STUDY OF NATURAL AND INDUCED TISSUE RESPONSE IN *MACROBRACHIUM ROSENBERGII* DE MAN

by

Gary L. Nash D.V.M. M.Sc. M.R.C.V.S

A thesis submitted for the Degree of Doctor of Philosophy

Institute of Aquaculture
University of Stirling
Stirling, Scotland

July 1990

DEDICATION

To my parents

DECLARATION

I declare that this dissertation has been composed by myself and that it embodies the results of my own research. It has neither been accepted nor is being submitted for any other degree. Where appropriate I have acknowledged the nature and extent of work carried out in collaboration with others which is included in the thesis.

Gary L Nash

Gary L. Nash

7/91

ABSTRACT

The tissue response of the giant freshwater prawn, *Macrobrachium rosenbergii* de Man, was studied histologically by investigating clinical cases of natural infectious and non - infectious diseases which occurred in cultured prawns and by carrying out the following experiments: intramuscular injection of alcian blue dye, intramuscular injection of BCG vaccine (also using TEM), intramuscular injection of complete Freund's adjuvant, intramuscular and systemic injection of heat-killed *Aeromonas hydrophila*, intramuscular and systemic injection of heat-killed and live *Vibrio parahaemolyticus* and *Vibrio anguillarum*, grafting or insertion of pieces of cuticle into the muscle of the same or different prawns and by cuticular traumatization or wounding of slight, moderate and severe degrees (also using SEM). Results demonstrated histologically the presence of a unified system of haemocytic cells participating in a range of defensive activities in varying degrees of intensity: viz. infiltration, aggregation, coagulation, melanization, phagocytosis, encapsulation, nodule formation and fibroplasia. More pronounced haemocytic activity occurred in the gills, hepatopancreas, heart and antennal gland, in addition to locally at the site of injection, wounding or grafting.

Electron microscopy revealed that the haemocytes which participate in the tissue inflammatory response consist of cell types belonging to all three main classes, namely agranular, semi-granular and granular haemocytes, although semi-granular and granular haemocytes appeared to predominate.

Findings of the experimentally induced tissue response were comparable to observations in the natural infectious and non-infectious disease conditions in *Macrobrachium* and in general, similar to those reported in natural infections and experimental challenges of other important wild and cultured crustaceans including penaeid shrimps, crabs and crayfish.

A routine study of the haemocytic tissue response in cultured prawns can be recommended as a useful means of monitoring their health status and identifying the early stages of a disease process. This can prove of assistance in developing disease control strategies.

ACKNOWLEDGMENTS

I am very grateful to Professor R. J. Roberts and Dr. R.H. Richards of the University of Stirling for their supervision of my dissertation and to other University of Stirling staff for their technical assistance: Mairi Beveridge and Paul Thomson for all my histological processing, Stuart Millar and Andrea Bundy for assistance with microbiology, Ron Stewart and Lewis Taylor for black and white photographic processing, Billy Struthers for assistance with scanning electron microscopy and Marilyn Nash for transmission electron micrographic processing for the clinical studies and for typing the first draft. I also thank Chantana Kongkaew of the Department of Anatomy, Faculty of Veterinary Medicine, Kasetsart University, Thailand for transmission electron microscopic processing for the experimental study. I am also indebted to Drs. M. Shariff, Ang Kok Jee and Ian Anderson and to Rosli Aslim and Saim Abu for providing facilities and for their assistance and hospitality during the course of my work at Universiti Pertanian Malaysia in Malaysia. Appreciation is also expressed to Drs. Kamonporn Tonguthai and Supranee Chinabut of National Inland Fisheries Institute, Bangkok, Thailand for providing facilities for photomicrography done in Thailand. This work was partly funded by EEC Science and Technology Development Tropical Agriculture grant TSDA 200 UK(H).

TABLE OF CONTENTS

pages

pages

pages

pages

LIST OF TABLES

pages

LIST OF FIGURES

pages

pages

pages

pages

pages

pages

pages

pages

pages

pages

pages

pages

pages

FORWARD

Like other arthropods, crustacea have an open vascular system which is more susceptible than that of vertebrates to any intrusions from the surrounding environment (Bauchau 1981). Haemocytes, the blood cells in the circulatory fluid, haemolymph, have been shown to play an important role in wound repair and defence mechanisms in arthropods and in a variety of crustaceans (Sindermann 1971, Bauchau 1981, Sparks 1985). Few basic studies, though, have been done of the giant freshwater prawn, *Macrobrachium rosenbergii* de Man. There is limited information available concerning the tissue changes associated with both natural disease and experimentally induced tissue insults in *Macrobrachium*. This knowledge is extremely important so that the pathogenesis, pathology and associated host tissue defence response can be understood in the context of various aetiologies. An understanding of these processes may also provide information useful for disease control strategies. The following investigations were initiated under the auspices of the EEC Prawn Project, the original terms of reference of which were to explore both the basic tissue responses to injury and carry out disease investigations in the field. *M. rosenbergii* was a good choice for a number of reasons. It is one of the most important cultured crustaceans throughout the tropics in its own right. With the growth of culture there has been a concomitant increase in disease problems. In addition it

has been shown in the course of these investigations to be, in general, an excellent model for other commercially important decapod crustaceans such as penaeids, and the relative ease of culture of the ongrowing stages in freshwater under unnatural conditions make it a good experimental animal.

The aims of this study are to define and compare the basic tissue responses of *Macrobrachium* to a range of biotic and abiotic insults, both experimentally induced and which occur in relation to diseases under typical culture conditions.

CHAPTER I: The Internal Defence System and the Diseases of Prawns

I. Introduction and Review of the Literature

General Introduction

The study of the pathology and diseases of the giant Malaysian freshwater prawn, *Macrobrachium rosenbergii*, is inextricably bound to the development of its culture. Over the past 25 or more years culture has moved from experimental to commercial production in numerous countries throughout the world. Prior to the establishment of commercial aquaculture, disease problems remained practically non-existent. As we have now reached the noon of commercial aquaculture, the dawn of culture-related disease problems has emerged. Under the artificial conditions created by aquaculture, the important role of environmental and other stressors in the inter-relationship between the pathogen and the host's immune response in the induction of disease cannot be underestimated (viz. stressors lead to host stress and increased susceptibility to the pathogen and disease). The culture of *Macrobrachium sp.* illustrates a case in point, as current major disease problems are apparently primarily related to commensal facultative opportunistic organisms, rather than obligate primary pathogens, which under imperfect and stressful environmental conditions are able to overcome the host defence mechanisms.

Before an examination of specific disease problems of *Macrobrachium sp.*, a description of the basic internal defence system of decapod crustaceans is

indicated in order to understand its vital role in the host's resistance.

I.1 The Basic Defence System of Crustaceans

The Epicuticle

The epicuticle forms the first defensive barrier - a mechanical barrier - against any potential pathogen present in the rich and varied microbial soup of a pond or tank environment, and thus, its integrity is of primary importance in the crustaceans' disease resistance (Johnson 1980). It is composed of lipids in the epicuticular outer layer (outer epicuticle) and proteins in the inner layer (inner epicuticle), as well as calcium. Phenolic tanning and melanization vary according to location. The epithelial cells secrete the cuticle, which is replaced at each moult. Any damage to, or abnormality of the epicuticle predisposes the cuticle and deeper tissues to subsequent invasion by micro-organisms (Brock 1983). The crucial barrier in the generally resistant crayfish is apparently the lipid epicuticle or the outer layers of the soft cuticle (Söderhäll & Unestam 1975). After each moult the cuticle undergoes hardening by phenolic tanning, a polyphenoloxidase located in the outermost layer (Dennell 1947) making it more rigid and resistant to infection (Nyhlén & Unestam 1980).

The Tegmental Glands

The tegmental glands originate from the ectodermis and are found in the cuticle between the

epidermis and the collagenous connective tissue layer (or within it), and in the base, stem and apex of the gills. The viscous, mucoid secretions of the glands are thought to be related to tanning and epicuticle production in forming the mechanical barrier against invasion by micro-organisms; they also agglutinate red blood cells and algae (Johnson 1980).

The tegmental glands of crustaceans may be functionally similar to the hypodermal glands of the horseshoe crab, *Limulus polyphemus*, a non-crustacean primitive marine arthropod. Glycoprotein exudates of the hypodermal glands secreted through the canals into the carapace serve as a mechanical barrier to pathogens due to viscosity and agglutinating properties. Like *Limulus* hetero-agglutinins, the mucosubstance immobilizes bacteria by binding flagella or rendering them inactive (Stagner & Redmond 1975).

The Branchial Podocytes

In addition to the important role played by the branchial haemocytes in cellular aggregation and the clearance of foreign particles such as bacteria, large vacuolar cells, known as branchial podocytes (nephrocytes, branchial excretory cells or arthrocytes) of the so-called branchial kidney or branchial gland, which are usually located in the efferent channels of the stems of the crab (the exact location is species-variable), are considered to participate in clearance of the gills, as well as ultrafiltration, excretion and

osmoregulation (Johnson 1980; Smith & Ratcliffe 1981). Smith and Ratcliffe (1981) demonstrated that the branchial podocytes, although not involved in the direct phagocytosis or removal of bacteria, were active in the removal of the haemocytes in response to the presence of bacteria and the accumulation of this haemocyte-derived debris in the large central vacuoles of the podocytes. They proposed that the podocytes augment the cellular immune reaction, not by phagocytosing the intact bacteria, but by removing much of the cellular debris which would otherwise occlude the lamellar sinuses of the gills and impair respiration.

I.2 The Internal Defence System of Crustaceans

Haemocyte Types, Morphology and Function

The blood cells of crustaceans present in the haemolymph are known as haemocytes (synonyms: amoebocytes, leukocytes, lymphoid cells). In addition to participating in manifold physiological activities, such as glucose and glycogen storage and metabolism (Johnston, Elder & Davies 1973; Williams & Lutz 1975 a,b; Bauchau 1981) and chitin synthesis and storage (Johnston & Davies 1972; Johnston *et al.*1973), they are also involved in haemocyanin synthesis (Stang-Voss 1971; Bauchau 1981), fabrication and resorption of the exoskeleton in ecdysis and in the pre-ecdysial growth of autotomized legs (Bauchau & Plaquet 1973), haemostasis - both cellular through aggregation, coagulin and fibrinogen secretion, and extracellular, through plasma clotting (Bang 1970;

Bauchau & de Brouwer 1972; Bauchau & Mengeot 1978; Johnson 1980). Haemocytes play a vital role in the cellular and humoral inflammatory reaction in response to physical injury and infectious disease (Bang 1970; Sindermann 1971; Johnson 1980).

There have been numerous investigations into haemocyte morphology in various crustacean species in order to establish a uniform system of classification (Johnson 1980). The first detailed description of crustacean haemocytes was presented by Haeckel (1857) (cited by Johnson 1980). Since then, though no definitive system has been constituted, some consensus has been reached. The granules within the cytoplasm of haemocytes are usually used as the primary criterion for classification: their presence or absence, their number, size, distribution, shape, staining reaction, refractile nature and ultrastructure; also used is nuclear/ cytoplasmic ratio.

Three major, basic types or forms of haemocytes have been identified in the haemolymph based on the absence or presence and relative size of granules which is considered to be the simplest and most consistent classification presently available (Martin & Graves 1985):

(1) cells with no cytoplasmic granules: <u>hyaline cells</u> (non-granular cells, non-granular haemocytes, hyalocytes).

(2) cells with a few or a variable number of small

cytoplasmic granules: semi-granulocytes (semi-granulated cells, semi-granular cells, intermediate cells, intermediate haemocytes, intermediate granulocytes).
(3) cells filled with many and/or large cytoplasmic granules: granulocytes (granular haemocytes, granular cells, eosinophilic granulocytes).
(Rabin 1970; Bauchau & de Brouwer 1972; Bodammer 1978; Bauchau & Mengeot 1978; Johnson 1980; Mix & Sparks 1980 a,b; Bauchau 1981). Bauchau (1981) reviewed the basic and a number of proposed alternate nomenclatures. Some investigators have recognised subtle cytological differences and further subdivided the classes. Thus, hyaline cells in the American lobster, *Homarus americanus*, have been subdivided into prohyalocytes and hyalocytes; and granular cells, into eosinophilic (early and late forms) and chromophobic granulocytes, whose granules do not resemble those of the hyalocyte or eosinophilic granulocyte morphologically or cytochemically (Cornick & Stewart 1978). In an earlier study, Hearing and Vernick (1967) investigated lobster haemocytes and identified eosinophilic granules. Stang-Voss (1971) recognized one cell type in *Astacus astacus* (L.), having three functional forms: the clotting cell, the phagocyte and the granular amoebocyte. Also emphasizing functional significance, as well as morphology, Hoarau (1976) described four haemocyte forms, three with variable granules and a fourth phagocytic type with few granules, but rich in rough endoplasmic reticulum and containing phagosomes. Additional rare

forms are also recognized: the lipoprotein haemocyte, found only in mature females undergoing vitellogenesis, possibly involved in reproduction and also epicuticle secretion (Bauchau & de Brouwer 1972) (also reviewed by Bauchau & Mengeot 1978; Bauchau 1981). Another form of cell, which lyses quickly upon withdrawing the haemolymph, is known as the "explosive corpuscle"; it is probably an effete form of a granulocyte or semi-granulocyte after granule extrusion (degranulation). It may represent the "clotting cell" described by Stang-Voss (1971) or perhaps be a different stage in various decapods and having no particular function (Johnson 1980). On the other hand, vacuoles have been identified which form to replace the space previously occupied by exploding granules in phagocytosing haemocytes, suggesting that they become agranular due to disappearance of their granules, a possible step in releasing hydrolytic enzymes from the granules to assist in breakdown of phagocytosed particles (Paterson & Stewart 1974).

It is the studies of prawn and shrimp haemocytes which are of most interest in this review. In cytological (using phase-contrast and Nomarski-interference optics) and cytochemical *in vitro* studies of preparations of live *Macrobrachium rosenbergii* haemocytes Blewett & Eble (1979) delineated three cytotypes -all containing varying numbers and distribution of four granule types (A,B,C and D):

Cytotype I, comprising 5.5% of the cell population, is not a true hyalocyte but contains the fewest number of granules, is quite motile and possibly represents the most immature stage;

Cytotypes II and III, comprising 70% of the cell population, contain more numerous granules and represent more mature cells. They considered it likely that only one basic cell type existed with the three cytotypes being different stages of that cell line. Degranulated cells ("explosive corpuscles") were also recognized and exhibited marked peripheral chromatin clumping and highly vacuolated cytoplasm. Acid phosphatase -and NADH dehydrogenase-positive centres were identified in haemocytes and assigned to granule types.

Ultrastructurally, Tsing, Arcier and Brehélin (1989) determined that *Penaeus japonicus, Penaeus monodon, Macrobrachium rosenbergii* and *Palaemon adspersus* had two to three main haemocytic cell types in the general circulation. All crustaceans possessed haemocytes with small granules and haemocytes with large granules. In addition, haemocytes with a low level of differentiation were present only in *P. japonicus* and *P. monodon*. The haemocytes with large granules were of two different subtypes in *P. monodon* and *M. rosenbergii*. In *P. monodon* some cells had large and dense inclusions. These variations were considered to be two physiological forms of the same cell type. Also in *P. adspersus* some of the haemocytes with small granules had vesicles which fused in large vacuoles. Another cell type, haemocytes

after natural lysis, appeared only at the level of an injury or at blood removal, but not in the circulation. The term "hyaline haemocyte" was recommended for use in histological rather than ultrastructural studies.

Martin & Graves (1985) examined by light and electron microscopy the structure of haemocytes from two species of penaeid shrimp, *Sicyonia ingentis* and *Penaeus californiensis*. Considering presence or absence and relative size of granules they identified agranular haemocytes (lacking cytoplasmic granules and composing 5-10% of the circulating haemocytes), small-granule haemocytes (with a variable number of small granules and the most abundant haemocyte - 75% of the cell population) and large-granule haemocytes (filled with large granules and composing 10-20% of the haemocytes). In cytochemical studies of the penaeid shrimp, *Sicyonia ingentis*, using specific stains for lysosomes, cytoplasmic contents and granule enzymes, agranular haemocytes and a subgroup of small granule haemocytes were found to contain extensive cytoplasmic glycoprotein deposits of coagulogen. No lysosomes were observed in agranular cells or were present rarely in small granule haemocytes with glycoprotein deposits. Numerous lysosomes with acid phosphatase, B-glucuronidase and nonspecific esterase activity were demonstrated in small granule haemocytes without glycoprotein deposits and in large granule haemocytes. Acid phosphatase was found in the Golgi body, small vesicles and small granules of the latter two cells. Prophenoloxidase activity was found to be

localized only in small granule haemocytes without cytoplasmic deposits and large granule haemocytes (Hose, Martin, Nguyen, Lucas & Rosenstein 1987). Based on results of morphological (Martin, Hose & Kim 1987) and cytochemical studies, Hose *et al.* (1987) divided shrimp haemocytes into two lines, the deposit line (composed of agranular and striated granule haemocytes) and the granulocyte line (comprising small and large granule haemocytes).

Martin *et al.* (1987) used light microscopy, methylene blue staining and transmission electron microscopy (TEM) to study the haematopoietic nodules in the ridgeback prawn, *Sicyonia ingentis*. With the former two methods, they identified four types of haemocytes: agranular, small granule, metachromatic (with intermediate stages between these two latter types) and large granule. With TEM they also identified four types of haemocytes: agranular, large granule (with three granular morphologies), small granule with cytoplasmic deposits (which may differ from agranular haemocytes only by the presence of one granule) and small granule without cytoplasmic deposits. The metachromatic haemocytes identified by light microscopy corresponded to small granule haemocytes with cytoplasmic deposits in TEM.

In general these cells are the same as circulating haemocytes described by Martin & Graves (1985). However, circulating haemocytes have ovoid to spherical shapes and cells in the walls of haematopoietic tubules have elongate and often irregular shapes.

TEM reveals that cells classified as agranular cells by light microscopy frequently contain one or two small granules; thus agranular cells can only be accurately classified by TEM. Cell division has only been observed in agranular and small granule haemocytes that lack deposits; also there is a greater proportion of agranular haemocytes in haematopoietic nodules than in the haemolymph, suggesting that agranular cells can mature after release to the haemolymph. The findings suggest that agranular cells mature into small granular cells with cytoplasmic deposits; also that small granule haemocytes mature to large granule cells within the haematopoietic nodules. Intermediate stages were observed between agranular haemocytes and small granule haemocytes with deposits and between small granule haemocytes without deposits and large granule haemocytes, suggesting the existence of two distinct haemocyte lines (Martin *et al.* 1987).

In the final evaluation of the morphological (cytological, histological or ultrastructural) similarities and variations of the haemocytes, researchers generally support Cuénot's (1891) original theory, agreeing that there is one basic blood cell type in the haemolymph and that the multiplicity of forms of the circulating haemocytes represent various maturation stages in a sequential developmental series of a single cell line (Stang-Voss 1971 ; Bauchau & de Brouwer 1972; Hoarau 1976; Bauchau & Mengeot 1978; Bodammer 1978; Blewett & Eble 1979; Johnson 1980; Mix & Sparks 1980

a,b). The three main haemocyte types apparently form a continuous differentiation, and may occur both in the haematopoietic tissues and in the general circulation (Bauchau 1981). The hyaline cells are considered to be the stem cells - they also originate and are discharged from the haematopoietic tissues (Bauchau 1981) - and the (eosinophilic) granulocytes the fully mature cells, the semi-granulocytes including partially differentiated cells (Cornick & Stewart 1978; Mix & Sparks 1980 a,b; Bauchau 1981).

The Role of Haemocytes in the Internal Defence System

In injury and in the defence against invading micro-organisms, haemocytes contribute to both the cellular and humoral immune response. These defence mechanisms are non-specific (Cornick & Stewart 1968). The haemocytic cellular defence mechanisms consist of haemocytosis, haemocytic infiltration, aggregation, coagulation, phagocytosis, encapsulation, nodule formation and melanization of the area of tissue damage (Sindermann 1971; Bauchau & Mengeot 1978; Johnson 1980; Bang 1983).

Coagulation

In direct haemocytic agglutination or coagulation involving cell aggregation, the formation of cellular clots which close wounds, hyalocytes and semi-granulocytes are the first and most active participants, although granulocytes also take part. Haemocytes provide a clotting factor, the enzyme, coagulin, which acts upon

another haemocyte component, plasma fibrinogen, in extracellular clotting or plasma gelation (Bauchau & de Brouwer 1974; Bauchau & Mengeot 1978). Extracellular clots can inhibit the movement of microbes and make them more susceptible to phagocytosis (Sindermann 1971).

In the American lobster, *Homarus americanus*, Hearing and Vernick (1967) concluded that the secretory products of granules are released from the basophilic granular haemocytes, initiating coagulation in other cells. Bauchau and de Brouwer (1974) also implicated the released contents of vacuoles in agglutination and plasma gelation - which quickly followed agglutination or occurred simultaneously. A similar mechanism occurs in phagocytosis. Stang-Voss' (1971) "clotting cells" had numbers of granules and supposedly stored haemagglutinin. Mürer, Levin and Holme (1975) demonstrated that the haemocytic granules in the horseshoe crab, *Limulus polyphemus*, contain all the necessary factors for blood coagulation, including clottable protein. Stagner and Redmond (1975) demonstrated that the hetero-agglutinin of horseshoe crab haemocytes functions both as an agglutinin and as a bactericidin. That these granules function both in coagulation and immunity supports the statement made by Levin (cited by Taylor 1969), discussing invertebrates, "coagulation and antibacterial mechanisms seem clearly associated, and may be inseparable". Bauchau & de Brouwer (1974) proposed that the hyaline cells, semi-granulocytes (less so the granulocytes) come together by means of elongated pseudopodia. Plasma

gelation first occurs upon agglutination of contents released from vacuoles in hyalocytes and semi-granulocytes; and the process in which granulocytes are involved occurs later in a more sustained fashion leading to more extensive gelation.

Phagocytosis

Metchnikoff (1893) was the first to recognise the significance of vigorous, successful phagocytosis in the prevention of disease by invading pathogens. Phagocytosis has been deemed the most important response in disease resistance (Schapiro 1975). Sindermann (1971, 1979) summarizes the fundamentals of crustacean immunity in his statement "phagocytosis, augmented by humoral factors with low specificity, constitutes the basic mechanism of internal protection in the large Crustacea". Thus, the cellular immune response is based upon certain forms of haemocytes free in the haemolymph or fixed at the bases of the appendages, in the lacunae of the gills and in the pericardial sinuses (Sindermann 1971, 1979). The immune responses are actually complex mixtures of cellular and humoral elements (Sindermann 1971; Schapiro 1975). Both systems have been shown to lack the specificity comparable to that of vertebrate immunity (Schapiro 1975; McKay & Jenkin 1969). Paterson & Stewart (1974) and Paterson, Stewart & Zwicker (1976) demonstrated that lobster cellular factors, both hyaline and granular haemocytes, accounted for the major portion of the animal's ability to remove and neutralize foreign

particles, such as sheep erythrocytes *in vitro* and *Aerococcus viridans* var. *homari* (formerly *Gaffkya homari*) bacteria *in vitro* and *in vivo* (which was also corroborated by Cornick & Stewart (1968). In the crayfish, *in vitro* studies demonstrated active phagocytosis only involving hyaline haemocytes (McKay & Jenkin 1970a).

Phagocytosis of smaller particles involves primarily hyalocytes and semi-granulocytes. These react most readily, although granulocytes occasionally participate and produce a more sustained reaction. Species differences exist, as Paterson & Stewart (1974) observed in the lobster, that both hyaline cells and granulocytes participate in phagocytosis, but in the freshwater crayfish, McKay & Jenkin (1970a) observed phagocytosis only in hyalocytes. All three cell forms have been recognized as partaking in encapsulation, the formation of a limiting haemocytic capsule around larger foreign material such as parasites, entire bacterial colonies and necrotic cellular debris which may also contain phagocytosed bacteria (Sindermann 1971; Johnson 1980).

Before further examining the role of the haemocyte in the cellular response and inflammation, the humoral immune system will be described.

Humoral Immunity

The humoral contribution of haemocytes to defence consists of haemocyte cell membrane components

and secretions or products released into the haemolymph on cell disruption. These include: natural agglutinins -in the lobster (Cornick & Stewart 1968, 1973, 1978), which are non-inducible (Paterson et al. 1976) and located in or on the haemocytes (Cornick & Stewart 1973), and may represent recognition factors (opsonins), which enhance phagocytosis, present both in the haemolymph and bound to the plasma membrane of haemocytes (Tyson & Jenkin 1974). Non-inducible serum agglutinins are also present in low titres in Macrobrachium haemolymph (Huang, Eble & Hammen 1981). Agglutinins serve as opsonins enhancing phagocytosis in the crayfish (McKay & Jenkin 1970a) and in the lobster (Paterson & Stewart 1974) and are located in the cell membrane of all haemocyte types. As mentioned previously, Stagner & Redmond (1975) demonstrated that the hetero-agglutinin in *Limulus* functions both as an agglutinin and as a bactericidin. Hetero-agglutinins have also been identified in the spiny lobster, *Panulirus interruptus* (Schapiro 1975). Other humoral factors have been described. There are haemagglutinins which may serve as opsonins, essential for efficient phagocytosis, and have a specificity similar to iso-haemagglutinin proteins in vertebrate sera (McKay & Jenkin 1969, 1970a). They have been also identified in the shore crab, *Carcinus maenas* (Smith & Ratcliffe 1976, 1978) and are both natural and inducible in crabs (Schapiro 1975).

Reported also are cytolysins and haemolysins which are naturally occurring (Sindermann 1971). Natural haemolysins have been identified in the West Indian spiny

lobster, *Panulirus argus* (Schapiro 1975). Precipitins have been identified in lobster haemolymph (Stewart & Foley 1969) and proved to be non-adaptive (Rabin 1970). Bactericidins have been determined to be of variable specificity (Schapiro 1975) and inducible in the lobster (Stewart & Zwicker 1972; Paterson *et al.* 1976) and a product of a plasma-haemocyte interaction (Stewart & Zwicker 1972); they have also been identified in *Limulus polyphemus* (Smith & Pistole 1985). An inducible bactericidin has been found in the West Indian spiny lobster, California spiny lobster and American lobster (Schapiro 1975). Lectins (carbohydrate receptor-specific proteins) have been identified which bind to the lipopolysaccharides of Gram-negative bacteria and are required for optimal bactericidal activity of *Limulus* amoebocytes (Smith & Pistole 1985). In *Macrobrachium rosenbergii* multiple serum lectins have been identified with more than two distinct specificities for binding foreign substances and have a role as potential recognition factors involved in defence mechanisms (Vasta, Warr & Marchalonis 1983). Multiple serum lectins have also been recognized in *Homarus americanus* and the coconut crab, *Birgus latro* (Hall & Rowlands and Vasta & Cohen cited by Vasta *et al.* 1983). Phenoloxidase, a haemocyte lysate protein, present in haemocytes was shown to be responsible for opsonizing fungal spores in an encapsulation reaction in the crayfish (Söderhäll, Vey & Ramstedt 1984). The role of phenoloxidase is described in greater detail below.

These factors are not invariably present in each species. They also have a low specificity compared with the response of vertebrate immunoglobulins and participate in short-term responses or act synergistically with haemocytes in the cellular immune reaction, enhancing adhesion and phagocytosis (McKay & Jenkin 1970a; Schapiro 1975; Steenbergen, Steenbergen & Schapiro 1978; Sindermann 1971, 1979). Specific immunoglobulins (serum proteins) and specific acquired immunity have not been identified in crustaceans (Bang 1970; Sindermann 1971; Schapiro 1975; Steenbergen *et al.* 1978; Johnson 1980). The vital role of the circulating opsonic factors in mediating the reaction between the haemocyte and foreign particle cannot be underestimated. McKay & Jenkin (1970a) demonstrated that specific serum opsonins in the crayfish were essential for efficient phagocytosis, these opsonic factors were found to be either free in the haemolymph or associated with the plasma membrane or the phagocytic cells (Tyson & Jenkin 1974; Cornick & Stewart 1973, 1978; Söderhäll *et al.* 1984).

In the lobster, Schapiro, Steenbergen & Fitzgerald (1977) did not observe successful phagocytosis of virulent and avirulent strains of *Aerococcus viridans* without prior opsonization. The non-inducible natural agglutinin in the haemocyte of the American lobster, *Homarus americanus*, which can be released into the haemolymph, stimulates elevated levels of phagocytosis (Cornick & Stewart 1968, 1973, 1978; Paterson & Stewart 1974; Paterson *et al.* 1976).

Protein hetero-agglutinins were found in horseshoe crab haemolymph by Stagner & Redmond (1975). These were bacteriostatic, aiding in the removal of bacteria from the haemolymph by preparing them for phagocytosis by haemocytes. McKay, Jenkin & Rowley (1969) and McKay & Jenkin (1970a) identified natural haemagglutinins in crayfish haemolymph which showed specific opsonic activity, enhancing adhesion and phagocytosis of foreign erythrocytes to phagocytic haemocytes, both *in vitro* and *in vivo*. These displayed a specificity comparable to the naturally-occurring iso-haemagglutinin proteins (immunoglobulins) in vertebrate serum.

Recognition of foreignness was shown to be mediated by a specific interaction of the particle with a factor in the haemolymph (McKay et al. 1969; McKay & Jenkin 1970a). Paterson *et al.* (1976) demonstrated that primary absorption of foreign particles to the lobster haemocyte depends to some degree upon a protein "receptor site". Some haemocytes may produce these opsonic agglutinins (Hearing & Vernick 1967; Cornick & Stewart 1973). In contrast to vertebrates, the level of these factors is not increased by prior immunization (McKay & Jenkin 1970a). Stewart & Zwicker (1972) demonstrated that in the lobster, *Homarus americanus*, the bactericidal activity in the haemolymph was a product of the interaction of the plasma components with material contained within, and released from, the haemocytes. The more variable plasma component could be enhanced <u>in vivo</u>

by immunization and the response was proportional to inoculum size and temperature. Studies indicated that there was more than one bactericidin. Further studying the crayfish, McKay & Jenkin (1970a,b,c) observed that following immunization, opsonins or haemagglutinins (McKay & Jenkin 1970a), although important in promoting phagocytosis, were not increased or responsible for an augmented rate of clearance. Rather, this was due to intensified phagocytosis due to increased numbers of haemocytes and an elevated level of activity by the haemocytes. The increase in the percentage of actively phagocytosing haemocytes parallelled the rise in protective immunity, thus, in the resistance to infection, the most important change in adaptive immune response of the crayfish consists of a change in the activity of existing phagocytic cells or the production of a new population of more active cells from stem cells (McKay & Jenkin 1970a). This non-specific adaptive immune response in the crayfish was dependent upon dose of vaccine, time of challenge following antigen administration and temperature (McKay & Jenkin 1969).

