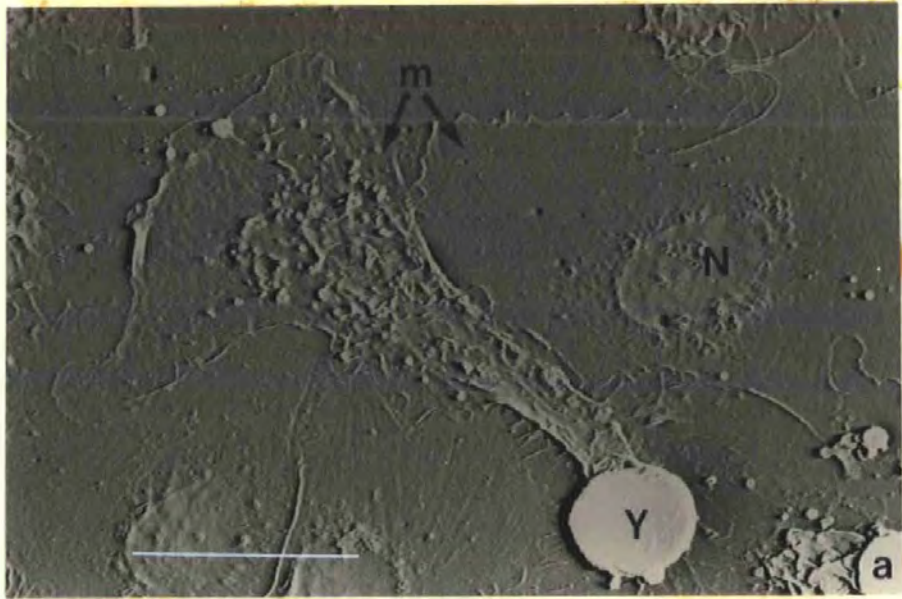
Plate 5

a) Type la granulocyte showing aggregation which is a common

feature of this cell type. TEM (x 2,697). Bar ≡ 10μ.

G_1 = granulocyte type la

M = monocyte

b) Type la granulocyte with extended pseudopodium. TEM (x 4,933).

Bar ≡ 2μ.

ps = pseudopodium

c) Type la granulocyte adhering to tissue culture plate.

SEM (x 5,661). Bar ≡ 1μ.

mf = microfilament effecting attachment

g = granules

v = vacuole

d) Type la granulocyte in peripheral blood smear. Note the

acidophilic granules. LM (x 576) Giemsa. Bar ≡ 10μ.

G_1 = Type la granulocyte

T_b = Type b thrombocyte

L = lymphocyte

e) Type la granulocyte showing granules. TEM (x 13,708). Bar ≡ 1μ.

g = granule

N = nucleus

m = mitochondrion
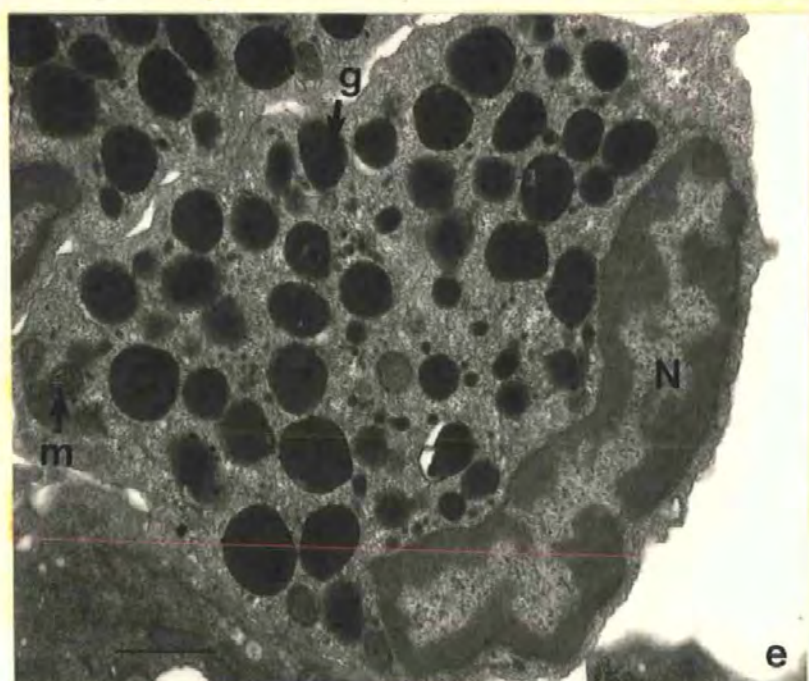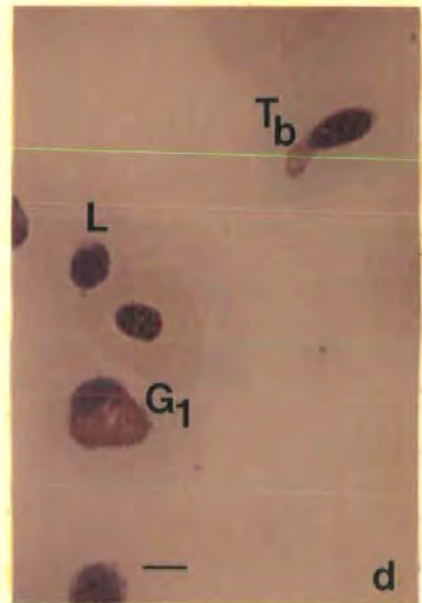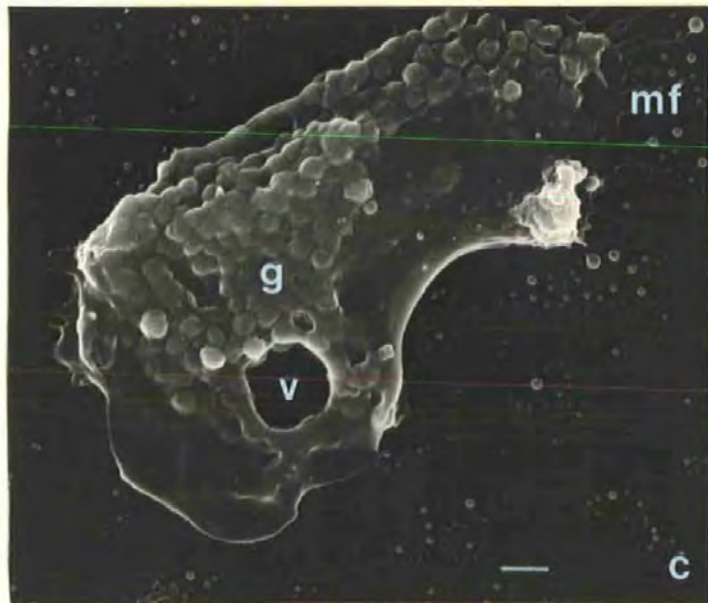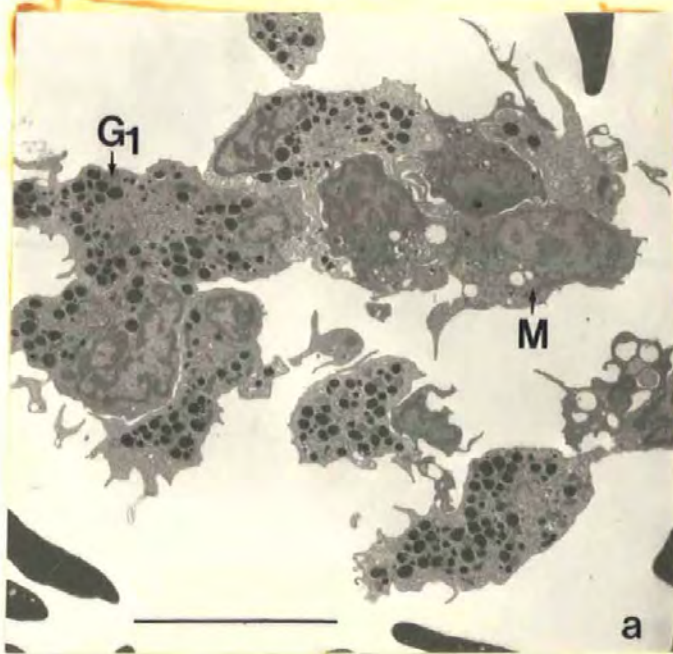
Plate 6

a)   Type lb granulocyte. TEM (x 17,333). Bar ≡ 1μ.

        N   =   nucleus

        g   =   granules

        er =   endoplasmic reticulum

        m   =   mitochondrion

b)   Type lb granulocyte in dogfish peripheral blood smear. Note .
the fainter staining properties of the granules. LM (x 1,431)
Giemsa. Bar ≡ 10μ.

        G_1 = type lb granulocyte

        T_b = type b thrombocyte

c)   Type lb granulocyte, with the more rarely seen polymorphic
nucleus. TEM (x 15,000). Bar ≡ 1μ.

        N   =   nucleus

        g   =   granules

a

b

c
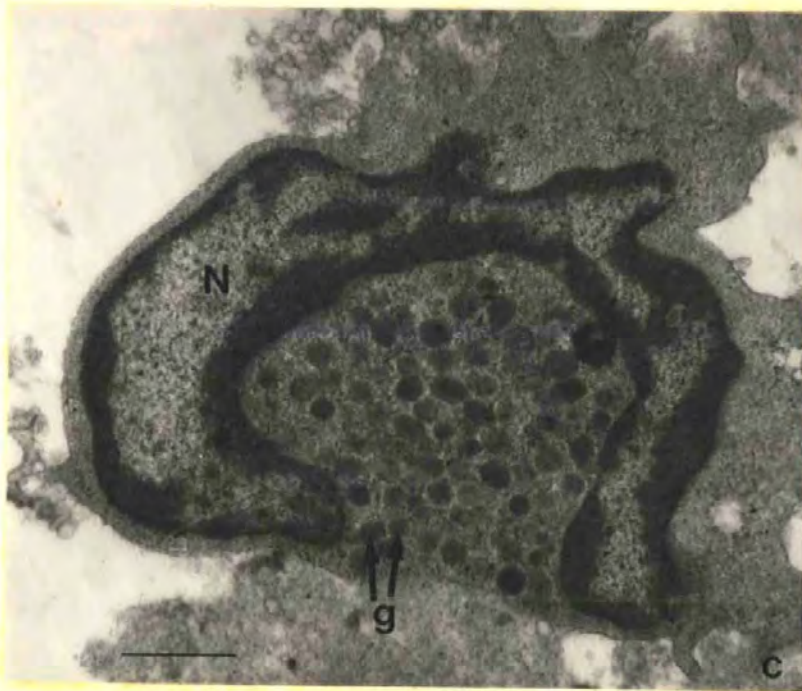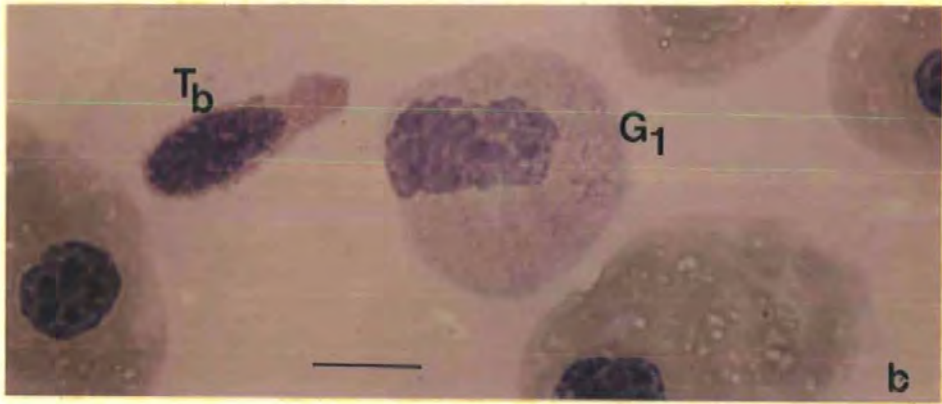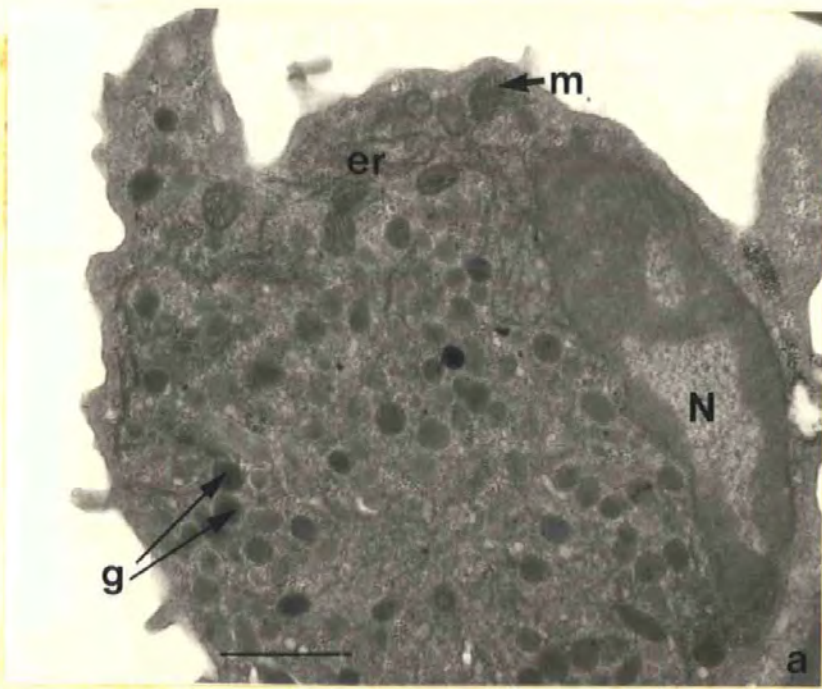
Plate 7

a) Type 2 granulocyte. Note the elongated granules in contrast to
    those of type 1 granulocytes. TEM (x 10,080). Bar ≡ 1μ.

                    N   =   nucleus

                    g   =   granule

                    cs =   centrosome

b) Type 2 granulocyte adherent on tissue culture plate. Note the
    different form of spreading to that of type 1 granulocytes.
    SEM (x 3,100). Bar ≡ 10μ.

c) Type 2 granulocyte in dogfish peripheral blood smear. Shows the
    polymorphic nucleus characteristic of this cell and prominent
    centrosome around which the granules are arranged radially.
    LM (x 1,431) Giemsa. Bar ≡ 10μ.

d) Type 3 granulocyte in dogfish peripheral blood smear. Note the
    bright, colourless granules. LM (x 1,431) Giemsa. Bar ≡ 10μ.

e) Type 3 granulocyte showing the vacuoles containing very small
    granules. Nucleus is typically arranged around the periphery
    of the cell, often assuming a dumb-bell shape. TEM (x 9,533).
    Bar ≡ 1μ.

                    v   =   vacuole

                    g   =   granule

                    N   =   nucleus
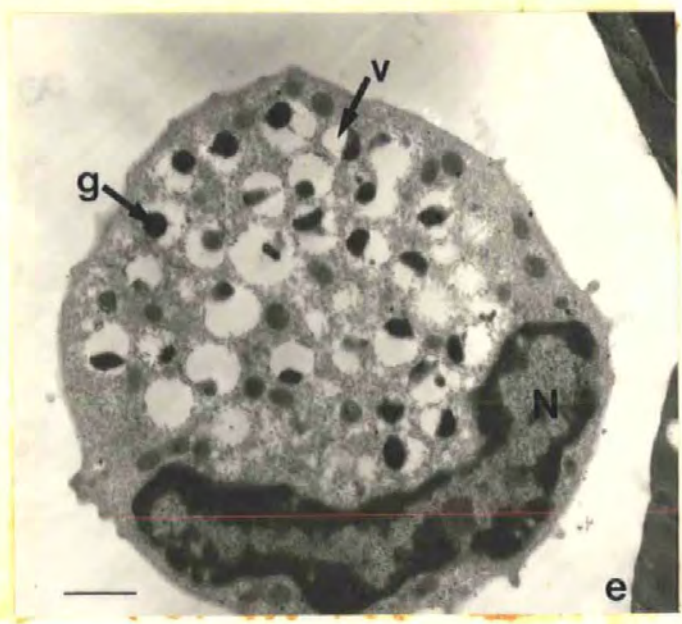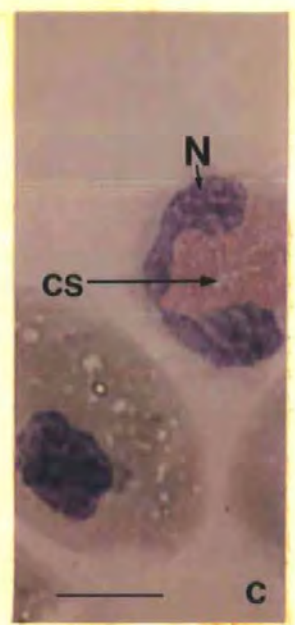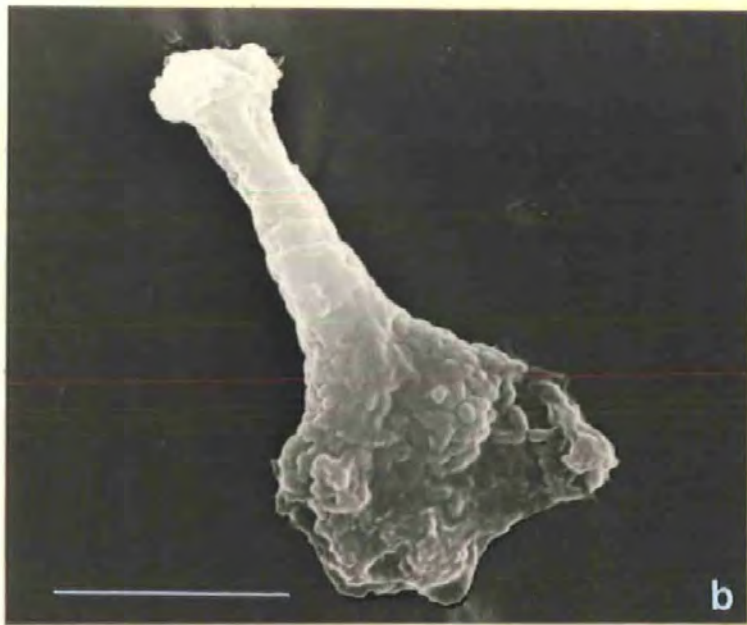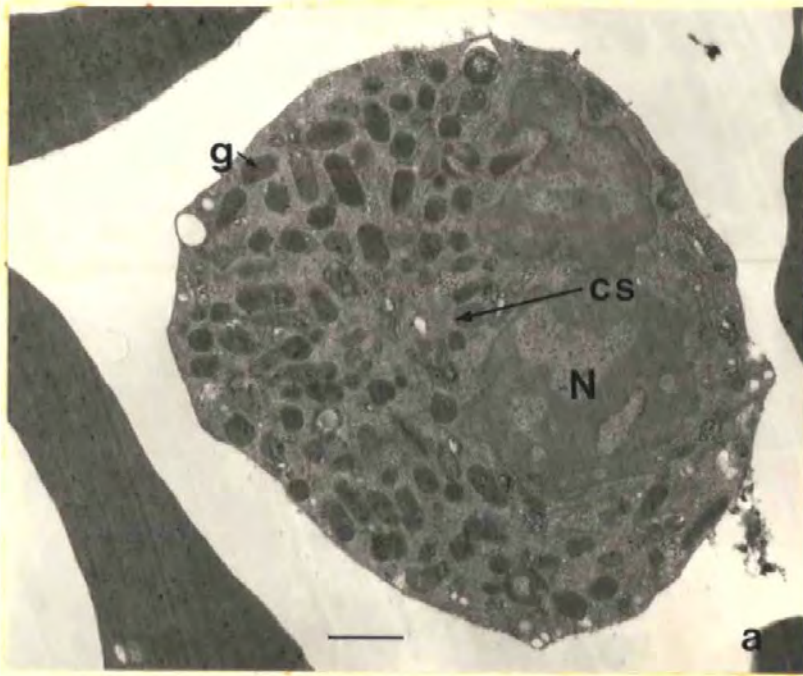
Plate 8

Granulocyte observed in the blood of only one dogfish. Note the
crystalloid-type inclusions in the granules. TEM (x 7,875).
Bar ≡ 1μ.

g = granule

N = nucleus

c = crystalloid inclusion
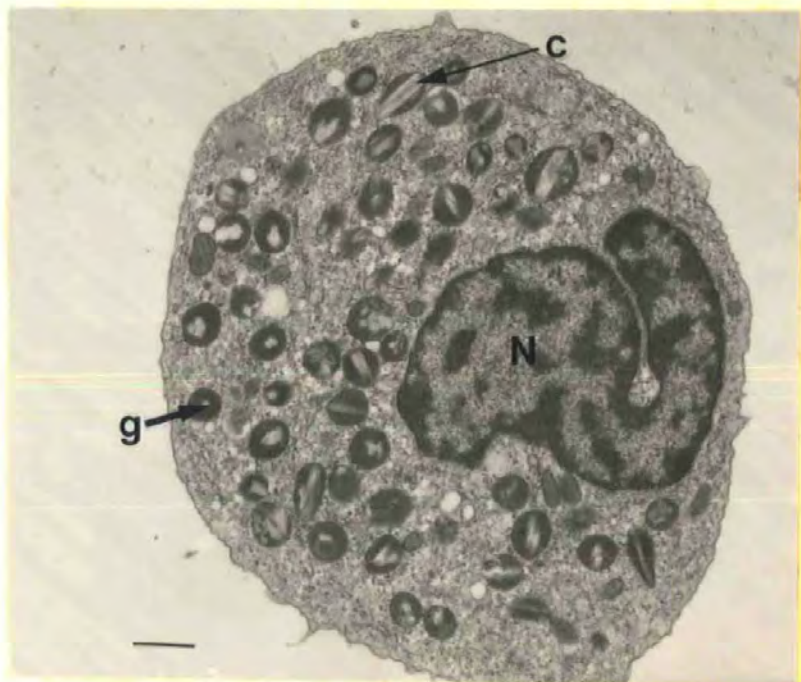
Plate 9

a) Dogfish spleen section showing ellipsoid areas associated with red pulp region and the lymphoid areas or white pulp. LM (x 229) Mallory's Triple Stain. Bar ≡ 100μ.

E = ellipsoid composed of macrophages

R = red pulp

W = white pulp

b) Dogfish spleen showing type a thrombocyte in red pulp region. TEM (x 7,970). Bar ≡ 1μ.

R = red blood cell

T_a = type a thrombocyte

c) Dogfish spleen showing type b thrombocyte in red pulp region. TEM (x 9,646). Bar ≡ 1μ.

T_b = type b thrombocyte

g = granules

Image labels: W, R, E (panel a); Ta, R (panel b); Tb, g (panel c)
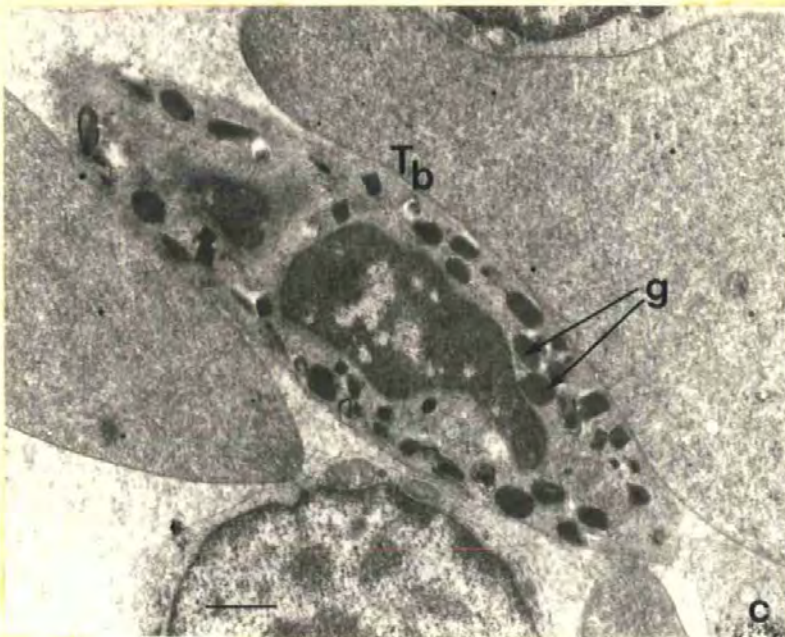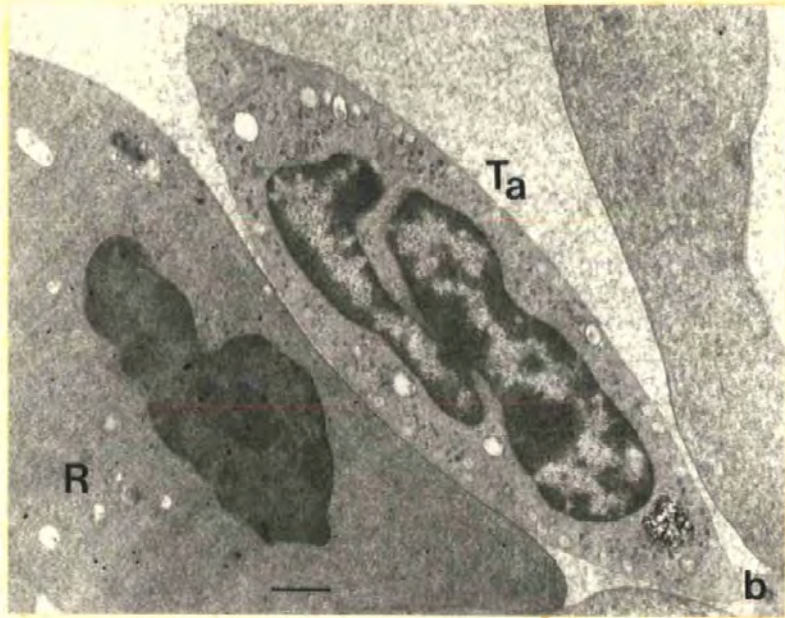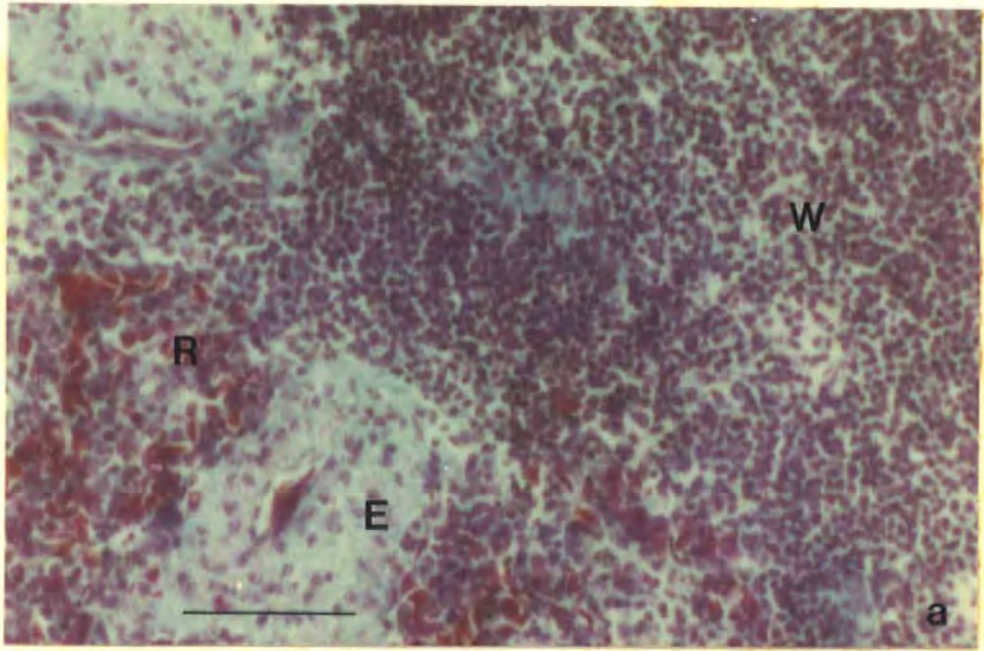
Plate 10

a)   Spleen of dogfish showing ellipsoid.   LM (x 573) Mallory's
     Triple Stain.   Bar ≡ 10μ.

               E = ellipsoid

               L = lymphoid cells

b)   Dogfish spleen showing macrophage aggregation.   Note the large
     amounts of cytoplasm present in these cells.   TEM (x 1,846).
     Bar ≡ 10μ.

               R = red cell

               M = macrophage
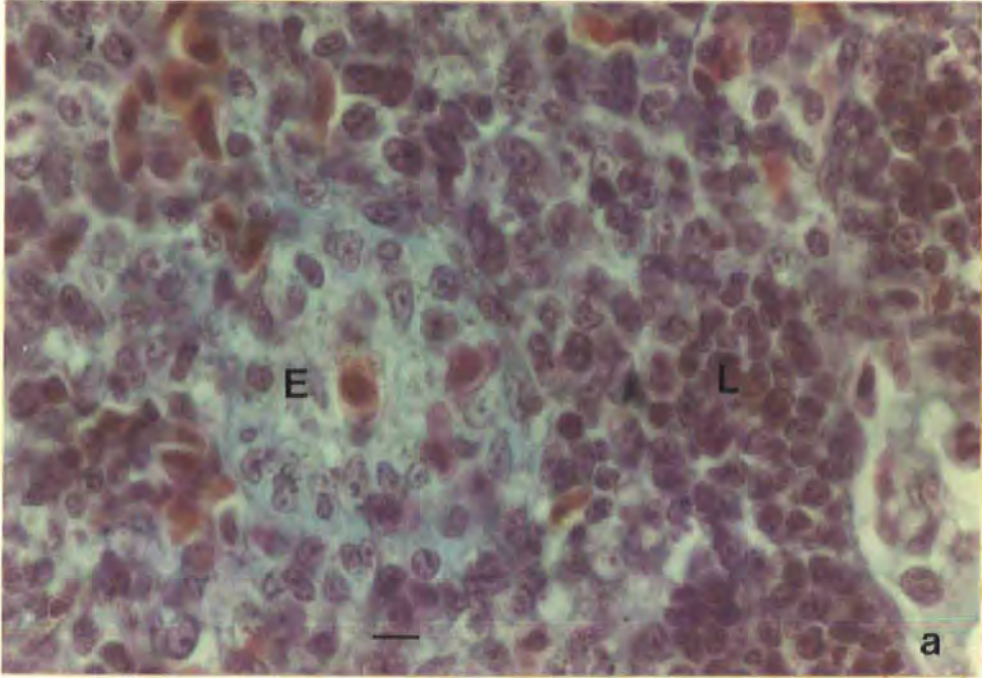
Plate 11

a)   Dogfish spleen showing ellipsoids. SEM (x 870). Bar ≡ 10μ.

E = ellipsoid

b)   Ellipsoid of dogfish spleen. Note the narrow lumen of the
arteriole allowing passage of only single blood cells at any
one time. SEM (x 4,800). Bar ≡ 2μ.

E = ellipsoid

R = red blood cell

c)   Melanomacrophage in dogfish spleen. Note that these are present
as isolated cells rather than accumulations of cells. LM (x 573)
Methyl Green. Bar ≡ 10μ.
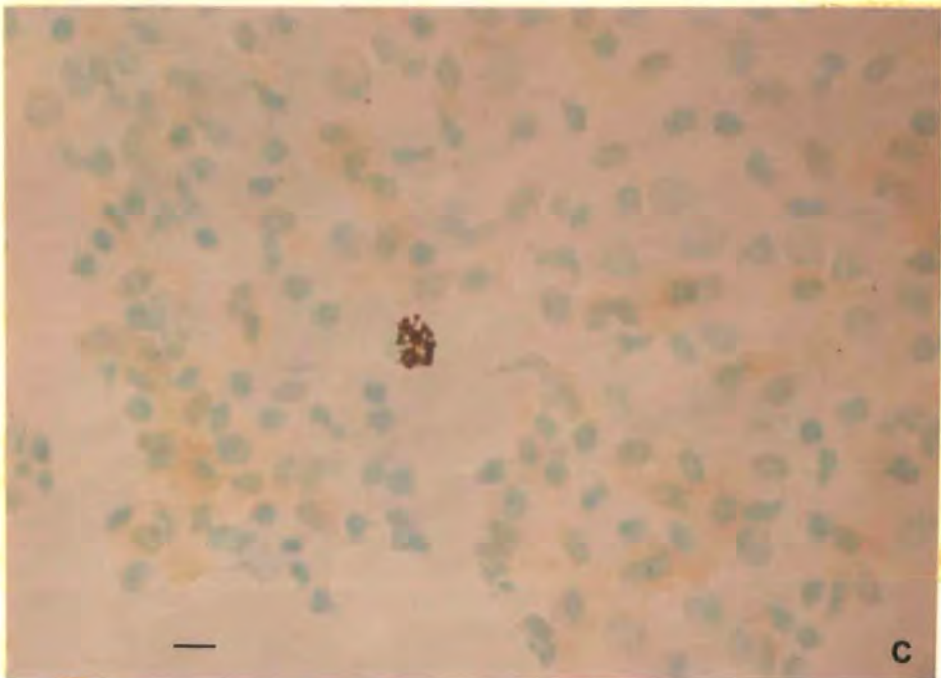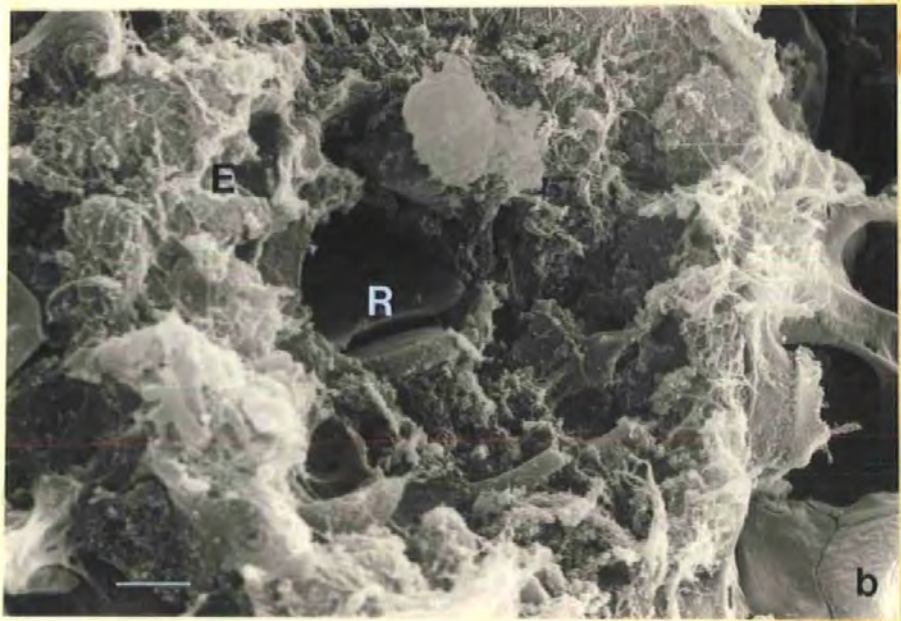
Plate 12

a) Dogfish liver showing open network designed for lipid storage. SEM (x 594). Bar ≡ 10μ.

   V = vacuoles normally filled with lipid

b) Transverse section of dogfish liver showing the open network of this tissue and demonstrating small aggregations of melanomacrophages. LM (x 573) Mallory's Triple Stain. Bar ≡ 10μ.

   M = melanomacrophage

   v = vacuoles normally filled with lipid

c) Dogfish liver cell containing melanin granules. Note the arrangement of pigment granules around the vacuoles and in some cases inside what appear to be digestive vacuoles. TEM (x 7,800). Bar ≡ 1μ.

   m = melanin granules

   v = vacuole

d) Melanin granules in liver cell. Note the granules appear to be confined by membranes around a vacuole. TEM (x 35,467). Bar ≡ 1μ.

   m = melanin granule

   mem = membranes enclosing melanin

   N = nucleus
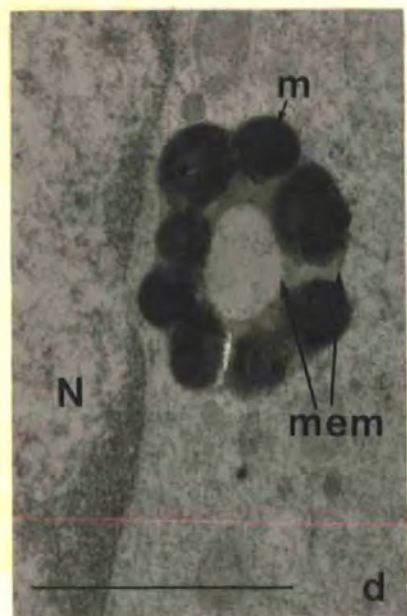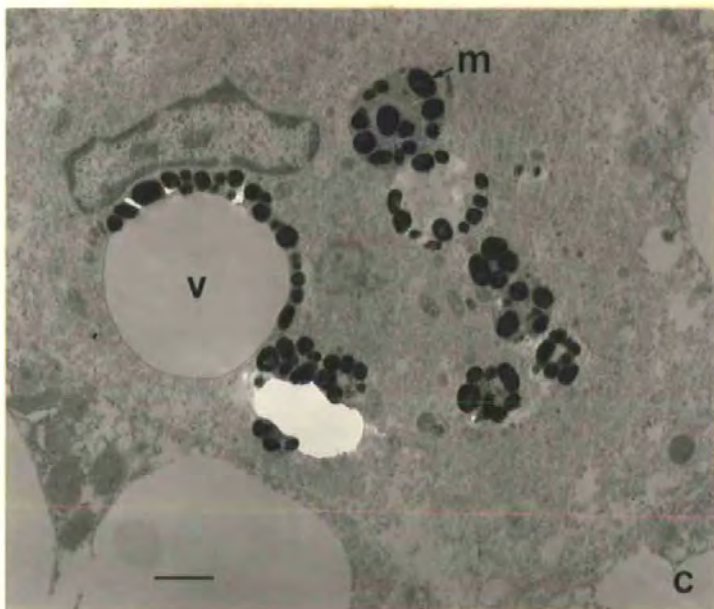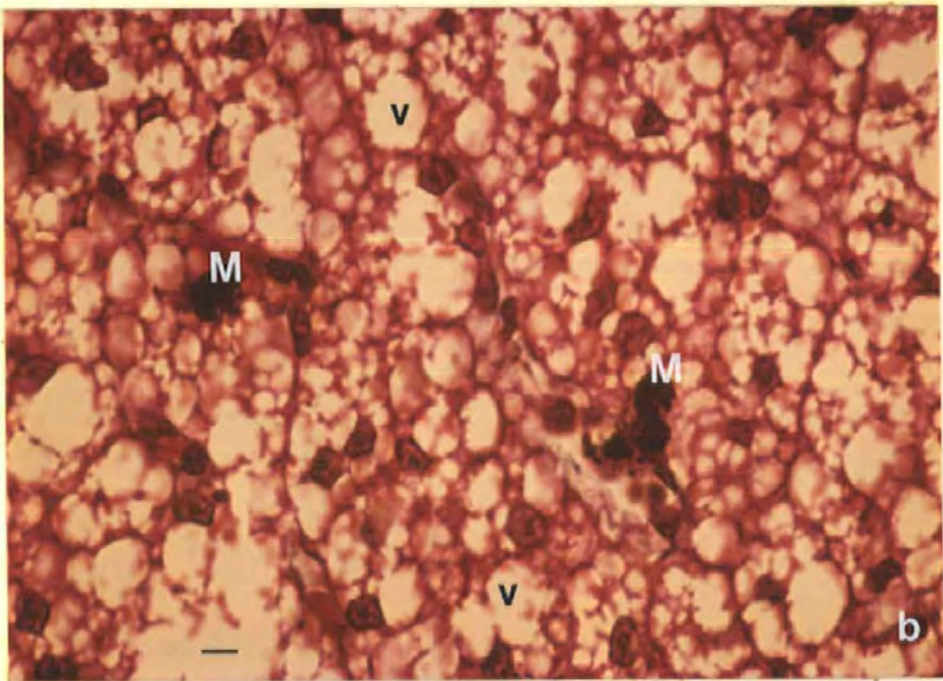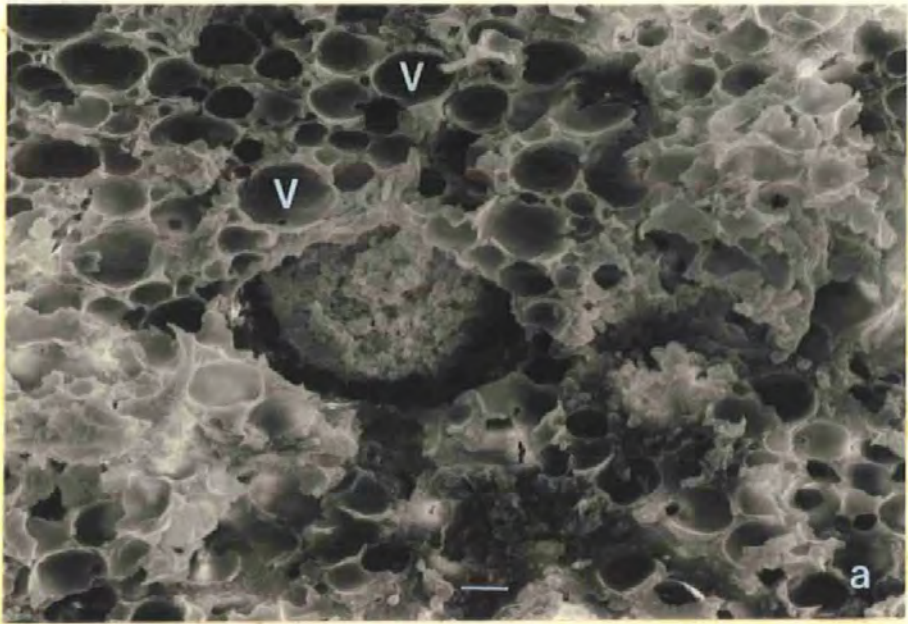
Plate 13

a)   Dogfish peripheral blood smear stained for peroxidase.   LM (x 573)

Giemsa.   Bar $\equiv$ 10μ.

