PHAGOCYTIC ACTIVITY IN FINFISH *Oreochromis mossambicus*

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CERTIFICATE

Certified that the dissertation entitled **"PHAGOCYTIC ACTIVITY IN FINFISH *Oreochromis mossambicus*"** is a bonafide record of work done by Shri. **MANOJ NAIR.R.**, under our guidance at the Central Marine Fisheries Research Institute during the tenure of his M.Sc. (Mariculture) Programme of 1994-'96 and that it has not previously formed the basis for the award of any other degree, diploma or other similar titles or for any publication.

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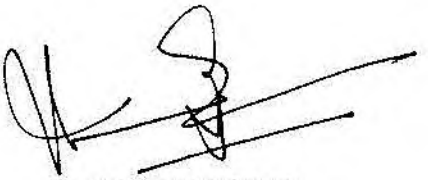

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सारांश

इनवाइवो और इन विट्रो तरीकों द्वारा ओरियोक्रोमिस मोसाम्बिकस में दिखाई जाने वाली अप्रमुख कोशिकीय सुरक्षा व्यवस्था फैगोसाइटोसिस का अध्ययन किया गया.

इनवाइवो में कोलोइडल कार्बन कणों के इन्जेक्शन द्वारा रेटिकुलोएन्डोथीलियल फैगोसाइटिक व्यवस्था का अध्ययन करने पर यह व्यक्त हो गया कि ओरियोक्रोमिस मोसाम्बिकस में ऐंटीजन स्थानीकरण के मुख्य अंग प्लीहा, वृक्क और परिकोष्ठ हैं. इन अंगों में प्लीहा में ऐंटीजन प्रग्रहण की अधिकतम क्षमता दिखाई पडी, जिसके बाद वृक्क में भी यह क्षमता देखी गई.

रक्त, वृक्क, प्लीहा और पेरिटोनियल नि:स्रवण कोशों को घनत्व प्रवणता अपकेंद्रीकरण, कांच के ढ़क्कन में आसंजन और निर्जीव यीस्ट कोशों के साथ ऊष्मायन करके वियुक्त किया गया और इन कोशों की इन विट्रो फैगोसाइटिक प्रक्रिया का अध्ययन किया गया.

ग्लास में आसंजित रक्त, वृक्क और प्लीहा के वियुक्त श्वेताणु आकृति में बृहत्भक्षकाणु (macrophages) थे. प्लीहा में कुछ कणिकाणुओं को भी दिखाया पडा. वृक्क और प्लीहा में कुछ कोशों को भी दिखाया पडा जिनकी भक्षकाणु क्षमता में कहनेलायक घटती नहीं है.

पेरिटोनियल नि:स्रवण कोश प्रमुखत: दो प्रकार के होते हैं बृहत्भक्षकाणु और कणिकाणु. कणिकाणु कुछ हद तक बृहत्भक्षकाणु होते है और अधिकांश कोशों में आसंजन देखा गया. भक्षकाणु कोशों में क्रोमैटिन का गुच्छन और कोशों के ऊपरि भाग में कणिकाओं का निर्गमन भी दिखाया पडा.

इस अध्ययन में रक्त के वियुक्त बृहत्भक्षकाणु की अंत:कोशिकीय हनन (intracellular killing ability) क्षमता भी दिखाया पडा.

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PREFACE

PREFACE

Aquaculture has grown into a significant industry in many parts of the world over the last twenty years. As fish farming is being done intensively under conditions of high stocking density, infectious diseases pose a constant and costly threat to successful aquaculture. Even when environmental conditions are good and fishes are healthy, certain infectious agents, if introduced into the farm can cause heavy mortalities. Though chemotherapy with chemicals and antibiotics have, to some extent helped to control a number of bacterial, fungal and parasitic infections, there is still no cure for viral infections.

Antibiotic treatment has a number of disadvantages such as, development of drug resistant strains of pathogens and public health hazards due to consumption of fish with antibiotic residues. It is against this background that **IMMUNOLOGY** has become an important tool in the hands of research workers and aquaculturists for developing devices aimed at prompt diagnosis and control of infectious diseases.

A competent immune system is essential for the fish to survive in a challenging environment where a variety of micro-organisms including virus, bacteria, fungi and protozoans also make their living. Even though Metchnikoff (1892, 1901, 1905) and his contemporaries reported that fish

are capable of mounting an immune response against foreign material late last century, knowledge about **FISH IMMUNOLOGY** is still in the developing stage and only in recent years has considerable progress made in this direction.

Fishes like other vertebrates, respond to infectious agents in both Specific and Non-specific manner, and appear to have similar cellular and humoral components as other higher vertebrates.

When a fish encounters a pathogenic micro-organism, the non-specific defence mechanism is more important than the specific defence mechanism because the former is least affected by temperature (Rijkers *et al.*, 1991) and the latter takes a longer time for antibody build up and specific cellular activation (Anderson, 1992a).

Thus the non-specific defence system especially the cellular defence mechanisms which includes phagocytosis and the intracellular killing by production of highly reactive Oxygen radicals, are quickly activated and protect the fish against invading pathogens.

While studies have been conducted for the successful collection of phagocytes and assay of their *in vivo* and *in vitro* activity, in various cold water fish species, such information is lacking in most tropical fishes. The fish *Oreochromis mossambicus* has been chosen for this study because it is

the most commonly cultured species in South-East Asia as a cheap protein source.

The study of the pagocytic activity of *Oreochromis mossambicus* was undertain with the aim of:-

1. Studying *in vivo,* The Reticuloendothelial Phagocytic system.

2. Isolation of Blood, Peritoneal, Spleenic and Kidney phagocytes.

3. Studing the *in vitro* activity of these cells.

INTRODUCTION

I. INTRODUCTION

Phagocytosis is the ingestion of particles by single cells. Phylogenetically, Phagocytosis is the oldest and the most fundamental defence mechanism in almost all animal species. In primitive organisms like amoeba, this process is directly related to nutrition as well as defence . With the evolution of metazoans, cells specially dedicated to the recognition and elimination of non-self materials emerged. The development of a vascular system also enhanced the speed with which these cells could be mobilized at the sites of injury to dispose off invading pathogens and damaged cells from the organism itself.

Although phagocytosis was first reported by Metchnikoff (1892, 1901, 1905) in Gold fish (*Carassius auratus*), studies on Phagocytic function in fish has been conducted mainly in Rainbow trout *(Oncorhynchus mykiss)*, Common carp *(Cyprinus carpio)*, Channel catfish *(Ictalurus punctatus)*, Plaice *(Pleuronectes platessa)*, and Japanese eel *(Anguilla japonica)* (Avtalion and Shahrabani 1975; Ellis *et al.*, 1976; Mori 1980; Braun - Nesje *et al.*, 1982; MacArthur *et al.*, 1983; Sakai 1984; Nagamura and Wakabayashi 1985; Scott *et al.*, 1985; Suzuki 1986; Sovenyi and Kusuda 1987; Chung and Secombes 1988; Kusuda and Taira 1989; Secombes 1990; Blazer 1991; Nakamura *et al.*, 1991; Sheldon and Blazer 1991; Jeney and Anderson 1993; Iida and Wakabayashi 1995).

The earliest study on the immune organs of *Oreochromis mossambicus* was done by Sailendri (1973) and Sailendri and Muthukkaruppan (1975), who studied the Histogenesis and morphology of the lymphoid organs. Jayaraman *et al.* (1979) studied the relationship between migration inhibition and plaque - forming cell responses to sheep erythrocytes in *Oreochromis mossambicus*.

Suzuki (1986), while studying the morphological and phagocytic characteristics of the peritoneal exudate cells in *Oreochromis niloticus*, reported the presence of three main cell types namely the Monocyte / Macrophage series cells, Neutrophils and Eosinophils. He further stated that these cells phagocytozed foreign materials added *in vivo* and *in vitro*, and the highest phagocytic capability was exhibited by the Neutrophils and the Monocyte / Macrophage cell types.

Doggett *et al.* (1987) after cytochemical studies with various stains reported the presence of four types of leucocytes in the peripheral blood of *Oreochromis mossambicus* namely Lymphocytes, Thrombocytes, Monocytes and Granulocytes. They stated that the granulocytes were of two types Type I and Type II, of which the Type I was phagocytic like the Monocytes.

Doggett and Harris (1989) examined the peripheral blood leucocytes of *Oreochromis mossambicus* electron microscopically for their structure and the *in vivo* response to colloidal carbon particles

and bacteria *Aeromonas salmonicida*. They reported a third type of granulocyte which was termed as Granulocyte Type III and this was found to be phagocytic.

Presence of lectin like receptors that bind to various sugars were noticed in macrophages isolated from gills, kidney and spleen of *Oreochromis spilurus*. These receptors were involved in the non-specific and specific recognition of the yeast *Candida guillerimondii* and the bacteria *Staphylococcus epidermis*. Complement recognizing receptors, which function in the recognition of opsonized micro-organisms, were also found in this fish (Saggers and Gould 1989).

Leung *et al.* (1995) studied *in* vitro the interaction of various virulent and avirulent strains of *Aeromonas hydrophila* with activated phagocytes. They found an increase in uptake of bacteria in the activated phagocytes. The intracellular replication of bacteria was also faster in activated phagocytes. Opsonized avirulent bacteria were sensitive to phagocyte mediated killing. Serum components and phagocytes were together found to prevent the growth of avirulent bacteria. They found the killing ability of phagocytes were different for the virulent and avirulent strains of *Aeromonas hydrophila*.

The modulation of phagocyte activity in *Oreochromis spp.* was studied by few workers. Low and Sin (1995) found significant decrease in phagocytosis and Chemiluminescent response in *Oreochromis aureus* phagocytes exposed *in vitro* to mercuric chloride.

Holladay *et al.* (1996) while studying the effect of organophosphate insecticide Chlorpyrifos (1 part per billion) *on Oreochromis niloticus* immune system, found significantly depressed phagocytic function and respiratory burst activity in isolated pronephric macrophages when compared with controls.

The Reticuloendothelial System

The reticuloendothelial system is the system of phagocytic cells widely dispersed throughout the body and is responsible for the removal of effete cells and particulate matter from the circulation.

In fishes, the reticuloendothelial system is the only means of defence during the early stages of development before immunocompetence is attained (Tatner and Manning, 1985).

The reticuloendothelial system has been studied by assessing the role of phagocytic cells in taking up various injected colloidal particles and substances which are used as models for the particulate or potentially harmful pathogens, the fishes encounter in their environment.

The cells of the teleost fish which are considered to comprise the reticuloendothelial system are the pro monocytes of the haemopoietic organs, the monocytes of the blood, the macrophages of the loose connective tissue, the free and fixed macrophages of the spleen, kidney

and the fixed macrophages of the atrial lining of the heart (Roberts 1978, 1989).

In teleosts, antigen uptake studies have revealed that kidney is the major organ for antigen localization. In kidney, the material is initially phagocytosed by the reticuloendothelial cell network within the haemopoietic parenchyma and then macrophages containing phagocytosed material aggregate in lymphoid areas often in the melanomacrophages (Mackmull and Michels 1932; Ellis *et al.*, 1976; Ellis 1980; Mori 1980; Secombes and Manning 1980; Ferguson *et al.*, 1982).

Cellular structure found throughout the teleost haemopoietic tissue and associated with the reticuloendothelial system are the melanomacrophage centers. These are pigment containing cells found either scattered or as aggregates in fish haemopoietic tissues.

The melanomacrophage centers are considered as metabolic dumps where circulating macrophages, replete with particulate matter of microbial or metabolic origin, home selectively (Roberts 1978; Agius 1980). The main pigment found in the melanomacrophages is melanin but variable amounts of lipofuscin and haemosiderin are also present (Ellis 1977; Agius 1980). There is no uniform agreement on the function of melanin in the melanomacrophage centres.

Several particles have been used to study the *in vivo* phagocytosis and organ localization of antigenic material in fish. They include Guinea pig erythrocytes, bacteria, vital dyes, silica and the most commonly used Indian ink (Metchnikoff 1892,1901,1905; Mesnil 1895; Wislocki 1917; Jansson and Waaler 1967; Ellis *et al.*, 1976).

Cells displaying Phagocytosis in fish

According to Ellis (1976), the basic criteria for identifying cell types in fishes are ontogenic, morphologic and functional. Ellis (1977) has reported morphologically different types of cells as being phagocytic in fish. From various studies undertaken, it has been concluded that the main phagocytic group of cells are the granulocytes and mononuclear phagocytes. The identification of fish phagocytes, especially granulocytes, is still controversial. Several possible reasons for this have been given by Ainsworth (1992).

Macrophages

Macrophages in teleosts have been reported from various fish tissues including the peritoneum, spleen, gills and atrium of the fish heart. They are also found in small numbers in the blood as monocytes but absent in the fish liver (Ellis *et al.*, 1976, Chilmonczyk and Monge 1980; Roberts 1989; Nakamura *et al.*, 1991).

According to Roberts (1989) monocytes in the kidney and blood are precursors of tissue macrophages which migrate to inflammatory sites, giving rise to macrophages when and where necessary.

Granulocytes

Ainsworth (1992) has described the presence of three types of granulocytes in fishes namely Neutrophils, Eosinophils and Basophils.