Steenbergen *et al.* (1978) found that diminished *in vitro* phagocytosis of a strain of *Aerococcus viridans* occurred in the lobster at elevated temperatures (>22°C) suitable for optimal growth, suggesting that high temperatures used in aquaculture potentially increase the risk of the lobster to disease.

Paterson *et al.* (1976) showed that vaccination led to higher bactericidin (inducible) titres (as Stewart

& Zwicker 1972), but had no effect on agglutinin (non-inducible) concentrations. Increased phagocytosis occurred both with opsonized and unopsonized sheep erythrocytes, demonstrating the cellular nature of the response and a qualitative and quantitative change in the animal after vaccination.

Huang *et al.* (1981) determined that the normal haemolymph of *Macrobrachium rosenbergii* contains agglutinins specific for certain bacteria and human type A erythrocytes. The agglutinins were active toward four serotypes of *Vibrio anguillarum* and a species of *Pseudomonas*, *Staphylococcus*, *Enterobacter* and *Klebsiella*, but not *Aerococcus viridans* var. *homari* or a chitinoclastic species isolated from a shell lesion of *Macrobrachium*. At least two populations of haemolymph agglutinins were considered capable of exerting a degree of antigenic binding specificity for the different cell types. The agglutinins were considered more similar to plant lectins than vertebrate antibodies by virtue of a heat stable (thus probably lipopolysaccharide rather than protein) reactive binding site and an agglutinin which was non-inducible by vaccination (although it may function as an opsonin).

Both Paterson *et al.* (1976) and McKay & Jenkin (1969, 1970c) observed a lack of absolute specificity in the lobster and crayfish in the phagocytic response following vaccination. A variety of vaccines and lipopolysaccharides from endotoxins of Gram-negative bacteria, antigenically unrelated to *Pseudomonas* were

able to increase the resistance of the crayfish to subsequent *Pseudomonas* infection (McKay & Jenkin 1969). Paterson *et al.* (1976) showed that the Gram-negative *Pseudomonas* bacterin induced higher numbers of haemocytes with increased phagocytic capability toward both sheep erythrocytes and Gram-positive *Aerococcus viridans*.

The lobster immune system can apparently control potential Gram-negative pathogens by phagocytosis and digestion, illustrated by the complete absence of known primary Gram-negative aetiologic agents of disease in these animals. Conversely, the Gram-positive *Aerococcus viridans* var. *homari* is both resistant to haemolymph agglutinins and even stimulated by bactericidin, and although phagocytosed, the bacteria multiply intracellularly, leading to cell death and eventually host death by septicaemia (Cornick & Stewart 1968). Thus, this disease is singular among diseases of Crustacea in its ability to overwhelm the host's intrinsic defence system. It is notable that Rittenburg & Bayer (1980) have developed a vaccine (or bacterin), an immunogenic but inactivated, non-pathogenic preparation of bacteria against gaffkemia in lobsters. This consists of an immunologically effective amount of antigens protective against gaffkemia (a toxoid of some type, possibly a cell wall precursor) free of living bacteria and substantially free of antibiotic (eg. vancomycin).

Lewis & Lawrence (1983) immunized *Penaeus setiferus* postlarvae 6-7 by placing them in an autoclaved

bacterin prepared from *Vibrio alginolyticus*. Mean weights of immunized shrimp were higher than that of non-immunized controls suggesting greater production efficiency of the immunized shrimp, possibly resulting from a degree of protection against low-level chronic infections. But other studies suggested higher survival but lower mean weights of immunized compared to non-immunized shrimp. Haemolymph derived from immune shrimp demonstrated induced but non-specific agglutinins toward Gram-negative bacteria, yet apparent specificity toward human B erythrocytes characteristic of a group of carbohydrate (i.e. galactose) receptor specific proteins classified as lectins. It was considered that immunoprophylaxis may be applicable in controlling certain bacterial diseases of penaeid shrimp and that haemolymph lectin agglutinin production in shrimp may represent the corollary of antibody production in the typical vertebrate immune response.

Recently a "Penaeid Multivalent Bacterin" (Argent Chemical Laboratories, Redmond, Washington, USA) has been developed and reported by the company to provide broad spectrum immunologic protection against many opportunistic bacterial infections encountered in larval rearing. Significant reduction in larval mortality and greatly enhanced growout survival is also reported by the company but not yet confirmed in the literature.

Haemocytic Aggregation and Encapsulation / Nodule Formation

In addition to phagocytosis by free and fixed

haemocytes, described as the most important crustacean response in disease resistance (Schapiro 1975; Schapiro *et al.* 1977), haemocytic clumping or aggregation and subsequent encapsulation and nodule formation throughout the body, especially localized in the gills, but also in the heart and hepatopancreas, plays a vital role in clearing foreign material such as bacteria and fungi from the circulation and tissues (Cornick & Stewart 1968; Smith and Ratcliffe 1976, 1980a,b). Following phagocytosis there is a reduction in cell numbers (haemocytopenia) as haemocytes migrate to and aggregate in the lamellar blood vessels of the gills, heart and hepatopancreas, and in the capillary and lacunar networks of the haemocoel, resulting in rapid clearance of invading micro-organisms (Johnson 1980; Smith & Ratcliffe 1980a,b). It is thought that the formation of haemocytic aggregates not only facilitates the haemocyte-bacteria interaction, but also prevents the spread of infection and, thus, is probably a vital part of the cellular defences (Smith & Ratcliffe 1980a,b,1981). Encapsulation and nodule formation are extremely effective in containing the spread of infective agents within the haemocoel and bring foreign material into close contact with the haemocytes thereby facilitating cell mediated killing or destruction of the invaders (Smith & Söderhäll 1986). The clumping of haemocytes and subsequent nodule formation are mediated by substances released from refractile granules (opsonins) in granulocytes (Tyson & Jenkin 1974) which enhance cell stickiness, cause

* Note: More recent <u>in vitro</u> experiments have determined that the factor, 76Kda glycoprotein, released from crayfish haemocytes enhanced their adhesion to foreign surfaces and also promoted semi-granular haemocytic encapsulation / nodule formation (V. J. Smith, personal communication, 1991, citing Johansson & Söderhäll 1988 and Kobayashi <u>et al</u>. 1990, respectively).

haemocytes to adhere together and entrap bacteria, as well as phagocytosing them (Smith & Ratcliffe 1980b). Stagner (1979) showed that phagocytosis and subsequent digestion of engulfed materials were preceded by cellular degranulation, local clumping of the antigen and bacterial attachment of the cell membrane, suggesting the participation of contributing humoral opsonic factors. Vacuoles form around phagocytosed particles replacing the space occupied by the exploding granules in phagocytic haemocytes. This suggests that granular cells, upon releasing hydrolytic enzymes from the granules to aid breakdown of phagocytosed particles, become agranular (Paterson & Stewart 1974). The localization of haemocyte aggregates containing foreign material in specific organs, especially gills, heart and hepatopancreas, has been observed in the lobster (Cornick & Stewart 1968), the crayfish, *Parachaeraps bicarinatus* (McKay & Jenkin 1970b), the white penaeid shrimp, *Penaeus setiferus* (Fontaine & Lightner 1974), the blue crab, *Callinectes sapidus* (Johnson 1976a, 1980) and the shore crab, *Carcinus maenas* (Smith & Ratcliffe 1980a,b). Sequestration isolates infection and facilitates degeneration and ultimate disposal of foreign material from the body. Enlargement of the cell clumps is followed by a gradual disappearance of these aggregates together with the diffuse haemocyte networks and numerous associated bacteria (Smith & Ratcliffe 1980a,b).

Unestam & Nylund (1972) studied the haemolymph reaction of crayfish, *Pacifastacus leniusculus* and

Astacus astacus, *in vitro* and *in vivo*, to the crayfish plague fungus, *Aphanomyces astaci*, demonstrating haemocyte agglomeration and clumping on fungal hyphae, stellate cells with pseudopodia which connected other cells, capsule formation, and the "explosion" or disintegration of granular cells, releasing the granulocytic granules and their contents, leading to the formation of a light refracting zone on the hyphal surface with subsequent zonal melanization. The particles which originated from the granules became attached to the wall of the fungus resulting in phenoloxidase activation, which is involved in the inhibition of mycelial growth and extracellular fungal enzyme activity enhancing the adhesion of bacteria or erythrocytes to haemocytes and the promotion of phagocytosis. Opsonic factors (haemagglutinins) of limited specificity present in the haemolymph, function in an analagous fashion to the immunoglobulins of vertebrates and are important in mediating the reaction between cell and particle (McKay & Jenkin 1969, 1970a; McKay *et al.* 1969; Tyson & Jenkin 1974; Schapiro *et al.* 1977). These opsonins were shown to be haemagglutinins which enhance the adherence of red cells to haemocytes (McKay *et al.* 1969). These recognition factors (opsonins) may be both associated with cell membranes and free in the haemolymph and are similar in nature (Tyson & Jenkin 1974).

Smith & Söderhäll (1986) describe the sequence of events in nodule/capsule formation as follows:

(a) The non-self molecules released from the surface of the foreign material trigger exocytotic release of prophenoloxidase (proPO) proteins from the semi-granular cells,

(b) Active proPO proteins released stimulate further degranulation of proPO components from the granular cells,

(c) Haemocyte adherence to the foreign material then commences and haemocytes continue to attach to the complex as long as proPO proteins are generated by degranulation,

(d) Equilibrium of the structure is finally reached when sufficient cells surround the capsule to effect "sealing".

Melanization

Melanization (derived from the Greek work melas meaning black) is the formation of an endogenous, non-haemoglobin-derived, brown-black pigment formed when the enzyme tyrosinase catalyses the oxidation of tyrosine to dihydroxyl phenylalanine (DOPA) (Robbins, Cotran & Kumar 1984). In the presence of oxygen, phenoloxidase normally oxidizes DOPA into a quinone (dihydroxyindole) which is rapidly converted into dopachrome; melanin is then formed by polymerization of the molecules (Unestam & Beskow 1976). In the integument of mammals and teleosts, melanin is formed in the melanosomes of melanocytes (cells of neural crest origin) and is a protective agent against ultraviolet radiation (Bloom & Fawcett 1968).

In crustaceans, the melanophores are the cells which contain melanin pigment.

In teleosts, melanin is also a component in numerous pathological processes throughout the tissues (Roberts 1975). Melanin is formed as part of the inflammatory response in the cuticle and tissues of many species of injured and diseased arthropods (Johnson 1980). These brown-black pigment deposits have been found in association with haemocytes in most cellular inflammatory conditions and diseases in penaeid shrimp (Lightner & Redman 1977) and *Macrobrachium* spp. (Brock 1983).

In their review of the literature on the cellular inflammatory responses of penaeids to diverse forms of injury, Fontaine & Lightner (1975) postulated the brown-black material to be melanin, but offered no proof. Using histochemical methods, Lightner & Redman (1977) positively identified as melanin the brown-black pigment deposits present in association with sites of haemocytic activity in penaeids in cellular inflammatory disease processes of non-infectious and infectious etiologies, including wounds, *Fusarium* infection, shell disease, "black gill disease", ascorbic acid deficiency, and copper and cadmium toxicites. In ultrastructural studies of blackening of the gills in penaeids due to cadmium exposure, Couch (1977) found the black deposits in association with necrosis of subcuticular tissues in the gills, with no evidence of melanosomes, melanocytes or melanophores.

Cadmium was found to induce gill blackening (melanization) and other histopathological changes in the

gills of the freshwater prawn, *Caridina rajadhari* (Ghate 1984). Mercury caused severe changes in gill structure, but no melanization. Although cadmium and mercury led to similar gill lesions in *Macrobrachium kistensis*, there was no melanization in *Caridina*; the absence of gill blackening in *Macrobrachium* exposed to both metals was inexplicable. It was thought that melanin may be protective against heavy metals since it can form complexes with them (Ghate 1984). Melanization of the gills has been reported to occur in response to gill damage in *Macrobrachium* (Johnson 1982; Brock 1983, 1988).

Unestam & Nylund (1972) were the first to demonstrate conclusively that in crustaceans, melanin is formed in blood reactions *in vitro*, and that both the enzyme, polyphenoloxidase, and the substrate for melanization are haemocytic in origin. Studying the reaction of crayfish blood to the hyphae of Aphanomyces astaci *in vitro* and *in vivo* Unestam & Nylund (1972) observed encapsulation of the hyphae by haemocytes, "explosion" or disintegration of certain cells - especially granular haemocytes- in the capsule and release of granules, formation of a light refracting zone on the hyphal surface and melanization of the surface. Following activation by the hyphal wall surface, the brown-black pigment was rapidly released from the granulocyte granules upon "explosion" and consisted of particles associated with polyphenoloxidase; these were deposited on the distal hyphal portions. The particles oxidize the substrate for melanization, which was slowly

released by the haemocytes or liberated by some factor in these cells (Unestam & Nylund 1972). In a later study, Unestam & Beskow (1976) demonstrated that activation of the phenoloxidase system was non-specific, being stimulated by various types of foreign matter, but was strongly enhanced by certain fungal cell walls. This more specific, stronger melanization was due to attachment of the enzyme in the particles to the wall surfaces, followed by activation by substances present in, or released by the wall, which also activated the enzyme still dispersed in the blood, inhibiting mycelial growth and extracellular fungal enzyme activity. The precise function of melanin is unknown, but it is probably involved in the defence reaction (Söderhäll & Ajaxon 1982).

It was subsequently demonstrated that B-1,3-glucans from fungal cell walls, as well as glycoproteins of *Aphanomyces astaci*, activated crayfish prophenol-oxidase (Söderhäll & Unestam cited by Söderhäll & Ajaxon 1982). A positive correlation has been found between the degree of melanization and crayfish resistance against *A. astaci* (Nyhlén & Unestam 1980). Heavily melanized hyphae of *A. astaci* in crayfish cuticle are still viable although mycelial growth is almost completely inhibited. Thus, the products of phenoloxidase activity are inhibitory to fungal growth, but not completely lethal. Phenoloxidase in crayfish haemocytes could become attached to many foreign surfaces such as fungal cell walls (Söderhäll, Hall, Unestam & Nyhlén cited by

Söderhäll & Ajaxon (1982). Another indication of the antifungal action of melanization is the denser mycelial growth which has been observed outside the wound area experimentally induced. The melanotic capsule around fungal hyphae might inhibit fungal extracellular enzymes or the uptake of nutrients, affecting their viability. Tanning of the wounded cuticle by phenols and their oxidation products might also make nutrients less available to a penetrating fungus (Nyhlén & Unestam 1980).

It has been established that L-DOPA itself is not toxic to *Aphanomyces*, but its oxidized products are inhibitory to fungal growth. Melanin has properties such as redox capacity, ability to bind toxic compounds and cationic exchange capacity which could affect the metabolism of a parasite and/or protect the host from toxins excreted by the pathogen (Söderhäll & Ajaxon 1982). Aromatic quinones, which are intermediates in the production of melanin, are strong oxidizing agents and they or their by-products produced in response to bacteria-induced damage are the non-specific bactericidal substances elaborated by many invertebrates (Taylor 1969). Quinones can inhibit many enzymes and melanin has been demonstrated to inhibit microbial proteases, as well as chitinase (Kuo, Alexander & Bull cited by Söderhäll & Ajaxon 1982). In the crayfish, as the attachment of phenoloxidase is dependent upon Ca^{2+} concentration and a serine protease, production of quinones and melanin *in vivo* is probably close to the cell wall of the parasite,

having more pronounced toxic effects than demonstrated in *in vitro* tests with *in vitro*-synthesized melanin precursors and melanin (Söderhäll & Ajaxon 1982). Having demonstrated that the phenoloxidase system generates toxic substances to certain fungi, Söderhäll & Ajaxon (1982) concluded that the system could be involved in defence reactions in arthropods against invading fungi. Phenolic tanning of the cuticle also alters the mechanical properties of the cuticle, making it more rigid and resistant to fungal penetration (Nyhlén & Unestam 1980).

The polyphenoloxidase system is considered to be a highly significant non-specific defence system common to both plants and invertebrates. The system functions in hardening and darkening and providing the protective colouration of the integument of many arthropods, and is at least partly defensive in nature, contributing to the haemocytic response in sealing and repairing traumatic wounds and lesions, isolating and encapsulating foreign bodies, resisting invading micro-organisms and parasites by the production of non-specific anti-microbial substances (Taylor 1969; Söderhäll & Ajaxon 1982). The blue crab, *Callinectes sapidus*, is one crustacean species that has not been found to produce melanin in response to tissue injury (Johnson 1980).

The Prophenoloxidase Activating System

In arthropods, including decapod crustaceans, the prophenoloxidase (proPO) activating system which

converts inactive prophenoloxidase into active phenoloxidase during the early stages of melanization and is ultimately responsible for melanin synthesis has now been recognized to be part of a single complex integrated complement-like recognition system towards non-self and for defence. The proPO system consists of a complex activating pathway or cascade of enzymes, mostly serine proteases, proteins and other factors (Söderhäll & Smith 1986a,b).

For crustaceans, the proPO system has been most extensively determined in the freshwater crayfish, *Astacus astacus*, but preliminary investigations on crabs, insects and several other groups of arthropods have indicated that the processes are probably common to the entire phylum (Söderhäll 1982; Söderhäll & Smith 1983, 1986a,b).

In arthropods, products of microbial origin such as Gram-negative bacterial cell walls and their constituent lipopolysaccharide (LPS) endotoxins (serving as indicators for Gram-negative bacteria) and B-1,3-glucans and carbohydrates from yeast and fungal cell walls (serving as indicators for yeasts and fungi) have been found to specifically activate proPO (Söderhäll & Smith 1986a,b). In this process, one calcium-dependent and highly specific serine protease, the activating enzyme, probably a zymogen, requiring pre-activation by proteolysis for activation, hydrolyses peptides with a specific structure. This reaction, constituting the

"alternate" pathway, operates in response to microbial invaders containing B-1,3-glucans or LPS (Söderhäll & Smith 1986a,b).

Other substances (including proteases, chymotrypsin, lipids, chloroform, detergents and organic solvents) and processes such as wounding and the aggregation of proPO subunits also elicit the non-specific activation of proPO. Gram-positive bacteria probably cause activation through receptors separate from those for LPS and glucans. Organic solvents, detergents and heating lead to activation of only the last component of proPO probably by causing conformational changes of proPO. It is unclear how lipids lead to activation; the initiating factors of protozoan and helminth parasites are also unknown (Söderhäll 1982; Söderhäll & Smith 1983, 1986a,b).

Low calcium concentration spontaneously triggers the conversion of proPO to phenoloxidase without the presence of non-self molecules such as B-1,3-glucans or LPS. In this reaction low calcium levels cause inactive serine protease S to become active serine protease S by a limited proteolysis involving removal of a peptide. This reaction, constituting the "classical" pathway, occurs in response to general forms of injury (Söderhäll & Smith 1983, 1986a,b).

Phenoloxidases, the serine protease terminal components of the system, are located, often in the inactive state as prophenoloxidase proenzymes, within the

hemocytes, particularly in the semi-granular and granular cells. (Söderhäll & Smith 1983) Hyaline cells lack both large intracytoplasmic granules and phenoloxidase activity (Söderhäll & Smith 1983). In this system, antigenic surfaces (such as those of an invading pathogen) contact the extremely labile semi-granular haemocytes causing their degranulation or exocytosis and subsequent lysis, during which the proPO system is activated. The semi-granular cells are therefore primarily responsible for the recognition of foreignness. The more stable granulocytes (which contain greater amounts of the proPO enzymes) are subseqently activated by proPO released by the semi-granulocytes and undergo exocytosis themselves. Phagocytosis of foreign particles by hyalocytes is opsonized by the released proPO proteins (Söderhäll 1982; Johansson & Söderhäll 1985; Söderhäll & Smith 1986a,b).

The activation of proPO is accompanied by a clotting process or coagulation in which phenoloxidase and four other serine proteases become sticky or attaching proteins. This process is calcium-dependent and is mediated by proteolysis. Activated serine protease converts coagulogen to coagulin by proteolysis and proPO to phenoloxidase which becomes a sticky protein attaching to the antigen and serving as an opsonin for phagocytosis (Söderhäll & Smith 1986a,b). Coagulation in crustaceans is considered to involve initial aggregation of haemocytes. The semi-granulocytes are extremely labile and rapidly lyse upon contact with foreign surfaces, releasing one or more proteins including

coagulin into the haemolymph. These agglutinating factors then gelate or form a modified clot which contributes to the immobilization of foreign material; the sticky proteins also coat the invading micro-organisms thereby opsonizing phagocytosis by hyalinocytes and/or semi-granulocytes (Söderhäll, Smith & Johansson 1986). Haemocytes subsequently attach to the clot leading to aggregation, encapsulation and nodule formation. There is thus evidence that in crustaceans, the proPO system upon activation produces a "degranulation factor", opsonin-like substances (Söderhäll et al. 1986) and a cell adhesive protein (Johansson & Söderhäll 1985) which enable the proPO system to have an important role in mediating cell-to-cell communication (Söderhäll & Smith 1983, 1986a,b).

Through the elaboration of phenol substrates, oxidizing phenols to quinones, proPO has also been found to lead to the production of antimicrobial (inhibiting microbial chitinases and proteases), cytotoxic and fungistatic substances, including melanin precursors such as 5,6-dihydroxyindole and quinones and melanin; quinones are converted to melanin through a series of nonenzymatic reactions (Söderhäll 1982; Söderhäll & Smith 1983). These also help protect against UV radiation (Söderhäll 1982). Wounding also causes melanization preventing bacterial and fungal growth at the wound site (Söderhäll 1982).

Regulatory factors identified in the crayfish which probably serve to modulate the reactions of the

proPO system include naturally occurring proteolytic inhibitors in the haemocytes and cuticle, a_2-macroglobulin activity which may sequester proteases and time-dependent spontaneous inactivation of the activating serine protease (Söderhäll 1986; Söderhäll & Smith 1986a, b).

The Inflammatory Response in Prawns and Shrimp

Although the basic inflammatory response in various crustacean species has been studied by various *in vitro* and *in vivo* methods, it is the *in vivo* and histological investigations of penaeid shrimp which best shed light upon the reactions in *Macrobrachium* sp.. Fontaine (1971) and Fontaine & Lightner (1973) studied wound repair processes in penaeid shrimp and Fontaine & Dyjak (1973) extended this in a study of scar tissue development after wounding with the Petersen disk tag. Further studies have investigated the inflammatory response of penaeid shrimp to injection with carmine particles (Fontaine & Lightner 1974) and turpentine (Fontaine, Bruss, Sanderson & Lightner 1975), and, exposure to *Fusarium* sp. by injection with and without prior wounding (Solangi & Lightner 1976). Fontaine & Lightner (1975) reviewed the cellular response to injury in penaeid shrimp.

Following injury or infection, the primary reaction is an infiltration of haemocytes into the damaged area. Haemocytes readily phagocytose smaller micro-organisms and non-living particles. Cells

resembling multinucleated giant cells and epithelioid cells (ie modified macrophages derived from blood monocytes) have also been observed in the reaction. Encapsulation, a walling off of the lesion by haemocytes, soon occurs. A multilayered capsule composed of fusiform haemocytes, toward the centre, and larger haemocytes round the periphery, eventually completely encloses the damaged area, containing any material too large for phagocytosis: fungi, parasites, necrotic cellular debris or necrotic haemocytes containing phagocytosed bacteria or other materials (Fontaine & Lightner 1973, 1974, 1975; Fontaine *et al.* 1975).

The process of encapsulation resembles granuloma formation in fish and other vertebrates, the haemocytes performing a macrophage and epithelioid cell function. "Open encapsulations" are formed by exoskeletal intrusions induced by Petersen disc tagging (Fontaine 1971).

Melanization of the lesion, as described previously, soon occurs. This brown-black pigmentation is common in many decapod crustacean species, is easily identified macroscopically and is a general clinical sign of damaged or diseased tissue (Fontaine & Lightner 1975).

In the crustacean chronic inflammatory reaction, fibrocytes (fibroblasts) have been shown to infiltrate the wound lesion caused by tagging in association with the haemocytic response, forming a dense network of fibroblasts and collagen-like fibres which

persists long after the injury, is stable and well organized and eventually forms a permanent scar (Fontaine & Lightner 1973, 1975; Fontaine & Dyjak 1973). Fibroblasts have also been demonstrated to participate in cellular infiltrations and encapsulations in damaged tissue resulting from carmine particle injection, polyvinyl chloride insertion and turpentine injection both at the site of injection and in the heart (Fontaine & Lightner 1974, 1975; Fontaine *et al.* 1975).

Penaeid shrimp react to plerocercoid larval infection in the hepatopancreas and muscle by developing a progressively denser cyst, composed of haemocytes, fibroblasts and collagen-like fibres around the parasite, which is eventually destroyed and resorbed, leaving a dense fibrous capsule. In the adjacent haemocoel, worms are encapsulated by a thin host cyst and less intense cellular infiltration and fibroplasia insufficient to destroy the parasite (Sparks & Fontaine 1973). Trematode metacercariae can be destroyed and resorbed by a similar reaction in *Macrobrachium* (Nash 1989). A marked hepatopancreatic intertubular haemocytic infiltration followed by encapsulation and fibrosis occurs in penaeids in response to aflatoxicosis (Lightner, Redman, Price & Wiseman 1982).

It has been found that granulocytes synthesized collagenous fibres in the encapsulation reaction in the shore crab, *Carcinus maenas*, (Hubert, Chassard-Bouchaud & Bocquet-Védrine 1976) contrary to the situation in vertebrates in which collagen is elaborated by

fibroblasts. Fontaine & Lightner (1974) cited Salt who stated that phagocytosis, nodule formation, encapsulation and melanization are all part of a general haemocyte response in insects; the process which occurs being related to particle size.

The gills constitute one of the primary sites of elimination of foreign and necrotic material in shrimp, phagocytosis and haemocytic aggregation readily occurring upon introduction of foreign material. The actual disposal occurs upon moulting of the gill cuticle (Fontaine & Lightner 1975).

The cuticular pores at the bases of setae on the appendages (pereiopods, pleopods, maxillae) provide additional ports of elimination, where particle-laden haemocytes may be discharged (Fontaine & Lightner 1973, 1975).

In addition to the free haemocytes in the haemolymph and circulation, the haemocytic sinuses of the heart (this supplementary system of weakly phagocytic cells or "nephrophagocytes" has not been observed in the hearts of crabs), gills, loose subcuticular connective tissues, abdomen and hepatopancreas (applied to the walls of the hepatic arterioles) of penaeids are lined with numerous "fixed" phagocytic haemocytes, providing a more enduring cellular response (Fontaine & Lightner 1975). The phagocytes are in the form of nodules or rosettes. In bacterial infections, both natural and experimental, activation of the phagocytes occurs, with phagocytosis

and sticking of bacteria to the rosettes. Certain viral infections also induce phagocytic activation. The fixed phagocyte system of decapod crustaceans is considered to be analagous to the mononuclear phagocyte system of mammals (Johnson 1980).

Fontaine (1975) found that the wound repair processes of the freshwater crayfish, *Procambarus* sp., were comparable to penaeid shrimp with two exceptions. Unlike the shrimp, the crayfish lost during molting the cuticular sheath formed around a stainless steel tag pin. Histologically, connective tissue did not form between migrating epidermis and the new cuticle. In addition, the crayfish scar tissue basal to the epidermis had a loose alveolar structure and was not as organized as in the shrimp.

1.3 Diseases and Related Problems of Cultured *Macrobrachium rosenbergii*

In the study of *Macrobrachium rosenbergii* culture it soon becomes apparent that good husbandry and environmental management are essential in the prevention of disease. Disease problems most commonly develop in hosts stressed and weakened by adverse environmental factors such as poor water quality, low dissolved oxygen, high carbon dioxide, unionized ammonia and nitrite concentrations, extremes in temperature, pH and salinity, heavy organic loads, pesticides, pollutants -such as heavy metals, contaminants-, overcrowding and inadequate or imbalanced nutrition. Facultative and opportunistic micro-organisms readily proliferate and exert their

effects in hosts weakened by marginal environmental factors or poor nutrition. There have been no reports, up to this time, of obligate pathogens, including viruses, rickettsia, chlamydia, bacteria, fungi, metazoan or protozoan parasites associated with disease problems in *Macrobrachium* culture. Neither viruses, chlamydia nor rickettsia have, as yet, been isolated from *Macrobrachium*; the first virus has recently been identified by EM (Anderson, Law, Shariff & Nash 1990). It is also the case that numerous diseases of *Macrobrachium* have an indeterminate aetiology. Although it has been demonstrated that *Macrobrachium rosenbergii* exhibits a strong resistance to infection (Huang *et al.* 1981), it is certain that further study into the diseases and pathology of *Macrobrachium* will identify previously unknown causes of disease and new aetiologic agents.

Disease is recently proving to be a limiting factor in *Macrobrachium* culture and significant disease-related decreased productivity and economic losses are possible. Health problems are unique to each of the hatchery, nursery and grow-out or pond production phases of *Macrobrachium* culture (Sindermann 1977a,b; Johnson 1982; Brock 1983, 1988).

Epibiont-related Diseases of *Macrobrachium*

Epibiotic or epicommensal organisms include a wide range of ubiquitous bacteria (*Aeromonas* sp., *Pseudomonas* sp., *Vibrio* sp.), filamentous bacteria (*Leucothrix* sp.); filamentous algae (the chlorophyte,

Oedongonium crassiusculum, and blue-green algae (the cyanophyte, *Lyngbya* sp.); phycomycetous fungi (*Achlya* sp. and *Aphanomyces* sp.); peritrichous ciliate protozoans (especially *Epistylis* sp., *Zoothamnium* sp., *Vorticella* sp., *Vaginicola* sp., *Lagenophrys* sp., *Corthunia* sp.) and suctorian protozoans (*Acineta* sp.); bryozoans; barnacles and the eggs of the diving beetle (water boatmen), *Ramphocorixa acuminata* (Hall 1979; Smith, Sandifer & Manzi 1979; Johnson 1982; Brock 1983, 1988). Many of these micro-organisms are filter feeders (biofilters) of organic particles, bacteria or protozoa and are the natural inhabitants in the aquatic environment. The numbers of organisms present under normal conditions have a minimal effect on the host and are shed regularly with the exuvia.

