A Study of the Carrageenin Granuloma in the Plaice (*Pleuronectes platessa* L.).

by

Metin Timur B.V.Sc.

A thesis submitted to the University of Stirling for the degree of Doctor of Philosophy

Unit of Aquatic Pathobiology
University of Stirling.

June, 1975

ABSTRACT

The inflammatory response is the cornerstone of pathology, because of the frequency of its occurrence, the fundamental nature of its component reactions, and the fact that most clinical diseases can be regarded as variants of it.

Although animal pathology has been studied over many years, there are still some aspects of the inflammatory process which have not been completely resolved, even for homeotherms, and the number of such aspects still undescribed in cold blooded animals, is very much greater, since there have been relatively few studies on any of the pathological responses in poikilotherms. A small number of workers have studied the inflammatory response in fishes, but they have almost exclusively dealt with the acute type of response (Finn and Nielson, 1971a; Roberts et.al. 1973a).

Aquaculture is developing into an important and rapidly expanding industry in many countries of the world. Fishes are being raised for food, for sport fisheries, and as ornamental aquarium fish. It is not surprising therefore, that there has been an increase of interest in fish pathology, and it was as a contribution towards a fundamental understanding of the mechanisms of the response of teleost fish to infection that this study was undertaken.

In mammalian pathology a great deal of information on the chronic inflammatory response has been obtained by injecting experimental animals with extracts of the marine seaweed Chondrus crispus (Carregeenin). In chronic inflammation, collagen fibre formation is a vital component of both chronic inflammation and of healing, and carrageenin, as well as being a potent irritant is a powerful stimulant to the production of collagen.

In this study, carrageenin was used, by inoculation, as a stimulus to the production of chronic inflammatory lesions in the teleost fish, in order to determine whether the teleost fish was capable of producing similar responses to those occurring in response to this model irritant in the higher animals. Throughout the study, the plaice (Pleuronectes platessa.L.) was used as the experimental fish. This was because the plaice is a relatively easy fish to keep, is readily obtainable and a considerable amount of base-line information on this marine teleost was already available from other studies (Wardle 1971; Roberts et.al. 1971; Ellis 1974).

Carrageenin was injected intramuscularly into plaice, to produce a chronic granulomatous inflammatory reaction. The main study was carried out at the fixed temperature of 10^{o}C., but studies were also carried out on the moderation of the carrageenin inflammatory response by reduction of temperature to 5^{o}C., since it is well recognized that the rate and to some extent the quality of the inflammatory response in poikilotherms is determined by the temperature at which the response is taking place (Finn and Nielson 1971).

After the carrageenin was injected into plaice, an active connective tissue response developed as part of the chronic granuloma, experiments were therefore also carried out to investigate the specific role of L-ascorbic acid (vitamin-C) in the enhancement of collagen synthesis. Within the developed granulomata, cells resembling the plasma cell and its precursors were observed. Since this suggested the production of serum antibodies within the granulomata, specific staining for plasma cells by Unna-Pappenheim stain was carried out, and a gel diffusion precipitation test (Ouchterlony 1947) was carried out to determine the antigenicity of carrageenin.

Since the granuloma contained large numbers of lymphycytes as well as macrophages, it was considered that there might well also be a delayed type of hypersensitivity (DTH) reaction taking place, in addition to the serum antibody response. Consequently a delayed type of hypersensitivity test (the migration inhibition test, MIT) was devised to measure this response. The histological observations were complemented by an electron microscope study of various stages of the developed granuloma, to determine the ultrastructure of those cells which played a significant role in this response.

ACKNOWLEDGEMENTS

This work was carried out while I was a research student in the Unit of Aquatic Pathobiology at the University of Stirling, Scotland, for the period September 1972 to July 1975.

Throughout the course of the project, I have received continuing help from both my supervisors, the Director of Nuffield Aquatic Pathobiology Unit, Dr R J Roberts and external supervisor, Dr A McQueen of the Department of Dermatology, Anderson College, University of Glasgow. I am extremely grateful for their encouragement, advice, valuable comments, and above all, their enthusiasm.

Most of the project work was carried out in the Nuffield Aquatic Pathobiology Unit at the University of Stirling, and I should like to thank the staff of the Unit, particularly its Chief Technician Mr I H McRae for providing me with a space and allowing me to use the facilities available at the Unit.

I would also like to thank the staff of the Electron Microscope Unit of the Biology Department of Stirling University, particularly the Chief Technician Mr T Forrest, who has given valuable assistance during the ultrastructural studies.

I would like to express my particular thanks to Mr R B Stewart of the Audio-Visual Aids Unit, University of Stirling for his photographic assistance.

Special thanks are due to Mrs M McNaughton who has typed the thesis with patience and great care.

My thanks also go to Gulsen Timur for her valuable assistance and suggestions both in her capacity as wife and colleague.

Finally, I wish to express my thanks to the Ministry of Education of the Turkish Government who provided a scholarship to enable me to carry out the work at the University of Stirling.

.....

The work presented in this thesis is the result of my own investigations and has neither been accepted nor is being submitted for any other degrees.

Metin Tinaz ..... Candidate

Ronald J. Roberts ..... Supervisor

5:8:75. ..... Date

A study of the Carrageenin Granuloma in the

Plaice (*Pleuronectes platessa*)

ABSTRACT

ACKNOWLEDGEMENTS

CONTENTS

SECTION A

SECTION B

SECTION C

SECTION A

REVIEW OF LITERATURE

INFLAMMATION IN MAMMALS

The term "inflammation" has been used since the earliest days of medicine, although its absolute definition did not take place till many years later. However, Celsus (30 BC to 38 AD) gave us its cardinal signs: heat, redness, swelling, and pain. To these Galen (AD 130 to 200) added loss of function. The cellular nature of the inflammatory response did not become apparent until the end of the 18th century and the beginning of the 19th century. The main disease on which discoveries were based was tuberculosis, which until the mid-20th century was one of the most frequent causes of mortality in man.

In the 19th century as the microscope came into general use, it began to be used for the routine study of disease, but it was not until 1882 that Robert Koch (1843-1910) discovered the cause of tuberculosis, which he showed to be an acid fast bacillary organism. In 1800, Edward Jenner had first described the phenomenon of delayed type hypersensitivity (DTH), although he did not of course use that term; this early history of inflammation is reviewed in detail by Robert and Ebert (1965).

Phagocytosis in its simplest terms is the transfer of a solid particle from an extracellular to an intracellular locus (Cohn 1965). The conditions leading to phagocytosis by inflammatory

cells were defined by Langhans (1839-1915), who observed that leukocytes phagocytosed fragments of affected blood cells found in tissue debris; Panum in 1874 and Rosser in 1881 suggested that this process might actually be a method of destruction of microbes. This early history of phagocytosis is reviewed by Mettler (1947). It remained for Metchnikoff to establish phagocytosis firmly as an important defence mechanism in the inflammatory response (Wright and Dodd 1955; Gordon and King 1960). Metchnikoff described his hypothesis as follows: "This reflection led me to make the following experiment: to wound and introduce spines beneath the skin of very transparent marine animals; if my hypothesis should be well founded this should bring about an accumulation of amoeboid cells at the injured spor. I selected for this purpose the large Bipinnarica larvae of starfish, so abundant at Messina, and inserted prickles of the rose into their bodies. Very shortly these prickles were found to be surrounded by a mass of amoeboid cells such as we see in human exudation as the result of the introduction of a spine or other foreign body. The whole process took place under my eyes in a transparent animal possessing neither blood nor other vessels, nor a nervous system. The first point was settled. The inflammatory exudation must be considered as a reaction against all kinds of lesions, the exudation being a more primitive and more ancient phenomenon in inflammation than are the functions of the nervous system or of the vessels" (Metchnikoff 1891). This was certainly the first evidence of study of the inflammatory response in lower animals.

Inflammatory changes are commonly the result of microbial infection but they are also a universal non-specific response to a wide variety of tissue traumata such as heat, radiation and chemicals. Inflammation is best defined as the sequence of events following injury to a living tissue, provided that the injury is not of such a nature as to destroy the tissues contemporaneously with the insult (Cappell and Anderson 1971).

The time scale over which the lesion develops depends on the nature of the insult and its persistence. If it is minimal then the tissue may return to normality rapidly but if it is prolonged there are usually proliferative changes which gradually supervene over the exudative and cellular changes of the acute reaction (Cappell and Anderson 1971).

HISTOPATHOLOGY OF THE INFLAMMATORY RESPONSE

The Acute Inflammatory Response:-

General View:-

Most often an acute inflammatory process is sudden in onset and of short duration. In any case, diseases classified as acute usually run a course of only a few days, or at most several weeks.

In any simple wound most of the immediately consequent tissue responses are related to the formation of an inflammatory exudate. Indeed, it would appear that the essence of the inflammatory reaction is to mobilize and transport both the humoral and the cellular components of blood to the site of the injury and to secure their passage through endothelial membranes into the tissue space (Morehead 1965). The cellular character of the exudate defines the morphologic stage of the inflammatory response as acute, subacute or chronic. The fluid substrate of the exudate may be water-rich (serous), protein-rich, fibrin-rich (fibrinous), and so forth. An exudate consisting primarily of neutrophils is termed a purulent exudate. One in which numerous neutrophils are associated with tissue destruction would be a suppurative exudate, as in the case of an abscess (La Via and Hill 1971). During the early phase of the inflammatory process, the predominant cell in all exudates is the neutrophil. As the inflammation progresses, however, the neutrophil granulocyte is replaced by the mononuclear cells. If the inflammatory process

is prolonged, lymphocytes may become quite numerous. Acute inflammatory processes may terminate in healing or death, or they may continue as either subacute or chronic inflammations (Cappell and Anderson 1971).

Changes taking place in an inflamed tissue:-

a. vascular changes:

As early as 1843 Addison demonstrated that when a region is inflamed, leukocytes adhere to the wall of the finer blood vessels and emigrate into the surrounding tissues.

In 1843, Addison experimented with the circulation in the web of a frog's (Rana pipiens, is a poikilotherm as it happens) foot. He applied a crystal of salt to the web and reported that the blood became stationary in the congested vessels at the site of application but that the blood was oscillating to and fro and circulating very rapidly in the adjacent areas.

In 1909, Adami presented his lectures on the experimental production of inflammation in vascular areas and said: "If an incision be made with a perfectly aseptic instrument into the skin, repair takes place with the minimal amount of change recognisable as inflammatory. Repair takes place indeed so rapidly that, if the divided structures have come or have been brought into immediate contact, there may be firm adhesion at

the end of twenty-four hours". In his experiments he noticed that the immediate neighbourhood of the wound was reddened and tumefied, and that there was a feeble exudation from between the opposed surfaces. He was also the first to notice that in the immediate neighbourhood of the lesion, there was a dilatation of the vessels, first of the arterioles, then of the veins.

In 1930, Menkin showed that when trypan blue was introduced into the circulation of previously affected animals, the trypan blue rapidly entered the inflamed area and fixed there. He also showed that the rapid accumulation of the dye in an inflamed area was the result of increased capillary permeability.

Marchesi and Florey (1960) and Marchesi (1961) have described the details of the passage of polymorphonuclear neutrophils, eosinophils, monocytes and red blood cells through the walls of inflamed venules in the mesentery of male and female Wistar rats as seen by electron microscopy. Emigrating leukocytes were found to extent a pseudopod into an intercellular junction of the endothelium.

Once the leukocytes have passed through the vessel wall into the tissue spaces, they have overcome the first barrier between them and the injurious agent. They then pass through the interstitial tissues to the site of the noxious stimulus. The process by which motile cells are attracted toward chemical substances in their environment is known as chemotaxis, and the attracting substances are called chemotactic agents (Morehead 1965).

In 1967-1970 Steele and Wilhelm studied vascular permeability changes. They injected various ions such as chloride and inorganic sodium salts into albino guinea pigs, and showed that there was increased vascular permeability as assessed by the extravasation of the dye at the injection sites. The permeability effects of the various chemicals fell into four main categories: (1) no reaction at isotonic concentration; (2) increased vascular permeability with minimal or no necrosis; (3) increased vascular permeability associated with necrosis; (4) necrosis with minimal effects on vascular permeability. In 1968 Ryan and Hurley studied vascular permeability following thermal injury, to male rats of the Sprague-Dawley strain. They applied a copper disc at 54^{o}C. to the abdominal skin of rats and showed that the effect of the thermal injury was on cells other than vascular endothelium and that these damaged cells released mediators that secondarily induced increased permeability of adjacent blood vessels. The evidence from these various workers indicated that the exudative changes of the inflammatory response resulting from acute tissue injury are mainly of vascular origin, and are responsible for the cardinal signs of heat, redness, swelling and pain.

The visible aspect of the vascular sequence during the inflammatory process is thus essentially as follows: arterial vasodilation, increased capillary flow, increased leakage of plasma protein into extravascular compartments, reduced venous outflow, slowed capillary flow, capillary and venular stasis, alteration of capillary wall proper, emigration of white cells, petechial haemorrhages, and eventually vasorhexis (Zweifach 1965).

b. cellular changes:

The majority of cellular changes in the early inflammatory lesion are due to migration of phagocytic leukocytes from the blood vessels, and are reviewed by Ebert and Florey (1939). They described the observations of two schools of thought on this phenomenon: von Recklinghausen 1863; Ziegler 1890; Grawitz 1890 and Marchand 1890 believed that the cells of the exudate originated from local fixed tissue cells, although Metchnikoff (1905) who made the first detailed study of the large mononuclear cells of the blood and fixed tissue phagocytes, grouped the two types together as functionally identical, and called them macrophages: another school believed the cells of the exudate had a dual origin (Maximow 1902-1927; Ranvier 1890-1899-1900; Renault 1906-1907; Dominici 1902). This group of investigators believed that lymphocytes and monocytes hypertrophied in the tissue to form macrophages, and at the same time, the "resting wandering cell" of Maximow, clasmatocytes of Ranvier, "cellules rhagiocrine" of Renault or "cellules lymphoconjuctive" of Dominici were mobilized as the result of irritation and formed active macrophages which became indistinguishable from the cells of haematogenous origin.

Maximow (1925) investigated the formation of the "tubercle", the typical lesion of tuberculosis infection in vitro. Cultures of various types of blood leukocytes were inoculated with tubercle bacilli and it was shown that the epithelioid and giant cells of the tuberculosis lesions have the same dual origin as the polyblast

or mononuclear exudate cells in common or purulent inflammation. They arise partly from local fixed elements - the histiocytes of the respective tissue (resting wandering cells or clasmatocytes, reticular cells, cells of Kupffer, endothelial cell of the sinuses in the spleen, etc.), partly from the migrated (or local, if available), non-granulated, i.e. lymphoid white blood corpuscles - both monocyte and lymphocyte.

To date the most convincing proof of the identify of the monocytes and macrophage has come from tissue - culture experiments and *in vivo* observations. Clark and Clark (1930a;b) made direct observations on the tails of living amphibian larvae. They found that the large mononuclears of the blood migrated into the tissues and changed into cells indistinguishable from large pigmented macrophages. They actually followed the change of individual blood cells marked with carmine, into pigmented macrophages.

In hanging-drop culture of blood from elasmobranches, teleosts, amphibia, reptiles, birds and mammals (including man), Lewis and Lewis(1925a;b; 1926) found that in the course of a few days the large mononuclears of the blood changed into cells indistinguishable from tissue histiocytes, epitheloid cells and giant cells. They concluded that all three cells were merely variations of the same cell type.

The ontogeny of mononuclear cells in inflammatory reactions has been studied extensively by Spector and his co-workers (Spector *et.al.* 1965; Spector and Willoughby 1968; Spector 1970). Their experimental animals

were Wistar albino rats and they used tritiated thymidine and particulate carbon to label various circulating mononuclear cells. They initiated a focal inflammatory response by intradermal injection of bovine fibrinogen and complete-incomplete Freund's adjuvant, and performed quantitative analysis of the labelled cells appearing in the exudate. Their results indicated that virtually all the exudative mononuclear cells were derived from cells, circularing in the blood of marrow origin at the time of injury and this conclusion has also been confirmed by van Furth (1970 a; b) in the study of the origin of monocytes and macrophages in normal mice.

The Chronic Inflammatory Response:

A disease that persists for more than a few weeks is said to be chronic. Such diseases may be preceded by an acute inflammatory phase, or they may present the features of chronicity from the beginning. Certain diseases such as pulmonary tuberculosis are characteristically chronic from their inception (Willis 1950; Morehead 1965; Cappell and Anderson 1971).

Chronic inflammatory lesions may contain cells of many kinds. There may be a mobilization of macrophages, lymphocytes and at the same time attempts at healing are shown by much fibroblastic activity. In addition giant cells may be formed and may accumulate especially around foreign bodies. The chronic stages of inflammation in contrast to the acute stages are usually characterized by dense collections of mononuclear cells of either local or systematic origin (Glynn 1968; Walter and Israel 1970; Gresham 1971).

The essential feature of the chronic inflammatory response is proliferation of local supporting tissues to produce a stroma of collagen. This proliferation also results from the successful resolution of an extensive acute inflammation insult in which case it develops into scar tissue, but if the acute inflammatory insult continues, the inflammatory and reparative responses take place together and chronic inflammation occurs (Cappell and Anderson 1971).

One of the best studied experimental models of the chronic inflammatory response in mammals has been the carrageenin granuloma (vide infra).

DELAYED TYPE HYPERSENSITIVITY (DTH)

Although the clinical response resulting from the delayed type hypersensitivity phenomenon was well recognised by Jenner (1749-1823) and Robert Koch (1843-1910) and the early history of the development of its study well documented by **Ebert** (1965), it is only recently that its significance as a constituent component of the chronic inflammatory response has been recognized (La Via and Hill 1971).

Delayed type hypersensitivity as described for mammals by Uhr et.al. 1955; Crowle 1962; Mackanes and Blandes 1967; Bloom and Bennett 1970; La Via 1971 and Roit 1972 is manifested histologically as a perivascular infiltrate of mononuclear cells, mainly of the lymphocyte

series, taking place towards the end of certain inflammatory responses, and qualitatively very different from the polymorphonuclear exudate of the early acute response. The mechanism by which delayed type hypersensitivity takes place is not completely understood even for mammals, but it is based on the activities of the "thymus derived" or T-lymphocytes. These are responsible for maintaining the production, by division, of the cell mediated immunity (CMI) cells, the lymphocytes which are found in the delayed type hypersensitivity response (Bloom and Bennett 1970; La Via 1971; Ellis 1974). The CMI cells are apparently stimulated to divide in the presence of certain types of foreign antigenic material, and migrate towards the antigen, bearing on their external surface antibodies which are specific for that particular antigen. They also release substances which have marked biological effects at the sites of activity. These are called lymphokines and among their varied components are the "macrophage inhibition factor" (MIF) which causes macrophages to remain at the sites of a delayed type hypersensitivity reaction, and "macrophage activation factor" (MAF) which acts on localized macrophages to increase their rate of activity phagocytosis and degradative enzyme production. The net effect of the release of lymphokines from CMI lymphocytes is to render the macrophages more aggressive to the offending antigens (Morley et.al. 1973). It is also possible that high levels of lymphokines, in persistent antigen presence, may stimulate the transformation of macrophages of an area into epithelioid cells and later, giant cells in the granulomatous hypersensitivity responses (Epstein et.al. 1962; Boros and Warren 1973; Spector 1967). This latter, however, is still a matter of investigation to determine its validity.

GRANULOMATOUS INFLAMMATION

Originally the term "granuloma" descriptively defined the tiny granular white bodies which could be seen in tissues such as the peritoneum, where they appeared as a result of systematic (hematogenous) spread of the tuberculous organism and the peculiar local inflammatory reaction to the mycobacterium. This regrettable definition has probably been handed down from Virchow (1821-1902) who defined a granuloma as essentially a tumor or neoplasm composed of granulation tissue (Morehead 1965). The term was later redefined according to certain microscopic criteria. At the present time, a chronic granulomatous inflammation is characterized by the presence of mononuclear phagocytes, macrophages, plasma cells, lymphocytes, fibroblasts, epithelioid cells and occasionally giant cells (Morehead 1965; Montgomery 1967; Spector 1969; Gresham 1971; Ward 1971).

The terms "mononuclear phagocyte", "macrophage" and "epithelioid cell" are now accepted as referring to different morphological appearances of the same cell type (Ebert and Florey 1939; Wright 1958).

As described above, granulomatous chronic inflammation is characterized, under the light microscope, by the accumulation of a variety of mononuclear cells, epithelioid cells, and giant cells. When the epithelioid cells assume a nodular arrangement, as they do in response to certain of the injurious agents, the nodules are

known as tubercles. The tubercles are a characteristic feature of certain phases of the inflammatory response in tuberculosis, although they may be seen in other conditions as well (Forbes 1955; Morehead 1965; Epstein 1967; Spector 1969). Giant cells also play a prominent role in granulomatous inflammation; wherever there is an insoluble or slowly soluble substance too large to be ingested by a single cell, a giant cell will sooner or later appear (Haythorn 1929). These large cells result from the fusion of macrophages or amitotic division of their nuclei and the arrangement of their nuclei within the cytoplasm is of some value in diagnosis.

A number of important diseases of animals and man elicit this response. Some of these, such as tuberculosis, leprosy or syphilis are of known etiology, whereas the aetiology of many others such as rheumatid arthritis and sarcoidiosis remains obscure.

GRANULATION TISSUE

According to Hadfield (1951), granulation tissue is the only convenient term in common use for the immature and highly fertile mesenchyme which invades and subsequently replaces, dead, dying, degenerate, ill-nourished, time-expired and useless tissue in any situation in the body, and under a multitude of pathological conditions.

The essential function of granulation tissue is to replace dead material by living mesenchyme. The newly-formed, active,

proliferating capillary blood vessels of granulation tissue are primarily responsible for its capacity to invade.

The general physiological process of mesenchymatous replacement would thus appear to have two phases, the first being one of capillary vascularisation which creates an environment in which cells can multiply. The second phase is characterized by a process of capillary devascularisation which is just as spectacular as the vascularisation of the first phase.

THE MORPHOLOGY OF THE CELLS IN THE INFLAMMATORY LESION

1. Macrophages:

Macrophages of mammals are mononuclear cells, usually relatively large, well rounded or elongated and varying in size from about 10 microns to many times this size. The eccentrically situated nucleus possesses a prominent membrane against which most of the scanty chromatin lies and which is seldom regular in contour, being less convex or even concave toward the main cytoplasmic mass (Evans 1915; Fedorko and Hirsch 1970; Carr 1973).

Dumont and Sheldon (1965) have studied the ultrastructure of hamsters (Mesocricetus auratus) macrophages both before and after phagocytosis. Prior to phagocytosis they were round or oval shaped cells with kidney-shaped nuclei whose chromatin was irregularly distributed around their periphery. Their cytoplasm was moderately abundant, electron dense and possessed rounded mitochondria. A well developed Golgi apparatus was usually found near the invagination of the nucleus.

After phagocytosis of tubercle bacilli, such cells underwent changes in nuclear morphology and showed a marked increase in numbers of mitochondria; there were also major modifications in the cytoplasm, which became generally less electron dense and contained a number of large granular and smaller vacuoles. The tubercle bacilli were contained within phagocytic vacuoles.

2. Polymorphonuclear leukocytes (neutrophils):

Morphologically neutrophils are characterized by the presence of numerous fine granules within their cytoplasm. In haematoxylin and eosin sections the cells appear much smaller than macrophages (10 to 12 micron in diameter). Not only are they capable of ameboid motion, but they are also readily attracted to the site of injury by bacteria and other chemotactic agents (Hirsch 1965; Morehead 1965; McDonald et.al. 1970).

Under the electron microscope the granules are seen as dense round structures, sprinkled liberally throughout the cytoplasm The endoplasmic reticulum is characteristically sparse in the cell and mitochondria generally few and small (Goodale et.al. 1962; Watanable et.al. 1967).

3. Lymphocytes:

In tissue sections lymphocytes are smaller than polymorphonuclear leukocytes but slightly larger than red blood cells (8 to 10 micron

in diameter. They consist for the most part of a relatively large, round, intensely basophilic nucleus, surrounded by a narrow rim of cytoplasm (Ham 1969; McDonald et.al. 1970).

Under the electron microscope, the nucleus usually contains one or more nucleoli and often shows a deep unilateral cleft of the membrane. In the cytoplasm, mitochondria are few and they are large and clear. The endoplasmic reticulum is absent or barely represented but free ribosomes are abundant. The Golgi apparatus is rudimentary or absent (Sutton and Weiss 1966). The cytoplasm of small lymphocytes contains only a small number of mitochondria and occasional vesicles. There is no endoplasmic reticulum or Golgi body and only isolated ribosomes. The nucleus often shows a narrow invagination and there is usually a small nucleus (Burnet 1970).

4. Plasma cells:

The plasma cell was first described by Unna and Pappenheim and the Unna-Pappenheim (pyronin and methyl green) method is the classical means of staining plasma cells (Movat and Fernando 1962; Burnet 1970). The plasma cell is derived from the lymphocyte and is present in the connective tissue and lympho-reticular tissue (Burnet 1970).

The plasma cell is roughly the size of a small or large lymphocyte (8 to 15 micron in diameter) circular or oval in shape. It has a dense characteristically eccentric nucleus, resembling a cart-wheel in appearance. The cytoplasm is deeply basophilic, homogenous with a high ribonucleic acid (RNA) content (Lever 1967; Ogilvie 1967; Abraham 1970; McDonald et.al. 1970).

Under the electron microscope, the main feature of the plasma cell is the extreme development and density of the endoplasmic reticulum and the comparable development of the Golgi apparatus with its centrioles. The nucleus of the mature plasma cell is extremely electron dense. There is condensation of the chromatin at the periphery of the nucleus, giving it the well known "cart-wheel" appearance. Well developed nucleoli are found only in immature cells. Round to oval dense bodies (cytosomes) are common in plasma cells. These bodies are probably microbodies or lysosomes. In plasma cells, the ergastoplasmic sacs may be flat or dilated; when dilated they may contain a finely floccular substance, small dense bodies, large dense bodies (Russel bodies), or crystals (Movat and Fernando 1962).

5. Fibroblasts:

The major role of the fibroblast appears to be synthesis, maintenance and repair of the connective tissue components (Schoenberg and Moore 1958). Its morphology is characteristically that of a spindle-shaped or stellate cell. It contains a single oval nucleus with one or more nucleoli. It has moderately abundant cytoplasm, which may be rich in ribonucleoprotein and may possess firmly ectoplasmic processes closely wrapped about bundles of fibres (Paul 1961; Chapman 1962).

Under the electron microscope, its elongated shape and well developed ER with extensive dilated cisternae and its association with collagen

fibres make it easily recognised (Chapman 1961). Vesicles occupy a considerable part of the cytoplasmic volume, and mitochondria are abundant (Sutton and Weiss 1966). The Golgi apparatus in the resting fibroblast is usually very small and small vesicles are found scattered throughout the cytoplasm (Movat and Fernando 1962), but among the mesenchymal cells it is the fibroblast and the plasma cell which have the largest and most complex Golgi areas (Movat and Fernando 1962).

6. Epithelioid cell:

The epithelioid cell is a characteristic feature of many types of granulomatous inflammation. Most observers accept that the cell is derived from the macrophage system (Maximow 1925; Epstein and Krasnobrod 1968; Spector 1969; Carter and Roberts 1971), but beyond that the factors that determine its origin are as yet undefined.

The epithelioid cell, in granulomatous chronic inflammation, is a large polygonal cell, which has a poorly defined cytoplasmic margin that merges with those of neighbouring cells. The abundant cytoplasm is eosinophilic and amorphous or finely granular. The nucleus appears large, ovoid and palely stained with a sharp, delicate nuclear membrane (Lewis et.al. 1925; Lewis and Lewis 1926; Epstein et.al. 1963; Sutton and Weiss 1966; Epstein 1967; Papadimitriou and Spector 1971).

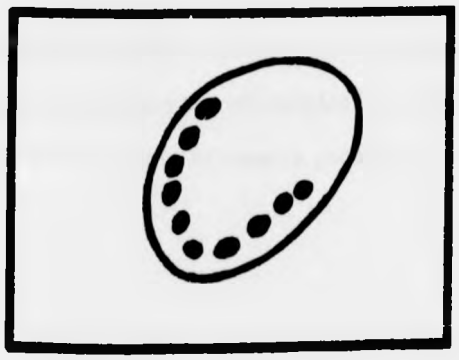
Limited information is available concerning the ultrastructure of epithelioid cells (Sutton and Weiss 1966; Wanstrup and Christensen 1966; Hirsch et.al. 1967; Epstein 1967). The prototype epithelioid cell is readily recognized by its very large, ovoid nucleus, with two or more nucleoli. The plasma membrane shows many pseudopodia and the cytoplasm usually contains numerous vacuoles. Some of these vacuoles appear to contain residual traces of phagocytosed material. The Golgi apparatus and a centrosome are seen frequently and a moderate number of mitochondria occur.

7. Giant cells:

There are three types of multinucleated giant cells encountered in granulomatous infiltrates. These large cells result from the fusion of macrophages (Poole and Florey 1970; Carter and Roberts 1971), or amitotic division of their nuclei without cell division (Forkner 1930).

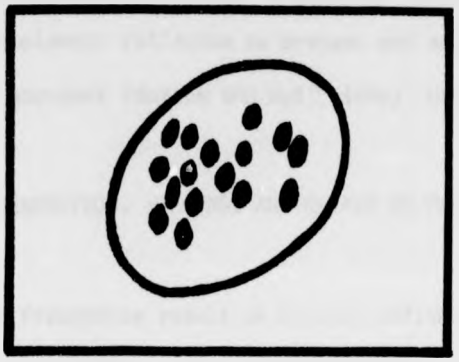
Morphologically they have a pale, eosinophilic cytoplasm and contain many nuclei. The three types recognized are:

(1)



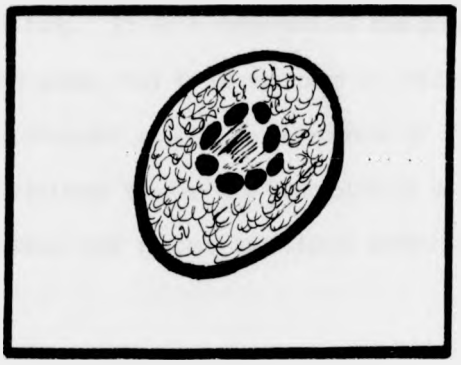
The Langhans cell which is seen in follicular granulomata, and consists of an oval or circular cell with a peripheral rim of nuclei arranged in a horse-shoe configuration, e.g. in tuberculosis.

(2)



The Foreign-Body type cell which tends to be irregular in shape and the nuclei are scattered at random throughout the cell. Foreign material may be seen within the cytoplasm, or lying free in the tissue surrounded by the giant cells.

(3)



The Touton type of giant cell which is seen most frequently in the granulomata formed as a reaction to lipids, e.g. in xanthoma. The cell is circular and contains a perfect ring of nuclei situated midway between the centre of the cell and periphery (Milne 1972).

The final disposition of giant cells probably depends largely on the relative inactivity of the substances which brought about their formation (Haythorn 1929).

Under the electron microscope, the nuclei of multinucleated giant cells have thin rims of marginal chromatin, the cytoplasm exhibits long interlocking filaments passing to other cells, and the

mitochondria are abundant and increase with age. Granular endoplasmic reticulum is present and smooth endoplasmic reticulum is abundant (Sutton and Weiss 1966; Hirsch et.al. 1967; Carr 1973).

REGENERATION, HEALING, AND REPAIR IN MAMMALS

The favourable result of chronic inflammation is resolution of the initial lesion. This may involve regeneration of damaged cells or more usually, replacement by the healing of the mesenchymatous tissues. Regeneration is a restorative process which, under normal conditions, is continually taking place in many of the tissues of the body. It is a component of the process of repair. The ability of a given cell to participate in this process depends largely upon its inherent ability to regenerate or multiply. If certain highly specialised tissues are incompletely destroyed the remaining cells multiply and restore the tissue essentially to its previous state.

Healing and Repair:

Healing and repair are practically synonymous terms and are commonly used interchangeably. Healing of a simple mammalian wound can occur with two types of histological manifestation which are referred to as primary and secondary unions (Boyd 1943; Morehead 1965; Montgomery 1967), and other more complicated healing processes are the result of the contemporaneous activities of the chronic inflammatory response and the healing mechanisms.

1. primary union (healing by first intention):

This occurs in uninfected lesions and it is characterized by the formation of only a limited amount of granulation tissue. At the beginning, epithelioid repair, vascular proliferation and fibrous tissue development take place and these progress to result in healing with a pale linear scar, although occasionally a hypertrophic scar or keloid forms (Mallory 1929; Willis 1950; Morehead 1965; McMinn and Pritchard 1969).

2. secondary union (healing by secondary intention):

This occurs in infected wounds, and is accompanied by an acute inflammatory reaction. In this case, the most obvious microscopic response is an accumulation of cells in the wound area. In the first 24 hours, these are mainly neutrophils and lymphocytes; thereafter, macrophages become more numerous, and from about the third day onwards, fibroblasts become prominent. New capillaries advance into the wound area by budding and other infiltrating cells constitute "granulation tissue" which grows from the base of the wound to fill the defect. At the beginning the neutrophils and macrophages together, by enzymic action and phagocytosis, remove the fibrin and debris. After the removal of blood, fibrin and dead cells from the wound, long spindle-shaped fibroblasts begin to proliferate. New permeable vessels and capillaries form. This fibrovascular granulation tissue continues to proliferate and to fill the wound space and further maturation of collagen which, over a period of months, becomes progressively less cellular (Mallory 1929; Willis 1950: Cliff 1963; 1966; Morehead 1965; McMinn and Pritchard 1969; Cappell and Anderson 1971).

COLLAGEN FORMATION

Collagen is the main structural component of the healed wound, and the fibroblast plays an essential role in the production of collagen fibrils and other extracellular components of connective tissue. Collagen fibrils are found in association with acid and neutral mucopolysaccharides. Collagen and its associated mucopolysaccharides are produced by fibroblasts. Berenson (1961) investigated the site of production of the mucopolysaccharides, and the factors regulating the synthesis of extracellular substances in vitro using growing chick embryo fibroblasts in a synthetic medium. He showed that the mucopolysaccharides were derived from the fibroblasts in sequence, hyaluronic acid, and three types of chondroitin sulphate being produced.

Fibroblasts are derived from resting fibrocytes or undifferentiated mesenchymal cells of perivascular disposition (McMinn and Pritchard 1969). These cells can be divided into two groups; a first group which produces the collagen of the connective tissue layers around the various tissues, and the other group comprising the chondroblasts and osteoblasts which produce the collagen of bone and cartilage (Finerty and Cowdry 1962; Ashhurst 1968; Ham 1969).

The morphology of the fibroblast has been studied extensively by Chapman (1961, 1962) and Movat and Fernando (1962). The active state is characterized by very extensively developed endoplasmic

reticulum with extensively dilated cisternae. The cell participating in healing is concerned largely with the production of collagen fibrils by secreting tropocollagen (Jackson 1957). The secretory mechanism has been studied by Chapman (1961), using the carrageenin granuloma as a model. The cell loses cytoplasmic content during fibrogenesis and his general conclusion from an ultrastructural study was that the secretory mechanism for collagen was similar to that occurring in apocrine gland cells. However Ross and Benditt (1965) on the basis of their autoradiographic studies, suggested the possibility that collagen was released from the cells as a form of merocrine secretion.

VITAMIN-C AND ITS INFLUENCE ON HEALING

The chemical name for vitamin-C is L-ascorbic acid. It is a white, odourless, crystalline compound, soluble in water, but insoluble in fat solvents. It is very heat labile and prone to atmospheric oxidation (Gresham and Jennings 1962; Harper 1971).