G_1 =   type la granulocyte

G_2 =   type 2 granulocyte

L   =   lymphocyte

p   =   peroxidase positive material

.

b)   Dogfish granulocytes and monocyte stained for peroxidase.   Note

that the type 1 granulocyte is positive.   LM (x 1,431) Giemsa.

Bar $\equiv$ 10μ.

G_1 =   type 1 granulocyte

G_2 =   type 2 granulocyte

M   =   monocyte

p   =   peroxidase positive material
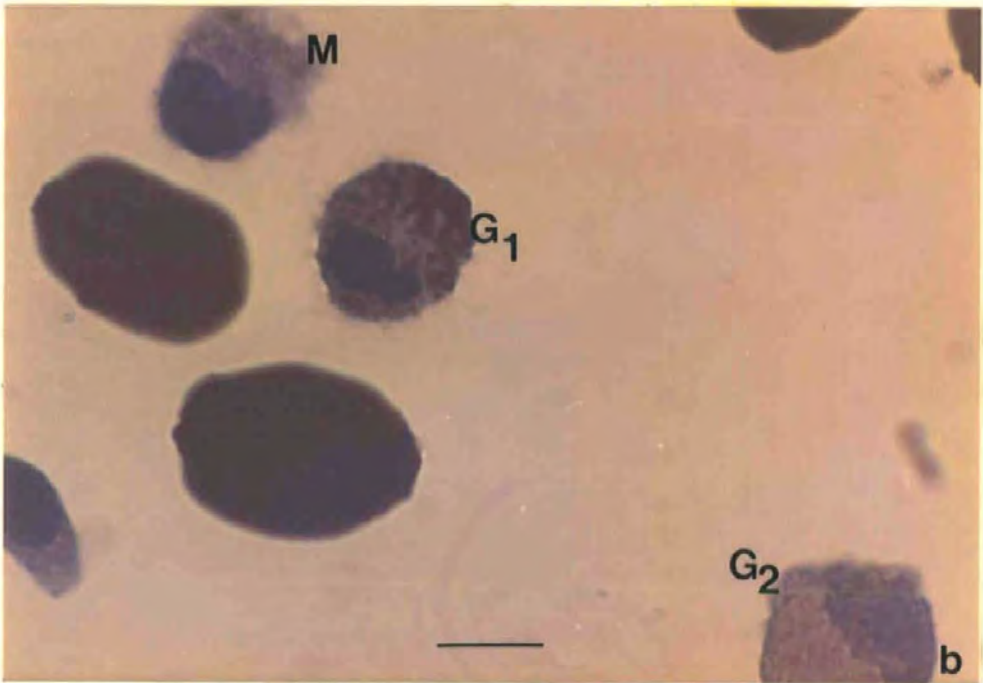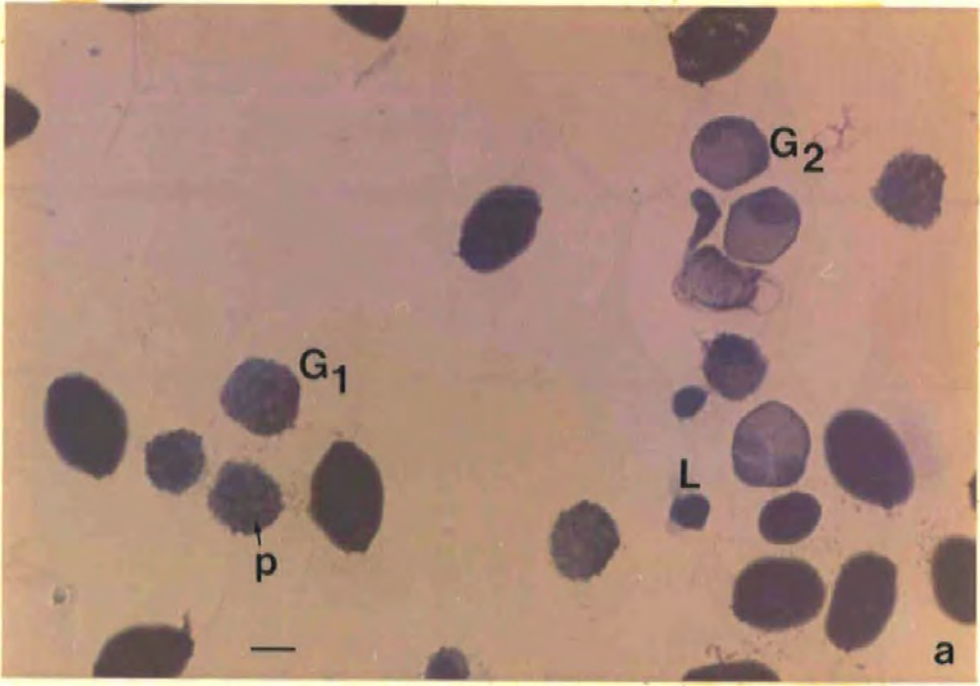
Plate 14

a)   Dogfish peripheral blood smear stained for acid phosphatase.
     LM (x 573) Methyl Green.   Bar ≡ 10μ.

$$G_1 \quad = \quad \text{type 1 granulocyte}$$

b)   Dogfish peripheral blood smear stained for acid phosphatase.
     LM (x 229).   Bar ≡ 100μ.

$$G_1 \quad = \quad \text{type 1 granulocyte}$$
$$G_2 \quad = \quad \text{type 2 granulocyte}$$

c)   Dogfish peripheral blood smear stained for acid phosphatase.
     LM (x 229).   Bar ≡ 100μ.

$$G_1 \quad = \quad \text{type 1 granulocyte}$$
$$G_2 \quad = \quad \text{type 2 granulocyte}$$
$$G_3 \quad = \quad \text{type 3 granulocyte}$$

a

b

c
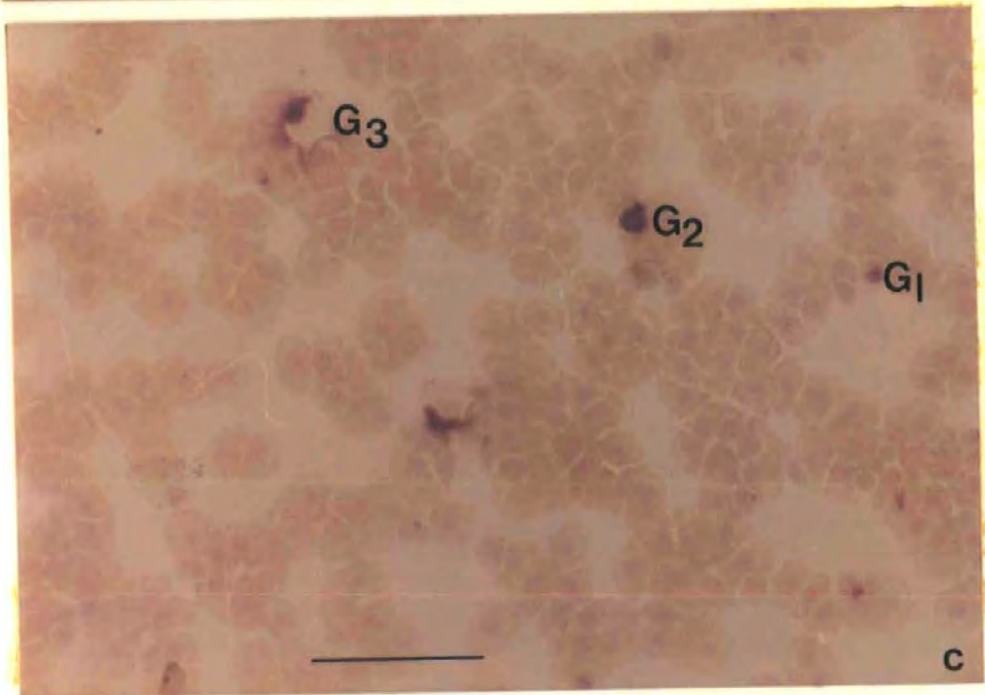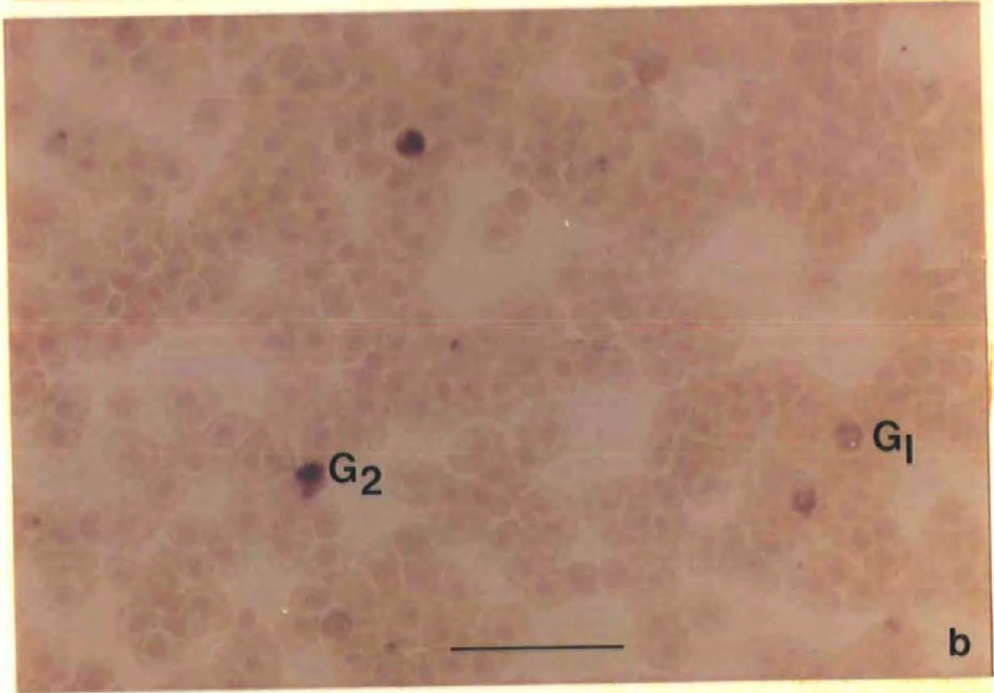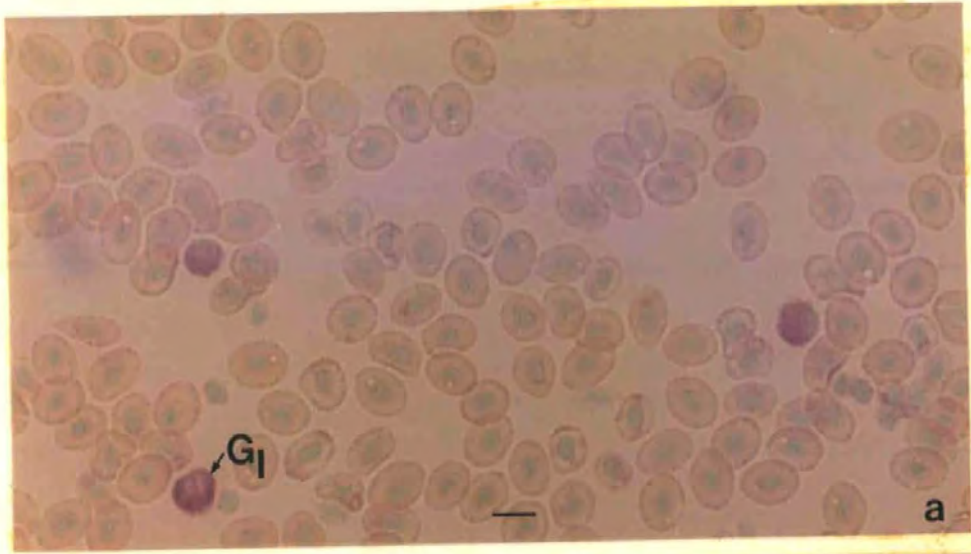
Plate 15

a)   Dogfish peripheral blood stained for non-specific esterase.

LM (x 573) Methyl Green.   Bar ≡ 10µ.

M   =   monocyte

g   =   granulocyte

b)   Adherent peripheral blood monocytes containing phagocytosed

yeast cells and stained for non-specific esterase.   LM (x 573)

Methyl Green.   Bar ≡ 10µ.

M   =   adherent monocyte

My   =   adherent monocyte containing yeast cell
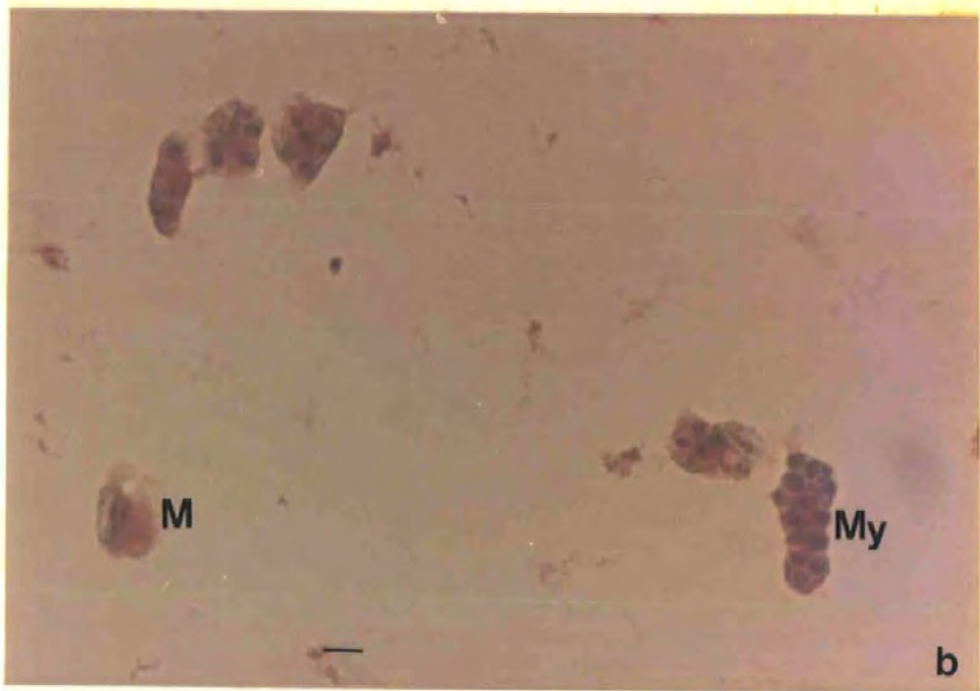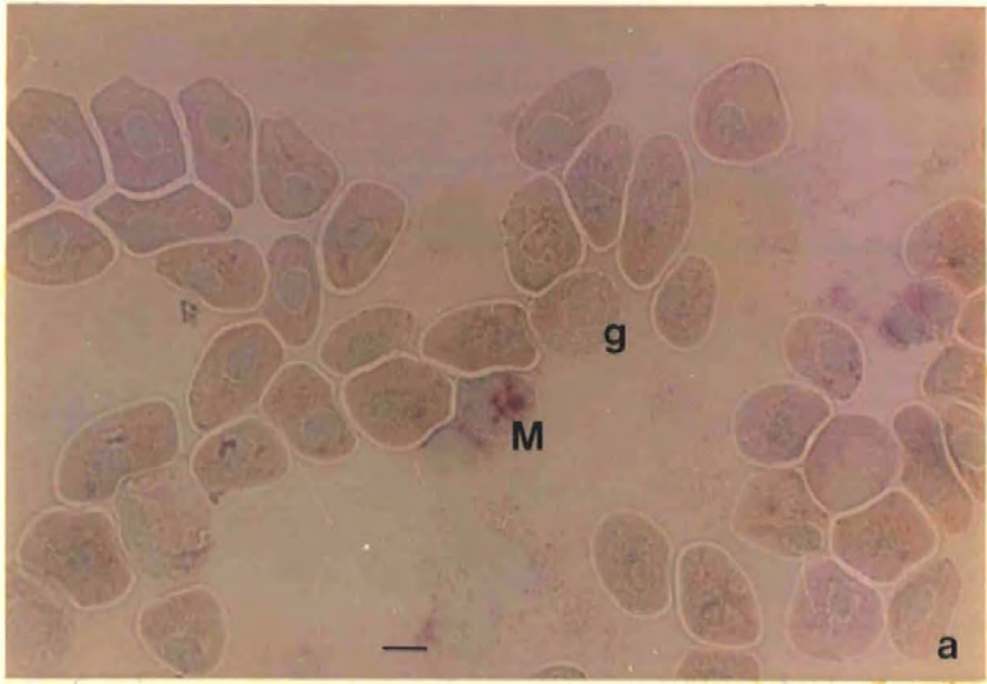
Plate 16

a)   Dogfish peripheral blood stained for sulphatase. A group of
      monocytes containing sulphatase positive material.   LM (x 1,431).
      Bar ≡ 10μ.

b)   Dogfish peripheral blood stained for sulphatase. LM (x 1,431).
      Bar ≡ 10μ.

                    L = lymphocyte

                    T = thrombocyte

c)   Dogfish peripheral blood stained for sulphatase. Type 1
      granulocyte showing positive reaction. LM (x 1,431). Bar ≡ 10μ.
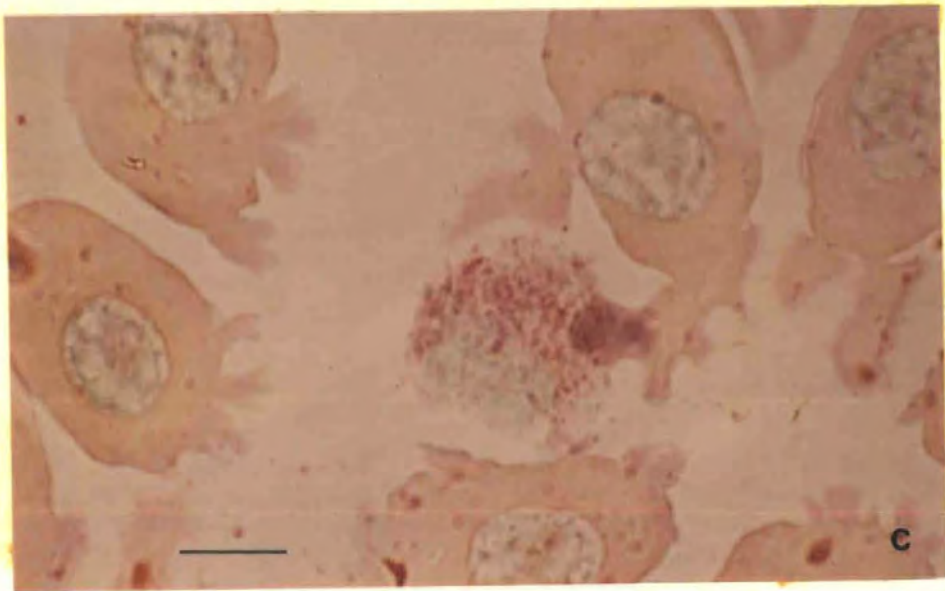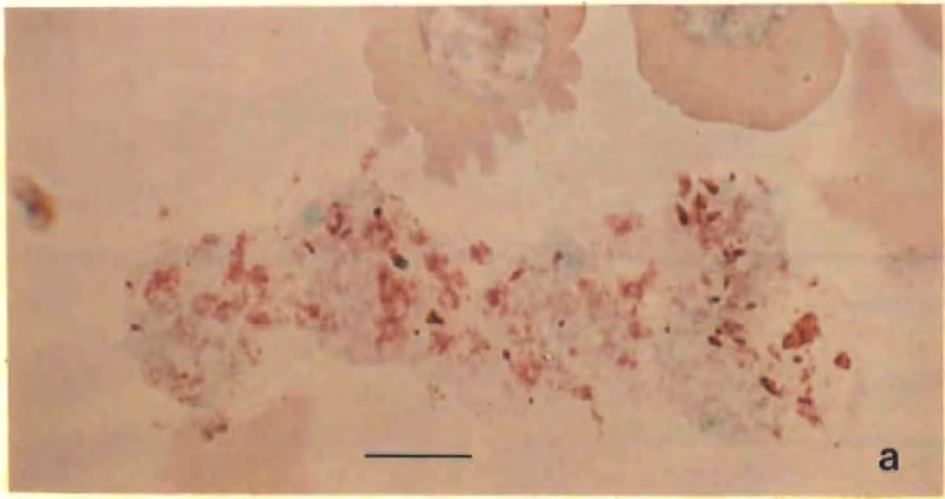
Plate 17

a)   Dogfish peripheral blood stained for P.A.S.   LM (x 229) Methyl

Green.   Bar $\equiv$ 100μ.

G_1 = type 1 granulocyte

G_2 = type 2 granulocyte

G_3 = type 3 granulocyte

b)   Dogfish peripheral blood stained for P.A.S.   LM (x 573) Methyl

Green.   Bar $\equiv$ 10μ.

M   = monocyte

G_1 = type 1 granulocyte

G_3 = type 3 granulocyte

c)   Dogfish peripheral blood monocyte vitally stained by the method

of Whitby and Hynes.   LM (x 573).   Bar $\equiv$ 10μ.

d)   Dogfish peripheral blood type 1 granulocyte vitally stained by

the method of Whitby and Hynes.   LM (x 573).   Bar $\equiv$ 10μ.

e)   Dogfish peripheral blood vitally stained by the method of Whitby

and Hynes.   LM (x 573).   Bar $\equiv$ 10μ.

M   = monocyte

G_1 = type 1 granulocyte

G_2 = type 2 granulocyte

G_3 = type 3 granulocyte
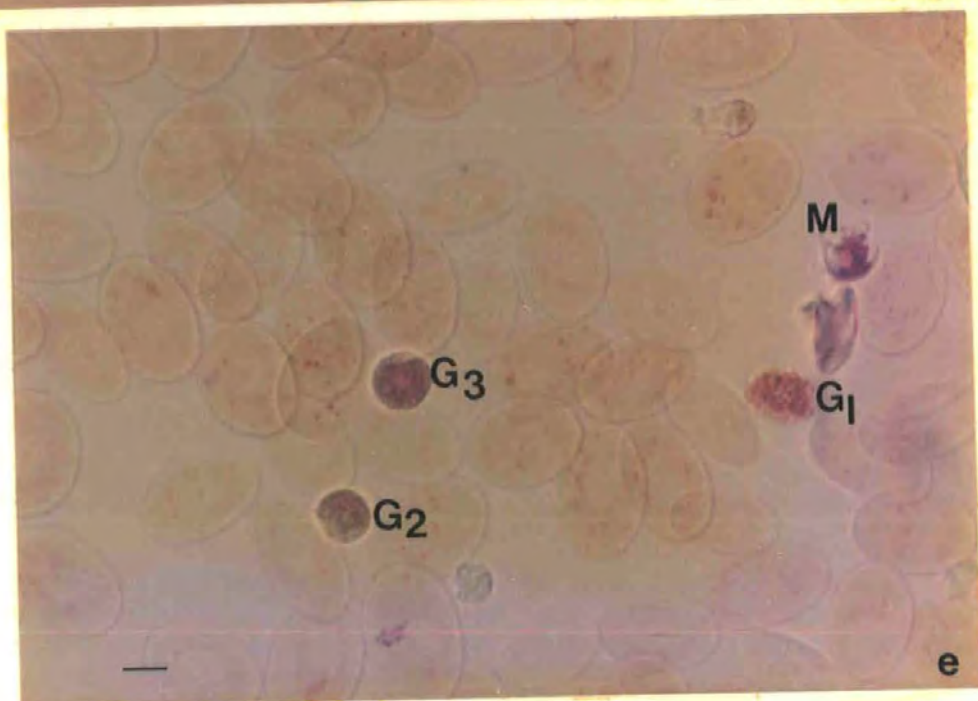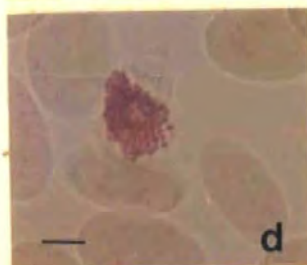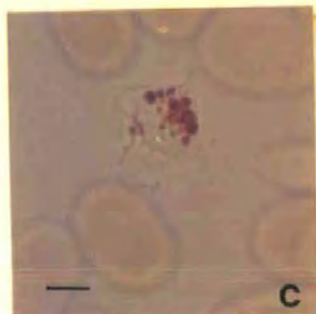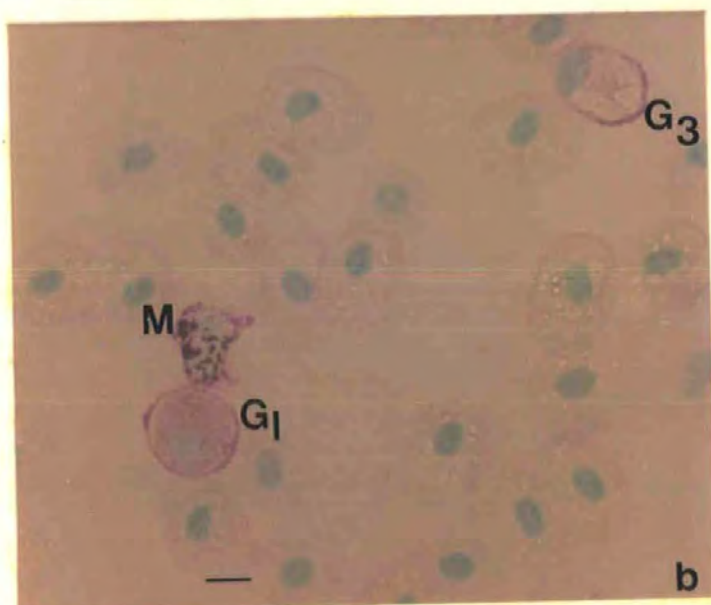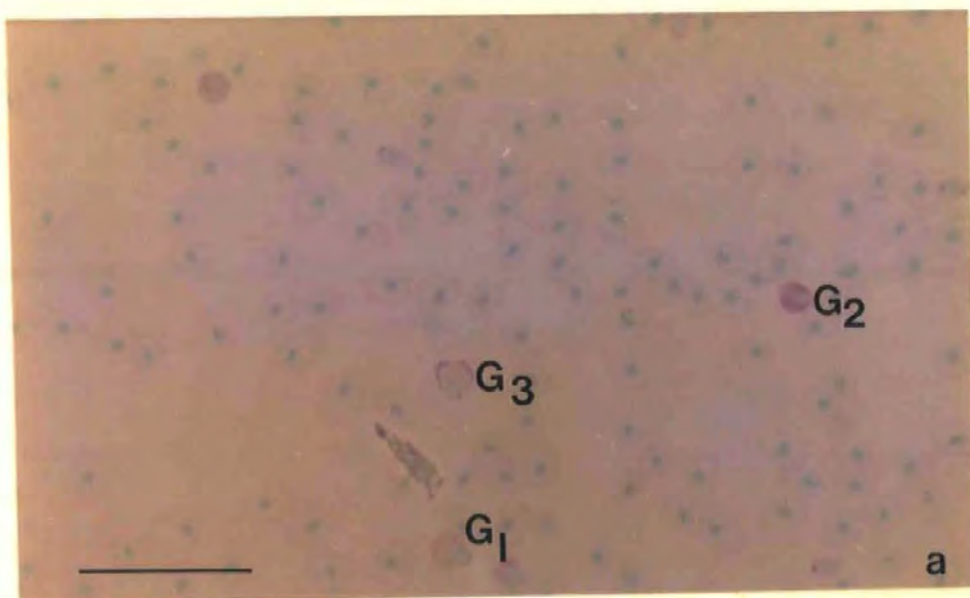
Plate 18

a) Dogfish liver imprint stained for β-glucuronidase. Note that
    only one cell reacted positively (arrow). LM (x 573) Methyl
    Green. Bar ≡ 10μ.

b) Dogfish spleen imprint stained for sulphatase. LM (x 573) Methyl
    Green. Bar ≡ 10μ.

                M = macrophage
                g = type 2 granulocyte

c) Dogfish liver imprint stained for sulphatase showing a great deal
    of general activity not associated with any particular cell
    type. LM (x 229) Methyl Green. Bar ≡ 100μ.

d) Dogfish liver macrophage (arrow) stained for sulphatase,
    LM (x 573) Methyl Green. Bar ≡ 10μ.
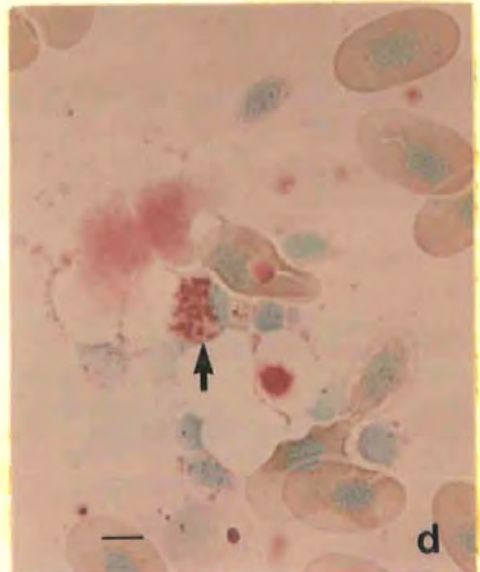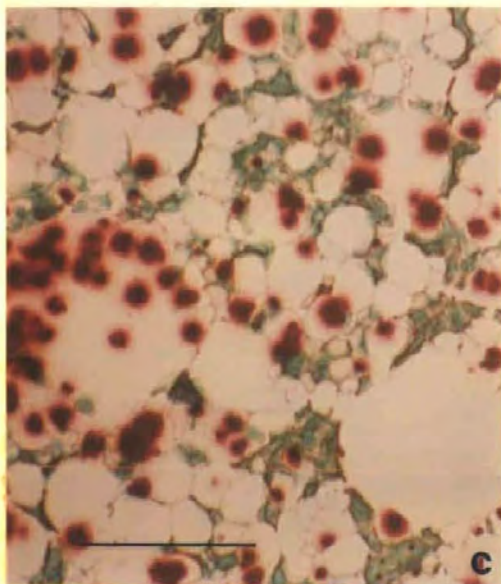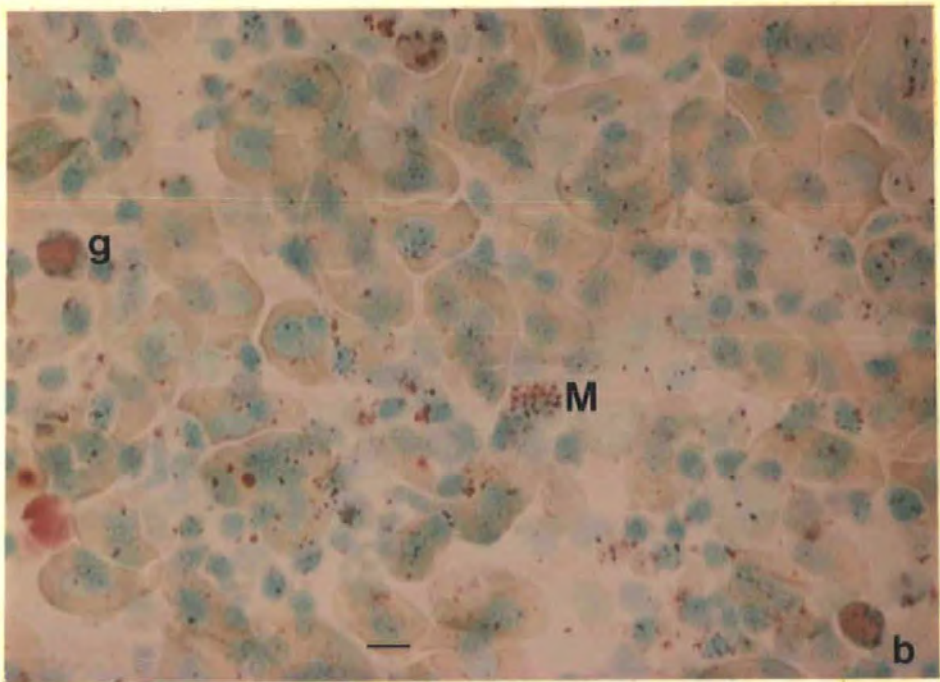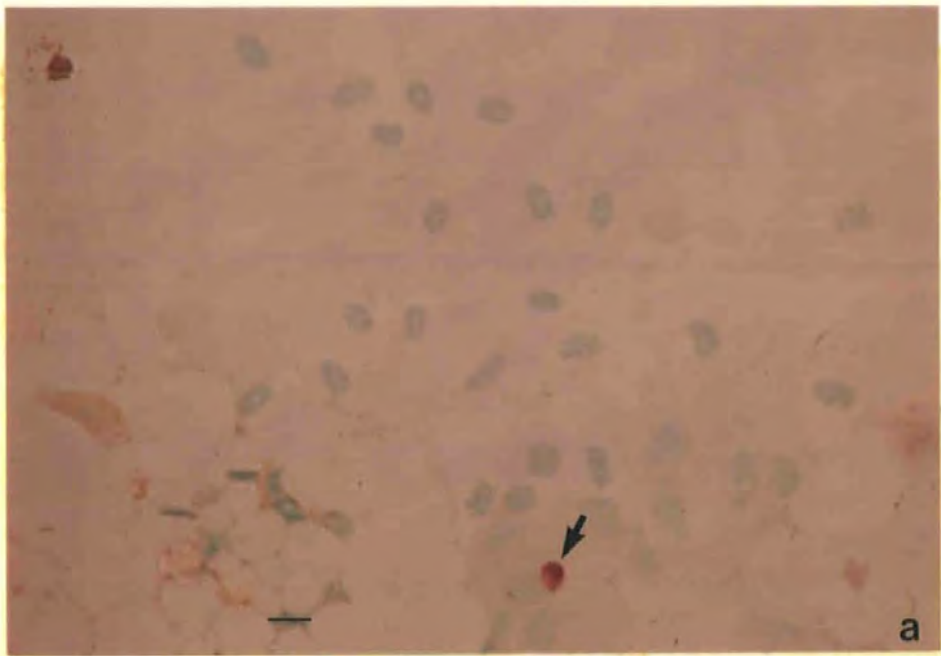
Discussion .

From the details presented in the results it is evident that most of the principal mammalian leucocyte types are represented in dogfish blood.

The lymphocyte takes the same general form as those in other fish species and as shown by mammalian lymphocytes. A plasma cell was not encountered in this study in the blood of dogfish, such as was demonstrated by an electron microscope investigation by Morrow and Pulsford (1980). However these authors themselves commented on the scarcity of these cells.

The thrombocyte took the same form as that described in other fish species. Morrow and Pulsford (1980) designated the cell, here described as a type b) thrombocyte, a granulocyte. The main criterion used here to classify this cell as a thrombocyte, was that it formed the same spindle, spiked and ovoid shapes observed in type a) thrombocytes, and also the presence of what appeared to be intermediates between types a) and b). Ellis (1976) described the thrombocytes of plaice as appearing in four forms:- spiked, spindle, ovoid and lone nucleus depending on the degree of stress the fish underwent during bleeding.

Most reports in the literature make no mention of granules in the cytoplasm but often refer to vacuoles particularly at the two poles of the cell (Karmarkar and Gazdar (1967, 1968); Saunders (1968); Ellis (1976); Ferguson (1976)). However two reports include descriptions of granular cytoplasm in fish thrombocytes (Shepro et al. (1966); Boomker (1980)) the former referring to an elasmobranch - Mustelus canis.

Further evidence against this cell being a form of granulocyte is that although the granules are eosinophilic as in type 1 and 2

granulocytes, they show none of the histochemical reactions of these granulocytes, although it may be argued that they could represent a developmental form of granulocyte. The function of thrombocytes is thought to include an involvement in the clotting mechanism as are platelets in mammals. Their observed adherent and aggregating capacity no doubt assists in cell clumping during clot formation.

Monocytes were positively identified in only small numbers in blood smears, whereas allowing blood to adhere to glass or plastic facilitated identification of these cells. Electron microscopy frequently showed these cells containing internalised nuclei from dead cells and as actively engaged in endocytic activity. It seemed reasonable to assume that these cells functioned by endocytosing foreign and dead material in the blood stream.

Monocytes have been reported in various investigations on fish leucocytes. They have been reported in plaice (Ellis (1976a); Ferguson (1976)), spanish mackerel - Scomberomorus maculatus (Pitombeira and Martins, 1970) in Carcharinus melanopterus and Chiloscyllium greshium (Karmarkar and Gazdar (1967, 1968)) and in S. canicula (Morrow and Pulsford, 1980). They were reported absent from carp (Weinreb, 1963), brown trout (Blaxhall and Daisley, 1973) ten species of elasmobranch (Saunders, 1966) and Squalus acanthias (Sherburne, 1974). It is possible that in the latter reports the monocyte has been identified as a lymphocyte and the best means of demonstrating these cells may be to allow blood cell suspensions to adhere to tissue culture plates to allow them to spread.

The granulocytes described include one which can reasonably be assumed to be an analogue of the mammalian neutrophil - that is the type 1 granulocyte. This cell corresponds to the types 1 and 2 granulocytes of Morrow and Pulsford (1980) who distinguish between the two round-granuled cells. These investigators however agree that

both are phagocytic. It is possible that the 'b' form is a precursor of the 'a' form. The small-granuled, 'b' form was the only granulocyte seen to contain carbon and this may mean that their discriminating capacity is not yet fully developed.

As described in the literature survey, there is some controversy over leucocyte nomenclature, particularly with regard to neutrophil equivalents in fish. They have also been called heterophils; however some workers use heterophil as interchangeable with neutrophil (Barber and Mills Westermann, 1978), whereas others distinguish between the two (Saunders, 1966). Furthermore, although investigators such as Saunders differentiate between the two cell types they also maintain that any one species has only one or other of these cells and so presumably suggests that they fulfill similar functions. Sherburne (1974) however, described both heterophils and neutrophils in Squalus acanthias. He could not detect monocytes in this species and as the heterophil has been described as a non-granulated neutrophil, his heterophil may be what others would classify as a monocyte.

The two other granulocytes described in this study do not appear to correlate with other mammalian granulocytes.

The type 2 granulocytes with their brightly staining rod-shaped granules bear some resemblance to mammalian eosinophils. The fact that they spread on glass but are not actively phagocytic is further evidence that they may represent this cell type. They do not have the crystalline inclusions observed in mammalian eosinophils but these have rarely been demonstrated in the granulocytes of lower vertebrates.

The cell in plate 8 however contains granules with what appear to be crystalline inclusions. This cell was encountered in the

blood of only one fish and so may represent a granulocyte normally only found in the tissues. There is therefore the possibility that type 2 granulocytes (or any of the other types) are precursors of cells such as that shown in Plate 8. Morrow and Pulsford (1980) agree with the observation that the type 2 granulocyte is not phagocytic.

Eosinophils have been described in various fish species such as carp (Weinreb, 1963), Spanish mackerel (Pitombeira and Martins, 1970), five elasmobranch species (Saunders, 1966), and C. melanopterus and Ch. greshium (Karmarkar and Gazdar, 1967, 1968). However, they have been classified merely by their staining properties. As in the present study no functional characteristics have been observed to support the hypothesis that they represent true eosinophils.