All stages, but especially larval prawns, are susceptible. High population densities and frequent daily feedings lead to heavy organic loading, providing substrates for the proliferation of these opportunistic organisms. Infrequent or inadequate water exchange can worsen the problem. Other water quality-related factors serve to further stress the larvae, increasing their susceptibility to epibiont fouling. Exuvia or food wastage and dead *Artemia* must be removed or siphoned regularly to prevent a proliferation of microbial opportunists. Extremely high water concentrations of calcium carbonate ($CaCO_3$ > 300 ppm) may lead to precipitation of $CaCO_3$ on the shell, as well as decreasing the frequency of moulting -hence decreasing

growth, which may be accompanied in pond culture by a substantial build-up of cuticular ectocommensals which would otherwise be periodically shed with the exuvia (Cripps & Nakamura 1979). This may also be a problem in older individuals wich moult infrequently (Johnson 1978). In addition physiological disturbances, ageing or moult cycle disruption may elicit reduced cleaning of the exoskeleton and resultant epibiont fouling (Hall 1979). It is also important to maintain optimum temperature as it directly influences moulting rate (Johnson 1982).

Inadequate nutrition may induce abnormalities of the epicuticle, suppress cleaning behaviour and activity and increase susceptibility to epibiont fouling (Johnson 1982). Facultative bacteria initially attach to the setae and setaeules of the appendages, and in severe chronic cases may be found on all epicuticular surfaces, including the proximal appendages, gills or body, restricting moulting, respiration, vision, feeding and movement. Epibiont fouling of larvae with nonfilamentous bacteria is apparently more serious and associated more frequently with mortality than filamentous bacterial fouling (Fisher 1977).

Johnson (1978) and Hall (1979) have reported that unlike other non-selective protozoa, *Zoothamnium* sp., preferentially populates the gills. Common sites of infestation are usually proximal, including the body, eyestalks, antennal scales, uropods and egg masses on the adult female (Hall 1979; Brock 1983). Mortality is considered to result from asphyxiation due to epibiont

occlusion of respiratory surfaces (Fisher 1977), with no penetration or inflammatory response in the cuticle (Brock 1983). During three years of observation, Hall (1979) found that *Corthunia* sp., *Epistylis* sp. and especially *Vorticella* sp., were the most common peritrichous ciliates. Infestations in adults were associated with extensive algal growth on the shell surfaces. Only rare incidences of heavy fouling affected the prawns adversely. Crustaceans, including prawns, have an increased oxygen demand just prior to moulting, and epibiont fouling can be particularly associated with mortality due to anoxia at this time (Fisher 1977; Mangum, McMahon, de Fur & Wheatley 1985).

Epibiont fouling may be apparent grossly as cotton wool-like masses on the affected prawns (known as "moss backs" in severe cases) or require examination of wet mount preparations for microscopic detection. Complete diagnosis involves both identification of the immediate fouling organism and the underlying primary problem, which may be associated with suboptimal water quality, too high a population density, inadequate nutrition or poor sanitation. The most effective prevention and control involves the appropriate prophylactic measures (Johnson 1978, 1982, Brock 1983). Immediate treatments of *Leucothrix* sp. infesting larvae, consists of copper sulfate at 0.05 mg Cu/l for 12 hours as a static bath; and for *Epistylis* sp. or *Zoothamnium* sp., a static bath of formalin at 25 to 125 mg/l for 12 hours, though usage may be associated with toxicity,

moulting deformities and anorexia (Sindermann 1977b; Brock 1983, 1988). Formalin as a 24 hour static bath at 20 ppm has been demonstrated by Roegge, Rutledge & Guest (1979) as totally effective and safe in controlling larval *Zoothamnium* infestation. Acetic acid is recommended at 2.0 ppt as a one-minute dip as the treatment of choice for peritrichous ciliates such as *Epistylis* on larvae because of low toxicity and consistent efficacy. Repeat treatments may be required because of re-infestation (Sindermann 1977a,b; Brock 1983, 1988).

That indiscriminate use of antibiotics must always be avoided was demonstrated by Nilson, Fisher & Shleser (1975), who found that antibiotic treatment of bacteria resulted in 100% mortality in treated animals due to *Lagenidium* (fungal) infection. Salinity alterations of 20 ppt as a 15 - 30 minute dip causing osmotic shock have been found effective against fungus (Dugan, Hagood & Frakes 1975) and hydrozoa (Chao & Liao cited by Johnson 1982) and should also be useful against some protozoa (Johnson 1982).

It is recommended that biofiltration units be established away from the actual rearing area so as to localize the process away from the animals; prawns may serve as substrates for biofiltration in waters where epibionts flourish (Johnson 1982).

Shell Disease (Brown Spot Disease, Black Spot Disease, Burn Spot Disease)

The disease syndrome known as shell disease, a

progressive necrosis (erosion, ulceration), inflammation (haemocytic infiltration) and subequent melanization of the cuticle in a focal to multifocal pattern on any aspect of the body or appendages (which may lead to autotomy) results from what is considered to be a multifactorial complex of epicuticle-cuticle damage or abnormality (mechanical, nutritional, chemical or otherwise) followed by secondary bacterial and/or fungal infection to which all life stages of *Macrobrachium* are susceptible. Mortalities may result; in any case, shell disease lesions make the prawns less marketable. Yet the definite pathogenesis of the disease remains unknown (Brock 1988) and Koch's postulates have not been fulfilled in association with a single aetiologic agent. Shell disease, along with epibiont fouling, is one of the most common disease problems affecting larval, juvenile and adult prawns both in culture and in the wild (Dugan, Hagood & Frakes 1975; Sandifer & Smith 1985), with incidence of infection related to handling, stress and frequency of prawn interactions (aggressiveness, cannibalism, difficulties during ecdysis) and is therefore more prevalent in intensive culture with high stocking density in which prawns are more likely to suffer physical damage (Delves-Broughton & Poupard 1976; Johnson 1978; Burns, Berrigan & Henderson 1979; Sandifer & Smith 1985).

Poor water quality and high organic loading are associated with higher concentrations of potentially shell lesion-inducing bacteria (Cook & Lofton 1973).

Once the epicuticle, which provides the primary physical boundary of defence, is breached, invasion of the cuticle and deeper tissue structures by shell disease-inducing bacteria and fungi becomes possible (Delves-Broughton & Poupard 1976; Fisher, Rosemark & Nilson 1976; Johnson 1980). As in epibiont fouling, epicuticular damage also may result from nutritional inadequacy, chemical treatments (such as malachite green), pollutants (such as heavy metals), nitrogenous waste products or developmental abnormalities (Fisher *et al.* 1976; Baross, Tester & Morita 1978; Johnson 1982; Brock 1983).

A variety of bacterial species, usually designated as "chitinolytic" or "chitinoclastic" or as producing extracellular lipases or proteases, have been implicated in shell disease, including *Aeromonas* spp., *Pseudomonas* spp., *Vibrio* spp., *Benekea* spp. (chitinoclastic *Vibrio* and *Pseudomonas* spp.), as well as the imperfect fungus, *Fusarium* spp.. The external surfaces of shell disease lesions also become populated with parasitic epibionts. All these facultative organisms have been repeatedly demonstrated to induce shell disease lesions in animals only after prior epicuticular injury (Rosen 1970; Fisher *et al.* 1976; Burns *et al.* 1979; Cipriani, Wheeler & Sizemore 1980 Brock 1983, 1988). Mixed infections are common and may occur after secondary pathogens invade lesions initiated by primary ones (Cipriani *et al.* 1980).

Secondary infections with facultative, potentially pathogenic organisms more commonly result

following concomitant traumatic cuticular damage and additional stress.

Black spot lesions usually remain localized, focally on the gills, carapace covering the gills (branchiostegites), appendages, uropods, telson or body cuticle, for example and are usually self-limiting, shed with the exuvia in otherwise healthy individuals, but, may in severe cases become diffuse, result in infection and loss of appendages, spread to the epithelium, muscle, viscera or result in septicaemia and mortalities (Brock 1983,1988). The imperfect fungus, *Fusarium* sp., has been especially associated with serious infections in some species of penaeids, as opposed to usually relatively minor disease conditions in *Macrobrachium* (Burns *et al.* 1979; Hose, Lightner, Redman & Danald 1984).

One possible variation of the condition known as branchiostegite shell disease, the specific cause of which is unknown, presents as bilaterally symmetrical focal or confluent and diffuse ulceration and melanization on the medial surfaces of the carapace overlying the gills (Johnson 1982; Brock 1983, 1988). Although numerous individuals in the same population may be affected (up to 20%), having experienced similar predisposing factors leading to epicuticular damage, the disease is not considered to be communicable, and any organisms which may be involved are ubiquitous in the environment (Brock 1983, 1988). Branchiostegite shell lesions, though, provide additional substrate for these organisms which may permit them to proliferate, hence

providing increased possibility for lateral infection.

As with epibiont fouling, prevention and control of shell disease involve improved sanitation, environmental and nutritional management and more careful handling and husbandry which may alleviate the primary initiating factors. Overcrowding may lead to increased agonistic encounters and injury, and should be avoided. Specific chemotherapeutic, rather than prophylactic, treatment of secondary bacterial and fungal pathogens may be indicated in some severe infections, as well as initiating the necessary changes in management and husbandry; on the other hand, general or prophylactic chemical treatments are not recommended (Delves-Broughton & Poupard 1976; Brock 1983, 1988). Fungal infections may be controlled by a 15 - 30 minute dip in 20 ppt seawater (Dugan *et al.* 1975; Sindermann 1977a,b. Maintaining broodstock prawns in slightly brackish water of 2 - 3 ppt has helped prevent shell disease and other problems (Hagood cited by Sindermann 1977a).

As the epicuticular repair mechanism relies upon precursor materials from the diet it has been found that scraped and wounded lobsters fed a complete diet remained invulnerable to shell disease, whilst those fed an insufficient diet were unable to synthesize an effective epicuticle and were susceptible (Fisher *et al.* 1976). The response of *Macrobrachium* is probably comparable. Antibiotic baths such as with oxolinic acid at 10 mg/l for one hour (El-Gamal, Alderman, Rodgers, Polglase &

Macintosh 1986) or furanace (Aquacop 1977a) may be useful in some cases.

The use of artificial habitats or microhabitats in providing shelter and refuge for prawns, especially pre- and early post-moult individuals, has been found effective in reducing the incidence of cannibalism and traumatic mechanical shell injuries. Aquatic plants, branches, gravel, shells or plastic pipes or nets have been utilized in an attempt to supply maximum "edge effect" which prawns prefer for refuge (Sandifer & Smith 1985).

Selective harvesting of larger prawns is considered to decrease the competition for territory, food and oxygen,reduce cannibalism and result in improved growth rates among remaining prawns (Sindermann 1977a; Sandifer & Smith 1985). *Macrobrachium* maintained in grow-out ponds for excessive periods of time have demonstrated increased fouling with organic debris probably associated with deterioration of water quality and the pond bottom and an associated increased incidence of shell disease (personal observation in Malaysia and Thailand).

Idiopathic Muscle Necrosis (IMN)

The disease known as idiopathic muscle necrosis (white muscle disease, muscle necrosis, spontaneous muscle necrosis, muscle opacity, milky prawn disease) affects especially *Macrobrachium* postlarvae and juveniles and occasionally adults, as well as *Penaeus* spp. (Rigdon

& Baxter 1970; Sindermann 1977a,b; Akiyama, Brock & Haley 1982; Brock 1983,1988; Nash, Chinabut & Limsuwan 1987). IMN appears grossly as focal, multifocal to diffuse opacity ("milkiness") of all striated body muscles (abdominal and gnathothoracic). Microscopically, varying degrees of myofibrillar necrosis, haemocyte infiltration, aggregation, encapsulation and myophagia, calcification and sarcolemmal nuclear proliferation (in regenerating lesions) are found with the same distribution (Rigdon & Baxter 1970; Sindermann 1977a,b; Akiyama *et al.* 1982; Brock 1983; Nash *et al.* 1987).

The disease is considered to be non-infectious, associated with exposure to one or more environmental stressors including exposure to air, extremes and fluctuations of temperature, pH and salinity, direct sunlight, quinaldine narcotization, hypoxia, overcrowding and hyperactivity due to handling (Rigdon & Baxter 1970; Venkataramiah 1971a,b; Sindermann 1977a,b; Lakshmi, Venkataramiah & Howse 1978; Brock 1983,1988; Nash *et al.* 1987).

It has been found that *Macrobrachium* with the disease also have a soft exoskeleton and show slow growth and high mortality (Delves-Broughton & Poupard 1976). Mortalities are associated with extensive necrosis of muscle fibres in individual prawns (Brock 1988). If the primary initiating factor(s) is (are) alleviated and necrosis has not progressed extensively, the disease has been found to be reversible (Venkataramiah 1971b; Rigdon & Baxter 1970; Brock 1983,1988).

Spotts & Lutz (1981) have verified that after handling stress there can be as much as a six-fold increase in the accumulation of L-lactic acid in the striated muscles of *Macrobrachium*. Reports in the literature have not related the possible role of lactic acidosis with the pathogenesis of muscle necrosis, but the vicious cycle of spasmodic contraction of muscle leading to insufficient perfusion of blood, subsequent hypoxia and the accumulation of lactic acid followed by further muscular contracture and so on, is well known from veterinary literature as initiating muscle necrosis in equine rhabdomyolysis (Jones & Hunt 1983). Muscle contraction is probably the cause of a gross severe distortion of body shape, present only when the prawn is alive, which has been reputed to be a problem of *Macrobrachium* in Malaysia -concomitant with extensive opacity of body muscle (Anderson, personal communication). Tissue hypoxia or anoxia is possibly a cause of this abnormality and idiopathic muscle necrosis in prawns.

Sarver, Malecha & Onizuka (1982) suggest that the prevalence of IMN in a population of postlarvae serves as a useful indicator of their general health and can be monitored when they leave the hatchery prior to stocking in ponds. Control consists in reducing environmental stressors, ensuring the optimum environmental conditions possible in a given culture situation and improving husbandry -notably during handling and transfer (Akiyama *et al.* 1982; Brock 1983, 1988).

IMN is also seen as a feature of other disease syndromes, such as white prawn disease, suggesting at least a partial relationship of initiating factors (Brock 1983).

Bacterial Necrosis

Aquacop (1977a) has reported a disease affecting *Macrobrachium* larvae in Tahiti which could cause up to 100% mortality in 48 hours for stages four and five, while other stages and postlarvae apparently showed increased resistance. The first abnormal signs in the larvae were a bluish discolouration, decreased food consumption (microscopically the stomachs appear empty) and slight cannibalistic activity, with weak larvae unable to swim and falling to the bottom of the tank.

Grossly, there were signs resembling shell disease, such as brown spots on the antennae and newly formed appendages (suggesting that the disease may not be a separate entity but perhaps a more advanced systematic form of this disease -reviewer's opinion).

Melanization (brown spots) indicates the host's reponse to injury, and subsequently, inability of the larvae to limit the course of the disease was manifested by appendage opacity, a necrosis which began at the bases of the appendages and which could result in partial or complete destruction of the member.

Mixed bacterial infections were observed with filamentous, *Leucothrix*-like organisms, non-filamentous

bacilli and cocci present on the setae, gills and appendages, more serious in younger larvae, especially stages four and five, in which sudden mortalities were most common and usually associated with moulting. In severe outbreaks, however, entire tanks containing more advanced larval stages could also be devastated. Lateral spread of the infection occurred, spreading from tank to tank in rearing factilities, the disease progressively affecting younger groups of prawns (stage VIII, then VI, then IV), other larval stages and postlarvae showed increased resistance, often regenerating necrosed appendages (Aquacop 1977a).

The disease was considered to resemble bacterial necrosis in penaeids and was found to respond to antibiotics, also useful in its control. A specific or primary aetiologic role of bacteria was, however, not confirmed. Bipenicillin-streptomycin at 2 IU/ml (2.0 ppm) proved to be the most efficacious treatment and was employed at this dosage prophylactically every third day or at twice the dosage daily during outbreaks. Treated prawns shed epibionts on setae and regenerated necrotic appendages at ecdysis. Other effective, but less successful treatments, were tetracycline chlorhydrate (1.0 ppm), erythromycin phosphate (0.65 - 1.0 ppm) and furanace (0.1 ppm) (Aquacop 1977a). Brock (1983) has reported similar diseases affecting prawns in Hawaii.

Mycobacterial Infection in *Macrobrachium rosenbergii*

Mycobacterium sp., Runyon Group II was

identified in an adult cultured (7-plus years) female prawn, *Macrobrachium rosenbergii*, in Hawaii (Brock, Nakagawa & Shimojo 1986). Necrotic nodular haemocytic lesions with encapsulation of Gram-positive, acid-fast bacilli were observed histologically predominantly in the hepatopancreas, heart, antennal gland, loose connective tissue of the gnathothorax, gill stem and gill lamellae and also in the hindgut submucosa, striated muscle and abdominal loose connective tissue. As acid-fast bacteria were not observed in the cytoplasm of fixed phagocytes but within capsules and nodules, haemocytic encapsulation rather than phagocytosis was considered to be the primary host response of *M. rosenbergii* to infection by mycobacteria (Brock *et al.* 1986).

Larval Mid-Cycle Disease (MCD)

The definite etiology of MCD has not yet been determined, but this important disease primarily associated with high mortalities in early larval stages (IV through XI) is considered to be infectious in nature. Possible causes include: bacteria, *Enterobacter aerogenes*, the species most frequently isolated from the digestive tract of affected larvae-, a virus or an unidentified toxin (Johnson 1978; Brock 1983, 1988).

Clinically, the disease first manifests itself during the second third (days 10 to 20) of the larval rearing cycle as a marked loss of appetite (decreased consumption of Artemia) and cannibalism of the moribund by healthier larvae. Histologically, this anorexia is

associated with absence of normal fat-glycogen vacuolation and atrophy of the hepatopancreas (which may be associated with starvation) (Rosemark, Bowser & Baum 1980), luminal dilatation and the occasional presence of coccobacilli within the digestive tract lumen. Larvae are often blue-grey in colour ("grey larvae"), may have epibiont fouling, swim weakly and often in spirals. Although the younger larvae (stages I through V) especially) are subject to epizootics with the acute high mortality syndrome, the more mature larvae (stage VI to IX through XI) are apparently more resistant, showing delayed metamorphosis, but with fewer mortalities. Postlarvae, juvenile and adult *Macrobrachium* are refractory to the disease (Brock 1983, 1988).

The aetiologic agent or reservoir has not been identified and is not considered to reside in the adult prawns (Brock 1983, 1988).

MCD causes reduced survival of larval cultures from a normal 50-70% to 5-10% at the postlarval stage (or from 10-25 to 1-2 postlarvae per litre). In infectivity experiments, an incubation period of four to five days has been determined (Brock 1983, 1988).

Mid-cycle disease or similar disease syndromes have reported in *Macrobrachium* hatcheries in Thailand, the Philippines, Hawaii, Mauritius, Northern Australia (Brock 1983, 1988) and Malaysia (Anderson, personal communication).

Antibiotic treatment with oxytetracycline (4

mg/l) or furanace (0.1 to 0.2 mg/l) daily added to the water for 10 to 15 days has sometimes improved larval survival and metamorphosis, if initiated immediately upon onset of larval anorexia. After noticeable mortalities begin, antibiotics have not proven useful (Brock 1983, 1988).

Control consists of depopulation of infected larval cultures, initiation of standard sanitation procedures, disinfection of contaminated or potentially contaminated tanks and equipment, controlling access to the hatchery, use of individual equipment for each larval rearing unit, conduction of larval rearing in distinct cycles with close adherence to separation of rearing tanks and clean up and disinfection between larval rearing cycles, between new batches of animals. These measures have proven to be effective in eliminating MCD from several hatcheries in Hawaii (Akita, Nakamura, Brock, Miyamoto, Fujimoto, Oishi, Onizuka & Sumikawa 1981; Brock 1983, 1988). Aquacop (1979b) advocated similar procedures, which could be applied to all aspects of *Macrobrachium* culture, calling this the "sanitary lot technique"(see Appendix II). Attention should also be paid to nutrition, ensuring that good quality *Artemia* are used (Johnson 1982).

Exuvia Entrapment Disease (EED)

Exuvia entrapment disease, also known as metamorphosis moult mortality syndrome, is a disease which primarily affects late stage (stage XI, occasionally stage X) larvae and early postlarvae

characterized by mortalities at moulting especially at metamorphosis. Affected larvae are unable to free appendages, eyes or rostrum from the exuvia in which they become entrapped. Other larvae which shed the exuvia have malformed appendages and die shortly after moulting or before the next moult (Brock 1983, 1988).

Moribund or dead larvae or postlarvae are found entrapped in the exuvia during the latter third of the hatchery rearing cycle. Larval mortalities, which are mot usually severe, may in some cases reach as high as 20 to 30%, usually associated with stocking densities exceeding 40 larvae per litre. The precise aetiology of EED is unknown, but it is thought that poor water quality (elevated unionized ammonia or nitrite) or nutritional inadequacy may cause or contribute to its occurrence (Brock 1983, 1988). Very hard water, as mentioned previously, has been associated with deposition of $CaCO_3$ on larval prawns and their inability to moult and increased *Epistylis* infestation (Cripps & Nakamura 1979). Calcium deposits on the inner surface of the exuvial exoskeleton have also been found to be associated with EED (Brock 1983) and probable nutrition-related, moult-associated mortalities in lobsters (Bowser & Rosemark 1981).

In Hawaii, the disease has been found to be more prevalent in systems not using algal cultures ("clearwater") then in "greenwater" systems where mixtures of zooplankton and phytoplankton are used (Brock 1983, 1988). Maddox & Manzi (1976), Manzi, Maddox &

Sandifer (1977) and Joseph (1977) have determined that use of algal cultures has led to increased growth and survival of *Macrobrachium* larvae and decreased length of time to metamorphosis. The precise mechanism through which algae cultures act is not known, but possibilities include direct nutrition of *Macrobrachium*, indirect nutrition through *Artemia salina* or water quality aspects in that algal cells could reduce the levels of nitrogenous compounds (detoxification) by utilizing ammonia and nitrite derived from metabolic wastes of *Macrobrachium* and *Artemia salina* or by providing an endo- or exometabolite which acts as either a growth factor for the larvae or as a conditioner for the larval media. Cohen, Finkel & Sussman (1976) concluded that algae facilitated the growth of *Macrobrachium* only indirectly by removing toxic material, notably ammonia, from the culture water.

Describing a similar condition in *Palaemon serratus* which resulted in exuvia entrapment during the metamorphosis moult, Wickens (1972) found that the disease was associated with the use of Utah *Artemia* but not with San Francisco Bay *Artemia* or when algal cultures were utilized. It was considered that the Utah strain was deficient in essential nutrients required by the larvae. Bowser & Rosemark (1981) determined that moult-associated mortalities (moult-death syndrome) in juvenile *Homarus* sp. were reduced by the addition of lecithin to the diet. Soybean curd (which contains lecithin) has been incorporated into larval diets and found to reduce

the incidence of EED when fed during the last week of the cycle. Control of EED includes use of "healthy" algal cultures, nutritional supplements containing lecithin, such as soybean curd, and maintenance of optimal water quality in the hatchery (Brock 1983, 1988).

Hard Water Associated Shell Disease

Nash and Brown (1988) have reported primarily nodular (but also ulcerative) melanized body and gill cuticular lesions in postlarval and juvenile *M. rosenbergii* accidentally exposed to total water hardness levels between 160 and 320 $CaCO_3$ mg/l in heated recirculation systems. This case is described in more detail in Chapter II.

Postlarval Stocking Mortality

Decreased survival and high prevalence of postlarvae with muscle opacity within one or two days following stocking in production ponds is considered to be associated with stressful hatchery and/or pond conditions, particularly low water temperature and/or high pH. Postlarvae have been shown to be intolerant of water temperatures of 19°C or lower and pH of 9.5 or higher, these and other stressful environmental factors may initiate this mortality syndrome (Sarver et al. 1982).

As with idiopathic muscle necrosis and other disease problems of *Macrobrachium*, prevention consists of improvement of environmental conditions (low oxygen, high pH or low water temperature), stocking postlarvae into

nursery ponds before release into grow-out ponds and monitoring the survival of postlarvae by placing a number in floating mesh bags when ponds are stocked (Sarver *et al.* 1982; Brock 1988). Aquacop (1979a) have reported mortalities of *Macrobrachium* in ponds, especially during moulting, associated with high pH (>10.5) due to phytoplankton blooms (*Chlorella* sp.). Treatments of the ponds with the algicide, Clarosan (Ciba Geigy), at 0.02 mg/l controlled the blooms; led to a rapid fall in pH, did not eliminate all algal species and was not harmful to the prawns.

Black Nodule Disease

The lesion consists of slight to extensive black fibrous nodules within the epidermis of the exoskeleton without apparent damage to the epicuticle. The melanized nodules are surrounded by infiltrating haemocytes and collagen. It has been reported only in laboratory-cultured *Macrobrachium* in the UK (Delves-Broughton & Poupard 1976). Although not conclusive, and though Koch's postulates were not satisfied, it was considered that the disease was systemic and bacterial in origin, since a number of bacteria were isolated and furanace at 0.09 mg/l controlled a moderate outbreak. Deficiency of dietary ascorbic acid has been reported to have induced similar lesions ("black death disease") in penaeid shrimp in which opportunisitic bacteria subsequently caused a fatal septicaemia (Lightner, Colvin, Brand & Danald 1977). A vitamin C deficiency-associated disease has not been reported in *Macrobrachium*

(Brock 1983). In the penaeids, dietary ascorbic acid supplements resulted in the cessation of "black death" among a vitamin C deficient population, and was demonstrated to be essential for normal collagen metabolism and wound repair (Lightner, Hunter, Magarelli & Colvin 1979).

Haemocytic Enteritis (HE)

Haemocytic enteritis is a disease syndrome recognized in the blue shrimp, *Penaeus stylirostris*, which has occurred in epizootic form, and is caused by the ingestion of the marine blue-green algae, *Spirolina subsalsa*, during algal blooms. Only postlarval, juvenile and subadult stages were affected. The principle lesion consists of necrosis and haemocytic infiltration of the mucosa of the midgut and occasionally dorsal caecum and hindgut gland, accompanied by a secondary terminal bacterial septicaemia (predominantly due to *Vibrio alginolyticus*) (Lightner, Danald, Redman, Brand, Salser & Rerpieta 1978). In Hawaii, sporadic cases of a similar disease, presumed to be caused by ingestion of blue-green algae (*Oscillatoria* spp.), have been reported in juvenile and subadult *Macrobrachium*; additional studies are required to verify the definite etiology of the disease in *Macrobrachium*. Control consists of maintaining adequate algal density to limit the growth of filamentous blue-green forms (Brock 1983).

Hepatopancreas Preservation

Hepatopancreas preservation or mummification

consists of the complete or nearly intact *Macrobrachium* hepatopancreata containing increased amounts of saturated fatty acids which have been recovered from production ponds in Hawaii during routine harvesting or draining (Brock 1983, 1988).

The precise cause of the occurrence is not known, however, the condition has been experimentally induced by exposing prawns to hypoxia, cold temperatures or lethal levels of organophosphate pesticides, such as rotenone. Hepatopancreata of juvenile through adult prawns have been recovered following pond hypoxia. The process which leads to hepatopancreas preservation is thought to represent a postmortem event. No abnormalities were found in the surviving prawns harvested from these ponds. Definite means of prevention are not known, but attention should be given to environmental parameters (dissolved oxygen, temperature, avoidance of pesticides) (Brock 1983, 1988).

Terminal Growth (TG)

Terminal growth or enzootic cachexia is an enzootic condition reported in all pond culture systems in Hawaii and also Puerto Rico, occurring only in large male, blue-claw *Macrobrachium rosenbergii* in the intermoult stage (moult stage C). It manifests as weakness, lethargy, pale discolouration, diffuse opacity and oedema and atrophy of the striated body musculature (which is soft or "mushy") and hepatopancreas atrophy - sometimes accompanied by generalized epibiont fouling and focal to multifocal shell disease lesions (Brock 1983, 1988).

Terminal growth is thought to be due to an endocrine change which interferes with the moult cycle in intermoult. *Macrobrachium* moult cycle cessation is considered to be analagous to the CT4 moult stage identified in other decapods such as the blue crab, *Callinectes sapidus*. Male prawns in the final intermoult stage are apparently unable to digest or metabolize ingested food, slowly reabsorbing body tissues and gradually weakening over an extended period of eight weeks or more, until death due to cachexia or secondary infection. The severely debilitated prawns commonly found under pond culture conditions are probably subject to and removed by predators under natural conditions in the wild (Brock 1983, 1988).

The presence of TG males in a pond havest indicates inefficient harvesting, as male prawns should be regularly removed during routine harvesting before they progress to the moribund TG states. Thus, control consists of efficient culling of these individuals during harvesting. TG prawns are unsuitable for marketing as they have shrunken, "mushy" flesh (Brock 1983, 1988).

Endocuticular Degeneration (ED)

Endocuticular degeneration (exoskeletal spotting) is a disease of unknown etiology which has only affected large adult females (10.5 cm or larger) in one pond, on one farm in Hawaii (Brock 1983). The reported prevalence of the disease was 38% with no associated mortality. The disease has also been reported to occur

occasionally among larger *Macrobrachium* cultured in Texas (Johnson 1978).

Gross lesions were observed on the cephalothorax and abdomen and consisted of focal to multifocal, irregularly shaped, non-bilaterally symmetrical, brown to orange-brown areas within the deeper layers of cuticle, which was not ulcerated but sometimes demonstrated increased flexibility (Brock 1983).

Histologically, areas of degeneration were seen within the membranous and endocuticular layers of the cuticle, with subcuticular amorphous deposits and disorganization and hypoplasia of the underlying epidermis (Brock 1983). Affected prawns have moulted successfully with no apparent macroscopic changes in the ED lesions (Brock 1983).

No infectious agent has been observed or isolated in association with the lesions (Johnson 1978; Brock 1983). Perhaps nutritional or endocrine etiology associated with egg production should be considered (Brock 1983).

White Prawn Disease (WPD)

White prawn disease (white syndrome, white *Macrobrachium*, whole prawn) is a chronic progressive disease of adult *Macrobrachium* (mainly females) maintined in a culture system lacking direct exposure to sunlight and fed a formulated, artificial diet. The etiology and pathogenesis are unknown. The disease has been reported in Hawaii (Brock 1983) and Texas (Johnson 1978).