Defects in collagen formation may result from generalized deficiency in vitamin-C. Deficiency of the vitamin appears to disturb the synthesis of collagen within the fibroblasts and also to arrest the maturation of much of the collagen at the stage of the argyrophil fibre. It is now accepted that vitamin-C is necessary for the gelation of an intercellular fluid matrix, secreted by the fibroblasts (Wolbach and Howe 1926; Robertson and Schwartz 1953; Knox and Goswami 1961).

reticulum with extensively dilated cisternae. The cell participating in healing is concerned largely with the production of collagen fibrils by secreting tropocollagen (Jackson 1957). The secretory mechanism has been studied by Chapman (1961), using the carrageenin granuloma as a model. The cell loses cytoplasmic content during fibrogenesis and his general conclusion from an ultrastructural study was that the secretory mechanism for collagen was similar to that occurring in apocrine gland cells. However Ross and Benditt (1965) on the basis of their autoradiographic studies, suggested the possibility that collagen was released from the cells as a form of merocrine secretion.

VITAMIN-C AND ITS INFLUENCE ON HEALING

The chemical name for vitamin-C is L-ascorbic acid. It is a white, odourless, crystalline compound, soluble in water, but insoluble in fat solvents. It is very heat labile and prone to atmospheric oxidation (Gresham and Jennings 1962; Harper 1971).

Defects in collagen formation may result from generalized deficiency in vitamin-C. Deficiency of the vitamin appears to disturb the synthesis of collagen within the fibroblasts and also to arrest the maturation of much of the collagen at the stage of the argyrophil fibre. It is now accepted that vitamin-C is necessary for the gelation of an intercellular fluid matrix, secreted by the fibroblasts (Wolbach and Howe 1926; Robertson and Schwartz 1953; Knox and Goswami 1961).

Ascorbic acid is widely required in metabolism; it can be synthesized in a variety of plants and animals except man, monkeys, guinea pigs and other primates. Those animals which are unable to synthesize the vitamin presumably lack the enzyme system necessary to convert L-gulonic acid to ascorbic acid (Knox and Goswami 1961; Keele et.al.1966).

The normal course of events in repair of a wound was described in the guinea pig by Dunphy et.al. (1956). Fibroblasts increased in large numbers and with invasion of capillaries, collagen formation proceeded. The tensile strength of the wound increased with collagen synthesis. By 12 days, the collagen content of the wound reached a maximum value. In contrast, studies on the effect of L-ascorbic acid deficiency on the sequence of wound repair in guinea pig showed that fibroblasts were poorly oriented and immature, and no collagen formation took place. By the twelfth day, the absence of collagen in the presence of immature fibroblasts was established.

CARRAGEENIN GRANULOMATA

Carrageenin has long been known as an excellent experimental inflammatory agent, and for studying connective tissue activity (Robertson and Schwartz 1953; Slack 1956; Williams 1957; Ryan and Spector 1969). The injection of carrageenin elicits a local response characterized by massive macrophage infiltration (so-called granuloma), followed by deposition and finally by resorption

of connective tissue (Jackson 1957; Chvapil and Cmuchalova 1961). The carrageenin lesions stay constant over a long period (Ryan and Spector 1969; Spector and Ryan 1969). Several investigators have found in various species that in the granulation tissue of carrageenin induced granulomas, extensive collagen synthesis commences only after the period of most active fibroblast proliferation has passed (Chvapil and Cmuchalova 1961; Menner 1971).

Histological studies of carrageenin granulomata have been restricted to date to mammalian species. The origin of the use of extracts of Chondrus crispus (Carrageenin) as an inflammatory agent lies in the work of Robertson and Schwartz (1953), who demonstrated that carrageenin provided the most satisfactory stimulus to fibrous tissue formation of several materials which they have tested.

Carrageenin has a high molecular weight (100,000 to 500,000) and is a long-chain polymer of sulphated galactose units (Smith and Cook 1953; Smith et.al. 1954) obtained from many members of the Rhodophyceae which are marine algae commonly found round the coast of the British Isles. They occur intercellularly and within cell walls. Species of Chondrus, Gigartina, Eucheuma, Polyides, Hypnea and Irideae all yield carrageenins or substances with carrageenin-like properties. Systematic nomenclature will doubtless emerge as the properties and structures of the carrageenins from the less familiar red sea-weeds are revealed and their relationship to those of the original carrageenins of Chondrus, and Gigartina species become apparent (Anderson 1967).

Most of the published work available has been concerned with carrageenin from species of Chondrus crispus, a reddish purple plant 7.5 to 15 cm. high with flat, wedge shaped segmented fronds. Under estuarine conditions the plant is much larger, lobed, fringed and parsley-like in form (Newton 1962). It is the common Irish Sea moss from which soups, jellies and other components requiring a gelatin component are manufactured. The name comes from Carrageenin, near Waterford, Ireland where Chondrus crispus abounds (Fig. 1).

Fig. 1. Dried carrageenin, as obtained from a health food shop, Edinburgh.

Biological activities:-

Carrageenins exhibit all of the general biological activities of the sulfated polysaccharides, such as interaction with proteins (Burns and Beighle 1960; Jacques 1962). They also have several other interesting biological effects. Anderson (1967) noted, for instance, that they reacted with fibrinogen resulting in an anticoagulant activity. Also, because of their strongly anionic structure, they are markedly metachromatic in histochemical tests. Williams 1957; Monis et.al. 1968; Gongolli et.al. 1973 all used Periodic acid-Schiff (PAS), and toluidine blue techniques to demonstrate metachromasia in carrageenin granulomata.

Another biological effect of carrageenin is its action on the vertebrate gastro-intestinal tract. Degraded and undegraded carrageenin, given orally or by stomach tube, has been shown to prevent or diminish histamine induced experimental peptic ulceration (Anderson and Soman 1967). Protection against gastric ulceration can be demonstrated in the guinea pig, when carrageenin is administered intraduodenally in an animal with a surgically ligated pylorus, but carrageenin does not appear in the gastric secretion, leading to the suggestion that some humoral mechanism is involved in the anti-ulcerative activity (Anderson and Soman 1963).

Degraded carrageenin can prevent experimental peptic ulceration when administered intravenously at a fraction of the oral dosage

(Anderson and Soman 1967), or by the subcutaneous route (Watt et.al. 1966), but undegraded carrageenin is not suitable either for intravenous administration because of its acute toxicity or for subcutaneous administration because of the marked tissue reaction, namely carrageenin granuloma, which ensues (Anderson 1967).

CARRAGEENIN AS AN INFLAMMATORY AGENT:

Carrageenin was introduced as an inflammatory agent by Robertson and Schwartz (1953), who demonstrated that it provided the most satisfactory stimulus to fibrous tissue formation of several materials which they tested. Their study was principally concerned with the analysis of collagen formation by a chemical means. However, after its introduction by Robertson and Schwartz, the carrageenin granuloma was used as a source of biological stimulus for a number of combined histological and biochemical studies of connective tissue development. Detailed histological studies of the carrageenin granuloma were furnished by Williams (1957), who studied the histological development of carrageenin granulomata in the guinea pig. He described two different types of reaction depending upon whether the carrageenin was injected subcutaneously or intra-dermally.

When he injected the material subcutaneously into the abdomen of skin there was an oedematous reaction with polymorphonuclear and mononuclear leukocytes appearing within the inflammatory response by the third day but by the 5th day granulation tissue consisting

of capillaries and fibroblasts had developed in the carrageenin mass. By the 10th day much of the extracellular carrageenin had disappeared, to leave a highly cellular tissue, consisting predominantly of macrophages and fibroblasts, surrounded by a fine reticulum of fibrous tissue. By approximately 14 days, collagen fibres had appeared in the proliferation zone of the granuloma and from then on the amount of collagen increased towards the centre of the granuloma. As the collagen developed in control areas, there was unexpected simultaneous resorption of the previously developed collagen fibres. By the 28th day, most of the fibres had been resorbed and this was completed by the sixth week, with no obvious inflammatory response remaining to the initial injection.

By contrast, when the carrageenin was injected into the dense interlacing collagen fibres of the dermis of the nape of the cavy's neck, there was progressive loss of dermal collagen tissue manifested by atrophy, until only a thin connective tissue was left. He did not see plasma cells in those histopathological studies on the subcutaneously and intradermally developed carrageenin granulomata. He expressed the view that carrageenin was a large molecular compound with a slow rate of diffusion, acting as a space-occupying foreign body and that the granuloma represented a reaction on the part of the organism to remove it by means of various forms of macrophage activity and fibrous replacement.

In 1959 Benitz and Hall described the histological features of the connective tissue reaction to carrageenin in the rat. From the first to the 4th day there was an acute polymorphonuclear infiltrate which later (5th to the 28th day) was gradually transformed into a polysaccharide containing granuloma. This observation differed somewhat from Williams' finding in the guinea pig, since Williams had found only a moderate polymorphonuclear leukocytic response in the early stages of the induced lesions. These differences indicated that carrageenin stimulated a more violent acute inflammatory response in the rat, than in the guinea pig, where it reacted by forming a pronounced granulomatous lesion.

Gardner (1960) employed carrageenin to produce experimental arthritis in rabbits and guinea pigs. He expressed the view that the response to this material depended entirely upon local stimulus to an inflammatory process, and that carrageenin was not antigenic, produced no systemic effects, and produced a reaction with a high degree of reproducibility.

McCandles and Lehoczky-Mona (1964) carried out a small study on the stimulus to connective tissue growth by carrageenin. They injected carrageenin into the guinea pig, and the animals were sacrificed 14 days after injection. Their histological observations were that the lesion comprised a collagenous network containing fibroblasts, macrophages and epithelioid cells. The macrophages were replete with carrageenin as demonstrated by the alcian blue staining technique.

Johnston and McCandles (1968) studied the immunologic response of the rabbit to carrageenin, and showed that despite Gardner's (1960) assertions to the contrary, carrageenin did elicit the formation of precipitating antobodies, at least in the rabbit.

In mammals, the electron microscope features of carrageenin granulomata have been studied by Chapman (1961, 1962), who described the fine structure of collagen and fibroblasts of the guinea pig, in carrageenin granuloma. After seven days the granuloma contained many fibroblasts and exhibited rapid production of collagen. The fibroblasts were characterized by an extensively developed endoplasmic reticulum and showed numbers of fine unstriated filaments in the outer regions of the cytoplasm. The cytoplasmic membrane was frequently disrupted, particularly bordering regions in which filaments occurred. He suggested that connective tissue precursors were released from fibroblasts by the disintigration or dissolution of the cytoplasmic membrane as in apocrine gland cells.

Monis et.al. (1968) set up experiments to study granulomatous lesions in the rat, following a single subcutaneous injection of carrageenin. In their study they found that on the 7th day the granulation tissue was rich in macrophages which were enlarged, and possessed one or several nuclei.

Spector and Ryan (1969) studied the life-span of macrophages in the carrageenin granulomata of the rat. The macrophage precursor cells

had been previously labelled *in vivo* with tritiated thymidine, and a low turnover rate was found in the macrophages within the reaction to carrageenin. They attempted a more extensive study of the life-span of macrophages in chronic inflammatory lesions (Ryan and Spector 1969). Using complete and incomplete Freund's adjuvant and *Bordetella pertussis* vaccine as an inflammatory agent. They found they could divide macrophages into two categories, low-turnover and high-turnover. Low-turnover macrophages were specifically the ones which formed the mature granuloma and were laden with carrageenin.

Perez-Tamayo (1970a) in a study of *in vitro* tissue explants showed that carrageenin granulomata possessed specific collagenolytic activity. He showed that the collagenolytic activity was present from the earliest stages in the development of the carrageenin granuloma, and increased progressively to reach a maximum in 14 day-old granulomas. He also studied (Perez-Tamayo 1970b) the ultrastructure of collagen resorption in the carrageenin granuloma. He carried out the study on the rabbit and the guinea pig, and showed that, at early stages the heterogeneous cell population consisted mainly of polymorphonuclear leukocytes, macrophages, and fibroblasts. In rabbits many giant cells were present at light microscopic level. These had four nuclei or more and were observed from three days after injection. Otherwise there was no significant difference in the microscopic evolution of the carrageenin granuloma in the guinea pig and in the rabbit.

Papadimitriou and Spector (1971) studied the origin and fate of

epithelioid cells in mice. They used several inflammatory agents such as Bordetella pertussis vaccine, BCG, colloidal gold, heat-killed Staphylococus albus, and carrageenin. They extracted the granulomata surgically and cultured them in vitro. They were then attached to strips of cellophane and the cellophane was surgically inserted into the irradiated mice. The animals were killed one week later and the macrophages subjected to electron microscopy. This showed that the macrophages were replete with carrageenin with hypertrophied Golgi apparatus but no lysosomes. Their experiments indicated that the life-span of the epithelioid cells was normally 1-4 weeks; and they considered that the epithelioid cells probably developed in the granulomata when the influx of macrophages exceeded the number required to ingest the non-digested irritant, or when the irritant was digested , not acutely toxic to the macrophage but continuously available so that new macrophages were in constant supply and lived long enough to develop epithelioid features.

INFLAMMATION IN FISHES:

As early as 1891 Metchnikoff wrote "we might turn our attention to the class of fishes, where we find inflammatory processes similar to those that are known the study of higher animals"; and in 1905 he injected red blood corpuscles of the blood of a guinea pig into the peritoneal cavity of a goldfish (Carassius auratus.L.) and observed that the numerous leukocytes that were found in the peritoneal fluid seized them and ingested them. However, despite

this early reference to the inflammatory response in fishes by the father of cellular pathology, subsequent studies of inflammation in teleosts have been very few in number. Indeed, fish pathology as a science is at present, in a very early stage of development, in comparison with mammalian or even avian pathology. This was highlighted by Snieszko (1972) who, in an analysis of current references to aspects of fish pathology, found that 48 per cent dealt with taxonomy of parasites of fishes, 14 per cent with fish biology, 9 per cent with the life-cycle of parasites and only 29 per cent had the word "disease" in their title. Thus, true pathological studies comprise a minute component of the total of the research which is being carried out at present under the imprecise umbrella term "disease of fish". There have, however, been occasional references to the inflammatory response in teleost fish since Metchnikoff's histological description.

Sutherland (1922) studied a tuberculosis-like disease in halibut (Pleuronectes hippoglossus vulgaris.L.), but he failed to find the typical giant cells of the tuberculosis lesion. Hodgkiss and Shewan (1950) described a "suppurative" sic and necrotic lesion in the muscle of a free-living plaice (Pleuronectes platessa.L.) putatively caused by a Pseudomonas sp..

Jakowska and Nigrelli (1953) produced experimental infections of female guppies (Lebistes reticulatus.L.), with a strain of Mycobacterium sp .. The cellular response was studied at intervals. She found that most bacteria remained extracellular during the first

two hours, but within 24 hours a few macrophages had appeared, and some of them had ingested micro-organisms. At 48 hours the number of macrophages had reached a peak, then rapidly diminished.

Wood and Yasutake (1956) and Wood et.al. (1956) studied a mycosis like granulomata of brook trout (Savelinus fontinalis.L.). The lesions were detected histologically in the walls of the stomach, the gills, liver, spleen. They were characterized microscopically by circular masses of fibrous tissue with foreign body cells and resembled tubercles.

Nigrelli and Vogel (1963) described spontaneous tuberculosis in 151 species of fishes, 11 amphibians and 24 species of reptiles, the result of a large number of investigations over many years. They showed that tuberculosis was much more prevalent in poikilothermic animals than had previously been suspected, but did not describe the occurrence of giant cells in such lesions.

Kluge (1965) studied flavobacterial infections on the black mollie (Molliensia sphenops.L.). The disease required several months to kill the fish, and at necropsy histological examination showed the presence of granulomatous lesions. Cyst-like structures and nodules were observed in the liver of infected fish, the latter consisting of macrophages surrounded by a zone of lymphocytes which in turn was surrounded by connective tissue.

Janssen and Waaler (1967) reported that both the cod (Gadus morrhua.L.) and the goldfish, after receiving intramuscular injection of two per cent silica at high temperature, responded with a definite

cellular reaction comprising lymphocytes, macrophages, and a few fibroblasts, with the accumulation of doubly refractile granules within the cells.

The first detailed histological studies on the inflammatory response of fishes which took into account the effect of temperature under controlled conditions was a study on rainbow trout (Salmo gairdnerii.L.) by Finn and Nielson (1971). They carried out a series of experiments involving injections of Freund's adjuvant, a suspension of heat-killed Staphylococcus aureus, and in addition produced tissue damage by various physical traumata. Fish were held at $15^{o}C$ and sampled over a period of 16 days. The cellular response consisted of polymorphonuclear leukocytes (PMN), macrophages, fibroblasts, lymphocytes and giant cells. There were no essential differences in the type of inflammatory response observed.

Wolke and Trainer (1971) described foreign body and Langhans type of giant cells in granulomatous enteritis in the White Sucker (Catostomus commersoni.L.) associated with the presence of diatoms (Chrysophyta) in the intestinal submucosa.

Mawdesley-Thomas and Bucke (1973) studied the healing reaction on a mechanical injury of the skin, connective tissue, muscle and spine of the goldfish. Fish were injured by the passage of a hypodermic needle through the dorsal musculature and sacrificed after 28 days. Throughout the experiment, water temperature was

kept between 17 and 21°C. The cellular response consisted of both acute and chronic inflammatory cells (macrophages, lymphocytes, plasma cells), with no evidence of microabscess formation, but the fibroblastic response was marked, particularly in the peritoneal areas. Around the site of injury, degenerating muscle fibres, muscle giant cells, and oseoclasts were seen. Joy and Jones (1973) studied the chronic inflammatory response to Lernea infection in a seranid fish (Morone chrysops.L.). This was characterized by extensive proliferation of fibrous connective tissue in the dermis and muscle.

Roberts et.al. (1973a) carried out studies on the tagging lesions in newly tagged salmon parr (Salmo salar.L.) as a model for the study for the inflammatory response within the tissue of the Atlantic salmon. The trauma used to produce the lesion was the introduction into the tissues of a plastic monofilament trace, carrying a plastic identification tag. Histological examination of the tagged area revealed that the early lesion comprised PMN and later severely affected muscle fibres were replaced by fibrogranulation tissue, but that the extension of the muscle lesion was limited by the perimysium and intermyotomal fascial planes. In the ensuing period the lesions reached maturity at 25 days when they consisted of follicular granulomata with multinucleated giant cells of foreign body type. The macrophages of the granuloma were swollen and many had melanin pigment and large vacuoles. They also studied the longer term features of this tagging lesion in the returning adult fish, and observed that the arterial supply to the area showed severe endarteritis obliterans.

The fibrogranulation tissue extended down through the fatty hypodermis to the subjacent muscle tissue. They showed several differences from the long term chronic inflammatory response in homeotherms, including the invasion of the chronic inflammatory tissue by eosinophil granule cells of unknown function and also the migration into the inflammatory lesion of melanin containing cells of the pigment cell series (Roberts et.al. 1973b).

In plaice chronic granulomatous inflammatory lesions were experimentally induced at 10°C, by injecting Freund's complete and incomplete adjuvant, talc, beryllium oxide and Mycobacterium marinum intramuscularly into the fish (Timur, G. 1975). After histological examination, granulomatous lesions composed of macrophages, lymphocytes, plasma cells, epithelioid cells, giant cells (Foreign body and Langhans type), and fibroblasts were found.

Relationship between temperature and defence mechanisms:-

Fishes are cold-blooded piokilothermic vertebrates. Their body temperature adjusts passively to that of the surrounding water. As early as 1891 Metchnikoff had shown that temperature was an important variable in the immunologic responsiveness of piokilothermic animals, and more complete studies on the temperature dependence of defence mechanisms in fishes were reported by Cushing (1969).

That the defence mechanisms of fish may vary with temperature was shown by Bisset (1946). He reported that goldfish at 10°C

could be infected with Ps. fluorescens which was normally considered to be saprophytic, but when the temperature was raised to 23°C., the bacteria were rapidly cleared from the fish tissues.

Janssen and Waaler (1967) reported that both the goldfish and the cod, after receiving intramuscular injection of two per cent silica at 6°C., responded with only minor degenerative changes and surprizingly they observed no inflammation. Finn and Nielson (1971) however, carried out experimental studies on rainbow trout, with Freund's complete adjuvant and a suspension of heat-killed Staphylococcus aureus at 5°C and 15°C and while they observed a delay in the appearance of macrophages within myotomes, the clearance of necrotic tissue from the lesions and the advent of fibroplasia in the inflammatory response of the fish held at 5°C relative to those at 15°C they still showed a qualitatively similar response.

Roberts et.al. (1973a) reported on the tagging lesion in newly tagged salmon parr, at three temperatures 12°, 8° and 4°C. Again, although the inflammatory response was similar at each temperature, it was inhibited at lower temperatures in its time scale of development. At the lower temperatures PMN appeared to play a slightly more dominant role in the lesion. The fibro-granulation tissue, in both the higher temperature specimens, was dominated by fibroblasts and fibrous tissue whereas in the 4°C fish even at 41 days fibrogranulation tissue consisted of a much smaller population of fibroblasts and was composed mainly of eosinophilic ground substance with considerable numbers of monocytes, myophagic macrophages, and in its early stages, PMN.

McQueen et.al. (1973) studied temperature effects in plaice, following infection with cercariae of the digenean fluke (Cryptocotyle lingua). They observed that the rates of cyst and host capsule development were consideraly lessened by the temperature reduction of 10^{o}C., but there was apparently no qualitative difference between infections at 15^{o}C and 5^{o}C., and the response at either temperature was slight, indicating that the fluke was a particularly well adapted parasite.

Anderson and Roberts (1975) compared the inflammatory response and the epithelial healing response of the Atlantic salmon and the white mountain cloud minnow (Tanichthyes albonubes.L.) a tropical species, in order to compare the response of a temperate and tropical teleost. Results of this study showed that the rate of healing in each species was similar at their optimum temperature, that elevation of temperature above the optimum did not markedly inhibit the response, but at low temperature the response was very much slower.

The teleost in normal health has a balance at a given temperature with the potential pathogens in its environment. Changes occurring in ambient temperature force it to reorient its defensive mechanism to deal with the new situation, and this continuing fluctuation in the relationship between the host and its pathogen is a vital factor in the study of the epizootology of fish diseases. Its effects are manifested in the histological

changes found in the response to such diseases as has been shown by Roberts (1974).

Timur, G. (1975), in order to determine the effect of temperature variation, carried out experimental studies of plaice with Freund's complete adjuvant at $5^{o}C$ and $10^{o}C$. After histological examination of the fish subjected to the insult at the lower temperature, she found that the response was characterized by the presence of PMN for much longer periods in the time scale of the experiment. The appearance of epithelioid cells and fibroblasts was delayed and no giant cells were observed.

THE MORPHOLOGY OF CELLS IN INFLAMMATORY LESIONS IN FISHES:

The histological details of most of the cells involved in the chronic inflammatory response of plaice have been described by Russell (1974), in a study of naturally occurring lymphocystis infection; by Ellis (1974) in a study of reticuloendothelial systems in the plaice and by Ferguson (1975), in a study of the ultrastructure of buffy-coat (leukocytes) cells. The electron microscopic details of the plasma cells of fish have only been described by Clawson et.al. (1966) in the paddlefish (Polyodon spathula). The following is a composite description based on these studies.

1. macrophages:-

These were circular in shape with a rounded or bean shaped eccentric nucleus, with large macrophages having an irregular shape and a foamy cytoplasma.

2. polymorphonuclear leukocytes (neutrophils):-

These were pale cells, with round bean-shaped nuclei occupying one third to one half of the cell, the cytoplasm usually being of a pale-pink colour.

Under the electron microscope, its outline was more irregular. The nuclear chromatin was dense. The cytoplasm was distinguished by its numerous large, round to elongated granules. The Golgi apparatus was not prominent.

3. lymphocytes:-

These were round shaped with only a rim of basophilic cytoplasm. Under the electron microscope, the plasma membrane was plicated and occasional small pseudopodia were evident. The nuclear chromatin peripherally was dense. The cytoplasm of the cell showed small vesicles, rough endoplasmic reticulum and large, often elongated, mitochondria.

4. plasma cells:-

These were oval shaped cells with the nucleus eccentric and occupying one half to one third of the cytoplasm. The nucleus was blue and the cytoplasm a purple colour when stained by Unna-Pappenheim's method (methyl green pyronin).

Under the electron microscope, the cells were studied in the

paddlefish and identified by their cytoplasm which was dominated in its fine structure by an open, vesicular, rough endoplasmic reticulum. Many free ribosomes were scattered about the cytoplasm. The nucleus was irregular in shape but without deep indentations or clefts. The surface of the cells was generally smooth to only slightly irregular and revealed no true microvillous processes.

5. fibroblasts:-

These were spindle shaped with a wide variation in nuclear density.

In addition the following cells have been described in teleost chronic inflammation.

6. epithelioid cells:-

These were large polygonal cells with an oval nucleus and indistinct cell boundaries. Such cells are closely related to their neighbouring epithelioid cells (McQueen, A. 1974; Roberts, R.J. 1974 pers. comm.).

7. giant cells:-

So far in fish pathology, only the foreign body type and Langhans type of giant cells have been reported (Aranson 1926; Amlacher 1970;

Dunbar and Herman 1971; Wood and Yasutake 1956; Wolke and Trainer 1971; Russell 1974; Roberts et.al. 1973a; Timur, G. 1975). According to Timur, G. (1975), foreign body type giant cells have an irregular shape and their nuclei are scattered at random throughout the cell; Langhans type of giant cells are oval or circular, with a peripheral rim of nuclei arranged in a horse-shoe configuration.

REGENERATION, HEALING AND REPAIR IN TELEOST FISHES:

Healing and reparative ability is of extreme ecological significance in free living wild animals. While much attention has been given to this process in the homeothermic animals, little attention has been paid to poikilothermic animals. Healing is an essential component of the inflammatory process; the basic protective mechanism against tissue damage of whatever cause. Again, in poikilothermic vertebrates, the rate of healing and repair is temperature depending (Halver 1972).

The rate of poikilotherm wound healing has been studied at warm and cold temperatures in a variety of freshwater and seawater species (Finn and Nielson 1971a:b; Mawdesley-Thomas and Bucke 1973; McQueen et.al. 1973; Roberts et.al. 1973a:b; Anderson and Roberts 1975).

The general results of these studies show that compared with mammalian wound healing:

(1) there is a delay in the appearance of cells involved in phagocytosis in the inflammatory lesion (Finn and Nielson 1971a:b; Anderson and Roberts 1975; Roberts et.al. 1973a:b).

(2) mechanical injuries produce a cellular response showing marked fibroblastic activity (Mawdesley-Thomas and Bucke 1973).

(3) less fibrous tissue is laid down in wound at low temperatures (McQueen et.al. 1973; Roberts et.al. 1973b).

(4) there is qualitative difference in wound histology at temperatures low in the range for the species being studied (Finn and Nielson 1971a:b; Roberts et.al. 1973a:b).

(5) the rate of wound healing is proportional to temperature (Anderson and Roberts 1975).

VITAMIN-C AND ITS INFLUENCE ON HEALING IN FISHES:

The first report of a specific vitamin-C deficiency in fish was in 1941, when Schneberger at the Thunder River Hatchery in Wisconsin reported that its deficiency caused paralysis in rainbow trout (Schneberger 1941). Subsequently, it was suggested that carp (Cyprinus carpio) did not require an exogenous source of vitamin-C but that rainbow trout and coho salmon did (Halver et.al. 1969). It is now considered that most fish have a requirement for the vitamin (Halver 1968; Love 1970).

Experimental wound repair studies in rainbow trout have been directly related to the vitamin-C content of the diet. When wound repair experiments were initiated or when fish were exposed to other stresses the requirement for vitamin-C doubled or trebled (Halver 1972). The vitamin-C requirement of growing fish is, according to Halver (1972):-

Species	requirement of dry ration	water temperature (°C)
Rainbow trout (*Salmo gairdnerii*.)	100-150mg./kg.	10-15
Chinook salmon (*Oncorhynchus tschawytscha*)	100-150mg./kg.	10-15
Coho salmon (*Oncorhynchus kisutch*)	50-80mg./kg.	10-15

(from Halver (1972))

THE PLAICE:

The plaice is a heterosomatid benthic flatfish inhabiting sandy areas of seabed. Its classification, according to Bagenal (1972) is:

CLASS	:	PISCES
ORDER	:	Heterosomata
Family	:	Pleuronectidae
Species	:	Pleuronectes platessa.L.

The plaice family (Pleuronectidae) contains most of the commonly captured species of British flatfish. All species of this family including the Dab (Limanda limanda.L.), Flounder (Platichthys flesus.L.), Lemon sole (Microstomus kitt.L.), Witch (Glyptocephalus cynoglossus.L.), Long rough dab (Hippoglossoides platessoides.L.) and Halibut (Hippoglossus hippoglossus.L.) normally lie on their sides and the left eye moves up over the upper right side at metamorphosis.

The plaice can be identified easily because the upper eyed side is smooth without any scales on the lateral line or on the dorsal and anal fin margins. The upper side of the fish is usually an olive greenish brown colour with bright red or orange spots which usually have a white or blue halo, although the species possesses a remarkable capacity for protective coloration adaptation. There are also a few dark brown spots and some cream ones. The underside is a dull cream-white. Plaice can grow to 60 cm. (24 inches) in length, Bagenal (1972).

The plaice is found abundantly all round the British Isles, and because of its economic importance to commercial fisheries, there have been many investigations by fishery biologists into its natural history. Plaice spawn from December to April around the British Isles and one of the largest spawning areas is in the Southern North Sea (Shelbourne 1964).

Food:-

The food of adult plaice consists mainly of bivalves, such as common mussels (Mytilus edulis), common scallops (Pecten maximus), other animals such as swimming crabs (Portunus depurator), shore-crabs (Carcinus meanas): common hermit-crabs (Eupagurus bernbardus), and common lugworms (Arenicola marina) are also eaten (Wimpenny 1953).

General Anatomy:

Skeleton:-

The skeleton consists of the skull, trunk bones and appendage bones.

The vertebral ribs, according to location are of two types - dorsal and ventral. They develop within the connective tissue portions (myosepta) of the lateral muscle bundles (myomeres) at the peritoneum, Lagler et.al. (1962).

Muscle:-

As in other vertebrates, there are three kinds of muscles. These

are smooth (gut muscle), cardiac (heart muscle), and skeletal muscle. The gut of the plaice is clearly differentiated anatomically into stomach, intestine, and a wide rectum (Edwards 1971). The trunk muscles are effectively attached to the firm skeleton, and muscle perimysium has an important role as a retaining barrier among the muscle bundles (Finn and Nielson 1971a). Generally in teleost fish, skeletal muscle comprise white and dark (red) muscle fibres. Those fibres are more clearly distinct from another in certain pelagic fishes, because the cells of dark muscles are narrower than those of white muscle (Love 1970). However, since the plaice is a sluggish, bottom-dwelling fish (Marshall 1972), it has a minute amount of red muscle (Tytler, P. and Pride, M. pers. comm. 1974).

Skin:-

Fish skin comprises an outer cuticle of mucous and cellular breakdown squames overlying a non-keratinizing epidermis with mucous cells; and a dermis containing scales, pigment cells and blood and lymph vessels. The skin of the plaice was studied in detail by Roberts et.al. (1971) who showed that the structure and ultrastructure of normal plaice skin is similar to other teleosts, divisible into epidermis, dermis and hypodermis.

They showed that the dermis can be divided into two strata; stratum spongiosum (upper) and stratum compactum (Henrikson & Matoltsy 1968). These correspond to the papillare and reticulare of mammalian skin. Throughout the basal layer of the epidermis, the eosinophilic granular

cells (dendritic secretory cells) previously undescribed, are found (Roberts et.al. 1971).

Mast cells are very common in the dermis, and a melanophore and guanophore system is described. Although no melanophores or melanocytes are found in the unpigmented areas of partially pigmented hatchery reared fish, the integrity of the guanophore system is complete in such fish (Roberts et.al. 1971).

Lymph system:-

In fishes, as in the other vertebrates, lymph is collected from all parts of the body by a lymph system of ducts and sinuses which finally return in to the main blood stream. In the plaice the main ducts are ventral; dorsal and lateral: ducts do not pass lymph to the head but act as collecting sinuses; the lymph flows from these sinuses to the neural lymph duct via the interspinal ducts (Wardle 1971). Wardle used the plaice as his model for lymph flow studies and found that as in the higher vertebrates, lymph was formed when filtered through capillary walls.

Blood system:-

Fish blood circulates, as in higher vertebrates, through the blood vessels; i.e., the arteries and veins, the main interchange between the blood and the tissues taking place across thin-walled capillaries and the main transfer of gases and ions across the capillary beds of the gills (Roberts and Shepherd 1975).

Fish blood consists of fluid plasma and blood cells. The blood contains several types of white cells (leukocytes), their range generally in teleost fishes lying between 20.000 to 150.000 per cubic millimeter in different groups of fishes. Among the white cells, granulocytes may make up between 4-40 per cent of all white cells (Lagler et.al. 1962). Thrombocytes are circulating thromboplastin containing cells which have a similar function to the platelets of mammals (Wardle 1971).

Since as early as the 1900's, several studies have been performed on the blood cells of fish. Most of the studies have been anatomical and have been largely based on mammalian terminology on grounds of morphology and staining affinities. However, some differences of opinion as to classification have appeared. Some workers considered that monocytes did not exist in fish blood (Catton 1951; Klontz 1972) although the majority do not concur with this somewhat idiosyncratic view. Ellis (1974); Ezzat et.al. (1974) by light microscopy studies, and Ferguson (1975) who carried out an ultrastructural study of the leukocytes of plaice, all showed the existence of such monocytes.

SECTION B

MATERIALS AND METHODS

General

Fish:-

Experiments were carried out on 1+ and 2+ year class plaice (Pleuronectes platessa.L.). The plaice was chosen because there is a considerable amount of information available on its physiology, e.g. Wardle (1971), its pathology, e.g. Roberts et.al. (1973), and its husbandry (Richardson 1972) and because at the outset it was readily available from aquaculture establishments. In this study the majority of specimens were obtained from the one year class of the White Fish Authority's stock at the nuclear power station effluent heated marine cultivation unit, Hunterston, Ayrshire. They were between 8 and 12 cm. in length. Towards the end of the experimental work it was found that stock from that source had become unsuitable due to infection by the fungus Ichthyophonous hoferi so that it became necessary to use wild specimens obtained from the SMBA Marine Laboratory, Oban, Argyll, and by trawling in Morecambe Bay, Lancs.

The fish obtained from the wild were slightly older than the rapidly growing farm fish, but of similar size.

Aquaria:-

Fish were held in fibreglass tanks (43 cm. x 46 cm. x 74 cm.) in recirculating saltwater. They were held at 10^{o}C. in constant temperature rooms as required, and acclimated for at least 14 days prior to experimentation. They were fed on a prepared pelleted

diet (WFA1) kindly provided by Mr C D Anderson of the White Fish Authority.

Experimental Materials

Carrageenin inoculum:-

Carrageenin* was obtained as a powder commercially. It was prepared as a solution of 0.3% carrageenin in 0.9% physiologic saline and autoclaved before use (Williams 1957).

Inocula of 0.05 ml. of carrageenin were used. Injections were made into the superficial myotomal muscle on the aboral side of the unanaesthetized fish using a 25 gauge needle. The exact site of inoculation was marked by clipping out the lateral fin web in line with the site of injection (Fig. 2). After administration of the irritant, all animals showed temporary difficulty in maintaining their balance and refused food for two or three days.

L-ascorbic acid injection:-

L-ascorbic acid was obtained from the Boots Company Ltd., Nottingham, and was prepared as a 1% solution with sterilized 0.9 per cent physiologic saline. The fish received daily 0.1 ml. L-ascorbic acid via the peritoneal cavity using a 25 gauge needle.

*Marine Colloids Ltd., (Viscarin. Batch No. 113302) Springfield, USA.

Fig. 2. This diagram shows the site of injection of the carrageenin inoculum and the fin clipping site which allowed its definition after the lesion had developed.