Finally, the type 3 granulocyte represents a type not described by Morrow and Pulsford (1980). In the present study it was quite common in that it was invariably observed in blood smears but not in large numbers. It did not adhere to glass or endocytose material. At first it was thought that it may represent a basophil as these cells are known to contain granules which are highly water soluble. If the cell had 'degranulated' in this way, this would account for the lack of its response to Giemsa stain. However, they were always present in the form illustrated by Plate 7d & e, never partially degranulated and the cells appearing intact which degranulated basophils and mast cells do not. This information when considered in addition to facts such as a) fish not possessing IgE (which mediates granule release in mast cells and basophils). b) Fish cells not possessing Fc receptors (see Chapter 5) for autologous immunoglobulin and so granule release could presumably not be mediated by antibody. c) Histamine having no effect on elasmobranch

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smooth muscle (Dreyer and King, 1948; Jensen and Festa, 1980) and
d) there being no suggestion of immediate hypersensitivity reactions
in elasmobranchs (Jensen and Festa, 1980). On considering the above
data it seems highly unlikely that the dogfish possesses a
basophil or mast cell.

Only one report has been discovered which describes a cell
similar to the type 3 granulocyte and this again concerns an
elasmobranch species - Etmopterus. Fänge (1968) described 'a small
percentage of granulocytes which had a cytoplasm filled with
colourless granules or vacuoles but otherwise looked like coarse
eosinophilic granulocytes', in imprints of Leydig's organ.

The numbers of blood cells counted from ten fish show that
erythrocytes tend to be present at about 1.5×10^8 cm^{-3} and
leucocytes at 2×10^7 cm^{-3}. The variation from fish to fish for
total erythrocytes or leucocytes is small, however the proportion
of each leucocyte type of the total is highly variable.

Lymphocytes and thrombocytes are the commonest cell types,
which appears to be the case in other fish species investigated
(Weinreb, 1963; Saunders, 1966; Pitombeira and Martins, 1970).
The other leucocytes are highly variable from one species to another.
When a monocyte is present it comprises only a small proportion of
the leucocytes. In plaice it represents 0.1 - 0.2% of the total
leucocyte population (Ellis, 1976). Morrow and Pulsford (1980)
reported that monocytes represented 13% of the leucocytes in
S. canicula which is very high (the proportion was 1-6% in the
present study), but this may have been stimulated by intravascular
injection of carbon.

The spleen was evidently one of the main leucopoietic organs
in the dogfish. Large accumulations of lymphoid cells are present

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and these surround the efferent splenic blood vessels to a greater extent than afferent blood vessels which terminate in the ellipsoids. This is in accordance with observations on the spleens of Raja clavata and Torpedo marmorata (Zapata, 1980). While red and white pulps exist in the dogfish spleen they appear to be less clearly defined than in higher vertebrates. Ellis et al. (1976) described the arteries and veins of the plaice spleen as running adjacent to each other. The arteries branch into the splenic pulp as arterioles with narrow lumens and thick walls composed of phagocytic cells - the ellipsoids. The lumen of the arteriole is so narrow as to only allow cells to pass through in single file. This is similar to the organisation shown by the dogfish spleen. In the plaice, melano-macrophage centres are present close to the ellipsoids (Ellis et al. 1976). This is in contrast to the situation in dogfish where only single melanin-containing cells are seen, although also often close to the ellipsoids. There is little evidence for the presence of germinal centres in the spleen of fish (Pitchappan, 1980) and this author in reviewing the status of the spleen in vertebrates notes that white pulp morphology has evolved from a diffuse tissue in primitive vertebrates to a concentrated follicular arrangement of lymphocytes surrounding arteries in advanced vertebrates.

The liver of dogfish while not apparently a leucopoietic organ, is the main organ containing pigmented macrophages. This has also been shown by Agius (1980) who further discovered that from the Agnatha to the Chondrichthyes to the Osteichthyes there is a progressive shift of pigmented cells from liver to spleen and kidney and from individual pigmented cells to organised aggregations of cells - the melanomacrophage centres.

The cells of the dogfish gill appeared not to be specially modified

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to phagocytose foreign matter but this process was effected by the pillar cells which are structural elements of the gill. Chilmonczyk and Monge (1980) showed similar activity in the gills of rainbow trout.

The heart possessed no leucocytic activity but was examined, as other workers have demonstrated the presence of phagocytic cells in the hearts of some teleost fish (Ferguson, 1975; Ellis et al., 1976).

Leydig's organ was demonstrated within the connective tissue of the walls of the oesophagus.

Fänge (1968) has studied this organ in various elasmobranchs and noted that in Etmopterus this organ contains eosinophilic granulocytes and non-granulated cells of various sizes and with basophilic cytoplasm. He suggested that granulocytes and lymphocytes developed in Leydig's organ.

The dogfish used in the present study, S. canicula invariably contained large numbers of the nematode parasite Proleptus obtusus Duj. in various parts of the gut including the oesophagus and the intestine anterior to the spiral valve. Since eosinophils are thought to be involved in the reaction of mammalian hosts to parasites, it was considered possible that in fact the large numbers of granulocytes in the oesophagus may constitute a host response to the attached worms. However, the organ of Leydig consists of well-delineated 'sacs' of cells and could not be considered inflammatory sites. If the type 2 granulocytes are however, a piscine equivalent of the mammalian eosinophil the relatively large numbers of this cell may be explained by the large numbers of Proleptus present in the gut and also possibly by the presence of trypanosomes which are again invariably demonstrated in

the blood stream of the dogfish.

Histochemistry

Table 9 summarises some of the reactions of various mammalian leucocytes and Table 10 the results obtained using other fish species. They serve as a comparison for the results described in this study.

The presence of peroxidase in type 1 granulocytes was further evidence for this cell being a neutrophil-equivalent. Mammalian neutrophils possess an 'iodination' system for killing ingested bacteria which involves hydrogen peroxide, peroxidase and a halide ion (Klebanoff, 1967). Plaice granulocytes are also peroxidase positive (Ellis, 1976) indicating that these cells may possess a similar mechanism to that observed in mammals.

Monocytes very occasionally contained a single peroxidase positive granule which may have been a phagocytosed neutrophil granule. Van Furth et al. (1970) demonstrated that as mammalian mononuclear phagocytes developed from promonocyte to monocyte to macrophage they progressively lost their peroxidase activity. No other fish monocytes have been shown to be peroxidase positive.

The acid phosphatase reaction was only appreciably positive in the granulocytes of dogfish whereas mammalian lymphocytes and mononuclear phagocytes also show acid phosphatase activity. Neutrophils of plaice are also acid phosphatase positive (Ellis, 1976).

Non-specific esterase is maintained to be specific for mononuclear phagocytes (Koski, Poplack and Blaese, 1976). In the present study monocytes and macrophages were strongly positive although granulocytes stained slightly pink also.

Table 9

The histochemical reactions of various mammalian leucocytes

HISTOCHEMICAL METHOD	MONOCYTE	NEUTROPHIL	EOSINOPHIL	BASOPHIL	MACROPHAGE	LYMPHOCYTE	THROMBOCYTE
Peroxidase	Can be slightly +ve, generally -ve	+ve	Very +ve	No data	-ve	-ve	No data
β-Glucuronidase	Very +ve	Moderately +ve	Moderately +ve	No data	+ve (variable reaction depending on source	Some +ve (Plasma cells +)	Moderately +ve
Sulphatase	+ve	+ve	+ve	No data	No data	-ve	+ve
Non-specific Esterase	Very +ve	Variable	-ve	No data	+ve	Slightly +ve	No data
Acid Phosphatase	+ve	Slightly +ve	No data	No data	Variable but +ve according to organ source	Slightly +ve	No data
Alkaline Phosphatase	No data	+ve	No data	No data	No data	No data	No data
Whitby + Hynes	-	Yellow granules	Orange granules	Maroon granules	-	-	-
Lipase	No data	-ve	No data	No data	+ve	No data	No data
Acridine orange	Orange fluorescent lysosomes, green nucleus	Red cytoplasmic fluorescence, green nuclei	No data	No data	More lysosomes than monocytes, these perinuclear	Intense green fluorescent nuclei, orange lysosomes	No data

Table 9 Continued

HISTOCHEMICAL METHOD	MONOCYTE	NEUTROPHIL	EOSINOPHIL	BASOPHIL	MACROPHAGE	LYMPHOCYTE	THROMBOCYTE
Lysozyme	+ve	+ve	No data	No data	Variably +ve depending on organ + animal	No data	No data
Histamine	-	-	1/3 of total blood histamine	1/2 of total blood histamine	-	-	-
P.A.S.	+ve	+ve	No data	No data (-ve?)	+ve (often resistant to amylase digestion)	Slightly +ve	No data

Table compiled from data of:- Austin & Bischel, 1961; Rytomaa, 1962; Bennet & Cohn, 1966; Bainton & Farqhar, 1968; Hayhoe & Flemens, 1969; Van Furth, 1970; Van Furth et al., 1970; Syren & Raeste, 1971; Yam, Li & Crosby, 1971; Vernon-Roberts, 1972; Van Furth, 1975; Allison, 1976; Breton-Gorius, Guichard, Vainchenker & Vilde, 1980.

Table 10

The histochemical reactions of the leucocytes of various fish species

	P.A.S.	Acid Phosphatase	Alkaline Phosphatase	Sudan Black B	Esterase	Lysozyme
Lymphocytes	Slightly + (plaice) Some +ve granules (S. trutta) -ve (S. canicula + S. stellare) 15% +ve (S. maculatus)	-ve (plaice) -ve (S. trutta) -ve (S. canicula S. stellare)	-ve (plaice) Slightly +ve (S. canicula & S. stellare)	-ve (plaice) -ve (S. trutta)	Slightly +ve (S. canicula & S. stellare)	-
Thrombocytes	Some +ve granules (plaice) -ve (S. trutta) +ve (S. canicula + S. stellare) Some +ve granules (S. maculatus)	-ve (plaice) -ve (S. trutta) -ve (S. canicula S. stellare)	-ve (plaice) Slightly +ve (S. canicula & S. stellare)	-ve (plaice) -ve (S. trutta)	+ve (S. canicula & S. stellare)	-
Monocytes	Slight +ve (plaice) +ve (S. canicula + S. stellare) Slight +ve (S. maculatus)	-ve (S. canicula & S. stellare) -ve (M. canis)	-ve (plaice) Slightly +ve (S. canicula & S. stellare)	-ve (plaice)	Slightly +ve (S. canicula & S. stellare)	+ve (plaice)
Neutrophils	Very +ve (plaice) Very +ve (S. trutta) +ve (S. canicula S. stellare) Very +ve (S. maculatus)	+ve (plaice) +ve (S. trutta) +ve (S. canicula & S. stellare) +ve (I. punctatus) -ve (M. canis)	Faintly +ve (plaice) Slightly +ve (S. canicula & S. stellare)	+ve (plaice) Very +ve (S. trutta)	+ve (S. canicula S. stellare)	+ve (plaice)

Table 10 Continued

	P.A.S.	Acid Phosphatase	Alkaline Phosphatase	Sudan Black B	Esterase	Lysozyme
Eosinophils	+ve (S. canicula + S. stellare) Slightly +ve (S. maculatus)	+ve (carp, bleak tench) -ve (S. canicula & S. stellare)	Slightly +ve (S. canicula & S. stellare)	-	+ve (S. canicula & S. stellare)	-
Basophils	+ve (S. canicula S. stellare) Very +ve (S. maculatus)	-ve (S. canicula & S. stellare)	Slightly +ve (S. canicula & S. stellare)	-	Slightly +ve (S. canicula & S. stellare)	-

Table compiled from the data of:- Fey, 1960; Kelenyi and Nemeth, 1969; Pitombeira and Martins, 1970; Blaxhall and Daisley, 1973; Fletcher and White, 1973; Weissman et. al., 1975; Ellis, 1976; Cannon, Mollenhauer, Cannon, Eurell and Lewis, 1980.

Sulphatase appears to be a broadly distributed enzyme in both mammalian and dogfish leucocytes and although the stain demonstrates active metabolism it is of relatively little use as a means of identifying specific leucocytes.

β-glucuronidase was absent from all fish leucocytes whereas it is widely distributed in mammalian leucocytes.

Type 2 granulocytes showed a small amount of lipase activity but otherwise this enzyme was generally absent. Vernon-Roberts (1972) reported its presence in mammalian macrophages.

The PAS reaction is a relatively non-specific reaction as it stains all types of polysaccharide. It is interesting that the most intense reactions are particularly seen at the cell membrane and also that a positive reaction is mainly associated with cells capable of adhering to glass. Vernon-Roberts (1972) has described a surface coat on macrophage plasma membranes, a part of which exhibits the staining characteristics of acid mucopolysaccharide which he suggests may be responsible for the adhesive properties of macrophages.

In contrast to plaice and brown trout neutrophils (Ellis, 1976; Blaxhall and Daisley, 1973) the leucocytes of the dogfish showed little lipid content.

The application of Csaba's mast cell stain to dogfish leucocytes showed no reactive cells suggesting that no basophil equivalent was present. By Romanowsky stains basophils have been described in four elasmobranchs, i.e. Carcharinus melanopterus and Chiloscyllium greshium (Karmarkar and Gazdar, 1967, 1968) and S. canicula and S. stellare (Fey, 1960). The results of Fey are curious as leucocytes with basophilic granules were not observed throughout the present study of S. canicula.

As may be seen in Table 8 the imprints of spleen and liver

showed that enzymes found in blood leucocytes were also present in similar cells in the tissues. That is with the exception of β-glucuronidase which was found in rare, isolated globules in both spleen and liver. In the liver some pink reaction was noted in melanomacrophage cells. The lack of any reaction in blood may reflect the immaturity of monocytic cells in that this enzyme may only be present in a few mature tissue macrophages.

Haemosiderin could not be demonstrated in spleen or liver sections from fish exposed to various antigens (i.e. bacteria or yeast) or control fish. This is in contrast to teleosts in which large deposits of haemosiderin are found in the spleen melanomacrophage centres and rarely in the liver and kidney MMC. Under conditions of starvation, the deposition is increased in splenic centres (Agius, 1979). By comparison the dogfish does not appear to deposit haemosiderin in the same way (Agius, pers. comm.).

Fänge (1968) studying S. canicula and Raja species found high acid phosphatase activity in the epigonal organ, Leydig's organ and the spleen and also in the thymus of Raja. He proposed that, since the former organs mainly comprised eosinophilic granulocytes and the latter organs - lymphocytes, both granulocytes and lymphocytes were rich in acid phosphatase.

From the present study it appears more likely that the acid phosphatase activity is attributable to granulocytes only (as lymphocytes were negative).

The lack of lysozyme in the dogfish was perhaps surprising as this is an important 'first-line' defence mechanism in clearing the circulation of susceptible (principally G +ve) bacteria. Fletcher and White (1973) detected lysozyme in white cell suspensions of plaice and found it was present in the monocytes and granulocytes of

this fish, as is true in mammalian leucocytes (Ghoos and
Vantrappen, 1970; Syren and Raeste, 1971; Way et al., 1980).
Lundblad et al. (1979) found large amounts of lysozyme in the cranial
lymphomyeloid tissue of Chimaera monstrosa; in Leydig's organ and
spleen of Etmopterus spinax and in Leydig's organ, the epigonal
tissue and spleen of Raja radiata. Little or no activity was
found in Leydig's and epigonal organ of Squalus acanthias or in
the thymus of C. monstrosa, R. radiata or Lophius piscatorius.
Lysozyme therefore seems to be a variable attribute of the defence
system of elasmobranchs.

Superoxide was found in very small amounts in dogfish monocytes,
as the reduction of nitroblue tetrazolium (NBT) was only slightly
inhibited by superoxide dismutase (SOD). The method using NBT
reduction is non-specific as this reagent may be reduced by a variety
of metabolic products in active cells. More specific assays employ
cytochrome oxidase as the substrate to be reduced. The principal
source of superoxide activity is, however in neutrophils (although
Karnovsky (1975) reports that mouse macrophages produce comparable
amounts of superoxide as PMN leucocytes) and if pure cultures of
these cells could have been obtained, larger amounts of superoxide
may have been detectable.

It is as yet unknown whether superoxide plays a direct role in
bactericidal processes as its metabolic product H_2O_2 is known to do
so, both directly and via the H_2O_2-myeloperoxidase-halide system of
neutrophils. Fig. 5 shows the metabolic cycle in which superoxide
and hydrogen peroxide are involved.

The detection of peroxidase in type 1 granulocytes of dogfish
suggests that a similar 'iodination' of bacteria could occur in these
cells as occurs in mammalian PMN leucocytes. However the dogfish

Fig. 5

Metabolic reactions in human polymorphonuclear leucocytes showing role of superoxide and its derivatives. (From "A comprehensive review of phagocytic function", Baehner, R.L. and Boxer, L.A. (1979))
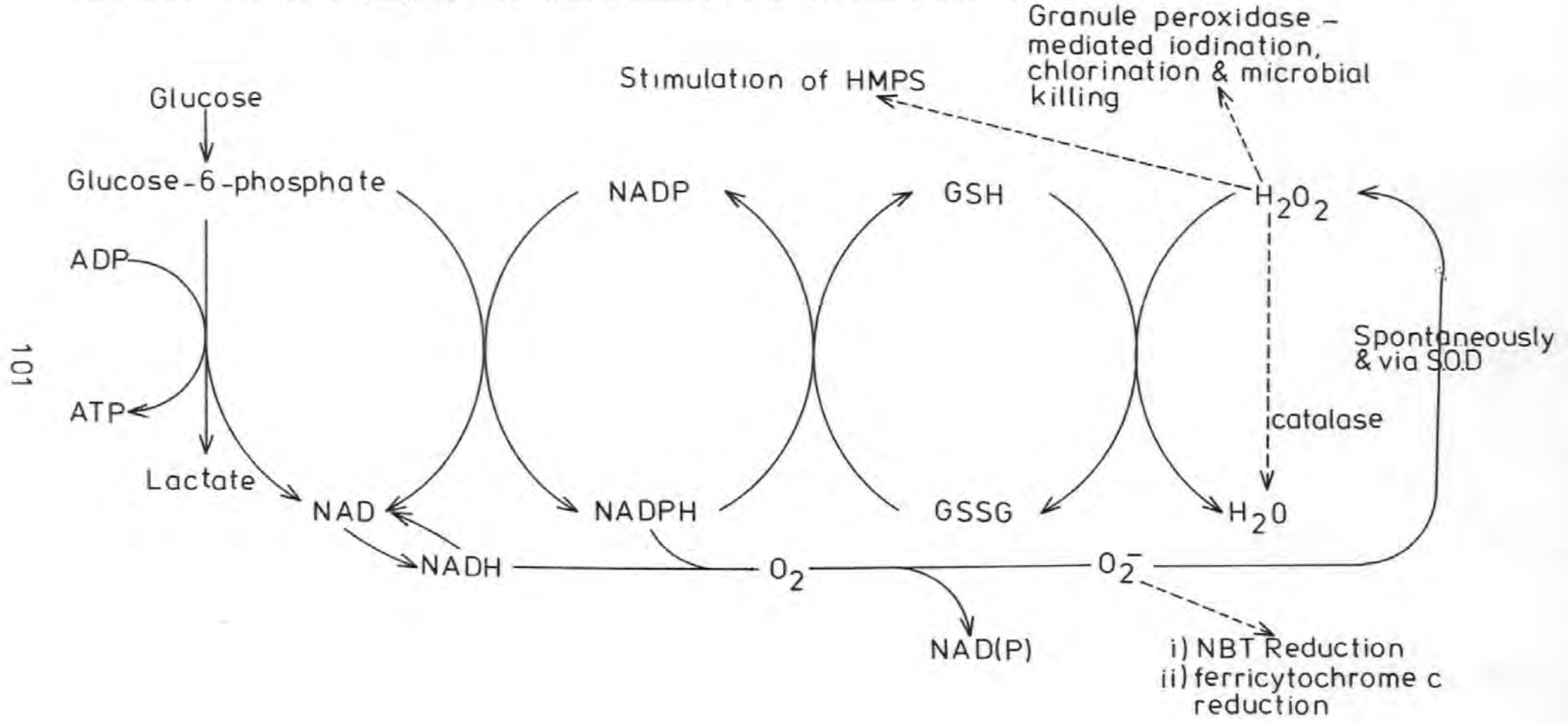
does not appear to be particularly efficient at clearing bacteria either *in vitro* or *in vivo* (see following chapter) implying that the enzyme systems are less active in dogfish than mammalian granulocytes. Furthermore, bacteria themselves can counteract many of these systems. For example, many can produce catalase which catalyses hydrogen peroxide to water and oxygen; others can withstand hydrogen peroxide and all aerobic bacteria possess the enzyme superoxide dismutase.

Chapter 4

Endocytic Studies

a)   A qualitative study of in vitro endocytosis

Blood leucocytes and splenic macrophages from dogfish took up a variety of antigens and particulate material to different degrees (see Table 11).

Monocytes appeared to be less discriminating than type 1 granulocytes (or neutrophils) but where neutrophils phagocytosed a particle it appeared to be more avidly than the monocyte. However the neutrophils seemed to become more fragile having undergone phagocytosis and would often be observed at various stages of degranulation.

Heat-killed yeasts were always readily phagocytosed by both monocytes and neutrophils (see Plates 19a & b).

Bacteria in all but a few cases were employed as heat-killed, fluoresceinated particles. The uptake of these may not necessarily indicate however that viable bacteria are endocytosed. In two cases, where bacteria were tested as live preparations, blood cells were not observed with ingested bacteria. However, in these two cases the phagocytic cells were in suspension cultures as opposed to being adherent. The impression gained from a comparison of suspended with adherent cells was that the adherent cells were more avidly phagocytic. This was not however tested quantitatively.

Adherent, dogfish cells given Streptococcus faecalis contained internalised bacteria (see Plate 19c), but it is not known whether these phagocytosed bacteria were in fact viable. /This bacterial species decreased in number when placed in TCM199 suplemented with urea and salt, and the presence of dogfish, blood phagocytes did not accelerate this decrease - see in vitro, quantitative results/ It may therefore be that the cells were taking up the non-viable cells

Table 11

Endocytosis of Various Antigens and Particles by Dogfish Phagocytes.

+ = cells observed containing particle or antigen

- = cells not observed containing particle or antigen

φ = phagocytes tested as adherent monolayers, otherwise tested as cell suspensions

* = FITC - conjugated

NT = Not tested

Antigen or particle	Blood Monocytes	Blood Neutrophils	Spleen Macrophages
Yeasts:- S. cerevisiae	+ φ	+ φ	+ φ
Bacteria:-			
Micrococcus lysodeikticus*	+	+	+
Bacillus subtilis	+ φ	+ φ	+ φ
V. anguillarum UNH 569	+ φ	+ φ	+ φ
" " COB 408	+ φ	+ φ	+ φ
S. typhimurium*	+	+	+
Strep. faecalis	+	+	+
E. coli*	+	+	+
Proteus vulgaris*	+	+	+
Staph. aureus*	+	+	+

Table 11 Continued

Antigen or particle	Blood Monocytes	Blood Neutrophils	Spleen Macrophages
Erythrocytes:-			
Sheep	+ φ	+ φ	+ φ
Calf	+ φ	+ φ	+ φ
Horse	+ φ	+ φ	+ φ
Latex:-			
0.81μ	+ φ	- φ	+ φ
1.1μ	+ φ	- φ	+ φ
Carbon:-	+	-	+
	also thrombocytes + ve		
Parasite:-			
Kodoa sp (myxosporidial parasite)	-	-	NT
Soluble Antigen:-			
KLH*	+ φ	+ φ	NT
BGG*	+ φ	+ φ	NT

and not viable bacteria.

All types of mammalian erythrocytes tested were phagocytosed, apparently as avidly as yeasts.

Latex and carbon were taken up by monocytes and carbon occasionally by neutrophils. Thrombocytes were often seen containing carbon.

Kodoa sp. (a myxosporidial parasite) spores were not observed to be phagocytosed by phagocytes in suspension, and were not tested on adherent cells.

Fluoresceinated, soluble antigens such as Keyhole Limpet Haemocyanin (KLH) and Bovine Gamma Globulin (BGG) were taken up by small numbers of adherent cells.

SEM of in vitro uptake of yeasts and bacteria

The method by which yeasts are engulfed is best demonstrated by EM (Plate 20). The phagocyte membrane extends around all sides of the yeast particle and eventually causes complete internalisation.

Plate 19c shows Streptococcus faecalis being taken up by an adherent blood monocyte.

As may be seen from Plate 19c, the monocyte appears to form an invagination immediately beneath a group of several bacteria as opposed to single cells.

b) A qualitative study of in vivo phagocytosis

i) Carbon

Carbon injected intravascularly was principally cleared by the spleen and gills. Blood cells rarely contained carbon by comparison, but when they were observed it was the monocytes which most commonly contained carbon (Plate 21a), less commonly thrombocytes (Plate 21b) and occasionally neutrophils (Plate 21c).

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The liver, kidney, heart and Leydig's organ did not appear to phagocytose carbon.

Wax sections of spleen showed that the active cells of this organ were located in the ellipsoids (see Plate 22a). After a week large amounts of carbon had accumulated here. Spleen imprints showed that there was also a large amount of extracellular carbon. It was often present as discrete vacuolated, accumulations not within cells but apparently where a cell which originally engulfed it had subsequently died around its undegraded contents. Plate 22b shows an electron micrograph of a splenic macrophage containing carbon.

The gill, in addition, was an active site for phagocytosis (Plates 22c and 23a). Large numbers of circulatory cells containing carbon were attached to epithelial surfaces of the vessels at the base of gill filaments. Also, cells in the gill filaments contained carbon; these cells included cells in the circulation and also the pillar cells of the gill itself.

A particularly salient feature was the large numbers of circulatory cells containing carbon in the gill as compared with blood smear preparations. The lumen of any particular gill vessel was clear of carbon.

ii) <u>Yeasts</u>

Yeasts were taken up by a few blood monocytes and by ellipsoidal cells in the spleen. Very much fewer yeasts were seen in the ellipsoidal cells however, by comparison with uptake of carbon by these cells.

A few circulatory cells were seen containing yeasts in the liver, and relatively large numbers of pigment-containing cells were seen in this organ four days after injection (see Table 12).

Most cells were seen with endocytosed yeasts in the gill, these

being mainly circulatory cells but occasionally pillar cells and the epithelial cells of the gill filament had also taken up yeasts (see Fig. 6).

iii) Bacteria

Very few cells in blood smears were observed containing bacteria. Those which did were identified as monocytes and neutrophils which formed large aggregations in the blood smears.

Two to three weeks after injection with BCG, monocytes were observed, by both light and electron microscopy, to contain large deposits of pigment (Plate 23b). In addition some cells were seen which evidently contained the remains of bacteria which were surrounded by an outer rim of dark pigment (Plates 23c and 24a). Furthermore, some cells showed bacteria – notably Salmonella typhimurium – present in vacuoles which had large membranous 'whorls' in evidence. In some cases these membranous areas were fusing with what appeared to be lysosomes (Plates 24b & c).

Thrombocytes also appeared to have the membranous whorls within their cytoplasmic vacuoles (Plate 25a). In addition, although granulocytes were not seen containing bacteria under the electron microscope, some appeared to have increased lysosomal activity.

Bacteria were apparently taken up by ellipsoidal cells of the spleen (Plate 25b), and even after one month numbers of particles were seen in these cells. These may have been undegraded bacteria but appeared more likely to be small pigment particles. The ellipsoids of spleens of fish which had received S. typhimurium six weeks previously appeared to contain few phagocytic cells.

The electron microscopic investigation showed splenic cells to have large numbers of the same 'membranous whorls' as observed in blood monocytes. This was only seen in the case of S. typhimurium

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Circulatory phagocyte containing yeasts.

Pillar cell containing yeast.

Gill epithelial cell.

Fig. 6

Diagrammatic representation of dogfish gill filament showing circulatory and structural cells containing yeasts.
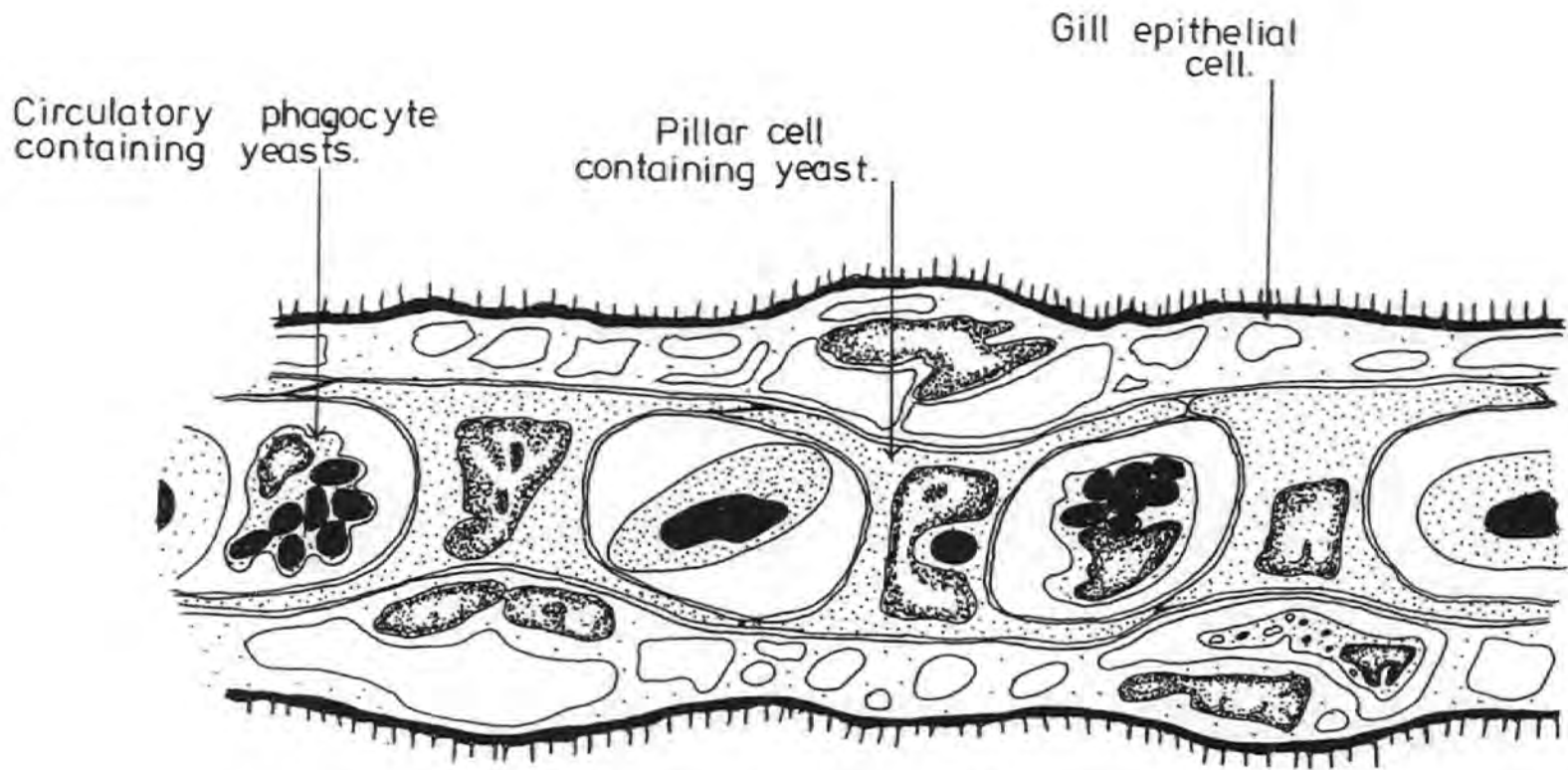
uptake (Plates 25c and 26a). Escherichia coli - injected fish appeared to have small accumulations of bacteria not obviously within any particular cell in the spleen (Plates 26b & c). Both yeasts and bacteria were not phagocytosed by liver or heart cells.

Table 12 shows the numbers of pigmented cells per 10 fields (x400) in wax sections (7μ) of liver and spleen 4 days after injection with various particles and antigens. The table shows the ratio of the numbers per unit area in treated tissue over the numbers in untreated control tissue. The number of pigmented cells in the liver increased in fish which had been injected with yeasts or bacteria.

While no electron micrographs of melanomacrophage cells containing antigen were prepared they are evidently involved in tissue breakdown (Plate 27a).

The spleens from fish injected with bacteria, as previously noted, were occasionally observed to contain small pigment granules in the ellipsoids. Cells with large amounts of pigment inside were generally located outside the ellipsoids (Plate 27b), but not obviously among the lymphoid elements of the spleen.

In control spleen the ellipsoid cells contained neither bacteria nor pigment granules but there were a few pigment cells within the spleen. Fish injected with yeasts or Salmonellae showed no increase in the numbers of these splenic pigment cells after 4 days (Table 12).

A few pigment containing cells were observed in the gill of treated fish.

In the case of carbon it was difficult to differentiate between carbon and pigment-containing cells as the latter had not been decolourised by bleaching agents. Counts of pigment cells in the liver of dogfish injected 4 days previously with colloidal carbon

Table 12

Numbers of MMC in the Spleen and Liver of Dogfish 4 days post
i.v. injection with Particulate Material.

Treatment	Organ	MMC/10 Fields	$\frac{\text{Treatment}}{\text{Control}}$ x 100
1. Control	Liver	24.5	100
2. 4 days post S. typhimurium i.v.	Liver	67.5	276
3. 4 days post yeasts i.v.	Liver	103	420
4. 4 days post carbon i.v.	Liver	31	127
5. Control	Spleen	8	100
6. 4 days post S. typhimurium i.v.	Spleen	7	87.5
7. 4 days post yeast i.v.	Spleen	6	75.0

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revealed a small increase by comparison with antigen-treated fish (see Table 12), but this may be accounted for by small amounts of carbon being present.

c) A quantitative study of in vitro phagocytosis and the effects of various factors on this process.

Yeasts

The clearance of heat-killed yeasts by dogfish adherent blood phagocytes was used to study the conditions required for in vitro phagocytosis.

i) Effect of pH

The optimum pH for phagocytosis in vitro was approximately pH 7. However appreciable phagocytosis occurred within the range 5.5 → 8.5 (see Table 13).

Table 13

In vitro Phagocytosis at a range of pH values.

pH	% Phagocytosis
5.5	13.9
6.0	6.9
6.5	10.4
7.0	22.5
7.5	11.0
8.0	4.7
8.5	9.1

ii) Temperature

The optimum temperature for phagocytosis was between 10-20°C. At temperatures below or above this range the number of cells taking up yeasts decreased (see Fig. 7).

At extremes of temperature (particularly at 37°C and 45°C), cells evidently became detached or were killed, this effect being most pronounced on any neutrophils present. At very high or low temperatures relatively few cells were present, all of these being monocytes.

All subsequent experiments were carried out at room temperature (i.e. 18-20°C).

iii) Effect of plasma

Rates of yeast uptake were measured in the presence or absence of whole or heat-inactivated autologous plasma, over an incubation period of one hour. (Additional experiments were performed over longer incubation periods and using yeasts coated in plasma, rather than adding serum directly to the incubation medium. These experiments gave essentially similar results).

With reference to Fig. 8a it can be seen that the presence of heat-inactivated or whole plasma had no appreciable effect on rate of uptake of yeasts.

Cells were also treated with trypsin (0.2%) or rabbit anti DF Ig ([1]/10 dilution) in order to assess whether immunoglobulin was a surface feature of blood phagocytes. The cells were treated for thirty minutes prior to their phagocytic potential being monitored in the presence or absence of plasma.

A slight increase was noted in the presence of plasma as opposed to its absence, in the control cells (see Fig. 8b). Trypsin markedly decreased phagocytosis and addition of plasma did not restore this

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Fig. 7

In vitro phagocytosis of yeasts at various temperatures.
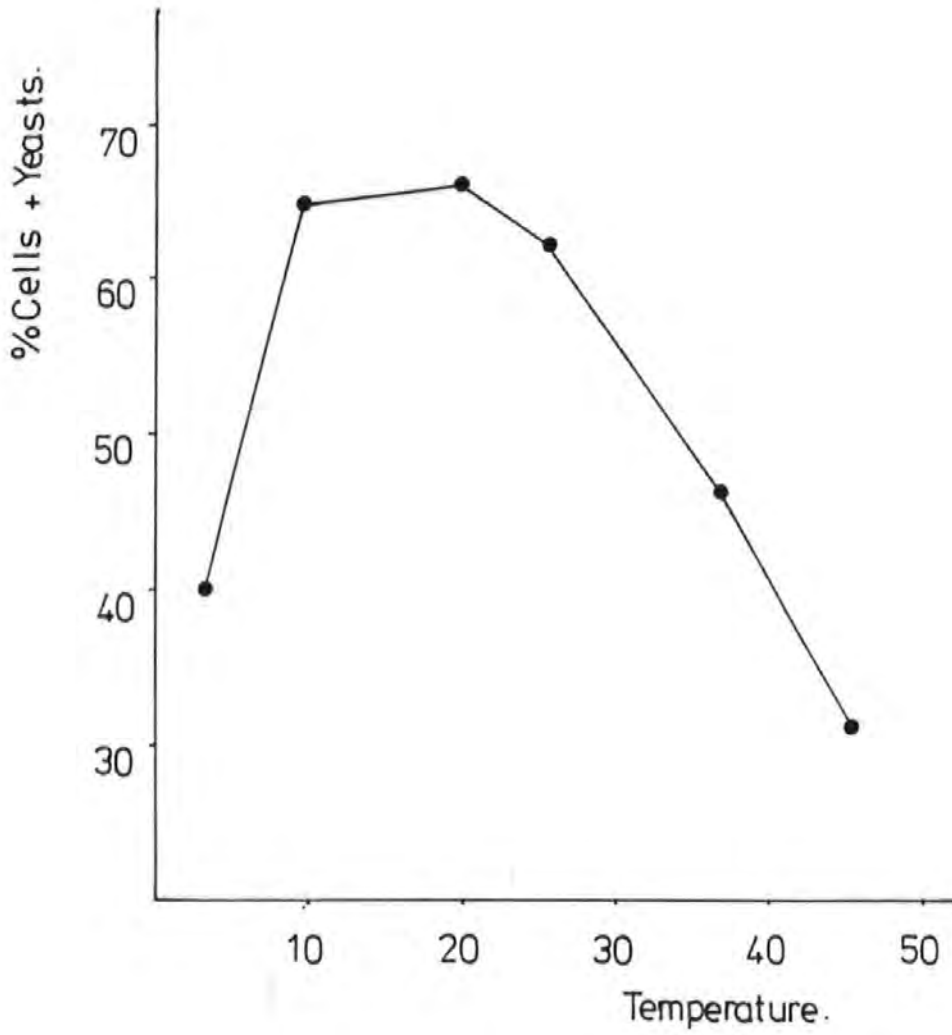
Fig. 8

a) <u>In vitro</u> phagocytosis of yeasts in the presence or absence of autologous plasma.