Affected prawns show a progressive loss of normal pigmentation (presumably due to atrophy of epidermal chromatophores) and softening of the cuticle. The syndrome presents initially as delayed shell hardening following ecdysis, progressing over six months or longer, until there is failure of proper exoskeleton hardening throughout intermoult. Microscopically, in some prawns the endocuticle shows apparent decreased thickness, the rest of the cuticle and epithelium are normal in appearance. Consistently accompanying lesions include hepatopancreas atrophy and diffuse body muscle oedema and atrophy, with focal to multifocal haemocytic nodules; and sometimes, additionally, epibiont fouling of the gills and body, cuticular ulceration and melanization and focal to multifocal melanization of the ovary, heart and green gland, due to haemocytic nodule formation. Prawns are also haemocytopenic, with prolonged clotting times. Mixed bacterial populations were isolated from 50% of haemolymph cultured postmortem (Brock 1983).

A nutritional or environmental aetiology is suggested, but additional studies are required (Brock 1983).

Johnson (1978) has reported a whitening in *Macrobrachium ohioni* adults in Texas, which progressively worsened in captivity. The subcuticular tissues appeared milky, but the muscles were normal. No micro-organisms were demonstrated.

Delves-Broughton & Poupard (1976) described a

disease in *M. rosenbergii* which they called "white syndrome" in which affected animals were a dense opaque white colour, with soft exoskeleton and showed slow growth and high mortalities. Microscopically, there was diffuse necrosis of striated body musculature, with haemocyte infiltration. The causes considered were a reaction against the presence of a foreign body or stress. White prawn disease has only been reported in prawns maintained under artificial lighting and fed formulated diets over extended periods and has not been recognized under pond conditions. A nutritional (vitamin, mineral?) cause seems likely. Until the disease is better understood control consists of the supplementation of artificial diets with fresh natural foods (Brock 1983) and exposure of the prawns to some direct sunlight if possible.

Red Discolouration of *Macrobrachium*

An uncommon condition of unknown aetiology consisting of reddish discoloration of the body associated with listlessness and handling mortalities has been reported in *Macrobrachium* in Texas by Johnson (1978). Most of the stock were affected. Johnson (1982) has reported a reddish abdominal discolouration affecting adult prawns reared in Florida. The abnormal pigmentation resulted from pigment that had dispersed from chromatophores which had lost their usual integrity. The cause was not determined (Johnson 1982).

Dying larvae have been seen to become reddish-

gold in colour, associated with chromatophore dysfunction (Johnson 1982). Too much light, diet and stress have been considered to be responsible for many of the chromatophore abnormalities which occur in larval culture and post-transport (Johnson 1982).

A Blood Ciliate in *Macrobrachium*

One or two blood ciliates have been reported which resulted in mortalities in adult *Macrobrachium*. The disease was associated with haemocytopenia (Quick cited by Sindermann 1977a).

A Parvo-like Virus in *Macrobrachium rosenbergii*

The first virus in *M. rosenbergii* was recently found. Intranuclear basophilic, PAS-negative and Feulgen-positive inclusions were identified in the hepatopancreatocytes in clinically healthy *M. rosenbergii* postlarvae in Malaysia in 1988. These intranuclear inclusions were thought to resemble hepatopancreatic parvo-like virus (HPV) in penaeids histologically. Electron dense spherical viral particles in the inclusion body were 29.1 nm in diameter. Unusual forms had an additional spherical electron-dense wall (Anderson *et al.* 1990).

Metazoan Parasite Infection/Infestation

Various metazoan parasites have been reported in Macrobrachium, usually in the wild and in grow-out ponds, none causing serious problems under culture conditions (Brock 1983).

Macrobrachium spp. may serve as second intermediate hosts for the metacercariae of the microphallid trematode, *Carnaeophallus choanophallus*, reported in the Gulf Coast area of the USA (Johnson 1978). Snails serve as first intermediate hosts and raccoons or rats as the final hosts. Metacercariae of an unidentified species of trematode have been found in adult *Macrobrachium* raised in grow-out ponds in Thailand (Nash 1989). The trematode metacercariae encyst in the body muscles and tissues of the prawn, causing a localized inflammatory reaction. Intact parasites are encapsulated by haemocytes and fibroblasts, killed and undergo gradual resorption. Though prawns are usually clinically normal, slight infections going unnoticed and not affecting marketability, prawns should be properly cooked to eliminate the possibility of zoonotic infections. Control of snails in ponds should be undertaken in the case of a severe pond infestation (Nash 1989).

Adult *Temnocephala* (Turbellaria: Rhabdocoela) which locate on the body surfaces and within the branchial chambers of *Macrobrachium* may restrict movement of the gills, water flow over them and decrease respiratory efficiency, when present in sufficient numbers. Formalin at 50-100 ppm would probably be an effective control. Prevention should include quarantine and careful examination of animals and treatment when required (Johnson 1978; Brock 1983). Bellon-Humbert (1983) reported mechanical structural and possibly enzymatic effects on the ovary and hepatopancreas of the

prawn, *Palaemon serratus*, due to turbellaria infestation.

Bopyrid isopods and those of the genus *Probopyrus* (reported in brackish waters of the Gulf Coast of the USA) may locate within the branchial cavity of *Macrobrachium* leading to discolouration of the area and causing a characteristic bulge of the cephalothorax. The much larger female parasites are usually found concomitantly with the smaller males. Prawns are the definitive hosts whilst copepods are the intermediate hosts. When found, the isopods should be mechanically removed from the infested prawns. Newly introduced animals should be quarantined and examined for the presence of isopods which should be removed to prevent spread (Johnson 1978; Brock 1983).

Corallinid isopods (*Austoargathona* spp.) attach to the cephalothorx and have been reported as parasites on *Macrobrachium* in Australia. Control is similar to that for bopyrids (Brock 1983).

Gas-Bubble Disease

Gas-bubble disease or gas supersaturation has been reported in penaeid shrimp (Lightner, Salser & Wheeler 1974; Brisson 1985) and in *Macrobrachium rosenbergii* (Brock 1988), as well as the blue crab, *Callinectes sapidus* (Johnson 1976b), and lobsters (Hughes cited by Brisson 1985). In the penaeids, the initiating cause was air and water mixing under pressure due either to air leaks in the circulating system, or to partial biofouling inside the water supply system between the

pump and the tanks (Brisson 1985) and the water warmed in a closed heater that did not allow excess gas to escape (Lightner *et al.* 1974).

In the single case described in *Macrobrachium* (Brock 1988), adult prawns were held in tanks, for several days prior to marketing, in which gas supersaturation of inflowing water resulted from pump cavitation. Larger prawns aand those kept for longer periods were apparently more susceptible. Clinical signs consisted of weakness and lethargy, with short bursts of erratic swimming. Large gas bubbles were macroscopically visible under the cuticle. Gas emboli presumably led to tissue damage and mortality. Permitting the incoming supersaturated water to splash onto the water surface in the holding tanks and repair of the pump cavitation improved the situation (Brock 1988).

Predation, Competition and Cannibalism

Predation, competition and cannibalism result in considerable losses in *Macrobrachium* culture (Johnson 1982). Reductions of prawns stocked in the hatchery and grow-out ponds of 40 to 60% have been related to a large extent to cannibalism, and to a lesser extent, to predation (Brock 1983). *Macrobrachium rosenbergii* reared in captivity are notorious as a rather aggressive, territorial and cannibalistic species (Forster & Beard 1974; Segal & Roe 1975). Increasing population density is directly associated with more frequent antagonistic and cannibalistic encounters for food and shelter

(microhabitats) among prawns of different sizes and moult stages, leading to increased acute mortalities of progressively smaller individuals (Sandifer & Smith 1975; Peebles 1978, 1980). Pre- and postmoult prawns and those of a smaller size or weakened by disease (Johnson 1982) are more susceptible to death through cannibalism than are intermoult prawns (Peebles 1978). During metamorphosis to postlarvae, the highest mortalities have been attributed to cannibalism (Aquacop 1977b).

Survival and growth have been found to be enhanced by the provision of additional substrate in the form of artificially constructed habitats (artificial habitats) composed of aquatic plants, branches, gravel or shells, offering the maximum possible edge area available for prawns ("the edge effect") (Smith & Sandifer 1975; Maddox & Manzi 1976; Ling cited by Sandifer & Smith 1985). The development of intensive, high density nursery systems for juvenile prawns has been facilitated by the provision of artificial habitats (Sandifer & Smith 1985). Additional preventive measures include attempting to rear animals of similar size in segregated groups by continual selective harvesting of the larger, faster growing individuals and lowering stocking density when necessary. These measures should aid in reducing competition for available territory, food and (indirectly for) oxygen, resulting in increased survival and growth rate (Smith & Sandifer 1975; Brock 1983; Sandifer & Smith 1985).

Predators can cause major losses at all stages

of production. Competitive organisms which utilize the food and other pond resources of prawns, rather than directly preying on them, can also be responsible for production losses (Sandifer & Smith 1985). Predation by a different species is referred to as interspecific predation. Manifold species have been implicated including fish (catfish in Malaysia; American eel; red-breasted, bluegill, warmouth and pumpkinseed sunfish and largemouth bass in South Carolina and mosquito fish (*Gambusia* sp.) in Hawaii); amphibians (bull frogs (*Rana* sp.) in South Carolina and Hawaii); reptiles (the brown watersnake, cottonmouth, Florida cooter and chicken turtle in South Carolina; monitor lizards in Malaysia); also alligators; birds (American anhinga, snowy and American eggret, belted kingfisher, redbreasted merganser, great blue heron and green heron in South Carolina and the black crown night heron and cattle egret in Hawaii); and mammals (river otters in South Carolina; also raccoons and man) (Green, Richards & Singh 1977; Willis & Berrigan 1977; Smith, Sandifer, Jenkins, Stokes & Murray 1982; Brock 1983). Insects may be a problem in the grow-out phase (Fujimura & Okamoto cited by Johnson 1982); damsel- and dragonfly nymphs have been reported to prey upon early postlarval prawns stocked in shallow dirt ponds in Hawaii (Green *et al.* 1977; Fujimura cited by Brock 1983). They may be controlled by *Gambusia* sp. and larger prawns (Green *et al.* 1977; Brock 1983). Air breathing insects can be controlled by repeated treatments with diesel fuel and motor oil mixed 10:1 (Johnson 1982). The medusan stage of small hydrozoan

jellyfish has been reported as a serious predator on *Macrobrachium* larvae and competitor for *Artemia* nauplii and are extremely difficult to eradicate; using 250 ppm formalin for one hour is useful, but preventing their introduction into hatchery systems is preferred. Potential hydroid vehicular material (such as detritus) should be excluded or sterilized before introduction into the system and only artificial or treated seawater utilized (in closed recirculation systems). Land crabs have been reported as predators on Macrobrachium and competitors for food and space in ponds. Crab holes can cause loss of pond water and trap prawns when they hide in them. Baited earthen jars have been recommended as a means of crab eradication (Tunsutapanich 1982).

Eradication is best accomplished by sacrificing the infested prawn cultures and disinfecting the tanks and filter beds by scrubbing and flushing with hot water and allowing them to dry for several days (Sandifer, Smith & Calder 1974). Tilapia infesting production ponds in Hawaii have proven to be competitors for available feed and even oxygen and also interfere with net harvesting. Draining and drying ponds or the use of piscicides such as rotenone has been suggested as a means of control (Brock 1988). Commercial preparations of rotenone such as derris powder containing 4-5% of active product have been recommended at 1.0 to 2.0 g/m^3 as effective piscicides when spread evenly throughout the pond (New & Singholka 1985). Crude saponin extracted from tea seed (*Camellia* sp.) used at 1.1 ppm has been

found to effectively eradicate indigenous predatory fish without harming crustaceans including penaeus shrimp under brackish water and marine conditions (Terazaki, Tharnbuppa & Nakayama 1980). Its use has not been reported in *Macrobrachium* culture (New & Singholka 1985).

Screens may be used on the inflow/intake water lines to prevent fish and other aquatic predators and competitors from entering ponds. Cleaning and drying of harvest nets used in ponds containing tilapia is recommended before use in a tilapia-free pond (Brock 1988). Construction of prawn ponds in areas which flood (leading to tilapia entering ponds) should be avoided (Brock 1988). Ponds may require draining for the eradication of fish predators (Sandifer & Smith 1985; Brock 1988). Birds may be controlled by protective netting hung over ponds (Singholka, New & Vorasayan 1980; Brock 1988).

Oxygen Depletion

Low dissolved oxygen (DO) can result in acute pond hypoxia, particularly a problem during the early morning and associated with a high biological oxygen demand (B.O.D.) following the sequence of a phytoplankton bloom and die-off. Exposure to levels of 2 to 3 ppm or lower (depending on temperature) may lead to acute incidences of pond hypoxia, inducing stressful behaviour in prawns, which congregate in the shallows along pond edges and subsequently die if conditions are not quickly improved. Green *et al.* (1977) reported an occurrence of

acute pond hypoxia in Malaysia over a three day period which resulted in 45% *Macrobrachium* mortality and was caused by over fertilization of the pond water with a mash-type supplemental food, bottom turnover due to heavy rains and a subsequent algal bloom.

Diffuse muscle opacity is prominent in affected prawns (Brock 1988). Oxygen actually becomes limiting at about 2 ppm at 23°C, 2.9 ppm at 28°C and 4.7 ppm at 33°C (Sandifer & Smith 1985).

All life stages of *Macrobrachium* are susceptible to hypoxia, but larger and more valuable prawns require more oxygen than smaller ones and are thus more susceptible to low DO. These are usually terminal growth prawns in grow-out ponds; moulting animals are also particularly susceptible.

Treatment consists of increasing DO levels as quickly as possible to levels of 5 ppm or more using mechanical aerators and paddle wheels and rapidly flushing a large volume of water into the system. Reduction of feed levels should also be considered. Pond conditions and algal concentrations must be continually monitored ensuring secchi disc readings of at least 25 to 40 cm. Secchi disc readings below 25 cm indicate phytoplankton levels are above beneficial levels of oxygen production. Phytoplankton may also be controlled by periodic flushing with clear water and proper use of algicides (Sandifer & Smith 1985; Brock 1983, 1988). Once all dead phytoplankton, dead prawns and organic debris

have been removed and pond conditions are back to normal, ponds may be reinoculated with algae from adjacent "healthy" appearing ponds (Brock 1983).

Phylactolaemate bryozoans or other sessile colonial organisms have been reported as a possible nuisance to prawn farmers as they may grow luxuriantly inside pipes and conduits, slowing the flow rate to the ponds. This would surely have a less drastic effect on oxygen levels than an algal bloom. On the other hand, prawns not fed commercial feeds have been observed to graze on the bryozoans, although their nutritive value was not determined (Bailey-Brock & Hayward 1984).

Other Important Water Quality Parameters

In addition to dissolved oxygen, other important water quality parameters include: temperature, CO_2, salinity, pH, water hardness, nitrogenous wastes and pollutants.

Temperature

Although *Macrobrachium* can withstand temperature extremes of 18°C and 33°C, reductions in growth, activity and survival generally occur outside the range of 22-33°C, with a range of 26-31°C being satisfactory (Sandifer & Smith 1985). Low water temperatures can result in growth suppression or death. It has been recognized as a problem in Hawaii during the winter, usually in ponds at a higher elevation or those exposed to evaporative cooling by strong winds. Location

should be considered when constructing ponds; also the use of wind breaks (Brock 1988).

Salinity

As *Macrobrachium* larvae require saline water for development, a salinity range of 12-16 ppt is generally used throughout the hatchery stage. Though usually reared in freshwater, postlarvae are euryhaline, but mortalities commence at salinities of 25 ppt or greater. Salinities of up to 10 ppt are suitable for pond culture of adults. Maintaining adult broodstock in 2 - 3 ppt has been found to reduce black spot and related problems (Hagood cited by Sindermann 1977a).

The higher the temperature, the lower the salinity at which stress manifests itself in depressed metabolism (Sandifer & Smith 1985). It has been found that "delayed shipping" of seven-day-old metamorphosed postlarvae (rather than one-day-old) in brackish water of 15 ppt (rather than freshwater) has reduced overall mortalities (Harrison & Lutz 1980).

pH

A pH range of 7.0 to 8.5 is considered suitable for pond culture of *Macrobrachium*. Extremes below 6.5 and above 9.0 should be avoided. Precipitation of calcium carbonate on prawns interferes with moulting, occludes gill surfaces and results in mortalities and has been associated with chronic high pH levels (Cripps & Nakamura 1979; Smith & Sandifer 1985). High pH levels (>10.5) due

to phytoplankton blooms led to mortality of prawns, particularly when moulting, in ponds in Tahiti (Aquacop 1979a).

Water Hardness

A $CaCO_3$ level of 50 - 100 mg/l is gene ally considered an optimum range for *Macrobrachium* (Cripps & Nakamura 1979). Additional calcium uptake from the water is required by prawns for complete calcification of the exoskeleton (Fieber & Lutz 1982), and below 50 ppm soft exoskeletons, poor growth and decreased survival have been reported (Cripps & Nakamura 1979; Sandifer & Smith 1985). Cripps (cited by Brock 1983) reported that a total hardness of 200 mg/l $CaCO_3$ was associated with reduced growth rate in juvenile *Macrobrachium rosenbergii*. At levels greater than 300 mg/l $CaCO_3$, slow growth of adults in grow-out ponds has also been observed, with deposition of $CaCO_3$ on the shell, decreased moulting and increased *Epistylis* sp. and *Bryzoa* sp. infestation on the shell, which normally would have been shed with the exuvia. $CaCO_3$ deposition was also observed on larvae in ponds with levels above 300 mg/l (Cripps & Nakamura 1979).

Nitrogenous Wastes

Nitrite (NO_2) and unionized ammonia (NH_3) are the most toxic forms of nitrogen; the levels of NH_3 are proportional to increas ing pH and temperature. Wickens (1976) determined that *Macrobrachium* was more susceptible to nitrite and nitrate than penaeids.

Prawns, particularly larvae, are susceptible to ammonia and nitrite toxicity from increased levels, which result in growth suppression and mortality (Brock 1983; Smith & Sandifer 1985). Sublethal effects of nitrite, which could be fatal in chronic exposure, may occur at less than 2 mg/l (Armstrong, Stephenson & Knight 1976).

Levels of nitrogenous compounds should be routinely monitored. Precipitating chemicals and nitrogenous waste products have been implicated in melanization of the gills and branchiostegites (Johnson 1982). Data concerning the toxicity of nitrogenous wastes to various stages of *Macrobrachium* have been summarized by Brock (1983) and Smith & Sandifer (1985).

Carbon Dioxide

Carbon dioxide may be toxic in rearing units with a low pH and limited aeration (Johnson 1982).

Contaminants

Pollutants, pesticides and heavy metals should be avoided as they can only have a negative impact on *Macrobrachium* culture (Smith & Sandifer 1985). A mass mortality of *M. rosenbergii* larve, possibly associated with pollutants such as heavy metals, has recently been reported in Malaysia (Piyan, Law & Chea 1985). In situations in which potential biotoxin-producing dinoflagellates are present the use of sentinel animals may be indicated (Johnson 1982).

Mortalities, decreased moulting and failures

have been demonstrated in postlarvae in association with exposure to mirex, a chlorinated hydrocarbon insecticide (Summer & Eversole 1978). For successful larval rearing of *M. rosenbergii* it has been recommended that the hatchery should contain less than 41 ppb mercury, the safety level being below 4 ppb mercury (Piyan *et al.* 1985). Larvae, particularly stages I to IV, are especially susceptible to mercury toxicity (Piyan *et al.* 1985).

The freshwater prawn, *Macrobrachium kitensis*, was found to be most sensitive to the toxic effects of sevimol in the late pre-ecdysial and early postecdysial stages. Sevimol also had the effect of inducing increased moulting frequency (Sarojini & Reddy 1984).

Dithiocarbamates, which are widely employed as agricultural herbicides and industrial fungicides, have been demonstrated to cause numerous degenerative changes in grass shrimp, *Palaemonetes pugio*, including branchial degeneration and melanization, irreversible necrosis of the hepatopancreas and antennal gland necrosis (Doughtie & Rao 1983).

Organic Matter

Increased levels may particularly be a problem in the hatchery in which larvae are reared at high population densities and fed several times a day (Brock 1983).

The accumulation of organic matter and bottom

debris such as decomposing food wastage, dead *Artemia* and exuvia should be discouraged by regular cleaning and siphoning and adequate water exchange. These practices should improve water quality and oxygen levels, reducing B.O.D and eliminating substrates and debris that can permit epibiont proliferation or interfere with respiration. Improved prawn survival and growth should result from strict attention to water quality parameters (Johnson 1982).

Emphasizing disease prevention, a health management programme for cultured prawns, as well as specific treatments, are described in Appendix II.

Lexicon of Some Pathological Terms Used in the Text

(a) Karyorrhexis: rupture of the cell nucleus in which the chromatin disintegrates into formless granules which are extruded from the cell.

(b) pyknosis: degeneration of a cell in which the nucleus shrinks in size and the chromatin condenses to a solid, structureless mass or masses.

(c) focal: the change described has a very localized distribution in one circumbscribed focus or a few foci.

(d) multifocal: the change described is more widely distributed or scattered in more numerous circumscribed foci.

(e) diffuse: the change described has a more evenly widespread distribution involving a larger area or areas of tissue or an entire organ or tissue.

(f) degree of severity grade or extent of a histological or pathological change was denoted by minimal, mild (+), slight (++), moderate (+++), severe (++++) or extensive (+++++) as determined subjectively.

CHAPTER II: Clinical and Pathological Observations on Cultured Prawns in Southeast Asia

II.1 Clinical Data

During the course of this study numerous clinical cases of disease in cultured *Macrobrachium* prawns and penaeid shrimp were investigated in the field in Malaysia and Thailand. Prawns cultured in the Tropical Prawn Unit of the Institute of Aquaculture and samples received from prawn farm managers or farm biologists in Southeast Asia were also examined. The diseases described are representative of important infectious and noninfectious conditions affecting cultured prawns. Pathology cases 6 and 7 have not been previously described.

Whenever possible, the investigation included obtaining a clinical history from the pond or hatchery manager or biologist, observations of the pond or hatchery conditions, followed by collection of prawn specimens, examination of fresh specimens microscopically, preservation/fixation of specimens for histology and/or culture for bacteriology followed by histological and sometimes ultrastructural examination and bacteriological testing and identification.

Some of the clinical cases of disease in *Macrobrachium* and the histopathological, ultrastructural and bacteriological findings are discussed in this chapter. Of primary interest was the natural tissue response which occurred due to injury and infection in the prawn which was studied histopathologically and is described. These are later compared in the discussion with the experimentally induced responses described in Chapter III.

Methods of prevention, treatment and control of the individual disease conditions are described more fully in the introductory chapter and in the appendices,

in which published papers concerning two of the clinical cases can also be found.

II.1.1 Case 1

Stage XI *M. rosenbergii* larvae cultured in a greenwater system in fibreglass tanks in a hatchery in Melaka, Southern Penisular Malaysia displayed increased morbidities and mortalities of up tp 50%. Treatment with 20 ppm formalin every third day did not lead to an improvement in survival rate. Microscopic examination of fresh moribund prawn larvae revealed multiple brick-red to brown foci throughout the body, with a loss of appendages and/or antennae. Healthy and moribund larvae were preserved and processed for histology and subsequent histopathological examination.

II. 1.2 Case 2

This case comprised 13 clinically normal adult market-sized *M. rosenbergii* which originated from one pond population cultured in Suphanburi Province in Thailand. The prawns were received already fixed in 10% neutral buffered formalin, except for one which was already processed to histological slides. Histological processing of the others and histopathological examination was done (Nash 1989, see Appendix I).

II. 1.3 Case 3

An adult female broodstock *M. rosenbergii* recently transported from Thailand was maintained in a

tank in the Tropical Prawn Unit of the Institute of Aquaculture. Grossly, the cuticle of the fifth to sixth abdominal segments was ulcerated so that 1 cm of the underlying soft tissues were exposed. The surface of the damaged area become brown to black in colour after a one hour bath treatment with oxolinic acid at 10 mg/l. Death occurred within 24 hours after treatment, with the distal abdomen a pink colour at autopsy. Sampling for bacteriology and histology was carried out.

II. 1.4 Case 4

This case consisted of six adult and four juvenile *M. rosenbergii* from growout ponds in Thailand.

The adult prawns had multiple brown-black shell erosions and ulcerations down to the soft subcuticular tissues on the cephalothorax and abdomen, with the loss of some of the appendages, leaving brown-black stumps (Fig.1a). The juvenile prawns' macroscopic sign was a diffuse opaque white discolouration of the abdominal musculature.

Microscopic examination of fresh gill and shell mounts from the adult found *Leucothrix*-like filamentous bacteria and *Epistylis sp.* ciliates fouling the surface of melanized lesions. Microscopic examination of fresh squash preparations of abdominal muscle from the juveniles revealed rod-shaped bacteria.

Specimens were prepared for bacteriology and histology.

Fig. 1a Moribund adult *M. rosenbergii* with numerous brown-black shell erosions, ulcerations and appendage stumps (arrow).

II. 1.5 Case 5

In a prawn hatchery in the vicinity of Bangkok, Thailand, the *M. rosenbergii* postlarvae, 28 days post-hatching, had a mortality rate of up to 60%. The prawns were stocked at 170 pieces/l in circular concrete tanks. The hatchery used a clearwater system with the salinity of the water brought to 10 ppt by mixing seawater with freshwater from a canal. The water was pretreated with chlorine and formalin prior to use.

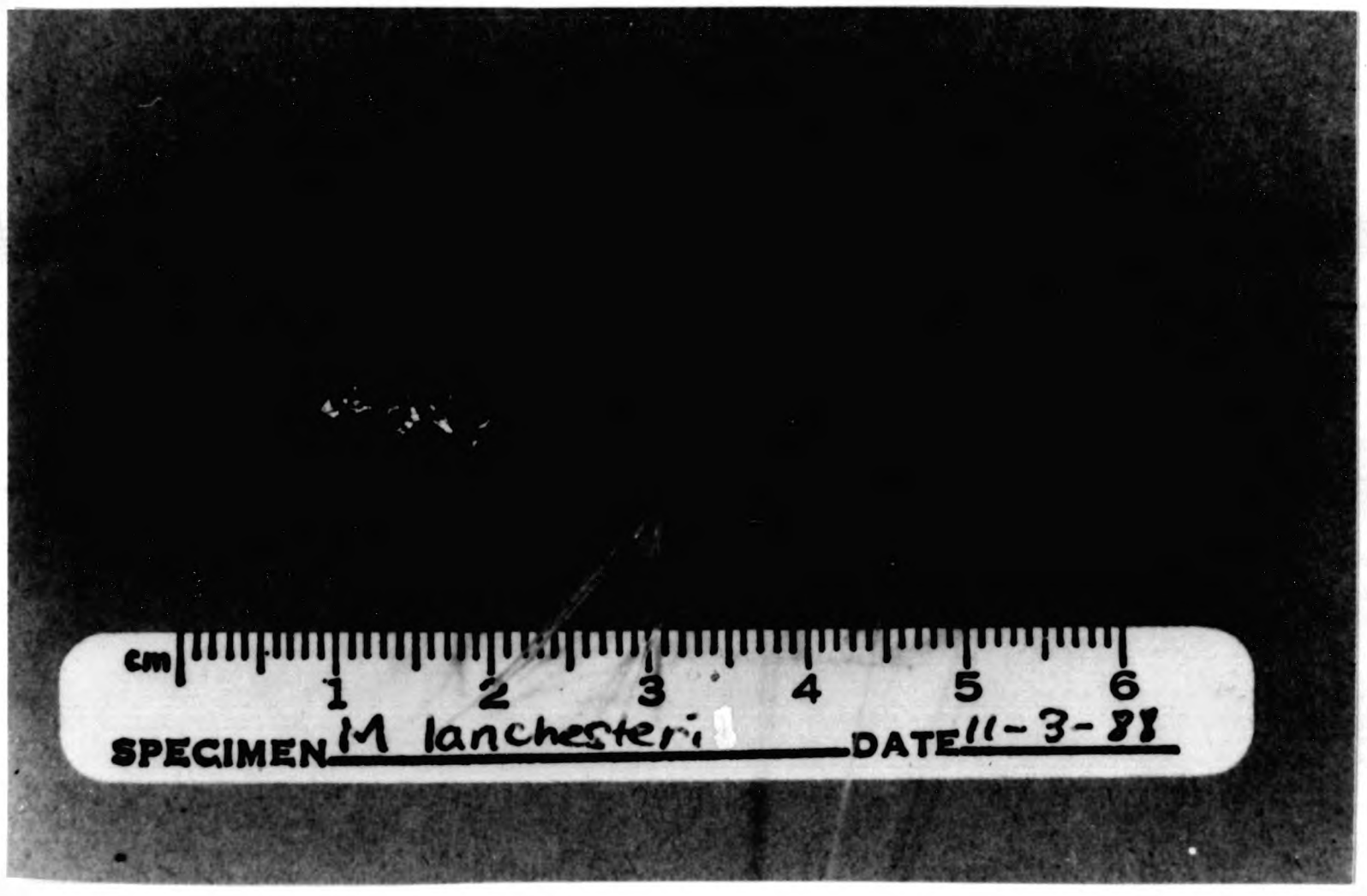
Large numbers of prawns showed the clinical sign of diffuse "milky" white opacity of the entire body which was rapidly followed by death.

Seven clinically normal and moribund "milky" prawns were sampled for histology and subsequent TEM (Nash *et al.*1987, see Appendix I).

II. 1.6 Case 6

A group of ten *M. lanchesteri* which originated from Singapore were recently obtained from an aquarium shop and maintained in an 80 l glass aquarium in the Tropical Prawn Unit, Institute of Aquaculture. All the prawns displayed varying degrees of brown-black discolouration of the branchiostegite cuticle and gills (Fig.1b). Mortalities of one to two prawns per day were occurring. Microscopic examination of fresh gill and shell mounts showed melanization of gill and shell cuticle, gill swelling and a few algal cells. Samples were taken for histology and subsequently for TEM.

Fig. 1b *Macrobrachium rosenbergii* with brown-black discolouration of the brachiostegite (arrow).


cm
1
2
3
4
5
6
SPECIMEN M lanchesteri
DATE 11-3-81

II. 1.7 Case 7

This case consisted of 42 post larval and juvenile *M. rosenbergii* maintained in 1.0 m diameter fibreglass tanks in a heated recirculation system in the Tropical Prawn Unit, Institute of Aquaculture.