Sampling

Experimental fish were sampled at the requisite times during the various experiments for histological examination. At least two fish were killed at each sampling time. Details of exact sampling times are described for the specific experiments. Fish were killed by a blow on the head followed by severance of the spinal-cord, immediately behind the head. The lesion bearing areas were then removed with sterilized forceps and scalpel and sliced into thin parallel strips prior to fixation. Tissues were fixed in 10 per cent formal saline at 4°C. for 12 hours. The jar containing the fixative was agitated once or twice during the next few hours as this was found by Morgan (Morgan, R.I.G. 1974 pers. comm.) to greatly enhance the fixation for fish skin. Zenker fixative, commonly used in mammals for fixation of mucopolysaccharides, was not successful in this situation and it was also found to be advantageous to fix the tissue at 4°C. rather than room temperature (Monis et.al. 1968).

I. Histological Procedures.

For Light Microscopy:

Tissues were processed in an automatic tissue processor.* The embedding programme used, passed the tissues through ascending grades of alcohols, two changes of absolute alcohol, two changes of chloroform, followed by two changes of wax (56^{o}C. melting point). The paraffin embedded tissues were cut at 4-5 microns on a Leitz rotary microtome.

Staining procedures:-

A variety of staining procedures were used. Table I indicates the methods, the detailed protocols and the purposes for which they were used.

*Shandon-Elliott Ltd., Automatic processor, UK.

TABLE I

THE MAIN TYPES OF STAINING PROCESSES USED FOR LIGHT MICROSCOPY ON PARAFFIN SECTIONS

Demonstration of	Stain
General view	Haematoxylin and eosin
Mucopolysaccharides	Alcian green method Alcian blue method Hale's iron technique
Metachromasia	Periodic-Acid-Schiff Toluidine blue
Connective tissue	van-Gieson Masson's trichrome
Elastic fibres	Verhoeff's method
Reticulin fibres	Gordon and Sweet method
Plasma cells	Unna-Pappenheim's stain
Mucopolysaccharides & Elastic fibres & Collagen fibres	Putt and Hukill stain

II. Histologic Procedures.

For Electron Microscopy:

Source of tissues:-

Small pieces of tissue (0.5 mm. x 0.5 mm. x 0.5 mm.) were removed by very rapid excision from the lesion area of freshly sacrificed animals.

In order to cut very thin slices of tissue without too severe mechanical deformation, sharp single edged razor blades were used.

Fixation:-

The tissue samples were fixed according to the method described by Roberts et.al. (1971) in their ultrastructural study of plaice (Pleuronectes platessa.L.) skin. Samples were fixed quickly in freshly prepared Karnovsky's fixative at pH 7.4 and fixed for at least 5 hours up to 12 hours at room temperature. They were then washed in 0.2 M sodium cacodylate buffer for ½ hour to overnight at 4°C., post fixed in 2 per cent Osmium tetroxide (OsO_4) for one hour and washed in 0.1 M sodium cacodylate buffer for ½ hour.

Dehydration and embedding:-

Dehydration was effected by passing the samples through a series

of ethanols of increasing concentration. For embedding dehydrated samples were polymerized in Luft's Epon 812 embedding mixture for 48 hours at 60°C. in a hot air oven.

Cutting and staining:-

Solid resin embedded material was first trimmed by means of razor blades under a light microscope. Trimmed sections were cut at 0.5 µ with glass-knives on an LKB III automatic ultra-microtome and cut sections floated on hot 1.0 per cent toluidine blue and stained for light microscopy for one minute prior to washing with distilled water. When the inflammatory areas had been identified by light microscopy, ultrathin sections were cut with 45° glass-knives for electron microscopy.

Selected resin embedded tissue samples were cut at 60-80 A° (silver colour) on an automatic ultra-microtome. Ultrathin sections, cut with great care, were mounted on to the dull face of uncoated copper grid s or onto the dull face of carbon film coated grid s, and left to dry in air.

In the studies three kinds of staining procedures were used:

1. Uranyl acetate (Papadimitriou and Spector 1971)
2. Lead hydroxide (Perez-Tamayo 1970a)
3. Uranyl acetate and lead citrate (Perez-Tamayo 1970a)

Microscopy:-

Stained grids were examined in an AEI Corinth 275 electron microscope. The instrument magnification varied between 6×10^2 and 100×10^3. resolution 9 A^o and accelerating voltage 60 KV. The negatives were further enlarged photographically with a Durst enlarger.

III. Immunologic Procedures.

For Immunological Studies:

Two qualitative immunological tests were used to indicate the production of an immune response against the carrageenin. These were the Ouchterlony gel-diffusion technique as modified for fish sera by Bullock (1971) and subsequently used by Harris (1973) and Russel (1974), and the migration inhibition test as described by George and Vaughan (1962) for determination of the presence of a cell mediated immune response in mammals.

1. Ouchterlony double gel-diffusion test:-

On the 28th day after initiation of the granuloma, the blood required for serology was withdrawn from the renal portal vein of the unanaesthetized fish by means of a 25 gauge needle, into an unheparinized tube, and left for 6 hours in the refrigerator at $4^{o}C$. By this time the blood had clotted to release a clear white-yellow serum.

This test was performed on Hyland immuno-diffusion plates* which were filled with 3 ml. of hot buffered 1.0% No. 2 Ionagar** in 0.85 per cent saline and allowed to cool for 2-3 hours. Wells were cut by means of 4 mm. and 2 mm. cork borers on a square template.

*Hyland Ltd, UK.

**Oxoid Ltd, UK.

Antiserum was placed in the central wells and test and control antigens in the peripheral wells. The test antigen used was a 0.3% solution of carrageenin in physiological saline. The control antigens used were bovine serum of Freund's complete adjuvant. The test plates were kept at 4^{o}C. in a moist chamber for 2-3 days (Roberts 1968; Russell 1974) prior to being examined and photographed.

2. Migration Inhibition Test (MIT), in vitro:-

For this test, the fish was sampled 28 days after injection of 0.05 ml. of 0.3 per cent carrageenin saline solution. The blood for the test was withdrawn into a plastic syringe (heparinized), from the renal portal vein of the unanaesthetized fish by the method described by Wardle (1971). The blood was then placed in micro-haematocrit tubes* which were sealed in a bunsen flame. These were centrifuged for five minutes at 15.000 r.p.m. (McCarthy and Stevenson 1973), and the supernatant serum removed. The tube containing the remaining cellular fraction was broken at the buffy coat-serum interface, and the portion of tube containing the red and white cells placed in a petri-dish containing tissue culture fluid at room temperature (Clem et.al. 1961; Paul 1970). The tissue culture fluid was prepared as follows:

*A R Horwell Ltd., Great Britain.

100 ml. of chilled distilled water.

10 ml. of Earle's BSS (Balanced Salt Solution).

2 ml. of MEM AA (Minimal Essential Medium - Amino Acid).

1 ml. of NE AA (Non-essential amino acids).

1 ml. of MEM VITS (Vitamins).

2 ml. of Penicillin/Streptomycin.

2 ml. of Sodium bicarbonate.

Before use ½ ml. of Glutamine and 5 ml. of Feotal Bovine Serum (FBS)* were added to each 6 ml. mixed batch as prepared above. The medium was prepared and batched according to Wood (1973).

Excess of antigen, in this case 0.3 per cent carrageenin saline solution of 1 ml. was added to the medium under test. The result was read six hours later.

*Flow Laboratories, UK.

SECTION C

RESULTS

Experiment I. Definition of the tissue response of the plaice to carrageenin at environmental temperature of 10°C.

The Study:

The purpose of the work was to define the standard chronic inflammatory response in the plaice, using carrageenin as the irritant. Williams (1957) had carried out a somewhat similar study in defining the carrageenin granuloma in guinea pigs and in order to compare his findings with those of the present study, the timing of sampling and dose rates of carrageenin used were based, for the pilot experiments, on Williams' experience.

The investigation is presented sequentially with the experimental protocol preceding the results.

Experimental Protocol:-

In order to determine the optimal sampling and dose rates and to allow a trial of the fixation technique and staining methods for mucopolysaccharides, a pilot experiment was carried out using 16 healthy plaice which were sacrificed at 1, 3, 5, 12, 14, 21 and 28 days.

The carrageenin was prepared as a 0.9 per cent solution in sterile saline and injected via a 25 gauge needle in a dose of 0.1 ml., or 0.05 ml. of a 0.3 per cent saline solution of carrageenin into the

dorsal myotomal area (Figs. 3:4). A 0.1 ml. injection of carrageenin was applied to the first three fish (1:3:5 days) and the rest of the fish were injected with 0.05 ml. of carrageenin. The site of inoculation was marked by clipping the fin web at the site. The fish were not anaesthetized, merely placed on a moist surface with the head covered with a wet cloth. No restraint was necessary and there was no evidence of any pain being induced by the procedures.

The pilot experiment showed that Zenker fixative was not a suitable fixative for the purpose, since it resulted in differential contraction of tissues, and muscle staining was poor. The stains used for the study were haematoxylin and eosin (H&E) for routine examination, alcian blue (Culling 1963), alcian green and Hale's iron method for mucopolysaccharides and Periodic acid Schiff for glycogen and other mucopolysaccharides (Putt and Hukill 1962; Drury and Wallington 1967). Another modification resulting from the result of the pilot experiment was that it was found that long periods of fixation at room temperature were not suitable for staining of mucopolysaccharides. With reduction of fixation time in formal saline solution to 12 hours and fixation at a temperature of 4^{o}C., excellent staining reactions were obtained.

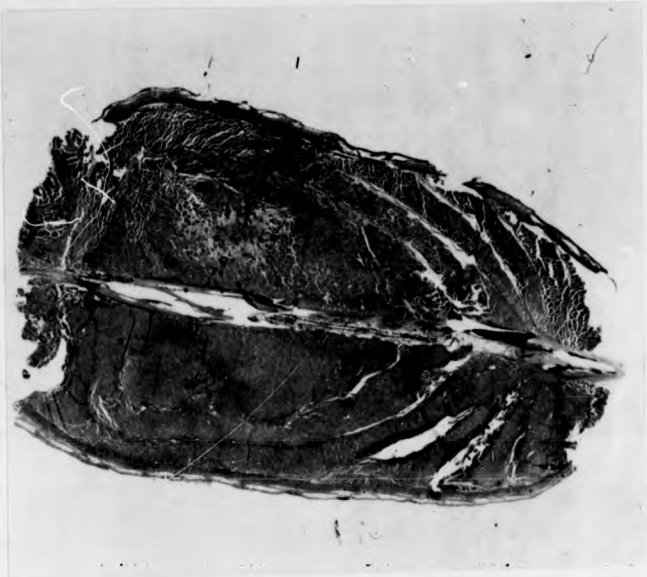
On the basis of the findings in the pilot experiment, 26 healthy plaice from the main stock were injected with 0.05 ml. of sterile saline solution of 0.3 per cent carrageenin as in the pilot experiment. Two fish were sacrificed at intervals of 1, 3, 5, 10, 12, 14, 21, 28, 35, 42, 56, and 80 days after injection of the irritant.

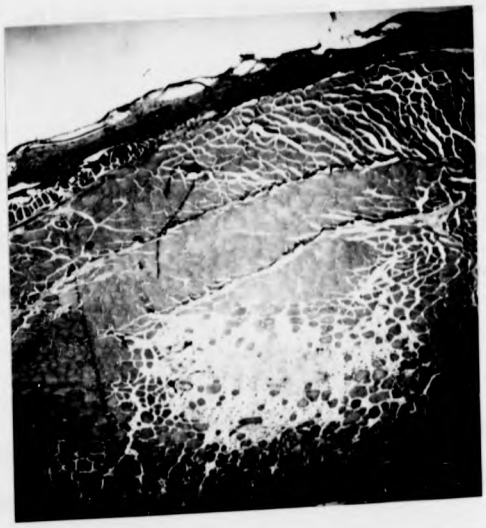
Fig. 3. Site of carrageenin injection into dorsal myotomal area. 14 days post-injection at $10^{o}C$.

(H&E) x 10

Fig. 4. Another picture of the figure 3, showing a close-up view of the injection site.

(H&E) x 20





At sampling, two fish at a time were sacrificed as described in the general materials and methods section. The four extra fish were held against any losses due to mortalities from other causes. The experimental inoculations did not affect the fish clinically in any way except that they did not feed for 24 hours post injection.

Light Microscopic Observations:-

By 24 hours after inoculation of carrageenin, the local inflammatory response consisted of neutrophil leukocytes, macrophages, and lymphocytes with a predominance of macrophages over neutrophils and lymphocytes. Some muscle fibres showed slight focal damage within the inflammatory area. The neutrophils (polymorphonuclear leukocytes) were circular in shape, with a three lobed or reniform nucleus. The nucleus occupied one third to one half of the cell. The size of the cell was 3-4 μ in diameter. In haematoxylin and eosin (H&E) stained sections, their cytoplasm was pink and the nucleus was very intensely basophilic. At this stage there was no evidence of intercellular carrageenin with the staining technique of alcian green, although carrageenin was in evidence over much of the extracellular component of the inflammatory area.

Necrosis of muscle fibres was first evident at 3-5 days. At this time the necrotic muscle fibres showed fragmentation of sarcoplasm and some macrophages were seen around and within the muscle bundles

(myophagia) (Fig. 5). By this time the cellular response consisted mainly of macrophages and lymphocytes. There were only a few polymorphonuclear leukocytes within the inflammatory exudate (Fig. 6). The extracellular exudative area contained stainable carrageenin, but the major portion was found within the numerous large macrophages which had distinct foamy cytoplasm. The material was easily identified within the vacuoles of these cells by its strongly positive reaction with Periodic-Acid-Schiff (PAS) and the alcian green technique. Small macrophages, which did not contain carrageenin, were circular in shape with a round, excentric nucleus. Their size was about 5.2-6 μ in diameter. The large macrophages contained large vacuoles within their cytoplasm and measured 6-10 μ in diameter. The lymphocytes were the smallest cells seen in the response. They had a rounded shape with only a rim of basophilic cytoplasm. The nucleus was round with a well defined margin. Their sizes were 2.5-3 μ in diameter. At this stage some of the blood vessels within the inflammatory area showed obvious diapedesis (Fig. 7). Many erythrocytes and leukocytes were seen either within the lumina of vessels or around them.

By the 10th day after inoculation of carrageenin the inflammatory response consisted predominantly of macrophages, fibroblasts and some lymphocytes. This was the first stage at which obvious fibroblastic activity was seen. The fibroblasts were spindle shaped cells with pale cytoplasm and rounded nuclei. They were found within the exudate, together with the macrophages.

(myophagia) (Fig. 5). By this time the cellular response consisted mainly of macrophages and lymphocytes. There were only a few polymorphonuclear leukocytes within the inflammatory exudate (Fig. 6). The extracellular exudative area contained stainable carrageenin, but the major portion was found within the numerous large macrophages which had distinct foamy cytoplasm. The material was easily identified within the vacuoles of these cells by its strongly positive reaction with Periodic-Acid-Schiff (PAS) and the alcian green technique. Small macrophages, which did not contain carrageenin, were circular in shape with a round, excentric nucleus. Their size was about 5.2-6 μ in diameter. The large macrophages contained large vacuoles within their cytoplasm and measured 6-10 μ in diameter. The lymphocytes were the smallest cells seen in the response. They had a rounded shape with only a rim of basophilic cytoplasm. The nucleus was round with a well defined margin. Their sizes were 2.5-3 μ in diameter. At this stage some of the blood vessels within the inflammatory area showed obvious diapedesis (Fig. 7). Many erythrocytes and leukocytes were seen either within the lumina of vessels or around them.

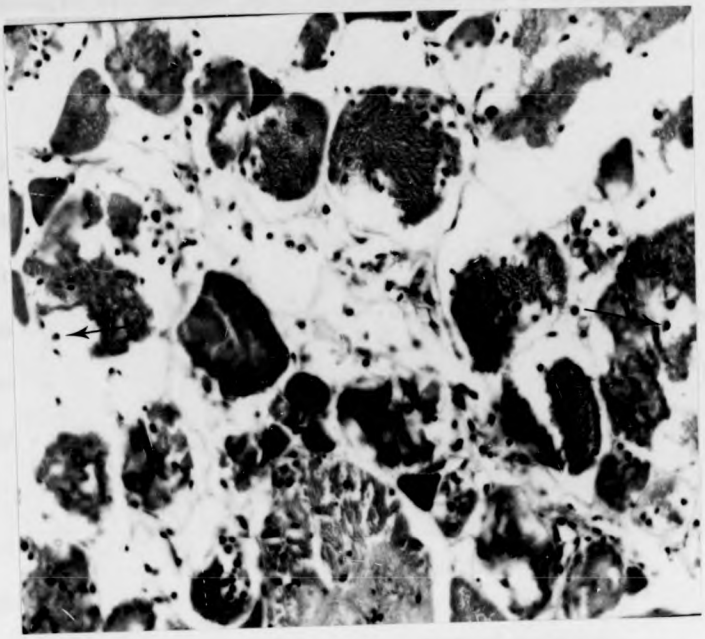
By the 10th day after inoculation of carrageenin the inflammatory response consisted predominantly of macrophages, fibroblasts and some lymphocytes. This was the first stage at which obvious fibroblastic activity was seen. The fibroblasts were spindle shaped cells with pale cytoplasm and rounded nuclei. They were found within the exudate, together with the macrophages.

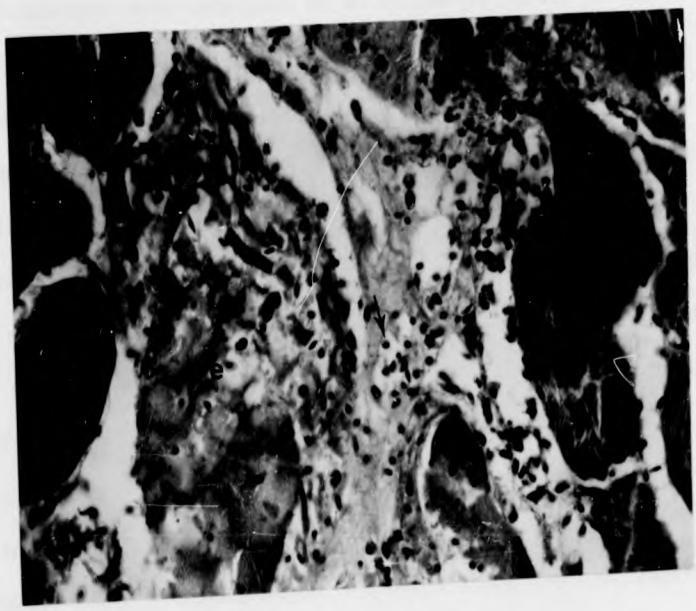
Fig. 5. The inflammatory area at 10°C. 3 days after injection showing early stages of myophagia. Observe the PMN leukocytes (arrowed).

(H&E) x 500

Fig. 6. The inflammatory area at 10°C 5 days after carrageenin injection showing myophagic macrophages. Note the PMN leukocytes (arrowed) and (e) showing an erythrocyte.

(H&E) x 500





By this stage some of the sarcoplasmic fibres had fragmented and myophagic macrophages were active around the sarcoplasm (Figs. 8:9). Vascularisation of the area had already begun. It was at this stage that plasma cells and their precursors were first seen with the Unna-Pappenheim's staining technique. The plasma cell had an oval or circular shape with the nucleus eccentric and occupying one half to one third of the cytoplasm. The nucleus was blue and the cytoplasm was bright purple coloured in Unna-Pappenheim's stained sections. They were about 3.4-4.5 μ in diameter. Their precursors were oval in shape, and had a bright purple colour cytoplasm, with a clock faced blue coloured nucleus. They were about 5.6-6.5 μ in diameter. By this time, the uptake of carrageenin by macrophages was very considerable as demonstrated by PAS and alcian green stained sections.

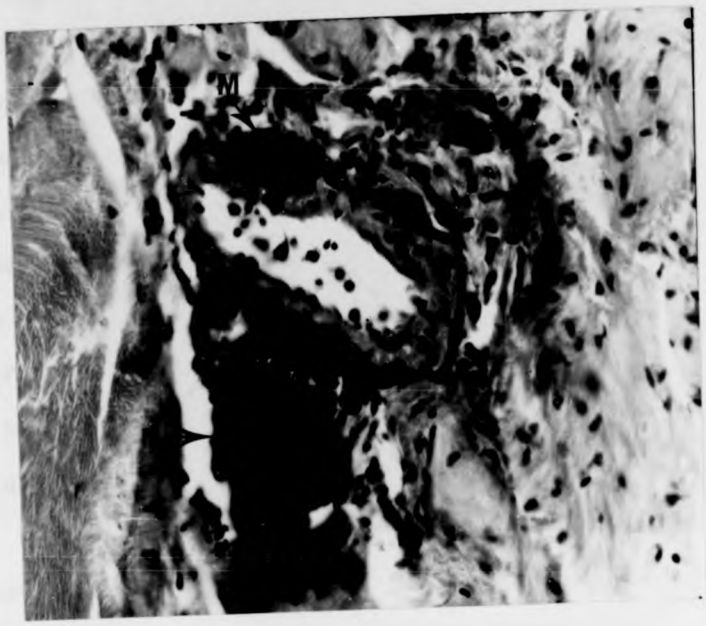
By the 12th and 14th days and initiation of the granuloma, the inflammatory area consisted of an increased number of macrophages, fibroblasts and some plasma cells and lymphocytes. Most of the macrophages at this stage maintained their foam-cell-like appearance after staining by the alcian green method (Figs. 10:11), but after the PAS method of staining, the phagocytosed polysaccharides appeared as multiple small droplets within the vacuoles of the macrophages (Fig. 12). At these stages vacuoles were seen at the edges of the lesions and were apparently sites of myophagia where the sarcoplasm had been digested leaving a large membrane bound space (Fig. 13) in which some remaining macrophages were often seen. Numerous thin-walled blood vessels were found throughout the inflammatory area. At these times the most striking

Fig. 7. A blood vessel showing diapedesis of leukocytes and extensive melanocytes distributed around the vessel (this is a normal feature).

(H&E) x 500

Fig. 8. Advanced stage of myophagia, showing clusters of macrophages within the fragmented muscle fibres. Observe that the (m) showing foam-cell-like macrophages. Carrageenin after 10 days at $10^{o}C$.

(H&E) x 500


M


m

cells were epithelioid cells and foreign body type giant cells (Figs. 14:15). The epithelioid cells were scattered randomly and showed a whorling pattern. They were large polygonal cells which had an oval nucleus and indistinct cell boundaries. The giant cells were spread throughout the inflammatory area. They were about 30-40 μ in diameter. The cells were irregular in shape and the nuclei were scattered at random throughout the cell (Fig. 16). Mucopolysaccharides were identified within the cytoplasm of the giant cells by alcian green sections. At this stage numerous active fibroblasts associated with the healing were present (Fig. 17). These were large, rounded and pale, whereas the fibroblasts of the normal collagen were dark blue in colour and flattened. The inflammatory area consisted of the carrageenin containing foam-cell-like macrophages, fibroblasts, young capillaries, epithelioid cells and giant cells, by the 12th and 14th days. This is the typical cellular composition of typical granulation tissues as seen in higher animals (Bethlem 1970; Gresham 1971).

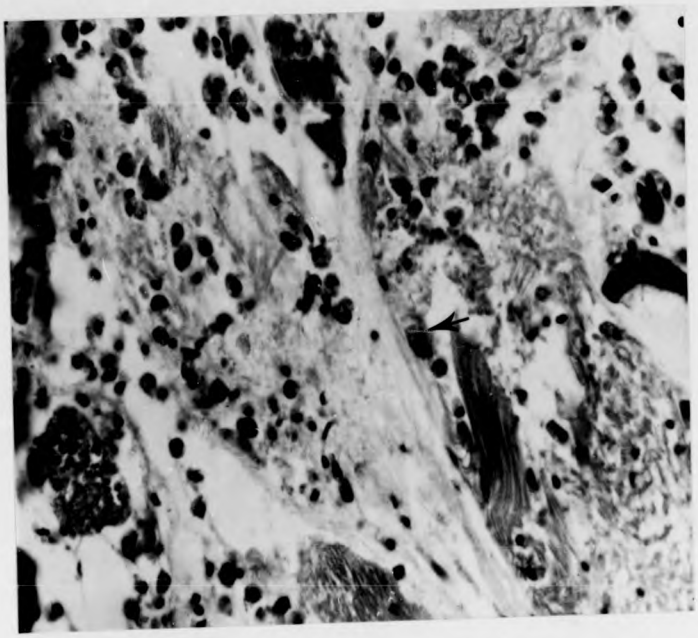
At the 21st day after inoculation of carrageenin, there was very little difference from the inflammatory response of the 14th day, but a new feature was the finding of evidence of regeneration of some muscle fibres within the inflammatory area with their bluish hue in H&E preparations (Roberts et.al. 1973a). Most of the macrophages were replete with carrageenin. The collagen fibres were found between the cellular elements. A few muscle fascicles showed necrotic fibres.

Fig. 9. Myophagia. Note the phagocytic cells are nearly foam-cell-like in appearance (arrowed).

(H&E) x 500

Fig. 10. Carrageenin replete foam-cell-like macrophages (arrowed).

(Alcian green) x 500



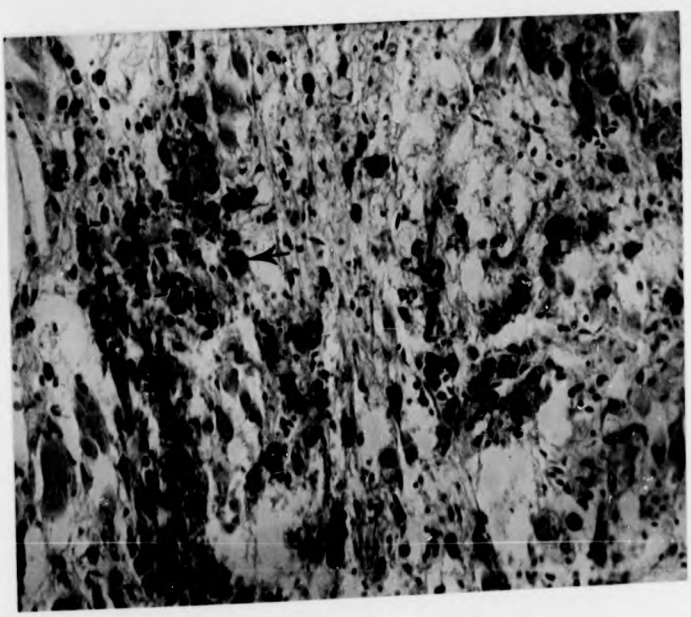


Fig. 11. The inflammatory area at 10°C., 14 days after carrageenin injection showing carrageenin replete foam-cell-like macrophages (arrowed). (Alcian green) x 800

Fig. 12. Mucopolysaccharides within the cytoplasm of macrophages. Carrageenin injection after 14 days at 10°C.

(PAS) x 800

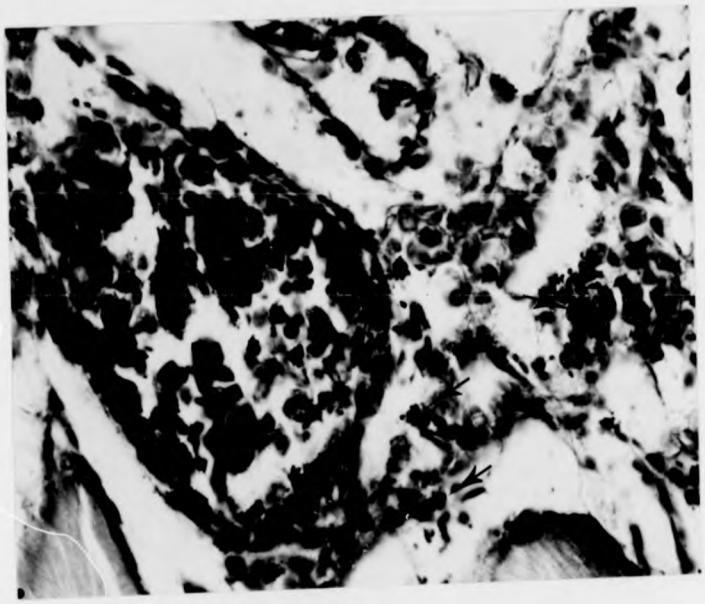
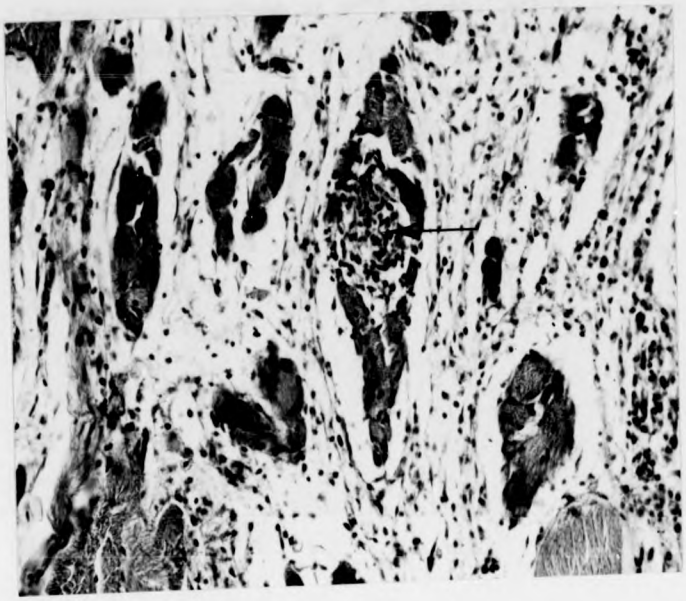


Fig. 13. Carrageenin granuloma after 14 days at 10°C., showing necrotic muscle bundles. Observe that the necrotic muscle had been digested leaving a large space in which macrophages are present (arrowed).

(H&E) x 500

Fig. 14. Carrageenin granuloma after 14 days at 10°C., showing whorling epithelioid cells.

(H&E) x 500



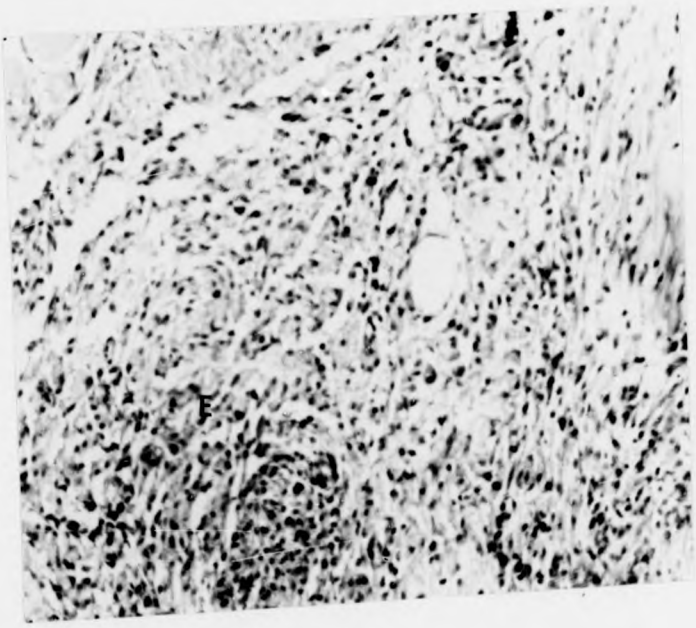
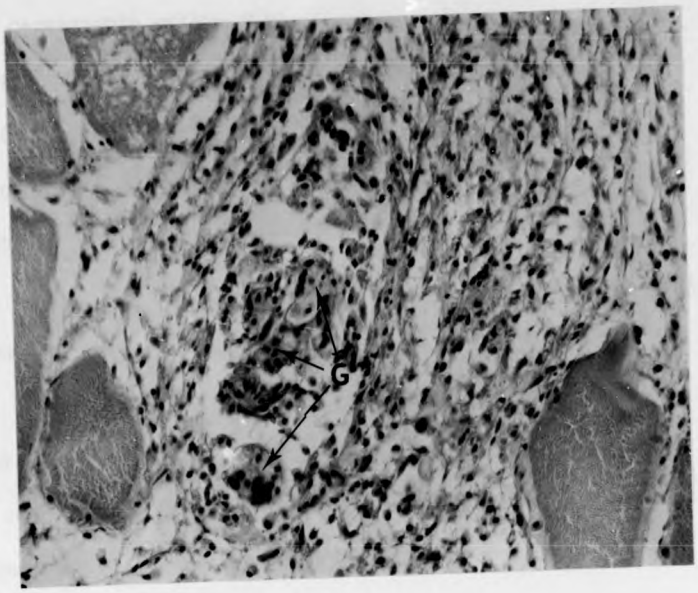


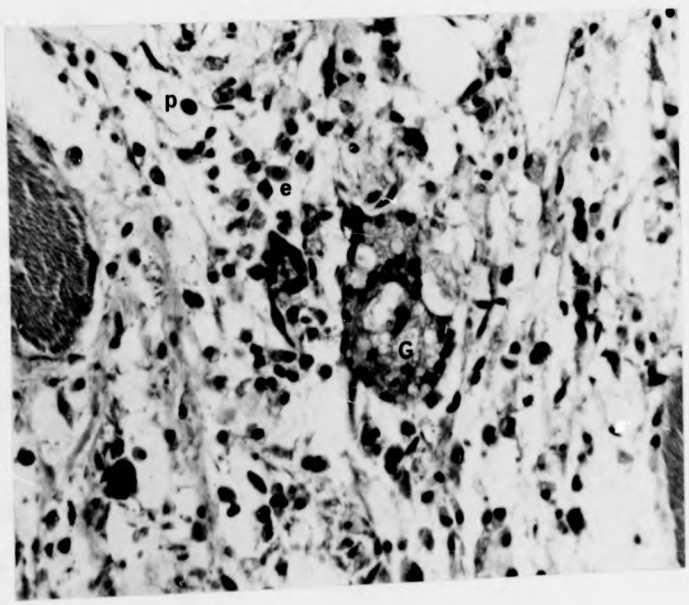
Fig. 15. Carrageenin granuloma at 10°C. 14 days after injection. Foreign body type giant cells are seen (G) within a stroma of loose connective tissue and macrophages and epithelioid cells.

(H&E) x 500

Fig. 16. Foreign body type giant cells (G) in early lesion, 14 days after injection at 10°C. The giant cells have considerable numbers of vacuoles. (P) plasma cell, (e) erythrocyte.

(H&E) x 800


G


p
e
G

By the 28th day after initiation of the granuloma, the inflammatory response showed similar numbers of macrophages, most of which showed carrageenin within their vacuoles. At this stage fibroblast numbers appeared to have increased, while lymphocytes, plasma cells (Fig. 18), epithelioid cells and small foreign body type giant cells were also increased in number. Some of the multi-nucleated giant cells were circular to oval in shape, and their nuclei were in a ring at the periphery of the cytoplasm (so-called Langhans type giant cells (Figs. 19:20), Roberts 1974; McQueen 1975; Campbell 1975 pers.comm.). The mucopolysaccharides were demonstrated within the cytoplasm of these cells by the alcian green staining technique (Fig. 21) and they were about 40-50 μ in diameter. No birefringence was detected within the giant cells by polarized light microscopy. New blood vessels and capillaries had considerably increased in number (Fig. 22), and some of the major blood vessels showed obliteration of their lumen (Fig. 23).

Staining by Verhoeff's elastica method and Masson's trichrome revealed that the elastic fibres and the collagen fibres of the granulation tissue were very thick, (Fig. 24) and appeared to form a retaining barrier to limit the spread of the irritant.

Although the regeneration of muscle fibres had only commenced on the 21st day, by this stage (28th day) the regeneration was very considerable, and no evidence of myophagia in muscle bundles was seen (Fig. 25).

Fig.17. Fibroblast development in carrageenin lesion at 10^{o}C., after 14 days.

(H&E) x 800

Fig. 18. Plasma cells (arrowed) in carrageenin granuloma at 10^{o}C after 28 days.