X = no plasma present
● = heat-inactivated plasma present
□ = whole plasma present
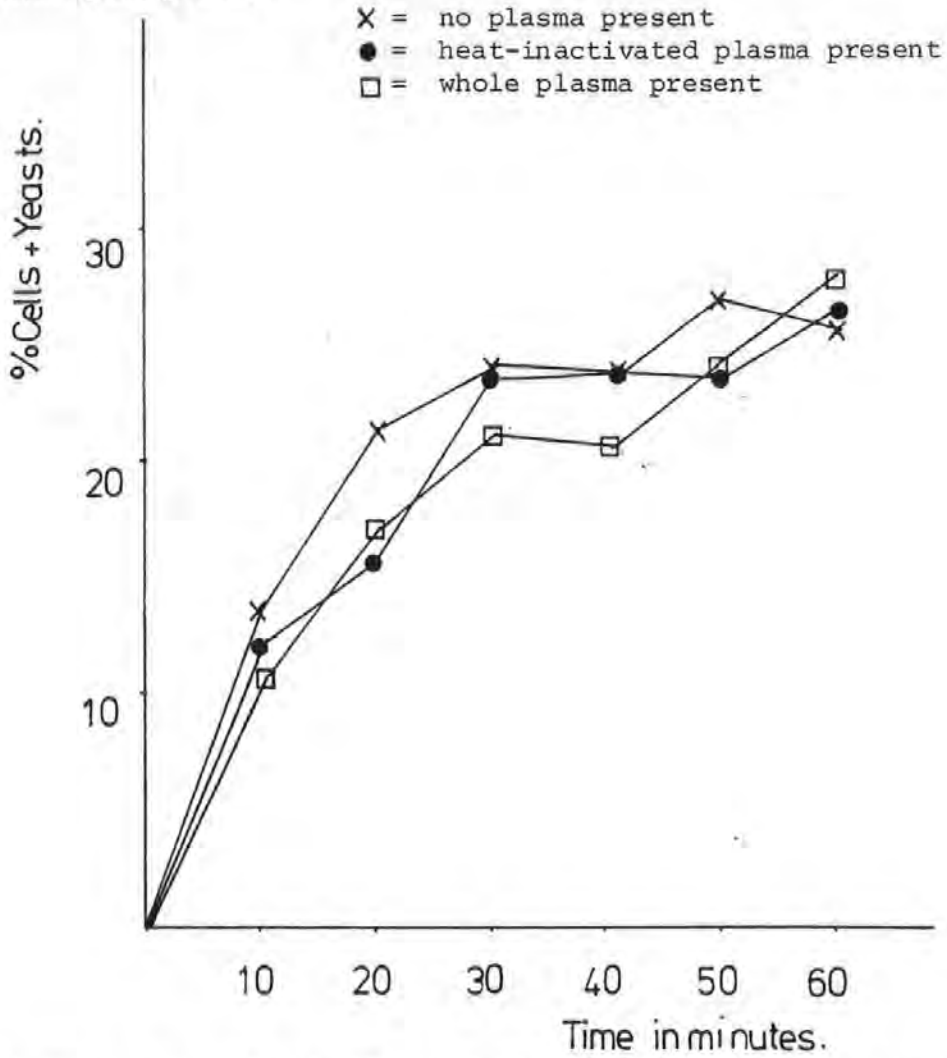
b) <u>In vitro</u> phagocytosis of yeasts in the presence or absence of plasma by dogfish blood phagocytes after various treatments.

Cells only. | Cells + plasma. | Cells + tryp-sin | Cells + plasma + tryp-sin | Cells + anti-Ig. | Cells + plasma + anti-Ig.

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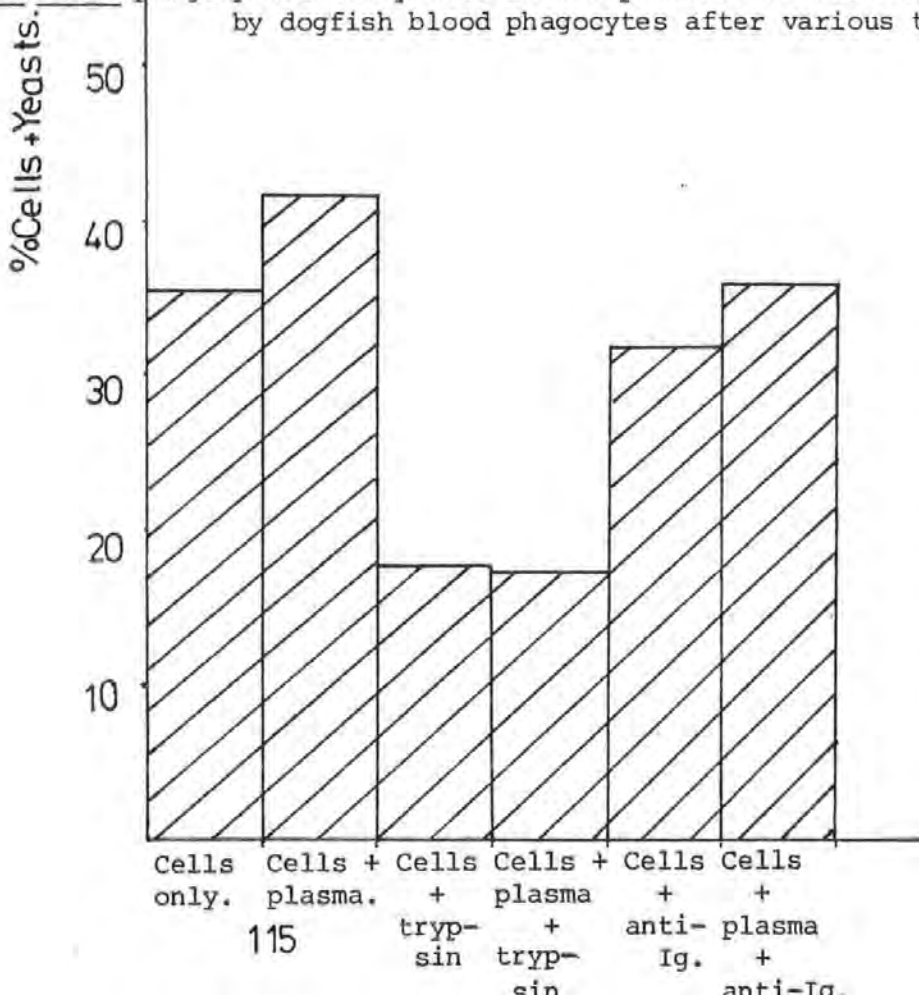
activity.

Addition of anti dogfish immunoglobulin slightly decreased activity which was partially restored by autologous plasma. The difference however was quite small indicating that there was probably not an appreciable amount of cell-associated immunoglobulin.

iv) Effect of metabolic inhibitors

Various concentrations of three different metabolic inhibitors were used to ascertain their effect on phagocytosis.

Iodoacetate

Iodoacetic acid was added to incubation medium at concentrations of 0.1, 1.0 and 10 mM and rates of yeast uptake compared to controls.

As shown in Fig. 9a, 0.1 mM iodoacetate had virtually no effect on the rate of uptake. However at the two higher concentrations a progressive decrease in phagocytic rate was observed, although even at 10 mM phagocytosis was not completely inhibited.

Azide

As with iodoacetate, the effects of azide on phagocytosis were monitored at concentrations of 0.1, 1.0 and 10 mM.

Generally no effect was noted on the rate of phagocytosis at all concentrations of azide (see Fig. 10a). However at 10 mM a more erratic pattern of uptake was observed, possibly due to directly toxic effects of this very high concentration.

Puromycin

Puromycin dihydrochloride was tested at concentrations of 0.1, 1.0 and 10 µg/ml.

Puromycin did not cause marked inhibition but again a very erratic pattern of uptake was always observed each time the

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Fig. 9

a) In _vitro_ phagocytosis of yeasts in the presence of varying

concentrations of iodoacetate.

    ● = control

    X = 0.1 mM

    O = 1.0 mM

    □ = 10 mM

b) In _vitro_ phagocytosis of yeasts in the presence or absence of

divalent ions.

    ● = control (i.e. complete Young's selachian saline (YSS))

    X = YSS without calcium salts

    O = YSS without magnesium salts

    □ = without calcium or magnesium salts

Fig. 9
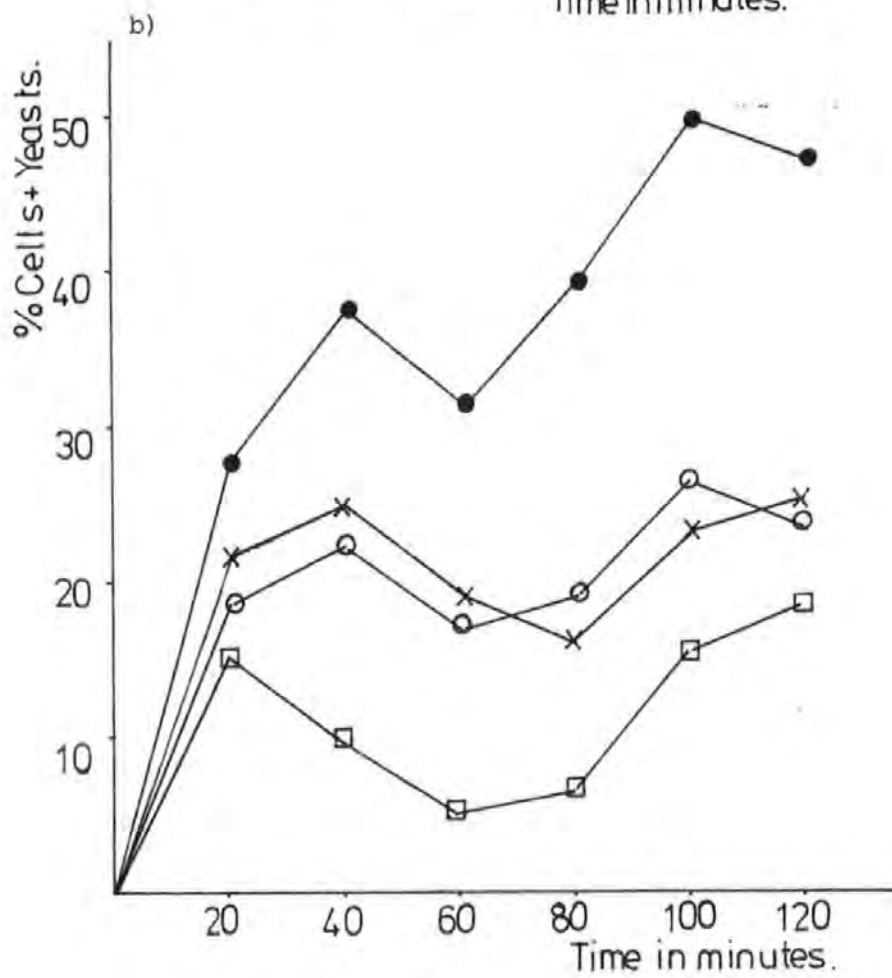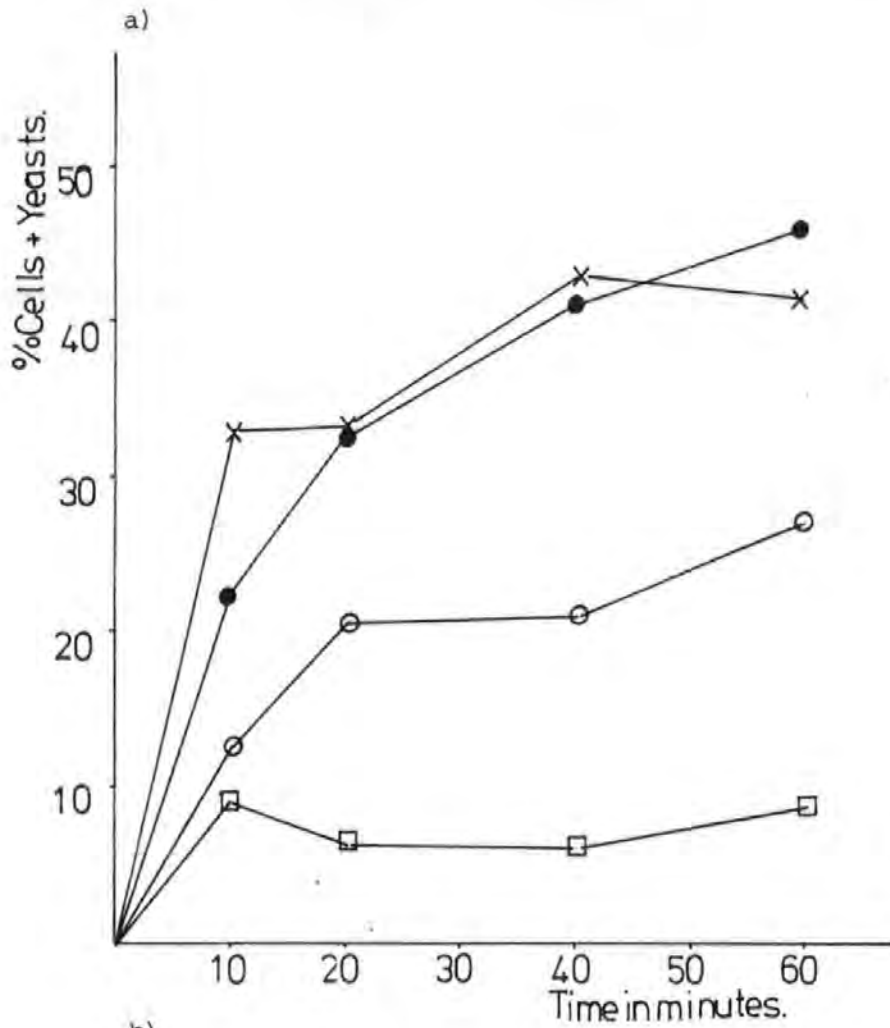
Fig. 10

a) In vitro phagocytosis of yeasts in the presence of varying

concentrations of sodium azide.

● = control

X = 0.1 mM

O = 1.0 mM

□ = 10 mM

b) In vitro phagocytosis of yeasts in the presence of varying

concentrations of puromycin dihydrochloride.

● = control

X = 0.1 µg cm^{-3}

O = 1.0 µg cm^{-3}

□ = 10 µg cm^{-3}
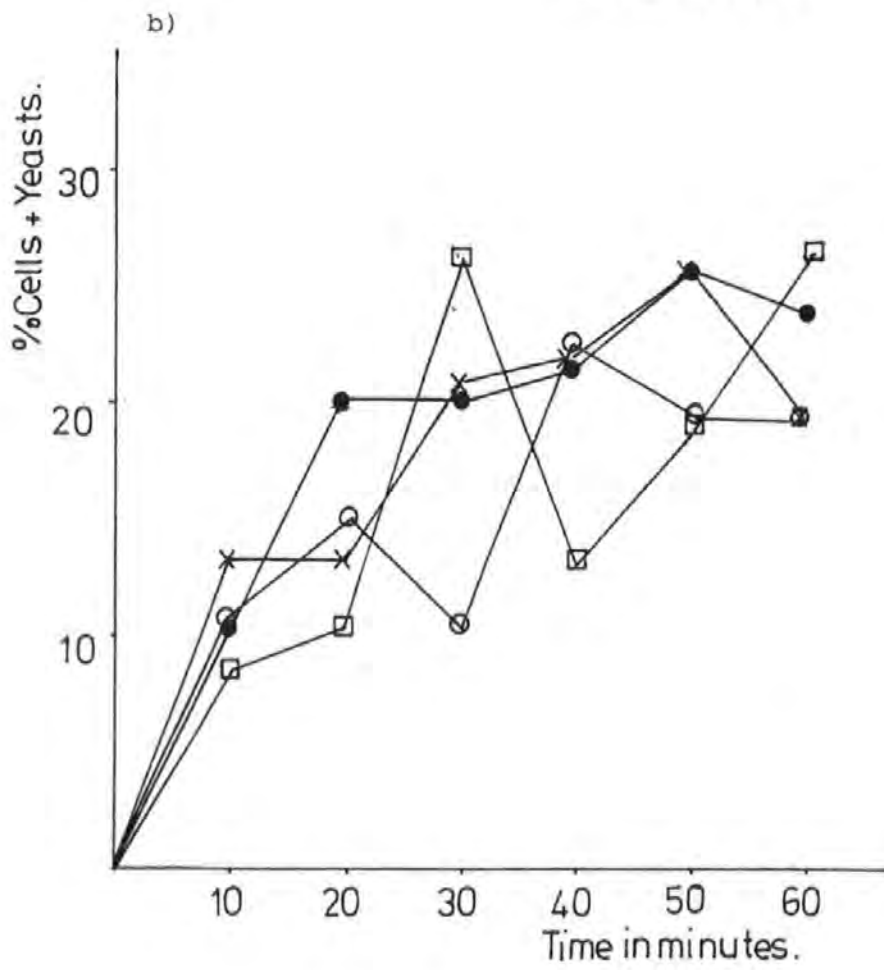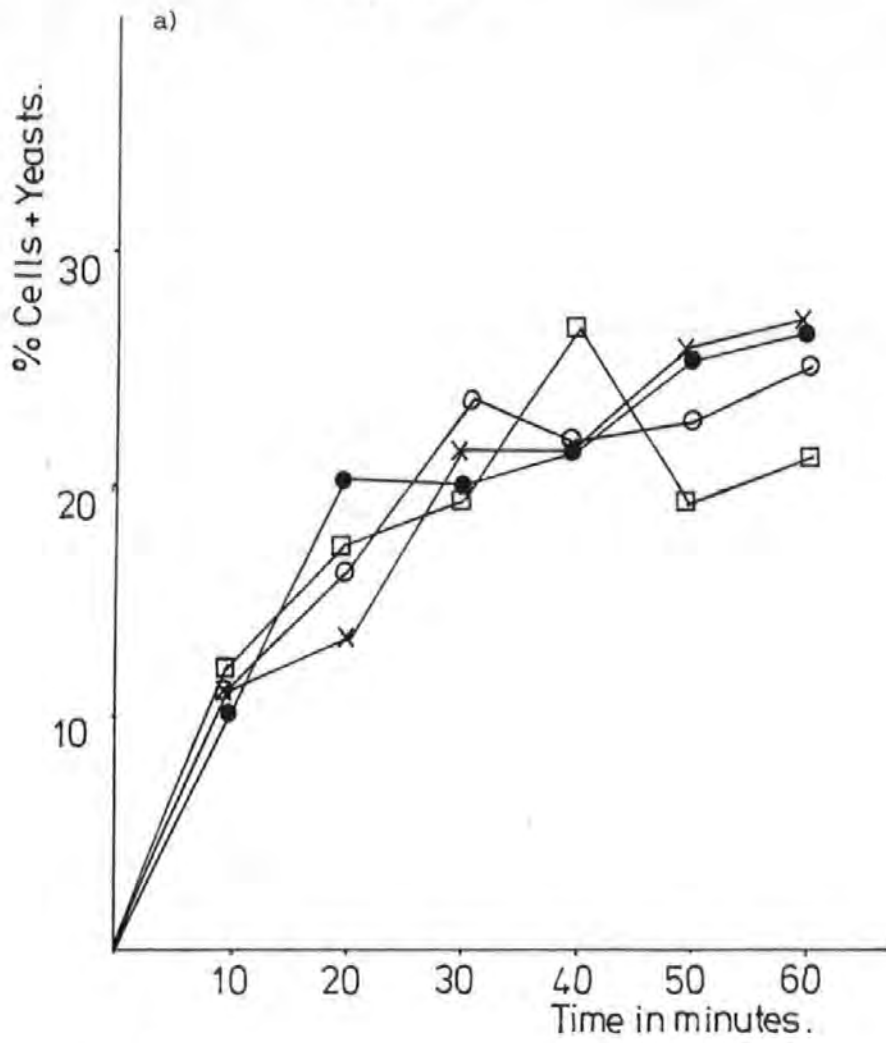
Fig. 10

experiment was performed (Fig. 10b).

v). Effect of divalent ions

The effects of divalent ions on phagocytosis were assessed by using Young's selachian saline, supplemented with 1% glucose and with constituents containing Ca^{++} or Mg^{++} omitted as appropriate.

The omission of either Ca or Mg ions had approximately similar effects, and a more pronounced effect was observed by omitting both (see Fig. 9b).

vi) Effects of "activation"

Cells from fish which were previously exposed to BCG, more actively phagocytosed yeasts than cells from control uninjected fish (see Fig. 11). This was shown to be a significant difference when statistical analysis was applied to the data at 5% and 1% levels. (i.e. analysis of variance - ANOVA)

In vitro uptake of KLH

Blood and spleen adherent cells were monitored for their ability to endocytose Keyhole Limpet Haemocyanin (KLH) (Calbiochem Ltd) in vitro. Samples were taken for periods of up to 24 h and proteins differentiated by Rocket Immunoelectrophoresis, on agarose (1g 100 cm^{-1}) containing appropriate dilutions of rabbit-anti KLH serum.

The experiment was repeated several times using a range of phagocytic cell numbers ($5x10^3$ to 10^6) per plate and varying KLH concentrations (250 to 40 µg cm^3).

In all experiments no decrease in the amount of KLH originally present could be observed. Cells were also, in one instance, incubated with fluoresceinated KLH and a few of the adherent cells showed positive fluorescence suggesting that these cells can in fact

Fig. 11

In _vitro_ phagocytosis of yeasts by phagocytes from BCG injected and
control fish.

    x=   'activated' cells

    •=   control cells
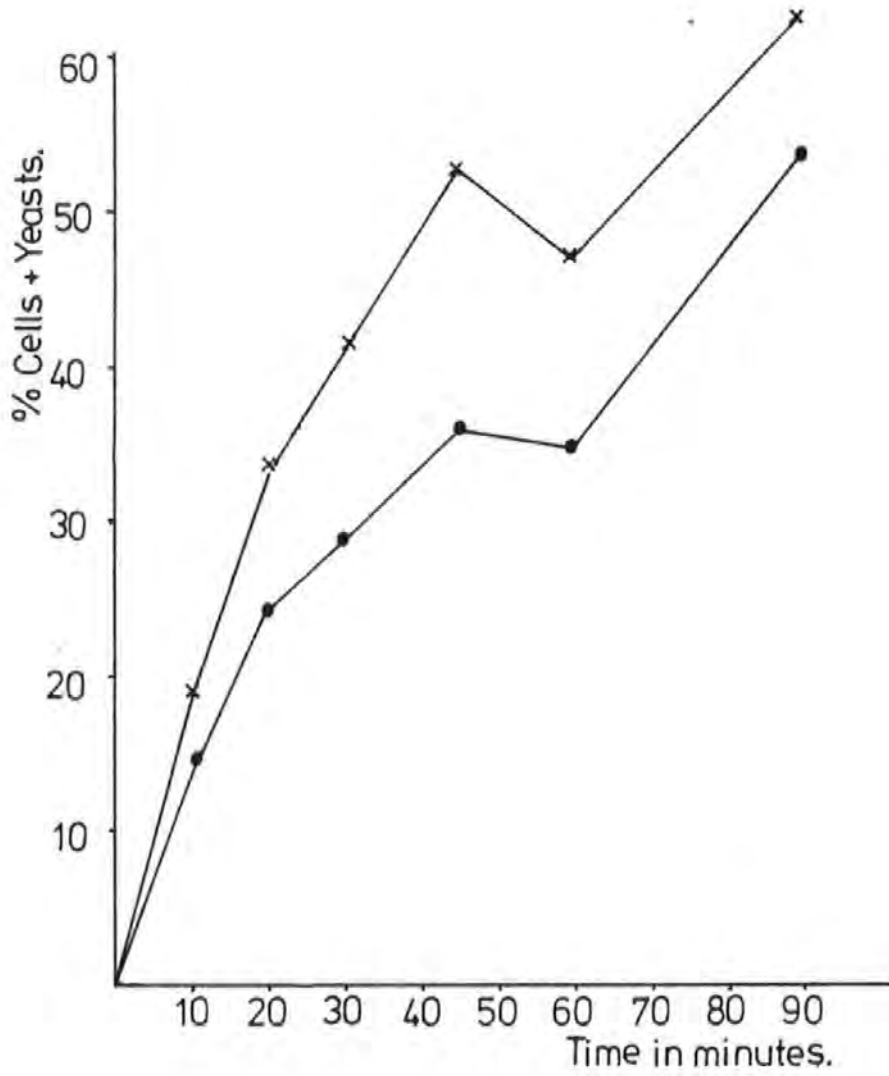
take up this antigen. It seems that the KLH does not decrease over the experimental period to any appreciable degree. The period of monitoring could not be increased further than 24h as the incubating medium needed to be renewed to control the level of toxic metabolites.

In vitro bacterial clearance

The uptake of bacteria with time was measured using essentially the same technique as with yeasts, but instead using viable bacteria and counting them by plating appropriate dilutions using a spiral plater.

In most cases the results obtained suggested that the presence of blood or spleen phagocytes had little or no effect on bacterial numbers.

For instance (see Fig. 12), Streptococcus faecalis showed a gradual decrease in numbers in TCM 199 supplemented with urea and salt, and the presence of blood or spleen phagocytes did not alter the rate of decrease in any way. Cells could be seen with internalised bacteria, but as the rate of decrease was no greater in their presence, it seemed likely that these cells were taking up only non-viable bacteria.

As iodoacetate had been shown to inhibit phagocytosis, one set of treatments included 1.5 mM iodoacetate in the incubation medium. Yet again the rate of decrease was not altered, although microscopic examination showed that the cells in fact contained fewer bacteria.

A virulent strain of Vibrio angullarum was tested against dogfish blood phagocytes and the bacteria multiplied in the medium during the incubation period. The presence of blood phagocytes, with or without iodoacetate, depressed the rate of bacterial proliferation to the same extent in each case (see Fig. 13).

Using an avirulent strain gave very different results. This

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Fig. 12.

In vitro clearance of S.faecalis in the presence or absence of iodoacetate.

● = Phagocytic cells and bacteria.
○ = Phagocytic cells and bacteria and iodoacetate.
□ = Bacteria only in TCM.
× = Bacteria in TCM + iodoacetate.
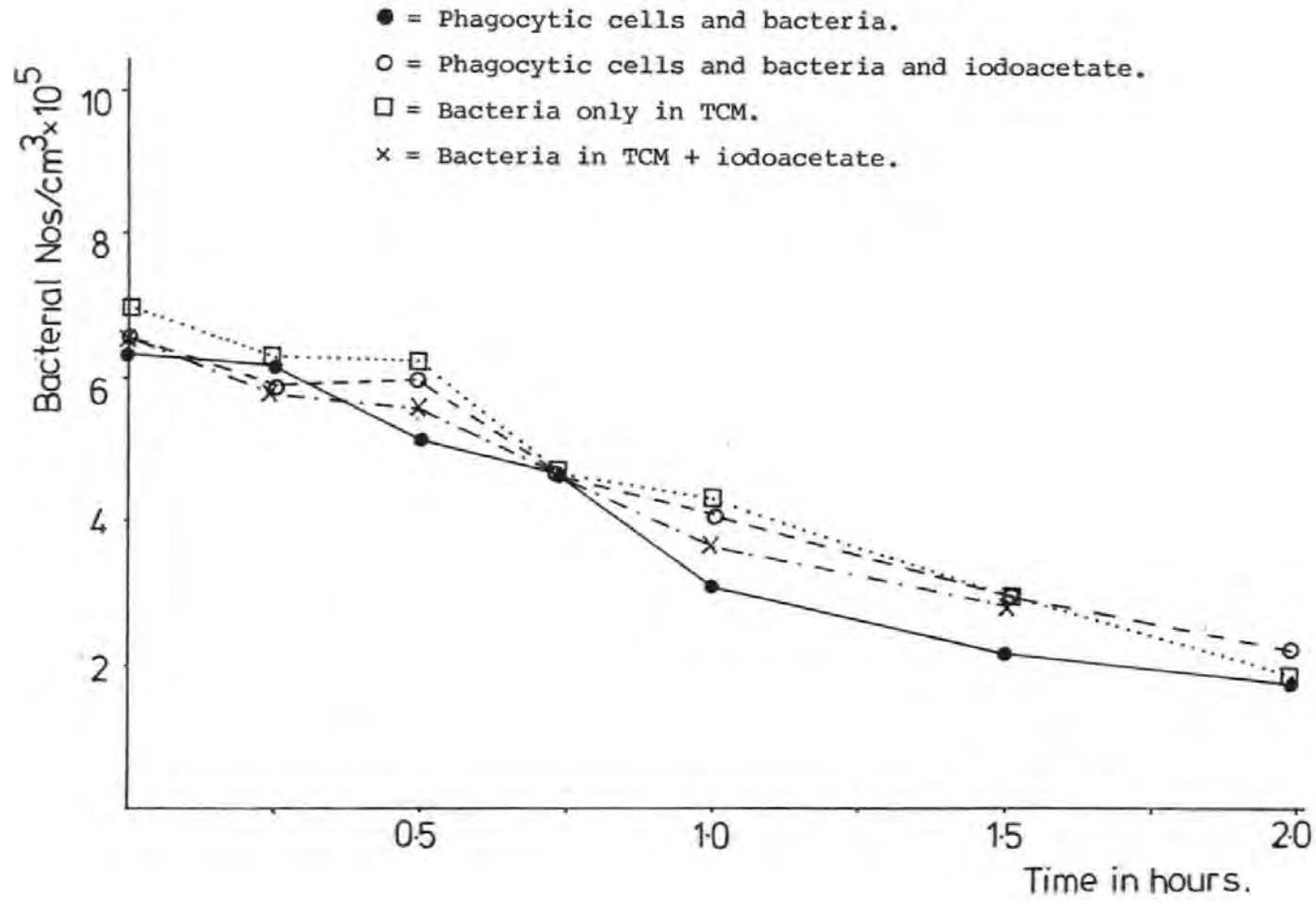
Fig. 13.

In vitro clearance of V. anguillarum (UNH 569).

● = Bacteria only in TCM.

O = Phagocytic cells + bacteria.

■ = Phagocytic cells + bacteria + iodoacetate.

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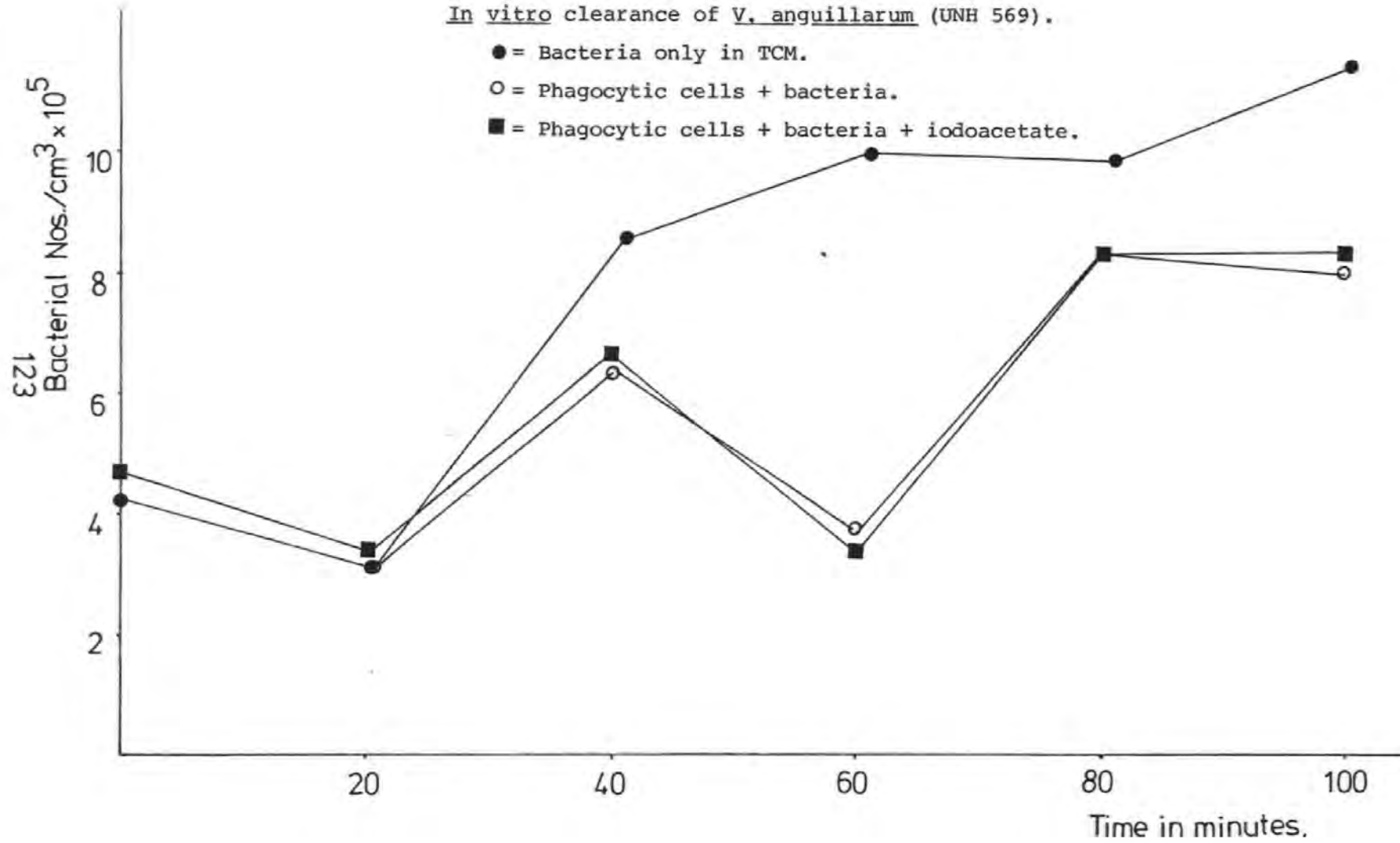
strain neither grew nor decreased in TCM, and no difference in numbers was noted between treatments with or without cells.

Several bacterial species have been monitored in this way (i.e. S. typhimurium, E. coli and a second avirulent strain of V. anguillarum), and in all cases, whether bacteria grew or not, the phagocytes in no way modified their activity.

d) A quantitative study of in vivo antigen clearance

The clearance patterns of various antigens or particles were monitored in the dogfish themselves. The inocula used and the figure numbers of the clearance rates to which they refer are summarised in Table 14.

i) Bacteria·

Bacteria were injected i.v. into dogfish and blood samples subsequently taken and diluted appropriately and plated on differential or selective media in order to assess the numbers of bacteria remaining in the circulation.

Salmonella typhimurium

This bacterium was chosen, as a suitable selective medium was readily available in 'Bismuth Sulphite Agar'.

A preliminary experiment monitored the clearance of S. typhimurium from the circulation of a single dogfish (see Fig. 14a). This experiment showed that after an initial sharp fall in bacterial numbers in the first three hours post-injection, the numbers remained relatively constant. Furthermore, at two days post-injection when the experiment was terminated the numbers appeared to be increasing.

Consequently a second experiment was performed in order to assess how long injected bacteria remained in the circulation. Two fish were

Table 14

Antigen inocula injected in order to monitor _in vivo_ clearance.

Fig. No	No. of fish	Antigen	Inoculum.
14a	1	S. typhimurium	6.5×10^8
14b	2	" "	2.5×10^{11}
15	2	" "	2.75×10^9
16a	2	" "	6.45×10^8
16b	3	" "	1.2×10^7
17a	2	Staph. albus	1.2×10^8
17b	2	Strep. faecalis	2.8×10^8
18a	2	E. coli	1.28×10^8
18b	1	" "	1×10^7
19a	2	V. anguillarum	6.45×10^8
19b	2	" "	3.5×10^7
20a	2	" "	4.6×10^9
20b	2	S. cerevisiae	2.8×10^7
21a	2	KLH	75 mg
21b	2	"	"
22a	2	"	"

Fig. 14

a) In vivo clearance of S. typhimurium from a single dogfish.
Inoculum = 6.5×10^8
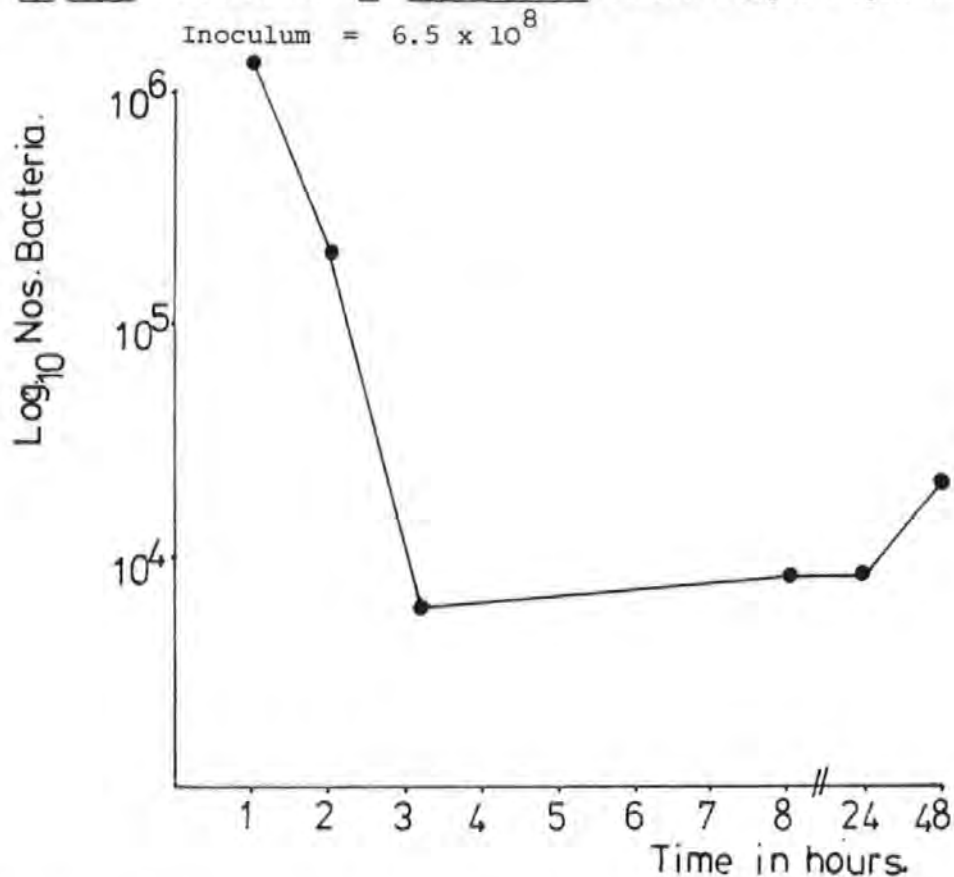
b) In vivo clearance of S. typhimurium from two dogfish previously immunized with Salmonella typhi H antigen.

Inoculum = 2.5×10^{11}
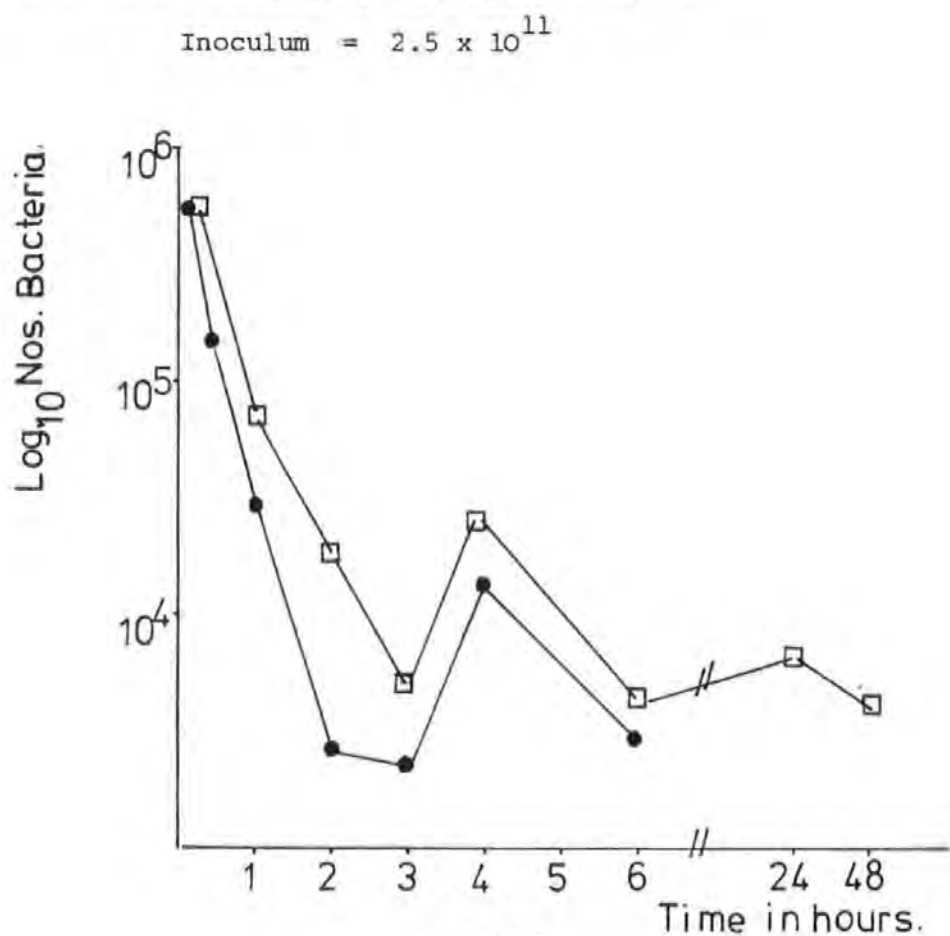
injected with approximately 10^9 bacteria intravascularly (i.v.) and their blood sampled at intervals of up to 36 days when the experiment was terminated (see Fig. 15). Again, after an initial, sharp decrease in bacterial numbers during the first day, the numbers of surviving bacteria remained between 10^4 and 10^5 per cm^3 of blood. The fish showed no obvious signs of distress, but at 36 days there was no suggestion that bacterial numbers were declining. The fish were killed at this point and their tissues processed for histology.