Neutrophils:- Neutrophils in fish are present in the kidney, blood and in inflammatory lesions. Phagocytosis by fish neutrophils is not yet demonstrated conclusively and even contradictory results have been reported (Ellis 1976; Ellis *et al.*, 1976; MacArthur and Fletcher 1985). Griffin (1983) has suggested that a certain stage of maturation is required before the neutrophils acquire the phagocytic competence. With the development of light and electron microscopy, the phagocytosis and intracellular killing ability of fish neutrophils became more clear (Ainsworth, 1992).

Eosinophils:- Although mammalian eosinophils are capable of phagocytosis, it is not their primary function. They are mainly involved in allergic reactions and antiparasitic immunity.

In fishes, Ezeasor and Stokoe (1980) hypothesized that the eosinophilic granular cells in the rainbow trout (*Oncorhynchus mykiss*)

were involved in the immune defence mechanism. In gold fish, artificially infected with a trematode parasite, the eosinophils adhere to the parasite, neutralize parasitic products, attract leucocytes to that area, and kill the parasite (Huizinga, 1980).

Though Hyder *et al.* (1983) has reported that the peripheral blood eosinophils of the nurse shark were not phagocytic, Bodammer (1986) found that the peritoneal exudate eosinophilic cells, of striped bass (*Morone saxatilis*) were phagocytic.

In *Oreochromis mossambicus*, three types of granulocytes have been reported namely, Type I, Type II and Type III out of which Type I and Type III were phagocytic (Doggett *et al.*, 1987; Doggett and Harris 1989).

Basophils:- Mammalian basophils play an important role in immediate hypersensitivity reactions. They also participate in delayed type hypersensitivity and perform endocytosis. According to Ainsworth (1992), in fishes, the functional role of Basophils is unclear and is still being studied.

Mechanism of Phagocytosis

The mechanism of phagocytosis proceeds in two steps namely, Phagocytosis and Intracellular Killing.

According to Blazer (1991) the phagocytosis in fish also proceeds through the series of steps similar to mammalian phagocytosis namely Signal, Pursuit, Surface recognition, Adherence and Engulfment.

When the phagocytes encounter invading micro-organisms, or any other stimuli, they produce a group of powerful oxidizing agents, which are their most important killing weapons (Babior, 1984). The intracellular killing is either **Oxygen Dependent** or **Oxygen Independent.**

Babior (1984) after studying the role of reactive oxidants in the intracellular killing by mammalian phagocytes, reported that reactive oxidants like Superoxide (O_2), Hydrogen Peroxide (H_2O_2), Singlet Oxygen (O_2), Hydroxyl radical (OH) and Hypohalite ions (XO) are produced from Oxygen through a special metabolic pathway called "RESPIRATORY BURST" that is unique to phagocytes. These highly reactive Oxygen containing compounds are responsible for the Oxygen dependent microbicidal activity of phagocytes.

The presence of Oxygen dependent mechanisms has been reported in a number of fishes. (Kanner and Kinsella 1983; Chung and Secombes 1988; Nagelkerke *et al.*, 1990; Sharp and Secombes 1993; Iida and Wakabayashi 1995).

Rowley *et al.* (1988) reported the presence of Oxygen independent microbicidal mechanism in fish consisting of cationic proteins, lysozymes,

lactoferrin and proteolytic enzymes. The bactericidal and larvicidal activity of fish phagocytes has been reviewed by Secombes and Fletcher (1992).

In addition to the earlier mentioned ***in vivo*** methods, ***in vitro*** methods have also been used to study phagocytosis.

Phagocytic leucocyte population is abundant in the spleen and anterior kidney (Pronephros). They are found in small quantities in the peripheral blood and in the peritoneal cavity. Several procedures have been adopted to isolate, enrich and concentrate these cells from the blood using single discontinuous density gradient (Blaxhall 1985; Waterstrat *et al.*, 1988).

Multiple layer discontinuous density gradients have also been used to separate leucocytes from the anterior kidney (Pronephros), peritoneal cavity and the spleen of fish (Braun - Nesje *et al.*, 1981; Chung and Secombes 1988; Sakai 1984; Anderson 1992b).

Blazer (1991) has reported that both **Direct** and **Indirect** methods have been used to study *in vitro* phagocytosis and the subsequent intracellular killing in fishes.

The most commonly used technique to evaluate *in vitro* phagocytosis is by calculating the **Phagocytic Index**. This is calculated

by counting, the mean number of particles ingested per phagocyte. Usually 100 or 200 phagocytes are counted to calculate the phagocytic index (Blazer 1991).

In fish the commonly used particles to study *in vitro* phagocytosis are yeast, sheep red blood cells, bacteria and latex beads (Sovenyi and Kusuda 1987; Thuvander *et al.*, 1987; Saggers and Gould 1989; Sheldon and Blazer 1991)

The other direct methods used include Flow Cytometry, Colony Forming Units (CFU), Fluorescent Technique and Bacterial Thin-Layer Method (Olivier *et al.*, 1986; Thuvander *et al.*, 1987; Blazer *et al.*, 1991; Nakayasu *et al.*, 1995).

In the indirect methods, the indirect measurements of various enzymes or the products of respiratory burst or both are used to quantify phagocytic activity as well as intracellular killing. The methods used are Nitroblue tetrazolium (NBT) reduction, Horseradish Peroxidase-dependent conversion of phenol red by hydrogen peroxide (H_2O_2), Reduction of Ferricytochrome C., Spectrophotometric measurement of congo red stained yeast cells and Chemiluminescence assay (Scott and Klesius 1981; Chung and Secombes 1988; Secombes *et al.*, 1988; Seeley *et al.*, 1990; Anderson *et al.*, 1991).

MATERIALS & METHODS

II. MATERIALS AND METHODS

2.1 Experimental Protocol

Healthy Oreochromis mossambicus with mean body weight of 200 g caught from Ajantha fish farm ,Narakkal were used in this study. About 40 fish were maintained in a 10 Ton round fibre glass tank. The fishes caught from brackish water were gradually acclimatized to fresh water and fed with a formulated pelleted feed @ 2% of body weight twice daily. 75 % of the tank water was changed daily. During the two week acclimatization period the fishes were observed daily for unusual behaviour, morphological changes and mortality. Water quality parameteres were also checked on alternate days. The mean water temperature during the trial was 27° C.

2.2 Studies on the Reticuloendothelial System

2.2.1. Intraperitoneal injection of colloidal carbon particles.

Indian ink (Camlin Ltd) was diluted in Normal saline (0.85 % NaCl) in the ratio 1:10. The thoroughly mixed suspension was centrifuged and then autoclaved. From this 0.5 ml was injected intraperitoneally to 16 fish and two fish each were killed at 2 hr., 8 hr., 16 hr., 24 hr., 48 hr., 5th day, 8th day and 10th day after the injection.

2.2.2 Histological and Cytological studies.

Blood and peritoneal touch smears were prepared on clean, grease free and sterilized glass slides. Kidney, spleen, peritoneum and heart were dissected and fixed in 10% buffered formalin for 24 hours. The tissues were dehydrated and embedded in paraffin wax. Sections of 6 µm thickness were cut using a rotary microtome. Deparaffinized sections were bleached in 20% Hydrogen Peroxide for 36-48 h to decolourize the melanin. (Ellis *et al* ., 1976) The sections were stained with Haematoxylin and Eosin. The slides were examined under the microscope and photomicrographs taken

2.3. *In Vitro* Studies

2.3.1 Isolation of Blood Leucocytes

Blood from the heart of healthy fishes were collected using heparinized 20G needles and 2ml sterilized, heparinized (0.1 ml of 25,000 I.U. ml^{-1} Heparin) plastic syringes.

Leucocytes from whole blood were separated by density gradient centrifugation as per the method of Blaxhall (1985).

Four ml of lymphocyte separation medium Histopaque -1077 (Sigma chemicals) was taken in a 10 ml sterilized glass centrifuge tube. 2 ml

of the extracted blood was carefully layered over the separation medium and centrifuged for 30 minutes at 400 *g* in a refrigerated centrifuge [Plate XIV]. A grey ring at the interface of blood plasma and the separation media was formed [Plate XV]. Using sterile pasteur pipette the supernatent of blood plasma down to the upper surface of the grey ring was carefully aspirated and discarded. With another sterilized pasteur pipette the leucocyte layer (grey ring) was aspirated without the separation medium.

The Leucocyte layer was transferred to another clean, sterilized 10 ml centrifuge tube containing three times the volume of chilled (4°C) Hanks balanced salt solution (Hi Media Chemicals) with 2% Fetal Calf Serum (Hi Media Chemicals), 100 I.U. ml^{-1} Penicillin , 100 µg ml^{-1} Streptomycin and 100 I.U. ml^{-1} Heparin. An even suspension made, was then centrifuged at 100 *g* for 5 minutes. The supernatent was aspirated and discarded. The pellet was again resuspended in cold Hanks balanced salt solution and the earlier step was repeated twice until the cells were free of the separation medium. The cells were finally suspended in cold Hanks balanced salt solution at a cell concentration of 2×10^6 cells ml^{-1} and used for the phagocytosis assay.

2.3.2 Isolation of Head Kidney Macrophages

Macrophages from kidney were isolated using the technique of Chung and Secombes (1988). The anterior kidney /Head Kidney of an

anaesthetised fish was carefully dissected out aseptically. The head kidney was pushed through a nylon mesh with cold Hanks balanced salt solution (Hi media Ltd.) containing 2% Fetal Calf Serum (Hi media), 100 I.U. ml^{-1} Penicillin, 100 µg ml^{-1} Streptomycin and 100 I.U. ml^{-1} Heparin (Himedia).

The resultant cell suspension was carefully layered over a 34 - 51% Percoll gradient separation medium and centrifuged at 400 *g* for 30 minutes at 4°C. The band of cells lying at the 34-51% interface was collected carefully using a sterilized pasteur pipette and transferred to another centrifuge tube containing cold Hanks balanced salt solution with 2% Fetal Calf Serum, 100 I.U. ml^{-1} Penicillin, 100 µg ml -1 Streptomycin and 100 I.U. ml^{-1} Heparin and centrifuged at 200 *g* for 5 minutes. This process was repeated three times to remove all traces of the separation medium. The pellet obtained after the final washing was resuspended in cold Hanks balanced salt solution containing 2 % Fetal calf serum, 100 I.U. ml^{-1} Penicillin and 100 µg ml^{-1} Streptomycin. The cell count was adjusted to $2x10^{6}$ cells ml^{-1} and used for the phagocytosis assay.

2.3.3 Isolation of Spleenic Macrophages

The method adopted here was modified from Anderson (1992b).

Spleen cell suspension was prepared from spleen aseptically removed from the fish killed by a cervical blow. Spleen was trimmed free of adherent fat and tissue was transferred to a sterile petri dish containing 5 ml cold Hanks balanced salt solution containing 2% Fetal Calf Serum (Hi Media Chemicals), 100 I.U. ml^{-1} Penicillin , 100 µg ml^{-1} Streptomycin and 100 I.U. ml^{-1} Heparin. A single cell suspension was made by tearing the spleen (after tearing the capsule) with a sterile curved toothed forceps. The cell suspension was transferred to a 15 ml centrifuge tube. The cell suspension was allowed to stand for 5 minutes for large cell clumps to settle. All but the settled debris was transferred into a second sterile centrifuge tube containing Histopaque -1077 (Sigma Chemicals) separation medium.

The cells were collected by centrifuging at 400 *g* for 30 minutes at 4° C. The Leucocyte band between the upper supernatent and the lower separation medium was carefully removed by means of a sterile 20 G spinal needle and transferred to another centrifuge tube. Cold Hanks Balanced salt Solution was added and then centrifuged at 200 *g* for 5 minutes. This washing was done three times to remove all traces of the separation medium. The pellet, after the final washing, was resuspended in cold Hanks balanced salt solution containing 2% Fetal Calf Serum, 100 I.U. ml^{-1} Penicillin and 100µg ml^{-1} Streptomycin. The cell concentration was adjusted to $2x10^{6}$ cells ml^{-1} and used for the phagocytosis assay

2.3.4 Isolation of Peritoneal Exudate Cells

The method followed was modified from Sakai (1984).

Sterilized liquid Paraffin (0.5ml/Fish) was injected intraperitoneally into six fishes. The fishes were maintained in 750 litre fibre glass tanks containing fresh water (Dechlorinated tap water) at 27° C and fed @ 2% body weight on formulated pelleted feed.

Two fish each were sacrificed on days 4, 5 and 11 after injection. The body surface of the anaesthetized fish was disinfected with 70% Alcohol and as much blood as possible was withdrawn from the heart and the caudal blood vessel.

The peritioneal cells were harvested under aseptic procedures by the following steps:

Ten milliliters of Hanks balanced salt solution containing 2% Fetal Calf Serum ml^{-1}, 100 I.U. ml^{-1} Penicillin , 100 µg ml^{-1} Streptomycin and 100 I.U. ml^{-1} Heparin was injected into the abdominal cavity of the bled fish. The abdominal region of the fish was gently massaged.

The medium containing the peritoneal exudate cells was aspirated using a sterilized pasteur pipette inserted through an incision made by a sterilized knife on one side of the fish.