(Unna-Pappenheim) x 800

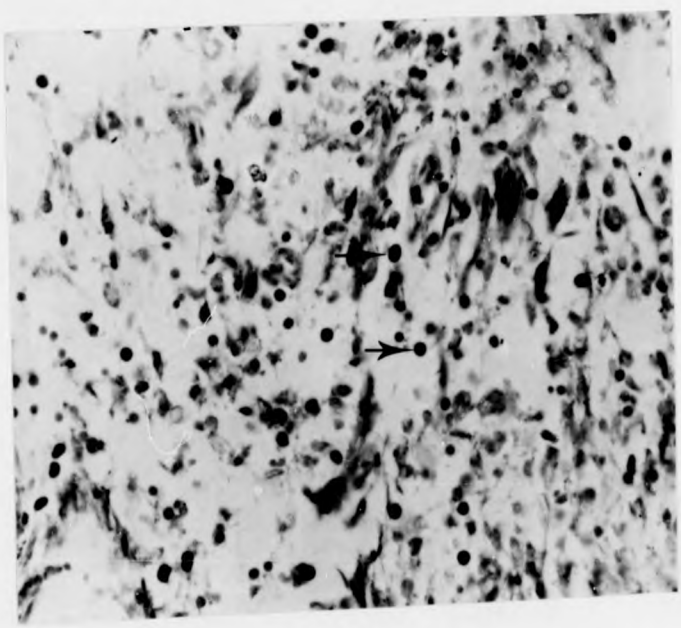
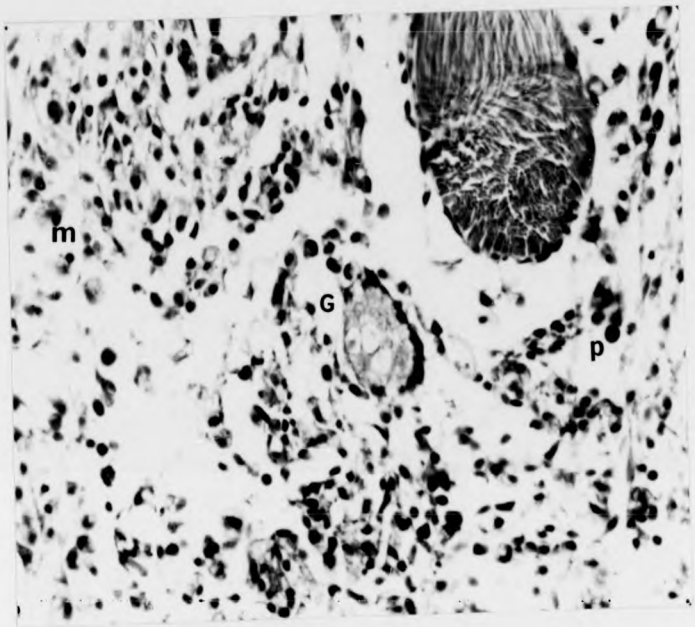


Fig. 19. The lesion area showing a Langhans type giant cell (G). Note the foam-cell-like macrophages (m) and large pyroninophil (plasma cell precursor - P).

(H&E) x 500

Fig. 20. Langhans type giant cell in 28 day old carrageenin granuloma at 10°C.

(H&E) x 500


m
G
p

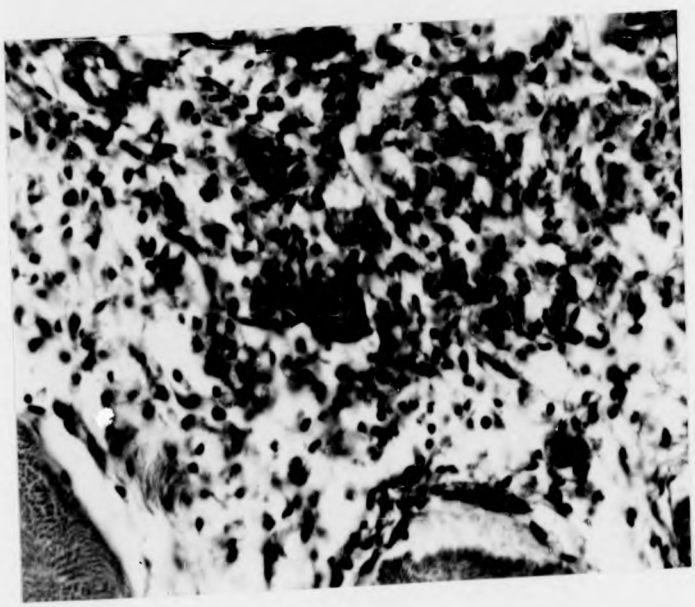


Fig. 21. The inflammatory area at 10°C., 28 days after carrageenin injection showing carrageenin replete Langhans type multinucleated giant cell.

(Alcian green) x 800

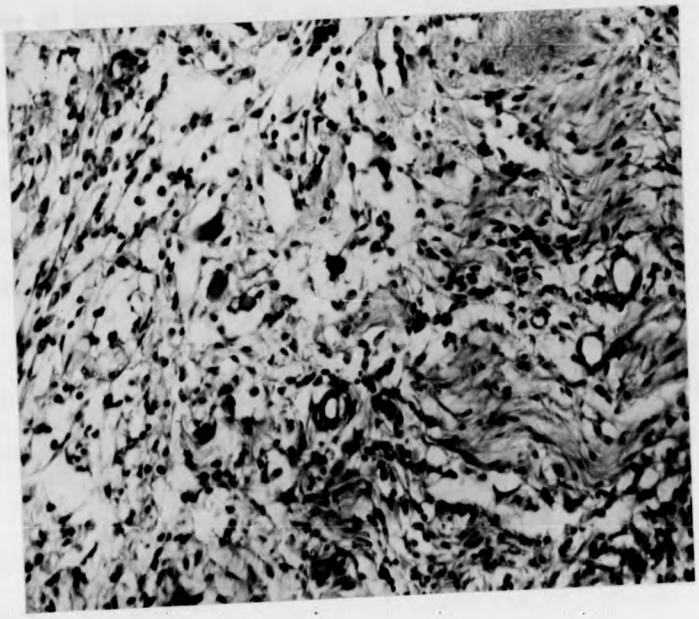
Fig. 22. Newly formed thin-walled blood vessels in the granulation tissue of 28 days old carrageenin granuloma at 10°C.

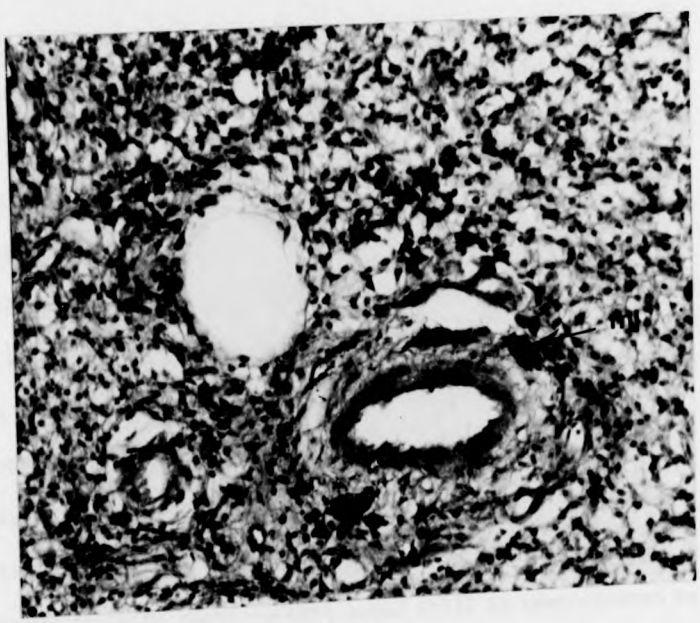
(H&E) x 500

Fig. 23. Degenerative changes in wall of blood vessel of 28 day old carrageenin lesion at 10°C.

(M1) melanin granules.

(H&E) x 800





By the 35th day after inoculation of carrageenin, the inflammatory response consisted of similar types of cells to those of the 28th day. The collagen fibres of the healing area showed increased density (Fig. 26) and the regeneration of muscle fibres had continued to develop but carrageenin was still readily observed within the macrophages. Lymphocytes had accumulated in some numbers at the site of the inflammatory area (Figs. 27:28).

By the 42nd day after initiation of the granuloma, the inflammatory response consisted of similar types of cells to those of the 35th day. The extracellular components of the inflammatory area comprised cellular debris and fibrin, but almost no extracellular carrageenin was present. No giant cells were seen but in some areas the muscle bundles were unusually variable in size. Regenerating fibres were numerous in such areas, readily distinguishable by their bluish hue in H&E preparations, and their active sarcolemmal nuclei.

By the 56th day the granuloma was less compact. The response consisted of large macrophages, epithelioid cells, with a whorling pattern, and foreign body and Langhans type giant cells, which were still clearly seen (Figs. 29:30:31). The granulation tissue at this stage consisted of a fairly dense collagen fibre network together with thin fibres around numbers of small vascular channels which were weakly argyrophilic. The cellular infiltrate within the granulation tissue was as described above, and plasma cells in small numbers could still be demonstrated by the Unna-Pappenheim method.

Fig. 24. Extensive development of elastic fibres of carrageenin lesion at 10°C. after 28 days, stained by Verhoeff's elastica method.

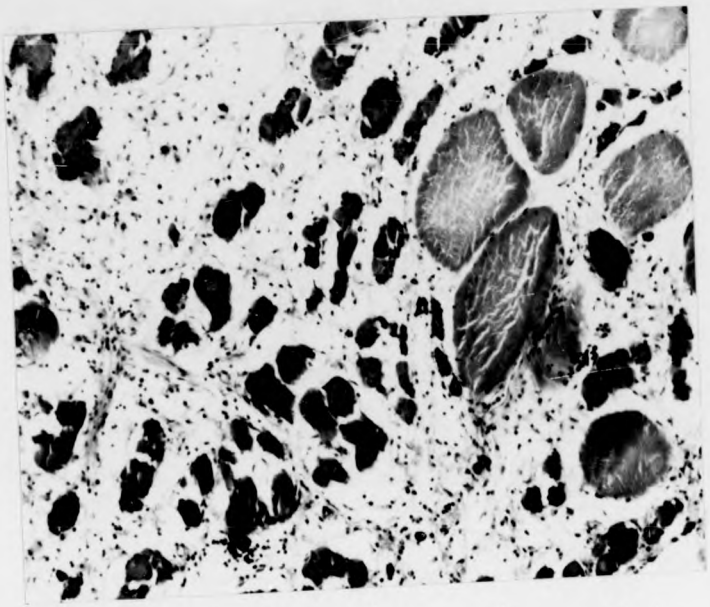
x 500

Fig. 25. Regeneration of muscle fibres in later stages (28 days) of carrageenin lesion at 10°C.

(H&E) x 500

Fig. 26. Collagen fibre network of carrageenin lesion at 10°C. after 35 days.

(H&E) x 500



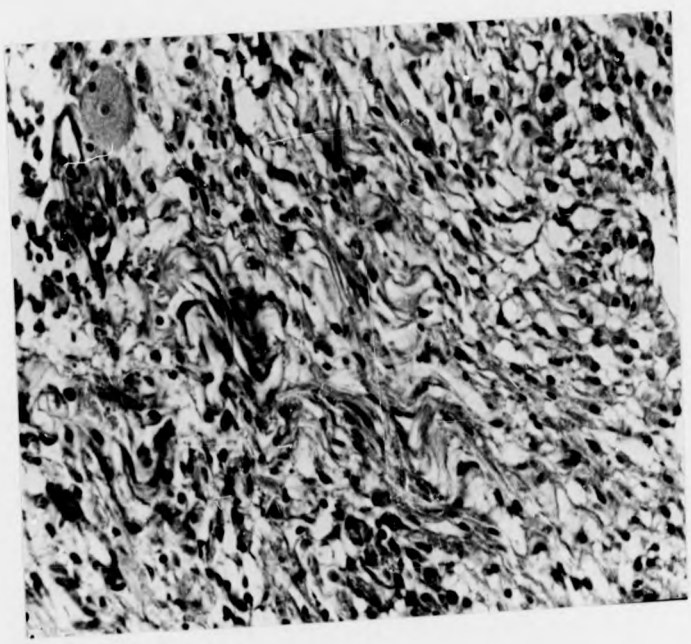
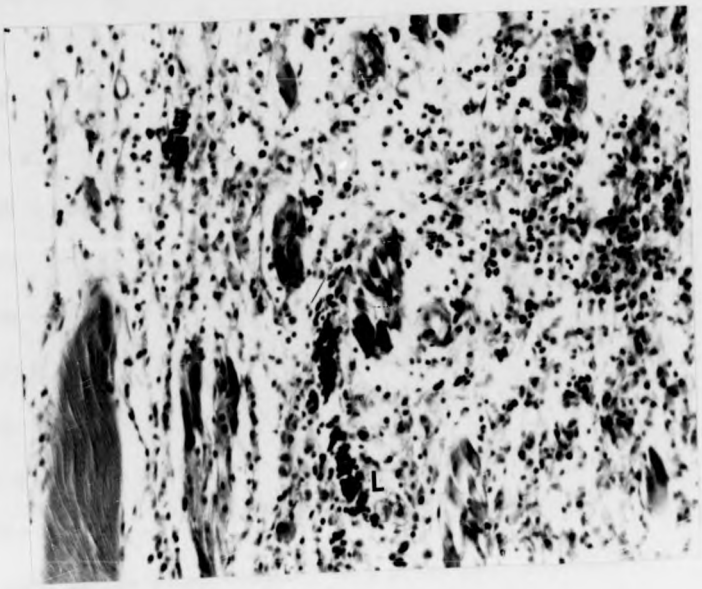


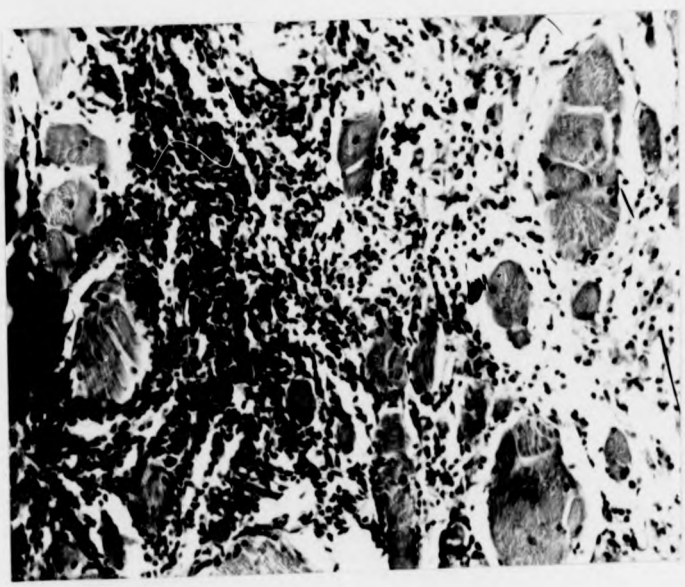
Fig. 27. Accumulation of lymphocytes with carrageenin lesion at 10°C. after 35 days.

(H&E) x 500

Fig. 28. Another area of the same lesion as Fig. 27, showing denser focus of lymphocytes.

(H&E) x 500





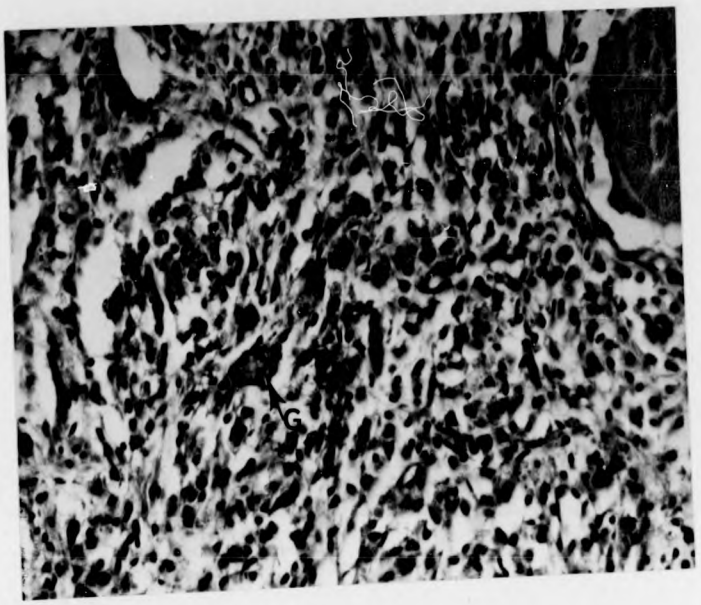
By the 80th day after initiation of the granuloma, the inflammatory response consisted of a similar type of cells to those of the 56th day, but no giant cells were seen. The few remaining macrophages had retained their foam-cell-like appearance and still contained phagocytosed carrageenin within their vacuoles. Extracellular carrageenin was not observed. Although most of the regeneration of muscle appeared to have been completed at previous samplings, there were still occasional small regenerating fibres noted. There was marked variation in sarcolemmal fibre diameter in H&E stained sections of this tissue. Instead of the rich network of thin walled blood vessels, only a few well established arterial and venous channels could now be seen, and it was apparent that a slow form of healing was still taking place (Fig. 32).

Fig. 29. Granulation tissue at 10°C. after 56 days showing a multinucleated giant cell (Langhans type) (G).

(H&E) x 500

Fig. 30. Granulation tissue at 10°C. after 56 days showing a complete network of epithelioid cells.

(H&E) x 500



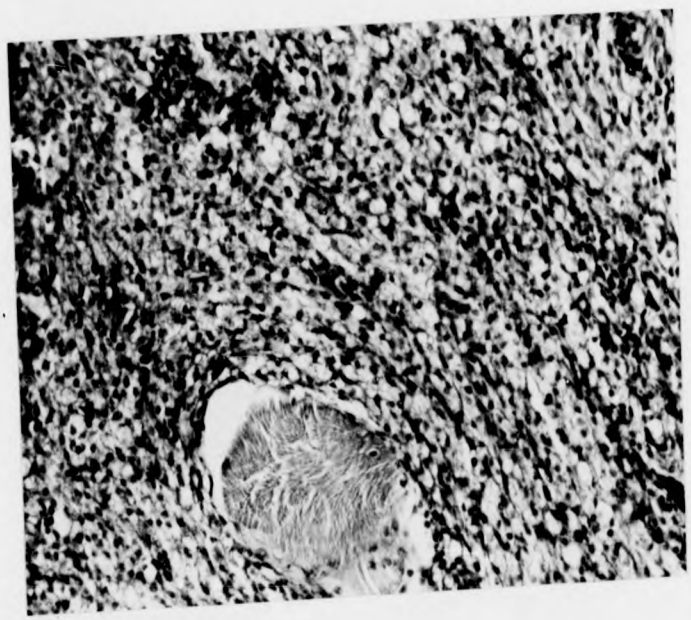
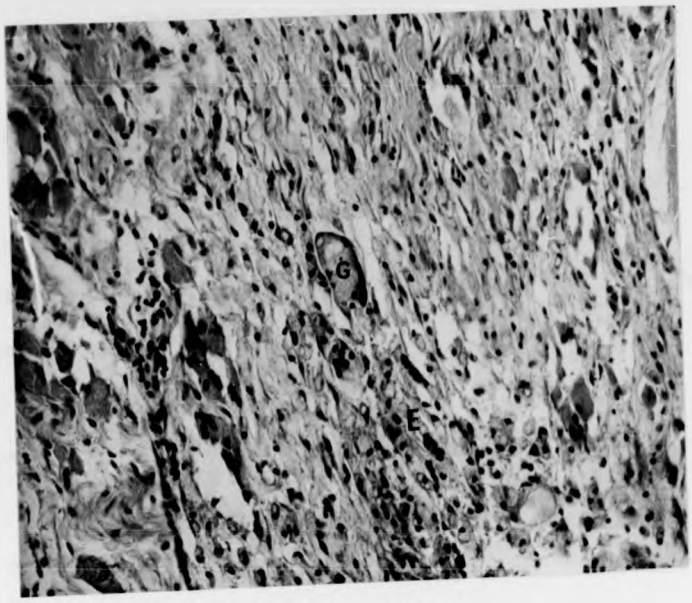
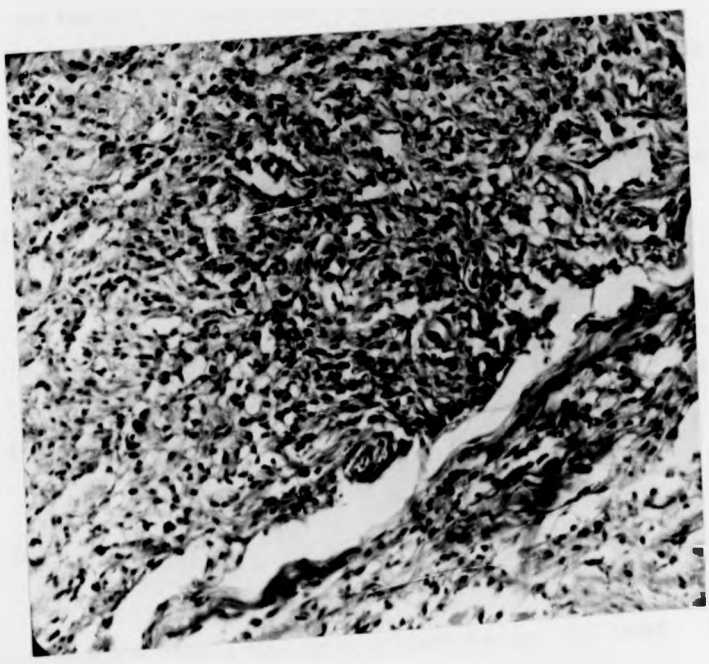


Fig. 31. Advanced stage (56 days) of granulation tissue showing multinucleated giant cell (G), epithelioid cells (E) and active fibroplasia.
(H&E) x 500

Fig. 32. Granulomatous healing of the lesion area after 80 days, at 10°C.
(H&E) x 500


G
E



Experiment II. The effect of temperature reduction on the carrageenin granuloma in the plaice.

The Study:

The study was designed to determine the effect of temperature reduction on the rate and qualitative nature of the inflammatory response and healing of the experimental carrageenin granuloma in the plaice in view of previous work on the effect of temperature on acute inflammation in teleosts (Finn and Nielson 1971; Roberts et.al. 1973; Queen et.al. 1973; Anderson and Roberts 1975).

Materials and Methods:-

Twenty-two healthy 2+ plaice were transferred to a coldroom and held at a temperature of 5°C. They were fed on a pelleted (White Fish Authority) diet, and acclimatised for three weeks prior to inoculation of carrageenin. The fish were inoculated with 0.05 ml. of sterile saline solution of 0.3 per cent carrageenin into the dorsal muscular area, and fin clipping marked the exact site of the injection point. Two fish at a time were sacrificed on days 1, 3, 5, 10, 18, 21, 35, 42, 56 and 85. Two extra fish were held against any losses due to mortalities from other causes.

Light Microscopic Observations:-

Twenty-four hours after inoculation of carrageenin, the local

inflammatory response consisted of a few inflammatory cells. Mucopolysaccharides were clearly demonstrated by the alcian green method, but none of it had been phagocytosed.

After the 3rd and 5th days, the inflammatory response consisted of macrophages, polymorphonuclear leukocytes and lymphocytes. They were similar in morphology, size and tinctorial properties to those of the higher temperature experiment, but they were quantitatively less in number. The loose connective tissue of the endomysium of the inflammatory area was considerably swollen. Some of the muscle fibres showed floccular sarcoplasm which was surrounded by phagocytic macrophages and a few polymorphonuclear leukocytes (Fig. 33). Extracellular mucopolysaccharide was present but again, none of the phagocytic cells had taken it up at these stages.

By the 10th day, the inflammatory cells were beginning to increase in number. They were almost entirely macrophages and some lymphocytes. The macrophages still had a diameter about 4.2-5.6 µ. It was at this stage that phagocytosed carrageenin was first observed in sections stained by the alcian blue and alcian green methods, but only a few were so stained. At this stage some of the blood vessels within the inflammatory area showed diapedesis (Fig. 34). First evidence of diapedesis was seen by the 3rd day after inoculation at 10°C.

By the 18th day after inoculation of carrageenin, some of the

macrophages appeared foam-cell-like. These matured cells had a diameter ranging from about 5.3-7.3 μ, but although in the 10°C. experiment by the 18th day all of the macrophages appeared foam-cell-like and were replete with carrageenin, this was not the case at 5°C. Myophagia was still continuing and the degenerated muscle fibres were surrounded by clusters of macrophages and some polymorphonuclear leukocytes (Figs. 35:36). This stage also marked the appearance of new fibroblasts, plasma cells and their precursors (Fig. 37). The carrageenin was taken up by some of the foam-cell-like macrophages but not to the extent seen at the higher temperature.

The microscopic picture of the inflammatory response had altered very little by the 21st and 35th days from that seen at the 18th day. The myophagia was not complete, but capillary loops and macrophages were increased within the inflammatory exudate. Loose collagen fibres had developed by the 21st day, and by the 35th day, in addition, weak elastic fibres were seen by the Verhoeff's staining method. Polymorphonuclear leukocytes were still present though in very small numbers. The mucopolysaccharides were still very poorly phagocytosed, and very few plasma cells and their precursors were seen by Unna-Pappenheim's method.

Epithelioid cells were first seen on the 42nd day after the inoculation of carrageenin (Fig. 38). By this time most of the macrophages had a foam-cell-like appearance, but the carrageenin was only very weakly demonstrated within the vacuoles of phagocytosed

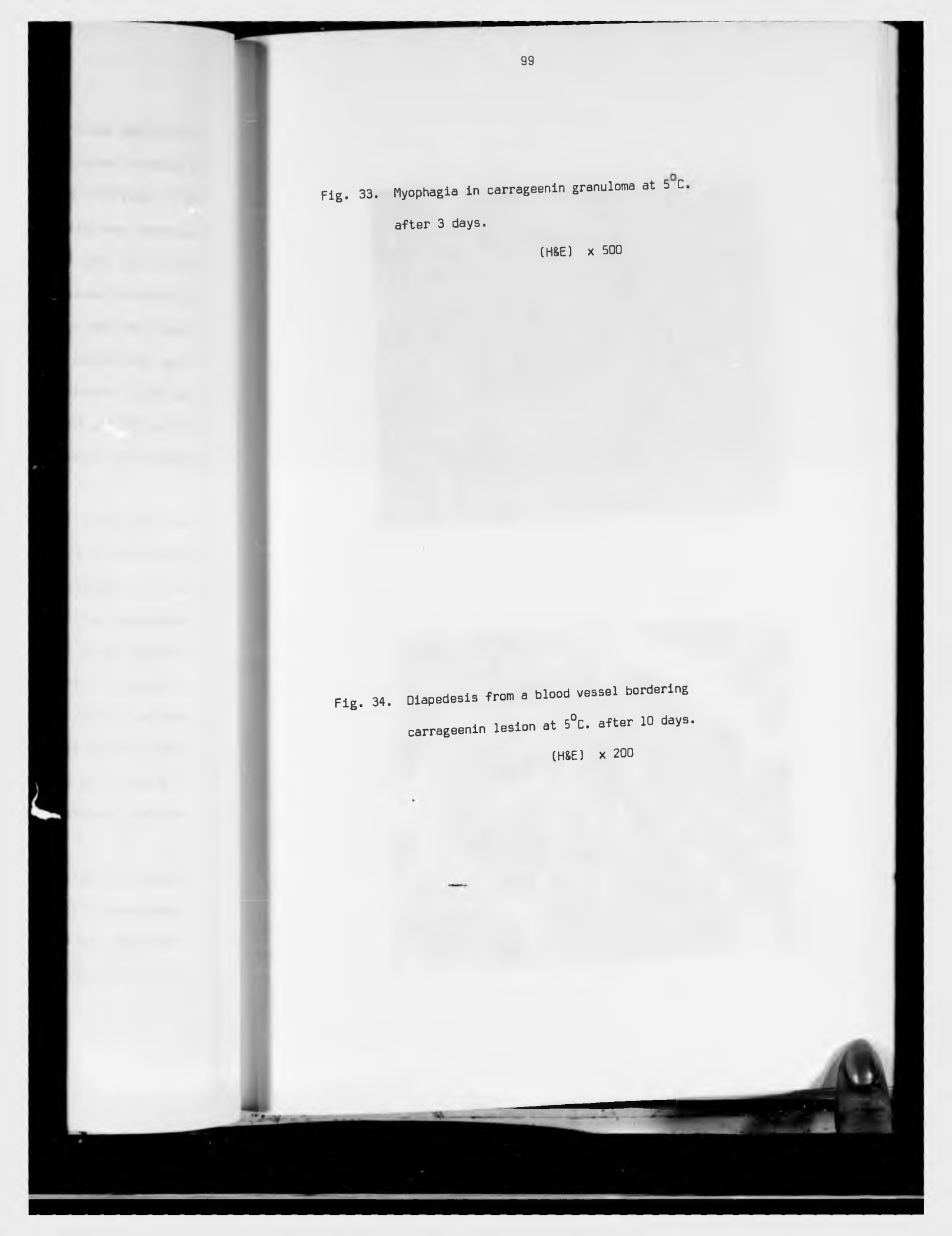
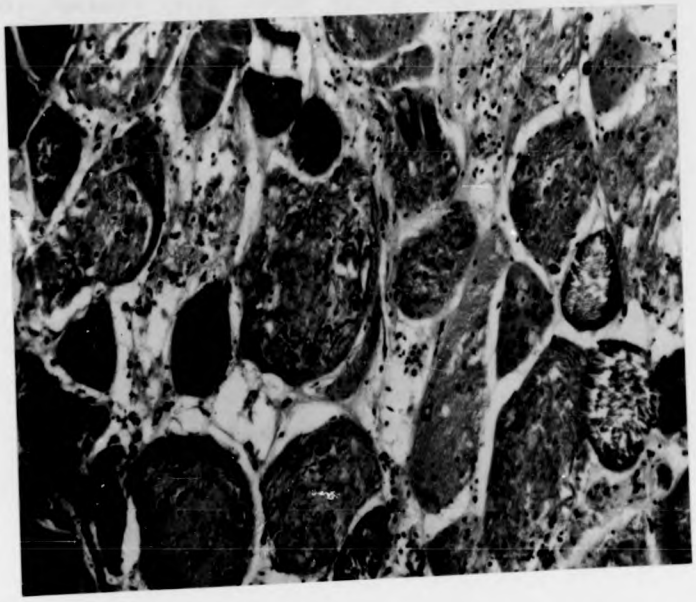


Fig. 33. Myophagia in carrageenin granuloma at 5°C. after 3 days.

(H&E) x 500

Fig. 34. Diapedesis from a blood vessel bordering carrageenin lesion at 5°C. after 10 days.

(H&E) x 200



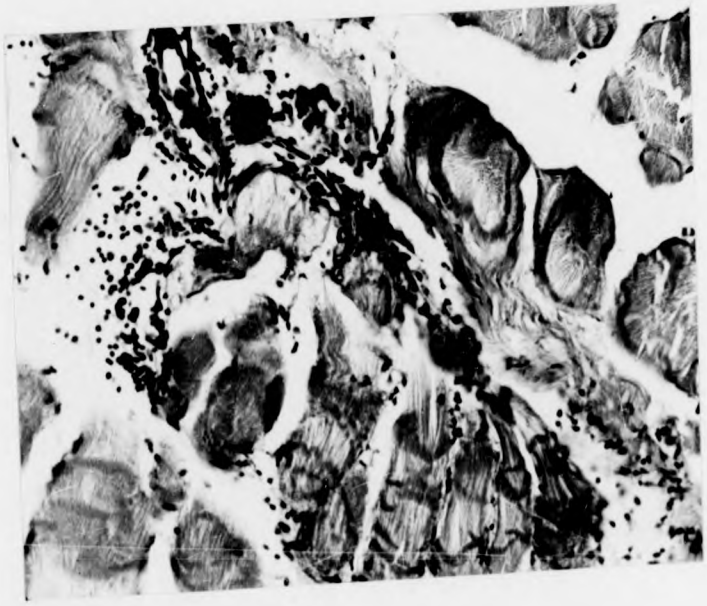
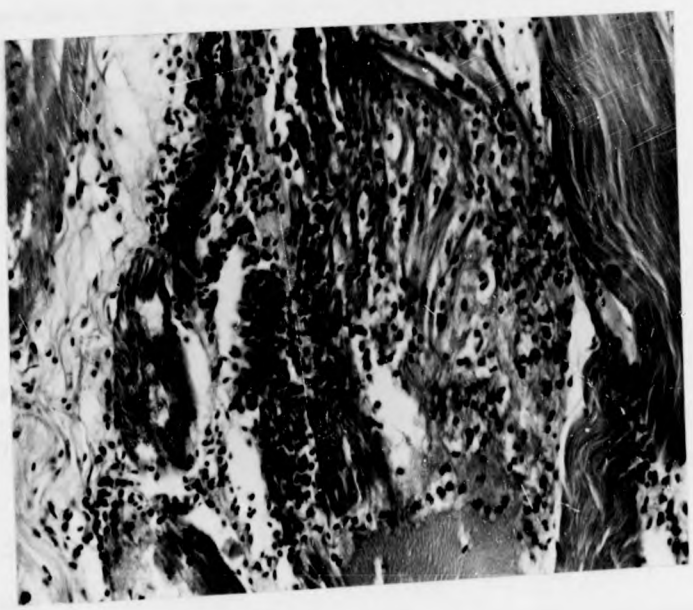


Fig. 35. Myotomal muscle fibres showing myophagia at 5°C. Note the large number of macrophages. There are also a few polymorphonuclear leukocytes (arrowed).

(H&E) x 800

Fig. 36. Extensive myophagia of myotomal muscles, 18 days after inoculation of carrageenin at 5°C.

(H&E) x 400



macrophages, although extracellular mucopolysacchardies were still very clearly stained by the alcian blue and alcian green mucopolysaccharides staining methods. The vascularisation was slightly increased, and myophagia still evident. The loose collagen fibres seen on the 35th day had developed considerably by now, as seen in sections stained by Masson's trichrome staining technique. Plasma cells and their precursors were not seen to be present in significant numbers until the 42nd day after initiation of the granuloma (Fig. 39).

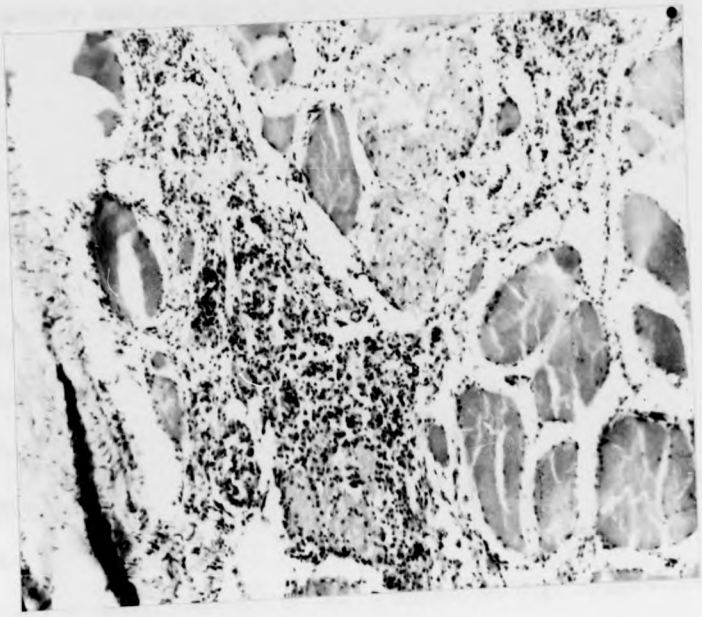
By the 56th day after initiation of the granuloma, the inflammatory response comprised the same cell types as on the 42nd day, but at this stage the phagocytosed muscle fibres started to be replaced by new collagen fibres, and some showed evidence of regeneration, viz bluish hue in haematoxylin and eosin (H&E) stained sections. The most interesting feature of this stage was that the epithelioid cells and myophagia of muscle bundles were seen side by side (Fig. 40), a feature not seen in the higher temperature experiment, because the clearance of muscle fibres by myophagia was completed as early as the 10th to 12th days in the previous experiment. The granulation tissue was highly vascularised and possessed numerous epithelioid cells and by this time the fibroblastic activity had reached its peak level (Figs. 41:42). The macrophages were decreased, a few foam-cell-like macrophages well endowed with carrageenin in their vacuoles were present, but most of the remainder were poorly endowed with carrageenin. Some lymphocytes had also accumulated at the site of injection.