The effect of 'blockade' by colloidal carbon was assessed by injecting colloidal carbon i.v. one day before monitoring clearance of S. typhimurium. As Figs. 16a & b demonstrate, the presence of carbon had no effect on the subsequent clearance of bacteria, and at 3-4 days post-injection appreciable numbers of bacteria remained in the circulation.

Finally, Salmonella typhimurium was injected into two fish which had previously been immunized with Salmonella typhi H antigen (see Fig. 14b). The general pattern of the graph was as comparable to the previous clearance patterns and at 2 days post-injection $10^3 - 10^4$ bacteria per cm^3 of blood remained. There therefore appeared to be little enhancement by previous exposure.

With reference to Figs. 17 and 18 it can be seen that E. coli, S. albus and S. faecalis had essentially similar clearance patterns to S. typhimurium, and that the bacteria were never fully cleared, at least over a limited period. Long-term clearance experiments were not performed on these bacterial species.

Vibrio anguillarum

This bacterium is a known fish pathogen which the previous bacteria mentioned are not. Three sets of experiments were performed using two 'avirulent' strains of V. anguillarum.

Fig. 15.

Long-term clearance of S.typhimurium from the circulations of two fish

Inoculum = 2.75×10^9
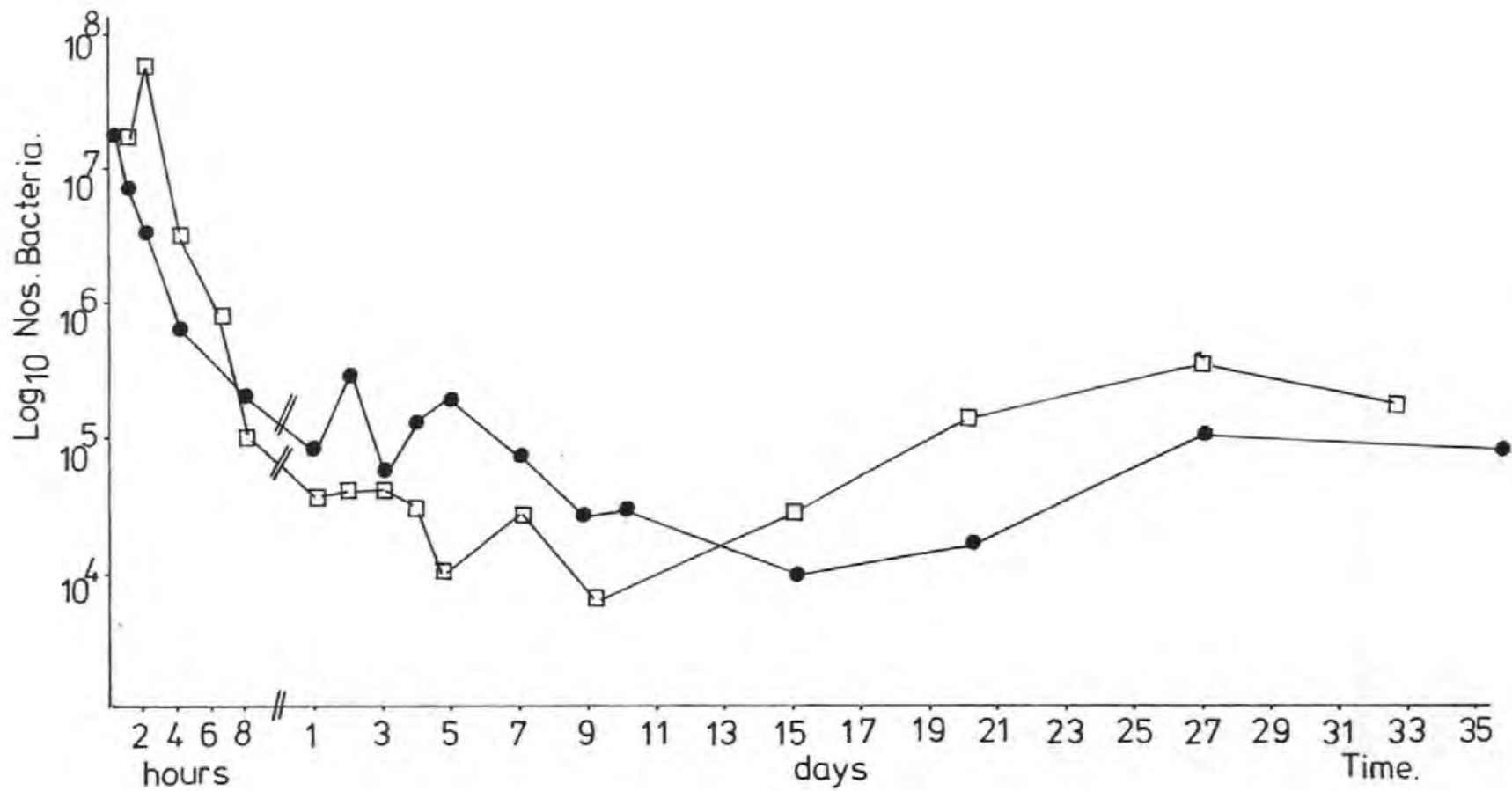
Fig. 16

a) In vivo clearance of S. typhimurium from the circulations of
    two dogfish, one of which was previously injected with colloidal
    carbon.

$$\text{Inoculum} \;=\; 6.45 \times 10^8$$

● = S. typhimurium + carbon

□ = S. typhimurium only

b) In vivo clearance of S. typhimurium from the circulations of
    three dogfish, one of which was previously injected with
    colloidal carbon.

$$\text{Inoculum} \;=\; 1.2 \times 10^7$$

● = S. typhimurium + carbon

O+■ = S. typhimurium only

Fig. 16
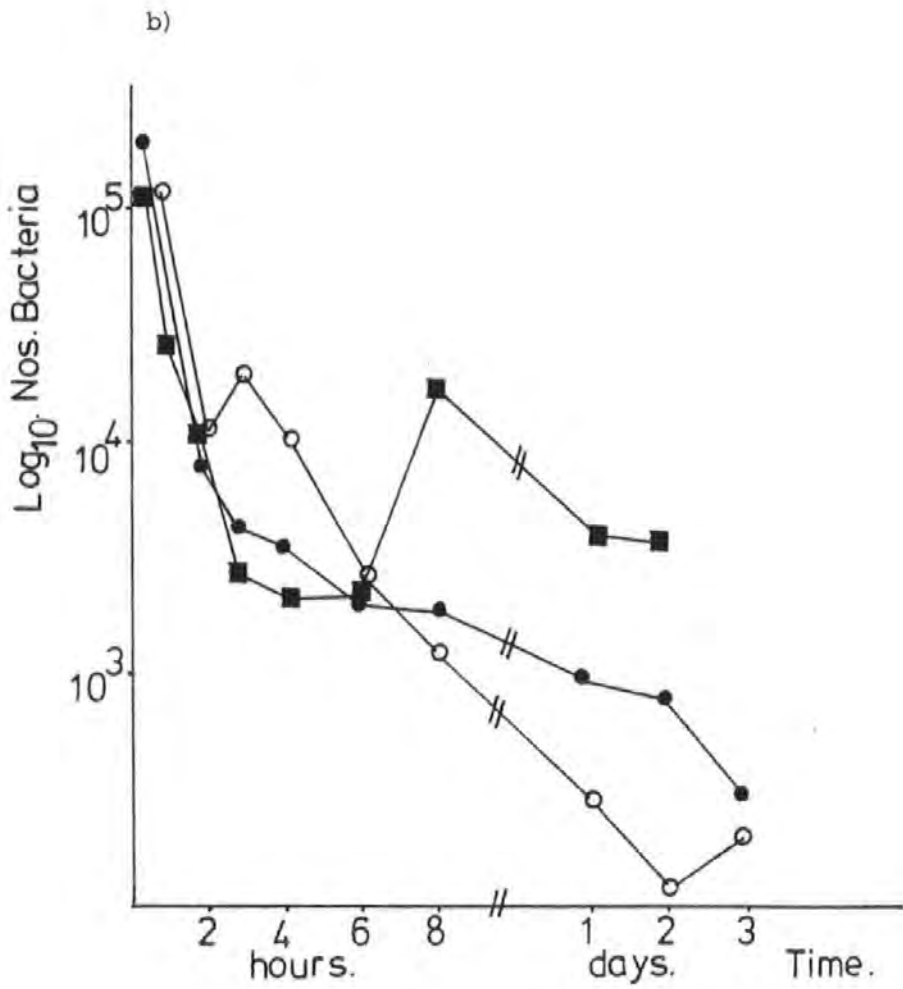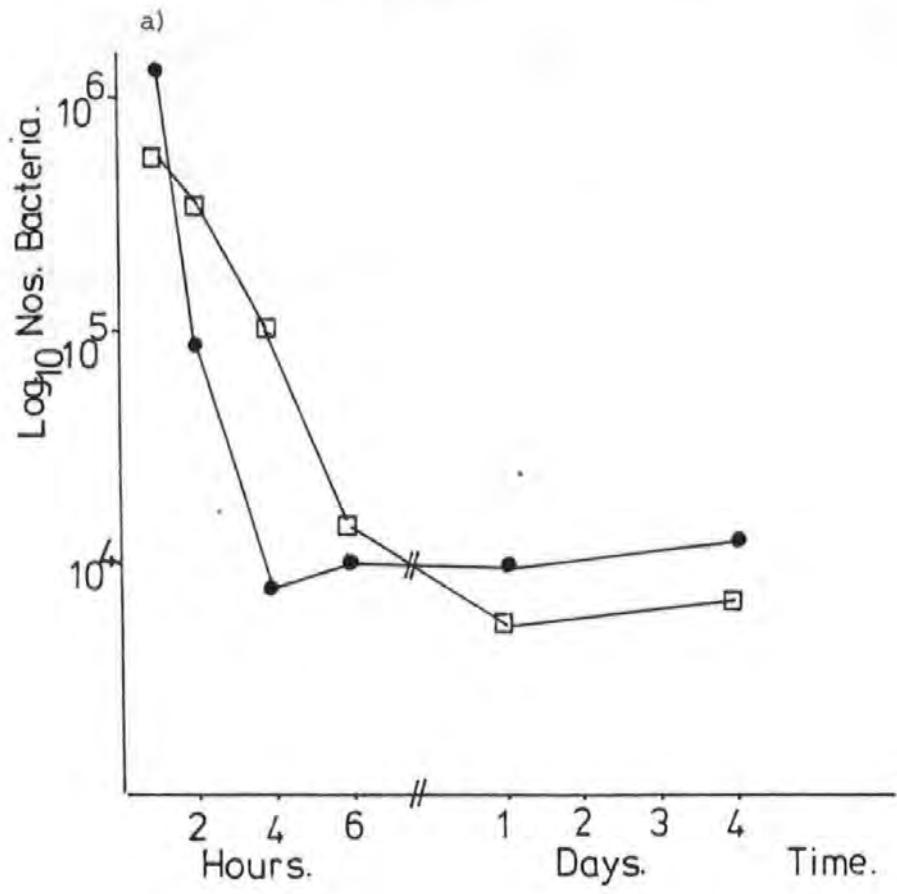
Fig. 17

a) In vivo clearance of Staphylococcus albus from the circulations of two dogfish.

Inoculum = 1.2×10^8
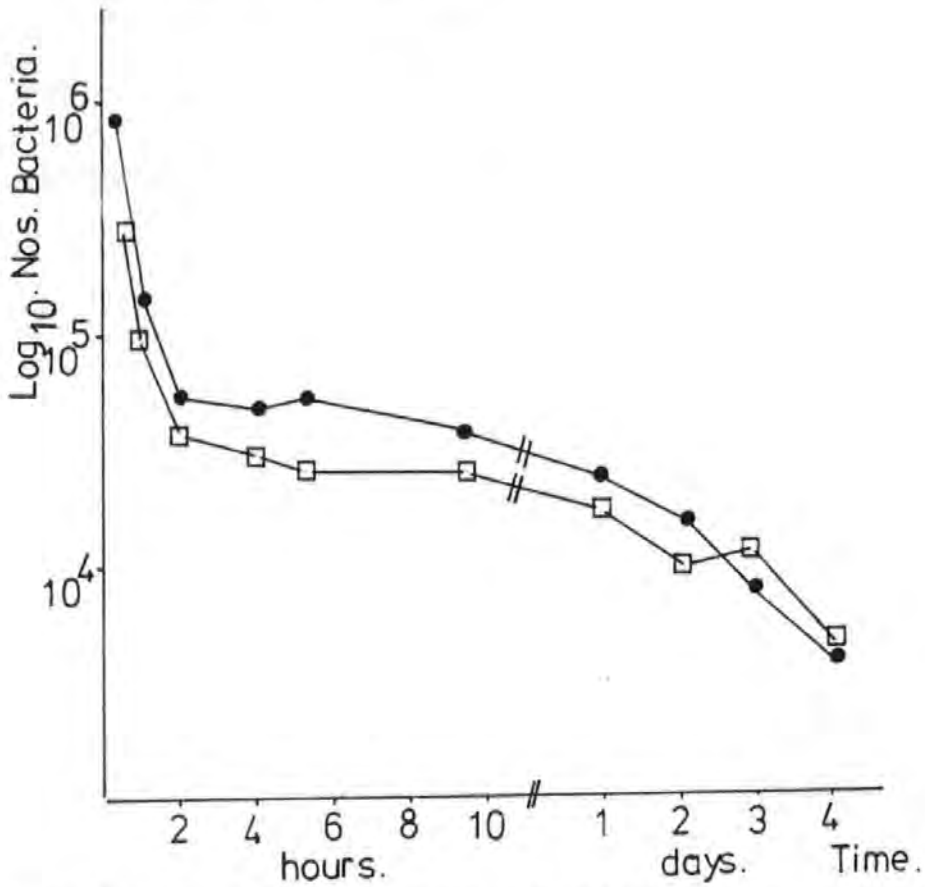
b) In vivo clearance of Streptococcus faecalis from the circulations of two dogfish.

Inoculum = 2.8×10^8

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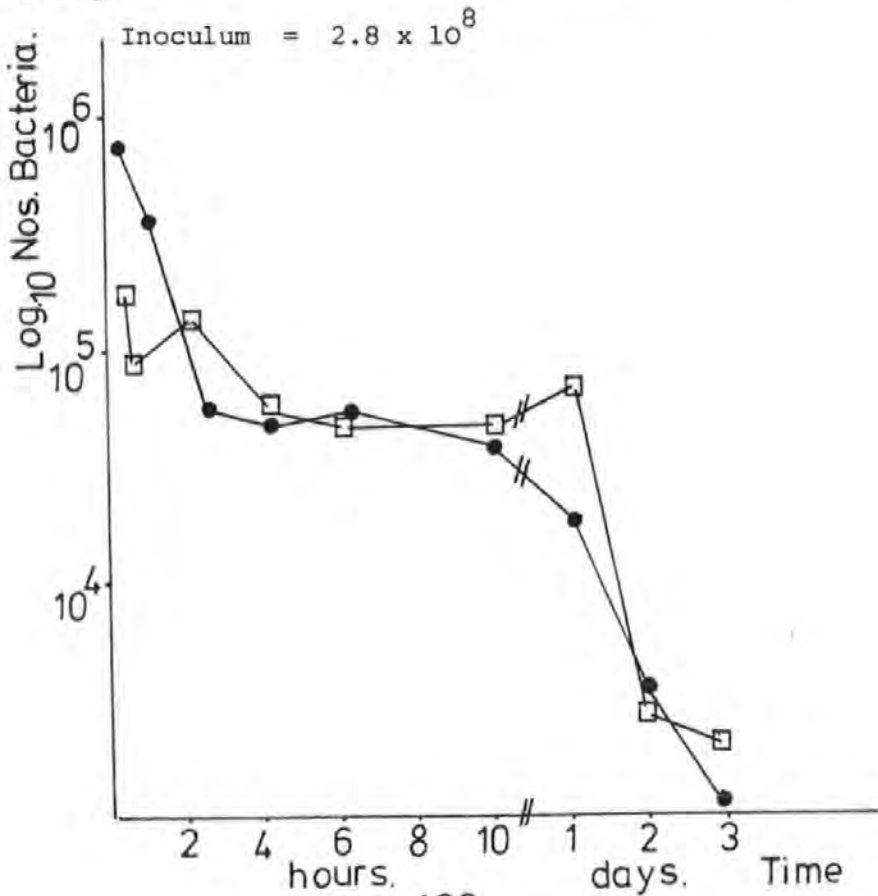
Fig. 18

a) In vivo clearance of E. coli from the circulations of two dogfish.
Inoculum = 1.28×10^8
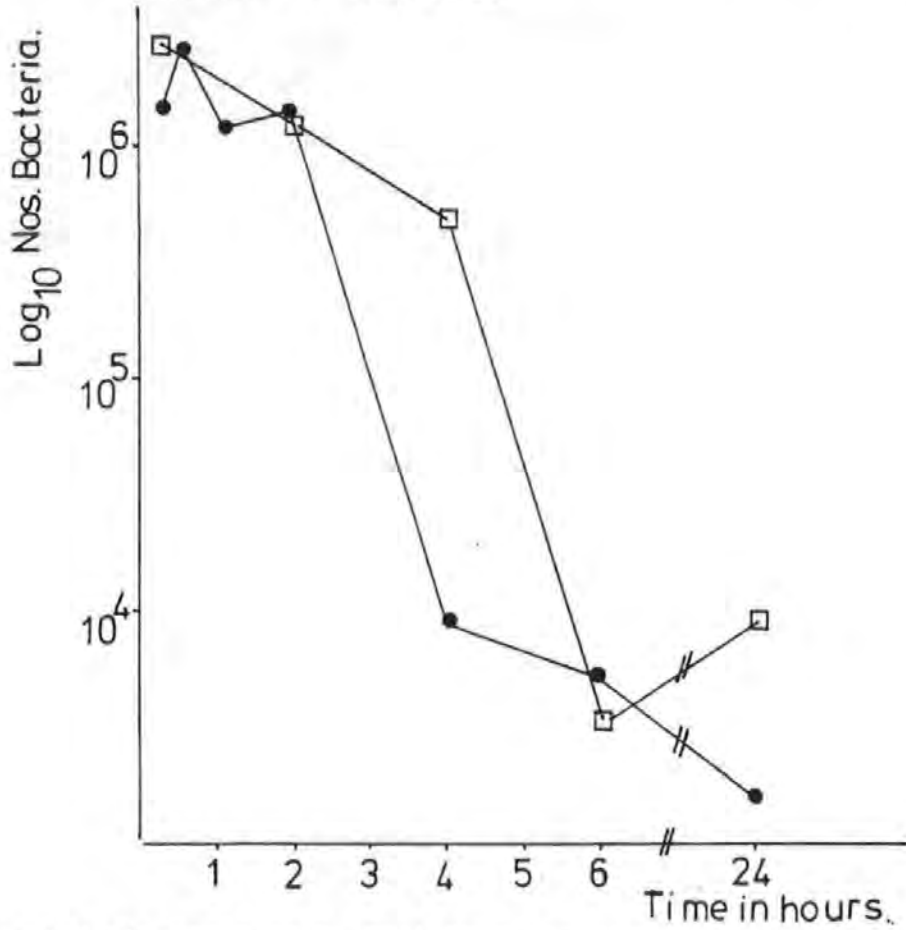
b) In vivo clearance of E. coli from the circulation of a single dogfish.
Inoculum = 1×10^7

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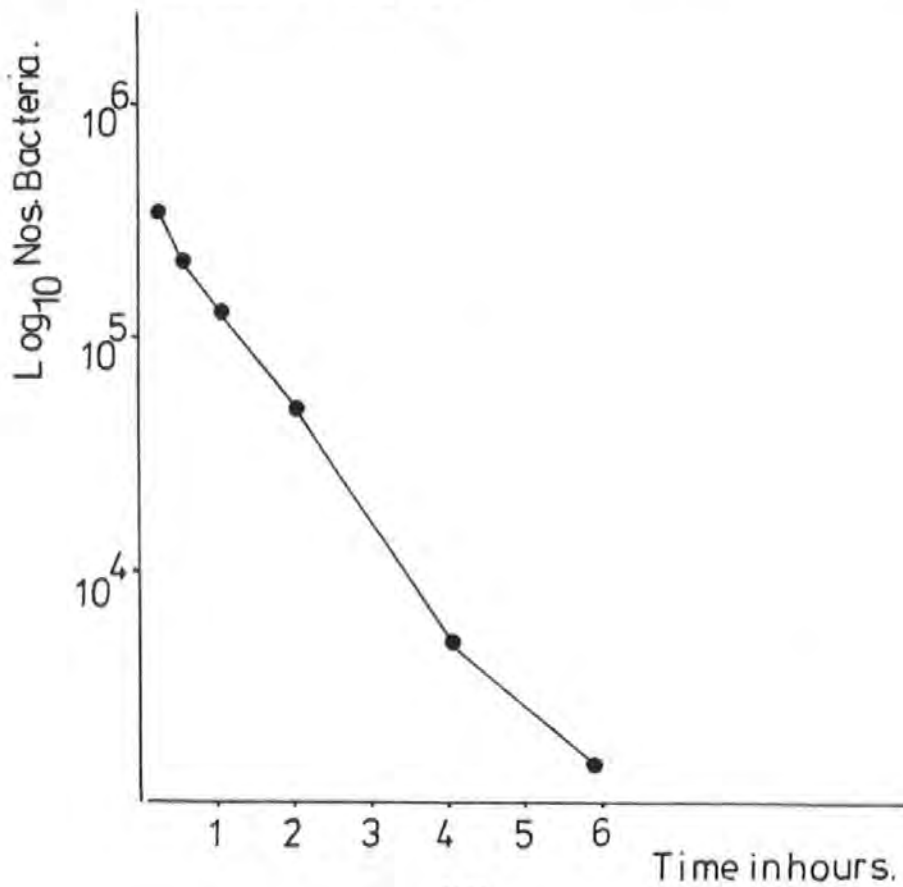
Initially two fish were injected with approximately 10^8 of strain A20/76 (see Fig. 19a). After 7 h the fish were killed, as they showed obvious signs of distress, and on post-mortem examination showed acute signs of vibriosis. Subsequently, lower numbers of a different strain (COB 408) were injected into a further two fish. In the 24 h of monitoring, the fish did not appear distressed. However, after an initial decrease in numbers a sharp increase occurred (see Fig. 19b).

The latter experiment was therefore repeated on two more dogfish and the bacteria in the blood monitored up to 6 days post-injection. These fish also did not succumb, but at 6 days 10^2 - 10^3 bacteria/cm^3 of blood remained (see Fig. 20a).

ii)   Yeasts

Saccharomyces cerevisiae

Yeasts injected i.v. were monitored in the blood of two dogfish for a period of 3 days. Between $\frac{1}{2}$ + 2 hours after injection a small decrease in yeast numbers occurred, but after this time the numbers remained constant at around 10^4 yeasts/cm^3 for up to 72 hours (see Fig. 20b).

iii)   Soluble antigen - KLH

75 mg of KLH in dogfish saline was injected into the caudal sinus. Blood samples were taken at various times post-injection from each of the six fish used.

The serum was examined by rocket immunoelectrophoresis into agarose containing appropriate volumes of rabbit anti KLH serum. With reference to suitable standards the residual KLH in the blood could be measured.

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Fig. 19

a) In vivo clearance of V. anguillarum (A20/76) from the circulations of two dogfish.

Inoculum = 6.45×10^8
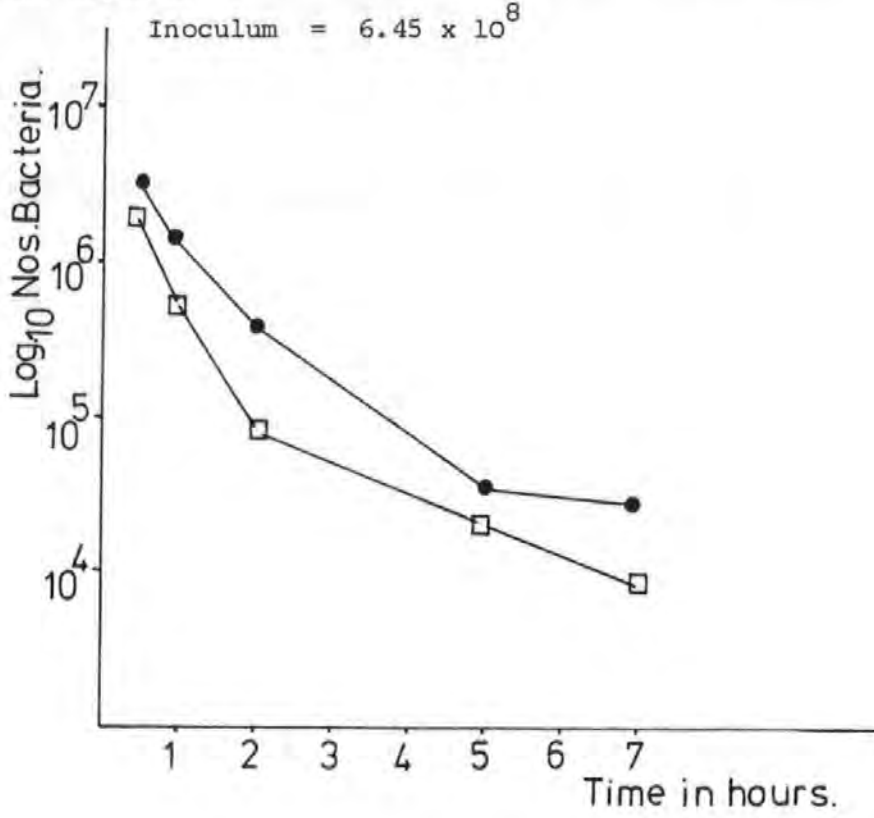
b) In vivo clearance of V. anguillarum (COB 408) from the circulations of two dogfish.

Inoculum = 3.5×10^7
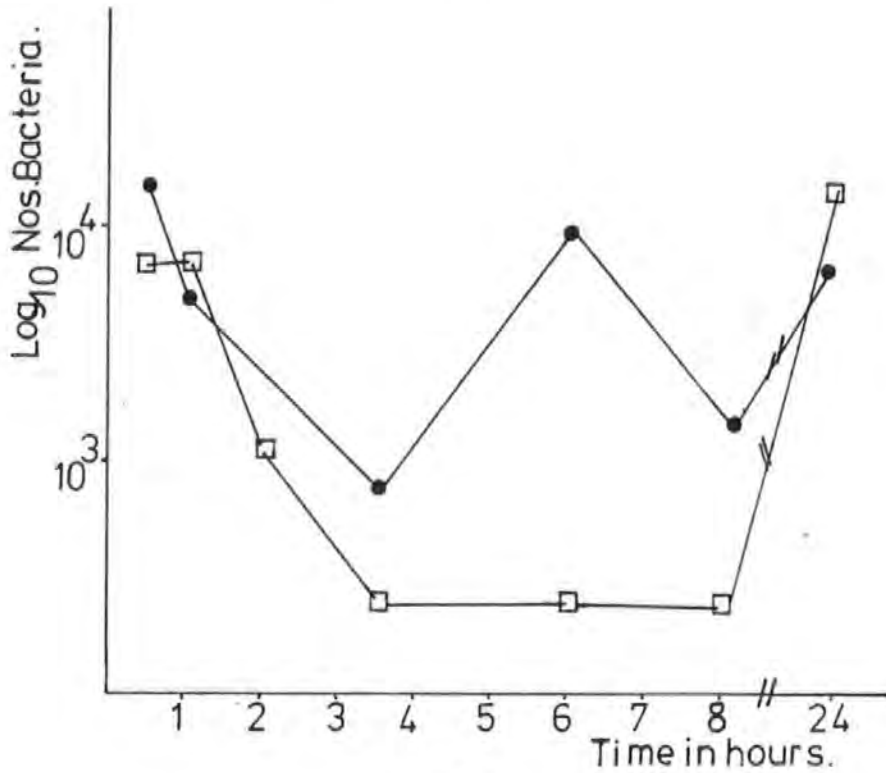
Fig. 20

a) In vivo clearance of V. anguillarum (COB 408) from the circulations of two dogfish.

Inoculum = 4.6×10^9
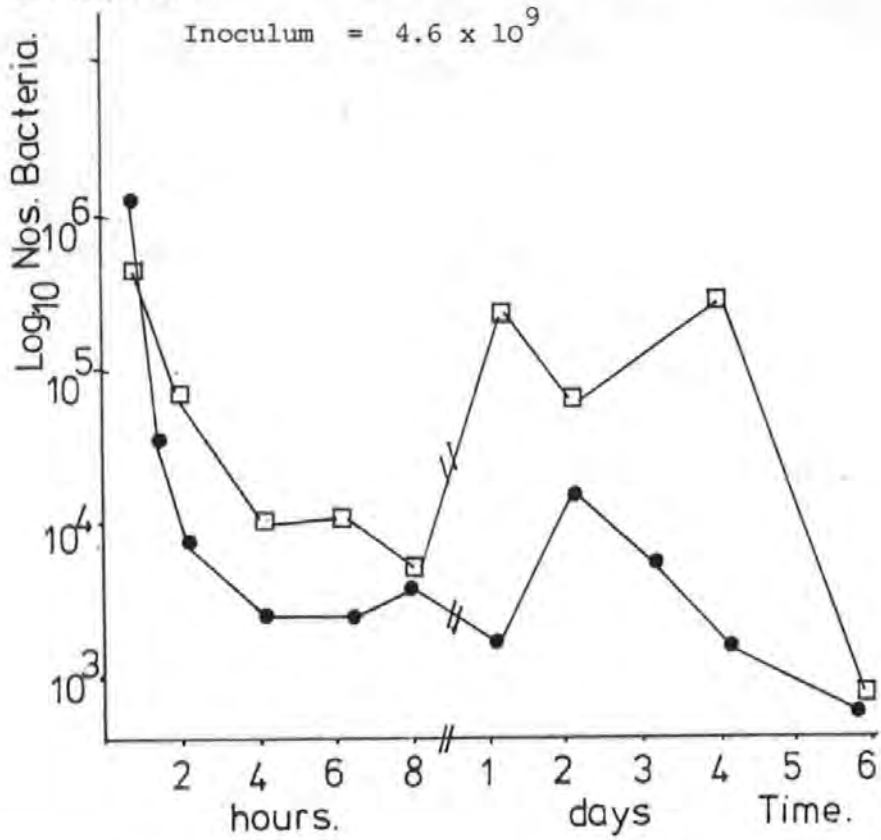
b) In vivo clearance of Saccharomyces cerevisiae from the circulations of two dogfish.

Inoculum = 2.8×10^7
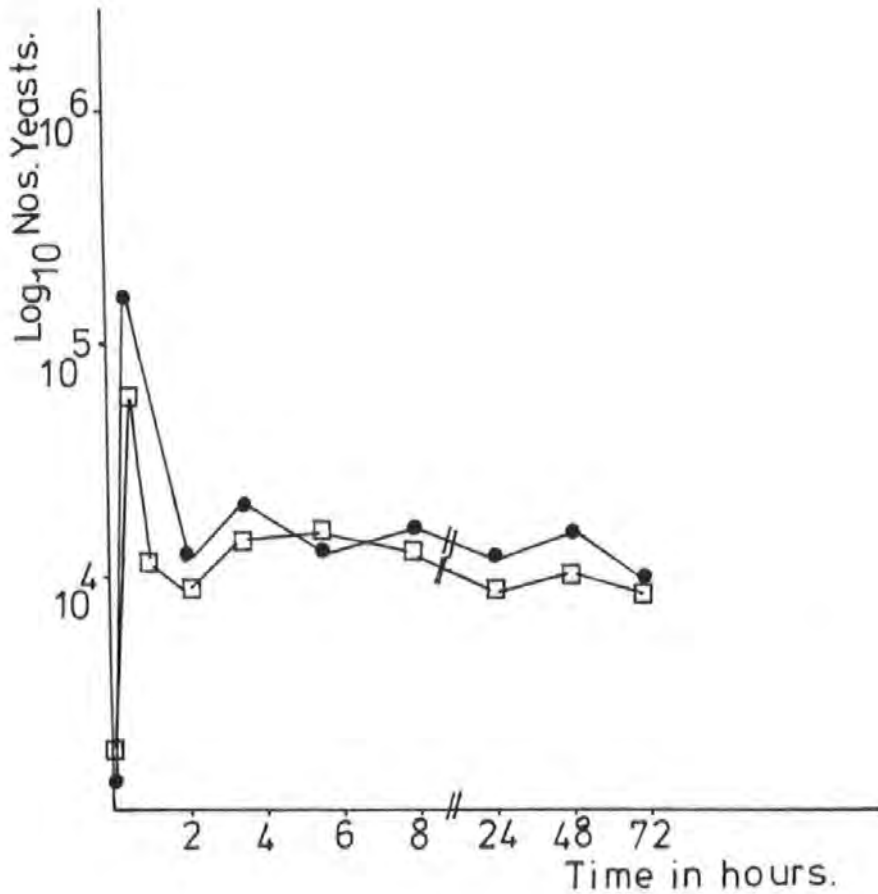
With reference to Figs. 21a & b and 22a it may be observed that five of the six fish showed a gradual decrease in the levels of KLH over the experimental period used. In the remaining fish the concentration of KLH remained fairly constant.

These graphs also show that previously immunized did not clear injected antigen any faster than control fish. In fact, the one fish which did not appear to clear the KLH at all was a fish previously primed with this antigen.

iv) Colloidal carbon

Carbon clearance was only monitored successfully in one fish (see Fig. 22b). This graph shows a steady decrease in the optical density of the blood samples monitored over 6 days.

Fig. 21

a) **In vivo** clearance of KLH from the circulations of two dogfish, one of which had been previously immunized with the antigen.

Inoculum = 75 mg

● = previously immunized fish

□ = control fish

b) **In vivo** clearance of KLH from the circulations of two dogfish, one of which had been previously immunized with the antigen.

Inoculum = 75 mg

□ = previously immunized fish

● = control fish

Fig. 21
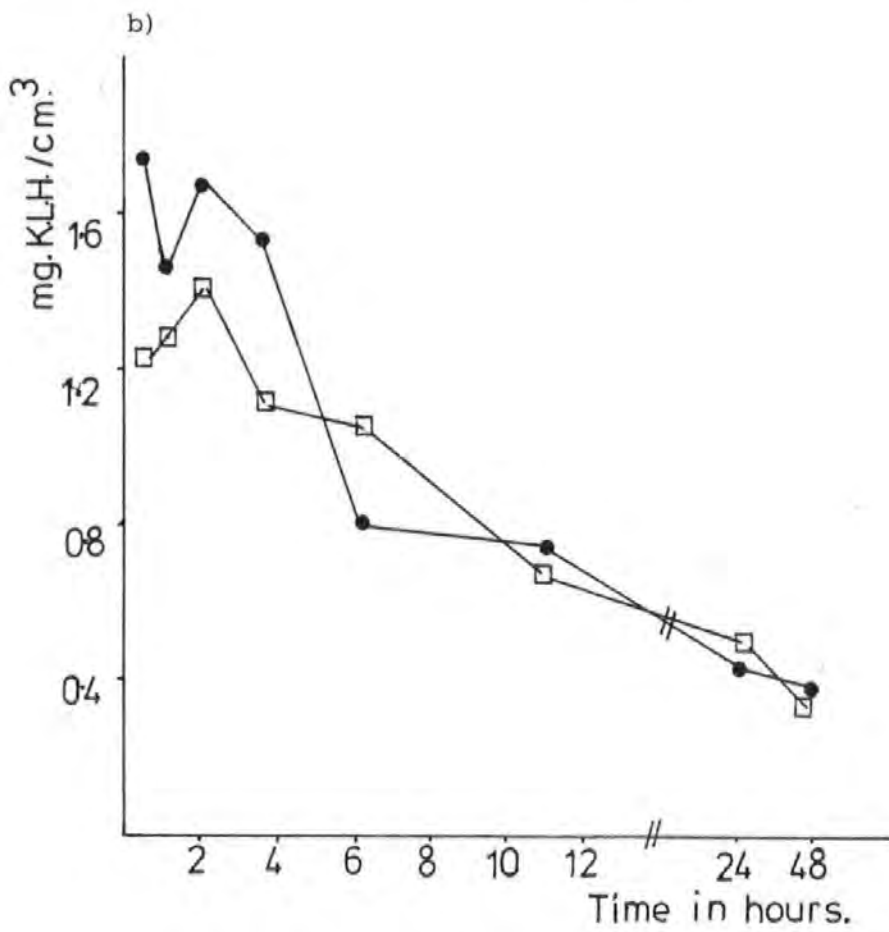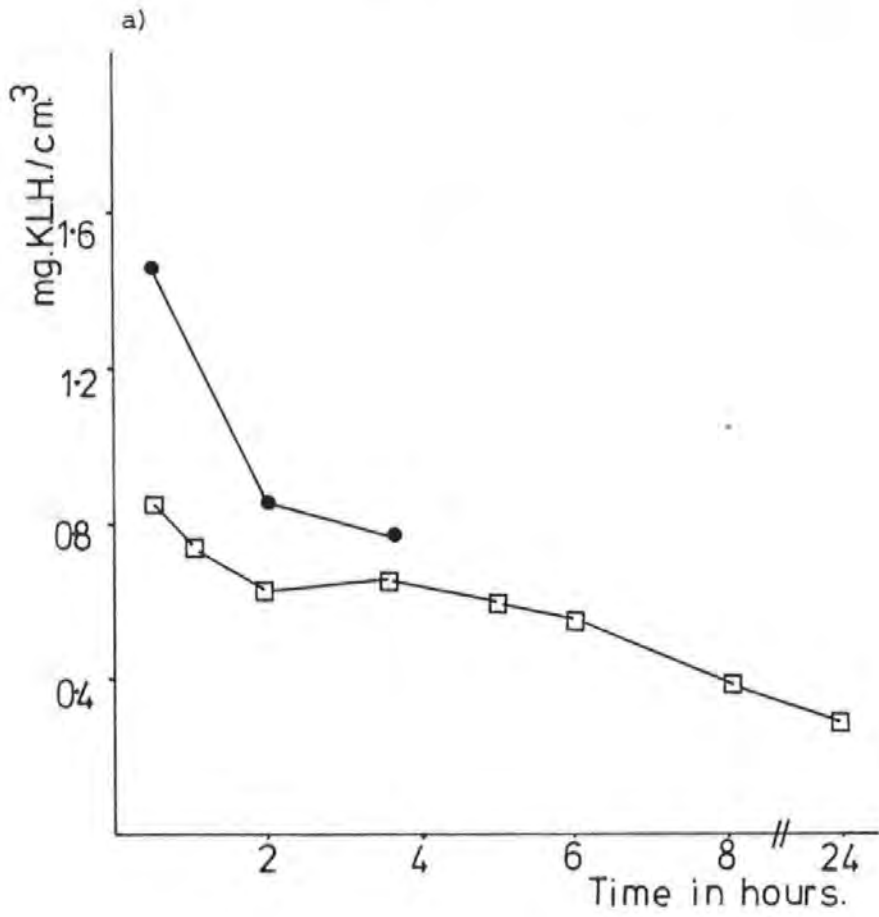
Fig. 22

a) In vivo clearance of KLH from the circulations of two dogfish, one of which had been previously immunized with the antigen.