Optimally the total calcium carbonate l vels of the water were kept at 60 to 100 mg/l $CaCO_3$, but over a period of four to six weeks in the initial setting up of the system the total hardness increased to 220-240 mg/l $CaCO_3$. Crushed oyster shell was used in the system as the water used in system was normally too soft.

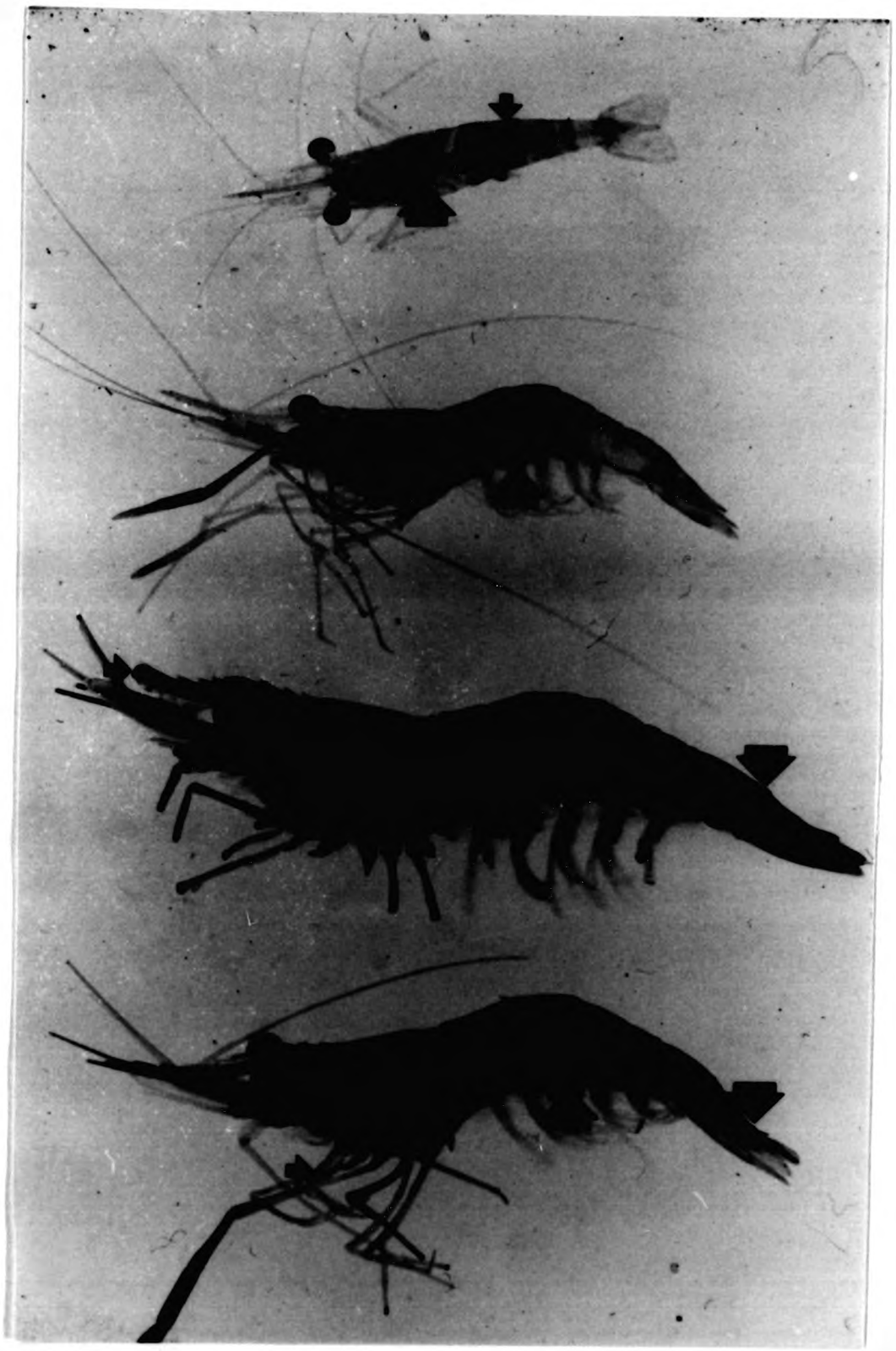
Macroscopically, the prawns displayed one or more randomly distributed circumscribed brown-black nodules on the body cuticle (Fig. 2). Occasionally there were also brown-black discolouration of the gills, shell erosions or ulcerations and appendage loss (Fig. 2). Specimens were prepared for histology.

II.2 Materials and Methods

Post-mortem Procedures and Histology

Prawns were autopsied and the tissues fixed in 10% neutral buffered formalin or Bouin's solution, processed routinely for histology to 5 micrometre paraffin sections. The paraffin sections were routinely stained with haematoxylin and eosin (HE) and selectively with von Kossa, Masson's trichrome and by the periodic acid-Schiff (PAS) reaction (Luna 1968). Specimens were examined microscopically with a BH-2 Olympus light

Fig. 2 Nodular (large arrow) and erosive/ulcerative (small arrow) brown-black cuticular lesions, also with appendage loss (note second prawn from bottom) in juvenile *M. rosenbergii*.



microscope and photomicrographed using the Olympus PM-10 AD system (both from the Olympus Optical Co., Ltd., Japan).

Transmission Electron Microscopy (TEM)

Formalin-fixed tissues were post-fixed in 2.5% phosphate buffered glutaraldehyde followed by 1% osmium tetroxide, dehydrated in graded alcohols, embedded in epoxy resin and sectioned. One micrometre semi-thin sections were stained with toluidine blue, thin sections with saturated uranyl acetate and lead citrate and then examined with a JEOL-100c transmission electron microscope (JEOL Ltd., Japan).

Bacteriology

For bacteriological sampling, portions of the affected tissue (eg shell, muscle) were carefully excised and the surfaces sterilized using 70% or 100% alcohol, rinsed in sterile water or saline, then cut into small pieces using sterilized scissors and forceps and cultured in tryptone soya broth (TSB, Oxoid, UK) for 24 hours at 22°C followed by subculture on tryptone soya agar (TSA, Oxoid, UK) under similar conditions.

Bacteria were subsequently isolated in pure culture and identified using standard bacteriological tests (Frerichs 1984) and the API 20 NE System (API Laboratory Products Ltd., UK).

microscope and photomicrographed using the Olympus PM-10 AD system (both from the Olympus Optical Co., Ltd., Japan).

Transmission Electron Microscopy (TEM)

Formalin-fixed tissues were post-fixed in 2.5% phosphate buffered glutaraldehyde followed by 1% osmium tetroxide, dehydrated in graded alcohols, embedded in epoxy resin and sectioned. One micrometre semi-thin sections were stained with toluidine blue, thin sections with saturated uranyl acetate and lead citrate and then examined with a JEOL-100c transmission electron microscope (JEOL Ltd., Japan).

Bacteriology

For bacteriological sampling, portions of the affected tissue (eg shell, muscle) were carefully excised and the surfaces sterilized using 70% or 100% alcohol, rinsed in sterile water or saline, then cut into small pieces using sterilized scissors and forceps and cultured in tryptone soya broth (TSB, Oxoid, UK) for 24 hours at 22°C followed by subculture on tryptone soya agar (TSA, Oxoid, UK) under similar conditions.

Bacteria were subsequently isolated in pure culture and identified using standard bacteriological tests (Frerichs 1984) and the API 20 NE System (API Laboratory Products Ltd., UK).

II.3 Results

II.3.1 Case 1 -Larval Bacterial Necrosis

Histopathological examination of moribund stage XI *M. rosenbergii* larvae revealed multifocal necrotic and inflammatory lesions in many organs and tissues, including gills, hepatopancreas, antennal gland, eye, nerves, muscle, cuticle, epithelium and connective tissue and especially the appendages. The tissue response consisted of haemocyte infiltration, aggregations, encapsulations and melanization; the multiple red-brown foci observed in the fresh larval specimen were melanized encapsulations. The lesions often contained numerous short rod-shaped bacteria. Some filamentous bacteria and *Epistylis* sp. ciliates were also observed fouling the cuticular surfaces of the body and appendages.

This case was diagnosed as larval bacterial necrosis.

It was considered that water of suboptimal quality and/or overcrowding, increased feed wastage and faeces led to excessive bacterial levels in the tanks. Overcrowding leading to increased prawn interactions could also have been a cause of injury to the shell of the larvae.

It was recommended to carry out bacteriological testing of both prawns and water to identify the causative organisms, their levels and antibiotic sensitivity and resistance. It would be also advisable to

ensure that the water was treated by prior filtration, chlorination and aeration. Overcrowding should be avoided and strict attention should be paid to sanitation in the hatchery especially through cleaning, disinfection and drying of tanks between batches of larvae (see the "sanitary lot technique", Appendix II).

II.3.2 Case 2-Metacercarial Infection

When present, one to seven encysted digenetic trematode metacercariae were found histopathologically per tissue section in eight out of the 13 prawns. The parasites were located mainly in the musculature of the cephalothorax and abdomen and occasionally the appendages, with only one case each with concurrent ovarian or hepatopancreatic infection (Appendix I, Nash 1989, Fig. 1a).

The encysted metacercariae, considered to resemble *Opecoeloides fimbriatus* (Linton, 1934) were encapsulated by one of eight concentric layers of haemocytes (Appendix I, Nash 1989, Fig. 1a), the nuclei usually compressed, elongated and orderly, but occasionally round to oval and disorganized, also displaying varying degrees of necrosis. Fibrous connective tissue was also variably present distal to the haemocyte layers.

The haemocytic encapsulations were most conspicuous in the muscle infections (Appendix I, Nash 1989, Fig. 1a). In some cases the centres of the cysts

were completely filled with a mass of amorphous eosinophilic material, with haemocyte nuclei. The cysts in the hepatopancreas and ovary (Appendix I, Nash 1989, Fig. 1a) led to some compression of the surrounding tissue.

Additional changes considered to have been caused by parasite migration consisted of muscle necrosis with haemocyte infiltration, aggregations and encapsulation, fibroplasia and calcification (Appendix I, Nash 1989, Fig. 2).

Findings unrelated to the trematodes included focal melanization of the gill cuticle and mild to moderate *Vorticella* sp. and *Lagenophrys* sp. infestations. Minimal gill fouling with non-filamentous bacteria was also present.

Nevertheless, the general condition of the prawns was considered to have been good as the hepatopancreases contained much stored lipid and the gonads were mature.

To prevent recurrence of the condition in subsequent prawn crops it was recommended that the ponds be drained, dredged, well dried and limed to help eliminate the snail first intermediate host of the digenetic trematode parasite. Controlling small mammals, aquatic birds and fish, possible definitive hosts of the parasite, would break the parasitic life cycle, in addition to eliminating potential predators of the prawns.

II. 3.3 Case 3-*Citrobacter* Infection

After 24 hours of bacteriological culture there was abundant beige opaque colonial growth, which after 48 hours had a wrinkled surface. The isolate was determined to be a Gram-negative bacillus, motile, oxidase negative, sensitive to novobiocin (10 µg/disc). Using the API 20 NE System the organism was positively identified as *Citrobacter freundii*, a member of the Enterobacteriaciae. This was reported to be a common facultative organism in the Institute Prawn Unit and a potential pathogen under certain conditions (Millar and Alexander, personal communications).

Histopathological examination discovered an area of extensive necrosis of the abdominal musculature associated with multiple large bacterial colonies and a diffuse necrosis and haemocyte infiltration, aggregations and encapsulations were observed throughout the surrounding and in the distant muscle tissues, as well as in the heart, hepatopancreas, gills, ovaries and nervous tissue, indicating a systemic infection.

There was also a slight to moderate fouling of the gill lamellae with filamentous *Leucothrix*- like organisms, non-filamentous bacteria and *Epistylis* sp. peritrichous ciliates and a few holotrichous ciliates.

This case illustrates the potential risk of a primary shell damage (erosion, ulceration) combined with the stress of recent transport in leading to localized

and subsequently to systemic bacterial infection. Treatment of such advanced cases rarely has a successful outcome.

Prevention is the most important means of disease control in prawn culture. Stress and injury should be avoided at all times, especially during transport and handling. Good nutrition, avoidance of overcrowding and maintenance of optimal water quality are essential to good culture. It can also be recommended that prawns showing early signs of shell damage should be treated with an efficacious broad spectrum antibiotic (eg oxolinic acid at 10 mg/l in a bath for one hour, repeated if necessary) as determined by antibiotic sensitivity and resistance testing. Recently transported prawns and introduced to the farm or hatchery should also be quarantined until they are determined to be healthy.

II.3.4 Case 4- *Aeromonas* Infection

Shiny yellow colonies were present on the TSA subculture from adult prawn shell lesion after 24 hours. The isolate was a Gram-negative motile bacillus, oxidase-positive and resistant to vibriostat 0/129 (150 µg/disc). It was tentatively identified as a serotype of *Aeromonas hydrophila*. The causative organism of the white discolouration of the juveniles was also determined to be *Aeromonas hydrophila*. Both isolates were sensitive to oxolinic acid and oxytetracycline in antibiotic sensitivity and resistance testing.

Histopathologically, the shell lesions were characterized by extensive erosion or ulceration of all the cuticular layers, after with necrosis of the underlying epidermis, connective tissue and muscle, with a prominent haemocytic infiltration. aggregations, encapsulations, nodules and melanization, in typical compressed multilaminations. The encapsulations and nodules often contained necrotic cellular debris and bacterial colonies.

The hepatopancreatic tubular epithelium was usually well vacuolated indicating a satisfactory nutritional status, but occasionally multifocal tubular necrosis and haemocytic aggregations were observed. The gills were sometimes swollen, with focal to multifocal haemocyte infiltration, aggregations and encapsulations.

In one prawn with a severe shell lesion, typical macroconidia of *Fusarium* sp fungus and a profusion of non-septate fungal hyphae were present in the affected cuticle and underlying soft tissues. Thus, in the adults the bacterial infection varied in severity from localized shell disease lesions to a systemic condition.

The juvenile prawns displayed a focal to extensive myofibre necrosis associated with bacterial colonies and a haemocytic response of infiltration, aggregation, phagocytosis and encapsulations were present in most organs and tissues, including the heart, gills, hepatopancreas, antennal gland and nervous tissue. This

case was diagnosed as a systemic bacterial infection.

These case demonstrated two clinical expressions of tissue damage in prawns - ie melanized shell disease lesions and opaque white discolouration indicative of necrosis of the muscle. These signs may or may not be infectious in nature. Thus microbiology and histopathology are required for a definitive diagnosis. In these cases the disease agent was *Aeromonas hydrophila*, one of the most common opportunistic bacterial pathogens of cultured prawns.

Prevention is again the preferred means of control. Optimal environmental, and management conditions and nutrition should be provided. The success of treatment is variable depending upon the severity and progression of the infection. If infection can be detected early and treatment carried out quickly there may be better chances of a favourable outcome. Bacterial isolation, sensitivity and resistance testing, followed by treatment of choice are indicated.

II. 3.5 Case 5 -Idiopathic Muscle Necrosis

The principal morphological change in the "milky" prawns consisted of slight to severe, multifocal to diffuse areas of body myofibre degeneration and necrosis (Appendix I, Nash *et al.* 1987, Figs. 1, 2, 3) accompanied by a variable haemocyte reaction of infiltration, aggregation, encapsulation and myophagia (Appendix I, Nash *et al.* 1987, Figs. 2, 3, 4). Focal to

multifocal dystrophic calcification of degenerating muscle tissue was also present (Appendix I, Nash *et al.* 1987, Fig. 3). No bacteria were observed in the muscle lesions indicating their sterile and idiopathic nature. Bacterial culture of affected muscle without resultant colonial growth would have been a necessary step to confirm this.

Some of the prawns also lacked normal hepato-pancreatocyte vacuolation indicating a suboptimal nutritional state.

TEM revealed that the myofibre necrosis involved one or more of the myofibrillar, sarcoplasmic or membranous systems (Appendix I, Nash *et al.* 1987, Figs. 5, 6, 7, 8). Concentric laminated bodies were also observed sequestering granular necrotic material, glycogen or mitochondria (Appendix I, Nash *et al.* 1987, Figs. 6, 7, 8).

Based on clinical, histopathological and TEM findings, a diagnosis of idiopathic muscle necrosis was made. This non-infectious condition is related to stressors in the culture situation. Avoidance of overstocking and increasing the level of dissolved oxygen in the tank water have been effective in preventing a recurrence of the disease in the hatchery.

II. 3.6 Case 6 -Systemic Parasitic Infection

Histopathologically, the affected *M. lanchesteri* branchiostegite epidermis and subcuticular

spongy connective tissue were thicked and structurally disorganized due to a massive infiltration of parasitic cells (Fig. 3). Epidermal cells and fibroblasts had one or more intracytoplasmic vacuoles containing a variable number of apparently unicellular organisms, often in clusters (Fig. 3). The parasitic cells appeared to have a round shape, were 2 to 6 micrometres in diameter with a single nucleus and a multivesicular cytoplasm (Fig. 4). Also present was a mild to severe necrosis of the cuticle and subcuticular tissues, haemocyte infiltration, aggregations and melanization (Fig. 3). The gill lamellae were markedly swollen and structurally disorganized due to the massive parasite infiltration (Fig. 4). Haemocyte infiltration was also noted (Fig. 4).

TEM demonstrated one or more intracytoplasmic elongated tapering parasitic organisms (Fig. 5). Culture of the organisms would have been necessary to make a definitive classification but it was not possible to obtain further samples of infected *M. lanchesteri*. The parasite may have been an amoeba or a form of lower fungus (Lom, personal communication).

This case was of the unusual "one off" variety. It does indicate the importance of quarantine of any newly acquired or introduced stock. With or without any abnormal clinical signs, they should be considered to be potentially infected until verified to be healthy.

Fig. 3 *M. lanchesteri* with marked infiltration of parasitic cells in the epidermis (E) and the subcuticular spongy connective tissue (T) of the branchiostegite cuticle (C) leading to structural disruption, vacuolation and swelling and haemocyte infiltration (h) (HE, X600).

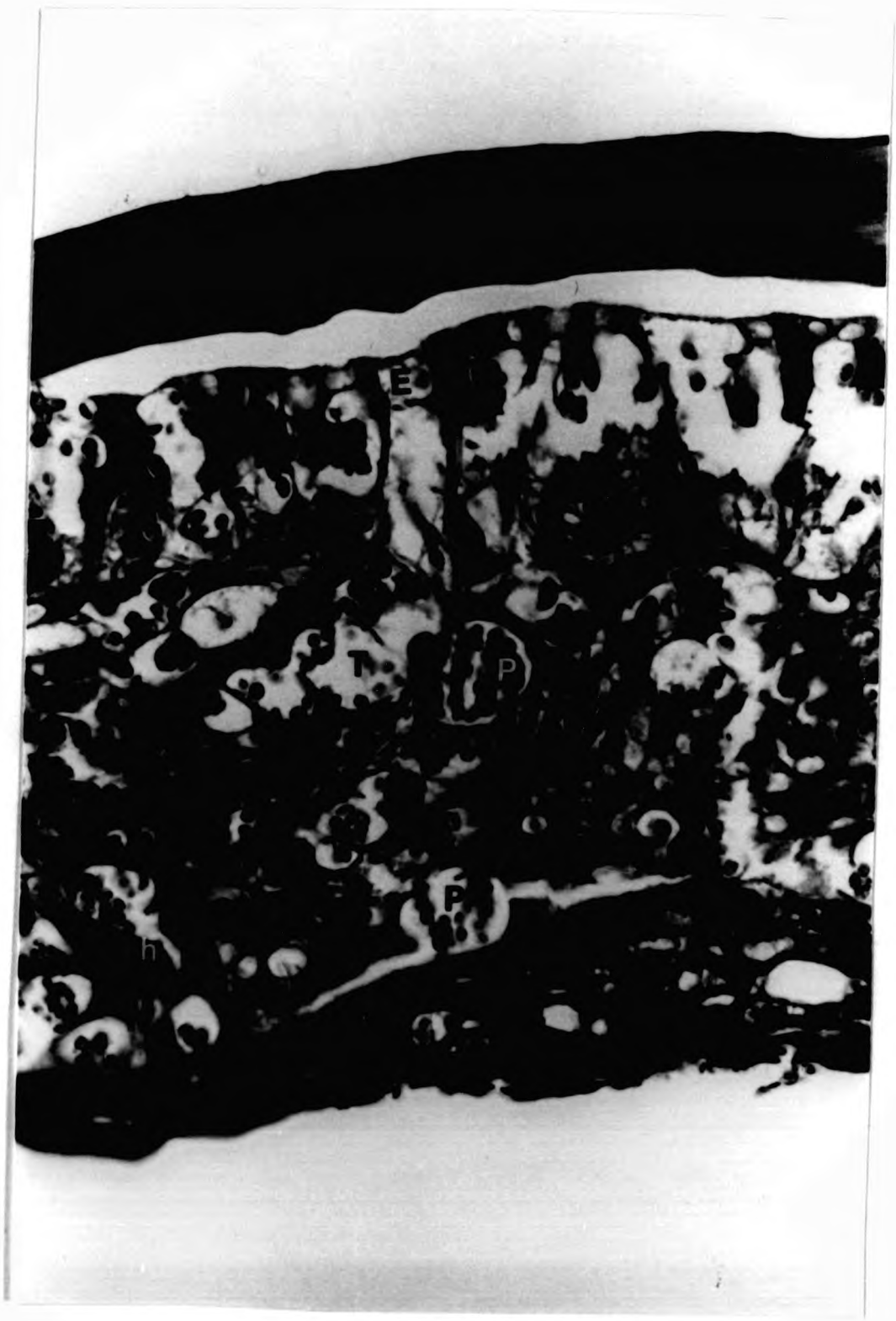

E
T
P
P

Fig. 4 *M. lanchesteri* gill lamella with a massive infiltration of parasite cells (p) causing severe tissue disruption and swelling and haemocyte infiltration (h) (HE, X1500).

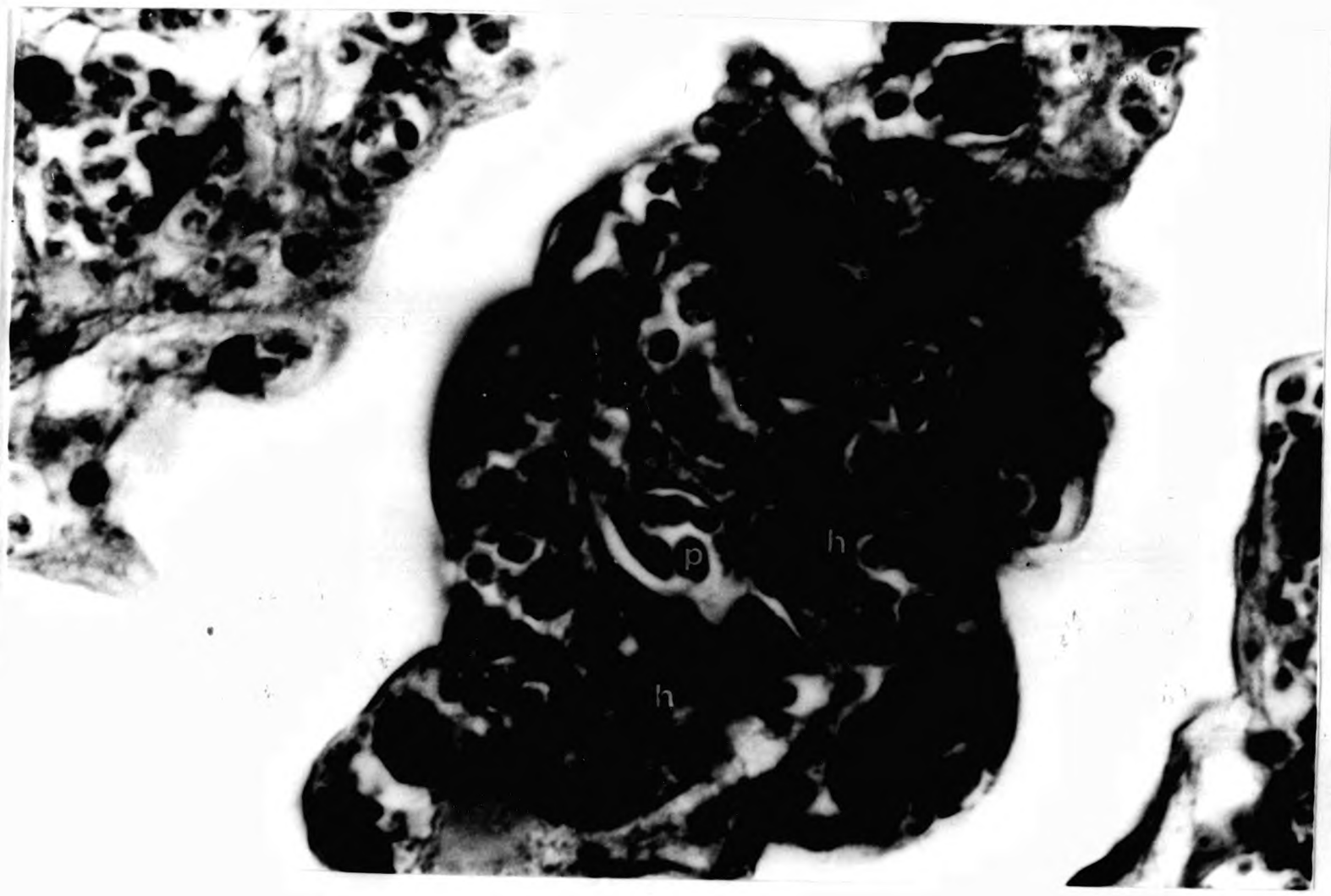
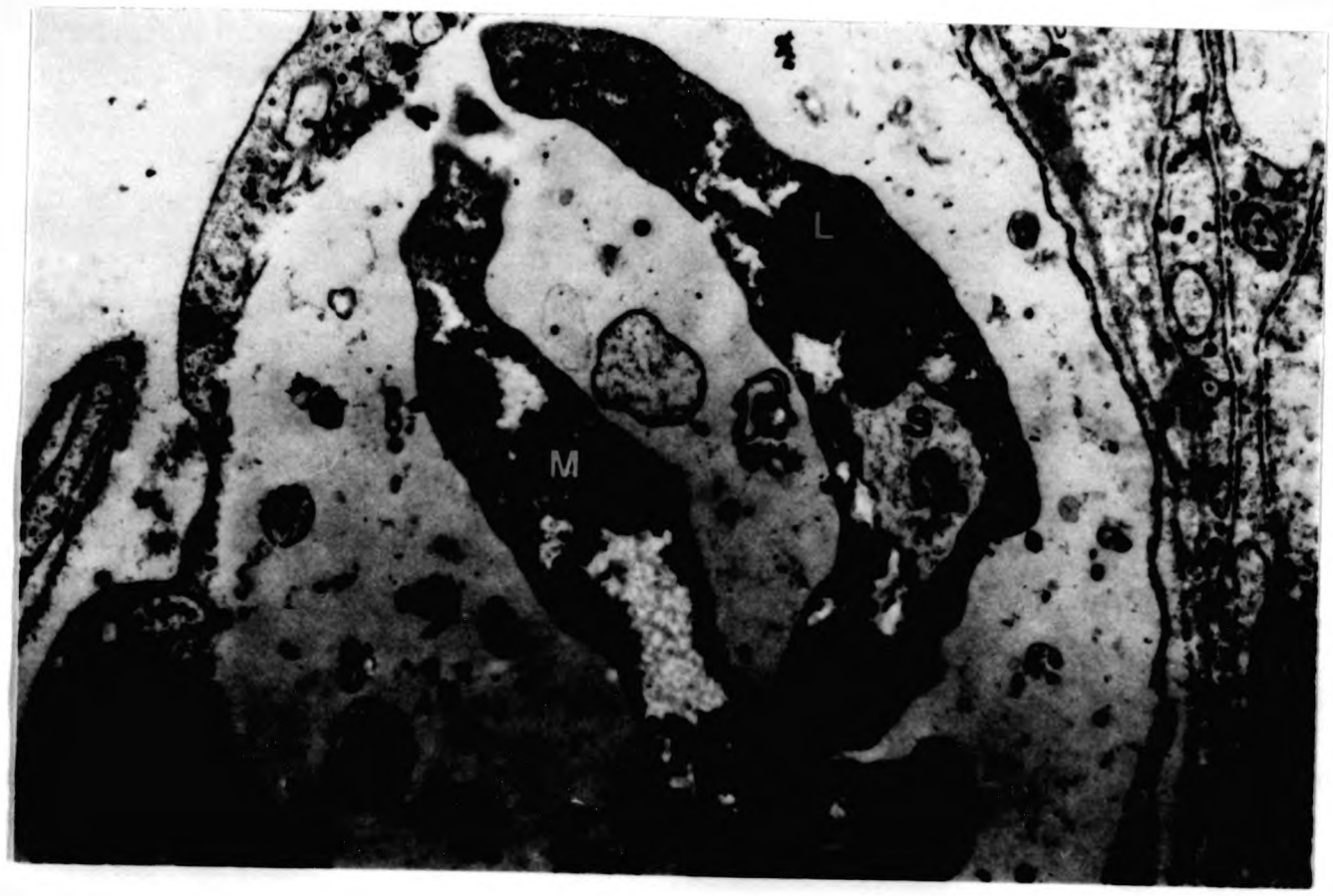

p
h
h

Fig. 5 Elongated tapering parasitic cell within cytoplasmic vacuole in epidermal cell of branchiostegite cuticle of *M. lanchesteri*. Lysosomes (L), mitochondria (M) and vacuolar secondary lysosomes are evident in the parasite (TEM, X8000).