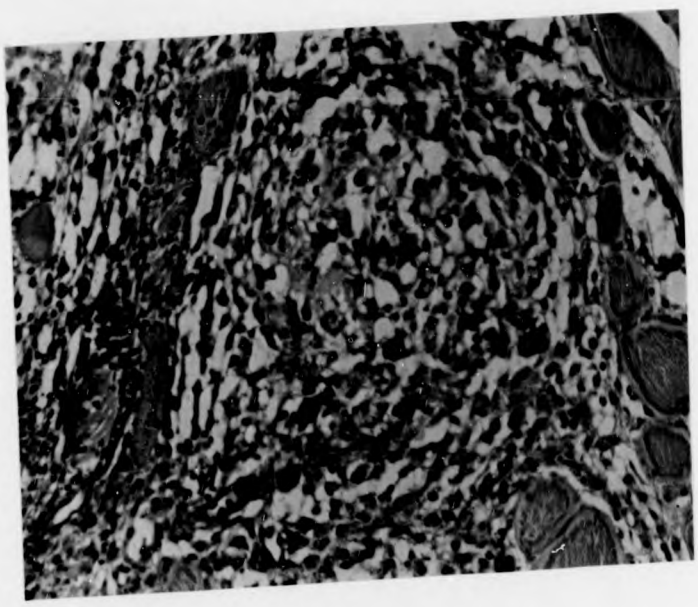
Fig. 37. General view of the carrageenin lesion at 5°C. after 18 days.

(H&E) x 200

Fig. 38. Whorls of epithelioid cells within a carrageenin granuloma after 42 days at 5°C.

(H&E) x 500





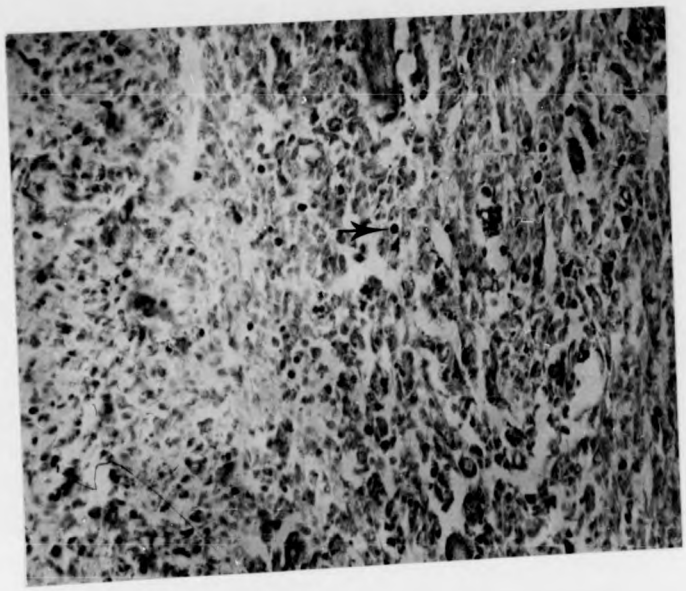
By the 85th day after initiation of the granuloma, the inflammatory response was composed predominantly of epithelioid cells. They were seen throughout the inflammatory area, and the few individual macrophages left within the inflammatory exudate were mostly foam-cell-like in appearance. In contrast, in the previous experiment at 10°C. extracellular mucopolysaccharides had been completely phagocytosed by the 42nd day. The most striking microscopic feature of this 85th day was the Langhans type giant cells (Fig. 43). They measured 21-30 μ in diameter, and their nuclei were distributed at the periphery of the cytoplasm. This was the first occasion on which evidence of giant cells occurring at the cold temperature was seen, and this was in sharp contrast to the situation at high temperature where they were present as early as the 12th day. The mature fibroblasts and vascular tissue still persisted around the edge of the granuloma, but plasma cell numbers had decreased.

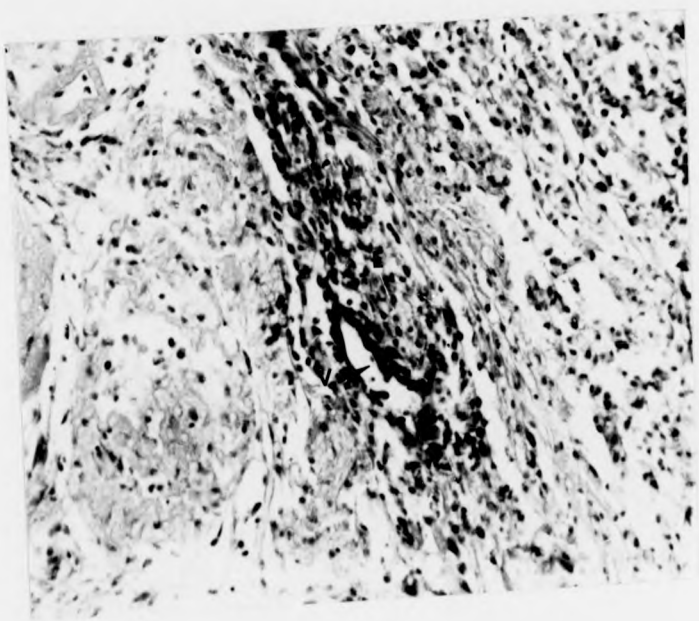
Fig. 39. Plasma cells (arrowed) within carrageenin granuloma at 5°C. after 42 days inoculation.

(Unna-Pappenheim) x 500

Fig. 40. Epithelioid cells, with concurrent myophagia. Carrageenin granuloma at 5°C. after 56 days. (E) epithelioid cells, (V) blood vessel.

(H&E) x 500





Figs. 41 and 42. Active fibroplasia with vascularisation in carrageenin lesion at 5°C. after 56 days. (H&E) x 500

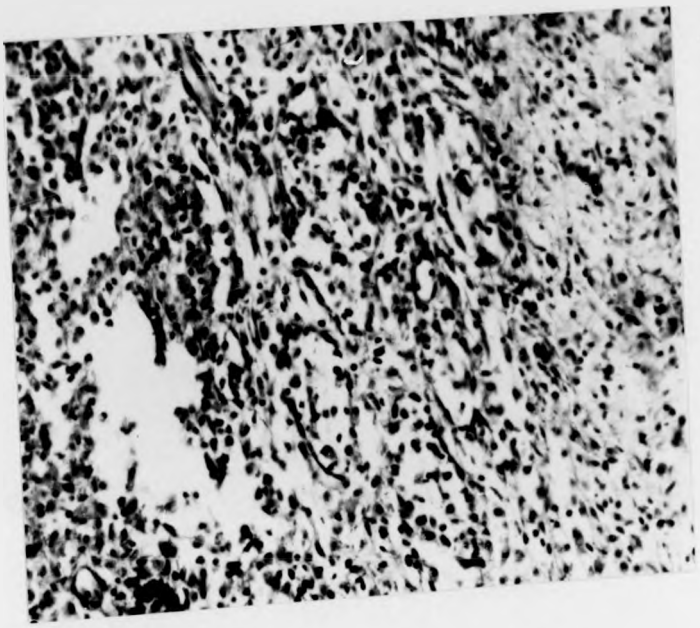
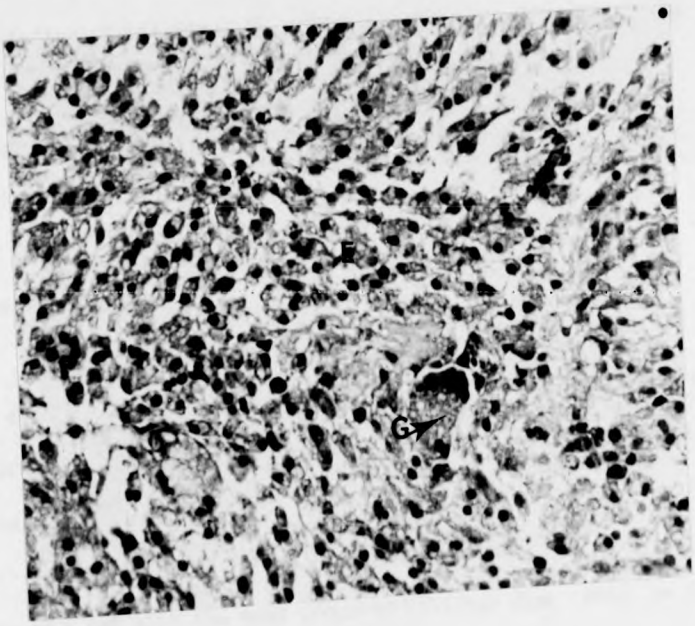


Fig. 43. Carrageenin granuloma at 5°C. after 85 days. The most significant feature is the giant cell (G) surrounded by epithelioid cells (E).

(H&E) x 800


G

Experiment III. The effect on the carrageenin granuloma of high L-ascorbic acid levels at the environmental temperature of 10°C.

The Study:

In view of the considerable contribution of fibroplasia to the latter stage of the lesion caused by carrageenin, and the known association of vitamin-C (L-ascorbic acid) with fibroplasia in fish (Halver 1972), an experiment was devised to study the pathogenesis of the carrageenin granuloma in vitamin-C supplemented fish.

Materials and Methods:-

The granuloma was established as in the previous experiments, but in order to determine the effect of extra vitamin-C on the healing of carrageenin inoculated fish, 0.1 ml. of a 1% saline solution of L-ascorbic acid was given daily, injected intraperitoneally. Fish were fed with the same prepared pelleted diet (WFAI) as in previous experiments. The feeding and injection of L-ascorbic acid was commenced two weeks before the beginning of the carrageenin inoculation, although on the actual day of carrageenin inoculation, the fish were not inoculated; inoculation was thereafter continued daily throughout the course of the experiment.

The dose of L-ascorbic acid was arrived at as a result of a small pilot experiment. Three 2+ plaice, of the same size, were injected with 0.1 ml., 0.3 ml., and 0.5 ml. L-ascorbic acid intraperitoneally, and they also received 0.05 ml. of carrageenin intramuscularly. The fish were identified by differential fin clipping. After 4-6 hours the fish which had received 0.5 ml. of L-ascorbic acid was found dead. The other two fish survived, but 0.3 ml. of L-ascorbic acid caused a large melanin pigmented granuloma in the peritoneal area, and the injected area was very swollen, so that 0.1 ml. of a 1% solution of L-ascorbic acid was chosen as the dose.

In the main experiment, 23 healthy fish from the main stock were inoculated with 0.05 ml. of sterile saline solution of 0.3 per cent carrageenin into the dorsal muscular area, and in addition received 0.1 ml. of L-ascorbic acid daily from two weeks before the inoculation, and daily thereafter. Three fish were held against any losses to mortalities from other causes. Two fish were sacrificed at intervals of 3, 5, 7, 10, 12, 14, 21, 28, 35 and 42 days after inoculation of the carrageenin.

Light Microscopic Observations:-

Three days after initiation of the inflammation, the area consisted mainly of macrophages, a few polymorphonuclear

leukocytes, and lymphocytes. A few of the macrophages were loaded with carrageenin and these were large, measuring 6.5-6.8 μ in diameter. The extracellular area contained much mucopolysaccharide and there was extensive necrosis of the muscle fibres as early as the 3rd day after initiation.

The fibroplastic activity was first seen at the 5th and the 7th days (Figs. 44:45:46). The cells were fusiform, large and pale coloured. Although the fibroblasts of normal connective tissue were also evident, these were flattened and dark blue coloured and situated mostly around the inflammatory area. In the standard experimental granuloma at environmental temperature of 10°C., this was seen at the 10th day. The exudative area was surrounded by a thick border of collagen fibres. The mass of necrosed muscle fibres showed fragmentation of sarcoplasm and was rich with macrophages. A few healthy muscle fibres were seen within the inflammatory area. The rest of the muscle fibres had degenerated. The extracellular exudative area contained stainable free carrageenin, while the majority of the macrophages had also taken up carrageenin, and were foam-cell-like in appearance. Vascularisation of the inflammatory area had already begun.

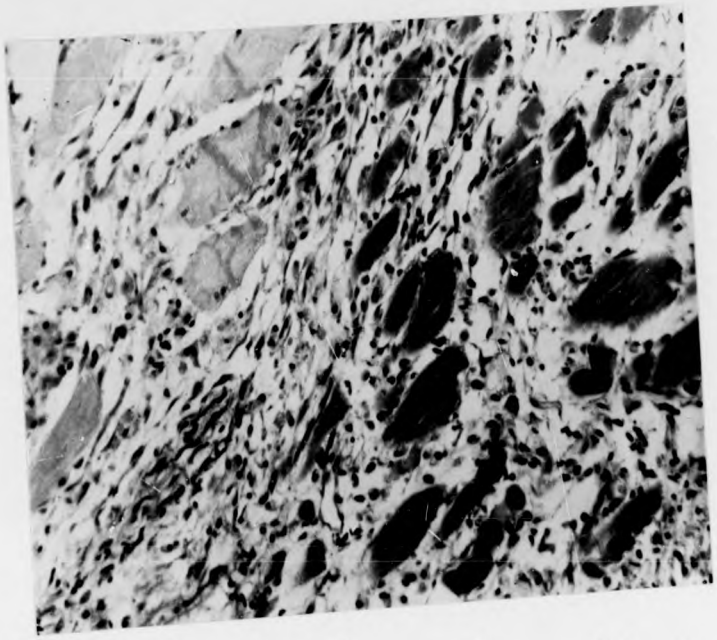
By the 10th, 12th and 14 days, the inflammatory response consisted predominantly of macrophages, fibroblasts, plasma cells and some lymphocytes (Fig. 47). In addition to these, the epithelioid

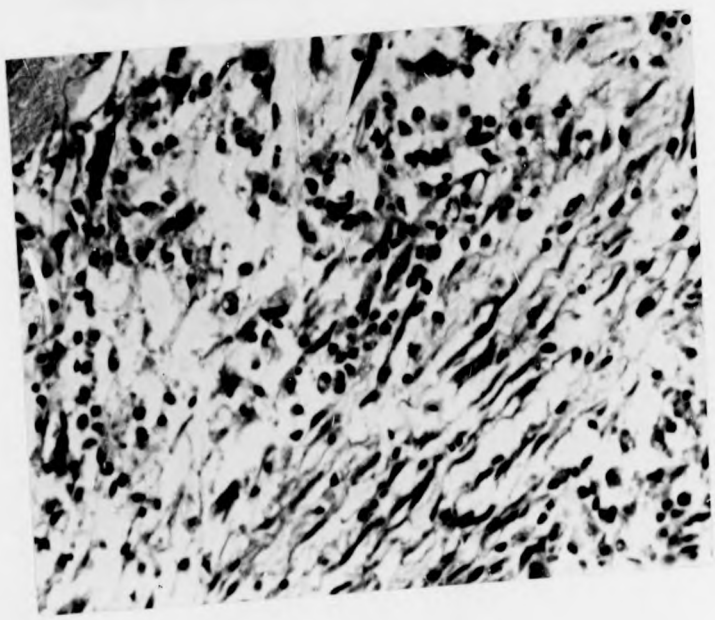
cells with their indistinct cell boundaries and the foreign body type giant cells were first evidenced by the 12th day as in the standard experiment. The epithelioid cells were scattered randomly, and were arranged in a whorling pattern. A few foreign body type giant cells, approximately 28-35 μ were seen by the 10th day after initiation of the granuloma (Fig. 48). Their number increased by the 14th day. On that day myophagia was almost completed, and previously necrosed areas were replaced by the new fibre networks with large collections of foam-cell-like macrophages (Figs. 49:50:51). The fibrous network by the 10th day consisted of very thin collagen fibres and elastic fibres. By the 14th day argyrophilic fibres were weakly stained by the Gordon and Sweet method (Fig. 52). The extracellular fluid showed strongly positive staining characteristics for mucopolysaccharides and contained foam-cell-like macrophages with carrageenin within their vacuoles. A high degree of vascularisation with numerous small capillaries was found throughout the new granulation tissue and was increased by the 14th day.

By the 21st and 28th days, the main feature of the lesion was regeneration of muscle fibres. The inflammatory cells consisted of epithelioid cells, macrophages, lymphocytes, plasma cells, fibroblasts and Langhans type giant cells (Figs. 53:54). Quantitatively, the number of epithelioid cells was considerable. They were arranged in a whorling pattern and were spread out throughout the new granulation tissue. Collagen fibre network was apparent between the cells and young capillaries were numerous.

Figs. 44 and 45. Early stage fibroblastic activity after 5 days. Some macrophages are already foam-cell-like in appearance. Vitamin-C supplemented at 10^{o}C.

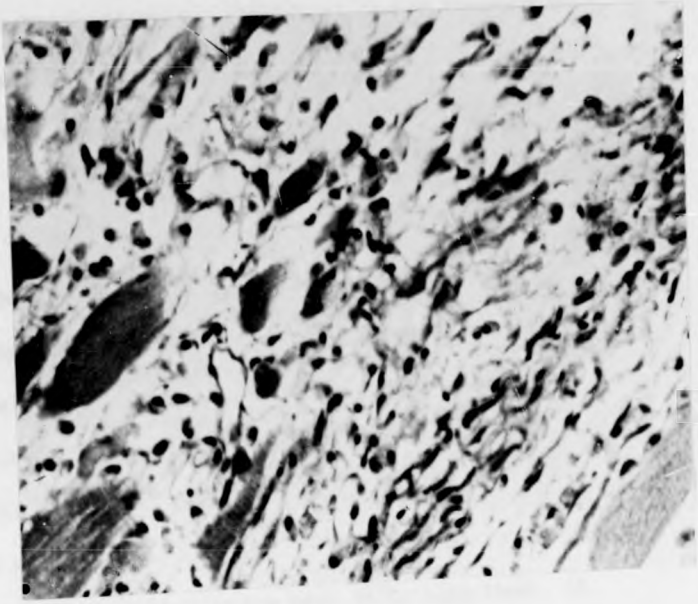
(H&E) x 500

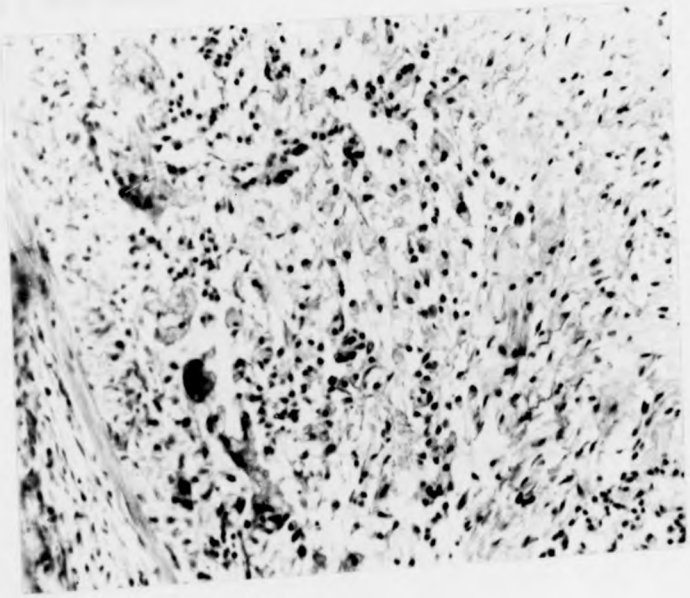




Figs. 46 and 47. Fibroblasts in healing granuloma, showing the characteristic shape and early formation of collagen, after 12 days (vitamin-C supplemented at 10^{o}C.).

(H&E) x 500





By the 35th day, the collagen fibres of the granulation tissue showed markedly increased density as in the standard 10°C. experiment (Fig. 55). The carrageenin was still extant within the extracellular area and within the foam-cell-like macrophages.

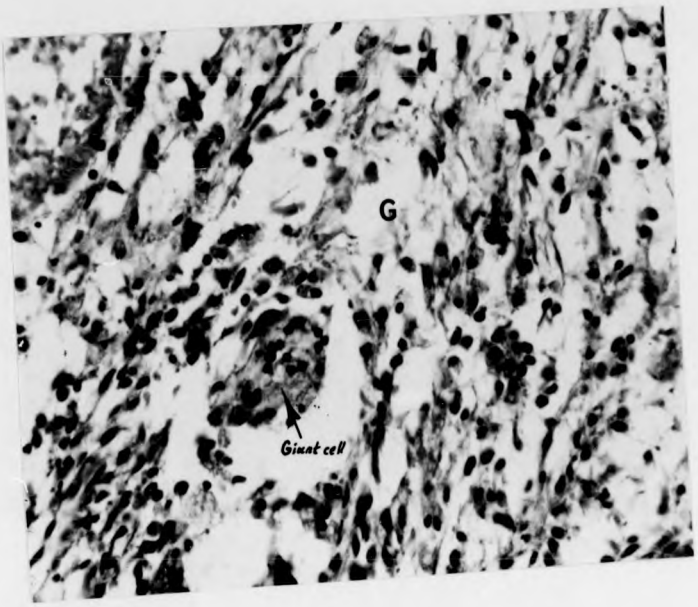
By the 42nd day after initiation of the granuloma, the cellular response consisted of similar types of cells to those of the 35th day. The carrageenin was almost all removed from the extracellular area, as in the 10°C. experiment, and between the macrophages in the inflammatory area, evidence of intercellular mucopolysaccharides by the alcian green staining method was seen. Although at this stage the feature of the granuloma were similar to the main 10°C. experiment, it appeared that the extra vitamin-C had stimulated fibroblastic activity 5 days earlier than in the standard 10°C. experiment. Subsequently, until the 42nd day, the fibroblastic activity (fibroplasia) was similar to that of the main 10°C. experiment. Additionally, both appearance and completion of myophagia was seen earlier than in the 10°C. main experiment.

Fig. 48. Young granulation tissue (G) in vitamin-C supplemented fish after 14 days, showing a multinucleated foreign body type giant cell.

(H&E) x 500

Fig. 49. Carrageenin granuloma of fish supplemented with vitamin-C after 14 days. Note the formation of collagen and abundance of phagocytic macrophages.

(H&E) x 500


G
Giant cell

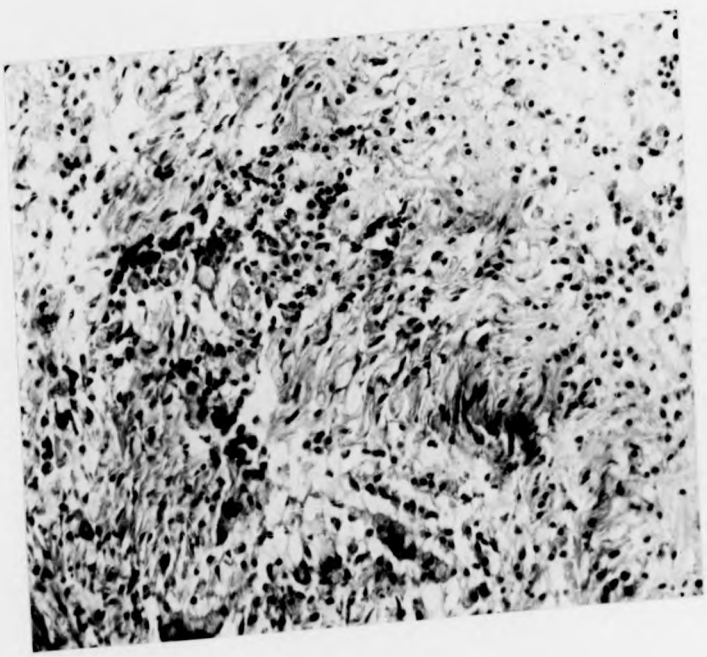
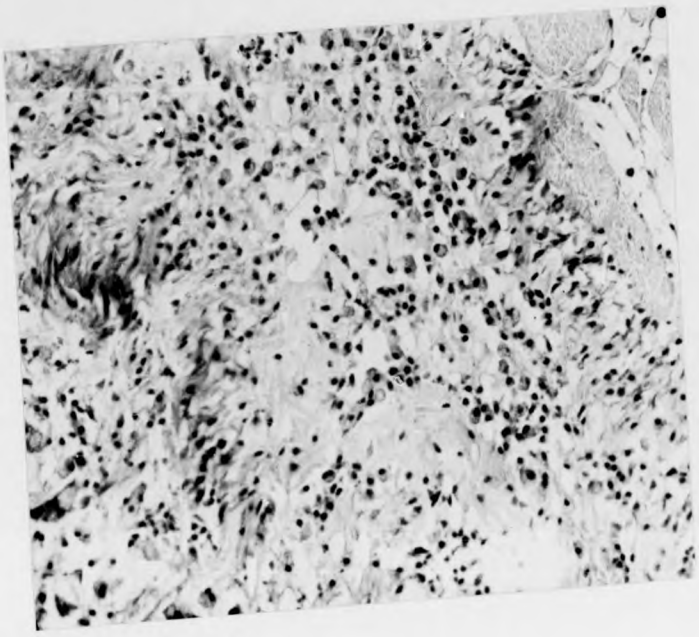


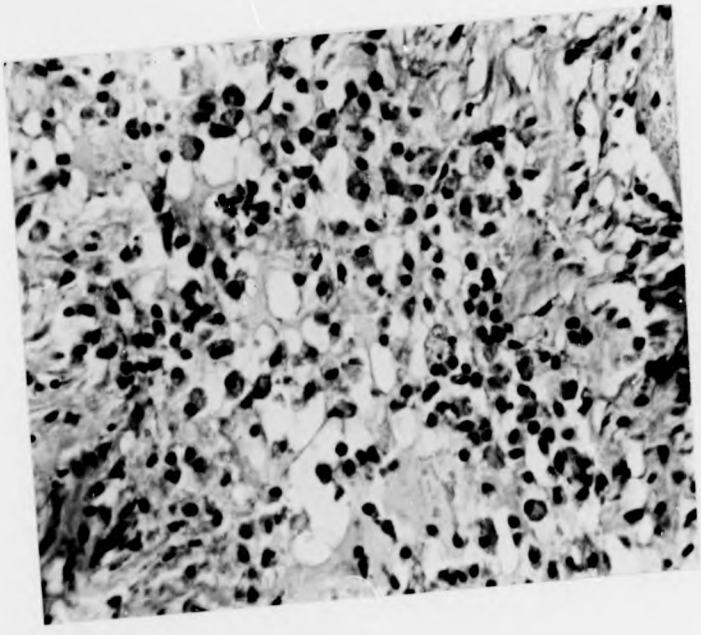
Fig. 50. Carrageenin granuloma of fish supplemented with vitamin-C after 14 days. Note the formation of collagen and clusters of phagocytic macrophages.

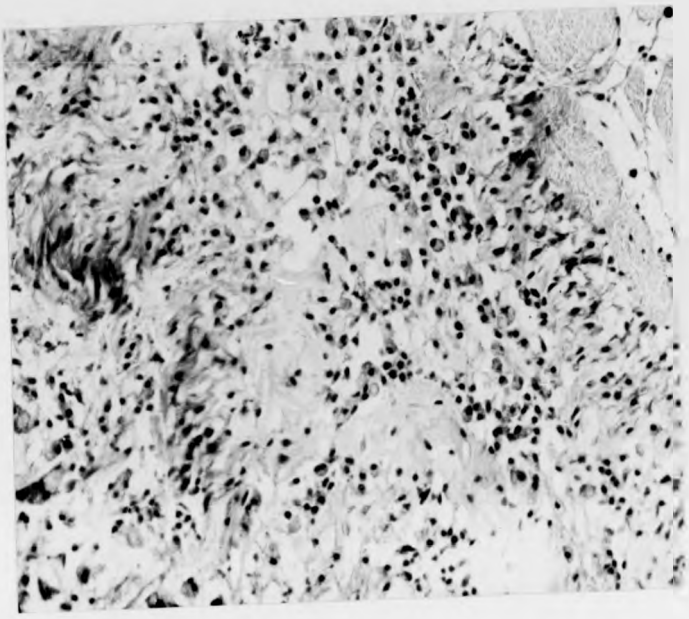
(H&E) x 500

Fig. 51. The granulation tissue, showing clusters of macrophages (vitamin-C supplemented fish at 10°C. after 14 days).

(H&E) x 800







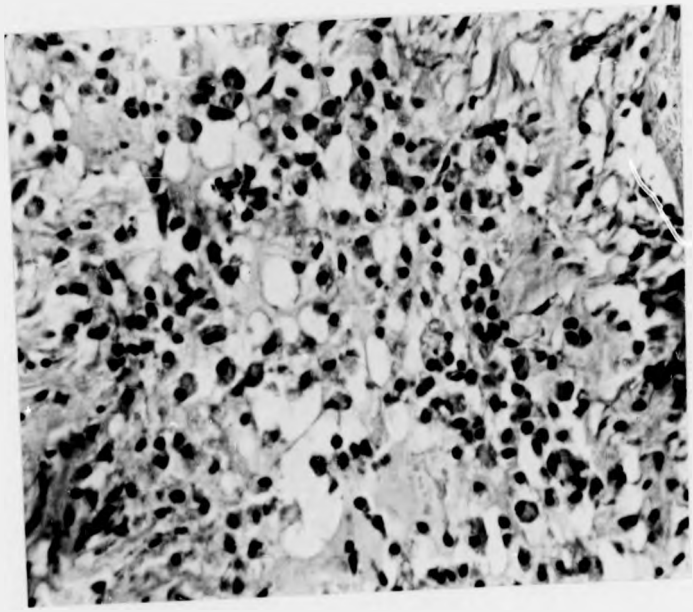


Fig. 52. Argyrophilic fibres of vitamin-C supplemented fish, showing affinity for silver salts in carrageenin granuloma after 14 days, at $10^{o}C$.

(Gordon and Sweet) x 500

Fig. 53. The granulation tissue, showing Langhans type (G), giant cell in vitamin-C supplemented, carrageenin injected fish after 21 days at $10^{o}C$.

(H&E) x 800

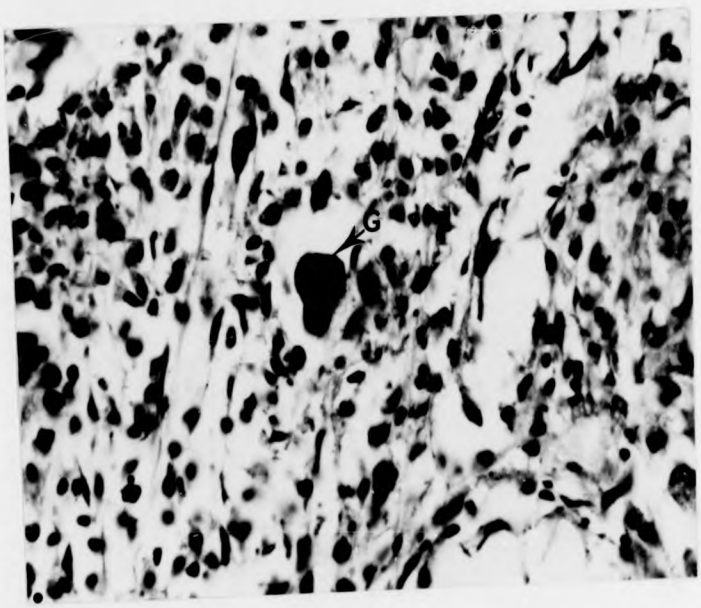
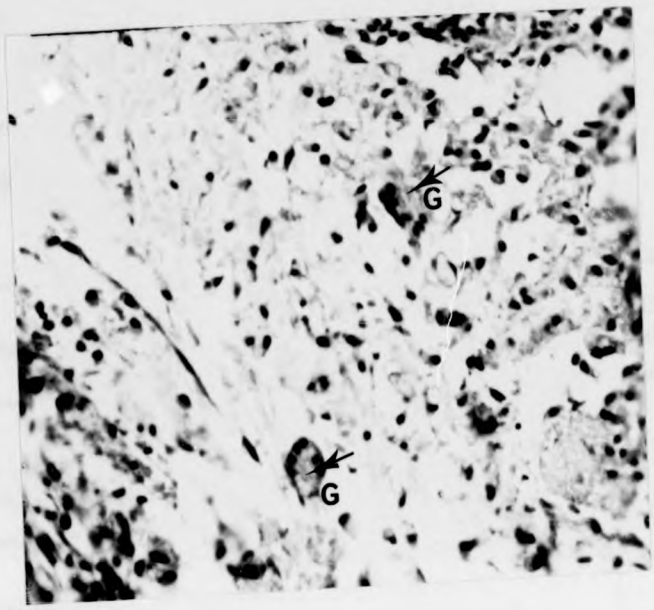

G

Fig. 54. The granulation tissue, showing multinucleated giant cell (Langhans type), (arrowed) in L-ascorbic acid supplemented carrageenin injected fish after 28 days at 10°C.

(H&E) x 800

Fig. 55. Collagen fibres and granulation tissue in vitamin-C supplemented fish at 10°C. after 35 days.

(H&E) x 500


G
G

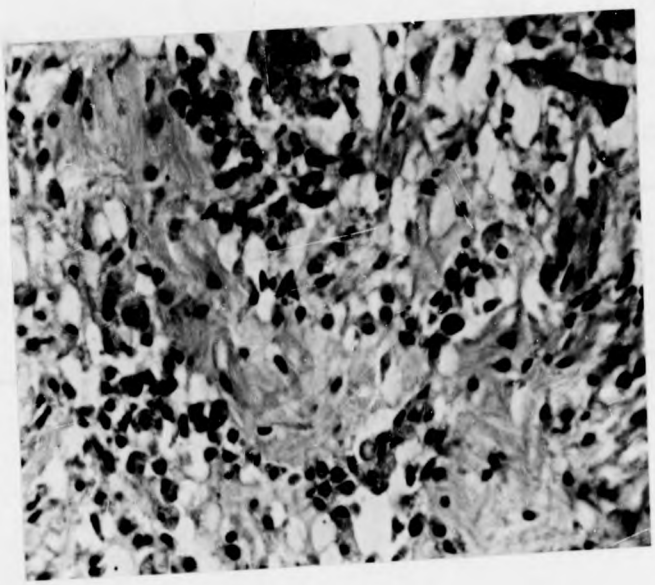


TABLE II

TIME OCCURRENCE OF KEY LESION FEATURES UNDER DIFFERENT EXPERIMENTAL CONDITIONS

Feature	10°C. lesion	5°C. lesion	10°C. with L-ascorbic acid supplement
First appearance of fibroblast	10 days	18 days	5 days
Myophagia a. beginning b. completed	 5 days 28 days	 5 days 85 days	 3 days 21 days
Collagen visible	14 days	21 days	12 days
Muscle regeneration first evident	21 days	56 days	21 days
Vascularisation beginning	10 days	21 days	7 days

Fig. 56. Duration of the inflammatory cell response within the carrageenin granulomata at 10°C.