Inoculum = 75 mg

● = previously immunized fish

□ = control fish
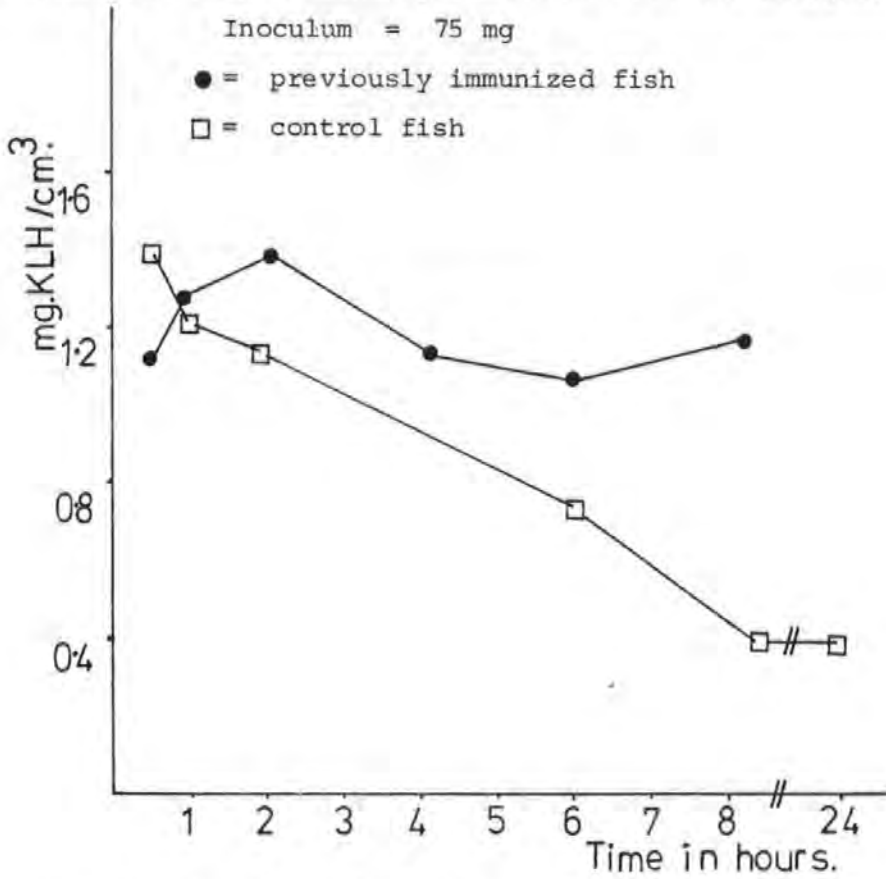
b) In vivo clearance of colloidal carbon from the circulation of a single dogfish.
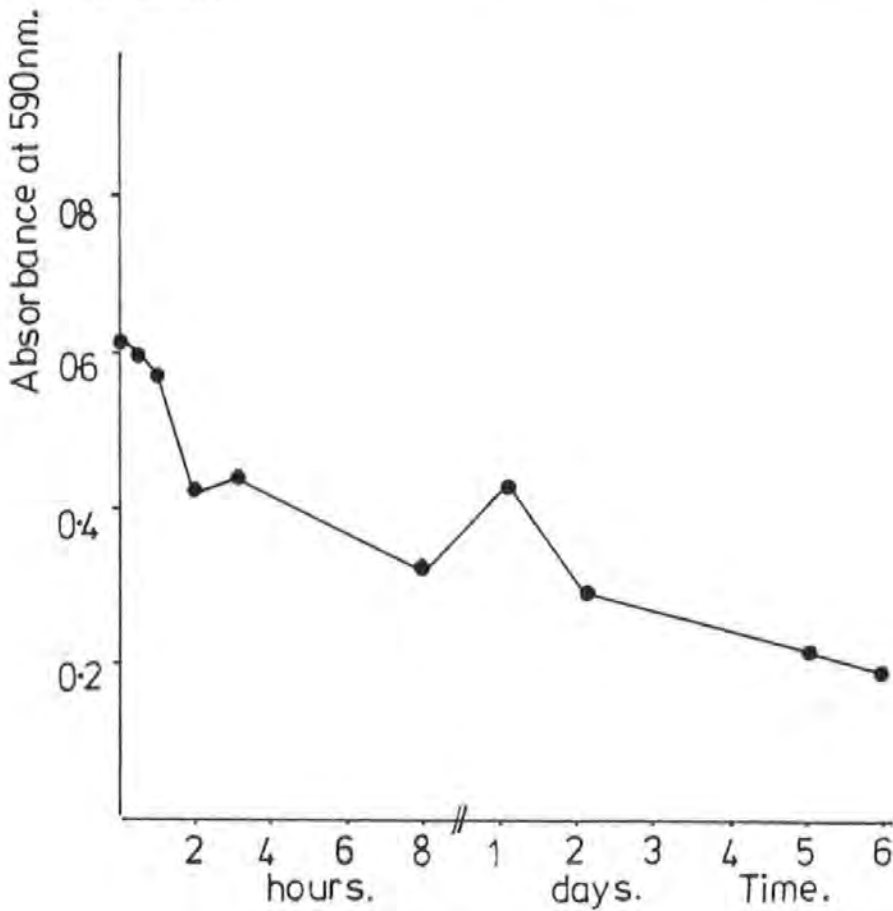
Plate 19

a) Dogfish adherent monocytes phagocytosing yeasts. LM (x 573)

    Giemsa. Bar ≡ 10μ.

b) Dogfish adherent monocyte containing, and actively phagocytosing,

    yeasts. SEM (x 2,400). Bar ≡ 10μ.

c) Dogfish adherent monocyte phagocytosing S. faecalis. SEM (x 3,300).

    Bar ≡ 10μ.
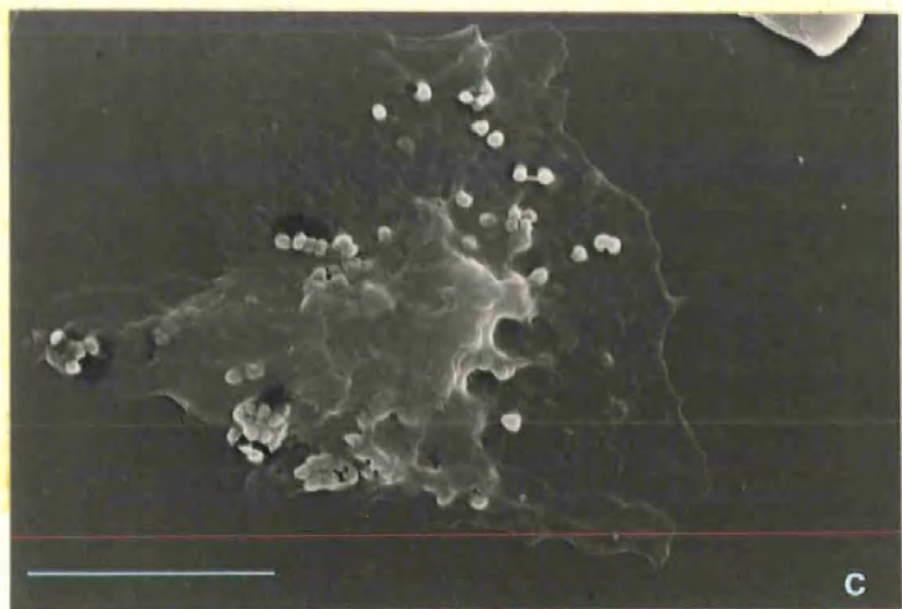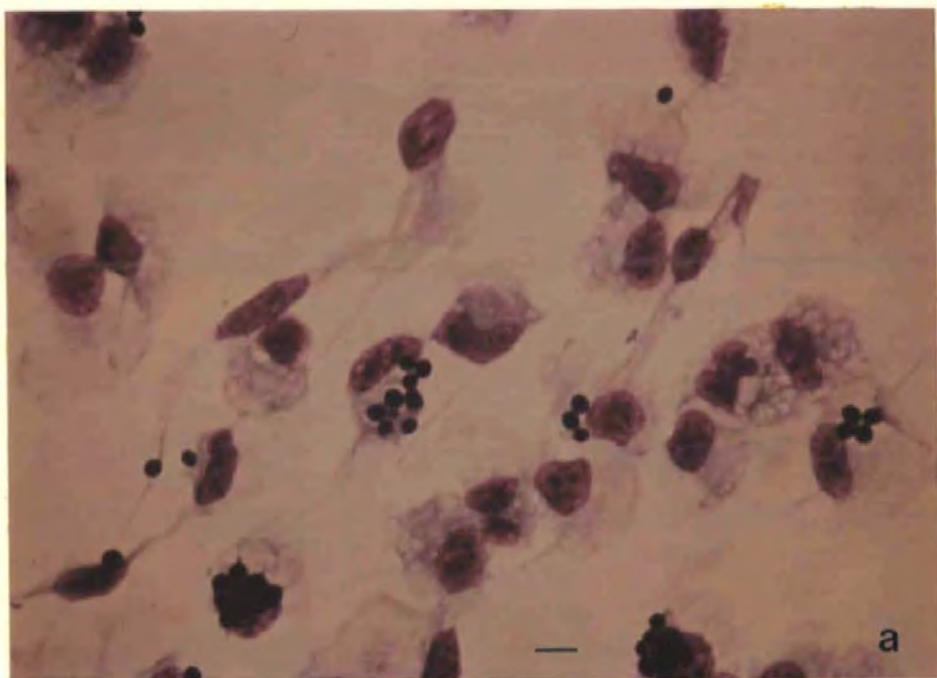
Plate 20

a)   Two adherent monocytes, one containing several endocytosed
       yeast cells and the other in the process of engulfing a single
       yeast cell. SEM (x 4,000). Bar ≡ 10μ.

b)   Phagocytosis of yeasts by adherent monocytes. SEM (x 8,420).
       Bar ≡ 2.5μ.

c)   Dogfish monocyte endocytosing yeast cell. TEM (x 26,607).
       Bar ≡ 1μ.
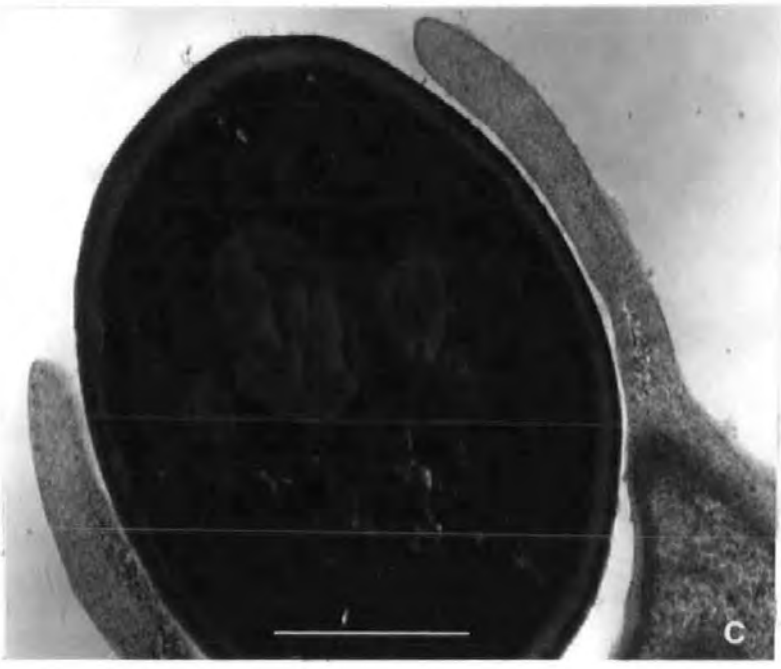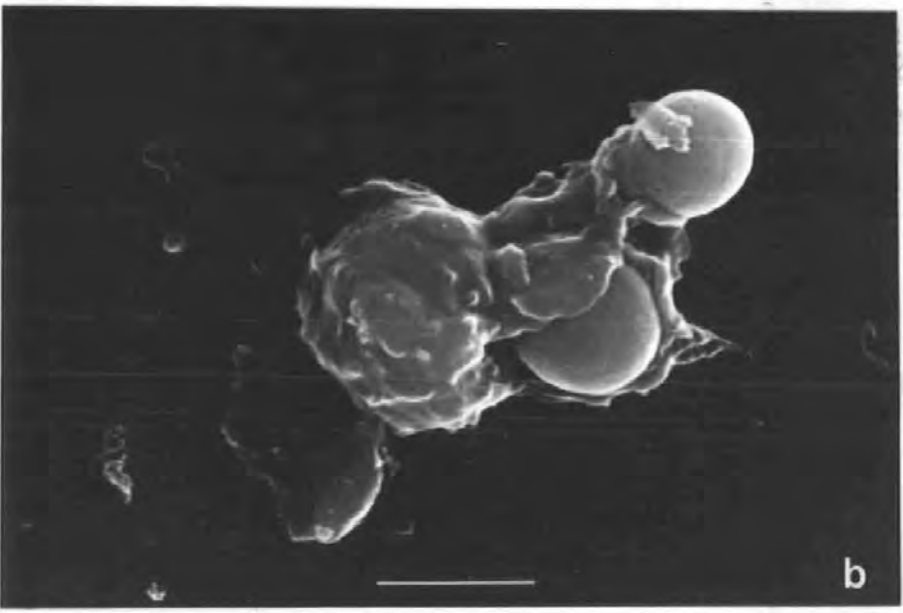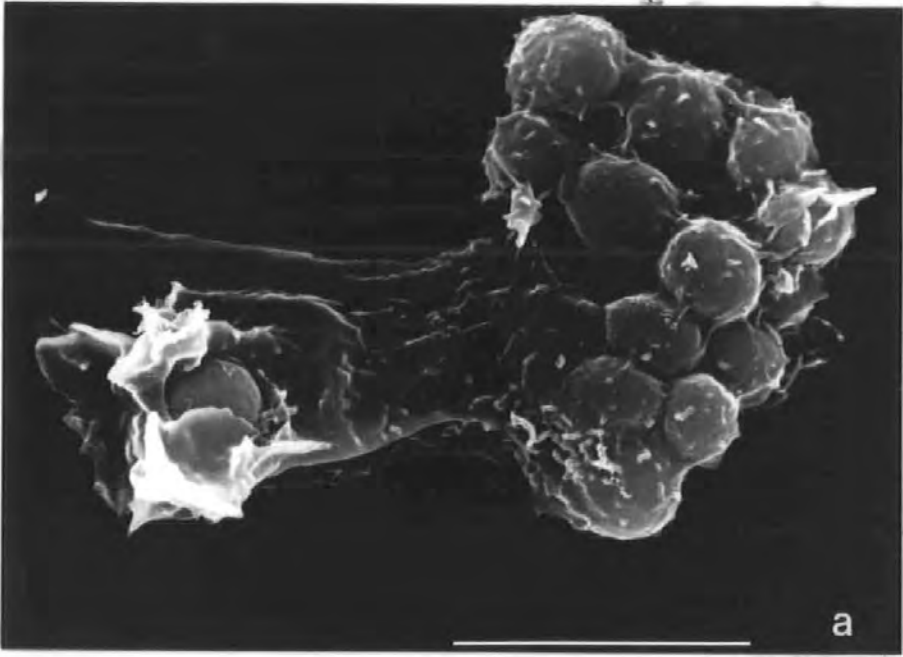
Plate 21

a) Dogfish peripheral blood monocyte containing carbon particles.

LM (x 1,431) Giemsa. Bar ≡ 10μ.

b) Dogfish peripheral blood thrombocyte (type a) containing carbon

in the vacuoles of the cytoplasm. LM (x 1,431) Giemsa.

Bar ≡ 10μ.

c) Dogfish peripheral blood smear showing type 1 granulocyte

containing carbon. LM (x 573) Giemsa. Bar ≡ 10μ.

G_1 = type 1 granulocyte

G_2 = type 2 granulocyte

T = thrombocyte

a

b

g_2

g_l

T

c
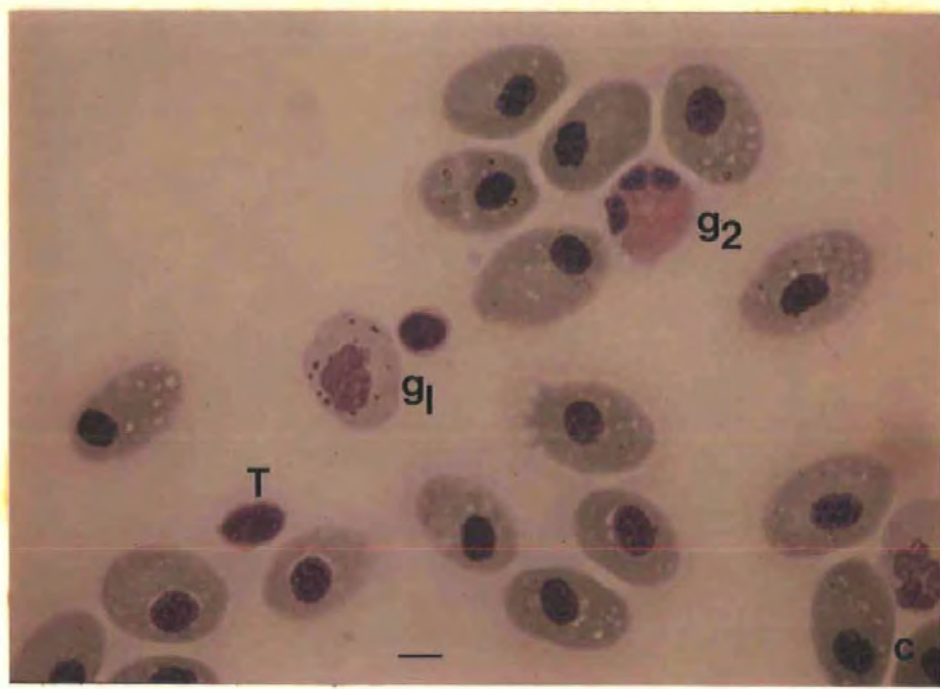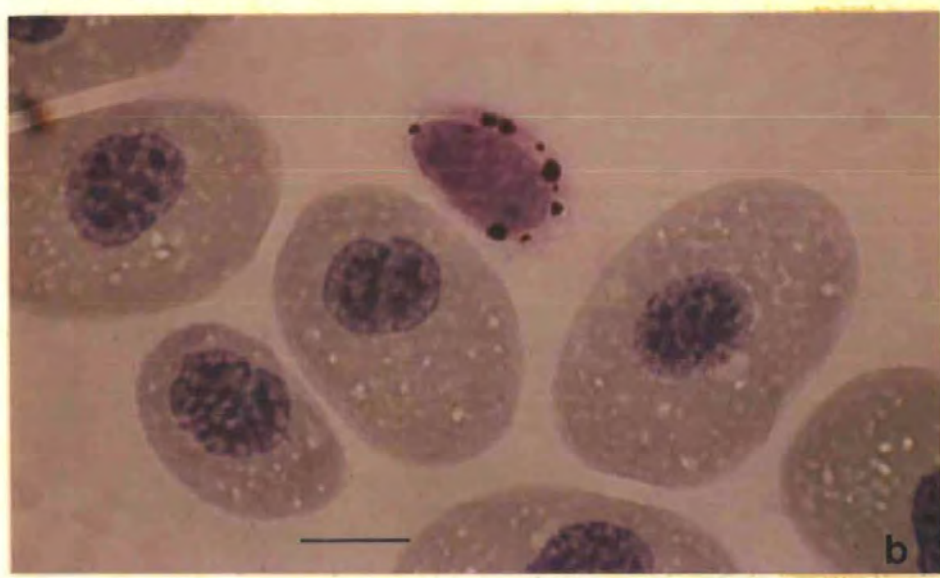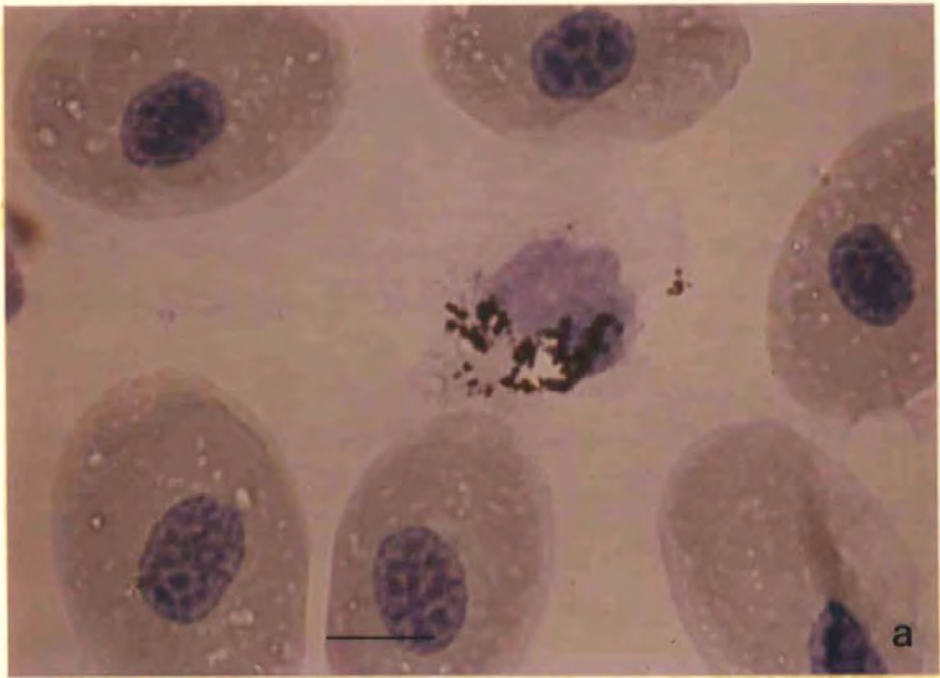
Plate 22

a)   Transverse section of dogfish spleen showing presence of
     carbon in ellipsoids. LM (x 573) Mallory's Triple Stain.
     Bar ≡ 10μ.

              E = ellipsoid
              arrow indicates carbon

b)   Splenic macrophage containing carbon TEM (x 9,157). Bar ≡ 1μ.

c)   Transverse section of dogfish gill showing carbon (arrow) in
     vessels at base of gill filaments. LM (x 229) Mallory's
     Triple Stain. Bar ≡ 100μ.

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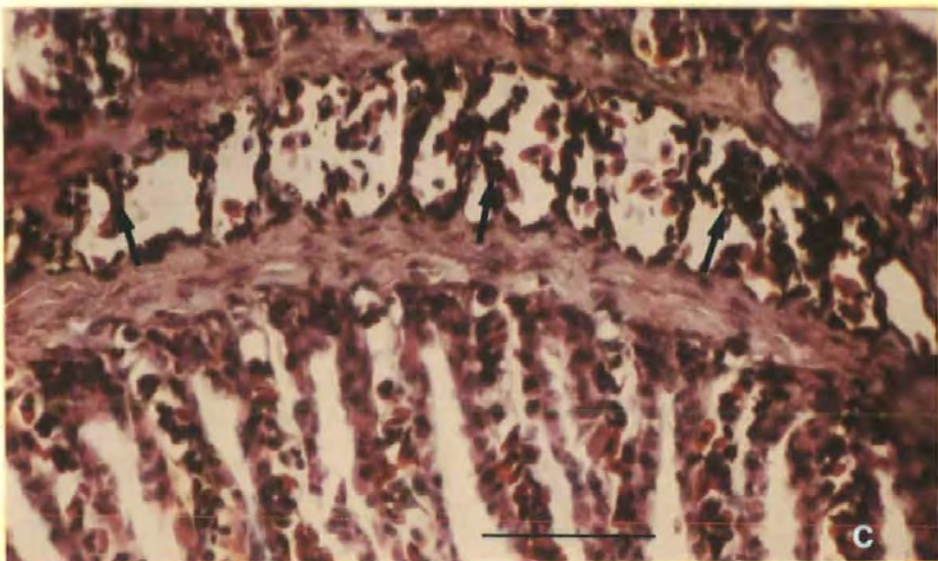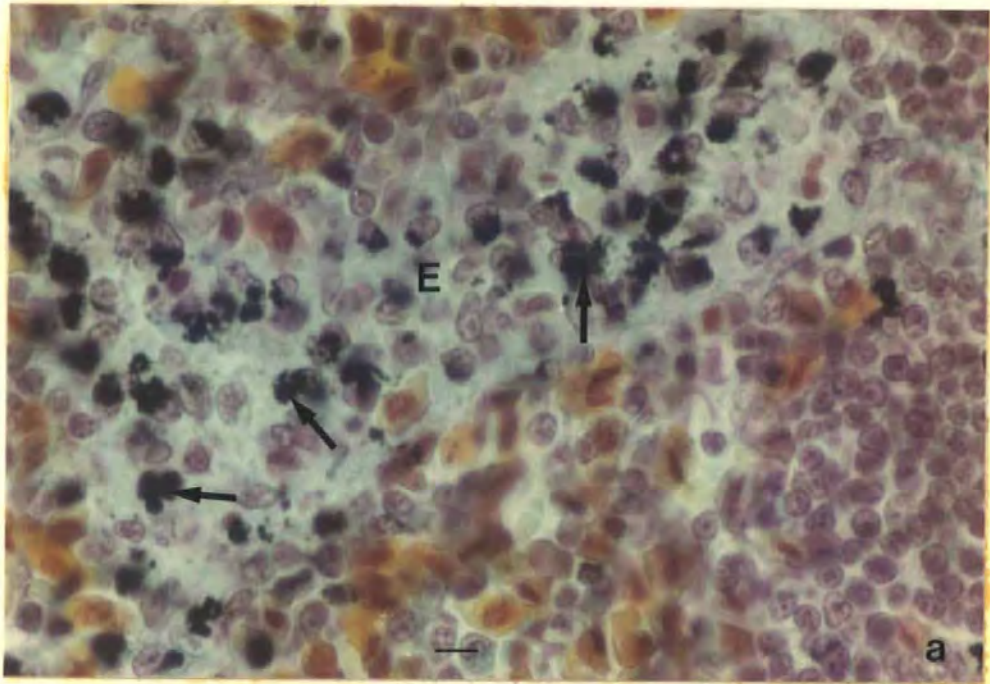
Plate 23

a)   Dogfish gill cells containing carbon particles (arrow).
      TEM (x 13,800). Bar ≡ 1μ.

b)   Dogfish blood monocytes containing large amounts of pigment
      two weeks post B.C.G. injection. LM (x 1,431) Giemsa.
      Bar ≡ 10μ.

c)   Dogfish peripheral blood monocyte with vacuoles containing cell
      debris and also B.C.G. bacterium which appears to be undergoing
      melanization (arrow). TEM (x 27,238). Bar ≡ 1μ.

                  V = vacuole
                  B = bacterium
                  N = nucleus
                  M = mitochondrion
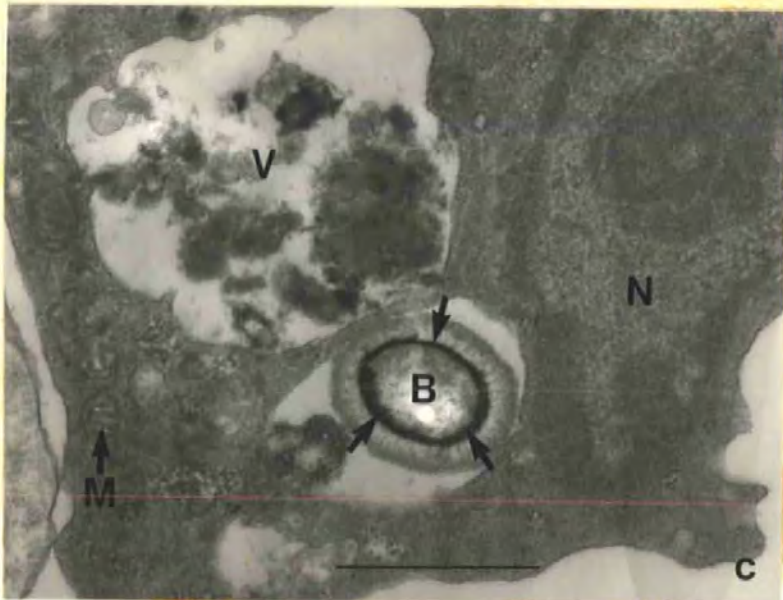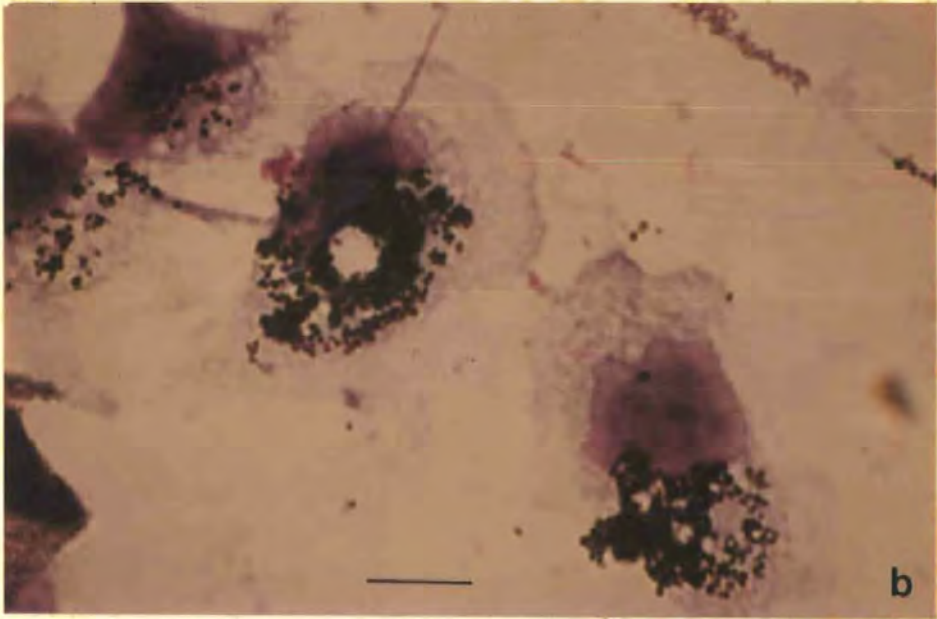
Plate 24

a) Dogfish monocyte containing a bacterium ($\underline{E}$. $\underline{coli}$) which is apparently undergoing melanization (arrow). TEM (x 96,970). Bar ≡ 0.1µ.

     B = bacterium

b) Dogfish monocyte after i.v. injection of $\underline{S}$. $\underline{faecalis}$. Note the highly vacuolated cytoplasm and the membranous whorls sometimes associated with lysosomes. TEM (x 17,600). Bar ≡ 1µ.

     N   = nucleus

     V   = vacuoles

     mw = membranous whorls

     l   = lysosomes

c) Membranous whorls associated with lysosomal enzymes in thrombocyte (see Plate 25a). TEM (x 45,000). Bar ≡ 0.2µ.

     mw = membranous whorls

     l   = lysosomal enzymes

     N   = nucleus
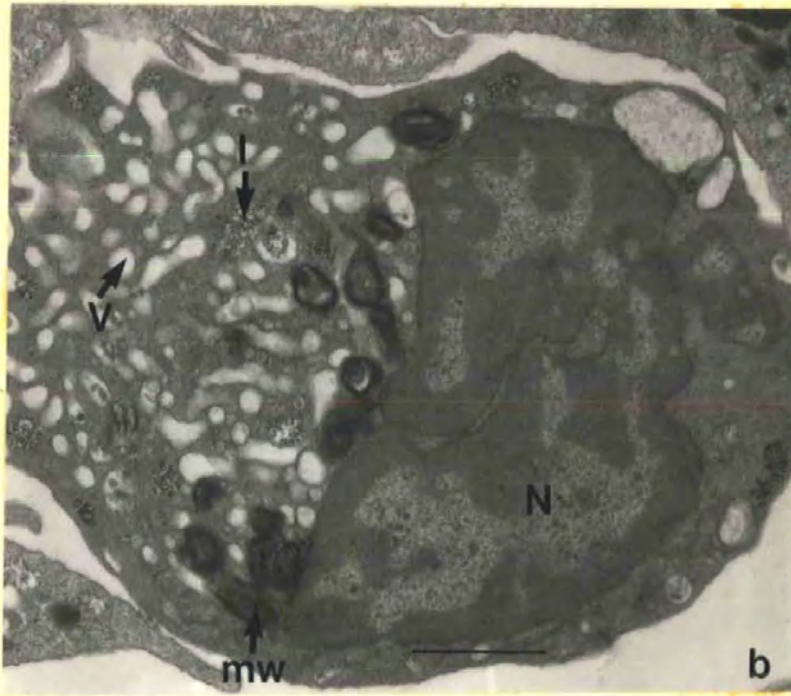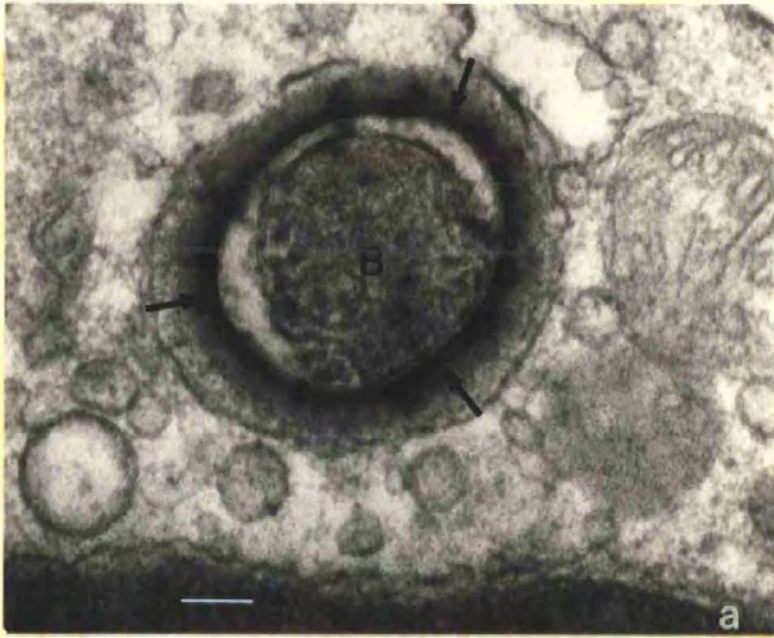
Plate 25

a) Thrombocyte from B.C.G.-injected fish apparently phagocytosing.

TEM (x 13,200). Bar ≡ 1μ.

mw = membranous whorl

l = lysosomes

v = vacuole

b) Ellipsoid in spleen of dogfish previously injected with B.C.G.

Note the small granules in the macrophages which may be pigment.

LM (x 573) Mallory's Triple Stain. Bar ≡ 10μ.

E = ellipsoid

arrows indicate 'pigment' granules

c) Spleen cell containing membranous whorls from dogfish previously

injected with S. typhimurium. TEM (x 7,832). Bar ≡ 1μ.
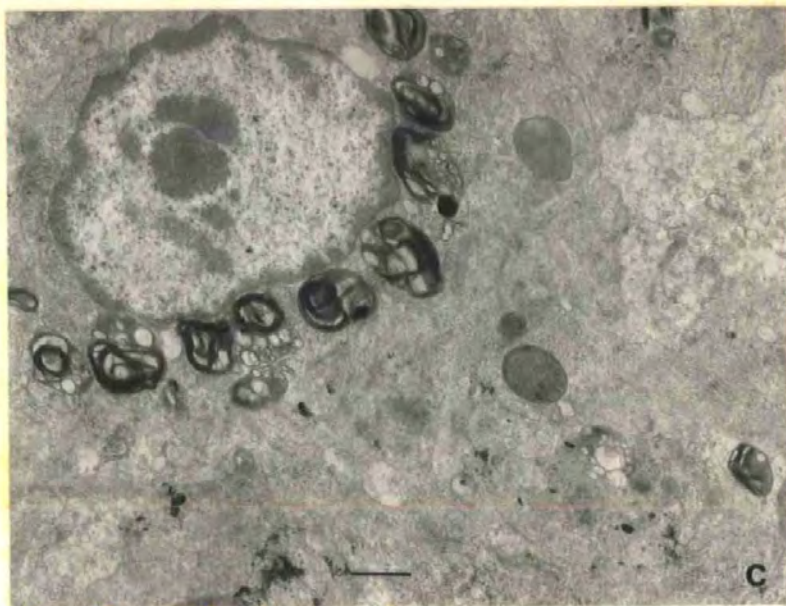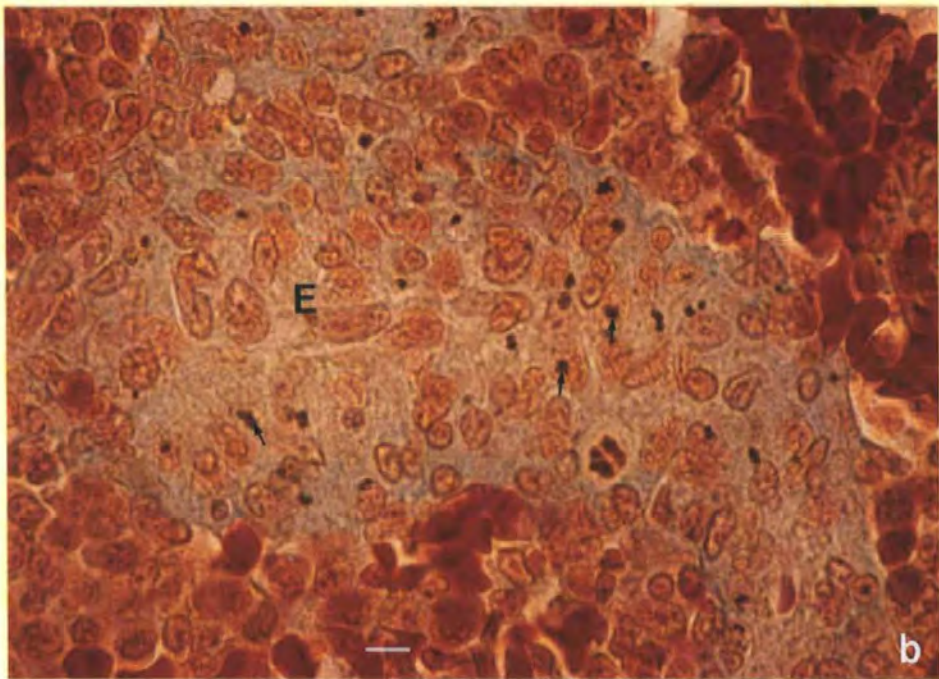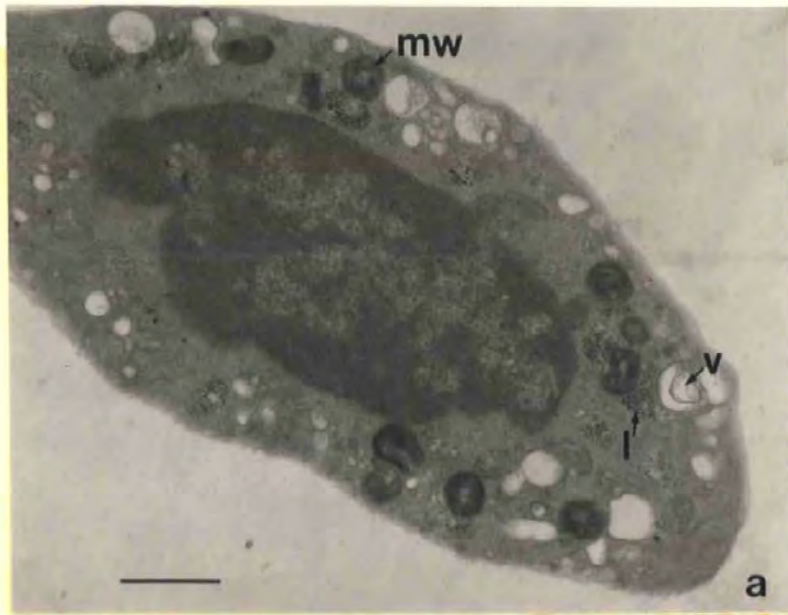
Plate 26

a) Membranous whorls in spleen cell of dogfish previously injected
    with S. typhimurium. Note the numerous layers of membranes
    (arrow). TEM (x 69,440). Bar ≡ 0.1µ.

b) Spleen cell from dogfish injected with E. coli. There appear to
    be large numbers of bacteria in the cell in which a well-defined
    cell membrane is apparently absent (open arrow). TEM (x 6,111).
    Bar ≡ 1µ.