L
M

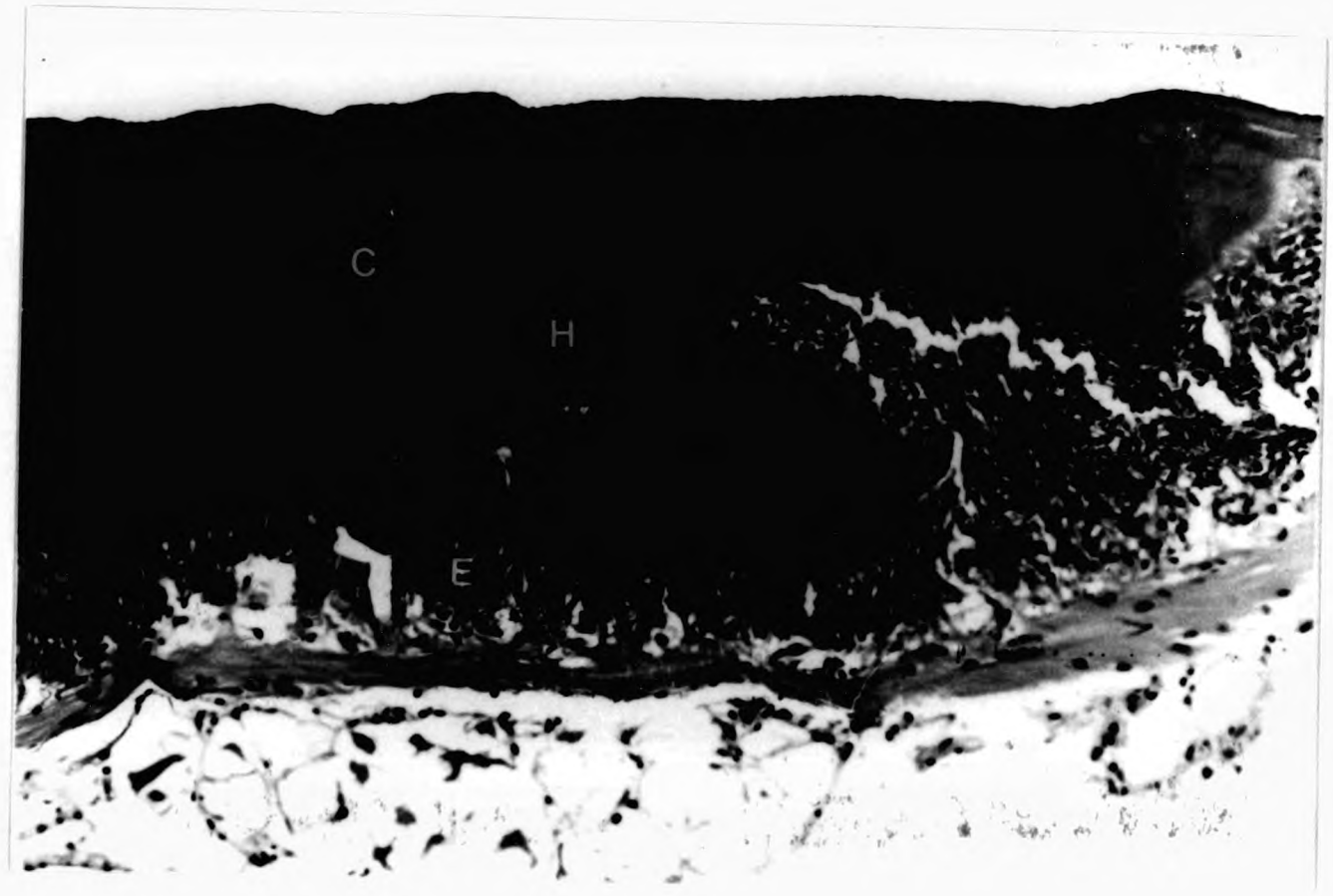
II. 3.7 Case 7 -Hardwater Shell Disease

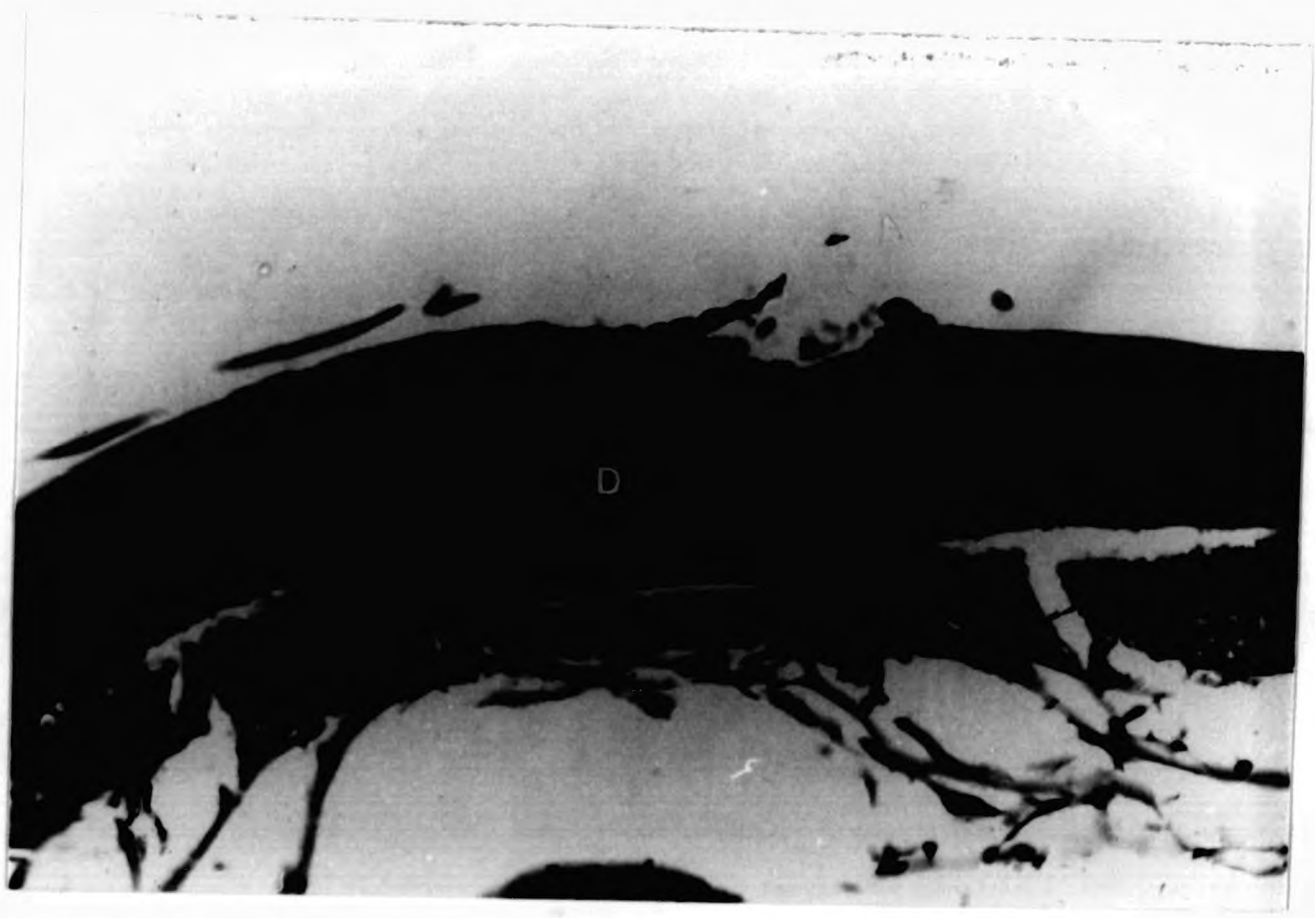
Histologically, the nodular cuticular alterations showed a thickening of the calcified portions of the cuticle, especially the deeper exocuticle and the endocuticle, which often impinged upon and compressed the underlying epidermis and spongy connective tissue (Figs. 6, 7). The thickening of the cuticle appeared to be associated with degenerative changes such as variations in structural density and vacuolation (Fig. 6) which were accompanied by an inflammatory reaction of haemocyte infiltration, aggregation, encapsulation and melanization (Figs. 6, 7). Malformation, erosion or ulceration of the epi-exocuticle were often also present (Fig. 7). There was focal to multifocal thickening and irregularity of the cuticle of gill lamellae, accompanied by a similar haemocytic response as observed in the body shell.

This condition was diagnosed as a form of shell disease related to extremely high water hardness (ie "hardwater shell disease"). With daily freshwater replacements of 3-5% over a three-month period, optimal water hardness levels (60-100 mg/l $CaCO_3$) were eventually reached and thereafter maintained with water replacements of about 0.5% every two days. The disease condition is now uncommon in the Tropical Prawn Unit.

Fig. 6 Increase in thickness of the cuticle (C) of *M. rosenbergii* with hard water shell disease impinges upon the epidermis (E) and is accompanied by degenerative and inflammatory changes, including variations in cuticular density and vacuolation and haemocyte infiltration (H) (HE, X125).

Fig. 7 Concurrent melanized epi-exocuticular erosion (E) and exo-endocuticular degeneration and melanization (D) in *M.rosenbergii* with hardwater shell disease (HE, X250).


C
H
E


D

CHAPTER III: Studies on the Tissue Response of Prawns to Experimentally Induced Insults

III.1 Introduction

Complementing the diagnostic studies, this series of experiments was designed to investigate and compare histologically *Macrobrachium rosenbergii*'s basic tissue response to injection of a number of biological and non-biological materials, as well as to traumatic injury and grafting.

A TEM study was also carried out to more precisely identify the types of haemocytes which participate in the tissue defence reaction. Scanning electron microscopy was done in addition to histology to study the cuticlar reaction to trauma.

III. 2 Materials and Methods

Prawns

For experiments carried out in Stirling, the juvenile giant Malaysian freshwater prawns, *Macrobrachium rosenbergii* de Man, the offspring of broodstock originating from Malaysia and Thailand, were reared in the tropical prawn aquarium facilities at the Institute of Aquaculture, University of Stirling, and ranged in total length from 6 cm to 10 cm and in weight from 3 g to 13 g.

For experiments carried out in Malaysia, juvenile and adult *M. rosenbergii*, the offspring of Malaysian broodstock, were reared in the greenwater tank systems and in the ponds at the Faculty of Fisheries and Marine Sciences, Universiti Pertanian Malaysia (UPM), and

ranged in total length from 10 cm to 13 cm and in weight from 10 g to 18 g (Fig. 8).

For the experiment done in Thailand, ongrowing juvenile *M. rosenbergii* were obtained from a private growout farm in Suphanburi Province. Prawns were 8 cm to 10 cm in length and 7 g to 11 g in weight.

Twenty-four to 48 hours after the aquarium and tank systems were established, a few prawns were introduced into each (see below) to ensure that the systems were functioning properly and that the prawns would survive; then, a further 24 hours later, after no abnormalities in the prawns or systems were observed, the remaining experimental prawn groups were acclimated for an additional 24 to 72 hours before commencement of the experiments.

The supply of suitably sized prawns available for experimentation at Stirling and UPM was limited but it was attempted to make the best use of those numbers available. Some experiments performed at UPM had to be terminated earlier than intended because of poaching of the prawns. Experimental animals consisted of treated prawns (received test inoculum for the injection experiments or undergoing procedures of grafting or traumatization) in groups of 20 to 36, positive control prawns (received same amount as treated prawn of sterile 0.85% phosphate buffered saline (SPBS), pH 7.2) in groups of 10 to 20, and/or negative control prawns (maintained without injection, grafting or traumatization) in groups

of 10 to 20. For all the injection experiments, sterile 1 ml tuberculin syringes and sterile 23 or 25 G needles were used.

After prawns had undergone the experimental injection (Fig. 9) or procedure at the beginning of each study, two to four prawns per sampling time, throughout the course of each experiment, were sequentially sacrificed and autopsied (see below) for histological, transmission electron microscopic (TEM) and/or scanning electron microscopic (SEM) processing and subsequent microscopic, TEM and SEM examination. Prawns were observed two or more times daily for clinical signs, morbidities and mortalities during all the experiments. Intact animals which had died were also handled and examined as above for histology, the ante-mortem lesions differentiated from post-mortem changes, but these findings were not included in the results report. Positive and negative controls were sacrificed, autopsied, processed and examined as above at the beginning (immediately after SPBS injection for positives), at 24 hours, at 7 and 14 weeks and at the end of the experiments.

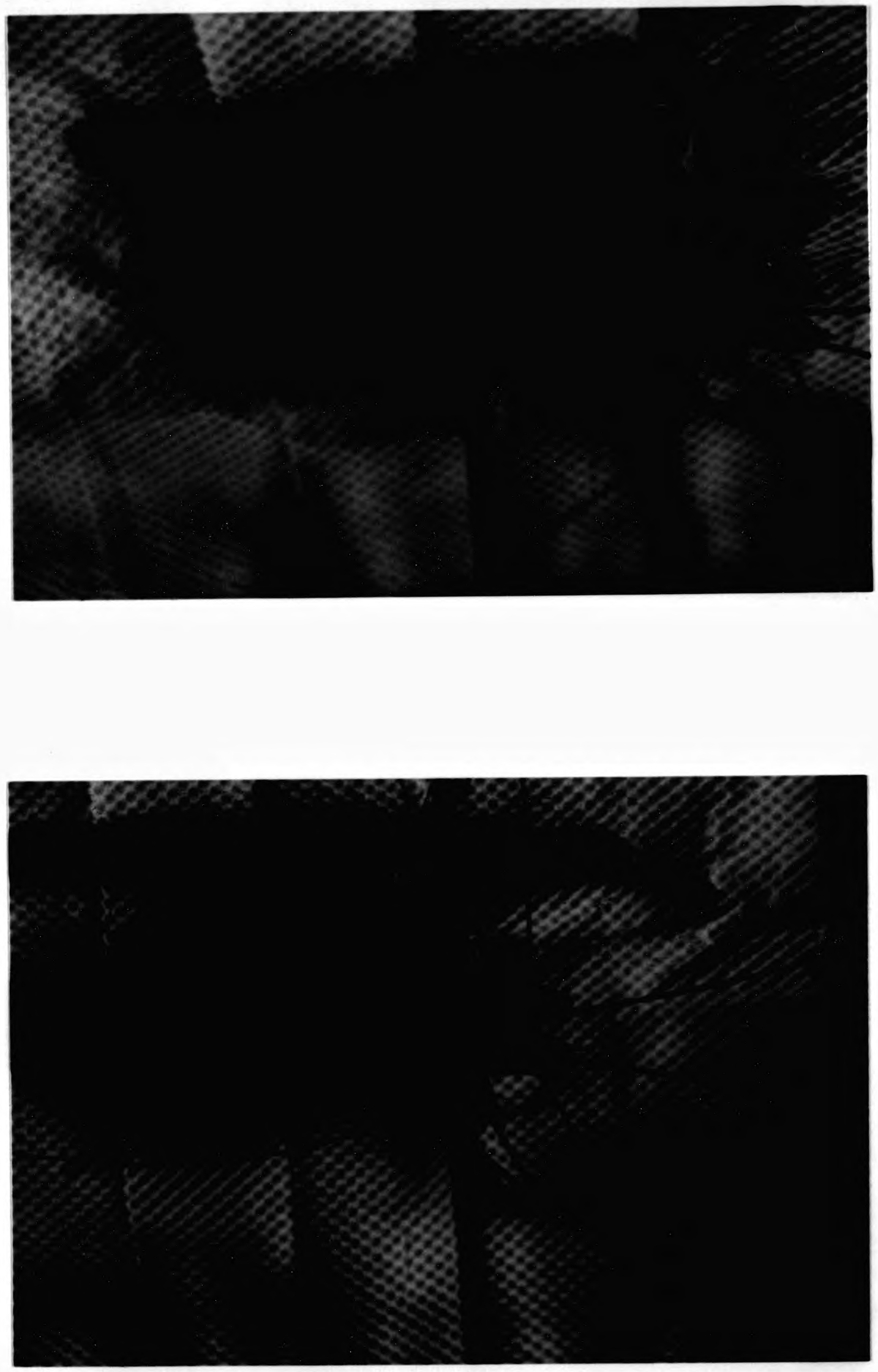
Aquaria, Tanks and Pond

Experiments at Stirling

For the auto-and allograft, *Vibrio*, alcian blue and BCG experiments four 80 or 100 l glass iodophor- or bleach- (sodium hypochlorite) disinfected tanks containing 60 or 80 l of water, respectively, covered

Fig. 8 Healthy untreated negative control *M. rosenbergii*.

Fig. 9 Healthy *M. rosenbergii* 24 hours after injection of *Aeromonas hydrophila* i.m. in the second abdominal segment, with focal cuticular melanization at the site of injection (arrow), present in all injected prawns. Melanization on the third segment (arrow), a common lesion in experimental animals, is usually caused by backward swimming and bumping into the side of the tank.



with plastic lids were used indoors under artificial lighting conditions (12 hours light, 12 hours darkness). Black plastic bags were also used to limit the amount of light entering the tanks to decrease stress.

All water used was copper- and chlorine-free, aged and aerated freshwater. Water temperature was maintained at 28 +/- 1°C using 150 and 200 W in-tank, thermostatic aquarium heaters (Armitages, Nimrod, Armitage Bros. PLC, UK) and monitored with a thermometer daily. Continuous supplementary aeration was supplied with piped-in air and airstones, one per aquarium.

Closed recirculation systems, Eheim filters (model 2013, Eheim, FRG), seeded with aged plastic media (known to have adsorbed *Nitrosomonas* and *Nitrobacter* spp.) from recirculation systems containing healthy Tilapia in the Institute tropical aquarium and packed with aquarium wool and activated charcoal or zeolite were used. Artificial habitats consisting of plastic pipes and plastic coated wire mini-platforms were used in the tanks as prawn hides to increase surface area and decrease prawn interactions and stress.

The excess uneaten food and faeces were siphoned daily from the aquaria using a plastic hose and about 10% of the aquarium water was replaced daily with aged, aerated, heated water. Aquarium water was monitored initially for unionized ammonia by the phenol-hypochlorite method (Golterman, Clymo & Ohnstad 1978), for pH using a pH meter (PW 9409 Digital PH meter,

Philips, UK) and regularly for total $CaCO_3$ hardness using test strips (Sofstix, Ames Co. Division, Miles Laboratories Inc., USA).

Experiments at UPM

For the *Aeromonas* and complete Freund's adjuvant experiments a series of six outdoor 203 1 (93 x24 cm) bleach- disinfected circular fibreglass tanks (Fig. 10) equipped with simple undergravel airlift pump filters (flow rate about 2 l/min) were employed using aged chlorine- and copper-free freshwater. Temperature varied from 24 to 28°C. Nylon nets were employed in the tanks as artificial habitats to reduce prawn interactions (Fig. 11).

For the shell traumatization experiment, the prawns were kept in four 1 m^2 nylon mesh cages secured on wooden poles in a 442 m^2 earthen pond with a water depth of about 0.8 m (Fig. 12). During pond preparation the pond had been fertilized using chicken manure at the rate of 260 kg/ha, limed with quicklime at the rate of 671 kg/ha and insects were controlled by applying 50 l of diesel oil with 50 ml of liquid detergent and 5 l of water per hectare of pond. The pond inlet was screened using 0.4 mm mesh size netting and the outlet using 1 mm mesh size netting . The pond had a one half horse power paddle wheel installed which was operated from 2200 to 0900 hours daily. The pond had been previously stocked with 3,500 animals or 7.9 juveniles/m^2. Temperature range was 24-28°C. Dissolved oxygen, pH, water hardness and temperature were monitored daily.

Fig. 10 Fibreglass tanks used for experiments carried out at UPM.

Fig. 11 Inside of one of the above tanks showing prawns with undergravel airlift pump and nylon net artificial habitat.

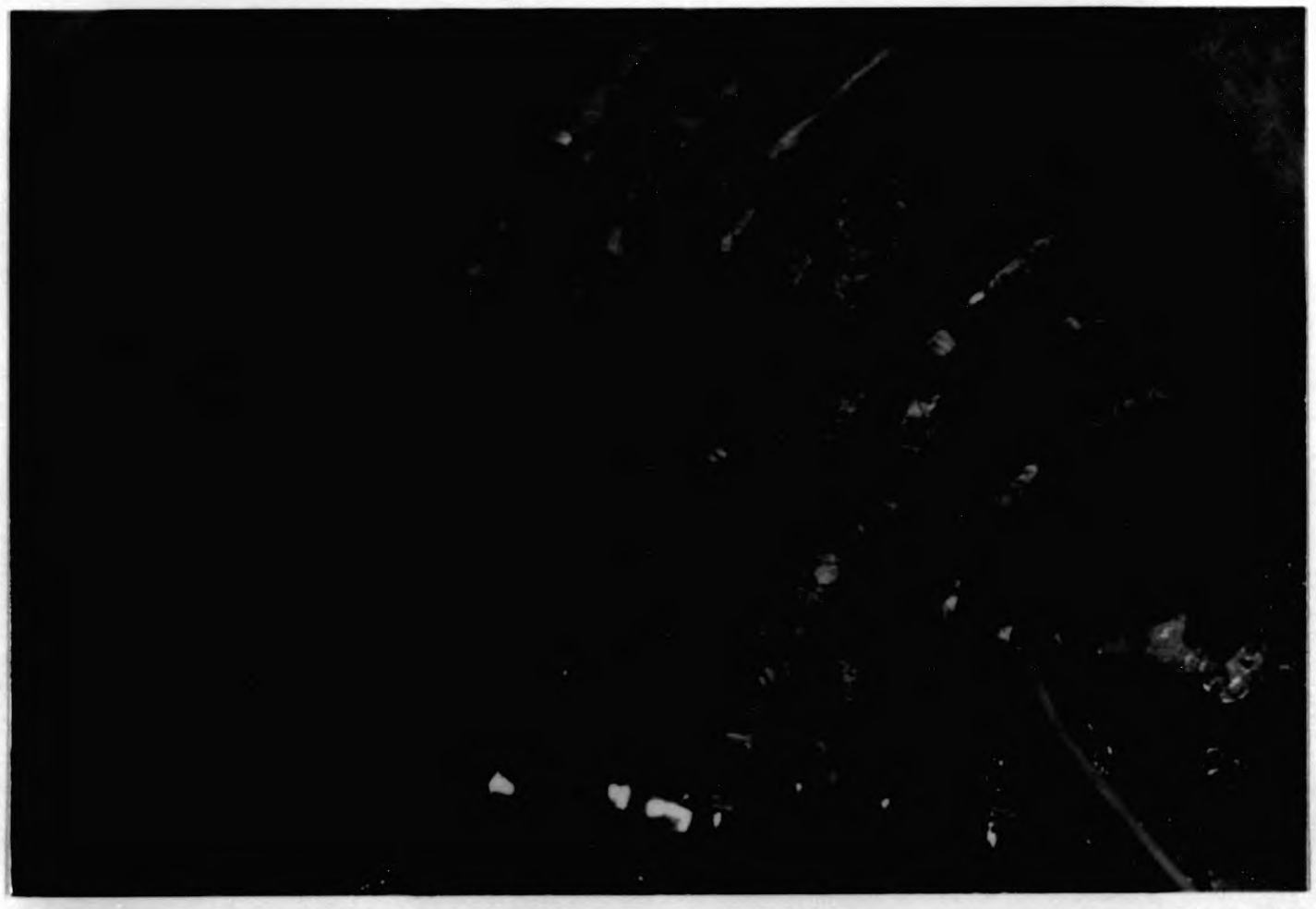


Fig. 12 Obtaining samples for the cuticular traumatization experiment from nylon net cages in the pond system at UPM.

Experiment in Thailand

For the BCG TEM experiment carried out in the Shrimp Health Centre, Mahachai wet laboratory, six bleach-disinfected 60 l aquaria, each with two undergravel airlift pump filters, were used. Underground fresh spring water which was previously aged and aerated was maintained at a temperature of 24-27°C. The aquaria and water were maintained as for the experiments done at Stirling, initially monitoring dissolved oxygen (DO), pH and total hardness.

Diet

A complete balanced pelleted prawn diet of 30%, 35% or 42% protein was fed once or twice daily in the aquaria and tanks and three times daily in the pond. In the aquaria (under artificial lighting conditions) this diet was supplemented with spinach and lettuce twice weekly.

Inocula and/or Experimental Techniques

(1) Intramuscular Injection of Alcian Blue

Alcian blue was shown previously to induce a systemic inflammatory response in fish (Nash 1983) and hence was chosen for use in this study.

Preliminary experiments determined the optimum concentration and volume of the inoculum for use in the main experiments and that for maximum animal survival, injection should cease when the gills were macroscopically replete with pigment. 0.01-0.02 ml of a

1% w/v solution of alcian blue 8GX (Ingram Blue 1, colour index 74240, Hopkins & Williams (GURR), UK) was injected very slowly through previously sterilized (using 70% alcohol, for this and all the injection experiments described below) cuticle into the first abdominal segment muscle. For two experiments, prawns were sampled at: immediately following (0 time), 5 and 15 minutes, 1, 4, 6, 8, 12, 24 hours, 2, 3, 4, 5, 6, 7, 14, 21 and 42 days after injection.

(2) Intramuscular Injection of BCG Vaccine

An immunogenic material, a suspension of Bacillus Calmette-Guerin (BCG) Vaccine BP (Evans, UK) (0.05-0.1 ml) containing a strain of live attenuated tubercle bacillus was injected via sterilized cuticle into the first abdominal segment muscle. For two experiments, prawns were sampled at 1, 4, 6, 12, 24 hours, 2, 3, 4, 5, 6, 7, 14, 21, 31 and 35 days after injection.

A third experiment was carried out in Thailand using the above procedure and sampling prawns at 1, 4, 8, 12, 24 hours and 2, 3, 5, 7, 14, 23, and 32 days after injection. Specimens were taken for histology and for transmission electron microscopy (see below).

(3) Intramuscular Injection of Complete Freund's Adjuvant

Well known for its ability to enhance the immune response, complete Freund's adjuvant (CFA) (each

1ml containing 1 mg of heat killed and dried *Mycobacterium tuberculosis* -H 37 Ra ATCC 25177-, 0.85 ml paraffin oil and 0/15 ml mannide monoalate) (Catalogue No. 17-5721, Grand Island Biological Co. Ltd., Grand Island, New York 14072, USA) was mechanically agitated for 5 minutes to mix the contents of the vial, then 0.1 ml was injected via sterilized cuticle into the muscle of the second abdominal segment. Sampling times were 1, 2, 3, 4, 6, 12, 24 hours and 2, 3, 5, 7, 8, 10, 12, 14, 17, and 21 days after injection.

(4) Cuticular Autografting and Allografting

The tissue response to cuticular grafts or inserts in the muscle was investigated. Cuticular grafts were prepared by incising small pieces (approx. 0.2 x 0.5 mm) of cuticle from the uropods and rinsing these in SPBS. The cuticular pieces were inserted via 0.2 mm wide similarly cleaned cuticular incisions into the muscle of the second and/or sixth abdominal segments of the same (autograft) or different (allograft) prawns. For two experiments, prawns were sampled at 3, 6, 12, 18, 24, 26 hours, 2, 2.5, 3, 4, 6, 7, 9, 10, 11, 14, 21 and 28 days after injection.

(5) Systemic and Intramuscular Injection of *Aeromonas hydrophila*

Aeromonas sp. and *Vibrio* sp. (below) were used in this study because they are important opportunistic pathogens of prawns and shrimps and usually cause an

inflammatory response in the tissues (Nash unpublished reports 1986-1990).

A pure culture of *Aeromonas hydrophila* identified using routine bacteriological methods (Frerichs 1984) and the API 2ONE System (API Laboratory Products Ltd., UK) previously isolated from the shell disease lesion of a moribund *Macrobrachium rosenbergii* female broodstock maintained in the Institute hatchery was used. The bacterium, maintained on a tryptone soya agar (TSA, Oxoid, UK) slant culture at 22 and 30°C was subcultured on TSA plates, examined after 24 hours for purity, then subcultured again in a 10 ml tryptone soya broth (TSB, Oxoid, UK) cultured for 24 hours at 30°C; the broth culture was centrifuged at 4°C (Denley BR401 refrigerated centrifuge, Denley Instruments Ltd., UK) for 15 minutes at 1,200 g, the supernatants discarded, the pellet washed twice and resuspended in 10 ml 0.85% SPBS. The final bacterial inoculum contained 1 x 10^8 colony forming units (CFU)/ml determined using a viable count method (Miles & Misra 1938). the bacterial suspension was killed before use by heating in a water bath for 10 minutes at 60 °C. As preliminary experiments using comparable dosages of live *Aeromonas* invariably led to 100% mortality within 24 hours, only heat-killed bacteria were used in the main experiments. Both systemic (via the haemocoel) and intramuscular routes were investigated. Systemic injection of the heat-killed *Aeromonas hydrophila* was performed into the ventral haemocoel adjacent to the fifth pereiopod and intramuscular

injection into the second abdominal segment muscle, both via sterilized cuticle. Sampling times were 1, 3, 6, 12, 18, 24 hours, 2, 3, 4, 5, 6, 7, 8, 10, 15, 17, 21, 25 and 28 days after injection.

(6) Systemic and Intramuscular Injection of Heat-killed and Live *Vibrio* spp.

Inocula of 0.1 ml containing live and heat-killed (chitinase, lipase and gelatinase positive) *Vibrio parahaemolyticus* (final concentration of 1 x 10^7 CFU/ml) and (chitinase and lipase positive) *Vibrio anguillarum* (final concentration of 1 x 10^8 CFU/ml) originally isolated from shell disease lesions of moribund *Penaeus monodon* Fabricius broodstock and identified as above were prepared and injected into prawns as described for the *Aeromonas* experiments (except that the cultures were maintained at 22°C on marine agar (MA, Difco, UK) and subcultured in marine broth (MB, Difco, UK) and the live bacterial suspensions were not heat-treated). For six replicate experiments, prawns were sampled at 30, 45 minutes, 1, 2.5, 6, 12, 18, 24 hours, 2, 3, 4, 5, 7, 10, 14, 21 and 28 days after injection.

(7) Cuticular Traumatization

The inflammatory response of the shell or cuticle and its associated tissues to trauma was studied. The cuticle of the second abdominal segment was disinfected with 70% alcohol and abraded with a sterile #22 scalpel blade in slight (epicuticular depth) and moderate (exocuticular/endocuticular depth) degrees;

marked damage consisted in cutting 1 mm^2 of cuticle (membranous layer/ epidermal depth) from the second abdominal segment using similarly disinfected surgical scissors. Control group prawns were not traumatized. Sampling times were 1, 2, 3, 6, 8, 10, 14, 21 and 28 days after traumatization. Specimens were also taken for scanning electron microscopy (see below).