The washings containing the peritoneal exudate cells with Hanks balanced salt solution and liquid paraffin were collected in silicon-coated centrifuge tubes and allowed to settle at 20° C for one hour. The upper layer of liquid paraffin in the tubes was discarded and the lower layer containing the peritoneal exudate cells with Hanks balanced salt solution was washed by centrifuging at 400 *g* for five minutes. The supernatent was discarded and the pellet was resuspended in Hanks balanced salt solution.

The washing procedure was done thrice to remove all traces of liquid paraffin.

The final pellet was resuspended in fresh chilled Hanks balanced salt solution with 2% Fetal calf serum, 100 I.U. ml^{-1} Penicillin , 100 µg ml^{-1} Streptomycin and 10 I.U. ml^{-1} Heparin. The cell concentration was adjusted to $2x10^{6}$ ml^{-1} and used for the phagocytosis assay.

2.3.5 Preparation of Antigens

2.3.5a *Preparation of Yeast Cell Suspension*

Commercial yeast *Saccharomyces cerviceae* was used for the preparation of yeast cell suspension. The yeast was ground finely into a powder using a clean, sterilized pestle and mortar to get individual cells. This powder was transferred into a sterile test tube and autoclaved at 120° C, 15 lbs for 30 minutes to kill the yeast cells. The killed cells were evenly suspended aseptically in cold Hanks balanced salt solution and the

cell concentration was adjusted to $4x10^7$ cells ml^{-1} and used for the phagocytosis assay.

2.3.5b Preparation of Formalinized Bacteria

Vibrio parahaemolyticus was used to prepare bacterial inocula and bacterial suspensions to be used as antigenic particulates for studies of phagocytosis. The bacteria cultured in TCBS agar were harvested during the log phase and washed twice with sterile Physiological saline (0.85% NaCl) by centrifuging at 400 g for 10 minutes. The washed pellet was resuspended in 0.5% formalin in physiological saline and kept undisturbed for 48 hours for killing the bacterial cells. After 48 hours the cells were again washed thrice by centrifuging at 400 g for 5 minutes each in sterile physiological saline until all traces of formalin was removed. The final bacterial pellet at a cell concentration of $4x10^7$ cells ml^{-1} was used for the phagocytosis assay.

2.3.6 *Phagocytosis Assay*

Phagocytosis Assay was conducted by two methodsnamely, **Phagocytosis by Glass Coverslip Adherent cells** and **Phagocytosis by Smear preparation.**

2.3.6a Phagocytosis by Glass Coverslip Adherent Cells

0.1 ml of separated leucocyte suspension (blood, kidney or spleen) was placed on a clean grease free sterilized glass coverslip, and incubated for 1 hour at room temperature (27° C) in a 5% CO_2 containing humid chamber. The coverslip was then washed with cold Hanks balanced salt solution gently to remove all non-adherent cells. Then the coverslip was covered by 0.2 ml of yeast cell suspension (to get Phagocyte : Yeast ratio, 1 : 40) and again incubated in a 5% CO_2 containing humid chamber for one hour. Then the coverslip was washed twice with cold Hanks balanced salt solution. The coverslip after air drying was fixed with Methanol for 1 minute and stained according to the procedure of Pappenheim (Schaperclaus, 1986).

2.3.6b Phagocytosis by Smear Preparation

Peritoneal exudate cell phagocytosis was conducted by smear preparation. Here, separated 0.5 ml peritoneal exudate cell suspension was thoroughly mixed with 1 ml cold yeast or bacterial cell suspension and incubated in a sterilized Eppendorf centrifuge tube with mild shaking. After one hour, thin smears were prepared on grease free, clean, sterilized glass coverslips and slides and stained according to Pappenheim (Shaperclaus, 1986).

2.3.7 Demonstration of Intracellular Killing by Nitroblue tetrazolium(NBT) Reduction Assay

Nitroblue tetrazolium Solution was used to determine levels of oxygen radical production by glass adherent neutrophils and macrophages.

The methodology followed was modified from Anderson *et al.* (1991).

0.1 ml suspension of the separated Blood leucocytes was placed on a grease free, clean sterilized square 22 mm coverslip and incubated in a 5% CO_2 containing humid chamber for 1 hour at 27° C for the cells to adhere. The non-adherent cells were washed off by Phosphate Buffered Saline (PBS) (pH 7.4). Then a 0.2 ml of yeast cell suspension (yeast cells ($4x10^7$ cells ml^{-1}) in PBS) was carefully layered over the coverslip which was then incubated in a 5% CO_2 containing humid chamber for 1 1/2 hours. Then the excess yeast suspension was washed off and the cover slip air dried. The filtered Nitroblue tetrazolium solution (0.2% in pH 7.4 PBS) was allowed to cover the adherent cells on the coverslip for 30 minutes in a 5% CO_2 containing humid chamber. The excess stain was decanted off and the cover slip was mounted in DPX on a clean, sterile glass slide and observed under the microscope (objective X 100 with oil immersion).

2.3.8 Phagocytic Index

Phagocytic indices of the blood, kidney, peritoneal and spleenic phagocytes were calculated according to the formula by Blazer (1991).

$$\text{PHAGOCYTIC INDEX (PI)} = \frac{\text{No. of ingested Yeast/Bacterial Cells}}{\text{Total No. of Cells}}$$

A total of 100 cells (with or without ingested particles) were counted to calculate the phagocytic index.

3.3.9 Staining procedure

The staining procedure adopted for both *in vivo* and *in vitro* cytological study was from Pappenheim (Combined May-Grunwald staining) (Schaperclaus,1986) for obtaining a differential count of the leucocytes.

Air dried smears and adhered cells on glass slides and glass coverslips were stained first with undiluted, filtered May-Grunwalds Giemsa stain (Merck Chemicals) for 3 minutes. Then the slides and coverslips were covered by de-ionized glass double distilled water , mixed carefully and kept for 1 minute. After decanting the stain -double distilled water mixture, the slides and cover slips were stained with filtered diluted Giemsa stain

(Merck Chemicals , 10 drops in 10 ml double distilled water) for 30-45 minutes. The slides were then thoroughly washed with de-ionized glass double distilled water, air dried, and mounted in DPX (Ranbaxy Chemicals) . The mounted slides and coverslips were observed under the microscope(objective X 100 with oil immmersion objective) and photomicrographs taken.

RESULTS

III. RESULTS

3.1 Studies on the Reticuloendothelial system

3.1.1 Histology

Spleen

Two hours after an intraperitoneal injection of colloidal carbon, the spleen revealed occasional carbon containing cells in the ellipsoids. By 8 hours moderate numbers of carbon containing cells were seen mostly restricted to the ellipsoids and ellipsoid walls.

After 16 hours, large number of carbon containing cells were observed in the ellipsoids. Occasionally, a few cells were seen migrating towards the parenchyma. After 24 hours large number of carbon containing cells were seen in the ellipsoids and a number of cells containing colloidal carbon were seen around the ellipsoid walls [Plate VI].

By 48 hours cells containing carbon were still found on the ellipsoids accompanied by an increase in the presence of more carbon containing cells in the parenchyma around the ellipsoids [Plate VII]. By day 4 a decrease in the cells containing carbon was noticed in the ellipsoids. Carbon containing cells were forming aggregates in the parenchyma and some cells were found to accumulate in the melano macrophage centres.

On day 8, carbon aggregates were seen mostly in association with the melanomacrophage centres. Carbon containing cells decreased in the ellipsoids and ellipsoid walls [Plate VIII]. On day 10 carbon containing cells were very much reduced even in the melanomacrophage centres and very few carbon containing cells were seen in the ellipsoids.

Kidney

Collidal carbon particles were seen in the blood vessel walls and sinuses 2 hours after an intraperitoneal injection of carbon particles [Plate IX]. Eight hours after injection of carbon particles, haemopoietic and interstitial tissues of the Kidney contained a number of cells with carbon particles.

After 16 hours, large amount of cells containing carbon particles were seen in the interstitial and haemopoietic areas of the kidney. In some areas they formed small aggregations.

Occasional cells containing carbon were seen in the haemopoietic as well as in the interstitial tissues after 24 hours [Plate X].

After 48 hours, carbon carrying cells accumulated in some areas of the haemopoietic tissues of the anterior kidney. Small numbers of carbon

containing cells were seen in the glomerular capillaries and intertubular blood vessels.

On day 4, more carbon containing cells were seen in the interstitial tissues. Occasional aggregation of cells was also noticed. This was more in number when compared to 24 and 48 hour kidney sections.

The aggregation of carbon containing cells reached a peak on day 8 [Plate XI]. On day 10, histological sections showed aggregation of carbon containing cells only in the interstitial tissue but the overall carbon content in the kidney decreased.

Heart

Carbon was mostly seen in the epicardial lining cells and also in free cells lying in the lumen of the heart 2 hours after an intraperitoneal injection of collodial carbon. No carbon was present in the endocardial lining of atrial trabeculae.

Eight hours after injection, the endothelial lining of ventral aorta revealed the presence of carbon. Occasional endocardial cells lining the atrium also revealed the presence of carbon.

After 16 hours, the entire valvular lining and ventricular endocardial lining contained large amount of carbon. Simultaneously this

time the endocardial linings of the atrial trabeculae started showing the presence of carbon and this was more when compared to the sections taken after 8 hours [Plate XII]

The cardiac sections taken after 24 hours showed accumulation of carbon in majority of the endocardial lining cells of atrium. The sections taken after 48 hours indicated more accumulation of carbon in the endocardial cells of the atrium. These cells were engorged with colloidal carbon.

The 4th day sections of the heart showed a mild reduction in the carbon present in the endocardial cells of the atrium. By day 8, further reduction of carbon in the atrial lining cells was evident. On day 10 most of the endocardial cells did not contain any carbon. Aggregation of carbon containing cells was not seen in the atrium at any time.

Peritoneum

Very few carbon containing cells were observed 2 hours after an intraperitoneal injection of colloidal carbon particles. After 8 hours, extensive carbon accumulation was seen in large number of cells. There was an increase in the carbon containing cells after 16 hours [PLATE XIII].

No further change was observed after 24 hours. Sub- cutaneous carbon accumulation was seen in the epidermis as well as in the melanomacrophage centres in the dermis after 48 hours.

Histological sections on the 4th day showed more carbon accumulation in the melanomacrophage centres in the dermis. Mild aggregation of carbon containing cells was seen along with individual cells containing carbon in the subcutaneous tissue. On day 8 also accumulation of carbon in the epidermis and subcutaneous tissue were seen. The aggregation of carbon containing cells showed an increase in number after day 10.

Thus among the three organs studied, carbon localization first occurred in spleen followed by kidney. Appearance of carbon occurred late in atrium. Massive amount of carbon appeared in spleen.

3.1.2 Cytology

Touch smears prepared from peritoneum indicated presence of carbon containing cells upto 48 hours [Plate IV]. The touch smears did not show any carbon containing cells on 4th, 8th and 10th days.

Blood smears prepared in fishes injected with colloidal carbon showed the presence of carbon containing macrophages and granulocytes until 48 hours. Adherence of carbon and uptake of carbon was also observed in some erythrocytes [Plate V]. Blood smears prepared after 4th, 8th and 10th days did not reveal any carbon particles in the blood cells.

3.2. *In Vitro* Studies

3.2.1 Coverslip Adhered Blood Leucocytes

Isolated cover slip adhered cells were elongated large cells with round to oval nucleus situated at one end and hence were designated as Macrophages. They constituted 100 % of the cells. These cells when incubated with yeast particles showed 85.7 % Phagocytosis [Table 4, Plate XVI].

3.2.2 Coverslip Adhered Kidney Leucocytes

The coverslip adhered kidney cells on microscopic examination appeared as elongated large cells with round to oval nucleus situated at one end. The cytoplasm stained light pink and contained yeast particles inside as well as adhered to the cell wall. These cells were identified as Macrophages due to there morphology. The macrophages were found to be of different sizes. 95 % of these cells were found to undertake phagocytosis of yeast particles when incubated with it [Table 4, Plate XVII].

3.2.3 Glass Coverslip Adhered Spleenic Leucocytes

The glass coverslip adhered spleenic cells were found to be of two types on microscopic examination One cell type was found to be

morphologically similar to macrophages having an elongate large cytoplasm with round to oval nucleus located at one end of the cytoplasm. The cytoplasm also revealed pseudopodia. Cells of different sizes were also observed. Macrophages constituted 75 % of the cells [Plate XVIII].

The second type of cells were spherical in shape and contained fine granules in the cytoplasm, and were designated as granulocytes. Granulocytes constituted about 25 % of the total cells.

The spleenic macrophage and granulocyte cells were phagocytic at 92.9 % and 71.4 % respectively [Table 4].

3.2.4 Isolated Peritoneal Exudate Cells

The peritoneal exudate cells were isolated on day 4, 5 and 11 after an intraperitoneal injection of liquid paraffin. The isolated cells incubated with yeast particles and then smeared on glass slides on microscopic examination revealed two cell types namely Macrophages and Granulocytes.

Macrophages

Cells which were elongate, large and with round to oval nucleus confined to one end of the cytoplasm which had pseudopodia were morphologically designated as macrophages [Plate XIX].