● : polymorphonuclear leukocytes

▲ : macrophages

✖ : lymphocytes

■ : fibroblasts

o : plasma cells

△ : epithelioid cells

□ : giant cells

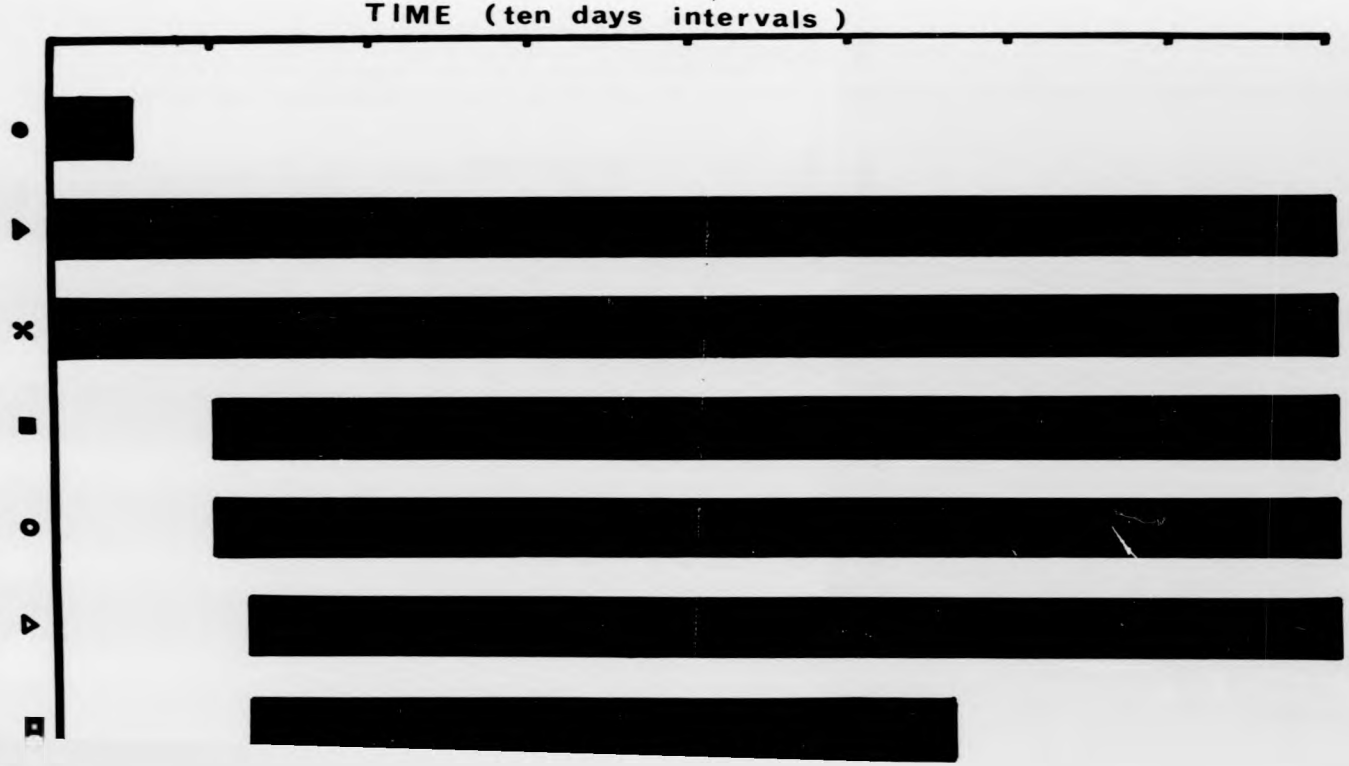

TIME (ten days intervals)
●
▶
×
■
○
△
□

Fig. 57. Duration of the inflammatory cell response within the carrageenin granulomata at $5^{o}C$.

● : polymorphonuclear leukocytes

▲ : macrophages

✖ : lymphocytes

■ : fibroblasts

o : plasma cells

▲ : epithelioid cells

□ : giant cells

TIME (five days intervals)

Fig. 58. Duration of the inflammatory cell response within the carrageenin granulomata at 10°C. with extra L-ascorbic acid supplement.
Commencement and termination of sampling days (3 days to 42 days) are shown by arrows (Vc).

●: polymorphonuclear leukocytes

▲: macrophages

✖: lymphocytes

■: fibroblasts

○: plasma cells

△: epithelioid cells

□: giant cells

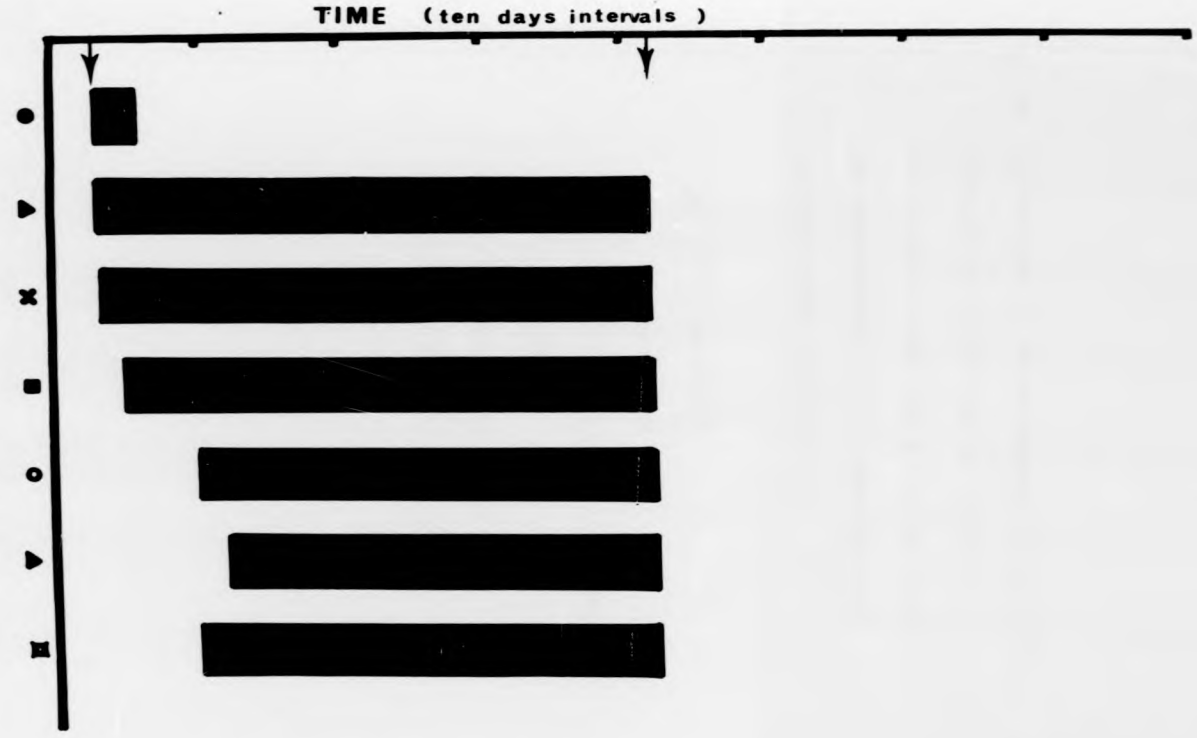

TIME (ten days intervals)

Fig. 59. Differences in the development of macrophage activity in the various experiments.
It should be noted that the arrow (Vc) shows the commencement of the L-ascorbic acid supplementation experiment.

✕: 10^{o}C. experiment

●: 5^{o}C. experiment

▲: 10^{o}C. with L-ascorbic acid supplement experiment

A : first appearance of the cell

M : maximum level of the cell

TIME (five days intervals)
A
M

Fig. 60. Differences in the development of lymphocyte activity in the various experiments.
It should be noted that the arrow (Vc) shows the commencement of the L-ascorbic acid supplementation experiment.

✖: 10°C. experiment

●: 5°C. experiment

▲: 10°C. with L-ascorbic acid supplement experiment

A : first appearance of the cell

M : maximum level of the cell

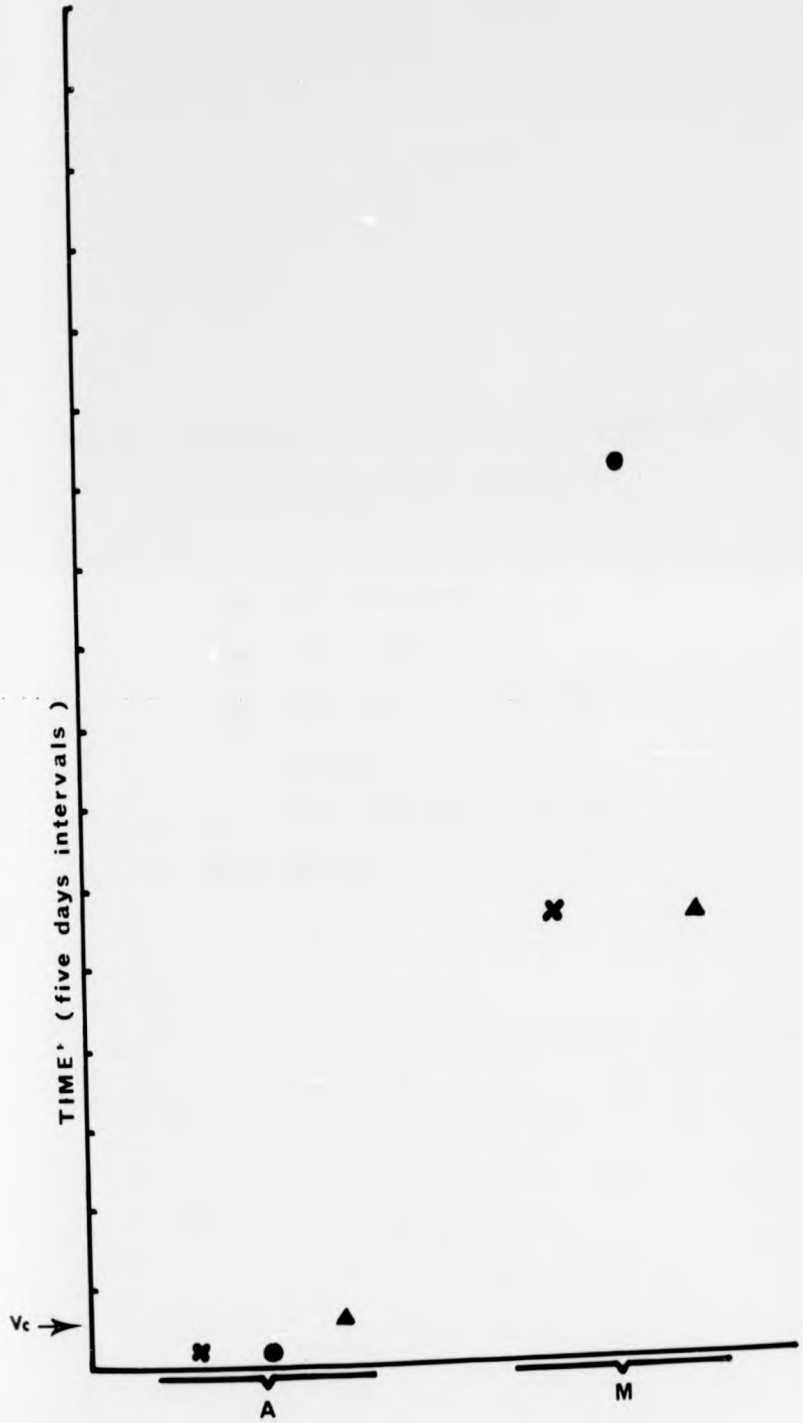

TIME' (five days intervals)
Vc
A
M

Fig. 61. Differences in the development of plasma cell activity in the various experiments.

✖: 10°C. experiment

●: 5°C. experiment

▲: 10°C. with L.ascorbic acid supplementation experiment

A : first appearance of the cell

M : maximum level of the cell

TIME (five days intervals)
Vc
A
M

Fig. 62. Differences in the development of fibroblast activity in the various experiments.

✖ : 10^{o}C. experiment

● : 5^{o}C. experiment

▲ : 10^{o}C. with L-ascorbic acid supplementation experiment

A : first appearance of the cell

M : maximum level of the cell

TIME (five days intervals)
Vc
A
M

Fig. 63. Differences in the development of epithelioid cell activity in the various experiments.

✖: 10°C. experiment

●: 5°C. experiment

▲: 10°C. with L-ascorbic acid supplementation experiment

A : first appearance of the cell

M : maximum level of the cell

TIME (five days intervals)
Vc
A
M

Fig. 64. Differences in the development of multinucleated giant cell activity in the various experiment.

✖: 10^{o}C. experiment

●: 5^{o}C. experiment

▲: 10^{o}C. with L-ascorbic acid supplementation experiment

A : first appearance of the cells

M : maximum level of the cells

TIME (five days intervals)
A
M

Experiment IV. Ultrastructural study of the carrageenin granuloma in the plaice at 10°C.

The Study:

The electron microscopy study was carried out to carry the light microscopic description of the morphology of the cellular elements of the inflammatory response to the carrageenin granuloma to the ultrastructural level. Ferguson (1975) described the leukocytes of the circulating blood cells of the plaice in some detail of the present study extending this to include the active inflammatory lesion and to describe the macrophages, epithelioid cells, fibroblasts and plasma cells, which are a major component of the carrageenin granuloma, but which are not found in circulating blood. The methods of experimentation, fixation and ultrastructural examination have been described earlier in the general materials and methods section.

Observations:

There were five major cell types in the carrageenin granuloma macrophages, epithelioid cells, lymphocytes, plasma cells and fibroblasts.

1. macrophages:-

The macrophages were similar in morphology to the published

descriptions for mammals (Dumond and Sheldon 1965). They were generally 8-10 µm in diameter (Figs. 65:66:67) and their surface was irregular and in section had several finger-like pseudopodia. The large nucleus was fairly irregular in outline with finely marginated nucleur chromatin. There was no prominent nucleolus. The cytoplasm was moderately abundant and several mitochondria of varying size were evident. The granular endoplasmic reticulum was quite prominent and was localised mainly on the periphery of the cells, with a well developed Golgi apparatus situated adjacent to the nucleus. The peripheral cytoplasm contained a number of small vesicles, but no lysosomes were evident, although they are regularly seen in the monocytes of plaice in circulating blood (Ferguson 1975). Monocytes were seen within blood vessels in the present study, and they contained a number of lysosomes (Figs. 68:69), suggesting that absence of lysosomes is a function of the macrophage associated with carrageenin. Phagocytic vacuoles were abundant and most of them were loaded with carrageenin. When such loaded vacuoles were subjected to high magnification, carrageenin strands were seen very clearly (Fig. 70).

2. lymphocytes:-

These cells were characterized by the large oval nucleus, which was surrounded by only a thin rim of cytoplasm (Fig. 71). Their plasma membrane was plicated and occasional small pesudopodia were evident.

Fig. 65. Macrophage from carrageenin granuloma showing carrageenin in its vacuoles, 21 days after inoculation.

(EM) x 10,000

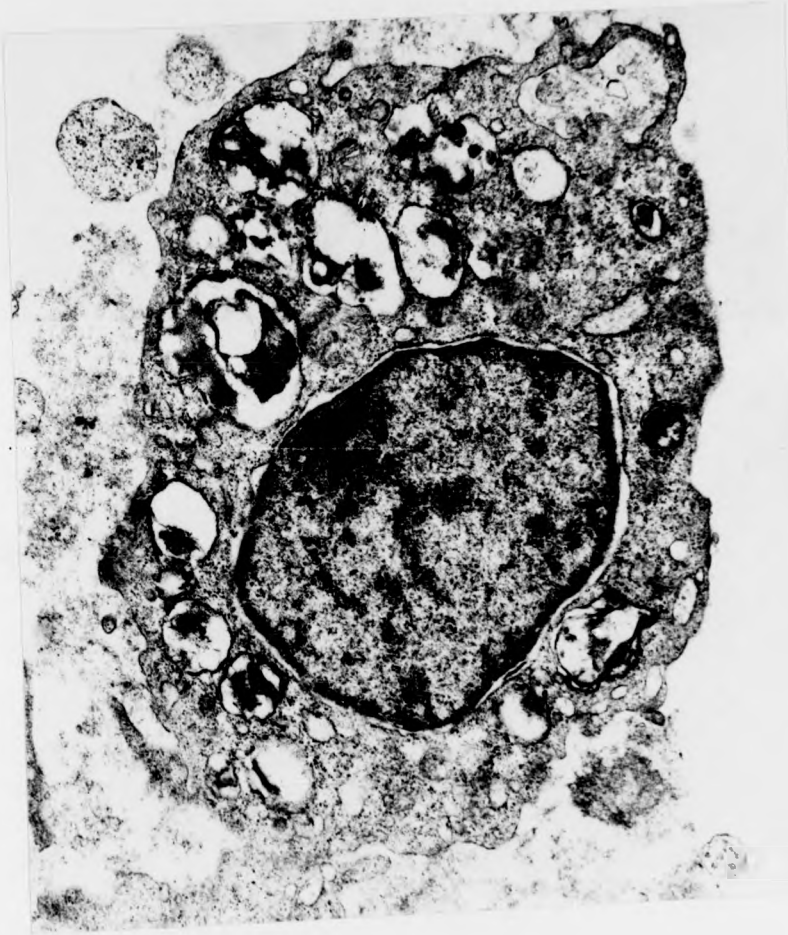
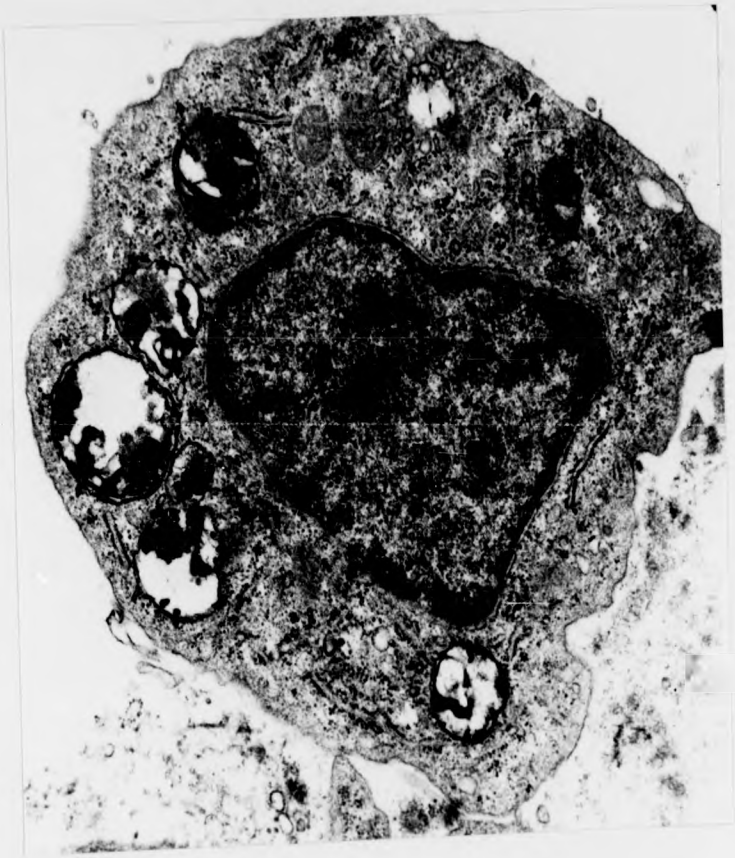


Fig. 66. Macrophage from carrageenin granuloma showing carrageenin in its vacuoles, 28 days after inoculation.

(EM) x 6,000



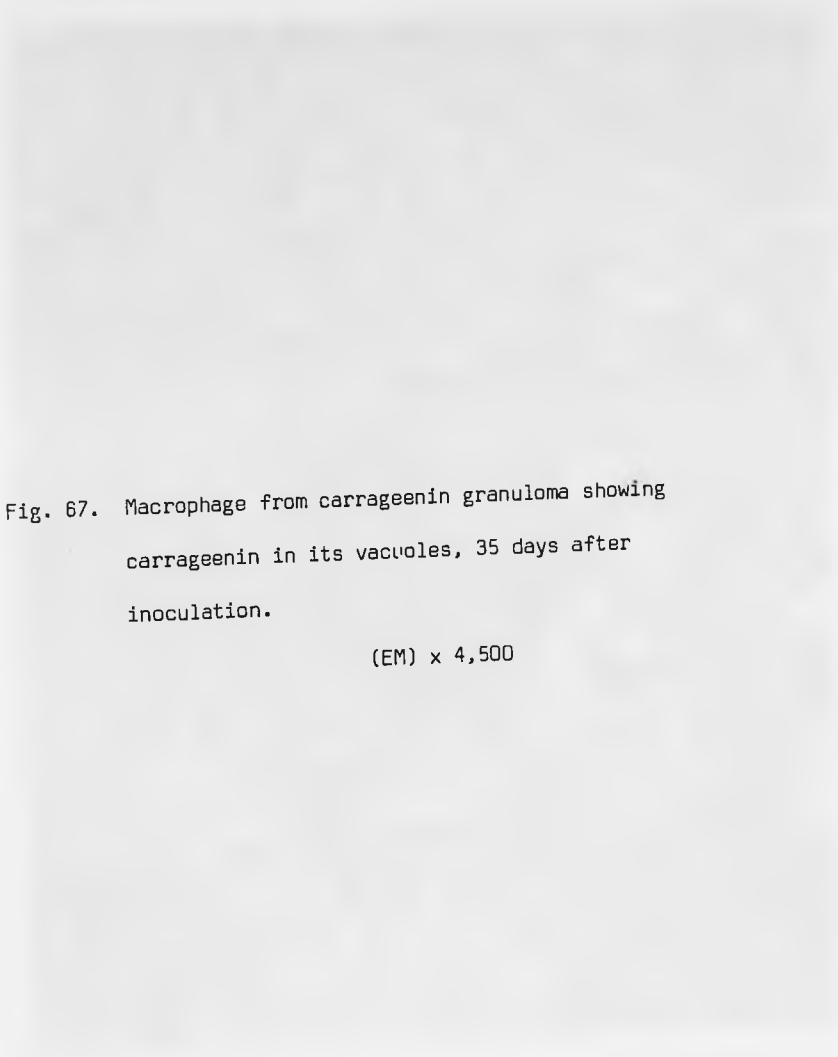


Fig. 67. Macrophage from carrageenin granuloma showing carrageenin in its vacuoles, 35 days after inoculation.

(EM) x 4,500

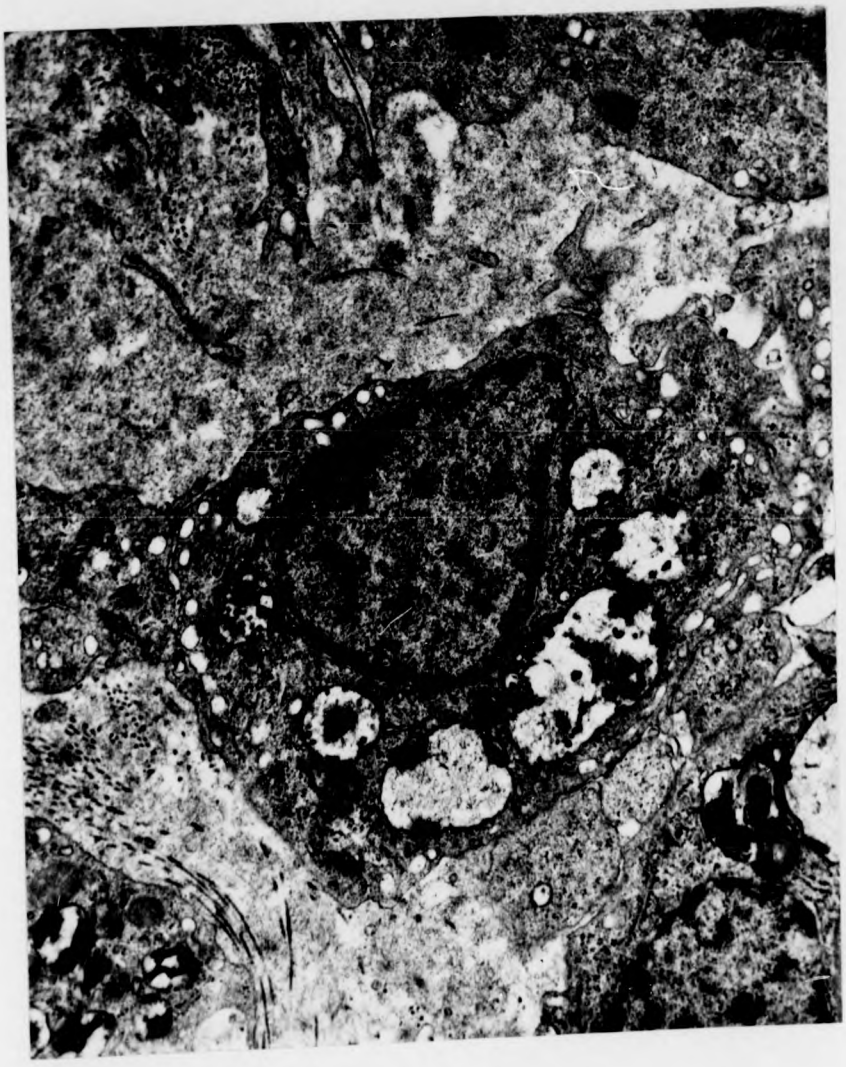


Fig. 68. A monocyte from a blood vessel adjacent to the granuloma from which Fig. 69 was taken. The major point of differentiation is the presence of lysosomes within the cytoplasm of the circulating macrophage which is not a feature of the macrophage within the carrageenin granuloma. (L) lysosome.

(EM) x 6,000

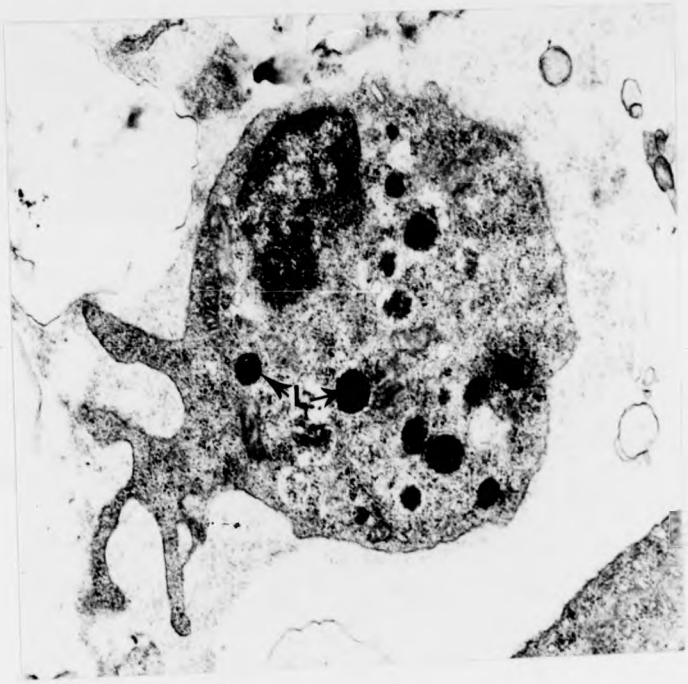


Fig. 69. A blood vessel adjacent to the granuloma.

(EM) x 2,500

Fig. 70. The ultrastructure of the macrophages of the carrageenin granulomata in the plaice.

1 : rough endoplasmic reticulum

2 : Golgi apparatus

3 : mitochondria

4 : vacuole replete with carrageenin

The Ultrastructure Features of the Macrophages of the

Carrageenin Granulomata in the Plaice

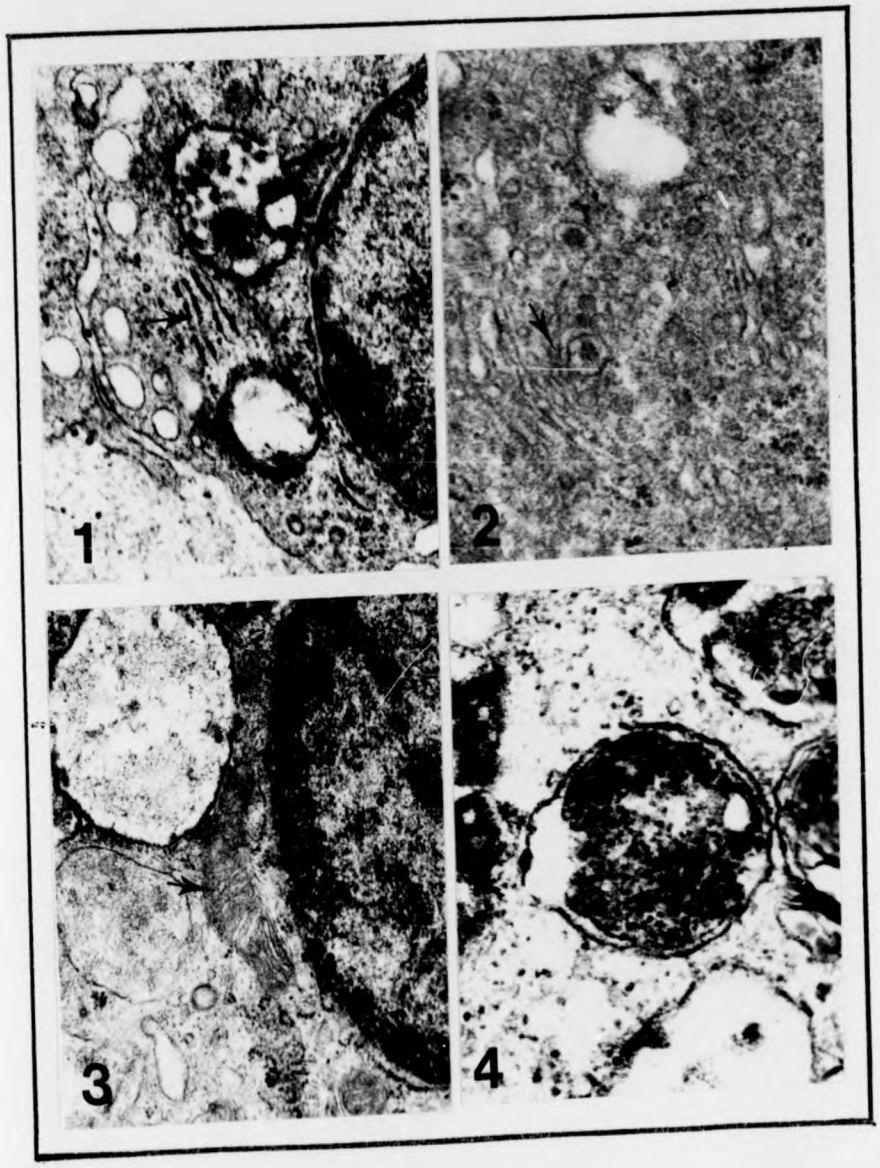
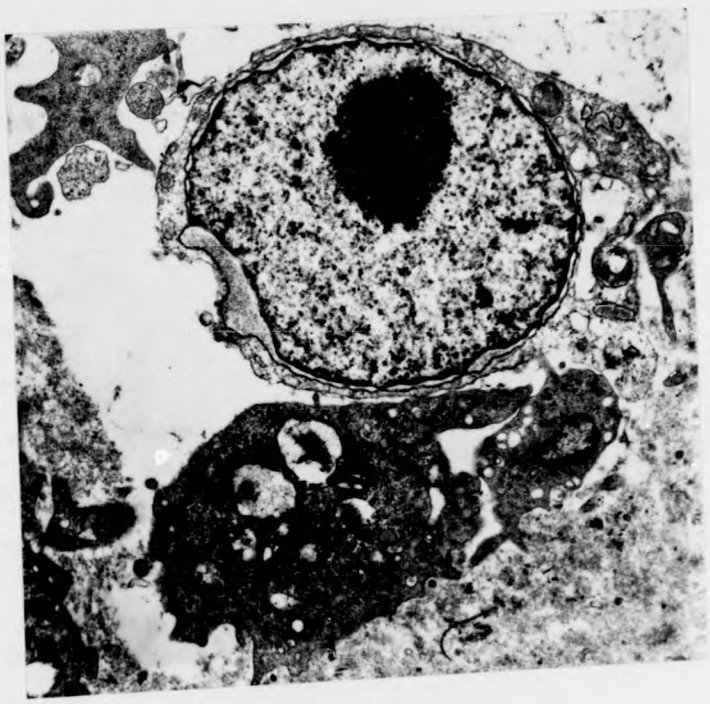


Fig. 71. Lymphocyte from carrageenin granuloma, 28 days after inoculation.

(EM) x 6,000



The granular endoplasmic reticulum was barely represented, but free ribosomes were abundant and occasional vesicles were seen throughout the cytoplasm. The Golgi apparatus was never apparent, the most prominent cytoplasmic feature being the extremely large and often oval shaped mitochondria.

3. plasma cells:-

These were more or less oval in outline, with a dense characteristically eccentric nucleus, which showed nuclear pores as described in mammals (Fig. 72). The nuclear chromatin was condensed around the periphery of the nucleus, and the cytoplasm had abundant granular endoplasmic reticulum which was often dilated into cisternae, and abundant free ribonucleoprotein particles, which were arranged in clumps within the cytoplasm. There were numerous rough surfaced vesicles near the nucleus. In the Golgi region, flat tubules were obvious. Mitochondria were numerous.

4. fibroblasts:-

After the granulomata had developed, many fibroblasts were seen (Fig. 73) and since they were highly active in the production of collagen fibres, they appeared swollen, elongated, and their nuclear chromatin was denser at the periphery. Their cytoplasm was characterized by extensive dilatation of cisternae of the endoplasmic reticulum. The cytoplasmic membrane was frequently

ill-defined or disrupted. A considerable part of the intracytoplasmic space was occupied with abundant vesicles, and electron dense lysosomes. The Golgi apparatus was large and juxtanuclear in position. Much cellular debris was present in the extracellular space, and the typical striated collagen bundles, showing regular cross banding, were numerous in the extracellular space (Fig. 74).

5. epithelioid cells:-

These cells were polygonal and their plasma membranes were contiguous. Their large nucleus was fairly regular, oval or polygonal in shape and contained finely marginated nuclear chromatin. The cytoplasm of the cell contained a variety of vesicles, large vacuoles, mitochondria, free ribosomes, microtubules and smooth and rough surfaced endoplasmic reticulum. Some of the vacuoles contained a little carrageenin, but the majority, although they possessed vacuoles, did not have ingested carrageenin within them (Fig. 75).

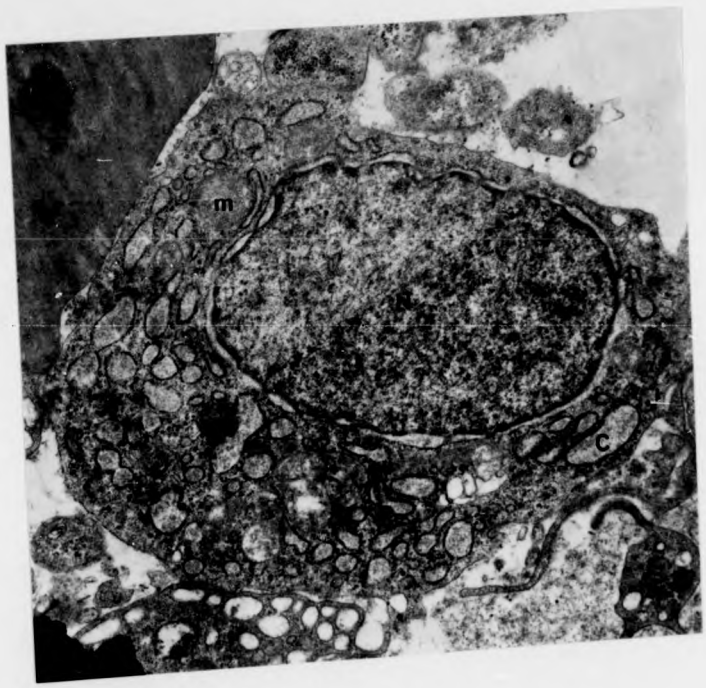
Fig. 72. Plasma cell from carrageenin granuloma, 28 days after inoculation.