Post-mortem Procedures and Histology

Prawns were sacrificed by first placing them in chilled water (when possible) and incising the supraoesophageal ganglion with scissors or scalpel. The entire cephalothothorax (with stomach, haemocoel, haematopoietic tissue, hepatopancreas, heart, antennal gland, connective tissue, muscle, nerve and ganglion) was then incised longitudinally for experiments 1, 2, 3, 5 and 6 and fixed along with the gills, first, second and/or fifth abdominal segments (with cuticle, epidermis, connective tissue, muscle and gut) in 10 % neutral buffered formalin for at least 48 hours. For experiments 6 and 7, only the abdominal segment with the graft or which had been traumatized was incised and similarly fixed. Fixed tissue specimens were later trimmed and cassetted. These were processed routinely for histology using an automatic tissue processor (Shandon Elliot, UK), embedding machine (Raymond A. Lamb, UK) and rotary microtome (Leitz, FRG) to 5 micrometres thick sections. Prior to cutting, the faces of blocks containing cuticular tissue were decalcified using rapid Decal

decalcifier (RDC, Bethlehem Instruments Ltd., UK). Tissue sections were routinely stained with haematoxylin and eosin (HE) and selectively by periodic acid Schiff and Masson's trichrome techniques (Luna 1968). Slides were examined microscopically with a BH-2 Olympus light microscope (Olympus Optical Co., Ltd., Japan) and photomicrographed using the Olympus PM-10AD system (Olympus Optical Co., Ltd., Japan).

Scanning Electron Microscopy

Formalin-fixed normal and traumatized (slight degree, 2 and 28 days; moderate degree, 2, 8, and 21 days; marked degree, 6, 14, 21 and 28 days) second abdominal segments were dehydrated in two 2 hourly changes each of 20, 40, 60, 70 and 100% acetone. The specimens were critically point dried using a Polaron (Watford, UK) critical point drying chamber, sputter coated (S150 sputter coater, Edwards, UK) and examined with an ISI-60A scanning electron microscope (International Scientific Instruments, Japan).

Transmission Electron Microscopy

Prawns were sacrificed as above and gills and muscle tissue at the site of BCG injection were dissected and cut with a sharp microtome blade into 1 mm square pieces. These were immediately fixed in chilled 2.5% glutaraldehyde in 0.1 N sodium cacodylate buffer, pH 7.4, and refrigerated at 4°C. Additionally, gill, hepatopancreas, muscle and heart were fixed in 10%

neutral buffered formalin as above. For further TEM processing, tissue specimens were secondarily fixed in 1% osmium tetroxide, dehydrated in graded alcohols, embedded in epon and sectioned with a LKB-Bromma Ultratome V 2088 ultramicrotome (LKB Produkter AB Research Instruments, Bromma, Sweden). One micrometre semi-thin sections were stained with 1% toluidine blue and thin 500-700 angstrom sections with aqueous uranyl acetate, counterstained with lead citrate, mounted on uncoated copper 300 mesh grids and then examined using a Hitachi HU-12A transmission electron microscope (Hitachi, Ltd., Tokyo, Japan).

III. 3 RESULTS

Gross Findings

For all injection experiments (i.e. alcian blue, BCG, complete Freund's adjuvant, *Aeromonas* and *Vibrio*) and SPBS controls the prawns' cuticular sites of injection displayed focal macroscopic brown discolouration (i.e. melanization) within 24 hours of treatment (Fig. 9). The melanization remained until moulting which occurred as early as one day, but usually 2 to 4 weeks later. The negative control prawns (Fig. 8) did not display this change.

Mortalities

Prawn mortalities in the experiments ranged from 0 to 37%. Histological results from mortalities were not included due to the high degree of post-mortem autolysis.

Controls

No significant changes were observed in the organs or tissues of the negative controls (Figs. 13, 14, 15, 16, 17, 18) or positive controls (including the gills as in Smith & Ratcliffe (1980b) except for normal minimal haemocytic activity (Fig.17) (considered to be base line) and slight to moderate muscle necrosis and haemocytic infiltration in the muscle considered to be due to trauma associated with needle insertion in the injection experiments. Gross cuticular melanization in the injection site was similar for all injected animals and all lesions were healed by melanized haemocytic multilaminations.

The organs and tissues of the controls (Figs. 13, 14, 15, 16, 17, 18) are considered to be "normal' and can be used for comparative purposes.

III. 3.1 The Tissue Response to Alcian Blue Dye Injection

Gross Findings

The blue dye spread rapidly throughout the prawns' tissues during injection imparting a deep blue colouration to most of the body. Most notably, the pigment appeared to accumulate in the ventral haemocoel and ventral abdominal vein, along the hind- and midgut and dorsal abdominal artery, into the gills, and to a lesser extent, into the dorsal haemocoel, pericardial sinus and heart and the bases of the antennae and antennules. The prawns remained variably blue in colour

Fig. 13 Normal intermoult cuticle in a clinically normal prawn. Epicuticle (P), exocuticle (X), endocuticle (N), membranous layer (M), epidermis (E), subcuticular spongy connective tissue (C) (HE, X500).

Fig. 14 Normal intermoult gills in a negative control prawn (BCG injection experiment 4 weeks). Gill stem (S), gill lamella (L), tegmental gland (T) (HE, X250).

FIG. 13

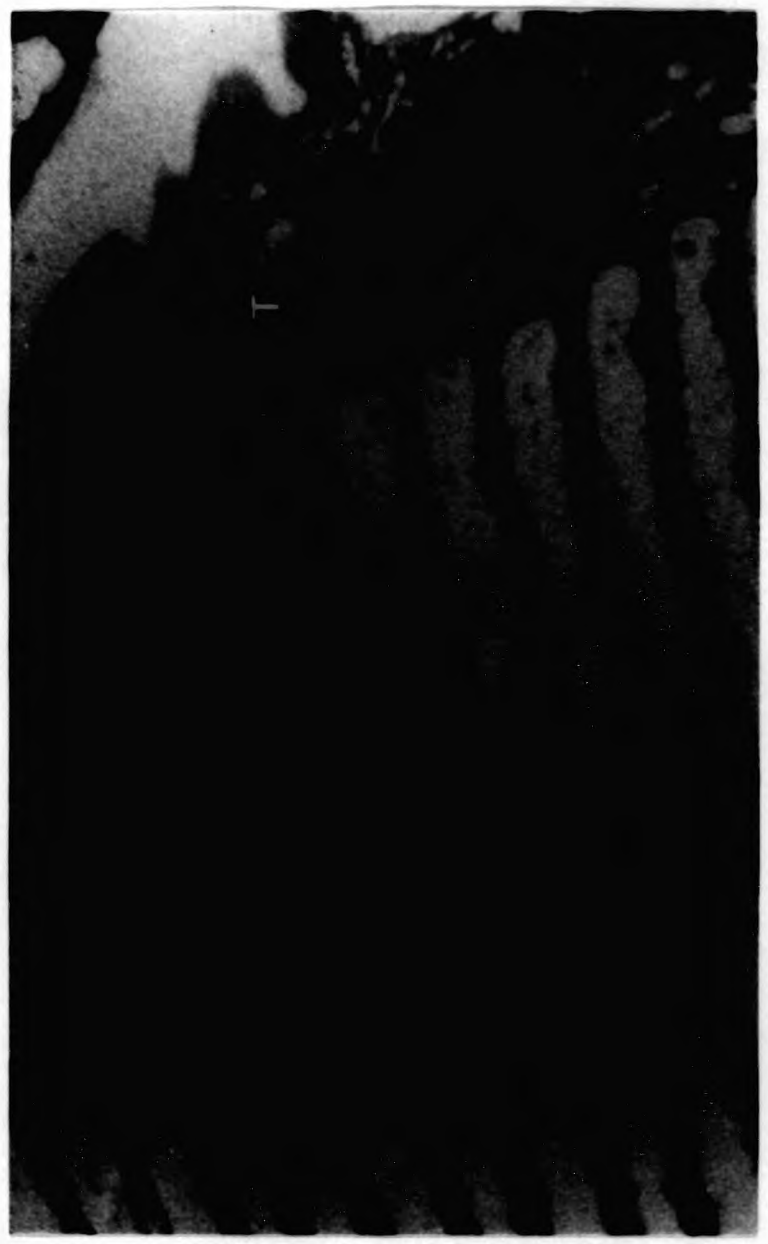


FIG. 14

Fig. 15 Normal heart in a negative control prawn (BCG injection experiment, 4 weeks). Peri-epicardium (E), myocardial bundles (M) (HE, X250).

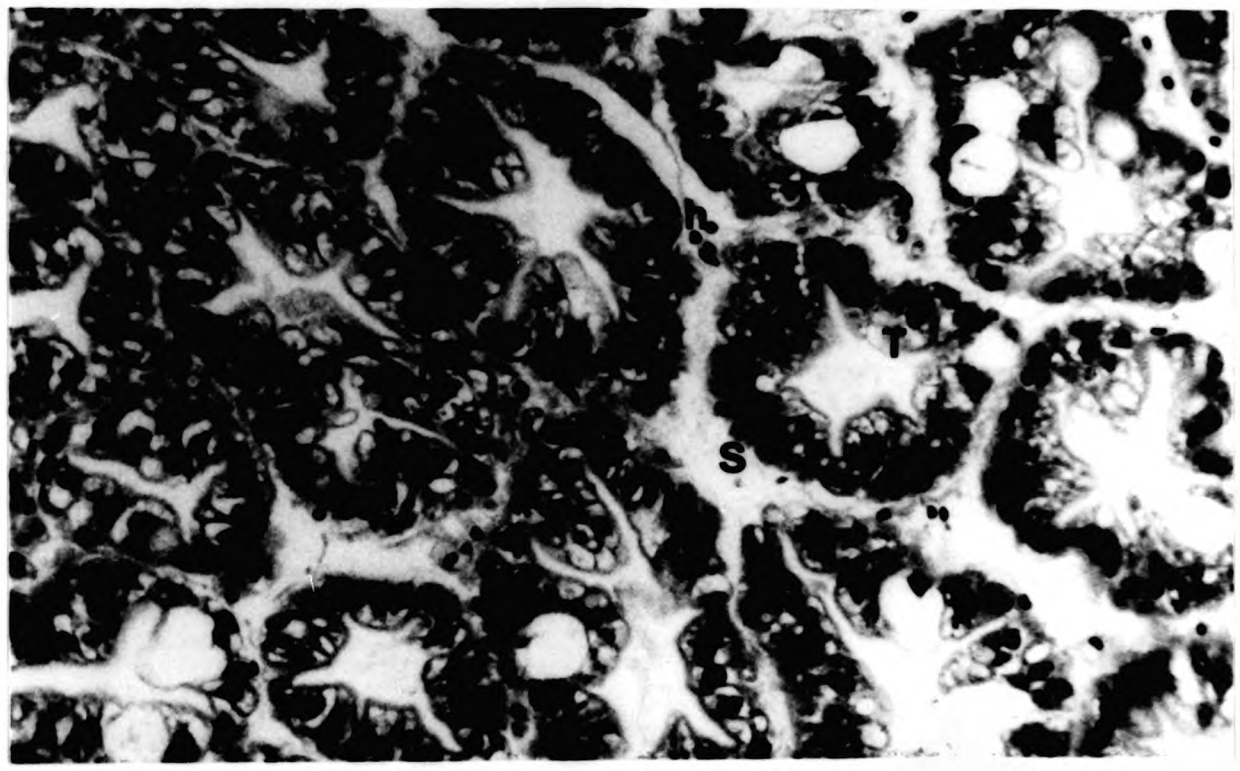
Fig. 16 Normal abdominal muscle tissue in a negative control prawn (BCG injection experiment, 4 weeks). Myofibres (M) (HE, X250).


M


M

Fig. 17 Normal hepatopancreas in a negative control prawn (BCG injection experiment, 4 weeks). Hepatopancreatic tubule (T), haemal sinus (S) with a few haemocytes (h) (HE, X250).

Fig. 18 Normal antennal gland labyrinth in negative control prawn (BCG injection experiment, 4 weeks). Tubules (T) are in close apposition so that the haemolymph sinuses (arrow) between them are not readily apparent (HE, X250).


S
T

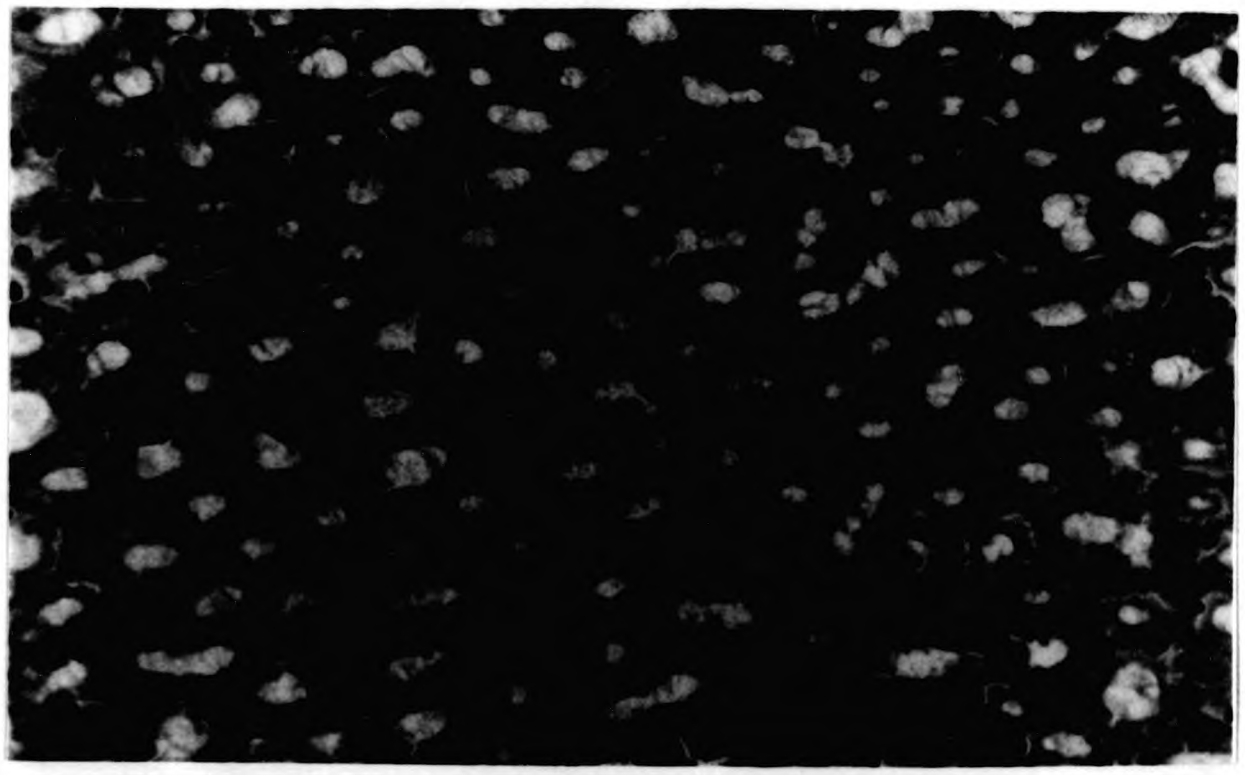
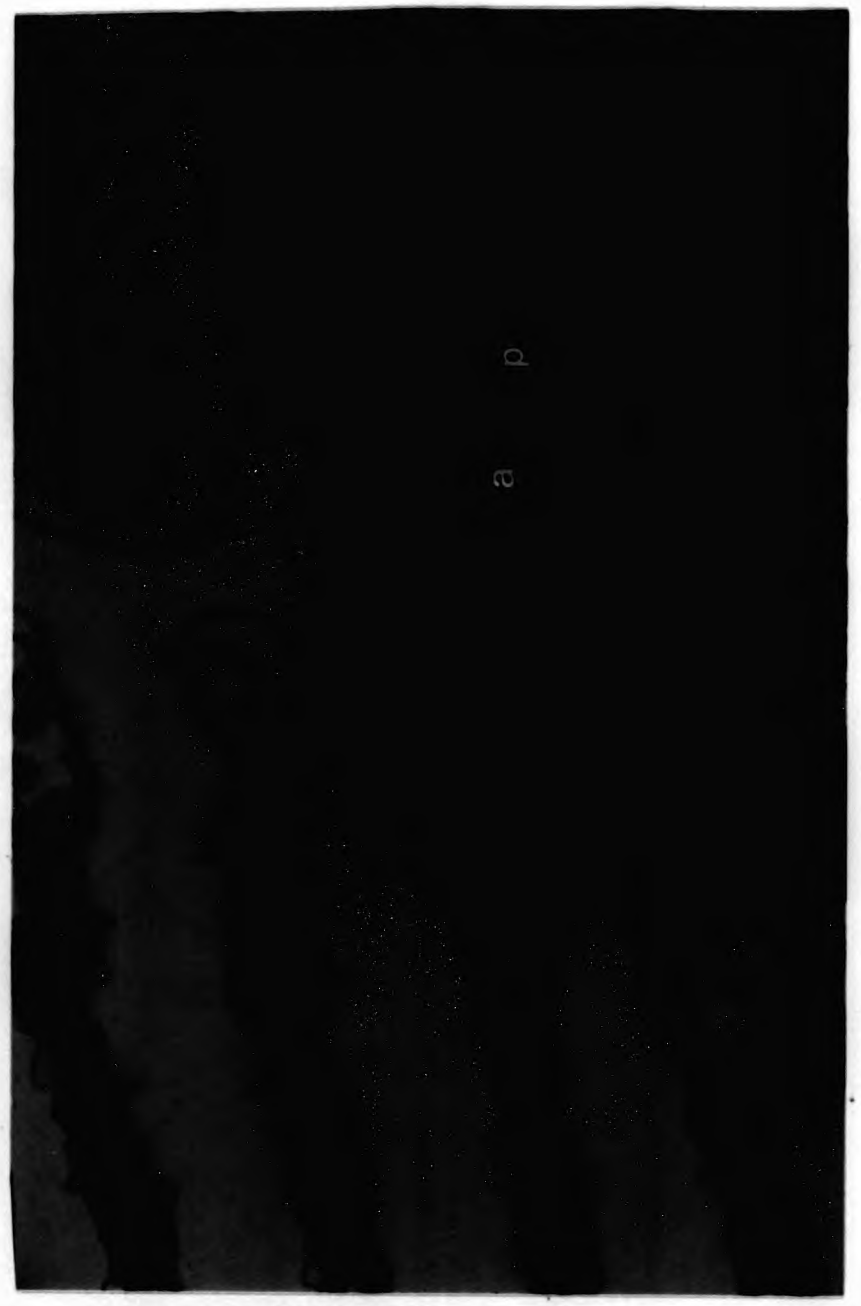
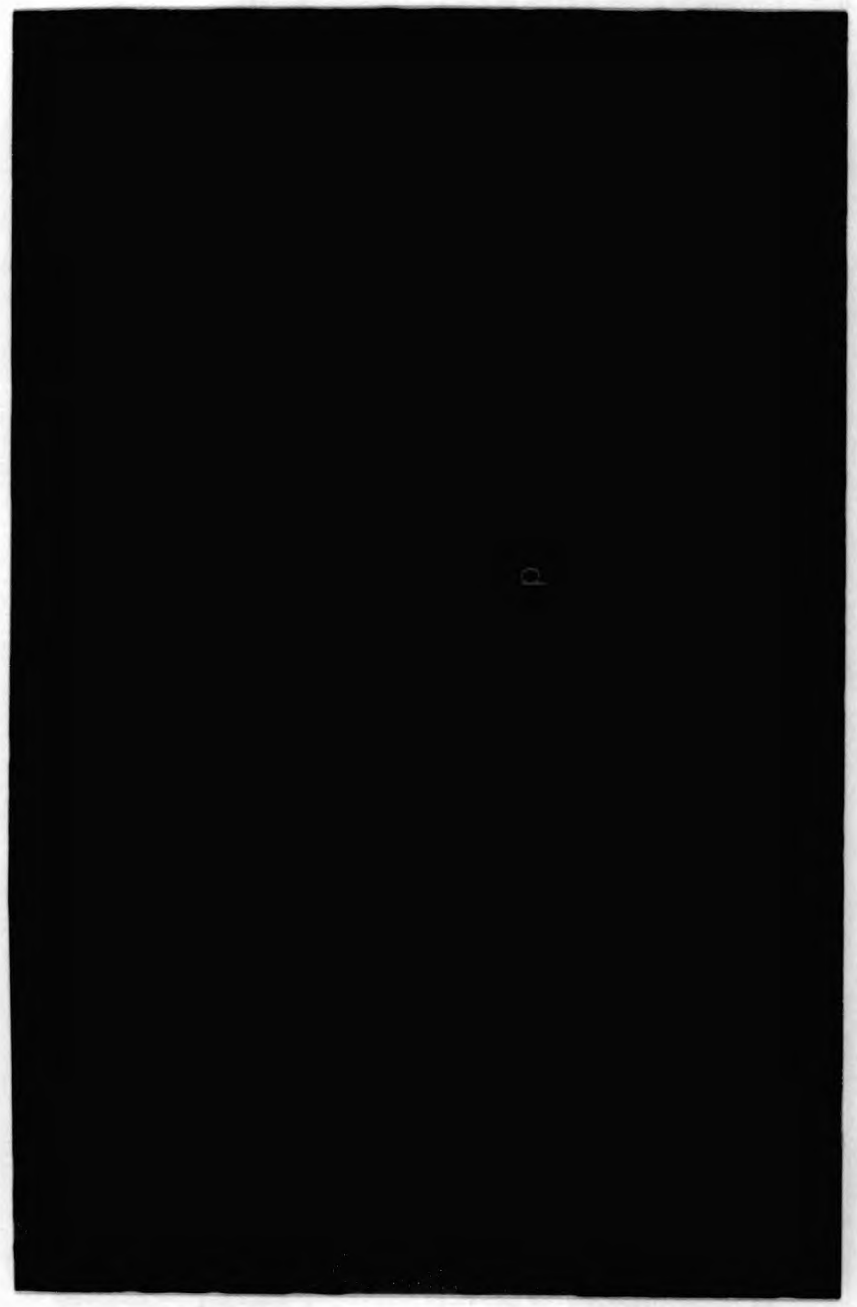


Fig. 19 Marked multifocal gill lamellar (L) free alcian blue pigment (P) accumulations asociated with aggregations of haemocytes (a). Alcian blue injection, 0 time (HE, X500).

Fig. 20 Free and phagocytosed accumulations of alcian blue pigment (P) in haemal sinuses of hepatopancreas associated with haemocyte infiltration and aggregations. Alcian blue injection, 15 minutes (HE, X250).





throughout the sampling period (including after ecdysis) which was up to 42 days after injection.

Histological Findings

From immediately following injection (0 time) to 4 hours after, there were minimal to extensive amounts of free pigment in most organs and tissues but especially in the gill stem and lamellae, hepatopancreas, heart and muscle injection site (Figs. 19, 20; Table 2). Free pigment persisted in the area of the muscle injection site up to 4-5 days and in the gills (minimally) up to 14 days (Table 2).

Haemocytic infiltrations and aggregations were initially associated with the pigment (Figs. 19, 20; Tables 1, 2) and phagocytosis occurred as early as 5 minutes in the gills and hepatopancreas (as Fig. 20; Tables 1, 2), and after 1 hour in the heart (Tables 1, 2). With the passage of time, more pronounced phagocytosis was observed in the heart, hepatopancreas (Fig. 21; Tables 1, 2) and gills, and after 6 hours in the haemocoel and after 8 hours in the antennal gland and other tissues.

Encapsulation of replete phagocytes was first seen in the gills (Fig. 22; Tables 1, 2) after 2 hours, after 6 hours in the haemocoel, after 24 hours in the hepatopancreas (Figs. 23, 24; Tables 1, 2) and after 8 hours in the muscle and connective tissues and with nodules after 12 hours (Tables 1, 2). Necrosis of

Fig. 21 Hepatopancreatic haemal sinus infiltration and aggregation of haemocytes containing phagocytosed (P) alcian blue pigment. Alcian blue injection, 14 days (HE, X500).

Fig. 22 Multilocular gill lamellar (L) pigment phagocytosis and encapsulation (e) with nuclear karyorrhexis (K). Alcian blue injection, 42 days (HE, X500).

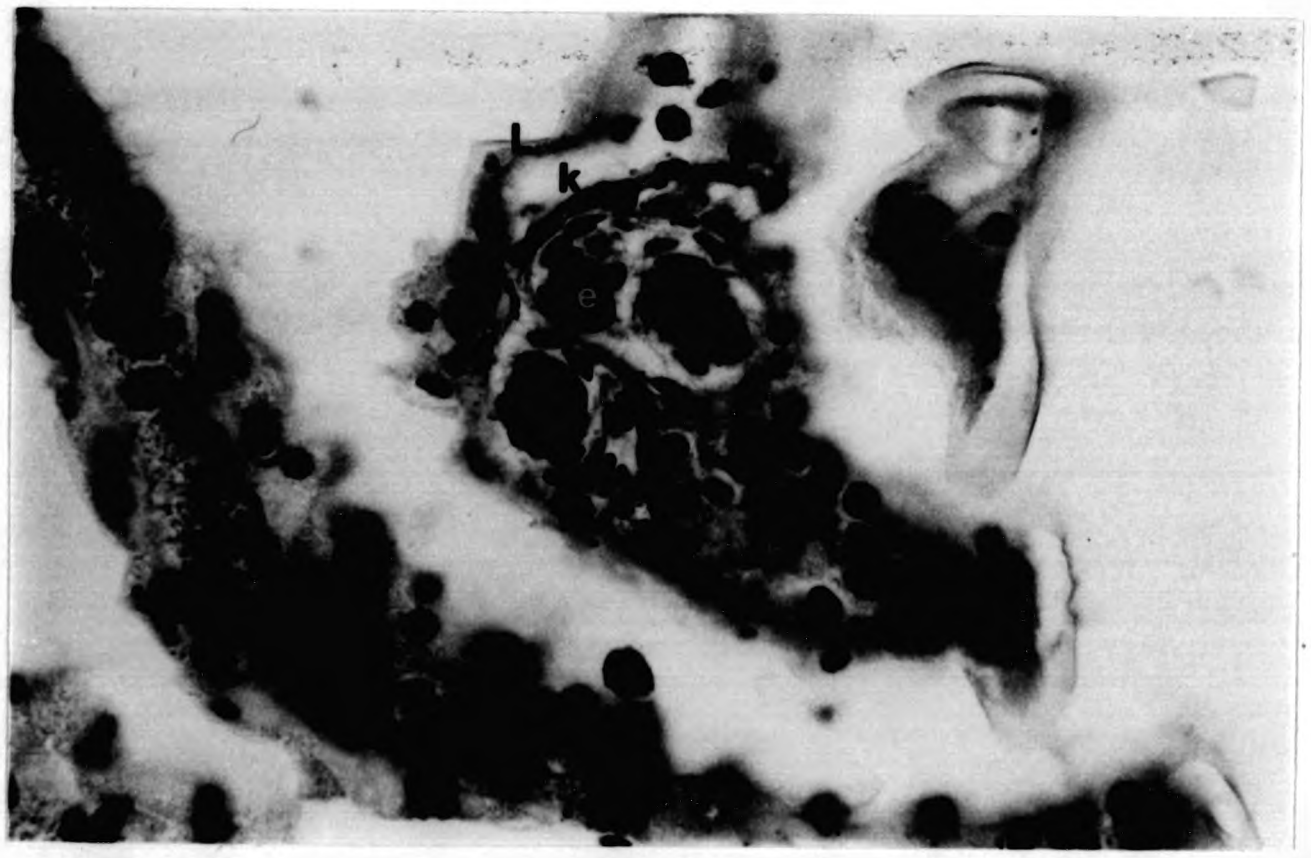
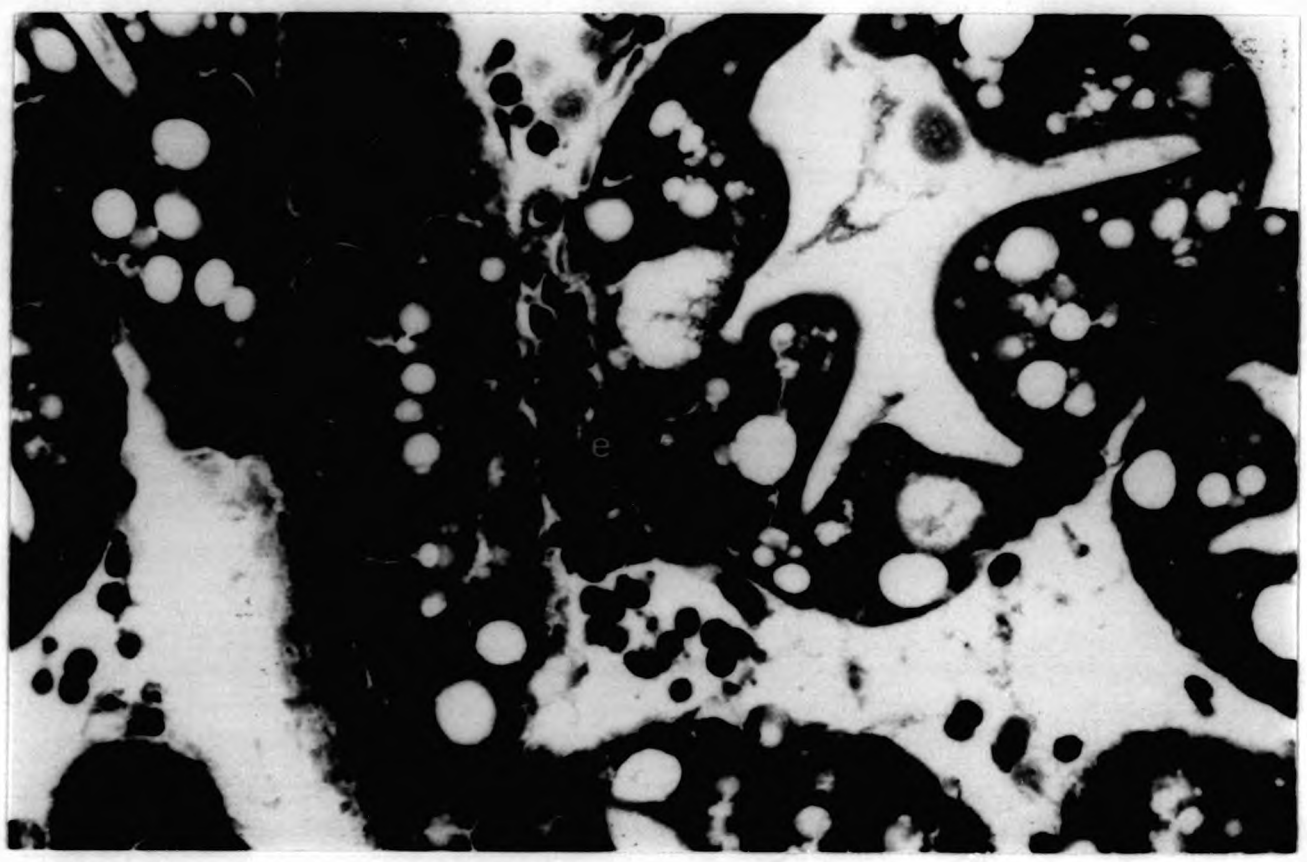

k
e

Fig. 23 Hepatopancreatic haemal sinus haemocyte infiltration and aggregation (a), with pigment phagocytosis and encapsulation (e). Alcian blue injection, 42 days (HE, X500).