Macrophages constituted 76 % of the total cells on day 4, decreased to 33 % on day 5 to increase marginally to 44.8 % of the total cells on day 11 [Fig. 1, Table 1]. Cells containing yeast decreased from 94.9 % on day 4 to 92.6 % on day 5 to 89 % on day 11 [Fig. 2, Table 2].

Phagocytic index calculated showed a progressive increase from day 4 to day 11.

Granulocytes

The second type of cells were spherical with fine basophilic cytoplasmic granules, hence morphologically designated as granulocytes [Plate XIX]. Such cells increased from 23.4 % on day 4 to a maximum of 66.4 % on day 5 to decrease slightly to 55 % of the total cells on day 11 [Fig. 1, Table 1].

Cells containing yeast particles (percentage of phagocytic cells) rose from 26.7 % on day 4 to 43.7 % on day 5 to decrease to 14.4 % on day 11 [Fig. 2, Table 2].

Phagocytic index calculated was maximum on day 5. In majority of cases, granulocytes did not show singnificant phagocytosis. However a few cells showed yeast particles inside the cytoplasm and in others yeast particles were adhered to the cell membrane.

Granulocytes which had ingested yeast particles, liberated their cytoplasmic granules, which were found around the cells [Plate XX]. The chromatin in the nuclei of the phagocytic granulocytes appeared clumped [Plate XXI].

3.2.5 Phagocytic Index

Phagocytic Indices of isolated and glass coverslip adhered blood, kidney and spleenic leucocytes incubated with yeast particles were calculated and presented in [Table 4].

The phagocytic index of the peritoneal exudate cells pre-incubated with yeast particles and then smeared on glass coverslips and glass slides showed the following pattern.

Phagocytic index of Macrophages showed a progressive increase from 2.55 on day 4 to 3.59 on day 5 to 3.89 on day 11 [Fig. 3, Table 3]. The maximum numbers of yeast particles ingested per macropage [20 yeast cells / Macrophage] was observed on day 5.

The granulocyte phagocytic index increased from 0.37 on day 4 to a maximum of 1.16 on day 5 then decreased to 0.28 on day 11 [Fig. 3, Table 3]. The maximum number of yeast particles per granulocyte [10 yeast cells / Granulocyte] was observed on day 5.

Peritoneal exudate cells isolated on day 4 and 5 were also incubated with killed *Vibrio parahaemolyticus*. Though the cells revealed phagocytosis of the bacteria, the culture used was found to be contaminated with fungal spores. Hence phagocytic index was not calculated in these slides.

3.2.6 Demonstration of Intracellular Killing by Nitroblue tetrazolium (NBT) Reduction assay

Isolated blood leucocytes adhered on glass coverslips, Pre-incubated with yeast and then stained with Nitroblue tetrazolium solution showed that cells with ingested the yeast particles had a blush black halo in the cytoplasm when compared with control cells [Plate XXII].

DISCUSSION

IV. DISCUSSION

The study of the Reticuloendothelial system in *Oreochromis mossambicus* by intraperitoneal injection of colloidal carbon particles revealed that the major organs of antigen localization were the spleen, kidney, atrium and to some extend the peritoneum.

It was observed that the main organ of antigen localization was the spleen where the particles first seen in the ellipsoids, migrated to the parenchymal tissues, to be finally taken up by the melanomacrophage centres and aggregated. This differs from the views of Ellis *et al.* (1976) and Ferguson *et al.* (1982) who observed kidney as the major organ of antigen localization.

MacArthur *et al.* (1983) studying the fate of injected 51 Cr-TRBC in plaice, *Pleuronectes platessa* reported that spleen was more active in TRBC uptake even though kidney had higher counts due to its larger size.

The atrial endocardial lining cells of *Oreochromis mossambicus* were phagocytic for carbon particles as reported by Ellis *et al.* (1976); Mori (1980); Nakamura *et al.* (1991); Nakamura *et al.* (1992) in various other fish species. In the present study it was noticed that there was not much accumulation of carbon intially, but by 48 hours there was a peak in the atrium. Another feature found was that the ventricular endocardial

lining cells were phagocytic. This is the first report in *Oreochromis mossambicus*. A similar observation was made by Woodhead (1981) who found that the endocardial cells in the ventricle of the Amazon molly (*Poecilia formosa*) were phagocytic for carbon particles.

Peritoneal exudate and blood smears revealed that the macrophages, granulocytes and erythrocytes were found to contain carbon particles in smears made up to 48 hours. The result agrees with the findings of Doggett *et al.* (1987) in the same species. However the work of Ellis *et al.* (1976) revealed carbon containing cells only after 48 hours, there after the smears failed to reveal any carbon containing cells. They observed changes in the morphology of the leucocytes containing carbon. In the present study no appreciable morphological changes were noted in the leucocytes which took carbon particles.

Tracing the pathway of the injected colloidal carbon particles, it is believed that the particles were taken up by the circulating blood phagocytes and transported to the various reticuloendothelial tissues. Similar views were expressed by Mackmull and Michels (1932) and Ellis *et al* (1976). As fish do not possess a diaphragm, they are capable of absorbing particles from the peritoneal fluid and then transporting them to the Reticuloendothelial tissues via the blood system.

This was substantiated by the fact that carbon particles were first seen in the blood and perintoneal smears after 2 hours and during this period the reticuloendothelial organs contained less carbon. Subsequently an increase in the carbon content was noticed in the reticuloendothelial organs especially the spleen which showed maximum phagocytic activity after 48 hours. The carbon particles were found to increase within blood vessel lumens in the viscera as free particles and was carried by the phagocytics cells to the reticuloendothelial organs. Ellis *et al.* (1976) also observed a similar phenomenon in the plaice.

In kidney also the carbon containing cells found in the blood vessels and sinuses, migrated to the interstitial tissue and formed aggregates. It was thought that the kidney, because of the renal portal system, may provide an extensive filter bed for returning venous blood and trap blood borne phagocytes.

Initially, carbon was seen in the ventricular wall of the heart. It was only later that the atrial endocardial cells were filled with carbon particles. There was no aggregation or storage of carbon in the atrium. The present observation indicated that the endocardial cells of the atrium were less phagocytic when compared to those of the spleen and kidney.

The aggregation of phagocytes in the melanomacrophage centres after taking up particulate materials or antigens was a common feature

observed by many workers. Melanomacrophage centre in addition to being functioning as a depot for metabolic wastes, also take part in antigen trapping and processing (Ellis 1980; Secombes and Manning 1980)

The main phagocytic cells in fishes are the macrophages and the granulocytes. The isolated, coverslip adhered blood leucocytes were mainly macrophages and they were phagocytic for yeast particles. This result was in agreement with the findings of Doggett *et al.* (1987) that monocytes precursors of macrophages were phagocytic in *Oreochromis mossambicus*.

The coverslip adhered kidney and spleenic leucocytes were predominantly macrophages. In the spleen, a small amount of granulocytes were also observed. It was noticed that the glass coverslip adhered spleenic and kidney macrophage population had cells of different sizes. These were thought to be cells at different stages of maturation. Kidney and spleen are the major haemopoietic and granulopoietic organs in the fish (Ellis 1982; Roberts 1989; Campbell and Murru 1990).

There was no significant difference in the phagocytic capability of the macrophages in relation to their size. From this study it was found that the maturation process and phagocytic capability were independent of each other. As no such reports are available on the effect of maturation on the phagocytic capability, it has to be further studied.

The peritoneal exudate cells of *Oreochromis mossambicus* obtained on days 4, 5 and 11 were mainly macrophages and granulocytes.

In mammals, neutrophils migrate more quickly than macrophages during the acute phase of infection (Robbins and Kumar 1987). This phenomenon has also been reported to be true in case of fish inflammation. (Finn and Nelsen 1971a, 1971b; MacAarthur *et al.* 1984; Suzuki and Hibiya 1986; Suzuki and Iida 1992).

In *Oreochromis mossambicus*, it was not possible to substantiate this view because, peak macrophage counts were obtained on day 4 which subsequently decreased on day 5 and day 11. In the case of granulocytes, an increase was observed from day 4 to day 5 and then a decrease on day 11. A similar result was obtained by Suzuki (1986) who got progressive decrease in macrophages and gradual increase of granulocytes (Neutrophils, Basophils and Eosinophils) in *Oreochromis niloticus*. However in these works since no characterization of the leucocytes within the first day after an induced peritoneal inflammation was done, it was not possible to conclude whether there is any departure from the views of other workers regarding leucocytic infiltration during inflammation in fishes. It cannot be concluded that in *Orechromis* spp. there is a major departure in the process of inflammation from other vertebrates.

Our results clearly show that there is a significant variation in the inflammatory process among various fish genera and even among different species of the same genus.

Data on phagocytic index and percentage of phagocytic cells of the peritoneal exudate showed that the macrophages were the main phagocytic cells and not the granulocytes which showed high variations in their phagocytic capability ranging from a majority of non phagocytic cells to some very active phagocytic cells.

In *Oreochromis mossambicus*, Doggett *et al.* (1987) and Doggett and Harris (1989) have reported the presence of three granulocyte cell types namely, Type I, Type II and Type III. They reported that Type I and Type III are phagocytic. In this study, the granulocytes were not characterized histochemically. Hence the poor phagocytosis observed on day 4 and day 11, and the active phagocytosis observed on day 5 in granulocytes could not be attributed to any specific types of leucocytes.

Another interesting phenomenon noticed was that the phagocytic granulocytes of the peritoneal exudate released their granular contents outside the cells. This could be due to the release of lysosomes. A similar finding was reported by Suzuki (1986) in the carp *Cyprinus carpio*. He suggested that the centrally vacuolated granular cells found in the peritoneal exudate was due to extraction of their granular contents.

SUMMARY

V. SUMMARY

1 Studies on the Reticuloendothelial phagocytic system *in vivo* by intraperitoneal injection of colloidal carbon particles revealed that the major organs of antigen localization in *Oreochromis mossambicus* were the Spleen, Kidney, Atrium and the Peritoneum.

2. Of the major organs of antigen localization, the Spleen had the maximum phagocytic activity followed by the kidney.

3 The ventricular endocardial cells were also phagocytic for carbon particles. This is the first report of this activity in *Oreochromis* spp.

4. The atrial endocardial cells also exhibited phagocytic activity No aggregation of carbon particles was found in the atrium.

5 Thus, tracing the pathway of the uptake and localization of carbon particles, it was found that they were taken from the peritoneal cavity by the blood and transported to the reticuloendothelial organs where they were deposited and aggregated.

6. In the spleen the particles were first found on the ellipsoid walls , migrated to the outside and then formed aggregations in the melanomacrophage centres.

7. In the kidney the carbon particles found first in the walls of blood vessels and sinuses, were taken to the interstitial haemopoietic tissue by the phagocytic cells where they formed aggregates in the melanomacrophage centres.

8. The isolated, coverslip adhered blood, kidney and spleenic leucocytes were mainly macrophages which had high phagocytic capability.

9. The kidney and spleenic macrophages were of different stages of maturity but did not show any significant difference in their *in vitro* phagocytic capability.

10. It is believed that the maturation process and the phagocytic capability are independent of each other.

11. The isolated and smeared peritoneal exudate cells harvested after day 4, 5 and 11 after an intraperitoneal injection of liquid paraffin revealed morphologically two cell types namely, macrophages and granulocytes.

12. The granulocytes of the peritoneal exudate were weakly phagocytic in a majority of cases, but some were highly phagocytic.

13. The phagocytic granulocytes of the peritoneal exudate showed chromatin clumping and liberation of granules to the outside of the cell. The significance of this phenomenon is not known and was not reported by other workers.