(EM) x 6,000

M : mitochondria

C : cisternae

N : nucleus


m
c

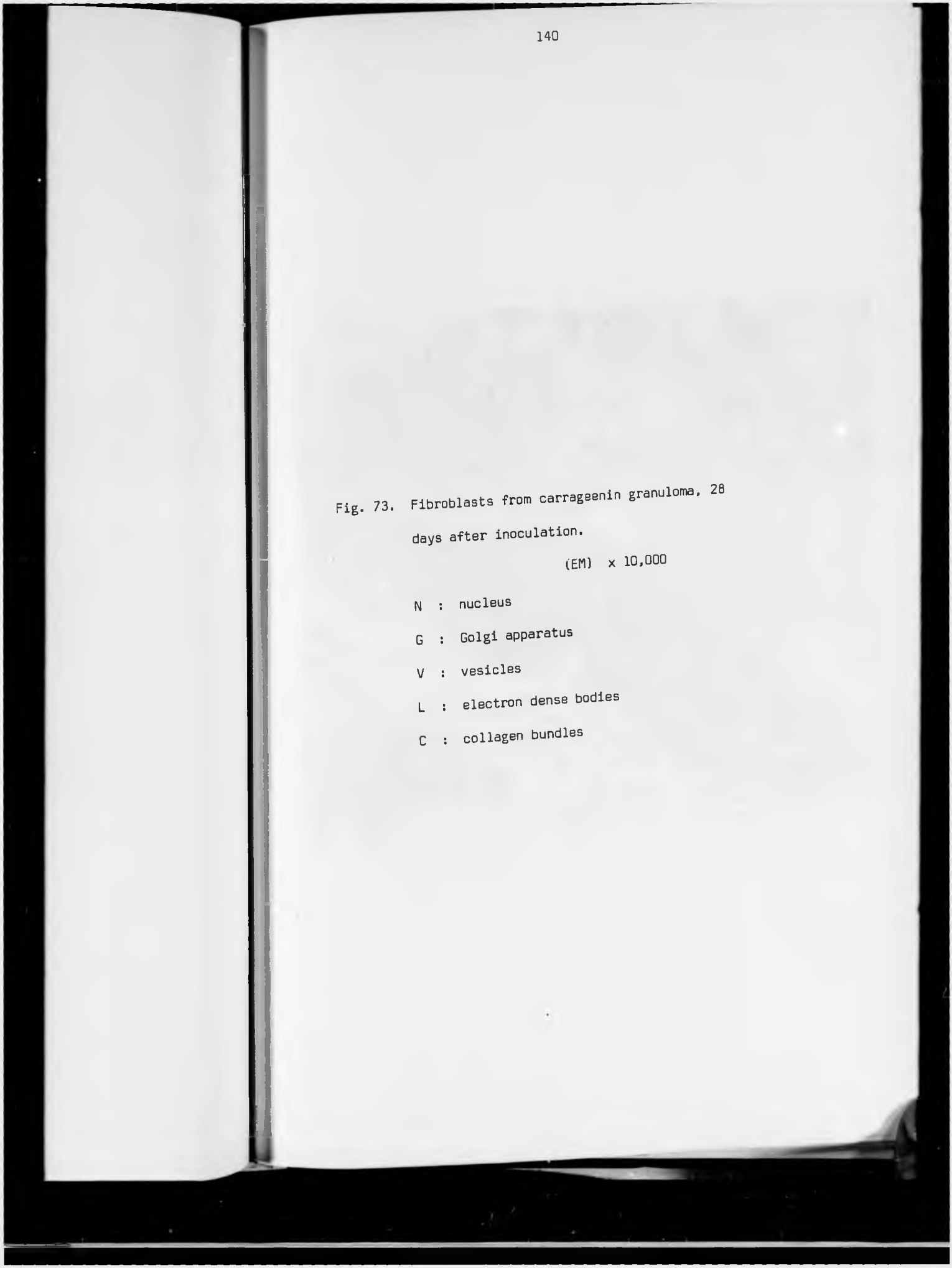


Fig. 73. Fibroblasts from carrageenin granuloma, 28 days after inoculation.

(EM) x 10,000

N : nucleus

G : Golgi apparatus

V : vesicles

L : electron dense bodies

C : collagen bundles

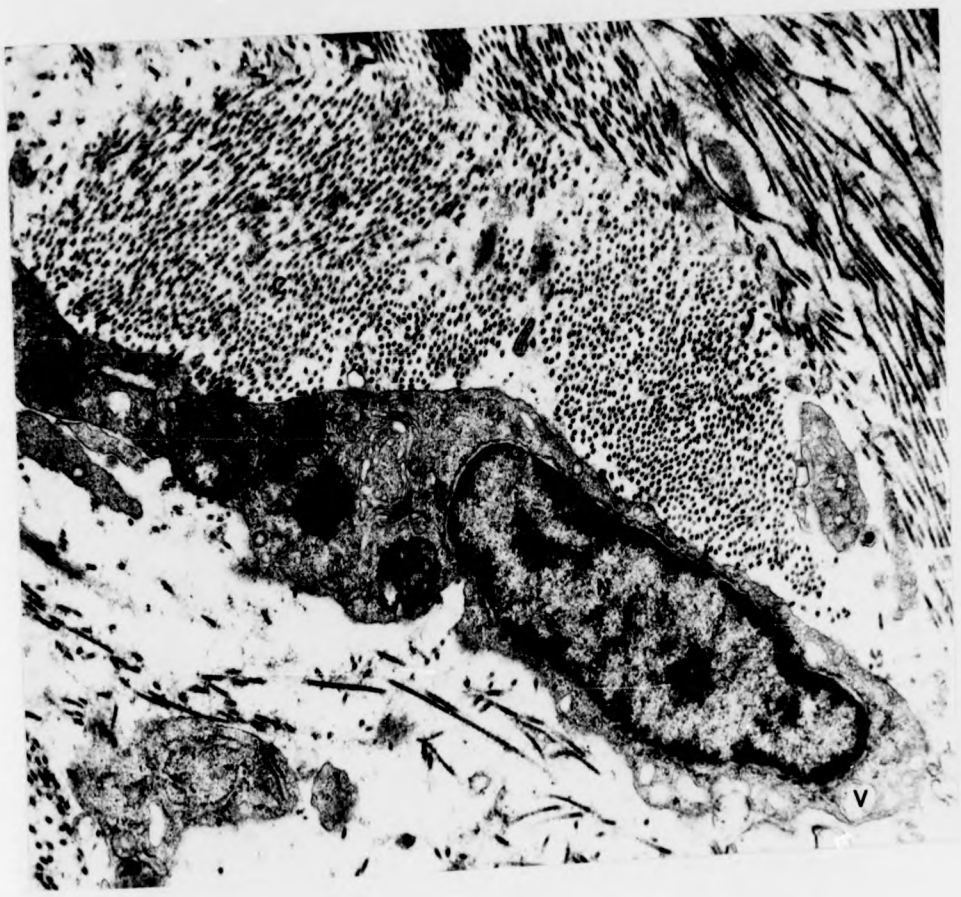

V

Fig. 74. Typical striated collagen bundles, showing cross banding, from carrageenin granuloma, 28 days after inoculation.

(EM) x 6,000

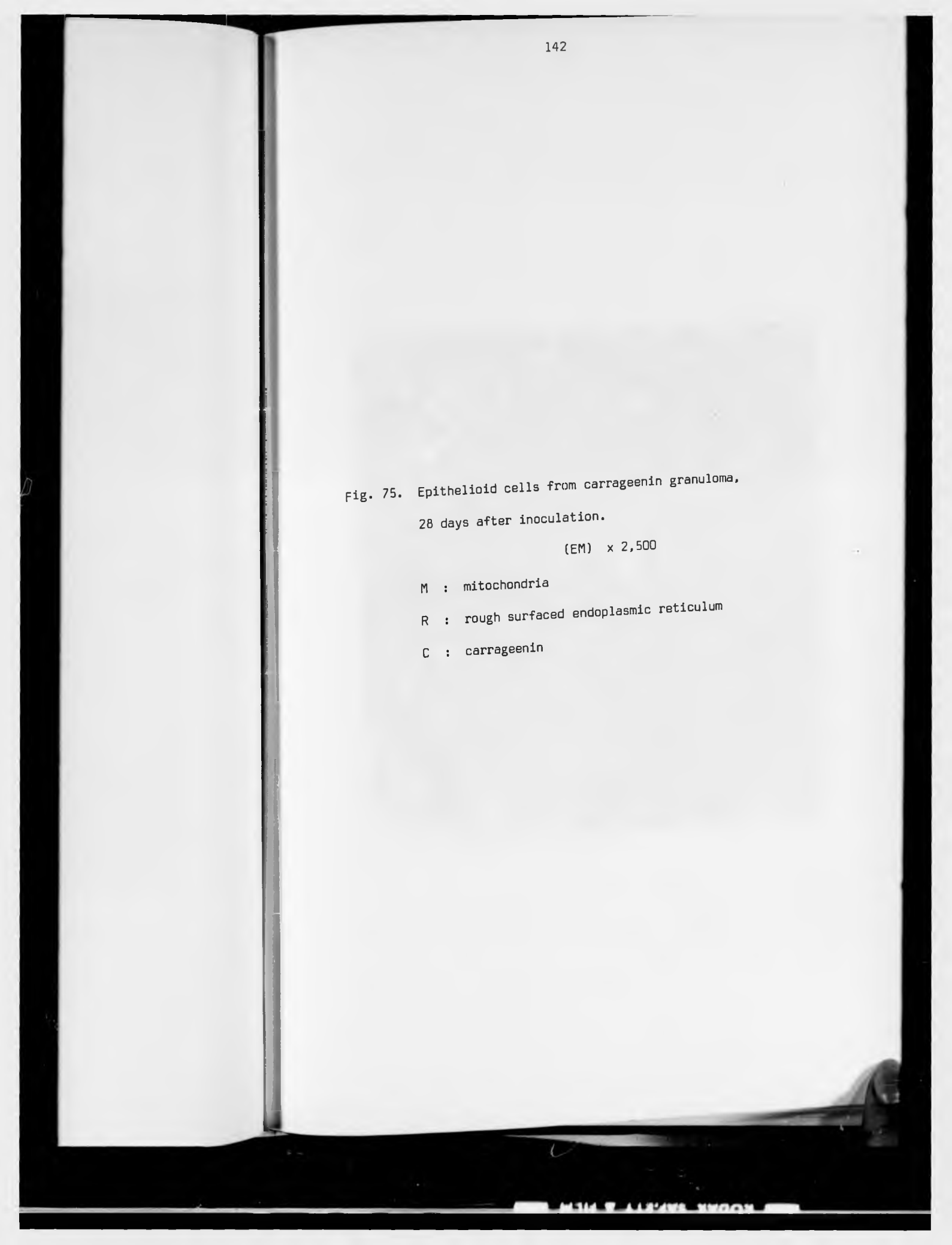


Fig. 75. Epithelioid cells from carrageenin granuloma, 28 days after inoculation.

(EM) x 2,500

M : mitochondria

R : rough surfaced endoplasmic reticulum

C : carrageenin

Immuno-Serologic Studies.

Introduction:

In view of the appearance of significant numbers of lymphocytes and plasma cells in the granuloma, experiments were devised to determine the antigenicity of the carrageenin in terms of humoral antibody, and of cell mediated antibody.

Experiment V. Determination of the antigenicity of carrageenin by means of the double gel-diffusion method of Ouchterlony, in vitro.

In the baseline 10°C. experiment, cells resembling the plasma cell and its precursors were observed by means of the specific staining method for plasma cells of Unna-Pappenheim. This finding suggested that the fish were producing humoral antibody against carrageenin and an attempt to prove this was made with the double gel diffusion method of Ouchterlony.

Materials and Methods:-

The gel diffusion precipitation test was chosen for the demonstration of humoral antibody and was carried out as described by Russell(1974), who used the technique to demonstrate humoral antibody production in plaice infected with lymphocystis virus.

Pilot experiments were carried out to determine whether there was any activity in serum of carrageenin affected fish. The gels were developed at 4°C. (Roberts 1968; Russell 1974) and at room temperature (Harris 1973). At both temperatures the result was positive, but at room temperature the agar was prone to deterioration, and although it gave a very quick result, it was less definite and was not suitable for photography.

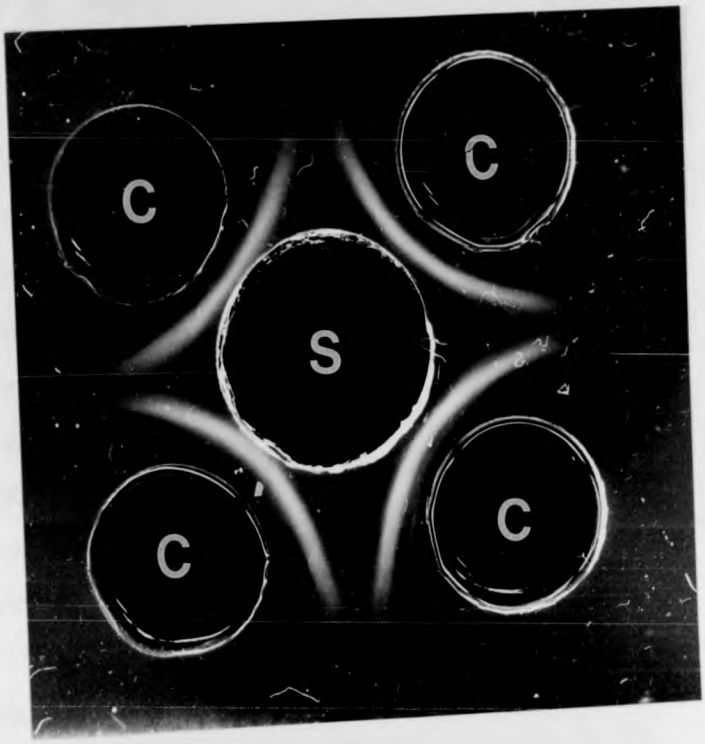
On the basis of the findings in the pilot experiments, the main experiments were carried out. Granulomata were initiated and after three weeks, serum samples taken. In order to show that the response was specific, control antigens of bovine serum and Freund's complete adjuvant, were used against the same sera and sera from two untreated plaice were exposed to the same antigens. The results were recorded photographically.

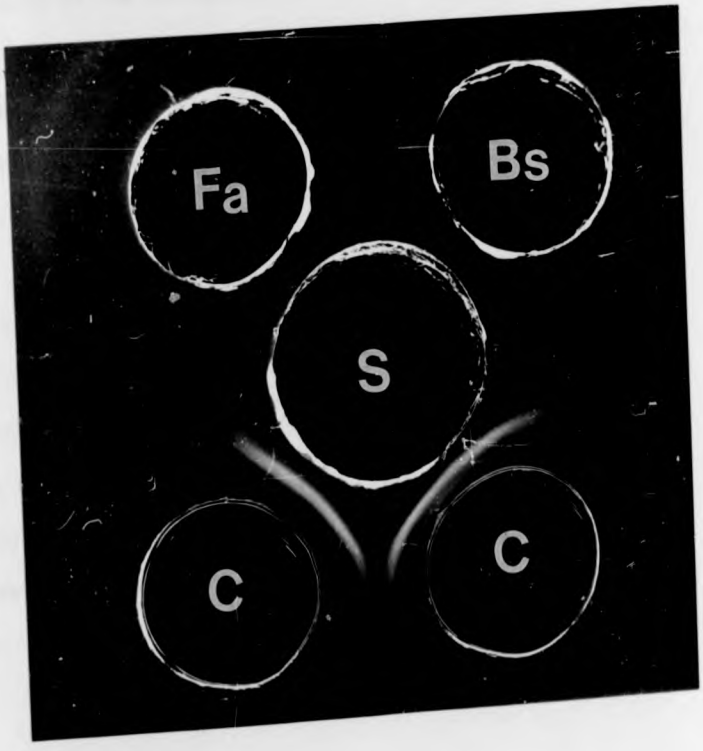
Result Observations:-

A specific single precipitation line was produced between the test sera and the carrageenin (Fig. 75). No lines were seen between the test serum and the other antigens (Fig. 76), and serum from the non-inoculated fish also failed to yield lines when tested. These results suggested that the inoculation of carrageenin into the teleost fish stimulated the production of a specific humoral antibody.

Fig. 75. Ouchterlony double diffusion test. Positive reaction indicated by distinct white precipitation lines to carrageenin. (C) carrageenin, (S) test sera.

Fig. 76. Ouchterlony double diffusion test. Positive reaction indicated by distinct white precipitation lines only to carrageenin (C). There is no reaction to the control antigens of bovine serum (Bs), or Freund's complete adjuvant (Fa).


C
C
S
C
C


Fa
Bs
S
C
C

Experiment VI. Determination of the presence of cell mediated immune lymphocytes in later stages of the carrageenin granuloma by the macrophage migration inhibition technique, *in vitro*.

At 10°C. lymphocytes play a part in the inflammatory response of the plaice to injected carrageenin from around the 15th day and the numbers seen in sections increase to a peak at 28th to 35th days. After this time their numbers, in common with other inflammatory cells, undergo a decline, although some long lived macrophages, replete with carrageenin, remain throughout the course of the experiments.

Accordingly, experiments were carried out to determine the immune status of the carrageenin granuloma in the plaice, with regard to cell mediated immunity, to allow an assessment as to whether such an immunological component could play a part in the fish granuloma as is putatively the case in granulomata of mammals.

Materials and Methods:-

The first approach to an *in vitro* model for cell mediated immunity was made by Rich and Lewis (1932), who observed that migration of cells from buffy coat preparations from hypersensitive animals did not occur in the presence of specific antigen. In subsequent years the technique was re-utilized and

described in detail by George and Vaughan (1962).

Prior to carrying out the main study, the suitability of the aposite mammalian techniques for use in fish systems had to be investigated and modifications as to suitable culture medium, source of white cells, temperature of incubation, etc. were made.

A number of small experiments showed that the culture medium of Wood (1973), which he had used for plaice tissue culture, a temperature of 20-22^{o}C. and buffy coat blood samples from the renal portal vein (Ferguson 1975) were suitable. Samples of blood from fish developing the granuloma after 10 days and 28 days, and from uninjected fish were used, and cell mediated immunity tests were carried out as described in detail and in the general materials and methods section.

Results Observations:-

In those tests where carrageenin antigen was added to a test involving sensitized 28th day blood cells, there was no migration of leukocytes out into the surrounding medium (Fig. 77), they merely formed a cap of buffy coat at the tip of the capillary tube. In the tubes unexposed to antigen and the tubes containing blood from fish inoculated less than 28 days previously, whether exposed to antigen or not, there was rapid migration of the buffy coat cells out into the culture medium .

(Fig. 78). These results indicated that there was inhibition of macrophage migration in the presence of specific antigen in those individuals inoculated sufficiently long to allow production of sensitized lymphocytes which were capable of releasing migration inhibition factor when they encountered their specific antigen.

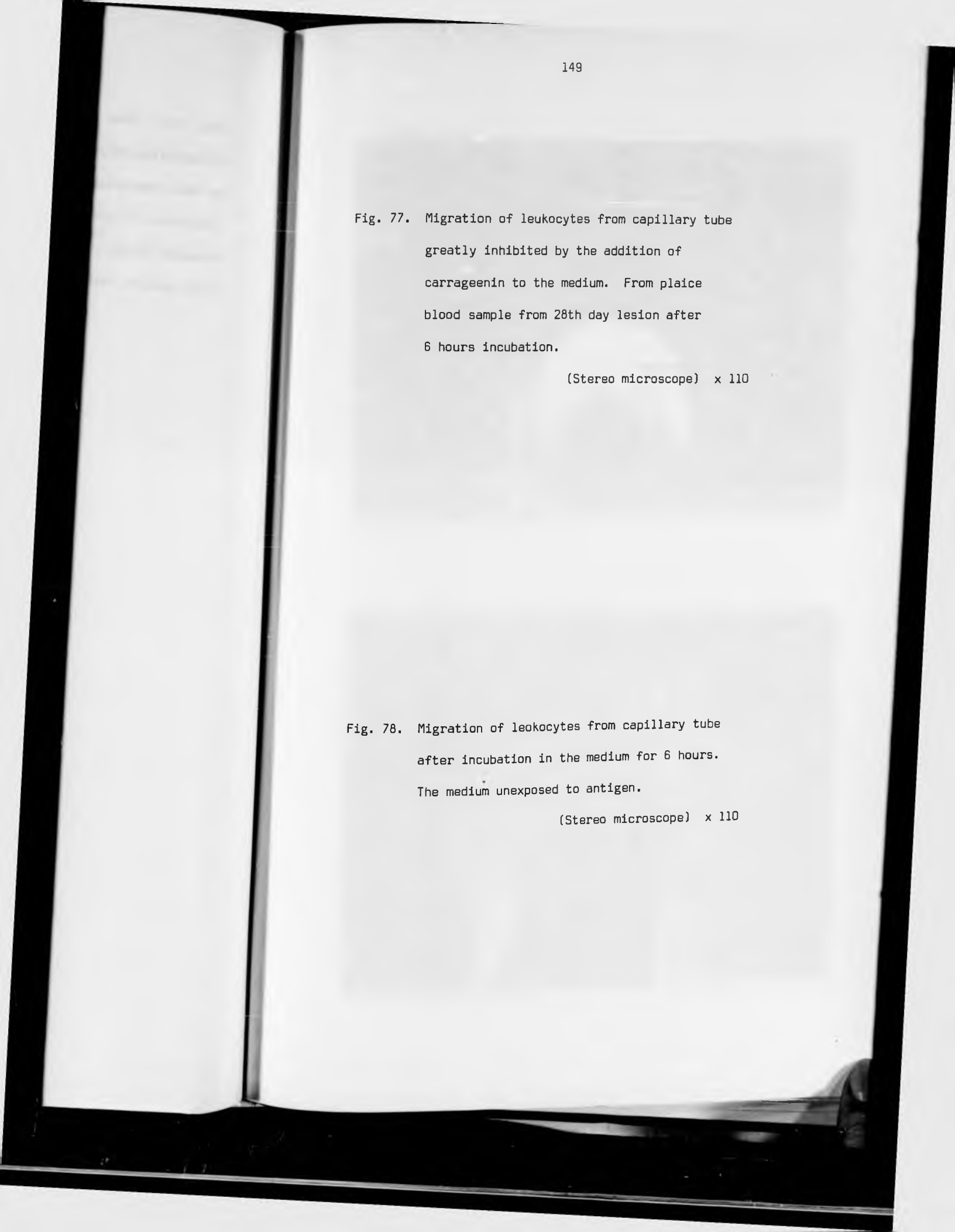
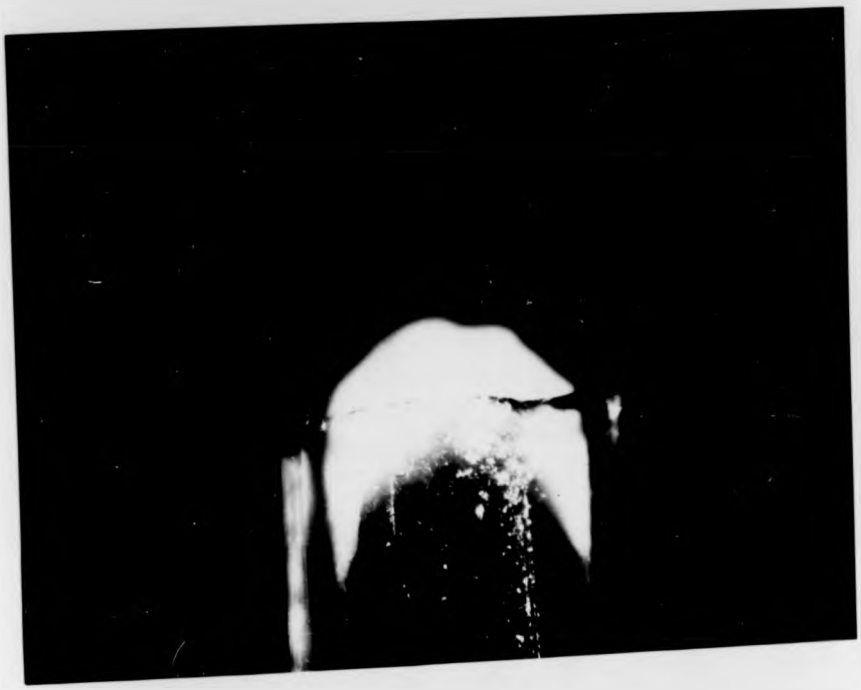


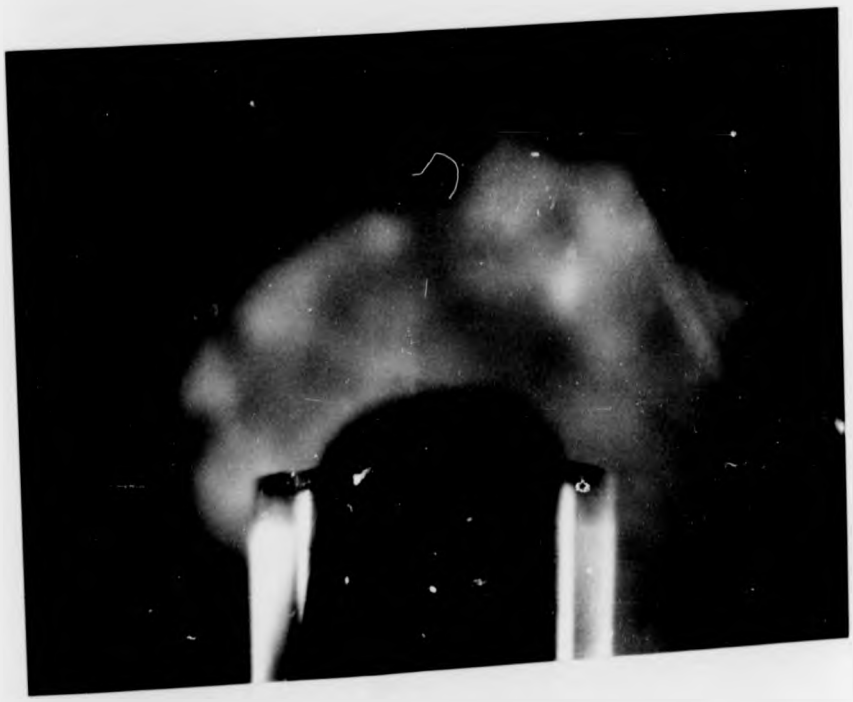
Fig. 77. Migration of leukocytes from capillary tube greatly inhibited by the addition of carrageenin to the medium. From plaice blood sample from 28th day lesion after 6 hours incubation.

(Stereo microscope) x 110

Fig. 78. Migration of leokocytes from capillary tube after incubation in the medium for 6 hours. The medium unexposed to antigen.

(Stereo microscope) x 110





SECTION D

DISCUSSION

The present study has shown that carrageenin is a potent stimulant of the chronic inflammatory response of teleost fish, and that a feature of that inflammation is the marked production of collagen fibres.

Although in mammals there is slight variation between species, the carrageenin granuloma is a relatively consistent lesion. In the guinea pig and in the rat, the mature granuloma is composed of polymorphonuclear leukocytes, macrophages, lymphocytes, fibroblasts (Williams 1957; Benitz and Hall 1959; Gardner 1960) and epithelioid cells (McCandles and Lehoczky-Mona 1964; Perez-Tamayo 1970). In addition to these cells, multinucleated giant cells have been described in the rat (Monis et.al. 1968) and in the rabbit (Perez-Tamayo 1970) and plasma cells have been found in the rabbit (Johnston and McCandles 1968).

In the homeothermic vertebrates, the inflammatory response to carrageenin appears to be very active with considerable macrophage infiltration, and phagocytosis of the extracellular mucopolysaccharides within 24 hours of the initiation of the inflammation. In the present study in the plaice, the response was not so marked and the macrophages had certainly not taken up any mucopolysaccharides for some 5 days, although the extracellular mucopolysaccharides could be defined relatively

easily by the PAS., alcian green and alcian blue staining methods. Macrophages only commenced phagocytosis on the 5th day after inoculation, by which time the response consisted of heavy macrophage infiltration. Carrageenin could then be very positively demonstrated within the vacuoles of the phagocytic cells.

In the guinea pig, the extracellular mucopolysaccharides were all cleared by the 10th day (Williams 1957; Perez-Tamayo 1970), but in the rat this was delayed until the 28th day (Benitz and Hall 1959). However, in the plaice at 10°C., the phagocytosis of the extracellular mucopolysaccharides was not completed until the 42nd day after initiation of the granuloma. This supports the consensus view that in the teleost fish, phagocytosis by macrophages is very much slower than in the homeothermic vertebrates. Finn and Nielson (1971) compared the rainbow trout and the laboratory mouse directly with Fruend's adjuvant as the phlogiston and showed that the inflammatory response produced in the fish, when compared with a mammal, was slower and the numbers of inflammatory cells produced, fewer. Comparison of the results of the present study with the published results for mammalian carrageenin granulomata suggests that the same relationship as that found for Freund's adjuvant by Finn and Nielson (1971) exists.

In the homeothermic vertebrates, the granuloma had resolved by the 28th day, and scar tissue developed (Williams 1957). Fibro-

plasia therefore developed very early and the gap of the exudative area was very quickly filled with new connective tissue. However, in the poikilothermic plaice, whose mean environment temperature is between 8° and 13°C., the healing was delayed and continued beyond the 80th day after initiation of the granuloma. The fibroplasia commenced around the 10th day, and some collagen fibres were visible by the 14th day, but although carrageenin appeared to be a potent collagen stimulant, as in the homeotherm, the healing process was appreciably longer. This appeared to be due as much to the greater time taken to resolve the inflammatory debris and phagocytose the carrageenin as to slowness in fibroblastic development.

In the plaice at 10°C. the macrophage infiltration began to decrease by the 56th day. By this time these cells were foam-cell-like in appearance and were replete with carrageenin. Some degree of muscle fibre regeneration had also occurred, but this was not a significant contribution to the healing of the granuloma. Mawdesley-Thomas and Bucke (1973) described tissue repair in the goldfish, and failed to show regeneration of muscle fibres, but this may well have been because they did not keep the fish alive sufficiently long after initiation of the wound. A comparison of the fibroblastic response to carrageenin in the plaice to other chronic inflammatory responses was possible due to the parallel study of Timur, G. (1975), who studied giant cell production in plaice granulomata. The results of such a comparison

showed that carrageenin was a more active stimulant to fibroplasia and to macrophage infiltration, than Freund's adjuvant and the other agents used by that worker.

As with the other components of the granuloma, development of epithelioid cells and giant cells was also delayed when compared with the mammal. Both cells were delayed some 10 days, compared with the mammal. The type of giant cell seen was not defined in any studies of the mammalian carrageenin granuloma, but in this study both Langhans and foreign body types, with from 5 to 15 nuclei, were seen up to the 56th day.

Vascularisation is an important factor in allowing the nutrition of the newly developing granulation tissue in all types of inflammatory lesions. It is not surprising therefore that it was prominent and apparently played a significant role in the development of the new granulation tissue of the carrageenin granuloma of the plaice. However, it was again delayed when compared with that of the mammal, beginning around the 10th day and approaching its peak by the 28th to 35th days. The process of development, maturation and ultimate degeneration was, however, similar, with hypertrophy of the media of the walls of the arterioles and ultimately occlusion and resorption, as the cellularity and hence nutritional requirements of the mature lesion decreased.

In the present study there was only a minimal epithelial lesion since the epidermal involvement was merely a puncture wound and was thus much less severe than the lesion which developed in the muscular area, where the carrageenin was deposited. Thus little comparison can be made between this lesion and the relatively insignificant ones produced by superficial skin incisions by Anderson and Roberts (1975).

The effects of deficiency of vitamin-C on wound healing in fish are well documented (Halver et.al. 1962; Halver 1972) and appear to be directly related to fibroblast development and fibroplasia. In the experiments of the present study, the maintenance of fish bearing carrageenin granulomas on an excess of L-ascorbic acid did not affect the general feature of the granuloma, except with regard to fibroplasia, and as well as increasing the degree of fibroplasia, it appeared to stimulate the fibroplasia five days earlier than in the "standard" granuloma.

The relationship between the temperature and the rate of development of the sequential changes of the inflammatory response has been the subject of several recent studies (Finn and Nielson 1971; McQueen et.al. 1973; Roberts et. al. 1973b; Anderson and Roberts 1975). All were agreed that the net effect was an approximate 50% reduction in rate of development for a net drop of temperature of 10^{o}C., but none of these studies examined the long term temperature effects on a major lesion.

The nearest studies to these criteria were those of Roberts and his colleagues, who studied the long term effects of temperature on the rate of healing of natural and surgically produced ulceration in salmon.

The present study, of a more severe and long-lasting lesion, showed a similar temperature response to that found by the other workers. The particularly significant feature of the carrageenin lesion was the fibroplastic activity and this was especially delayed by the reduction of temperature to $5^{o}C$. In addition, there was a less intensive inflammatory cell response, but one which was much longer lasting. This coincided with the much longer period of myophagic activity before the removal of necrotic sarcoplasmic debris. The only other long term study to look specifically at fibroplasia in relation to temperature was also carried out in the plaice (McQueen et.al. 1973) and was the study of the inflammatory reaction to the penetration of Cryptocotyle lingua, a digenean cercaria, over the range $5^{o}C$. to $15^{o}C$. The host response to this penetration is ultimately the development of a fibrous capsule, and this was again specifically remarked upon by the authors as being one of the main features to be dominated by temperature. Fibroplasia is a production process involving major protein metabolic pathways and it is possible that this metabolic requirement which is temperature controlled, is greater than that required for other aspects of the inflammation.

The effect of the cellular components of the inflammation caused by the temperature reduction in the present study, extended the findings of the other workers to the effect of temperature on epithelioid and giant cell development. The cellular feature of greatest disparity from the standard granuloma at 10°C. was the coexistence of epithelioid cells and active myophagic activity. At the higher temperature, the myophagia was complete before significant epithelioid cell development took place, whereas both were seen side by side from the 42nd to the 56th days at 5°C. The polymorphonuclear leukocyte, whose function in fish inflammation still remains somewhat enigmatic (Klontz 1972), was also prominent for a very much greater period at low temperature (35 days as opposed to 5 days at the higher temperature). This feature was also noted by Anderson and Roberts (1975), but in view of the lack of information available on the function of the polymorphonuclear leukocyte in fishes, it is not possible to hazard a suggestion as to the possible explanation for this.

The morphology of the cells involved in the carrageenin granuloma in plaice was very similar to that of their counterpart cells in higher animals, both at the light microscopic and the ultrastructural levels. An exception was the polymorphonuclear leukocyte. Ellis (1974) and Ferguson (1975) have described the structure of the polymorphonuclear leukocytes in the circulating blood of the plaice, and in the carrageenin granuloma it was very similar to their descriptions.

The effect of temperature on the production of antibody by fish has been well documented, with the best response being obtained at temperatures towards the upper limits of the temperature range over which a given species of fish can be maintained (Bisset 1946; Avtalion et.al. 1974; Ellis 1974). Although the present study was in no way intended to investigate this phenomenon, it was of possible significance to it that plasma cells, the antibody producing cells of the piokilothermic vertebrate, were first seen in any numbers at the 10th day at 10°C. Whereas they were not seen until 18-21 days at the lower temperature and never appeared in any numbers.

The antigenicity of carrageenin has received scant attention in the higher animals, and only Johnston and McCandles (1968) have given direct proof of its antigenicity by showing that it could stimulate the production of specific prepipitins in the rabbit, Gardner (1960) having previously expressed the view that carrageenin was not antigenic. Certainly the findings of the present study both in terms of plasma cell stimulation and precipitation production, support the contention of Johnston and McCandles (1968), and extend the range of carrageenin antigenicity to the lower vertebrates.

Antigenicity, especially in terms of a granuloma inducing agent, must not be considered in terms of serum antibody stimulation alone, since it is now considered (La Via and Hill 1971; Boros

and Warren 1973) that cell mediated immunity plays a considerable part in the pathogenesis of tuberculosis and many other granulomatous lesions, and the sequential histopathological features of the lesion were very similar to those of tuberculosis or skin graft rejection which are considered to be mediated via the delayed type hypersensitivity reaction (Cappell and Anderson 1971).

The positive results of the migration inhibition test, which is used as an indicator of the presence of sensitized, so-called T-lymphocytes, are the first demonstration in teleost fish, that cell-mediated-immunity plays a role in the poikilothermic granuloma. It is still a subject of speculation in higher animals (Epstein et.al. 1962; Spector 1967; Boros and Warren 1973), whether lymphokines from stimulated lymphocytes are responsible for the transformation of macrophages within an inflammatory area into epithelioid cells, and later, giant cells, but the present study certainly provides circumstantial morphological evidence which adds to the hypothesis.