            B = bacteria

c) Section of dogfish spleen containing what appears to be a
    'packet' of E. coli (arrow). These do not appear to be
    intracellular. TEM (x 4,054). Bar ≡ 2µ.
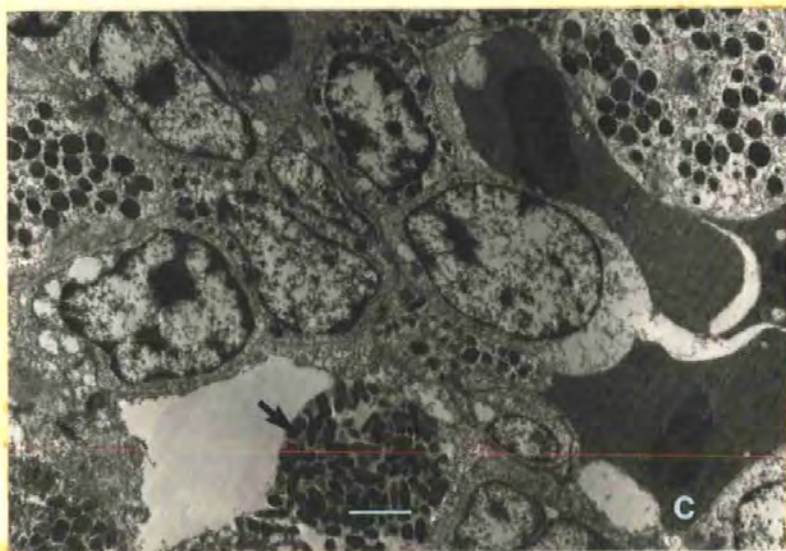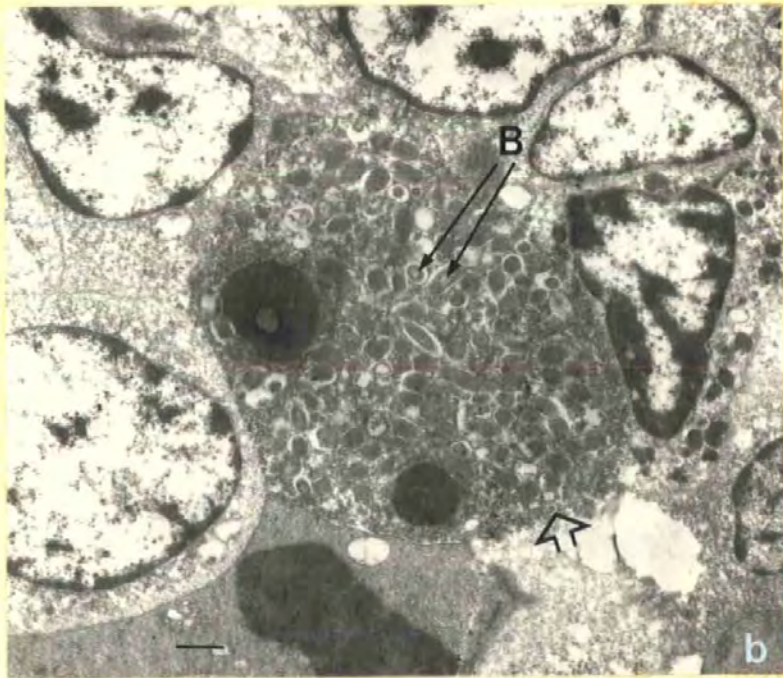
Plate 27

a) Liver cell in dogfish. Melanin granules appear to have been
    discharged or formed in a digestive vacuole containing cellular
    debris. TEM (x 9,822). Bar ≡ 1μ.

b) Spleen section of dogfish. Note the few isolated pigment cells
    (arrow) outside the ellipsoid but not in the lymphoid areas.
    LM (x 229) Mallory's Triple Stain. Bar ≡ 100μ.

                    E = ellipsoid

                    L = lymphoid areas
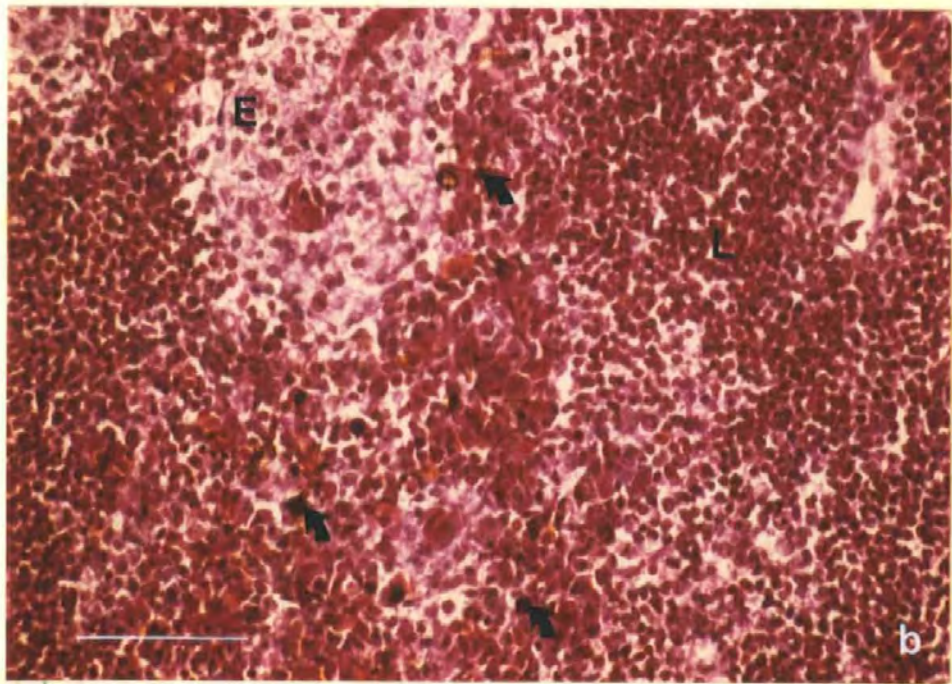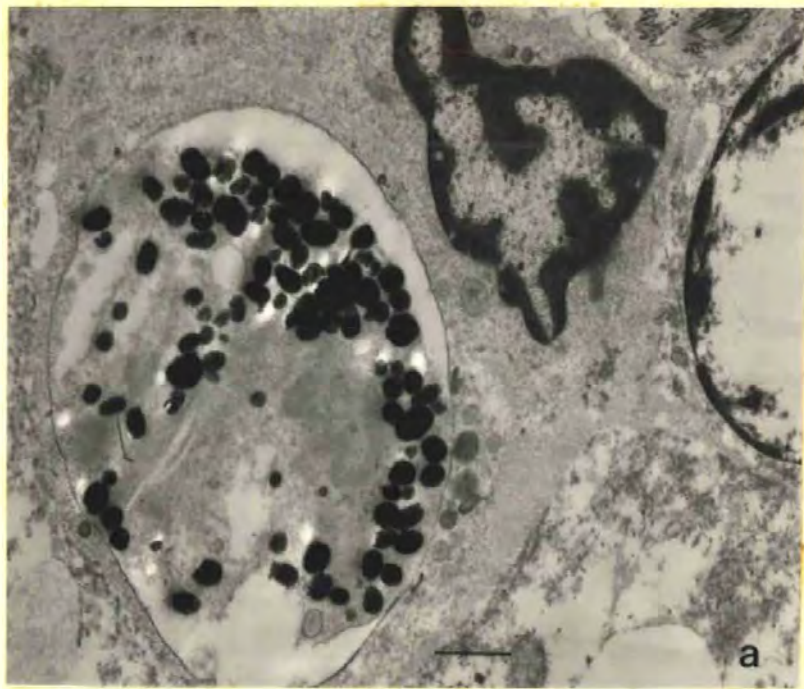
DISCUSSION

a) A qualitative study of _in vitro_ endocytosis

From the results it appears that two species of peripheral blood leucocyte of the dogfish are capable of active uptake of a variety of antigens and particulate material.

From these observations it appears that the monocyte is the least 'discriminatory' of the two cell types but the more resilient. The fragility of the neutrophils may in fact not be a real phenomenon but may reflect less than optimal tissue culture conditions. This is, to a certain extent, corroborated by Yoffey (1929) who noted that granulocytes in say spleen imprints were very fragile and the granules were often seen throughout the imprint. It may be that _in vivo_, when neutrophils have ingested material they migrate to areas such as the spleen where the material is deposited or further degraded. These cells are short-lived in mammals having a life span of only 1-2 days (Roitt, 1977). It is possible that there is a limit to the amount of material these cells are able to ingest, and when this has been reached they die, or conversely they may die as a result of phagocytosis-associated metabolism. In the artificial, and perhaps sub-optimal _in vitro_ conditions when they are challenged with relatively large quantities of antigen, this process may be even more rapid.

The yeasts and erythrocytes appeared to be the most avidly phagocytosed particles, and being of similar size it appeared that they were taken up to a greater extent than bacteria. However carbon and latex particles were often observed phagocytosed by monocytes and so any differences are more likely to be due to surface characteristics, which are highly variable in different bacterial species.

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The difference between adherent and suspended cells has been observed in other reports. It has been demonstrated that "it is particulate complexes (that is surfaces) which are involved in cell stimulation" and that "studies in vitro suggest that adherence and perhaps spreading, is an integral component in the process whereby a monocyte differentiates into a macrophage" (Henson et al, 1980). This may mean that the process whereby the phagocytes (or, at least monocytes) adhere to tissue culture plates may simulate the in vivo process of monocytes differentiating into mature macrophages with the consequent increase in metabolic and phagocytic activity. It is therefore possible that all in vitro work using adherent blood phagocytes may not represent what occurs in the circulation, but is more reminiscent of cells from organs containing macrophages.

The impression of lower activity in spleen cells may be attributable to differences in tissue culture requirements of phagocytic cells from different sites in the fish. It may however simply reflect that spleen cells took longer to adhere and spread than peripheral blood phagocytes and many were washed away. This would perhaps have left too few cells to show optimum phagocytosis as there appears to be a minimum critical number of phagocytes per unit area for phagocytosis to take place efficiently. Braun-Nesje et al. (1981) showed that two hours after 'seeding', salmonid pronephros macrophages were still very rounded and it was not until 24 hours that they were fully spread. Since all the above experiments were carried out within 2-3 hours of seeding this may explain the apparent reduction in activity of splenic phagocytes.

The less discriminatory nature of monocytes has also been noted by others. Ellis et al. (1976) observed that colloidal carbon injected intraperitoneally into plaice was only taken up by

mononuclear phagocytes never granulocytes. In fact the neutrophil

in plate 21 was the only granulocyte seen throughout this study

containing carbon and this was in a smear taken from an _in vivo_

injected fish.

The mechanism by which microorganisms are ingested as

demonstrated by Plates 19c & 20, appears to be analogous to the

processes observed in mammalian phagocytes (King, 1966). The

'enfoldment' mechanism noted by McKinney et al. (1977) in _Lepisosteus_

was never encountered during this study.

b) A qualitative study of _in vivo_ phagocytosis

The circulatory phagocytes generally seem to show little activity

in clearing particulate matter either antigenic or non-antigenic,

in vivo. The main active phagocyte of the blood with regard to

carbon uptake is the monocyte. This is endorsed by previous work by

Ellis (1976), Ferguson (1976) and Morrow and Pulsford (1980).

These workers also observed carbon inside thrombocytes, however

Ellis (1977) did not consider this an active uptake but a trapping of

material by vacuoles, which communicate with the environment via

stomata in the cell membrane.

Morrow and Pulsford (1980) also showed, by electron microscopy,

a granulocyte with small round granules containing carbon, from the

dogfish. This is the same type of granulocyte as observed in Plate 21,

though in this study these cells very rarely contained carbon.

The liver, kidney and heart showed no appreciable activity. The

lack of activity in the liver is in marked contrast to mammals where

the liver, Kupffer cells are the main cells of the Mononuclear

Phagocyte System responsible for clearance (Biozzi, Benacerraf and

Halpern, 1953; Spiegelberg, Miescher and Benacerraf, 1963). In fact

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there seem to be no cells resembling Kupffer cells in the liver, the main purpose of which appears to be in lipid storage - which is vital for buoyancy in elasmobranchs as they do not possess swimbladders (Hoar, 1966). The only other cells present in any number are the pigmented cells which play no direct part in carbon clearance. These observations differ from those of Hoskins and Hoskins (1918) who reported intense phagocytic activity for trypan blue by hepatic cells and Kupffer cells of dogfish.

In teleosts the anterior kidney is, with the spleen, the main clearance organ. In these fish the kidney is an active excretory organ, and in the intertubular regions there is abundant lymphoid tissue, phagocytic cells and melanomacrophage centres. In elasmobranchs the excretory function of the kidney is diminished as these fish do not drink the surrounding medium because they maintain osmotic equilibrium by means of retention of urea, trimethylamine oxide and other salts (Hoar, 1966). In these fish the kidney appears merely as a large, distended blood vessel. The lack of phagocytic activity in this organ in dogfish is therefore in contrast to similar work carried out by Mackmull and Michels (1932) and Ellis et al. (1976) on teleosts.

The heart again, has no phagocytic activity and all reports which demonstrate such activity in fish have dealt with teleosts. Ferguson (1975) and Mackmull and Michels (1932) both reported significant activity in the plaice and cunner (Tautogolabrus adspersus) respectively.

In the present study the heart of the grey mullet (Crenimugil labrosus) was shown to take up carbon but not the heart of the European eel.

This variable property of the heart to clear foreign matter may'

reflect differences between species in the requirements of a continuously recirculating blood supply. More active species such as formerly mentioned may require a greater amount of oxygen and if the heart was in any way impaired in its pumping mechanism then the fish would be put at a disadvantage. The eel and dogfish are generally more sluggish fish and this requirement may not be as important.

The spleen is the main area for phagocytosis of carbon in the dogfish. This is in agreement with Ellis et al. (1976) and Mackmull and Michels (1932) and shows that this organ in elasmobranchs performs a similar function to that in teleosts. Ellis et al. (1976) reported that over time carbon was cleared from the ellipsoids and was found in melanomacrophage centres. This was not observed in dogfish but comparable time course experiments were not performed.

The gill also showed a high degree of endocytic activity. Hoskins and Hoskins (1918) reported considerable phagocytic activity in the gills of dogfish, but since they were using trypan blue the results may not be comparable to results obtained using particulate matter. More recently Chilmonczyk and Monge (1980) showed gill pillar cells of 'sub-adult' rainbow trout capable of phagocytosis in some instances. Latex and carbon was taken up by approximately 4% of pillar cells and some of these cells contained pigment.

Injected yeasts, in this study, were also taken up by gill cells and in fact more yeasts were observable 4 days post-injection in the gill than any other site. In this case the yeasts were seen not only in circulatory cells in the vessels of the gill but also to a lesser extent in the pillar cells and epithelial cells.

This activity may merely be to ensure that nothing obstructs the circulation and hence inhibits uptake of oxygen. However the

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circulatory cells containing yeasts or carbon are at a much greater concentration in the gill blood vessels than in the general circulation, and so it almost seems feasible that these particles are transported to the gill for a purpose. It is possible that if certain disease organisms can pass from the external water via the gills into the fish as is suspected by some authorities (Ahne, 1978; Bowers and Alexander, 1981) then it may be equally possible for unwanted foreign matter to be eliminated via this route. This may help explain the large amounts of carbon and yeasts present in gills.

At four days post-injection of yeasts it is difficult to see yeasts in significant numbers in any organ other than the gill. In spleen a few yeasts were seen and in the liver a few circulatory cells contained them.

Since they are observed to be so avidly phagocytosed in vitro, it may be that they have already been dealt with by this time. However this seems unlikely as viable yeasts were still detectable in the blood at this point. By four days the numbers of melanomacrophages in the liver had increased by more than four-fold. Agius (1981) noted that starved fish (including the dogfish, S. canicula) possessed increased numbers of melanomacrophage centres with time, suggesting that this pigment is associated with cellular degradative processes.

While melanin/pigment containing cells were present in the spleen, no greater numbers were present in treated compared to control fish.

A few pigment cells were present within the gill. These cells containing degraded material may be migrating to the gill to release their contents. Archer (1978) noted melanin-containing cells

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discharging their contents into the peripheral mucus of the eel epidermis. A similar action may be occurring here.

In the case of bacteria, these were seen to be taken up in the ellipsoids of the spleen. As with the yeasts, 4 days after injection of _Salmonella typhimurium_, the number of pigment cells in the liver had increased by nearly three fold, again suggesting degradative activity.

Large amounts of pigment were rarely seen in the ellipsoid cells, but what appeared to be small pigment granules, in the case of BCG-activated fish, were present here.

It is proposed that antigen, taken up by ellipsoidal cells, is processed until the cells reach capacity and when replete with pigment they leave the ellipsoid and make their way to the main site of deposition of pigment cells, i.e. the liver. This would explain why fully-pigmented cells were rarely seen in ellipsoids and why they were not generally present in white pulp (i.e. lymphoid) areas but remained within the circulating blood cells of the spleen such that they could be carried to the liver via the circulation. It would also explain the large numbers of 'monocytes' containing melanin in the blood 2-3 weeks after BCG injection. However, it is also possible that the monocytes are themselves capable of producing pigment via degradative processes and so these cells may be blood as opposed to spleen-derived phagocytes. The few pigmented cells in the gill may equally be migrating from the gill to the liver.

Although this is a proposal based on circumstantial evidence, previous work on teleosts has demonstrated that replete macrophages (with carbon), rounded up and migrated to 'pigment areas' (Mackmull and Michels, 1932; Ellis _et al_., 1976; Ferguson, 1976).

In the dogfish as the site of pigment deposition is the liver macrophages would have further to travel and this perhaps makes the

proposal less feasible. Nevertheless, pigment cells increase in numbers in the liver after injection with antigen and the liver is not a primary clearance organ. Furthermore, six weeks after _Salmonella typhimurium_ injection the ellipsoids appear depleted of macrophages which would suggest that they are migrating away from this site.

The failure to show an increase in the number of pigment cells in the spleen after antigenic challenge may indicate that initially pigment cells are deposited in the liver and if this reaches capacity then an increase in pigment cells may occur in the spleen. This is corroborated by Agius (1981) who noted a greater increase in liver melanomacrophage centres than in the spleens of starving fish.

The methods by which bacteria are taken up and digested appear to vary between bacterial species. E. coli and BCG were observed in blood monocytes which contained melanin deposits. The bacterium in the phagocytic vacuole had a very dark pigmented halo around it, suggesting that the pigment itself was being used to degrade the bacteria or else being produced as a result of the action of lysosomal enzymes on the ingested bacteria.

Parallels are difficult to find in vertebrate literature but in the insects, pigments (in particular melanin) have a significant involvement in the defence system. Crossley (1979) reported that quinone polymers form melanin and that melanin or its more reactive precursors serve as bacteriostatic agents and inhibit the development of eucaryotic parasites. Giving a specific example he described _Mesolius_ a parasite of the insect _Strongylogaster._ The egg of this parasite is invariably encapsulated but the parasite is only able to develop if melanin fails to form round the capsule.

Ratcliffe and Rowley (1979) show electron micrographs of fungal spores in the larvae of _Chironomus lividus_ which have large

deposits of surrounding melanin. Again it does not appear to have been determined whether it is the melanin itself or its precursors which are active. Agius (1979a) reports that all ingested cellular debris is subjected to lysosomal enzymes and the indigestible residues give rise to pigments which gradually accumulate. In the same report it is suggested that the presence of pigments is related to the inability of fish to control their body temperature; and that as intracellular digestive processes are poorly developed in fish, accumulation of indigestible residues may occur in melanomacrophage centres.

Salmonella typhimurium has been observed inside blood monocytes and splenic cells, in vacuoles surrounded by multimembrane whorls. In the case of the blood cells these vacuoles or phagosomes are often associated with lysosomes whereas in the spleen cells lysosomes are generally absent as demonstrated by electronmicroscopy.

A few thrombocytes (or at least cells which had the general appearance of thrombocytes) also had ingested bacteria in membranous vacuoles.

This unusual effect of processing phagocytosed bacteria appears to have three possible analogies in the current literature.

Ratcliffe and Rowley (1979) show an electron micrograph of cells from Galleria mellonella taking up E. coli in vitro. After 12 hours incubation the bacterium is surrounded by what are described as 'myelin-like membranes' which the authors say are 'characteristic' structures present during digestion of various bacteria. While the myelin 'layers' are not as numerous as those observed in the present study they show some similarity to the observed phenomenon.

A further possibility is that a mechanism similar to the 'enfoldment' process McKinney et al. (1977) described in Lepisosteus

platyrhinclus cells phagocytosing yeasts is occurring. In this description one, long pseudopodium encircled the yeast particle several times before the cell membrane fused with the cell at several points along the coil. The mechanism may operate on a reduced scale in engulfing bacteria and the membranes may be what remains of multiply coiled 'micropseudopodia' around the bacterium.

Finally the third possibility is that while the bacterial membranes may be sheared and the cytoplasmic contents degraded, the membranes themselves may be less susceptible to the degradative processes available to the phagocytes and hence phagocytic vacuoles contain relatively intact bacterial cell walls. This has been observed in mouse and rabbit tissues experimentally injected with Streptococcal species. Up to fourteen days after injection streptococcal cell walls could be seen in vacuoles in muscle granulomata. By 35 days these are cleared (Ginsburg, Mitrani, Ne'eman and Lahav, 1975). It therefore takes in excess of two weeks for these membranes to be cleared in mammals. If the structures described are bacterial membranes they could persist for even longer periods in the fish due to the lower activity and the persistance of viable bacteria in the circulation. Melanin or melanin-like pigments appeared to be particularly involved in breakdown of BCG, as opposed to, for instance Salmonellae. This observation could reflect the large quantities of lipid present in the outer membranes of Mycobacteria (Wolinsky, 1980), i.e. up to 60% of their dry weight. The reactivity of pigments or their precursors may be via interaction with lipids. Agius (1979a) quotes lipofuscin as being derived from damaged cellular components through peroxidation of their unsaturated lipids. The cell walls of Mycobacteria contain true waxes which are fully saturated and chemically non-reactive, and

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glycolipids (Wolinsky, 1980) which would be the more likely target of reactive pigment precursors.

Finally the "accumulations" of bacteria in the spleens of dogfish injected with E. coli possibly demonstrate a mechanical trapping of bacteria in the splenic cell meshwork. This is a further method by which bacteria could be processed. Alternatively it may represent E. coli actively proliferating within the spleen.

c) A quantitative study of in vitro phagocytosis and the effects of various factors on this process

The in vitro method used to determine the conditions required for phagocytosis was chosen after investigating several different techniques.

Initially an objective method of quantifying endocytosis was required and the following methods were considered:-

1. Addition of C^{14}-labelled E. coli to leucocyte suspensions, differentially centrifuging and measuring the intracellular accumulation of the radio-label.

2. Addition of Staphylococcus aureus to leucocyte suspensions, sampling at various intervals and making intracellular and total bacterial counts using lysostaphin to lyse extracellular bacteria.

3. Addition of mammalian erythrocytes, and determining intra-leucocyte haemoglobin accumulation. Although these methods appear technically simple they were discarded for a variety of reasons.

The first two methods involve the use of bacteria which, throughout the experimental work were never observed to be taken up in large numbers. The numbers which the phagocytes appeared able to ingest were too low to be enumerated accurately by these methods.

In addition these methods used cell suspensions which, as previously discussed, do not seem as active as adherent cells, although no quantitative work was attempted.

In the third method, too little haemoglobin accumulated within the leucocytes to measure adequately by standard haemoglobin assays.

The method involving microscopic counting of yeast uptake by monolayers of blood phagocytes was therefore adopted, as with the small numbers of leucocytes available for each treatment, significant counts could be obtained. The subjectivity of the method was partially overcome by counting 'positive phagocytes' and 'yeasts per positive phagocyte' in up to ten fields.

The two phagocytic indices counted gave similar trends and so displaying percentage positive phagocytes was considered justified.

In all cases controls were run, as cells differed in phagocytic activity from fish to fish and also in a single fish from one period to another.

The results for pH require little further comment. The pH of dogfish serum is approximately pH 7 and so the optimal pH for phagocytosis would be expected to be around 7. The range between which phagocytosis would occur (i.e. pH 5.5 → 8.5) suggested that changes in the immediate environmental pH would not completely inhibit phagocytosis.

The effect of temperature on phagocytosis is in agreement with previous observations which suggest that optimal endocytosis occurs at body temperature in homeotherms (Mudd, McCutcheon and Lucké, 1934) and at optimal environmental temperature in poikilotherms.

Phagocytosis occurred at all temperatures tested (i.e. 4° - 45^oC). The cells appeared least viable at 37° and 45^oC, closely followed by 4^oC. It seems likely that any phagocytosis which occurred, took place

in the first few minutes when the cells were brought to experimental temperature. The optimum observed temperature between 10° and 20°C seems related to the optimum temperature for the fish. Dogfish show obvious signs of stress and invariably die when maintained at temperatures much above $15-16^{\circ}$C. Nevertheless, the phagocytes remained relatively active at 20°C and 25°C indicating that these cells have a greater temperature tolerance than the organism from which they derive. This is true in other animals, for instance, mammalian polymorphonuclear leucocytes have been observed to preserve their phagocytic activity in the body for as long as eleven days after death. This suggests that strict maintenance of body temperature is not vital for continued phagocytic activity (Mudd et al., 1934).

The lack of an effect of serum on phagocytic rate or capacity is of interest.

Serum has been shown to have an opsonic effect on phagocytosis by mammalian phagocytes. After the original observation of Wright and Douglas (1903), it was shown that fresh 'normal' serum possessed heat-labile opsonic activity while immune serum had a heat-stable component which acted opsonically on the test antigen. It was further shown that in certain instances the two factors could act cooperatively (Stossel, 1975). The heat-labile system was attributable to complement (or the properdin system) and the heat-stable component to antibody (Nelson and Lebrun, 1956; Nanni, 1958; Cohn and Morse, 1959).

In no fish species has any conclusive evidence so far emerged regarding the enhancement of phagocytosis by serum factors. In this study it seems that dogfish phagocytes do not require serum as long as all essential nutrients are provided in the culture media. Ruediger

and Davis (1908) showed that leucocytes from a variety of
poikilotherms including six fish species would phagocytose various
bacterial species in vitro, but if washed they lost this ability,
which was regained on addition of serum. These workers also
demonstrated that although autologous serum was most efficient in
promoting phagocytosis, heterologous serum was also active, even from
homoeotherms. The opsonic activity was lost or inhibited by heat
(55°C for 30 mins).

Contradictory evidence has emerged more recently. Holmquist
et. al. (1980) and Braun-Nesje et. al. (1981) showed that in four species
of teleost, mammalian immunoglobulins and complement did not
enhance phagocytic activity of peritoneal macrophages or pronephros
macrophages. Autologous serum was not tested in these studies, and
in view of the conflict of evidence over the use of heterologous sera
there seems to be a requirement for further work particularly on the
role of autologous sera.

One report exists where opsonic activity has been demonstrated
using fish (Mustelus canis) blood phagocytes (Weissmann et. al., 1975).
Liposomes containing peroxidase were more avidly engulfed when
coated with heat-aggregated, isologous 1gM than when uncoated or
coated with native 1gM.

In fact the dogfish (S. canicula) invariably possesses
substantial quantities of natural antibody in its serum and it may
be that due to its presence, the requirement for opsonic activity is
reduced. Since the natural antibody shows "specificity" towards many
antigens, once attached to a cellular antigen there is a strong
possibility that the cell would be lysed via complement. As this is
apparently a more efficient way of eliminating antigen it seems
unlikely that phagocytes would need to have receptors for antibody

and complement which act more efficiently on their own.

Avtalion and Shahrabani (1975) showed that blood cells from immunised carp were more efficient at clearing bacteria in vitro than cells from unimmunized carp. They showed that the rate of uptake was not influenced by the presence of specific as opposed to natural antibody, but that the pre-exposed cells were more efficient at inactivating or killing the bacteria once inside.

Pre-treating the cells with trypsin or anti-dogfish immunoglobulin was performed as a result of the work of Van der Knapp (1980). He showed that no opsonic activity could be demonstrated by adding serum to snail phagocytes but if the cells were first treated with trypsin or an anti-'snail serum' antibody, the addition of serum did increase phagocytosis indicating that the opsonin was associated with the phagocyte membrane.

In the case of trypsin a purely toxic effect was demonstrated and phagocytosis was irreversibly inhibited. This was probably due to too high a concentration of trypsin.

The anti-dogfish Ig serum inhibited phagocytosis to a slight degree and addition of serum returned this to normal.

While further investigation of this is necessary it seems unlikely that appreciable antibody is present on the surfaces of blood phagocytes.

A further observation is that the phagocytic rate experiments were performed using coated particles in addition to adding serum to the incubation medium. If surface immunoglobulin was the means by which antigen attached, then uptake of coated particles should have been slower than that of uncoated particles as the attached antibody would perhaps obscure the antigenic sites for phagocyte-bound antibody. This did not occur.

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The apparent absence of opsonic activity led to an investigation into the surface receptors of dogfish phagocytes (see following chapter).

Iodoacetic acid is a metabolic inhibitor of glycolysis. The inhibitory effect of this reagent, on phagocytosis suggests that energy for the process is supplied by glycolysis as opposed to aerobic respiration since azide did not inhibit phagocytosis (azide is an aerobic respiration inhibitor via its action on the cytochrome system). This observation is in general agreement with reports on phagocytosis in all types of animals. Ratcliffe and Rowley (1979) showed that insect haemocytes require glycolytic energy for phagocytosis. Cohn (1970) reported that 10^{-4}M iodoacetate inhibited phagocytosis by mouse macrophages by more than 80% whereas cyanide inhibited by less than 10%. By comparison the dogfish phagocytes were relatively resilient, as greater than 1 mM was required to substantially reduce phagocytosis.

Human, guinea-pig, canine, rabbit and rat polymorphonuclear leucocytes, human monocytes, mouse and guinea-pig peritoneal macrophages and rabbit, guinea-pig and human alveolar macrophages have been reported to be inhibited by glycolytic inhibitors but not aerobic respiration inhibitors (Cohn, 1968; Stossel, 1975).

However other reports have demonstrated mitochondrial inhibitors as effective inhibitors of human, guinea-pig and rabbit alveolar macrophages, Acanthaenoeba and mouse and rabbit peritoneal macrophages (Stossel, 1975). It appears that in mammals the energy requirements of phagocytosis may be supplied by aerobic respiration in some instances depending on the 'oxygen status' of the organs from which the phagocytes are derived.

When puromycin was added to phagocytosing cells a fluctuating

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pattern of uptake emerged. Puromycin inhibits protein synthesis and has been shown to inhibit pinocytosis in mouse macrophages by up to 90% (Cohn, 1970). However the same author observed less than 10% inhibition of phagocytosis by the same cells, and this result is endorsed by Rabinovitch (1970).

The dogfish phagocytes, while significantly affected by puromycin at all concentrations tested were not generally inhibited. The fluctuations in the clearance pattern may have been due to generalised toxic effects due to excessively high concentrations of the inhibitor (as was observed with high azide levels).

There is no obvious explanation in the literature for these differential effects of puromycin on pinocytosis and phagocytosis. Obviously when soluble antigen is pinocytosed the internalised cell membrane must be replaced and this involves protein synthesis. However it would be expected that the same would be needed during phagocytosis. The only explanations which seem possible are:-

1. Pinocytosis may occur all over the surface of the cell whereas phagocytosis occurs only at limited points on the cell surface, and so the requirement for new membrane may be less acute for phagocytosis than for pinocytosis.

2. The mechanisms of uptake may differ. It is possible that during pinocytosis the membrane is degraded along with the contents of the pinocytic vacuole whereas in phagocytosis there may be some means by which the membrane is recycled.

Calcium and magnesium ions are (apparently) of approximately equal importance in promoting phagocytosis by dogfish cells. However even in the total absence of calcium and magnesium, inhibition was not total. This suggests that these ions may perhaps be bound to the phagocyte membrane and consequently their absence from the surrounding

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medium is not totally inhibitory.

Divalent ions, particularly calcium and magnesium, promote the ingestion of a variety of particles by mammalian and non-mammalian phagocytes. Human neutrophils and rabbit alveolar macrophages have been reported as being unable to ingest albumin-coated paraffin oil particles without certain divalent cations present (Stossel, 1975).

Mudd et al. (1934) report a case where addition of calcium chloride not only to saline but also to serum increased phagocytosis indicating that in vivo it may be the availability of free calcium ions which controls in part at least, the degree of phagocytosis. The increase was due to an increase in the rate of phagocytosis instead of an increase in the numbers of cells phagocytosing.

The effect of previously injecting fish with BCG, was that these 'activated' cells showed greater activity than control cells. (The difference being statistically significant).

The only other report of enhanced phagocytosis following activation is that S. aureus-immunized carp were shown to have phagocytes which were more actively bactericidal than those of control carp (Avtalion and Shahrabani, 1975).

The term 'activation' refers to the phenomenon described by Mackaness (1964) in which there is enhancement of macrophage function during infection, which has an immunological basis involving lymphocytes (Karnovsky, Lazdins and Simmons, 1975).

Macrophages taken from animals at a particular time after infection, spread out more extensively on glass and appear to be more phagocytic than the normal cell. They also have enhanced, non-specific bactericidal activity. The specificity of the phenomenon lies in the fact that when 'activation' can no longer be demonstrated its return is dependent on the animal being challenged with the

original antigen (Karnovsky et al., 1975).

Mackaness (1970) compared peritoneal macrophages from normal and BCG-infected animals 15 mins after being placed on glass and demonstrated fully spread cells from the BCG animals whereas those from control animals had only just begun to spread. He noted that activated cells also had more free ribosomes and a more elaborate endoplasmic reticulum than unstimulated cells. Activated cells had a more mitochondria, lysosomes and pinocytic activity.

In BCG-infected dogfish the only typical property of activated cells was the increased phagocytic activity. 'Activated' cells have been observed under phase contrast and do not appear to show any change in the above mentioned organelles and do not adhere to tissue culture plates any faster than control cells. In addition numbers of blood borne monocytes do not increase after intravascular BCG injection and so it seems that not all characteristics of 'activation' are shown by fish phagocytes.

Mackaness (1970) reported that 'highly activated macrophages exist for only a limited period during the course of most infectious diseases' and so it is possible that the fish were not sampled during this critical time.

Certainly more knowledge of this phenomenon in fish is required as it is associated with cellular immunity as well as defence against infectious agents. Thus it would help to confirm the presence or absence of delayed hypersensitivity in fish.

Bacteria were difficult to demonstrate being phagocytosed. The work on Streptococcus faecalis shows the rate of decrease of bacterial numbers in the tissue culture medium was not increased by the presence of dogfish phagocytes and so it seems unlikely that these cells were taking up viable bacteria. In fact the results with bacteria

corroborate the results gained from in vivo bacterial phagocytosis experiments and further comment will be made in the next section.

KLH was never observed to be cleared in vitro by dogfish, adherent blood phagocytes. Even at the highest cell densities no detectable difference in KLH concentration was demonstrated. It therefore seems probable that if KLH is taken up it is in such small quantities to be indistinguishable by rocket immunoelectrophoresis. Possibly autoradiographic methods would have proved more useful as it was evident by immunofluorescence that some KLH was endocytosed.

d) A quantitative study of in vivo antigen clearance

The most obvious feature of the in vivo clearance experiments was the apparent inability of the dogfish to clear bacteria from the circulation in any reasonable time interval. This is in marked contrast to mammalian observations where bacteria injected i.v. are rapidly eliminated from the blood by fixed macrophages of the reticuloendothelial system, mainly in the liver and spleen (Spiegelberg, Miescher and Benacerraf, 1963).

The general pattern was that a sharp decrease in bacterial numbers occurred in the first 1-2 hours post-injection which could not be attributed to dilution in the blood (i.e. blood volume is approximately 5-6% body weight in elasmobranchs (Hoar, 1966) and so the dogfish used would have at most 50-60 mls blood).

This sharp decrease may be accounted for by the bacteria themselves adapting to the less than ideal conditions in the fish, i.e. high levels of urea, trimethylamine oxide and salt. Alternatively this decrease may be due to the action of natural antibody and complement which is invariably present in the dogfish.

After this decrease, bacterial numbers remained fairly constant

within a power of 10 (1 log unit). It is possible that numbers of antigenic particles must increase above a certain number to be recognised and cleared. It may also be that only dead microorganisms are phagocytosed to any degree and the fish relies on natural antibody and complement to clear its blood system normally. This is borne out by the in vitro observations where viable bacteria are not obviously phagocytosed (see graph of in vitro S. faecalis clearance).

This hypothesis is further endorsed by the fact that no difference in clearance pattern of S. typhimurium was observed if the fish was injected with carbon i.v. one day earlier. This may suggest that carbon clearance occurs via phagocytosis, but that antigen is cleared largely by humoral mechanisms. In mammals a previous injection of carbon slows the rate of clearance of subsequent carbon injections by 'reticuloendothelial blockade' (Biozzi, Benacerraf and Halpern, 1953).

Immunizing the Salmonella typhi H antigen also had little effect on clearance. Although the immunogen was a different species to the challenge bacterium (i.e. S. typhimurium), the antibody present in the serum cross-reacted with S. typhimurium though whether this was natural antibody or induced, specific antibody is not known.

Nelstrop et al. (1968) monitored the clearance of T, phage in three fish species including the dogfish S. canicula.

Dogfish given 4×10^6 p.f.u. showed no increase in clearance rate when monitored three times with 5 and 4 day intervals. Decreasing the dose to 4×10^5 p.f.u. had no effect and in fact secondary doses were cleared sometimes slower than primary doses. No neutralising antibody was detected in any of the fish used.

It was suggested that the results of clearance using dogfish were due to some kind of 'blockade' of the phagocytic system or

'immune paralysis'. Furthermore the graphs indicate that injected phages were by no means totally cleared by the end of the experiment. It would have been interesting from the point of view of the present work to know how long the dogfish took to completely clear the circulation of phages, if at all.

Wrathmell (unpublished observations) has observed sheep erythrocytes still within the dogfish circulation three weeks after injection for immunization purposes, showing that phagocytosis, even of non-proliferating antigen, is slow.

It seems that these fish can tolerate certain numbers of bacteria in the body while remaining apparently healthy. Bisset (1946) has shown that while some fish clear bacteria faster at higher than lower temperatures, at lower temperatures a condition of 'symptomless parasitaemia' may occur, in the absence of humoral antibody formation. This could be happening in the present case, as, even if natural antibody is present in the fish prior to injection the numbers may be sufficient to react with all the available antibody but leave a surplus of bacteria in the blood.

Tolerance of bacteria by fish has been reported elsewhere. Chung and Kou (1973) isolated predominantly Aeromonas, Pseudomonas and Enterobacteriaceae from gills, intestine, blood and viscera of apparently healthy eels (Anguilla japonica). Bullock and Sniezko (1969) demonstrated the presence of bacteria in three trout species at two hatcheries. At one hatchery 12.5% of fish carried bacteria in their blood and/or kidneys and at the other 26.1% of fish. Evelyn and McDermott (1961) also isolated bacteria from hearts, livers, kidneys, spleens, muscles and mucus of fresh water fish. In all the above cases the fish were apparently healthy and showed no signs of the diseases which might be associated with some of the bacterial species isolated (i.e. such as furunculosis - caused by

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Aeromonas salmonicida).

The various bacteria other than S. typhimurium were used in order to show any differences in clearance due to Gram reaction or catalase reaction. In mammals a condition known as 'Chronic Granulomatous Disease' (CGD) occurs in which the polymorphonuclear granulocytes are unable to kill certain bacteria due to an impaired myeloperoxidase system. Catalase negative, hydrogen peroxide-producing bacteria such as S. faecalis are killed whereas Staphylococcus aureus which is catalase positive is not killed (Baehner and Boxer, 1980). It was therefore decided to use G+ve and G-ve bacteria, also to test catalase positive and negative species in case fish leucocytes lacked a comparable system to the myeloperoxidase system of mammalian leucocytes.

The bacteria used and their Gram and catalase reactions are shown below.

Bacterium	Gram Reaction	Catalase Reaction
S. typhimurium	−	+
S. faecalis	+	−
S. albus	+	+
E. coli	−	+

From the results it is evident that there was little difference in the clearance pattern from one species to another; it was therefore improbablethat there was any correlation between gram and catalase reactions and clearance.

Vibrio anguillarum was used since it is a known marine fish pathogen and it was thought that since it was more likely that the fish

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had been previously exposed to this bacterium, it may have a different clearance pattern.

A highly virulent strain (UNH 569) killed two dogfish within 12 hours (virulence was judged by LD_{50} values on the European eel, Anguilla anguilla - Munn, Chart and Pearcey, in press).

Therefore an 'avirulent' strain (A20/76) was injected into two fish. The fish were monitored for seven hours and showed a steady drop in blood bacterial numbers, but by seven hours the fish were in an evidently stressed state and so were killed. All these fish showed signs of acute vibriosis, particularly those which received UNH 569 which on post-mortem examination had oedematous stomachs and dilated blood vessels around the gut and gonads.

Finally another 'avirulent' strain (COB 408) was used, which at the levels injected did not obviously stress the fish but was not completely cleared from the circulation even after six days.

It seems therefore that even potentially pathogenic organisms are not cleared from the circulation.

The results gained using yeasts were surprising in view of their apparently avid uptake in vitro. However if it is true that only non-viable antigens are phagocytosed to any significant degree, the in vitro work may be substantiated, since heat-killed yeasts were used throughout these experiments.

KLH was cleared in 5 out of 6 fish injected and evidently previous exposure made little difference to this clearance. From the in vitro work however, it seems unlikely that the clearance is due to phagocytic cells.