GRAPHS & TABLES

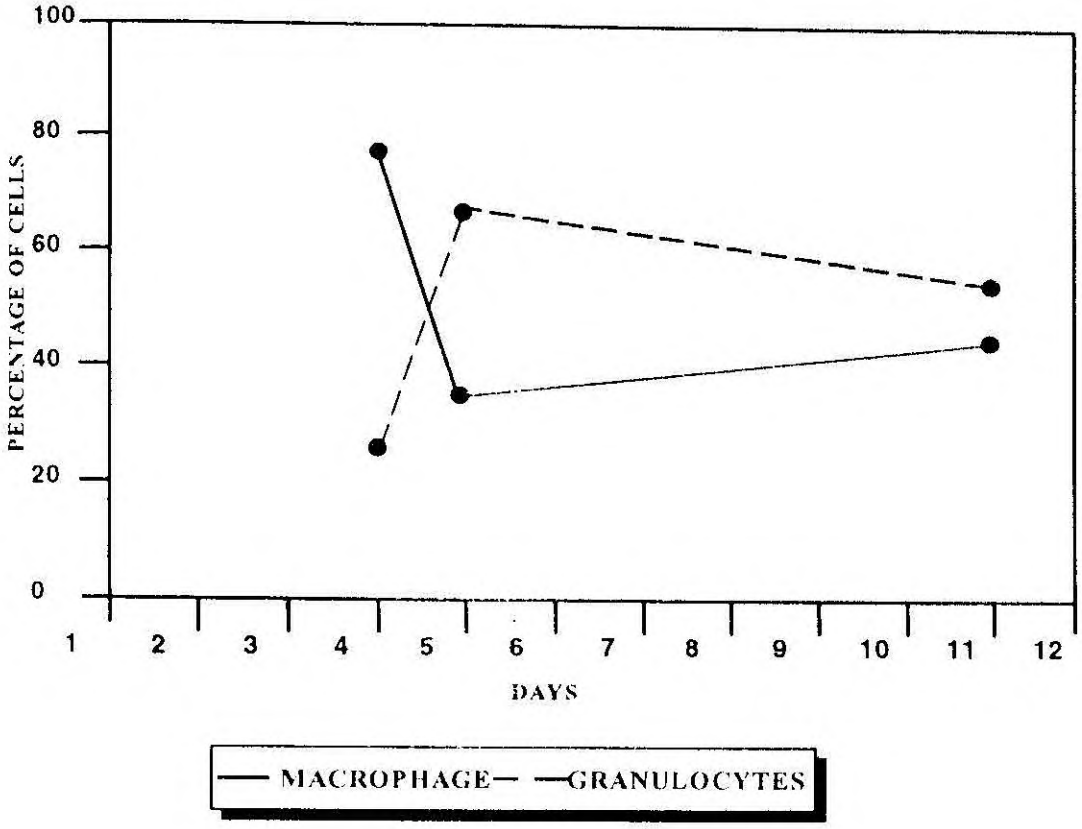

Fig. 1 Percentage composition of peritoneal exudate Cells

DAY	PERCENTAGE OF CELLS	
	MACROPHAGES	GRANULOCYTES
4	76.6	23.4
5	33.6	66.4
11	44.8	55.2

Table 1 : Percentage composition of peritoneal exudate cells

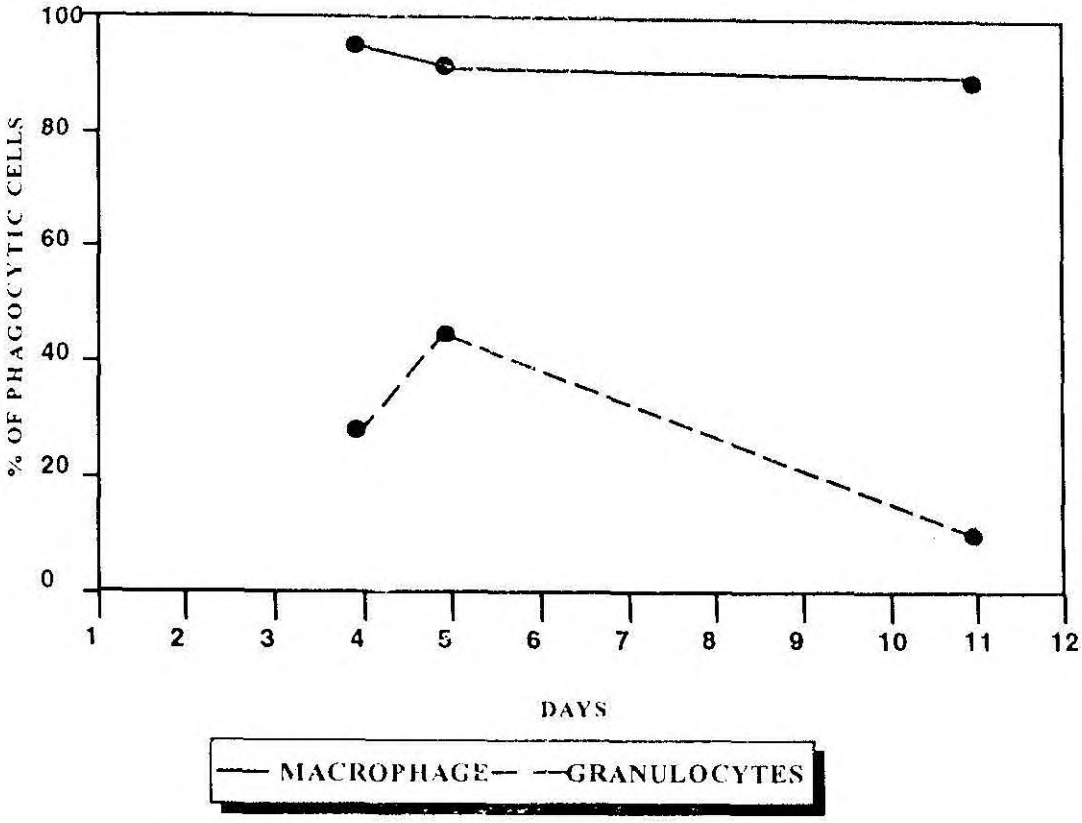

Fig. 2 Percentage of Phagocytic peritoneal exudate cells

DAY	PERCENTAGE OF PHAGOCYTIC CELLS	
	MACROPHAGES	GRANULOCYTES
4	94.9	26.7
5	92.6	43.7
11	89.0	14.4

Table 2 : Percentage of Phagocytic Peritoneal exudate cells

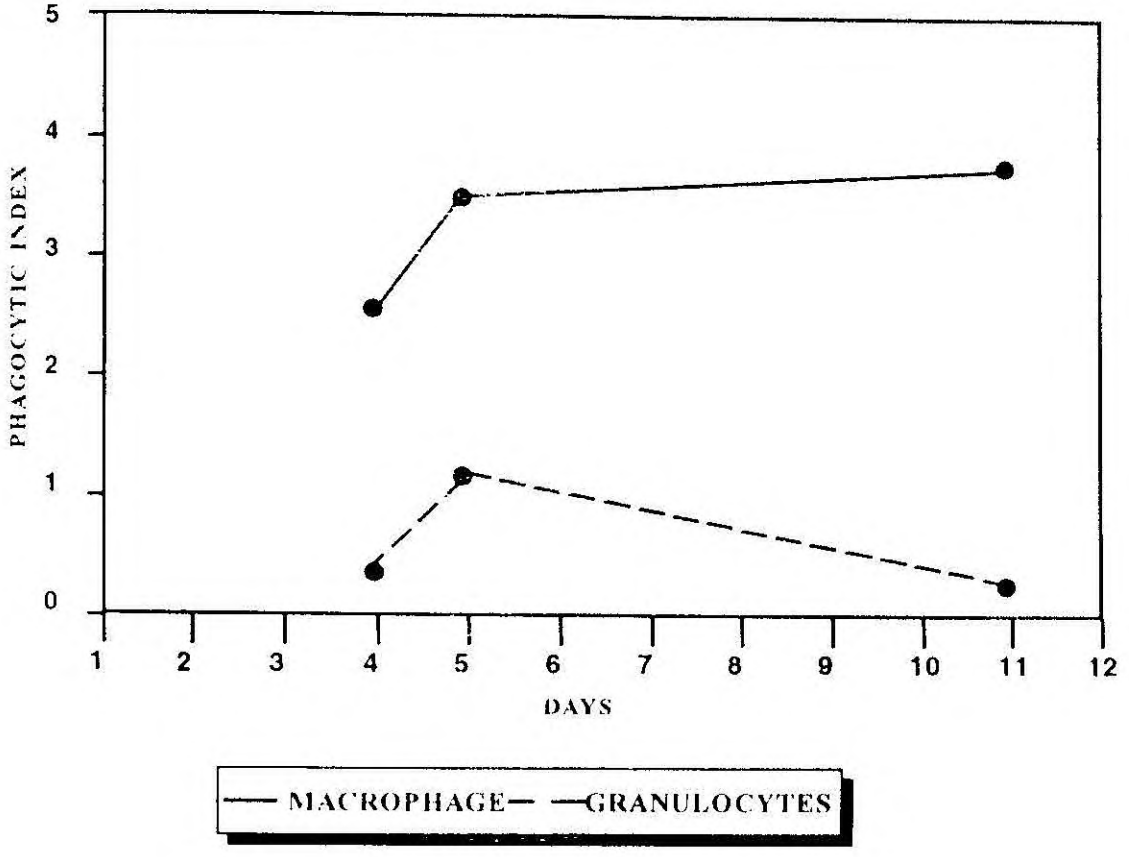

Fig. 3 Phagocytic index of peritoneal exudate cells

DAY	PHAGOCYTIC INDEX		MAX. NO. OF YEAST PER CELL	
	MACROPHAGES	GRANULOCYTES	MACROPHAGES	GRANULOCYTES
4	2.55	6.37	10	3
5	3.59	1.16	20	10
11	3.85	0.26	12	5

Table 3 : Phagocytic index of peritoneal exudate cells

	TYPE OF CELLS	PHAGOCYTIC INDEX	PERCENTAGE OF PHAGOCYTIC CELLS
BLOOD	MACROPHAGE	2.21	85.71
	GRANULOCYTES	--	--
KIDNEY	MACROPHAGE	2.10	95.00
	GRANULOCYTES	--	--
SPLEEN	MACROPHAGE	2.57	92.86
	GRANULOCYTES	1.43	71.43

Table 4 : Phagocytic index and percentage of phagocytic cells from Blood, Kidney and Spleen

PLATES

PLATE: I

Experimental Fish *Oreochromis mossambicus*

PLATE: II

Reticuloendothelial Organs of *Oreochromis mossambicus*. H. Heart K. Kidney S. Spleen.

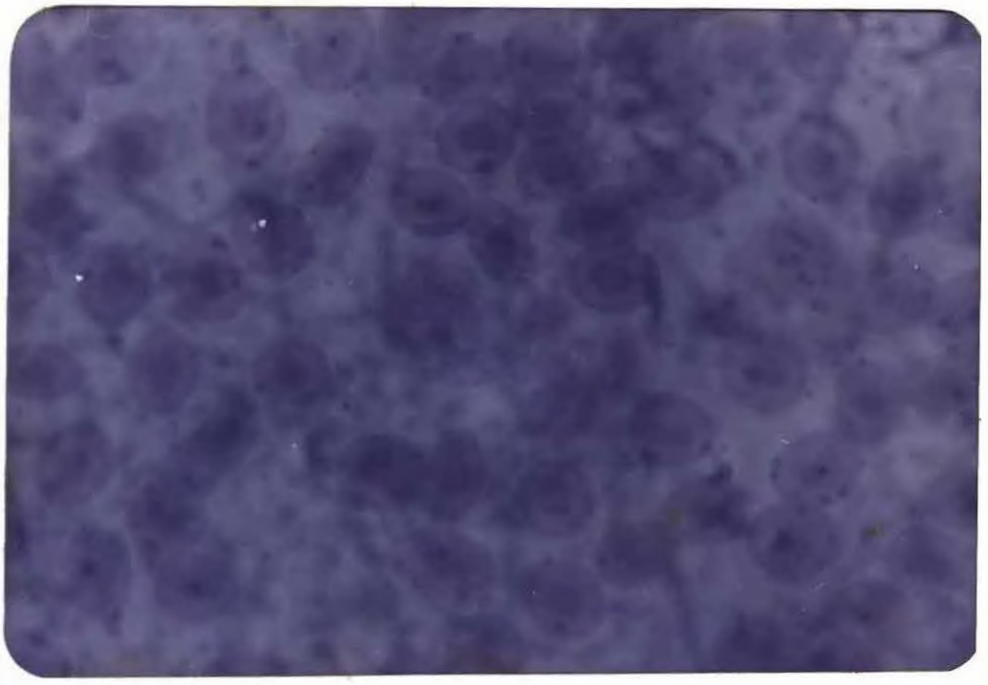

PLATE: III

Bloodsmear of *Oreochromis mossambicus* (x 400).

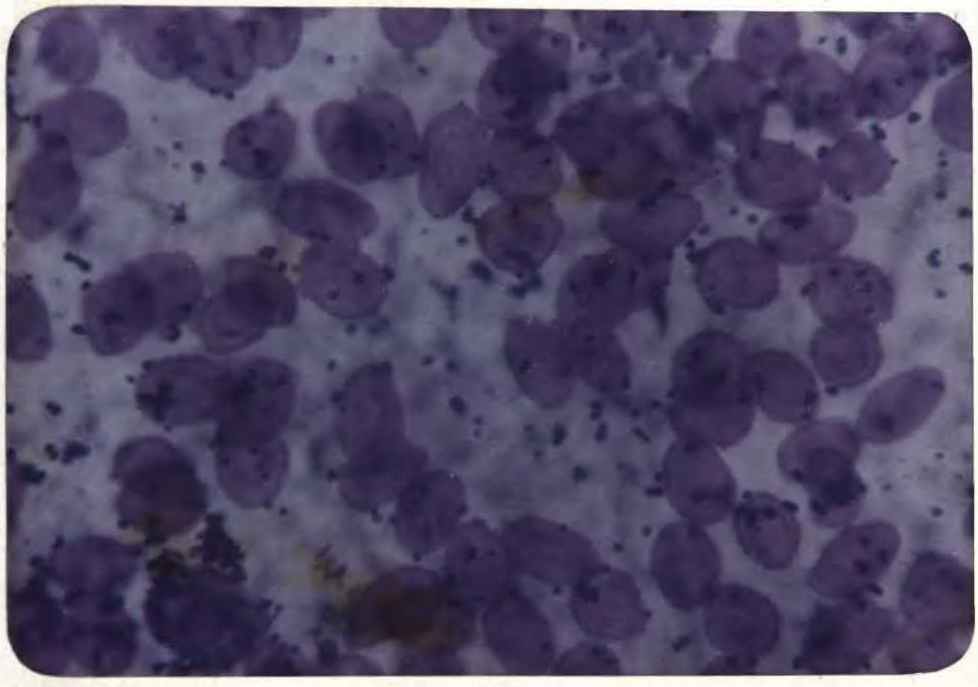

PLATE: IV

Twentyfour hour bloodsmear after intraperitoneal injection of collodial carbon particles (x 400).