The particular feature of the ultrastructure of the macrophage which takes part in the carrageenin granuloma in mammals is the absence of lysosomes (Papadimitriou and Spector 1971). The mechanism for this is not known, but it was also a feature of the plaice granuloma. The monocytes seen in blood vessels in the area and migrating into the lesion had them but no cell containing carrageenin in its cytoplasm had any evidence of a lysosomal complement.

During the present study many minor differences between the inflammatory process in the plaice compared with the higher animals have been noted, and the main dominating factor in the fish's poikilothermic existence, the effect of temperature on all aspects of its metabolism, has been shown to affect similarly its inflammatory processes, but the most striking feature of the entire study has been the remarkable similarity between the pathogenesis of the chronic granuloma in this fish and in the highest of vertebrates, suggesting that the inflammatory response is a process of considerable evolutionary antiquity in the development of vertebrates and has been modified to only a limited degree during that time.

SECTION E

CONCLUSIONS

1. The carrageenin granuloma in the plaice was a very suitable model for the study of the chronic inflammatory response of teleost fish.

2. The carrageenin granuloma of the plaice is morphologically very similar to that of higher animals.

3. The component cells of the inflammatory lesion, i.e. the polymorphonuclear leukocyte, the macrophage, the lymphocyte, the plasma cell, the epithelioid cell and the giant cell, all had the same histochemical, and ultrastructural features as their homologues in the carrageenin granuloma in higher animals.

4. The rate of development of the lesion was much slower than in mammals, even at the relatively advanced temperature of $10^{o}C$. Phagocytosis of the extracellular polysaccharides was slow but the macrophages which did phagocytose it lost their lysosomal configuration. This ultrastructural feature had also been reported in the higher animals.

5. The injected carrageenin was a good stimulant to the development of fibrous tissue but this feature was even more marked when the affected fish were kept on high levels of vitamin-C prior to and during the induction of the granuloma.

6. At low temperature the rate of development of the lesion was markedly reduced and several of the phases of development overlapped considerably, e.g. polymorphonuclear leukocytes were present for 35 days compared with 5 days at 10°C., and epithelioid cells were seen side by side.

7. In addition to being a potent stimulant to inflammation and fibroplasia, carrageenin was antigenic both for serum precipitin production and cell mediated immunity production at 10°C.

SECTION F

SUMMARY

The development of the carrageenin granuloma was studied in the plaice (Pleuronectes platessa.L.) at 10°C., as a model for the study of chronic inflammation in a poikilothermic vertebrate, and the results were compared with published histopathological studies on this granuloma in higher animals.

The effect of temperature reduction on the development of the plaice granuloma was studied in fish held at 5°C., and the effect of high level supplementation of L-ascorbic acid (vitamin-C) was also defined.

The microscopical, ultrastructural and histochemical features of the lesion at 10°C. were found to be similar to the lesion in higher animals except that they had a considerably longer time scale.

Reduction of temperature to 5°C. resulted in even further expansion of the time scale for the development and resolution of the lesion, and several phases of development which were separate in the lesion at 10°C., overlapped at 5°C. In particular, the phase of debris removal was concomitant with the development of the epithelioid cell response, which were both completely separate at 10°C.

High levels of ascorbic acid did not affect the general features of the granuloma but fibroplasia occurred earlier than in the standard granuloma.

Carrageenin was found to be highly antigenic and produced a specific serum precipitating antibody and also specific sensitized lymphocytes for a cell mediated immune response.

.....

SECTION G

REFERENCES

Abraham,E P (1970) The antigen and antibody reaction ; in General pathology.Ed.Florey,H W Oxford University Press Oxford. 1259 pp.

Adami,J G (1909) Inflammation ; an introduction to the study of pathology. McMillan & Co Ltd, London. 356 pp.

Addison,W (1843) Experimental and practical researches on the structure and function of blood corpuscles ; on inflammation; and on the origin and nature of tubercles in the lung . Trans.prov.med.surg.Ass. 2 , 233-306

Amlacher,E (1970) Textbook of fish diseases . Trans.Conroy,D A and Herman,R L . TFH Publications, New York,USA. 302 pp.

Anderson,W (1967) Carrageenin : structure and biological activity. Can.J.pharm.Sci. 2 , 81-90

Anderson,C D and Roberts,R J (1975) A comparison of the effects of temperature on wound healing in a tropical and temperate teleost . J Fish.Biol. 7 , 173-182

Anderson,W and Soman,P D (1963) Degraded carrageenin and experimental acute gastric ulceration in the guinea pig . Nature (London) . 199 , 389

Anderson,W and Soman,P D (1967) Intravenous degraded carrageenin and histamine gastric ulceration in the guinea pig . Nature (London) . 214 , 823-824

Aranson,J D (1926) Spontaneous tuberculosis in saltwater fish. J.infect.Dis. 39 , 315-320

Ashhurst,D E (1968) Fibroblasts-vertebrate and invertebrate; in Cell structure and its interpretation. Ed.McGee,S M ; Russell F ; Ross,K A . Edward Arnold Ltd.London. 433 pp.

Abraham,E P (1970) The antigen and antibody reaction ; in General pathology.Ed.Florey,H W Oxford University Press Oxford. 1259 pp.

Adami,J G (1909) Inflammation ; an introduction to the study of pathology. McMillan & Co Ltd, London. 356 pp.

Addison,W (1843) Experimental and practical researches on the structure and function of blood corpuscles ; on inflammation; and on the origin and nature of tubercles in the lung . Trans.prov.med.surg.Ass. 2 , 233-306

Amlacher,E (1970) Textbook of fish diseases . Trans.Conroy,D A and Herman,R L . TFH Publications, New York,USA. 302 pp.

Anderson,W (1967) Carrageenin : structure and biological activity. Can.J.pharm.Sci. 2 , 81-90

Anderson,C D and Roberts,R J (1975) A comparison of the effects of temperature on wound healing in a tropical and temperate teleost . J Fish.Biol. 7 , 173-182

Anderson,W and Soman,P D (1963) Degraded carrageenin and experimental acute gastric ulceration in the guinea pig . Nature (London) . 199 , 389

Anderson,W and Soman,P D (1967) Intravenous degraded carrageenin and histamine gastric ulceration in the guinea pig . Nature (London) . 214 , 823-824

Aranson,J D (1926) Spontaneous tuberculosis in saltwater fish. J.infect.Dis. 39 , 315-320

Ashhurst,D E (1968) Fibroblasts-vertebrate and invertebrate; in Cell structure and its interpretation. Ed.McGee,S M ; Russell, F ; Ross,K A . Edward Arnold Ltd.London. 433 pp.

Avtalion,R R; Wojdani,A ; Malik,Z ; Shahrabani,R ; Duczyminer,M (1974) Influence of environmental temperature on the immune response in fish . Curr.Top.in Microb & Immunol.61,1-10

Bagenal,T B (1972) The observer's book of sea fishes . Frederick Warne & Co Ltd, London. 184 pp.

Benitz,K F and Hall,L M (1959) Local morphological response following a single subcutaneous injection of carrageenin in the rat . Proc.Soc.exp.Biol.med. 102 , 442-445

Berenson,G D S (1961) Conferance on the biology of connective tissue cells. Arthritis and Rheumatism Foundation Conference Series. 7 , 209-218

Bethlem,J (1970) Muscle pathology ; introduction an atlas . North-Holland Publishing Co.Amsterdam-London. 132 pp.

Bisset,K A (1946) The effect of temperature on non-specific infections of fish . J.Path.Bact. 58 , 251-267

Bloom,B R and Bennett,B (1970) Macrophages and delayed type hypersensitivity . Seminars in haematology. 7 ,215-224

Boros,D L and Warren,K S (1973) The bentonite granuloma : charecterization of a model system for infectious and foreign body granulomatous inflammation using soluble mycobacterial , Histoplasma and Schistosoma antigens . Immun. 24 , 511-529

Boyd,W (1943) A textbook of pathology : an introduction to medicine . Henry Kimpton, London.1043 pp.

Bullock,G L (1971) Diseases of fish . TFH Publications , Jersey City , USA. 2 , 41 pp.

Burnet,M (1970) The origin of the plasma cells in cellular immunology . Cambridge University Press , Cambridge.725pp.

Burns,R and Beighle,H (1960) Experimental carrageenin granuloma of the cornea . Arch.Ophthal. (Chicago) . 64 , 712-719

Cappell,D F and Anderson,J R (1971) Inflammation ; in Muir's textbook of pathology . Edward Arnold Ltd. London. 976pp.

Carr,I (1973) The macrophage : a review of ultrastructure and function . Academic Press, London. 154 pp.

Carter,R L and Roberts,J D B (1971) Macrophages and multi-nucleated giant cells in nitrosoquinoline-induced granuloma in rats : an autoradiographic study . J.Path. 105 , 285-288

Catton,W T (1951) Blood cell formation in certain teleost fishes . Blood. 6 , 39-60

Chapman,J A (1961) Morphological and chemical studies of collagen formation I. The fine structure of guinea pig granulomata . J.biophys.biochem.Cytol. 9 ,639-651

Chapman,J A (1962) Fibroblasts and collagen. Br.med.Bull. 18 , 233-237

Chvapil,M and Cmuchalova,B (1961) Dynamics of changes in connective tissue components during development of carrageenin granuloma . Exp.med.surg. 19 , 171-182

Clark,E R and Clark,E L (1930a) Observations on the macrophages of living amphibia larvae . Am.J.Anat. 46 , 91-148

Clark.E R and Clark,E L (1930b) Relation of monocytes of the blood to the tissue macrophages . Am.J.Anat. 46 ,149-186

Clawson,C C; Finstad,J ; Good,R A (1966) Evolution of the immune response V. Electron microscopy of plasma cells and lymphoid tissue of the paddlefish . Lab.Invest. 115 , 1830-1847

Clem,W ; Moewus,L ; Sigel,M (1961) Studies with cells from marine fish in tissue culture . Proc.Soc.exp.Biol,(New York). 108 , 762-766

Cliff,W J (1963) Observation on healing tissue : a combined light and electron microscopic investigation . Phil.Trans.R.Soc.(London). 246 , 305-325

Cliff,W J (1966) The behaviour of macrophages labelled with collaidal carbon during wound healing in rabbit ear chambers. Quart.J.exp.Physiol. 51 , 112-119

Cohn,Z (1965) Phagocytosis ; in The inflammatory process . Ed.Zweifach,B W ; Grant,L ; McClusky,R T . Academic Press, London. 931 pp.

Crowle,A J (1962) Delayed hypersensitivity in health and diseases . Charles C Thomas, Springfield,Illinois. 131 pp.

Culling,R F A (1963) Handbook of histopathological techniques. Butterworths,London. 712 pp.

Cushing,J E (1969) Immunology of fish ; in Fish physiology . Academic Press.Ed.Hoar,W S ; Randall,D J. 4 , 532 pp.

Drury,R A B and Wallington,E A (1967) Carleton's histological technique. Oxford University Press, Oxford. 432 pp.

Dumont,A and Sheldon,H (1965) Changes in the fine structure of macrophages in experimentally produced tuberculosis granulomas in hamsters . Lab.Invest. 14 , 2034-2055

Dunbar,C E and Herman,R L (1971) Visceral granuloma in brook trout (Salvelinus fontinalis.L.) .Comp.Path.Bull. (Washington). 6 , 4

Dunphy,J E ; Udupa,K N ; Edwards,L G (1956) Wound healing : new pespective with particular reference to ascorbic acid deficiency . Ann.Surg. 144 , 304-317

Ebert,R H (1965) The experimental approach to inflammation ; in The inflammatory process . Ed.Zweifach,B W ; Grant,L ; McClusky,R T . Academic Press, New York. 931 pp.

Ebert,R H and Florey,H W (1939) The extravascular development of monocytes observed in vivo . Br.J.Exp.Path.20,342-356

Edwards,D J (1971) Effect of temperature on rate of passage of food through the alimentary canal of the plaice(Pleuronectes platessa.L.) . J.Fish.Biol. 3 , 433-439

Ellis,A E (1974) Aspects of the lymphoid and reticulo-endothelial systems in the plaice (Pleuronectes platessa.L.) . Ph.D Thesis, University of Aberdeen, Scotland. 224 pp.

Epstein,W L (1967) Granulomatous hypersensitivity . Prog.Allergy. 11 , 36-88

Epstein,W L ; Shaken,J R ; Krasnobrod,H (1962) Granulomatous hypersensitivity to zirconium : localisation of allergen in tissue and its role in formation of epithelioid cells . J.invest.Derm. 38 , 223-229

Epstein,W L ; Shaken,H ; Krasnobrod,H (1963) The organized epithelioid cell granuloma : differentiation of allergic (zirconium) from collaidal (silica) types. Am.J.Path. 43 , 391-405

Epstein,W L and Krasnobrod,H (1968) The origin of epithelioid cells in experimental granulomas of man. Lab.Invest.18,190-195

Evans,H M (1915) The macrophages of mammals .Am.J.Physiol. 37 , 243-258

Ezzat,A A ; Shabana,M B ; Farghaly,A M (1974) Studies on the blood characteristics of Tilapia zilli (Gervais) . J.Fish.Biol. 6 , 1-12

Federko,M E and Hirsch.J E (1970) Structure of monocytes and macrophages . Seminers in haematology . 7 ,109-124

Ferguson,H W (1975) The reticulo-endothelial system of teleost fish with special reference to the plaice (Pleuronectes-platessa.L.) . Ph.D Thesis , University of Stirling,Scotland. 115 pp.

Finerty,J C and Cowdry,E V (1962) A textbook of of histology . Lea & Febier , Philadelphia , USA .573 pp.

Finn,J P and Nielson,N O (1971a) The effect of temperature variation on the inflammatory response of rainbow trout . J.Path.105 ,257-268

Finn,J P and Nielson,N O (1971b) Inflammatory response in rainbow trout .J.Fish.Biol. 3 ,463-478

Forbes,W D (1955) Granulomatous inflammation : a clinical and pathologic challenge . Am.J.Clin.Path. 25 ,427-428

Forkner,C E (1930) The origin and fate of two types of multinucleated giant cells in the circulating blood . J.exp.Med. 52 , 279-286

Gardner,D L (1960) Production of arthritis in the rabbit by local injection of the mucopolysaccharide carrageenin . Ann.rheum.Dis. 19 , 369-376

George,M and Vaughan,J H (1962) In vitro cell migration as a model for delayed hypersensitivity .Proc.Soc.exp.Biol.Med. 21 ,514-521

Glynn,L E (1968) The chronicity of inflammation and its significance in rheumatoid arthritis . Ann.rheum.Dis.27,105

Gongolli,S D ; Wright,M G ; Grasso,P (1973) Identification of carrageenin in mammalian tissues : an analytical and histochemical study . Histochem.J. 5 , 37-48

Goodale,F ; Fillmore ,R ; Hillman,E (1962) Electron microscopic observations of human leukocytes I. Response in vitro to bacterial endotoxin .Exp.Molecular.Path. 1 ,229-250

Gordon,G B and King,D W (1960) Phagocytosis .Am.J.Path.37,279-292

Gresham,G A (1971) Responses to cellular damage : a colour atlas of general pathology . Wolfe Publishing Ltd ,London.365 pp.

Gresham,G A and Jennings,A R (1962) Nutritional disease ,vitamin-C; in An introduction to comparative pathology.Academic Press , London and New York . 412 pp.

Hadfield,G(1951) Granulation tissue .Ann.R.Coll.Surg.Engl.9,397-407

Halver,J E (1968) Vitamin requirements ; in Fish in research . Ed. Halver,J E . Academic Press ,London and New York. 713 pp.

Halver,J E ; Ashley,L M ; Smith,R R (1969) Ascorbic acid requirements of coho salmon and rainbow trout . Am.Fish.Soc. 98, 762-771

Ham,A W (1969) Histology , sixth edition , Pitman Medical Publishing Co Ltd , London . 1037 pp.

Harper,H A (1971) Review of physiological chemistry . Blackwell Scientific Publications , Oxford . 529 pp.

Harris,J E (1973) The immune response of (Leuciscus leuciscus) to injected antigenic material . J.Fish.Biol.15 ,261-276

Haythorn,S R (1929) General review : multinucleated giant cells. Arch.Path. 7 , 651-713

Henrikson,R C and Matoltsy,A G (1968) The fine structure of teleost epidermis I. Intruduction and filament cells . J.Ulstruc.Res. 21 , 194-212

Hirsch,J G (1965) Neutrophil and eosinophil leukocytes ; in The inflammatory process . Ed. Zweifach,B W ; Grant,L ; McClusky,R T . Academic Press, New York. 931 pp.

Hirsch,J G ; Federko,M E ; Dwyer,C M (1967) The ultrastructure of epithelioid and giant cells in positive Kveim test sites and sarcoid granulomata . La Sorcoidose Rapports , IV. Conference Internationale , Paris . 59-70

Hodgkiss,W and Shewan,J M (1950) Pseudomonas infection in a plaice . J.Path.Bact. 62 , 655-657

Jackson,D S (1957) Connective tissue growth stimulated by carrageenin I.The formation and removal of collagen . Biochem.J. 65 , 277-284

Jacques,L B (1962) Progress in medical chemistry. Ed.Ellis,G P and West,G B . Butterworth, London. 1, 262 pp.

Jakowska,S and Nigrelli,R F (1953) Localised responses in fish to experimental inflammation caused by pathogenic bacteria. Anat.Rec. 117, 526

Janssen, C W and Waaler, E (1967) Body temperature, antibody formation and inflammatory response. Acta.Path. et. Microbiol. Scand. 69, 557-566

Johnston, K H and McCandles, E L (1968) The immunologic response of rabbits to carrageenins, sulfated galactans extracted from marine algae. J.Immun. U.S.A. 101, 556-562

Joy, J E and Jones, L P (1973) Observations on the inflammatory response within the dermis of a white bass Morone chrysops (Rafinesque) infected with Larnea cruciata (Copepode: Caliqidea). J. Fish.Biol. 5, 21-23

Keele; C A, Neil, E; Jepson, J B (1966) Vitamin-C Samson Wright's applied physiology. Eleventh edition. Oxford Medical Publications,Oxford.576 pp.

Kitamura, S; Ohara, S; Suwa, T; Natagawa, K (1965) Studies on vitamin requirements of rainbow trout (Salmo gairdnerii. L) I. On the ascorbic acid. Bull.Jap.Soc.scient.Fish. 31, 818-826.

Klontz, G W (1972) Haematological studies and the immune response in rainbow trout. Symp.zool.Soc.(London) 30, 89-99.

Kluge, J (1965) A granulomatous disease of fish produced by Flavobacteria. Path.Vet. 2, 545-552

Knox, W E and Goswami, M N D (1961) Ascorbic acid in man and animals. Adv.Clin.Chem. 4, 122-187

Lagler, K F; Bardach, J E; Miller, R R (1962) Ichthyology: the study of fishes. John Wiley Inc. New York and London. 545pp.

LaVia, M F (1971) Immunologic injury; in Principles of pathology. Ed. LaVia, M F and Hill, R B. Oxford Medical Publications, Oxford. 281pp.

LaVia, M F and Hill, R B (1971) Principles of pathology. Oxford Medical Publications, Oxford. 281 pp.

Lever, W F (1967) Histopathology of the skin. Pitman Medical. London. 794 pp.

Lewis, M R and Lewis, W H (1926) The transormation of mononuclear blood cells into macrophages, epithelioid cells and giant cells in hanging-drop blood cultures from lower vertebrates. Contr.Embryol. Carneg. Instn. (Washington). 18, 95-120

Lewis, W H and Lewis, M R (1925) The transformation of leukocytes into macrophages, epithelioid cells and giant cells in cultures of pure blood. Am.J.Physiol. 72, 196

Lewis, M R; Willis, H S; Lewis, H W (1925) The epithelioid cells of tuberculosis lesion. Bull.Johns.Hopkins.Hosp. 36, 175-184

Love, R M (1970) Connective tissue; in The chemical biology of fishes. Academic Press, London. 547pp

Mallory, F B (1929) The principles of pathologic histology. Part I. Saunders W B, Philadelphia. 576pp

Marchesi, V T (1961) The site of leukocyte emigration during inflammation. Quart.J.exp.Physiol.Cognate. med.Sci. 46, 115-118

Marchesi, V T and Florey, H W (1960) Electron microscopic observations on the emigration of leukocytes. Quart.J.exp.Physiol. Cognate.med.Sci. 45, 343-347

Marshall, N B (1972) The early life of marine fishes; in The life of fishes. Weidenfeld & Nicolson, London. 402 pp.

Mawdesley-Thomas, L E and Bucke, D (1973) Tissue repair in a poikilothermic vertebrate Carassius auratus (L): a preliminary study. J.Fish.Biol. 5, 115-120

Maximow A A (1925) Role of the non-granular blood leukocytes in the formation of the tubercle. J.infect.Dis. 37, 418-429

McCandles, E L and Lehockzy-Mona, J (1964) Identification of active fraction of carrageenin in stimulation of connective tissue growth. Growth, 28 143-156.

McCarthy, D H; Stevenson, J P; Roberts, M S (1973) Some blood parameters of the rainbow trout (Salmo gairdnerii Richardson) I. The kaploops variety. J.Fish.Biol. 5, 1-8

McDonald, G A; Dodds, T C; Cruickshank, B (1970). Atlas of haematology. Third ed. E & S Livingstone Ltd, Edinburgh and London, 212pp

MacKanes, G B and Blandes, R V (1967) Cellular immunity. Progr.Allergy. Karger, Basle, New York, 11 89-140

McLaren, A; Keller, E; O'Donnell, D J; Elvehjem, C A (1947) The nutrition of rainbow trout. I. Studies of vitamin requirements. Arch.Biol.Chem. Biophys. 15, 169-185

McMinn, R M H and Pritchard, J J (1969) Connective tissue reactions; in Tissue repair. Ed.McMinn, R M H, Academic Press, New York and London. 432pp

McQueen, A; McKenzie, K; Roberts, R J (1973) Studies on the skin of plaice (Pleuronectes platessa L). III. The effect of temperature on the inflammatory response to the metacercariae of Cryptocotyle linguea. J.Fish.Biol. 5, 241-248

Menner, G (1971) Cell division and collagen synthesis in cultured fibroblast. Exp.Cell.Res: 65, 49-60

Menkin, V (1930) Studies on inflammation. J.exp.med.Rockefeller Institute, 52, 201-213

Metchnikoff, E (1891) Lectures on the comparative pathology of inflammation. Translated by Starling F A and Starling E H. Dover Publications Inc. New York, U.S.A. 218 pp

Metchnikoff E (1905) Resorption of the formed elements in immunity; in Immunity in infective diseases. Cambridge University Press republished by Johnson Reprint Corporation New York No. 61. 136pp.

Mettler, C C (1947) History of Medicine. Blackiston, New York, U.S.A. 1215 pp.

Milne, J A (1972) An introduction to the diagnostic histopathology of the skin. Edwards Arnold Ltd., London. 363pp.

Monis, B; Weinberg, T; Spector, G J (1968) The carrageenin granuloma in the rat: a model for the study of the structure and function of macrophages. Br.J.exp.Path. 49, 302-310

Montgomery, H (1967) Granulomas; in Dermatopathology. Harper and Row, New York, 650pp

Morehead, R P (1965) The inflammatory process; in Human pathology an introduction to medicine. McGraw-Hill, New York, 1676 pp.

Morley, J; Wolstencroft, R A; Dumonde, D C (1973) The measurement of lymphokines; in Handbook of experimental immunology. Ed.Weir D M, Blackwell, Oxford, 2, 1245pp

Movat, H Z and Fernando, N V P (1962a) The fine structure of connective tissue. II. The plasma cell. Exp.Molecular.Path. 1 , 535-553

Movat, H Z and Fernando N V P (1962b) The fine structure of connective tissue. I. fibroblast. Exp.Molecular Path. 1, 509-534.

Newton, L (1962) Chondrus crispus (carrageenin); in A handbook of British Seaweeds. British Museum London. 478 pp.

Nigrelli; R F and Vogel H (1963) Spontaneous tuberculosis in fish and in other cold-blooded vertebrates with special reference to Mycobacterium fortuitum Cruz from fish and human lesions. zoologica 48, 131-144.

Ouchterlony, O (1947). Antigen antibody reaction in gel. Acta.Pathol.Microbiol. Scand. 26, 507-512

Ogilvie, R F (1967) Inflammation and repair; in Histopatho logy. Sixth edition, E & S Livingstone Ltd, Edinburgh. 518 pp.

Papadimitriou, J M and Spector, W G (1971) The origin, properties and fate of epithelioid cells. J.Path.105, 187-203.

Paul, J (1961) Information from connective tissue cell cultures. Arthritis and Rheumatism Foundation Conference Series. 7, 52-59.

Paul, J (1970) Cell and tissue culture. Fourth ed. E & S Livingstone, Edinburgh, London. 403 pp.

Perez-Tamayo, R (1970a) Collagen resorption in carrageenin granulomas. I. Collagenolytic activity in vitro explants. Lab.Invest.22, 137-141

Perez-Tamayo, R (1970b) Collagen resorption in carrageenin granuloma. II. Ultrastructure of collagen resorption. Lab.Invest. 22. 142-159.

Poole, J C F & Florey H W (1970) Chronic inflammation and tuberculosis; in General pathology. Ed.Florey, H W Oxford University Press, Oxford. 1259 pp.

Putt, F A and Hukill, P B (1962) Alcian green. Arch.Path. 74, 169-170

Rich, A R and Lewis, M R (1932) The nature of allergy in tuberculosis as revealed by tissue culture studies. Bull.Johns. Hopkins.Hosp. 50, 115-131

Richardson, I D (1972) Rearing of marine fish-problems of husbandry, nutrition and disease. Sym.zool.Soc. (London) 30, 341-357

Roberts, R J (1968) A study of Corynebacterium pyogenes Ph.D Thesis, University of Glasgow 203pp.

Roberts, R J (1974) The effects of temperature on disease and their histopathological manifestations in fish; in The pathology of fishes. Ed.Ribelin, W E; Migaki, G. The University of Wisconsin Press.1004 pp.

Roberts, R J; McQueen, A; Shearer, W M; Young, H (1973a) The histology of salmon tagging. I. The tagging lesion in newly tagged parr. J.Fish.Biol. 5, 497-504

Roberts, R J; McQueen, A; Shearer, W M; Young H (1973b) The histopathology of salmon tagging II. The chronic tagging lesion in returning adult fish. J.Fish.Biol. 5, 615-620

Roberts, R J and Shepherd, C J (1975) Handbook of trout and salmon diseases. Fishing News (Books) Ltd. Surrey 168 pp.

Roberts, R J; Young, H; Milne, J A (1971) Studies on the skin of plaice (pleuronectes platessa.L) I. The structure and ultrastructure of normal plaice skin. J.Fish.Biol. 4, 87-98

Robertson, W and Schwartz, B V (1953) Ascorbic acid and the formation of collagen. J.Biol.Chem. 201, 689-696

Roitt, I (1972) Hypersensitivity; in Essential immunology. Blackwell Scientific Publications, Oxford, Edinburgh, London. 220 pp

Ross, R and Benditt, E P (1965) Wound healing and collagen formation. V. Quantitative electron microscope radioautographic observations of proline-H3 utilization by fibroblasts. J.Cell.Biol. 27, 83-106

Russell, P H (1974) Lymphocystis in wild plaice (pleuronectes platessa.L) and flounder (Platictithus flesus.L) in British coastal waters: a histopathological and serological study. J.Fish.Biol. 6, 771-778

Ryan, G B and Hurley, J V (1968) The drug inhibition of increased vascular permeability. J.Path.Bact. 9, 371-379

Ryan, G B and Spector, W G (1969) Natural selection of long-lived macrophages in experimental granulomata. J.Path.99, 139

Schneberger, E (1941) Fish research in Wisconsin. Prog.Fish. Cult. 56, 14-17

Schoenberg, M D and Moore, R D (1958) Studies on connective tissue cells. Arch.Path.65, 115-124

Shelbourne, J E (1964) The artificial propagation of marine fish; in Advances in marine biology. Ed.Russell, F S. Academic Press, London. 2, 274pp

Slack, H G B (1956) The metabolism of sulfated polysaccha sides in carrageenin-induced granuloma. Biochem.J. 64, 7

Smith, D B and Cook, W H (1953) Fraction of carrageenin. Arch.Biochem. 45, 232-233

Smith, D B; Cook, W H; Neal, J L (1954) Physiological studies on carrageenin and carrageenin fractions. Arch.Biochem.Biophys. 53, 192-204

Sniezko, S F (1972) Progress in fish pathology in this century; in Diseases of fish. Ed.Mawdesley-Thomas, L E. Academic Press, New York and London.
Symp.zool.Soc. (London) 30, 1-15

Spector, W G (1967) Histology of allergic inflammation. Br.med.Bull. 23, 35-38

Spector, W G (1969) The granulomatous inflammatory exudate Int.Rev.exp.Path. 8, 1-55

Spector, W G (1970) The macrophage in inflammation. Serology in haematology. 3, 132-144

Spector, W G and Ryan, G B (1969) New evidence for the existence of long-lived macrophages. Nature (London) 221, 860

Spector, W G; Walters, M N I; Willoughby, D A (1965) The origin of the mononuclear cells in inflammatory exudates induced by fibrinogen. J.Path.Bact. 90, 181-192

Spector, W G and Willoughby, D A (1968) The origin of mononuclear cells in chronic inflammation and tuberculin reactions in the rat. J.Path.Bact. 96, 389-399

Sutherland, P L (1922) A tuberculosis-like disease in a saltwater fish (Halibut) associated with the presence of an acid fast tubercle like bacillus. J.Path.Bact. 25,31-35

Sutton, J S and Weiss, L (1966) Transformation of monocytes in tissue culture into macrophages, epithelioid cells and multinucleated giant cells. J.Cell.Biol. 28, 303-330

Steele, R H and Wilhelm, D M (1967) The inflammatory reaction in chemical injury I. Increased vascular permeability and erythema induced by various chemicals. Br.J.exp.Path. 58, 592-607

Steele, R H and Wilhelm, D M (1970) The inflammation in chemical injury. III. Leukocytosis and other histological changes induced by superficial injury. Br.J.exp.Path.51, 265-276

Timur, G (1975) A study of giant cells in inflammatory lesions of the plaice (Pleuronectes platessa.L) Ph.D. Thesis. University of Stirling. Scotland. 144 pp.

Uhr. J W; Salvin, S B; Pappenheimer. A M (1955) Delayed hypersensitivity. II. Induction of hypersensitivity in guinea pigs by means of antigen-antibody complexes. J.exp.Med. 105, 11-24

van Furth, R (1970a) Origin and kinetics of monocytes and macrophages. Seminars in haematology. 7, 125-132

van Furth, R (1970b) The origin and turnover of promonocytes, monocytes and macrophages in normal mice; in Mononuclear phagocytes. Ed.vanFurth, R. Blackwell Scientific Publications, Oxford and Edinburgh. 654 pp.

Walter, J B and Israel, M S (1970) Chronic inflammation; in General pathology. J and A Churchill, London 1116 pp.

Wanstrup, J and Christensen, H E (1966) Sarcoidosis. I. Ultra-structural investigation on epithelioid cell granulomas. Acta-Path.et.Microbiol-Scand. 66, 169-185

Ward, P A (1971) Inflammation; in Principles of pathology. Ed. Lavia and Hill, R. Oxford University Press, Oxford. 281 pp.

Wardle, C S (1971) New observations on the lymph system of the plaice (Pleuronectes platessa.L.) and other teleosts. J.mar.biol.Ass. U.K. 51, 977-990

Watanable, I; Donahue, S; Hoggatt, A (1967) Method for electron microscepic studies of circulating human leukocytes and observations on their fine structure J.ulstruc.Res. 20, 366-382

Watt, J; Eagleton, G B; Marcus, R (1966) Effect of degraded carrageenin as gastric secretion stimulated by histamine and histology. Nature (London) 211, 989

Williams, G (1957) A histological study of the connective tissue reaction to carrageenin. J.Path.Bact. 73 557-563

Willis, R A (1950) Repair of an open wound or ulcer by granulation, or healing by second intention; in The principles of pathology. Butterworths, London. 667 pp.

Wimpenny, R S (1953) The plaice. Edward Arnold, London. 145 pp.

Wolbach, S B and Howe P R (1926) Intercellular substances in experimental scorbutus. Am.med.Ass.Arch.Path. 1 1-24

Wolke, R E and Trainer, F R (1971) Granulomatory enteritis in Catostomus commersoni associated with diatoms. J.Wildl.Dis. 7, 76-79

Wood, P (1973) Fish tissue culture, methods, problem and costs. M.Sc. Thesis, University of Stirling, Scotland 59 pp.

Wood, E M; Yasutake, W T; Lehman, W I (1956) A mycosis-like granuloma of fish. J.infect.Dis. 97, 262-267

Wood, E M,and Ysutake, W T (1956) Histopathology of fish. IV A granuloma of brook trout. Prog.Fish.Cult. 18 108-112

Wright C S and Dodd, M C (1955) Phagocytosis. Ann.Acad.Sci. (N.Y.) 59, 945-950

Wright, G P (1958) An introduction to pathology. Longmans Green and Co. Ltd. London. 635 pp.

Zweifach, B W (1965) Microvascular aspects of tissue injury; in The inflammatory process. Ed.Zweifach, B W; Grant L; McClusky, R T. Academic Press, London. 931 pp.

Fig. 75. Ouchterlony double diffusion test. Positive reaction indicated by distinct white precipitation lines to carrageenin. (C) carrageenin, (S) test sera.

Fig. 76. Ouchterlony double diffusion test. Positive reaction indicated by distinct white precipitation lines only to carrageenin (C). There is no reaction to the control antigens of bovine serum (Bs), or Freund's complete adjuvant (Fa).

Experiment VI. Determination of the presence of cell mediated immune lymphocytes in later stages of the carrageenin granuloma by the macrophage migration inhibition technique, *in vitro*.

At 10°C. lymphocytes play a part in the inflammatory response of the plaice to injected carrageenin from around the 15th day and the numbers seen in sections increase to a peak at 28th to 35th days. After this time their numbers, in common with other inflammatory cells, undergo a decline, although some long lived macrophages, replete with carrageenin, remain throughout the course of the experiments.

Accordingly, experiments were carried out to determine the immune status of the carrageenin granuloma in the plaice, with regard to cell mediated immunity, to allow an assessment as to whether such an immunological component could play a part in the fish granuloma as is putatively the case in granulomata of mammals.

Materials and Methods:-

The first approach to an *in vitro* model for cell mediated immunity was made by Rich and Lewis (1932), who observed that migration of cells from buffy coat preparations from hypersensitive animals did not occur in the presence of specific antigen. In subsequent years the technique was re-utilized and

described in detail by George and Vaughan (1962).

Prior to carrying out the main study, the suitability of the aposite mammalian techniques for use in fish systems had to be investigated and modifications as to suitable culture medium, source of white cells, temperature of incubation, etc. were made.

A number of small experiments showed that the culture medium of Wood (1973), which he had used for plaice tissue culture, a temperature of 20-22^{o}C. and buffy coat blood samples from the renal portal vein (Ferguson 1975) were suitable. Samples of blood from fish developing the granuloma after 10 days and 28 days, and from uninjected fish were used, and cell mediated immunity tests were carried out as described in detail and in the general materials and methods section.

Results Observations:-

In those tests where carrageenin antigen was added to a test involving sensitized 28th day blood cells, there was no migration of leukocytes out into the surrounding medium (Fig. 77), they merely formed a cap of buffy coat at the tip of the capillary tube. In the tubes unexposed to antigen and the tubes containing blood from fish inoculated less than 28 days previously, whether exposed to antigen or not, there was rapid migration of the buffy coat cells out into the culture medium .

(Fig. 78). These results indicated that there was inhibition of macrophage migration in the presence of specific antigen in those individuals inoculated sufficiently long to allow production of sensitized lymphocytes which were capable of releasing migration inhibition factor when they encountered their specific antigen.