Finally carbon clearance while appearing to be technically simple was not easy to perform.

The blood cells were difficult to lyse even in 1% acetic acid or

distilled water.   It was also not possible to spin cells down as
large amounts of carbon were trapped and sedimented with the cells.
Clearance took up to 24 hours (as opposed to approximately 1-2 hours
in rats - Hudson and Hay, 1980).   Possibly lysing blood samples by
ultrasonification would have yielded more meaningful results but the
main problem appeared to be due to the vastly extended clearance
time by comparison with mammals.

Chapter 5

Cell Surface Properties

Fc Receptors

i) <u>Receptors for particulate antigen-antibody complexes</u>

Results of experiments using antibody coated erythrocytes are
summarised in Table 15.

At no time were Fc rosettes observed using leucocytes from either
peripheral blood or the spleen of dogfish or plaice; nor were any
spontaneous rosettes formed with uncoated erythrocytes. All
phagocytic cells contained erythrocytes or were vacuolated regardless
of whether the erythrocytes were coated or not, indicating that
phagocytosis is independent of antibody.

Ray blood cells were only occasionally examined, as it was
difficult to obtain completely uncoagulated blood samples, however
when they were tested no rosettes were observed. In the case of
spleen cell suspensions, approximately 1% of cells formed rosettes but
these occurred spontaneously in the presence of uncoated erythrocytes
and the numbers of rosettes were not increased by coating erythrocytes
with dogfish antibody. The rosettes appeared to be associated with
lymphocytes, and since adherent spleen cells never exhibited rosette
activity it was assumed that a proportion of the lymphocytes could
interact directly with erythrocytes. (Rosettes were only observed in
2 out of 5 rays examined) Erythrocytes coated with ray natural
antibody were not tested, due to the difficulty of obtaining
unclotted blood samples. (When the blood clotted, the plasma
itself also gelled, even in the presence of heparin)

Eel blood and spleen cells were similarly negative when tested
with rat antibody-coated erythrocytes, eel natural antibody-coated

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Table 15

Results of rosette tests using antibody-coated sheep or bovine erythrocytes

Erythrocyte Complex	DOGFISH		PLAICE		RAY*		EEL		RAT
	Blood	Spleen	Blood	Spleen	Blood	Spleen	Blood	Spleen	Spleen
1. E	–	–	–	–	–	≃ 1%	–	–	–
2. EA$_{RAT}$	–	–	–	–	NT	≃ 1%	–	–	+
3. EA$_{IMMUNE\ DOGFISH}$	–	–	–	NT	NT	< 1%	NT	NT	NT
4. EA$_{DOGFISH\ NAT\ AB}$	–	–	–	–	–	≃ 1%	NT	NT	–
5. EA$_{DOGFISH\ MONOMERIC\ IgM}$	–	–	NT	NT	NT	< 1%	NT	NT	NT
6. EA$_{EEL}$	NT	NT	NT	NT	NT	NT	–	–	NT
7. EA$_{PLAICE}$	–	NT	–	–	NT	NT	NT	NT	–

E = erythrocytes

A$_x$ = antibody

x = source of ab

NT = not tested

* = 2 out of 5 rays tested had positive rosettes in spleen cell suspensions, these ·were identified as lymphocytes.

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erythrocytes and uncoated erythrocytes, except on one occasion when spleen cells were incubated with uncoated erythrocytes. In this case, a small number of rosettes were observed (< 1%). On another occasion eel blood and spleen cell suspensions were observed to be negative after 1 h and so were incubated overnight at 4°C. No rosettes were subsequently observed, but in the case of the spleen cells all the bovine erythrocytes had lysed. This was true of all treatments, i.e. even with uncoated erythrocytes. Erythrocytes incubated with eel blood leucocytes however were still present in large numbers even after overnight incubation.

Mammalian antibody-coated erythrocytes incubated with rat spleen cells served as a positive control, the percentage of rosette-forming cells varying between 20 and 35% of adherent macrophages.

ii) Receptors for soluble antigen-antibody complexes

Only dogfish leucocytes were tested for Fc receptors using soluble immune complexes.

Adherent blood cells took up fluoresceinated BGG alone to a limited extent as judged by fluorescence ($\sim$1%) and these cells appeared to be granulocytes.

With "dogfish anti BGG-BGG" complexes over 90% of the granulocytes showed fluorescence. In no case was the fluorescence arranged around the periphery of the cell as would be expected if Fc receptors were present.

Dogfish adherent blood cells were also tested with KLH-antibody complexes.

In the case of KLH alone and also 'KLH-natural antibody' complexes (i.e. using normal dogfish plasma) very little fluorescence was seen associated with the adherent cells.

With "rabbit anti KLH-KLH" and "dogfish anti KLH-KLH" complexes

(i.e. dogfish immunized with KLH) a great deal of fluorescence was associated with the cells though again not noticeably around the edge of the cell.

The explanation for the fluorescence appeared to be that both with BGG and KLH the immune complexes precipitated before being added to the phagocytic cells, it therefore seemed that the cells were endocytosing precipitate as opposed to binding soluble complexes to the membrane via receptors.

The experiment was subsequently repeated using dogfish and . rabbit antibody at a sub-precipitating dilution. In this case very little fluorescence was seen in any of the treatments and any that was present was associated with the granules of granulocytes.

iii) Fc receptors on trypsin-treated blood leucocytes

Dogfish blood leucocytes did not exhibit rosettes after treatment with trypsin when incubated with either dogfish, plaice or rat antibody-coated erythrocytes or with uncoated erythrocytes.

C_3 Receptors

The results of the attempts to demonstrate complement receptors are given in Table 16.

Negative results were obtained, using all methods described, for fish leucocytes. Rat spleen adherent cells were invariably positive though results varied from 20 to 75%.

As with Fc receptors prior treatment with trypsin did not reveal any previously hidden C_3 receptors.

Surface Immunoglobulin

A small percentage (< 25%) of dogfish adherent cells, whether

Table 16

Results of C_3 receptor test

Complement source	DOGFISH		PLAICE		EEL		RAT
	Blood	Spleen	Blood	Spleen	Blood	Spleen	Spleen
P	-	-	-	-	-	-	-
PC_{RAT}	-	-	-	-	NT	NT	+(40%)*
$PC_{DOGFISH}$	-	-	-	-	NT	NT	-
PC_{PLAICE}	-	-	-	-	NT	NT	-
PC_{EEL}	NT	NT	NT	NT	-	-	NT

P   =   particle (i.e. bacteria or yeasts)

C_c =   complement

x   =   source of complement

NT   =   not tested

*   =   ~40% is the mean of three observations

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from spleen, peritoneal cavity or blood showed surface fluorescence, indicative of surface immunoglobulin.

The "rabbit anti dogfish immunoglobulin" serum was absorbed against washed dogfish erythrocytes but even so several precipitin lines were revealed by agar-gel diffusion indicating that the serum was possibly not specific to dogfish immunoglobulin.

Discussion

The failure to demonstrate Fc and C_3 receptors on leucocytes of the fish species tested, may be correlated with the demonstration that phagocytosis by dogfish leucocytes is not significantly enhanced by the presence of plasma containing natural antibody.

The occasional positive rosettes observed with ray and eel spleen cells may have revealed lymphocytes producing natural antibody in these fish. However this is unlikely as plaice and dogfish also possess natural antibody and rosettes were not observed in these species. Furthermore, approximately 20% of ray thymus cells formed spontaneous rosettes (Wrathmell and Parish, 1980) further evidence against the rosettes being formed on antibody producing cells. Possibly a proportion of ray and eel lymphocytes express non-specific receptors for erythrocytes such as human T cells express for sheep erythrocytes.

The lysis of bovine erythrocytes which occurred when they were incubated with eel spleen cells was also occasionally observed with dogfish spleen cells (Wrathmell, personal communication). This may represent some natural cyto toxic ability amongst spleen cells similar to the 'natural killer' cells which exist in mammals.

Few reports exist in the literature which describe attempts to demonstrate receptors on fish leucocytes for antibody or complement.

Weissmann et al. (1975) demonstrated an opsonic effect of heat-aggregated, autologous IgM on the uptake of liposomes by blood phagocytes of the smooth dogfish (Mustelus canis). Aggregated IgM-coated liposomes were phagocytosed to a greater extent and more quickly than uncoated liposomes or liposomes coated with native IgM. The experiment was carried out at 30°C and this may have affected

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the result as phagocytes from S. canicula do not exhibit optimal

phagocytic activity at temperatures above 25°C. Presumably cells from

M. canis have a broader range of temperature tolerance than

S. canicula which is a cold-water species.

In addition, Weissmann et al. (1978) demonstrated a form of

ADCC, mediated again by heat-aggregated IgM, of M. canis phagocytes

against antibody-coated sea urchin (Arbacia punctata) eggs. These

reports suggest the presence of Fc receptors for this form of antibody

on the phagocytic cells of M. canis.

Earlier, in the literature review, it was suggested that there

would seem to be little value in possessing Fc receptors for so

artificial a form of antibody. However it has been suggested that

the way in which antigens mediate their effects is by aggregating

the antibodies to which they bind (Fewtrell and Metzger, 1980). If

this is, in fact, what occurs in vivo then the heat-aggregation may

not be as artificial as was formerly supposed. However, it would

therefore be expected that aggregation should occur in vitro if

antibody is complexed with antigen.

In the present study, heat-aggregated (62°C for 10 min) IgM was

not tested, although in one instance, to decomplement the serum it

was heated at 56°C for 30 min which may well be adequate to aggregate

fish antibodies.

Another report implicating the presence of Fc receptors

described a serum induced cell mediated cytotoxicity response of

nurse shark leucocytes (Pettey and McKinney, 1980). Antibody mediated

cell cytotoxicity in mammals requires the presence of Fc receptors;

however these were not investigated in the nurse shark, nor is it

certain that the mechanism involved is necessarily similar to that in

mammals.

Braun-Nesje et al. (1981) noted that salmonid pronephros macrophages did not form rosettes in the presence of sheep erythrocytes coated with rabbit IgG, rabbit IgM or mouse complement. In addition, Holmquist et al. (1980) reported that peritoneal macrophages of two marine teleosts were only minimally more phagocytic to sheep erythrocytes in the presence of rabbit antibody, and furthermore, coated erythrocytes were not lysed by these macrophages.

These two reports indicate that fish macrophages do not possess receptors for mammalian immunoglobulins (even IgM which resembles fish antibody). The evolutionary distance between fish and mammals may mean that immunoglobulin structure is so different to make them incompatible, alternatively they may support the premise that fish leucocytes do not possess Fc receptors.

In fact, receptors for IgM have only rarely been reported for mammalian phagocytes (Rhodes, 1973; Grewal et al., 1978), and in the former of these two reports, the IgM receptor was for the monomeric form of the antibody. The antibody class for which mammalian macrophages have receptors is predominantly IgG (Huber and Fudenberg, 1970; Bianco et al., 1975; Grewal et al., 1978; Montarosso and Myrvik, 1978; Zuckerman and Douglas, 1979).

Between different mammalian species the subclasses for which there are receptors vary. For instance, in humans these are IgG1 and IgG3, whereas in guinea-pigs IgG2 is the subclass with receptor binding capacity. Within a single species receptors are highly specific for immunoglobulin class and subclass, but will bind the appropriate immunoglobulin from another mammalian species (Zuckerman and Douglas, 1979).

Jean Davey and Asherson (1967) have shown that treating guinea-pig macrophages with trypsin resulted in increased numbers of

IgG rosettes. In the present study, prior treatment of dogfish leucocytes with trypsin did not reveal Fc receptors which were previously not demonstrable.

C_3 receptors have been demonstrated on mammalian leucocytes (Huber and Fudenberg, 1970; Bianco et al., 1975; Müller-Eberhard, 1975; Harmsen and Jeska, 1980). The methods using C_3-coated bacteria and yeasts incubated in serum to activate the alternate complement pathway, may not in retrospect have been applicable to the fish studied. Fish sera invariably contained natural antibody, and so there was presumably no requirement for properdin activation, as the binding of antibody would have activated complement via the classical pathway.

In addition, the method assumes that the complement system of fish is similar to that of mammals. A comprehensive study of the complement system of another elasmobranch the nurse shark (Ginglymostoma cirratum) has been carried out (Jensen and Festa, 1980). The complete system has been shown to consist of 6 components as opposed to the 9 of the mammalian system. There was no evidence for the existence of labile intermediate complexes, or for inhibitors or inactivators comparable to those found in the mammalian system. No C_3 or C_5-type activity could be demonstrated for any of the components or an alternate complement pathway. The authors suggested that the nurse shark may not need sophisticated control systems as it is insensitive to histamine and shows no hypersensitivity reactions such as are found in mammals and in which C_5 is known to be involved.

C_3-receptors are the means by which IgM-antigen complexes become bound to mammalian phagocytes, as IgM receptors are generally absent on these cells. Complement can have an additive effect in

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antigen binding when present with IgG, and it is interesting that the human IgG subclasses (1+3) which bind to monocytes and macrophages are also those which fix complement most efficiently.

The role of Fc and C_3 receptors on phagocytic cells appears to be in promoting phagocytosis, (C_3 being important early in the infective process when generally insufficient levels of specific antibody are available (Müller-Eberhard, 1975)) in mediating ADCC and possibly in interactions with B cells (Huber and Fudenberg, 1970; Zuckerman and Douglas, 1979). Fc receptors exist on a variety of cell types suggesting a heterogeneity of receptor function.

Rhodes (1973) observed that IgM has been demonstrated on the surfaces of T cells in a monomeric form, and has demonstrated receptors for monomeric IgM on guinea-pig splenic macrophages. He proposed therefore that the monomeric IgM receptor may be involved in the collaboration of macrophages with T cells. Feldmann (1972) postulated that binding by macrophages of monomeric IgM-antigen complexes released by T cells may facilitate the presentation to B cells of antigenic determinants in an appropriately spaced lattice.

There is no unequivocal evidence that fish have distinct B and T cell analogues and if, as seems possible, the lymphocytes of fish can perform both T and B type functions, there may be no requirement for the interactions described above and mediated by Fc and C_3 receptors.

Huber and Fudenberg (1970) have implicated Fc and C_3 receptors in the trapping of antigen by dendritic cells. They observed that IgG-antigen complexes bound to phagocytes and were ingested, whereas IgM-antigen complexes only bound to phagocytes in the presence of C_3 and even so were not ingested.

Via the latter system it seems that antigen could remain

extracellularly on the membrane of the dendritic cell in close contact with lymphoid cells.

In fish, where distinct germinal centres have not been demonstrated, it has been hypothesized that the melanomacrophage centres (MMC) could act as germinal centres in an immune reaction. It has been shown that as macrophages accumulate in MMC there is a concomitant increase in associated lymphoid cells (Ferguson, 1976b).

It is difficult to envisage how this could apply in the case of the dogfish as pigmented macrophages accumulate in the liver on antigenic stimulation and do not attract large numbers of associated lymphoid cells. The elasmobranch may therefore represent a more primitive stage in the development of the immune response at least in terms of antigen processing.

However, in the case of the teleosts, it would be useful to study the status of melanomacrophages with regard to their surface properties. Ellis (1976) found surface immunoglobulin on a proportion of splenic macrophages, a large number of which appeared to be pigmented. This may mean that permanently cell-bound antibody is important to these fish in antigen binding.

Chiller et al. (1969) described cells from the pronephros and spleen of rainbow trout with the morphological characteristics of macrophages, capable of forming rosettes with sheep erythrocytes. The erythrocytes formed a single layer around macrophages but multiple layers around lymphocytes leading the authors to suggest that macrophages had surface (cytophilic) antibody while the lymphocytes were actively producing antibody.

Only a small number of dogfish phagocytes were positively fluorescent when stained for surface immunoglobulin. This suggests however that a few spontaneous rosettes should be formed with uncoated erythrocytes.

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The only spontaneous rosettes formed, occurred in a few of the rays and eels tested and even here the cells involved had the morphology of lymphocytes and not macrophages.

In more recent work on mammalian cell surface receptors, purified antibody preparations and complement components have been employed to visualise receptors (Bianco, 1976). Had highly purified reagents been available in this study the work would perhaps have proved more conclusive, but as yet these components have only been isolated from a few fish species and even these have not been fully characterised.

Since phagocytosis occurs in the presence or absence of serum, and the current evidence seems to indicate that the cellular immune response of fish is less sophisticated than that of mammals, there would appear to be less demand for Fc and C_3 receptors.

Chapter 6

Delayed Hypersensitivity

Results

i) Skin tests

In all the fish used only two showed any lesion or swelling at the site of antigen deposition, and both of these were associated with BCG. This appeared to be a non-specific reaction as a lesion developed round a BCG challenge site in a BSA sensitised fish and also around the site of one of the BCG sensitising injections in a second fish.

Immunization with BCG and subsequent challenge with purified protein derivative (PPD) of this bacterium did not result in any lesion or swelling at the challenge site, again indicating a lack of specificity.

BSA and KLH did not cause typical hypersensitivity lesions at challenge sites in either previously sensitised or control fish.

The possibility that insufficient time was allowed for sensitisation was tested by performing skin tests using an extract of Proleptus obtusus, (the preparation consisted of an ammonium sulphate-precipitated protein extract) in addition to PPD and phosphate buffered saline (PBS).

However again no lesions were observed in the fish tested. (The fish were subsequently killed and dissected to confirm the presence of appreciable numbers of the nematode)

Only three plaice were tested. Two were BCG immunized and a third acted as a control. They were challenged with PPD, BSA and BCG but no hypersensitivity reactions were observed within three weeks. They were therefore re-challenged, but again up to three weeks

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afterwards no response was elicited.

Six eels were also examined for the development of hypersensitivity reactions. Two received BCG as sensitising antigen and two BSA. The remaining two fish acted as controls. They were all challenged intradermally with both BSA and BCG.

In less than two weeks all six fish exhibited a reaction which gradually ulcerated to form open lesions around the BCG challenge sites but not the BSA sites. In addition, one of the BCG sensitised eels also developed a large lesion around the site of the sensitising injection. The lesions were fixed for wax histology (see Plate 28) and on examination revealed that an acute inflammatory response was occurring with, what appeared to be, large numbers of granulocytes and monocytes infiltrating the musculature of the body and causing widespread destruction of muscle fibres leading to the formation of a cavity in the body wall. This occurred in all six fish to a greater or lesser extent but showed no correlation with the sensitising antigen.

The experiment was repeated by immunizing an eel with BCG and skin testing with PPD, which did not, however, lead to the formation of necrotic lesions.

Finally, eight eels were taken and two sensitised with sheep erythrocytes, two with sheep erythrocytes in Freund's Complete Adjuvant (FCA), two with BCG and two with FCA. The first four were challenged with sheep erythrocytes and the remaining four with BCG intradermally. No lesions developed in any of these fish.

ii) MIF Tests

The results of the various MIF tests are detailed in Table 17. All the results were obtained using blood leucocyte suspensions as splenic leucocytes from both dogfish and eels did not migrate from

Table 17

Results of 'Migration Inhibition Factor' Tests

Expt No. 1*	Fish Sp.	Immunogen	MIF Challenge AG	Challenge AG Concentration	M. Index[2]
2	Dogfish	BSA	BSA	O	1
				100 µg/ml[3]	1.12
				200　"	1.22
				300　"	1.29
3	Dogfish	BSA in FCA	BSA	O	1
				312.5 µg/ml	1.058
				625　"	0.917
				1250　"	0.987
4	Dogfish	BCG	BCG[4]	O	1
				1/800 *3	0.89
				1/400	0.87
				1/200	0.73
		BSA	BCG[5]	O	1
				1/800	1.08
				1/400	0.8879
				1/200	0.5846
		NONE	BCG[6]	O	1
				1/800	0.99
				1/400	1.09
				1/200	0.693
		BCG	BSA	O	1
				40 µg/ml	1.15
				100　"	1.106
				200　"	1.16

Table 17 Continued

Expt No	Fish Sp.	Immunogen	MIF Challenge AG	Challenge AG Concentration	M. Index
4 Cont	Dogfish	BSA	BSA	0	1
				40 µg/ml	1.04
				100 "	1.18
				200 "	1.08
		NONE	BSA	0	1
				40 µg/ml	0.958
				100 "	1.038
				200 "	0.85
SAME EXPERIMENT. MIF TESTS REPEATED 1 WEEK LATER					
		BCG	BCG[4]	0	1
				$^1/800$	1.385
				$^1/400$	1.15
				$^1/200$	1.32
		BSA	BCG[5]	0	1
				$^1/800$	1.007
				$^1/400$	0.83
				$^1/200$	0.62
		NONE	BCG[6]	0	1
				$^1/800$	1.17
				$^1/400$	0.96
				$^1/200$	1.17
		BSA	BSA	0	1
				40 µg/ml	1.57
				100 "	1.53
				200	1.43

Table 17 Continued

Expt No	Fish Sp.	Immunogen	MIF Challenge AG	Challenge AG Concentration	M. Index
4 Cont	Dogfish	BCG	BSA	0	1
				40 μg/ml	1.47
				100 "	1.84
				200 "	1.24
5	Dogfish[1]	BCG	PPD	0	1
				100 μg/ml	1.07
				200 "	0.8
				400 "	0.763
		BCG	BSA	0	1
				100	0.898
				200	0.7176
				400	0.685
REPEATED ON THIS FISH AND 3 OTHER CONTROL FISH					
		BCG	PPD	0	1
				100 μg/ml	1.34
				200 "	1.25
				400 "	0.95
		NONE	PPD	0	1
				100 μg/ml	0.8357
				200 "	0.728
				400 "	0.7
		NONE	PPD	0	1
				100	1.06
				200	1.17
				400	0.911

Table 17 Continued

Expt No	Fish Sp.	Immunogen	MIF Challenge AG	Challenge AG Concentration	M. Index
5 Cont	Dogfish	NONE	PPD	O	1
				100 µg/ml	0.86
				200 "	0.81
				400 "	0.81
6	Dogfish	KLH	KLH	O	1
				50 µg/ml	1.27
				100 "	1.27
				200 "	1.31
		KLH	KLH	O	1
				50 µg/ml	1.16
				100 "	1.13
				200 "	1.12
		NONE	KLH	O	1
				50 µg/ml	0.98
				100 "	0.97
				200 "	1.07
7	Dogfish	KLH	KLH	O	1
				100 µg/ml	1.22
				200 "	1.0
		KLH	KLH	O	1
				100 µg/ml	1.2
				200 "	1.36
		NONE	KLH	O	1
				100 µg/ml	1.41
				200 "	1.84

Table 17 Continued

Expt No	Fish Sp.	Immunogen	MIF Challenge AG	Challenge AG Concentration	M. Index
7 Cont	Dogfish	NONE	KLH	0	1
				100 µg/ml	1.15
				200 "	1.36
		KLH	BGG	0	1
				100 µg/ml	0.89
				200 "	1.16
		KLH	BGG	0	1
				100 µg/ml	1.15
				200 "	0.86
		NONE	BGG	0	1
				100 µg/ml	1.21
				200 "	1.33
		NONE	BGG	0	1
				100 µg/ml	1.27
				200 "	1.31
8	Eel	BSA	BSA	0	1
				50 µg/ml	0.967
				100 "	0.875
		BCG	BCG	0	1
				$^1/100$	0.87
				$^1/50$	0.517
		NONE	BCG	0	1
				$^1/100$	0.688
				$^1/50$	0.867
8	Eel	BCG	PPD	0	1
				200 µg/ml	0.985
				400 "	1.02

Table 17 Continued

Expt No	Fish Sp.	Immunogen	MIF Challenge AG	Challenge AG Concentration	M. Index
8 Cont	Eel	BCG	BSA	O	1
				200 µg/ml	0.77
				400 "	0.88

1. Refer to Material and Methods for expt. no.

2. Ml = migration index and is calculated by dividing the migration in test antigen dilution by the migration in the absence of antigen.

3. BCG (Glaxo) 1 ampoule made up to O.3 mls and dilutions made from this.

4. Same fish, tests performed at different times.

5. Same fish, tests performed at different times.

6. Same fish, tests performed at different times.

Underlined values are those showibg significant inhibition.

the capillary tubes even in the absence of antigen.

With reference to Table 17, it is evident that in most cases less than 80% of the control migration was not obtained (80% is taken as the level below which migration must be inhibited to give a positive result - see Hudson and Hay, 1980). When significant inhibition did occur it was not a specific reaction, as control cells and cells from fish having received unrelated sensitizing antigens, were inhibited. Generally inhibition occurred at the highest antigen concentration tested. Furthermore, a fish which showed significant inhibition on one occasion often showed no inhibition when tested a week later.

Certain antigens were consistently better than others at producing inhibition - notably BCG and PPD which elicited inhibition from several fish in this study though not necessarily specifically in fish presensitised with BCG.

Plate 28

a)   Longitudinal section through lesion caused by B.C.G. in dermis
      and underlying musculature of the eel. Note the massive
      inflammatory influx of leucocytes has caused muscle breakdown.
      LM (x 229) Mallory's Triple Stain. Bar ≡ 100µ.

                    M = muscle

                    L = leucocytic infiltrate

b)   Longitudinal section through B.C.G.-induced inflammatory lesion
      in the eel. Note that the action of the leucocytes has caused
      complete degradation of musculature leaving a 'hole' where the
      muscle was before. LM (x 91.6) Mallory's Triple Stain.
      Bar ≡ 100µ.
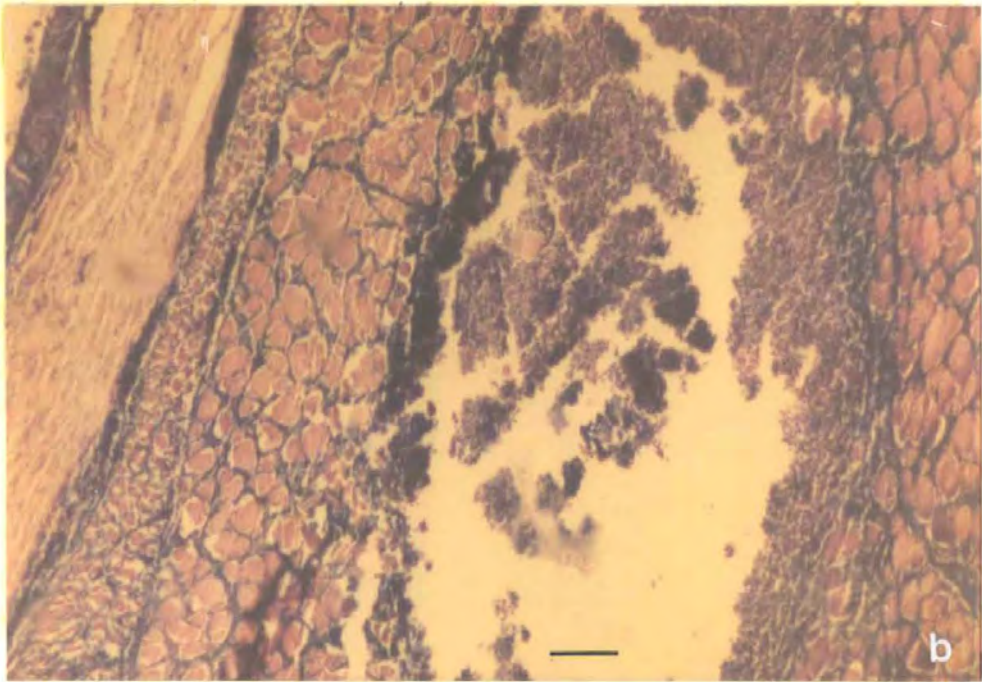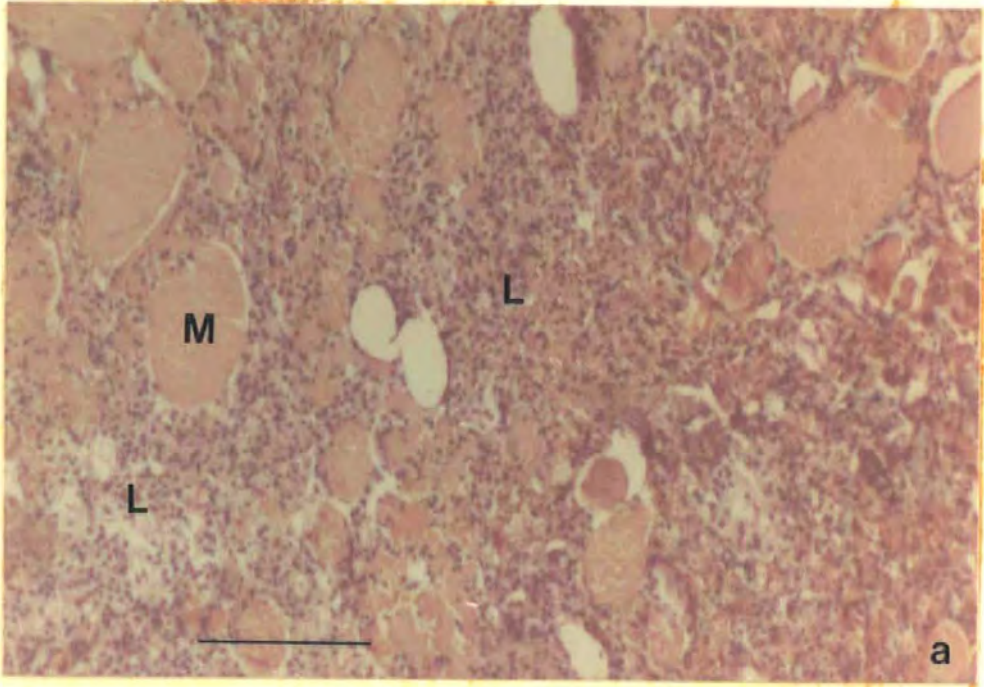
Discussion

The results showed that none of the fish tested demonstrated typical delayed hypersensitivity reactions. Where lesions or swellings occurred they were always associated with BCG. BCG is an attenuated bovine strain of Mycobacterium tuberculosis. The Mycobacteria contain large quantities of lipids (notably Wax D) in their cell envelopes, which contribute to their immunogenicity in mammals (Wolinsky, 1980). Since skin tests did not show positive reactions to PPD, it seems possible that the lesions which developed were non-specific inflammatory reactions induced by the large amounts of lipid present in BCG (as lipids have been shown to be relatively potent inflammatory agents).

Another salient point is that even with BCG not all fish reacted suggesting that individual fish, differ in their ability to mount an inflammatory response, possibly due to the degree of 'stress' they experience. The lack of response in dogfish does not appear to be due to insufficient time between sensitization and challenge as no response was elicited to the Proleptus antigen extract. Dogfish contain large numbers of these worms in their guts and where they attach there is a marked inflammatory reaction in the adjacent host tissue. Since the worm is therefore in contact with the cellular elements of the dogfish immune system it would seem likely that if sensitization was possible it would occur in these circumstances.

The BCG lesion in the eel was reminiscent of an acute inflammatory response with suppuration as has been described in mammals (Muir, 1964). In suppuration there is a progressive influx of polymorphonuclear leucocytes which fill the adjacent tissues followed by a gradual destruction and disappearance of tissue elements which become replaced by pus. Suppuration therefore differs

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from simple necrosis in that in the latter the tissue may retain its structural outline for some time.

The above described reaction is evidently occurring in the eel lesions (see Plate 28). Although the leucocytes of the eel have not been conclusively characterised, at high magnification the lesions were observed to be composed of some monocyte/macrophage type cells but predominantly of granular cells.

This latter observation is further evidence for the proposal that inflammation is occurring as opposed to a specific delayed hypersensitivity reaction. Dvorak (1974) in describing delayed hypersensitivity reactions notes that the infiltration of cells is essentially composed of mononuclear cells whereas in inflammation there is an initial exudation of neutrophils. Although he concedes that neutrophils may be involved in delayed hypersensitivity lesions he adds that caution should be exercised in evaluating their role, as delayed hypersensitivity may be accompanied by reactions which involve neutrophils. These include Arthus and anaphylactic responses, severe delayed responses which lead to tissue necrosis and also skin tests using antigens contaminated with irritants such as bacterial endotoxin.

From the above information, the presence in the eel lesions of large numbers of granulocytes would not be sufficient to discount delayed hypersensitivity, but when considered with the apparent non-specificity of the reaction, this fact becomes more significant.

Delayed hypersensitivity has been poorly studied in fish, however in the reports which exist, several authors have claimed to have demonstrated hypersensitivity reactions in elasmobranchs and teleosts but variably in agnatha.

Papermaster et al. (1963) reported that hag fish did not develop

delayed allergy to tuberculin, nor did they show an inflammatory response at the site of injection of Freund's Complete Adjuvant.

However, in the bullhead Ameriurus melas, a teleost, the same authors described delayed sensitivity following tuberculin sensitization.

Ridgway et al. (1966) reported a delayed corneal reaction to PPD in rainbow trout, one month after their sensitization with FCA. The reaction was maximal at 3-5 days but lasted 12 days in total as compared to a faint clouding of the cornea seen in control fish, which lasted only 2 days.

Finstad and Good (1966) showed that cyclostomes and lower fishes developed necrotic lesions at the sites of subcutaneous injections of FCA. Marchalonis (1977) reported similar results in the 'dogfish shark' and suggested that these observations may indicate that a phenomenon resembling delayed hypersensitivity occurs in primitive vertebrates. This observation does not appear to consider the lack of specificity of the reactions described or the fact that FCA is composed of mineral oil and heat-killed Mycobacterium bovis, both of which could cause non-specific inflammation.

Workers on mammalian cell-mediated immunity have defined delayed hypersensitivity in terms which may not be applicable to lower vertebrates, even though these animals may exhibit a functionally similar reaction.

Mackaness and Blanden (1970) described delayed hypersensitivity as having the following features:-

1) A maximum local response 18-24 hours after the injection of specific antigen.

2) Persistence of the response beyond this time.

3) A mononuclear cell infiltrate at the reaction site.

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4) A dependence on living cells for its passive transfer to normal recipients.

A fundamental property of the reaction is its dependence on sensitised lymphocytes and it is the transfer of living lymphoid cells which passively confers reactivity (Dvorak, 1974). These lymphoid cells are T lymphocytes (Roitt, 1977).

In poikilothermic animals the time for the emergence of a reaction may extend beyond the 24 - 48 h observed in mammals, thereby rendering at least by this criterion, the above definition unsuitable for fish.

Since the leucocytes of fish, in particular the lymphocytes, have not been conclusively identified and their functions made clear, it is not possible as yet to determine whether immune phenomena such as delayed hypersensitivity occur at this level of evolution.

The MIF tests on blood gave equally non-specific and random results with both eels and dogfish, and provided no more evidence than the skin tests to justify the proposal that fish (i.e. dogfish and eels) are capable of mounting a delayed hypersensitivity reaction.

The MIF test is an in vitro test for delayed hypersensitivity. Sensitised mammalian T-cells produce a lymphokine known as migration inhibition factor which is a glycoprotein of MW between 35,000 and 55,000. This acts on leucocytes particularly macrophages. In the test, leucocytes are prevented from migrating out of capillary tubes when specific antigen is present in the surrounding tissue culture medium. Migration is not inhibited if specific antigen is absent or an unrelated antigen is present.

The random response of dogfish and eel leucocytes when tested by this method suggests either that conditions were unsuitable or that there is no basis for assuming that such a mechanism occurs in fish.

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Few reports exist which describe the MIF tests on fish leucocytes.

McKinney and Sigel (1974) observed antigen specific inhibition of cell migration in a holostean fish, the gar (L. platyrhinchus).

Ellis (1978) quoted that in the virus condition lymphocystis, the late stages of the lesions are characterised by caseation and invasion of the tissues by lymphocytes and coincides with the development of a positive macrophage migration inhibition test in blood samples in vitro.

Smith et al. (1980) have used MIF tests to test the efficacy of various vaccination procedures against furunculosis in brown trout. They found that the percentage of fish producing a positive cellular response against a natural challenge strain of A. salmonicida (as measured by MIF tests) corresponded closely to the percentage found resistant to furunculosis in vaccine field trials.

They further found that the percentage fish showing cell-mediated immunity decreased with time, and at 18 months very few fish showed a positive response, although they did not know if this reflected a decrease in resistance to furunculosis.

From the above cited data there are evidently differences between fish species in their ability to mount delayed hypersensitivity reactions. Most of the more conclusive evidence concerns fish species of the Osteichthyes, and it is possible that well-defined hypersensitivity reactions do not emerge phylogenetically until this stage in the evolution of fish.

Evidently from the results observed here, it is impossible to draw any definite conclusions. More fish and a broader range of antigens require to be tested, but these preliminary findings do not support the hypothesis that a delayed hypersensitivity mechanism occurs in either dogfish or eels.

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Chapter 7

Conclusions

Initial examination of dogfish leucocytes showed that the cells
which were readily identified on morphological grounds (i.e.
lymphocytes, thrombocytes, monocytes and neutrophils) demonstrated
similar staining and functional characteristics to their mammalian
counterparts. However, the remaining granulocytes could not be
definitively characterised by any of the techniques used or by
comparison with mammalian leucocytes. This may suggest that these
remaining cells are either developmental stages of the mature, defined
leucocytes or quite distinct, piscine cells with no obvious parallels
in higher vertebrates.

To obtain more conclusive results with regard to these latter
cells, studies concerned only with characterisation of one cell type
would be useful. Enhanced cell separation techniques, such as those
used by Braun-Nesje et al. (1981) to obtain pure populations of
salmonid macrophages, might be usefully applied to these latter
cells in order to make a definitive characterisation.

The investigation of phagocytosis and antigen clearance both
in vitro and in vivo suggests that the dogfish has a poorly developed
phagocytic system, in that injected antigens remain in the circulation
for long periods. This species would seem, therefore, ill-equipped
to defend itself against external pathogens. However, a number of
environmental and physiological features should be taken into
consideration.

Firstly, dogfish live in deep sea-water which rarely rises
above 10°C in temperature. At this temperature, although their own
metabolism is slow, bacteria and other potentially invasive organisms
also multiply very slowly. It is highly unlikely that in the natural

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state the fish would face an influx of such large numbers of bacteria. Secondly, those which did gain entry would perhaps be adequately dealt with by the unfavourable internal environment where there are high levels of urea, trimethylamine oxide, salt and also natural antibodies present in the circulation.

The elements of the immune response may differ from one species to another, as may the environmental conditions required for the demonstration of immune phenomena (Ellis, 1974). Studies involving only a few fish species should not therefore lead to generalisations covering all fish, as this group contains more species than all other vertebrate groups combined (Ellis, 1974), and so the scope for variation is wide.

Phagocytosis evidently takes place but this could primarily be a mechanism to eliminate effete cells of intrinsic origin and antibody-neutralised antigens. The observation in the present study and also elsewhere that various cells other than typical phagocytes can phagocytose material was attributed in the literature survey, to an incompletely differentiated cell system in fish. However Boyden (1963) has observed that "it is not often appreciated that the capacity to phagocytose is shared by most cell types in the mammalian body. Phagocytosis has been reported to occur in fibroblasts, renal tubular cells, endodermal cells of the intestine, pigment cells of the retina, smooth muscle cells, skeletal muscle cells, certain epithelial cells and possibly platelets". The capacity for phagocytosis is therefore a fundamental characteristic of many cell types in addition to the specially modified granulocytes and mononuclear phagocytes.

The demonstration of melanin-containing cells in the blood system and the proposal that replete spleen phagocytes may depart via

the circulation to the liver, suggests that the mononuclear phagocyte system of fish is organised differently to that of higher vertebrates. The fundamental difference is evidently the lack of bone marrow in fish and so the precursors of macrophages must arise elsewhere in fish, possibly in the spleen? If the blood contains phagocytes which are in various stages of development, including fully mature cells, then it would appear that the system is arranged in a quite different way to that of mammals (Langevoort et al., 1970).

If the immune system of the dogfish is organised along relatively simple lines, then there may be no requirement for sophisticated mechanisms of cellular interactions, and this may explain the failure to demonstrate such immune phenomena as delayed hypersensitivity and the presence of surface receptors. Nevertheless, all the experimental techniques employed in this study were modified from methods described in the mammalian literature. The failure to demonstrate certain phenomena may therefore reflect that the conditions of assay were unsuitable for dogfish cells as opposed to suggesting truly negative results. For example, tissue culture media were modified from established mammalian media. Since the physiological status of most fish species is as yet ill-defined by comparison to mammals, it is impossible to ensure that in vitro conditions are suitable for the cells, tissues and body fluids of a particular fish species.

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