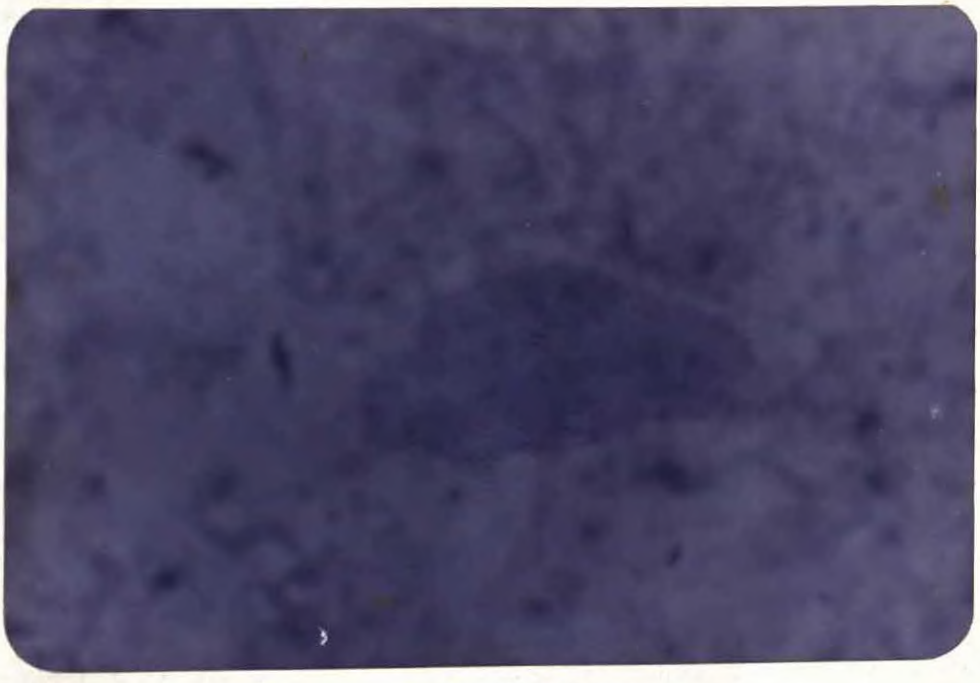

PLATE: V

Peritoneal Macrophage with ingested collodial carbon 24 hours after interperitoneal injection (x 1000).

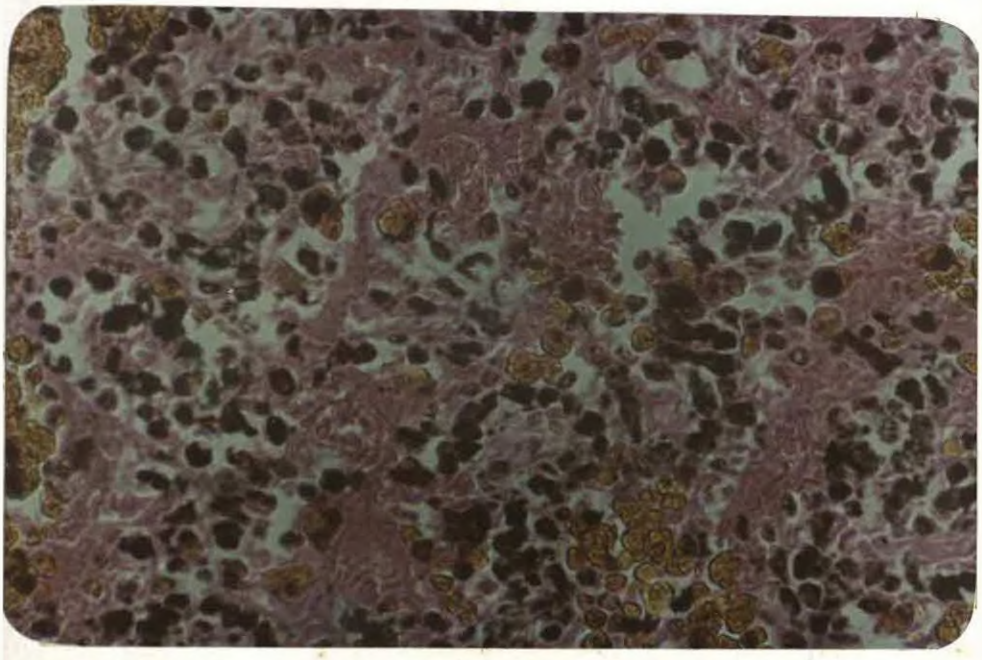

PLATE: VI

Spleenic section showing carbon particles in the ellipsoids (x 200).

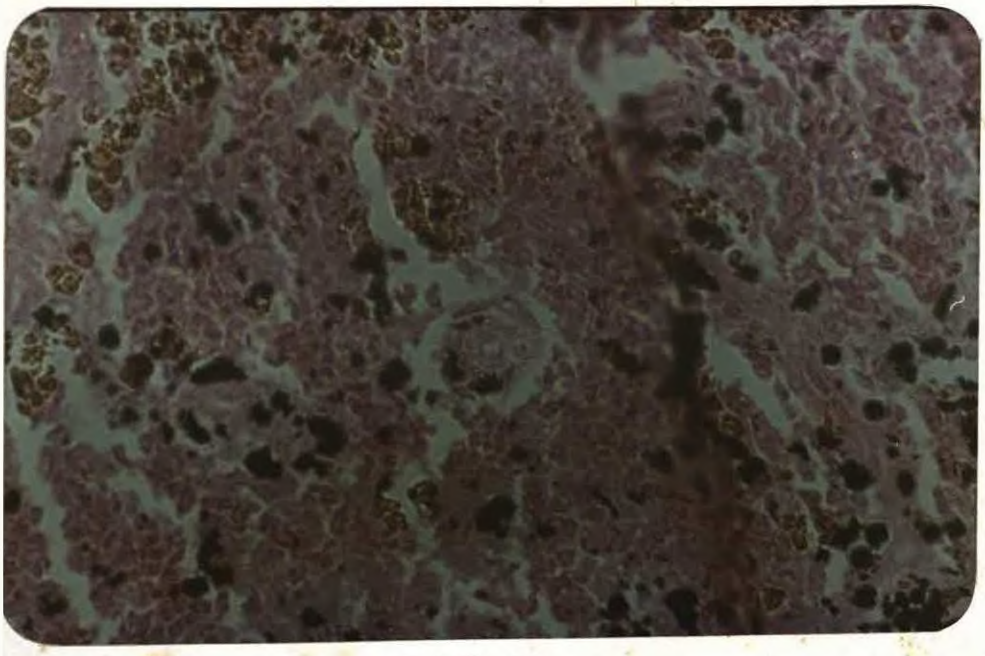

PLATE: VII

Spleenic section showing carbon aggregation in the parenchyma (x 200).

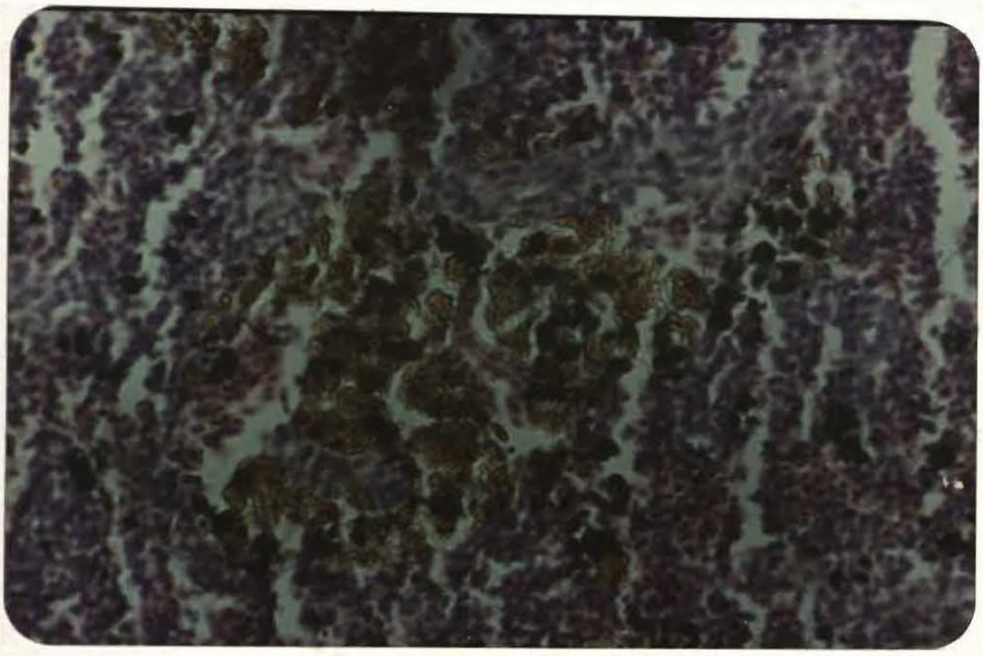

PLATE: VIII

Spleenic section showing carbon aggregation within the Melanomacrophage centres (x 400).

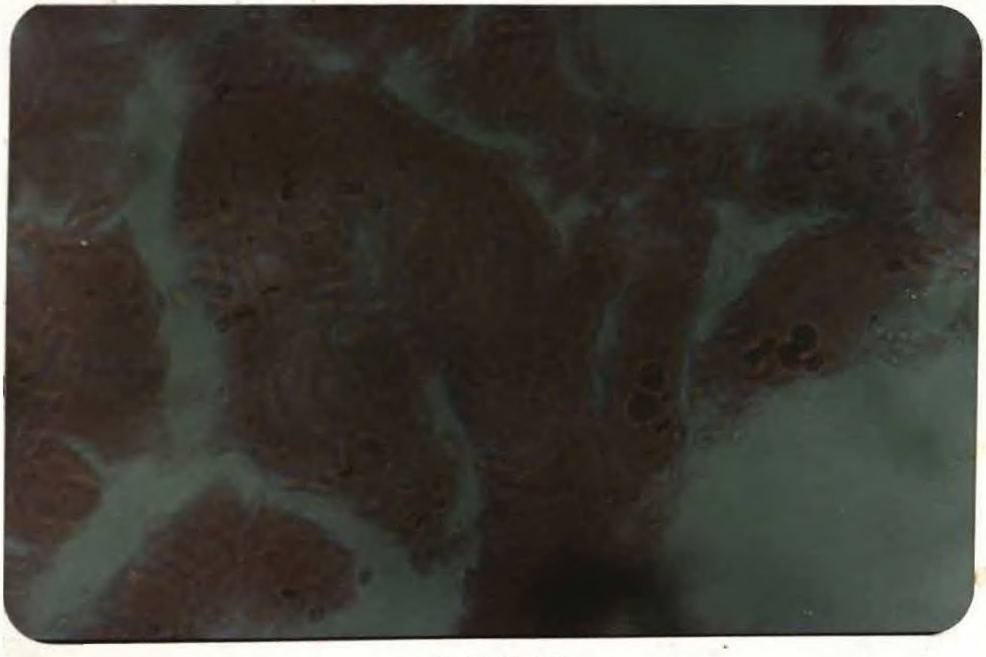

PLATE: IX

Section of the Kidney showing carbon particles in the blood vessel walls (x 400).

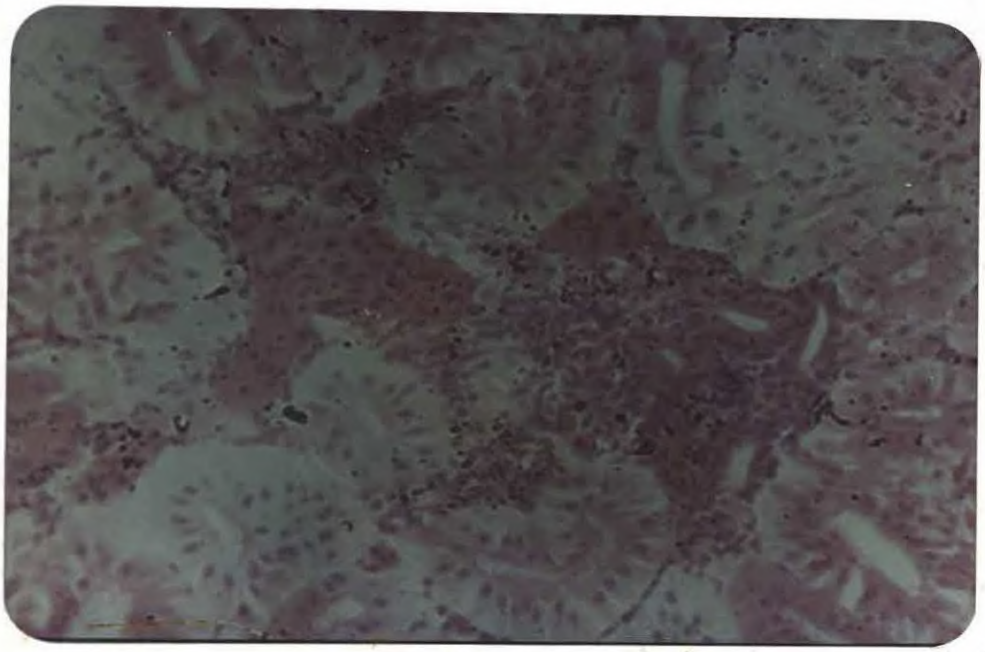

PLATE: X

Section of the Kidney showing carbon particles in the interstitial tissue (x 200).

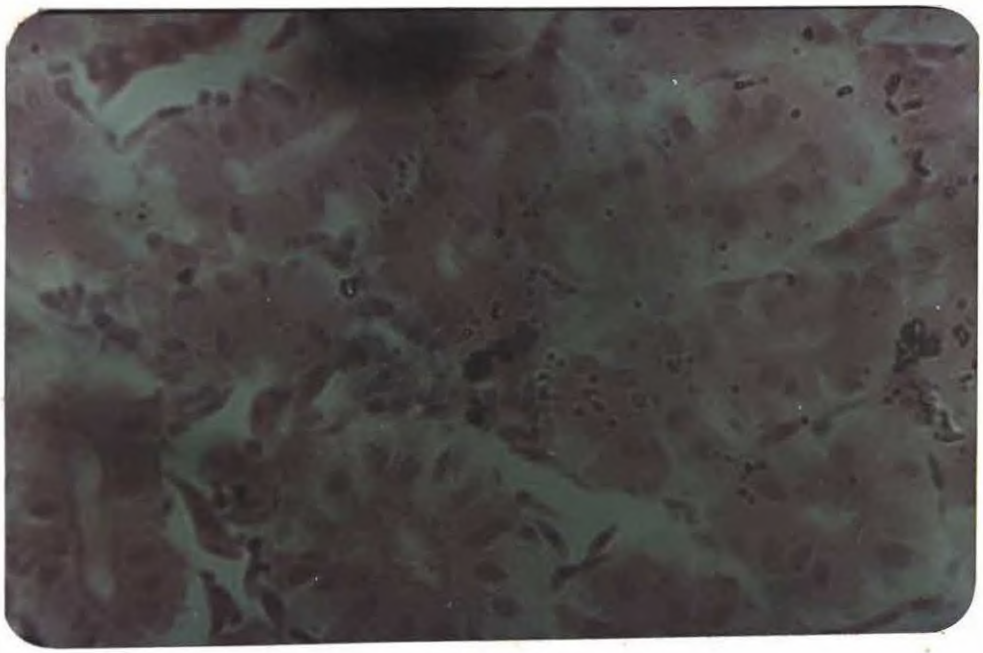

PLATE: XI

Section of the Kidney showing carbon aggregation (x 400).

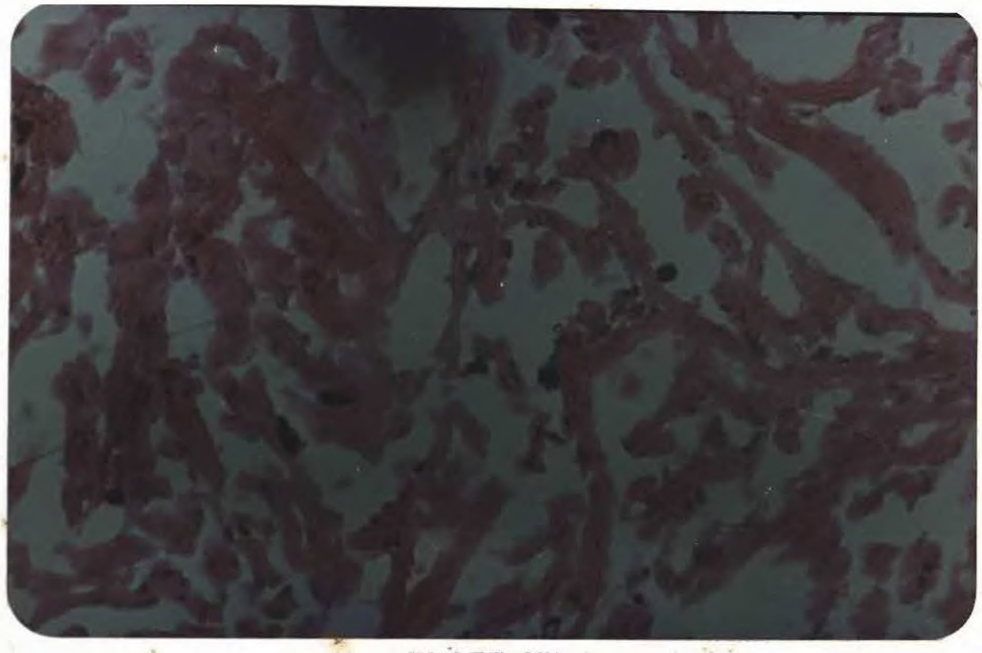

PLATE: XII

Cardiac section showing carbon particles trapped in the Atrial endocardial cells (x 200)

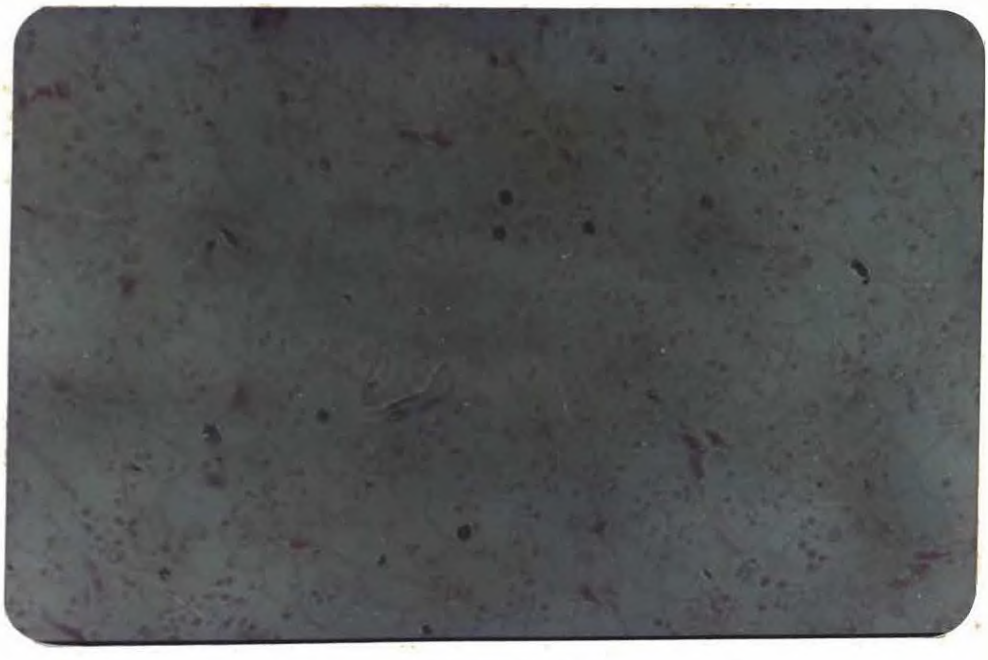

PLATE: XIII

Section of Peritoneum showing carbon particles (x 200).

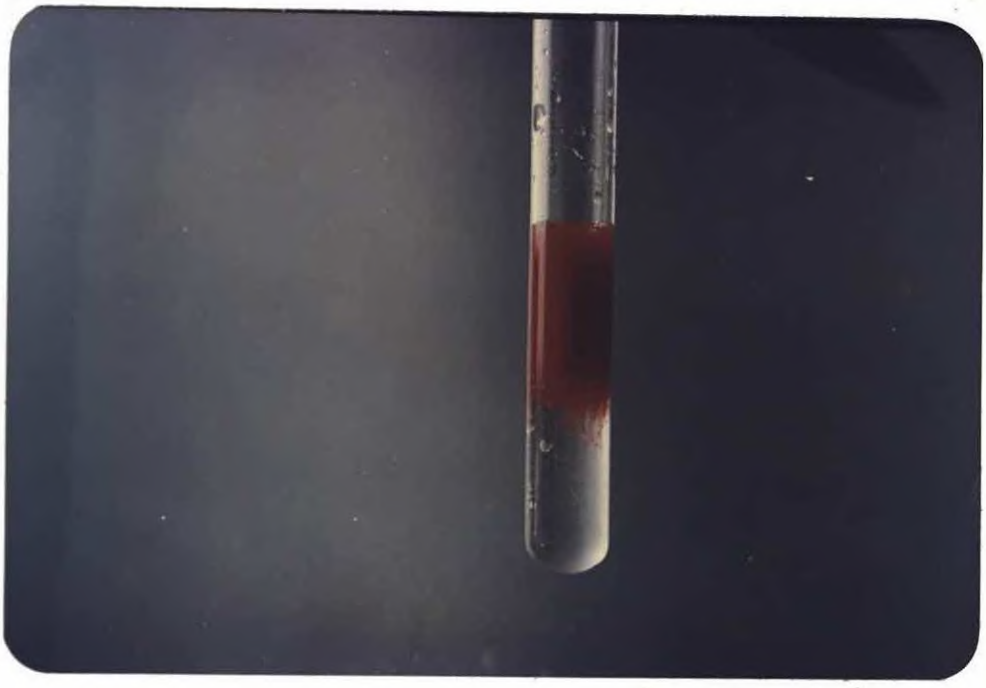

PLATE: XIV

Blood layered over Histopaque - 1077 separation medium.

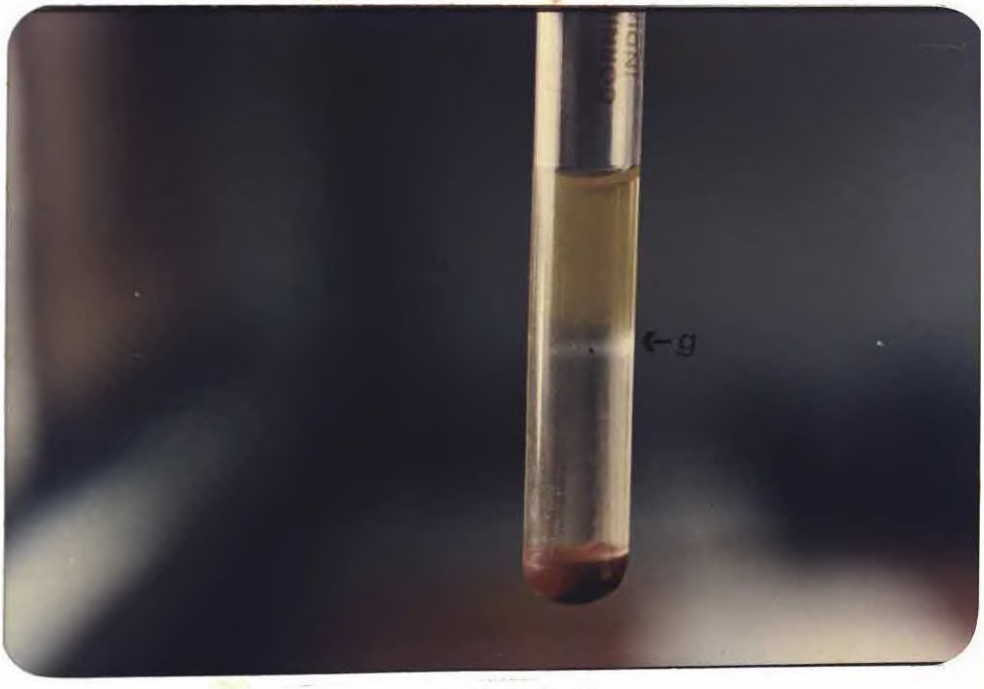

PLATE: XV

Centrifuged blood showing the grey ring (g) between the blood plasma and the separation medium.

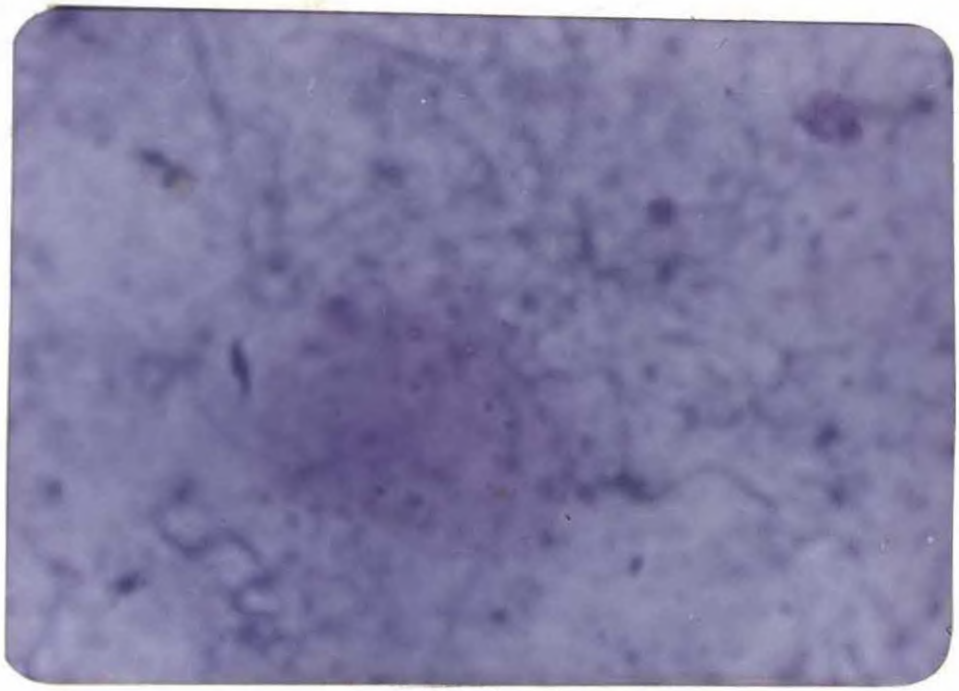

PLATE: XVI

Coverslip adhered Blood Macrophage with ingested yeast particles (x 1000).

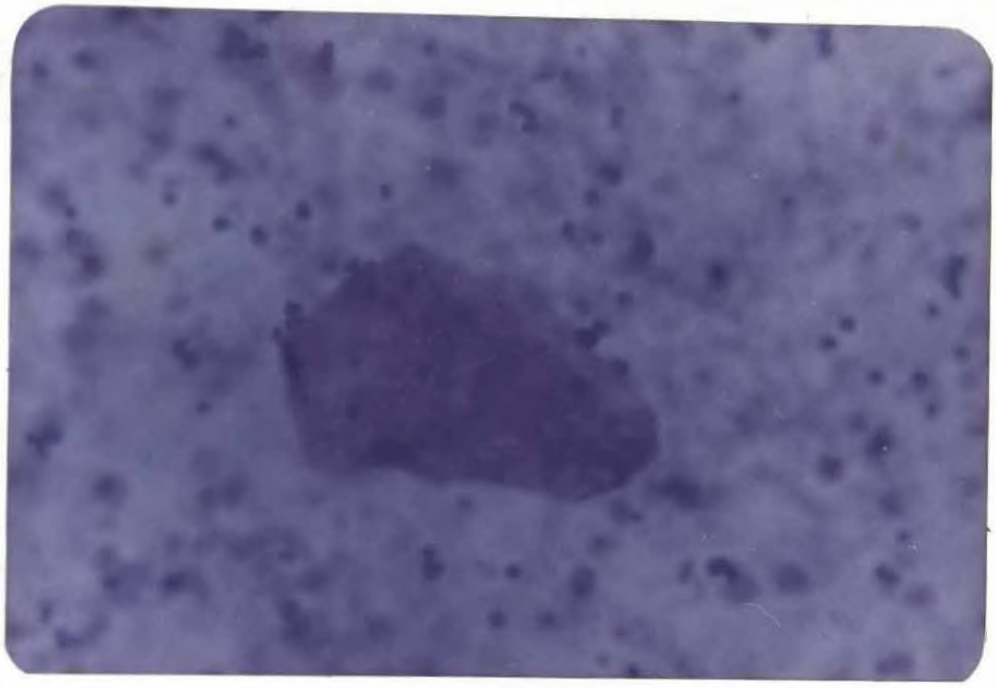

PLATE: XVII

Coverslip adhered Kidney Macrophage with ingested yeast particles (x 1000).

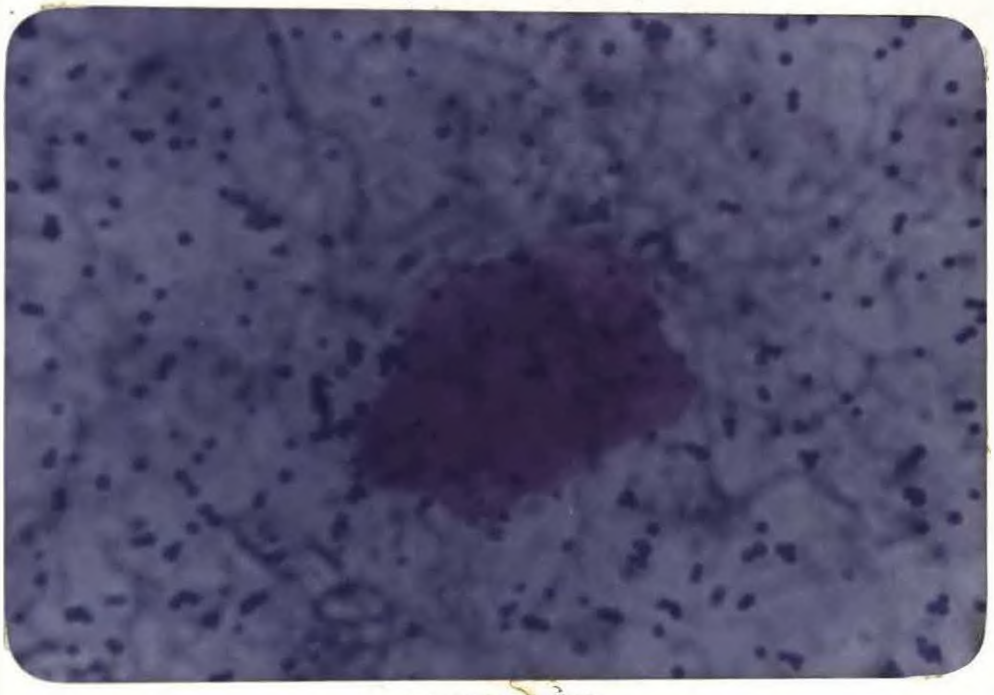

PLATE: XVIII

Coverslip adhered Spleenic Macrophage with ingested yeast particles (x 1000).

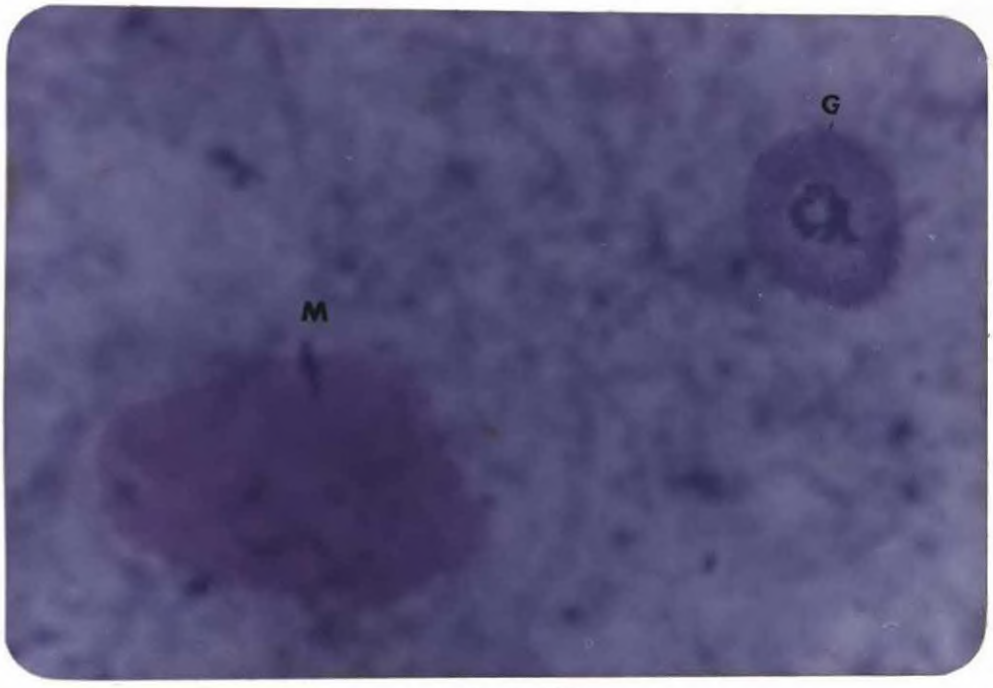

PLATE: XIX

Isolated Peritoneal Macrophage (M) and Granulocyte (G) (x 1000).

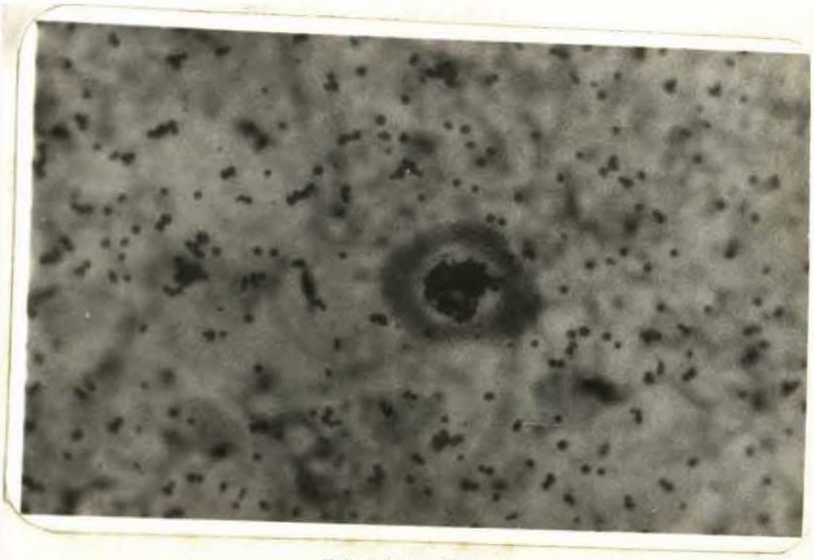

PLATE: XX

Peritoneal Granulocyte showing liberation of Granules outside the cell after phagocytosis of yeast particles (x 1000).

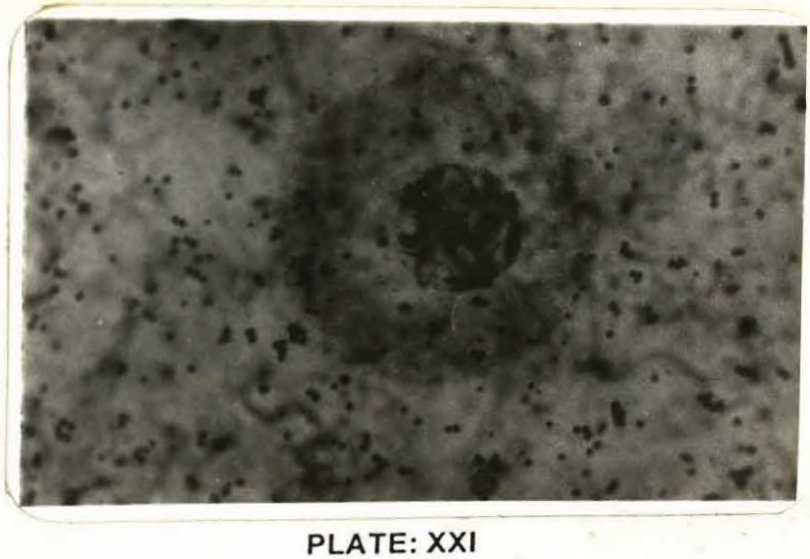

PLATE: XXI

Peritoneal Granulocyte exhibiting Chromatin clumping after phagocytosis of yeast particles (x 1000).

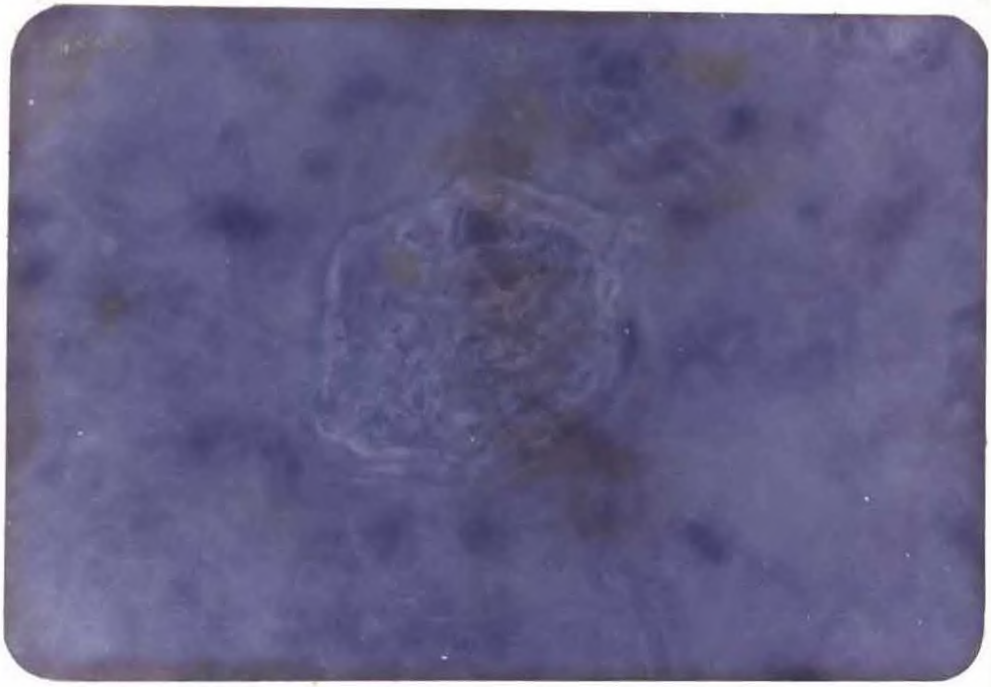

PLATE: XXII

Isolated coverslip adhered Blood Macrophage showing bluish granules in the cytoplasm after NBT reduction Assay (x 1000).

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