Fig. 24 Haemocytic phagocytes, aggregations and multilocular encapsulations and nodules (n) of alcian blue pigment in connective tissue of peripheral encapsulating sheath between hepatopancreas (H) and midgut (G). Melanization (m), mostly obscured by the pigment, is also present. Alcian blue injection, 42 days (HE, X500).


e

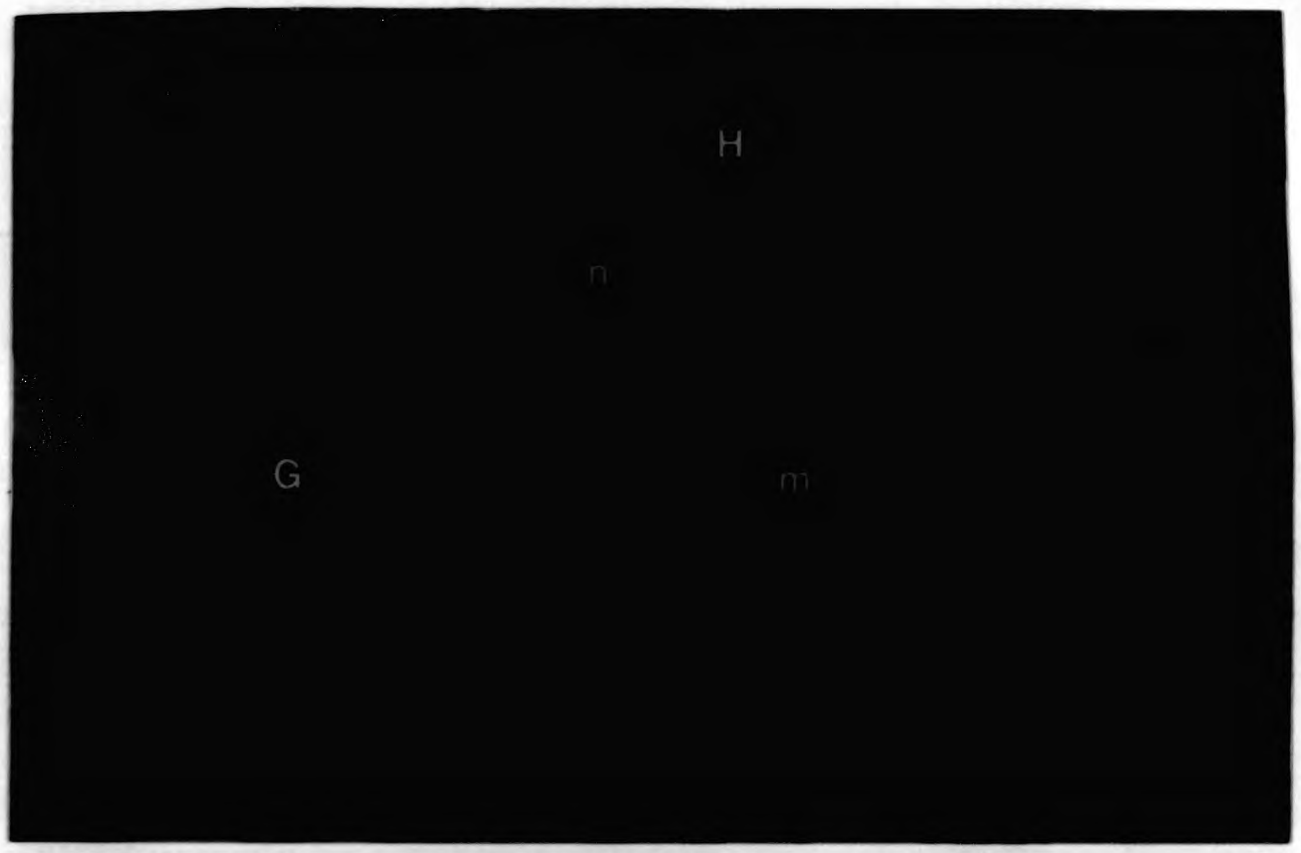
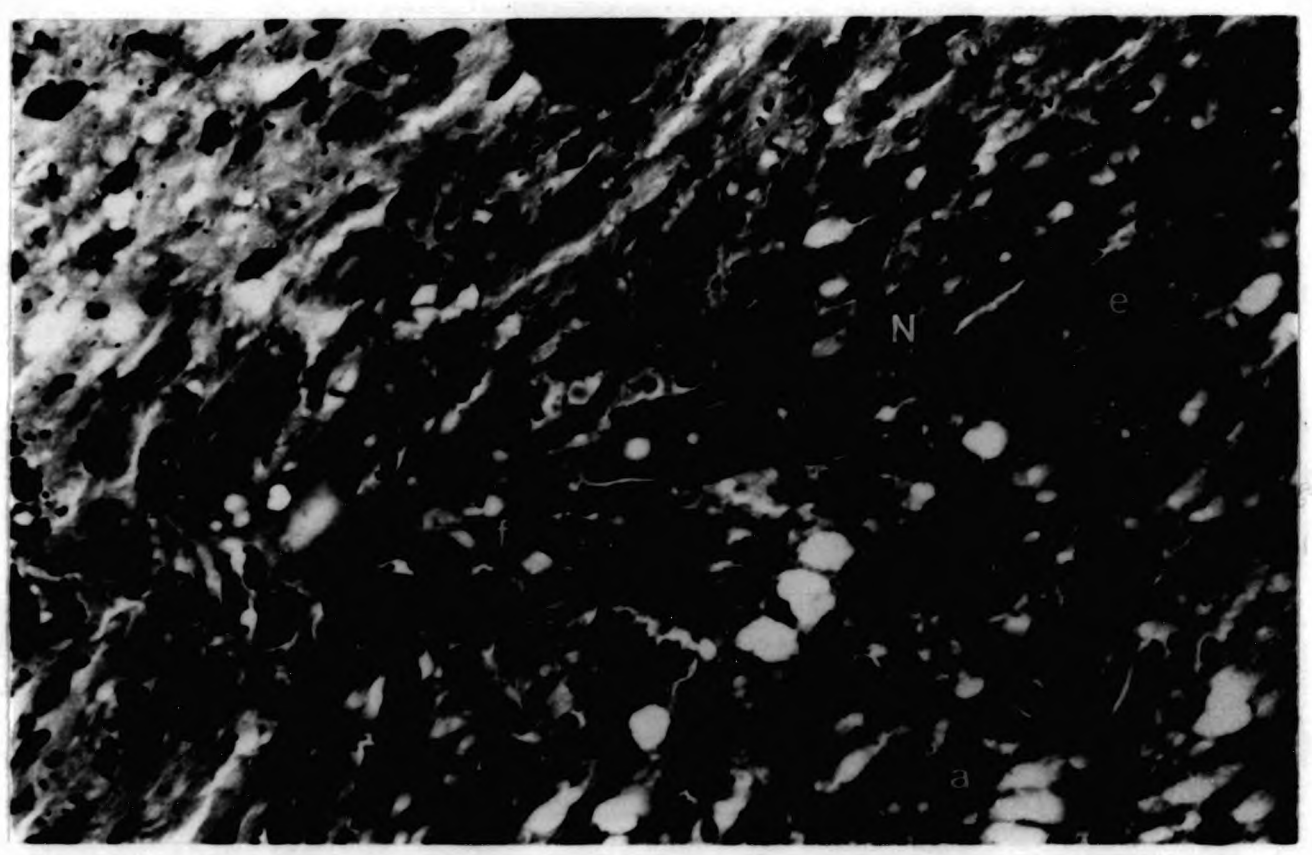

H
n
G
m

Fig. 25 a&b Diffuse muscle myofibre (M) necrosis (N) and fibroplasia (f), with haemocyte infiltrations, aggregations (a), pigment phagocytosis and encapsulations (e). Alcian blue injection, 42 days (HE, X500).



encapsulations was first noticed in the haemocoel after 8 hours (Table 2), and later in encapsulations in other organs and tissues (Table 2), with haemocytic nuclear karyorrhexis (Fig. 22) often present.

Melanization (Fig. 24) of encapsulations was found in the muscle injection site after 2 days, in gills after 3 days and in the general muscle and connective tissues after 6-7 days (and up to 42 days) (Tables 1, 2). Participation of phagocytic haemocytes in capsule formation was noted after 3 days in the muscle and connective tissues.

Fibroplasia and necrosis was observed in the muscle injection site after 4-5 days (Tables 1, 2) and persisted up to 42 days (Fig. 25; Table 2).

Slight to moderate haematopoietic activity (ie mitotic figures) was present in the haematopoietic tissue, noted from 12 hours to 42 days (Table 1). There was a persistence of haemocytic phagocytosis, aggregation and/or encapsulation up to 42 days in most organs, including the gills, hepatopancreas, heart, antennal gland, muscle and connective tissues and haemocoel (Figs. 22, 23, 24, 25; Tables 1, 2).

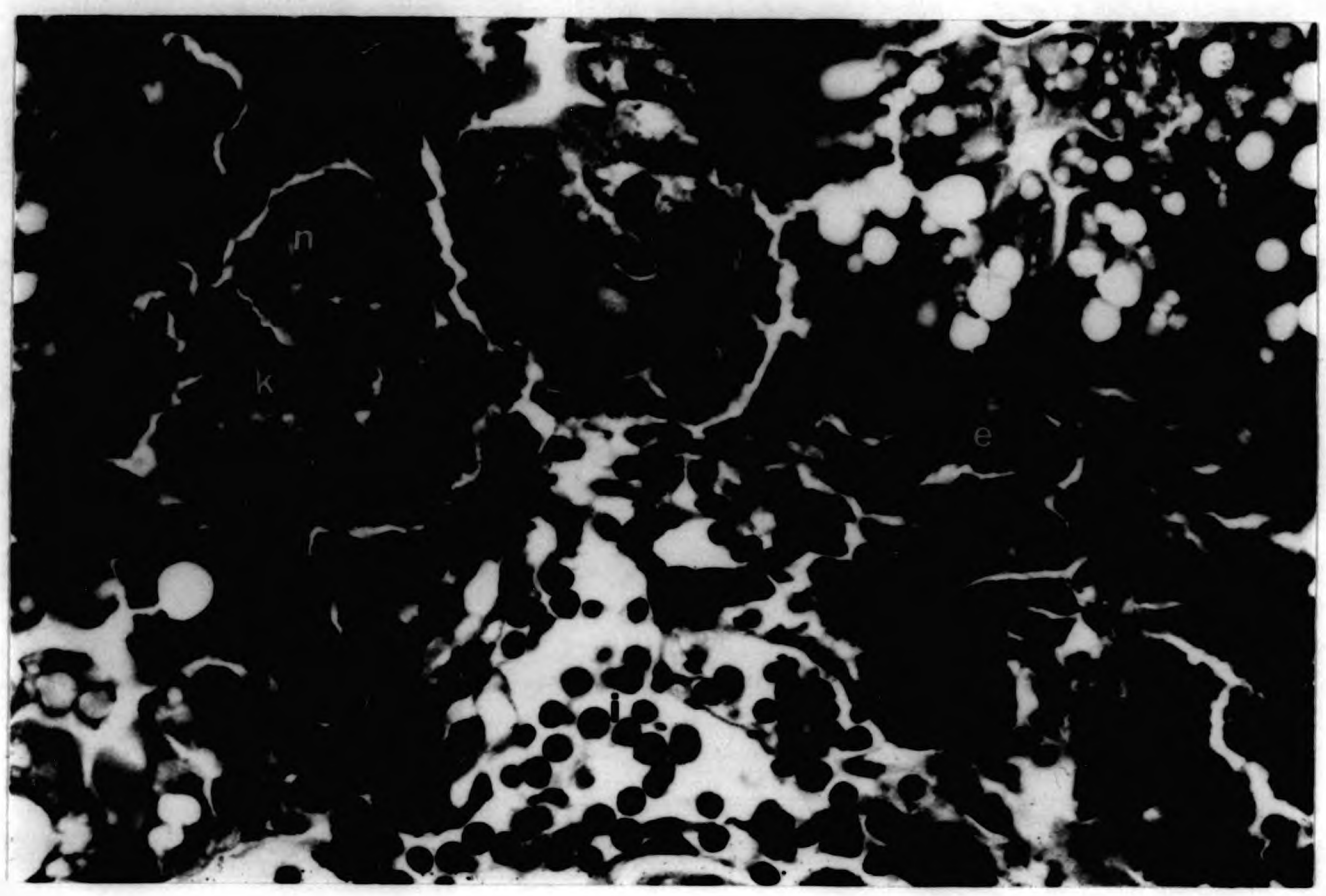
III. 3.2 The Tissue Response to BCG Injection

Gross Findings

Within 4 hours of injection the treated abdominal muscle became white in colour.

Fig. 26 Hepatopancreatic haemal sinus haemocytic infiltration (i), aggregations, encapsulations (e) and nodules (n), with phagocytosis, nuclear karyorrhexis (K) and early melanization. BCG injection, 6 hours (HE, X500).

Fig. 27 Muscle (M) injection site showing focal necrosis and fibroplasia (F) and melanization (m) of the cuticle (c), with area of diffuse haemocytic infiltration and aggregations forming multilaminar melanized sheets 1) and multifocal melanized encapsulations (e), epidermis (E). BCG injection, 3 days (HE, X300).


n
k
e

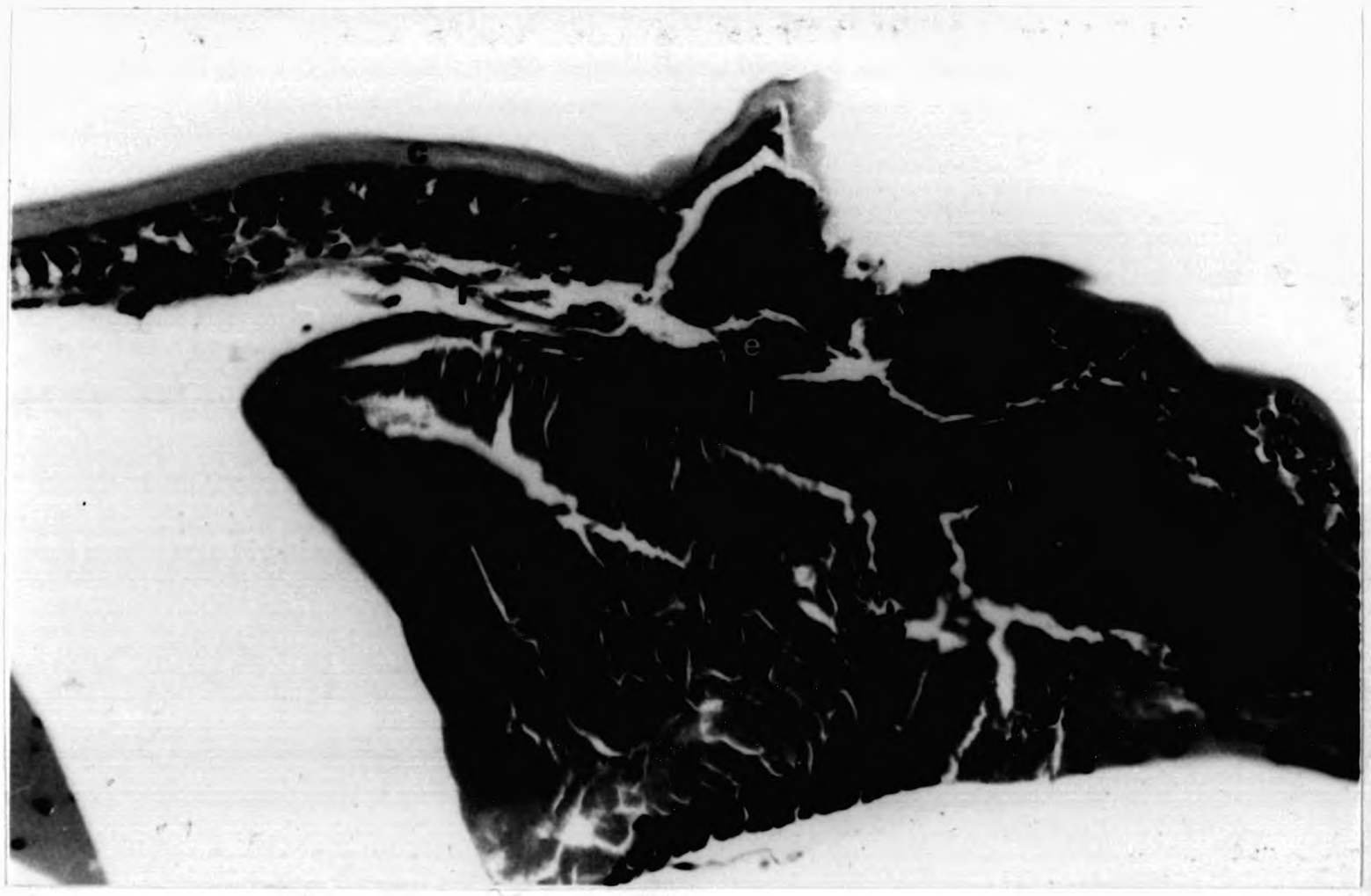
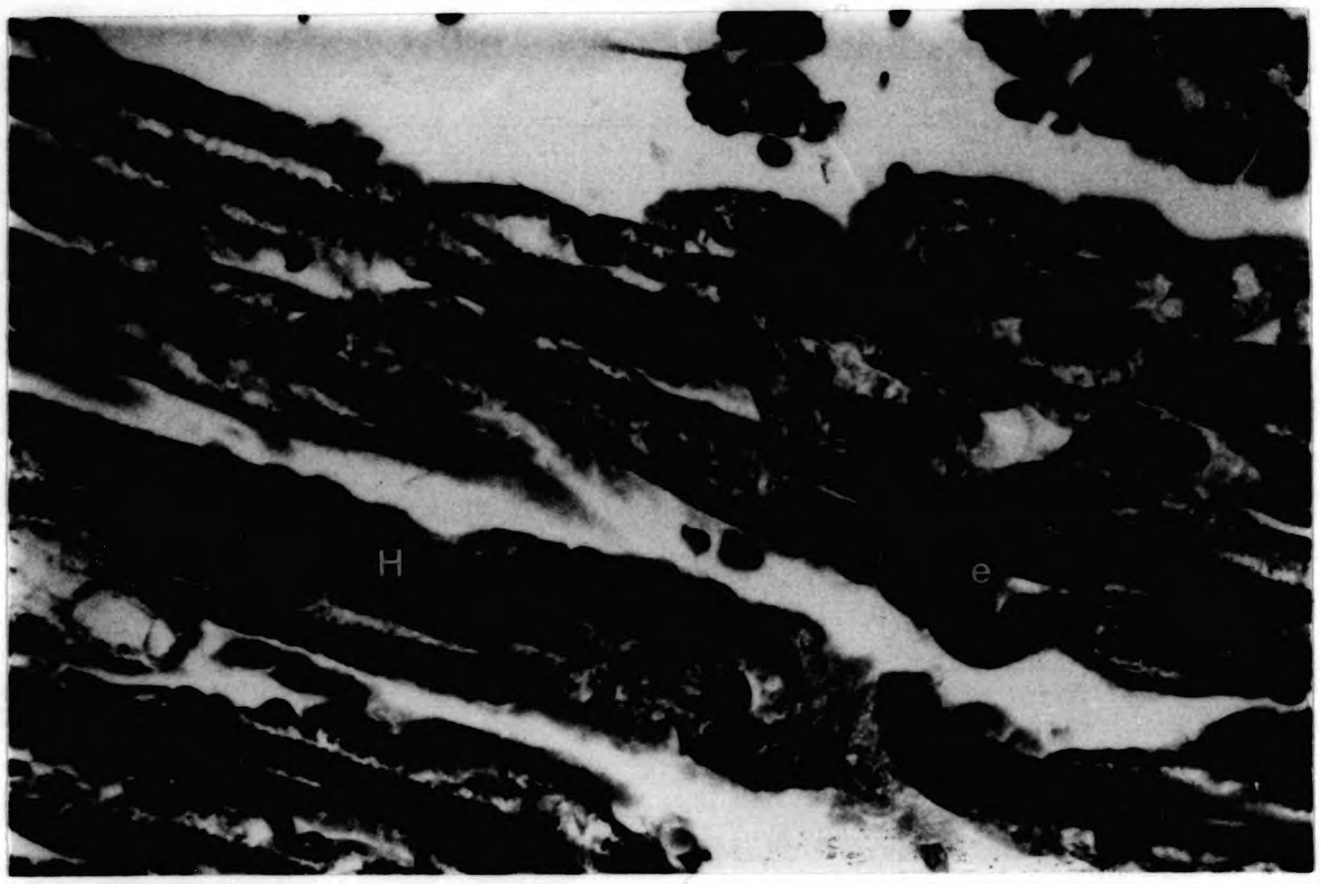

e

Fig. 28 Multiple cardiac (heart)(H) melanized myo- and endocardial trabecular melanized phagocytic encapsulations (e). BCG injection, 21 days (HE, X500).

Fig. 29 Multifocal gill lamellar (L) encapsulations (e) of melanized necrotic phagocytic haemocytes. BCG injection, 35 days (HE, bX600).


H
e

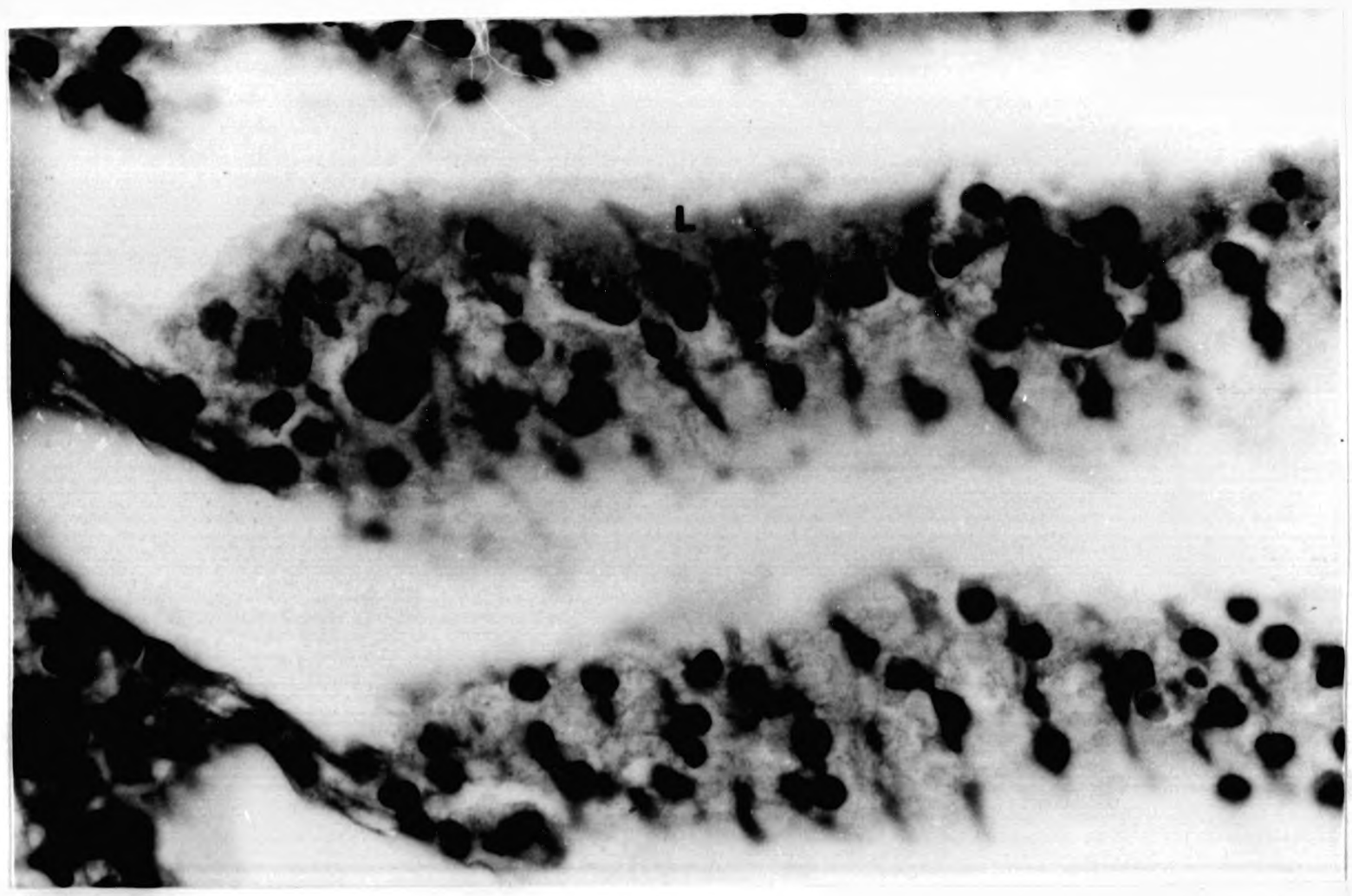
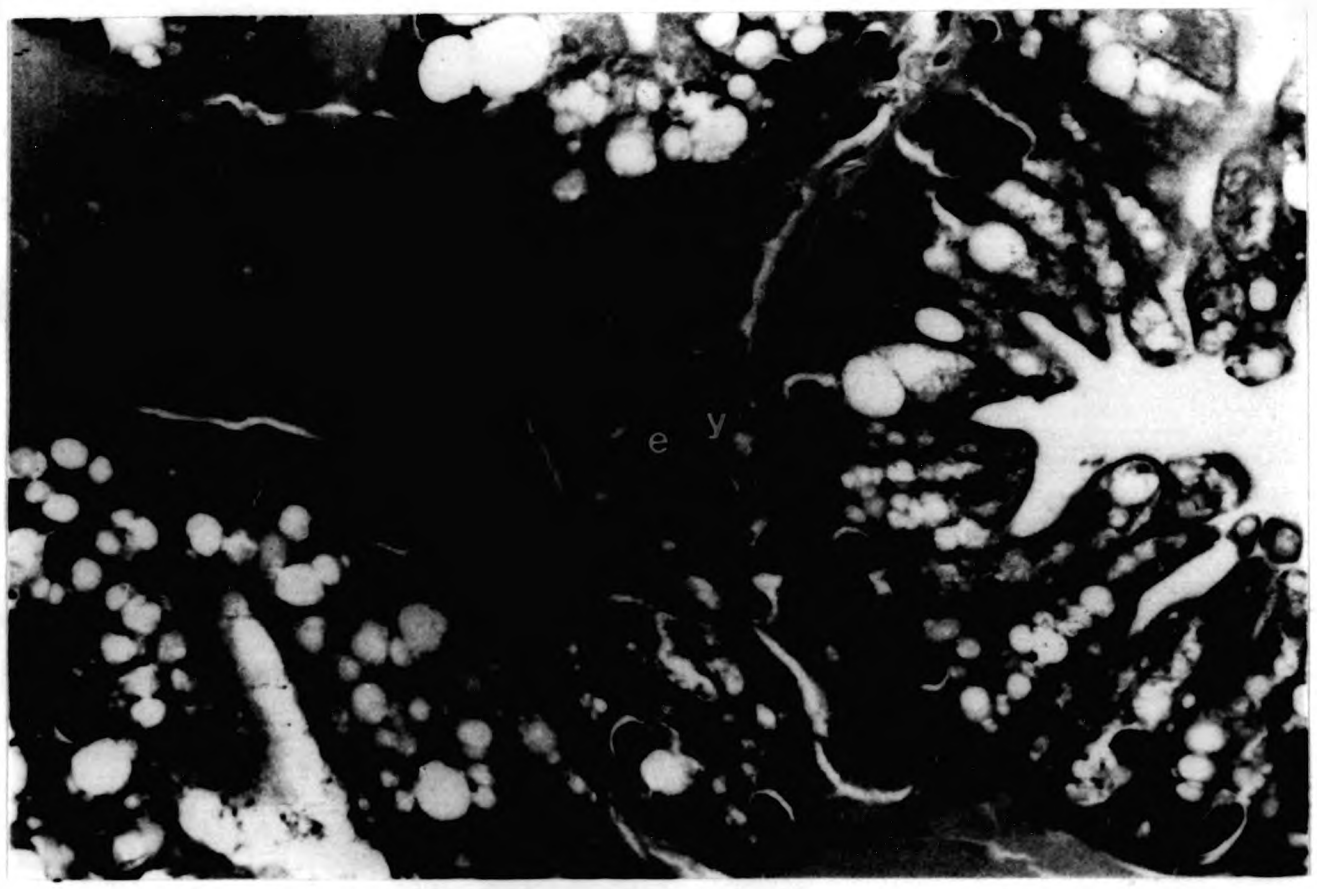

L

Fig. 30 Hepatopancreatic haemal sinus aggregation (a) and early encapsulation (e) of melanized phagocytes, with nuclear pyknosis (y). BCG injection, 35 days (HE, X500).



Histological Findings

Haemocytic activity (infiltration, aggregation) with encapsulation was first observed in the gills after 1 hour (Tables 1, 3). After 4 hours activity was also present in the hepatopancreas, with phagocytosis, and also in the muscle, connective tissue and haemocoel (Tables 1, 3). After 6 hours haemocytic activity was more marked with infiltration, aggregation and/or phagocytosis and encapsulations, which were also melanized and necrotic, in the hepatopancreas (Fig. 26; Tables 1, 3) and muscle injection site (as Fig. 27; Tables 1, 3) and after 12 hours in the gills, which continued up to 35 days in these and other organs (Figs. 28, 29, 30; Tables 1, 3).

Infiltration and aggregation were not observed in the antennal gland until 24 hours and not until 2 days in the heart, with phagocytosis; the phagocytes became necrotic after 10 days and occurred with melanization and encapsulation after 14 days and later (Figs. 28, 29, 30; Tables 1, 3).

Fibroplasia with nodule formation were present in the muscle, connective tissue and haemocoel after 3 days (Fig. 27; Tables 1, 3) and were especially pronounced in the muscle injection site from 7 to 35 days (Tables 1, 3).

A noticeable progression of the healing response in the muscle injection site was observed from

17 days with more compressed multilaminar melanized effete haemocytic sheets forming around a central area of necrotic nuclei and filling the cuticular ulcer, with the epidermis compressed but intact.

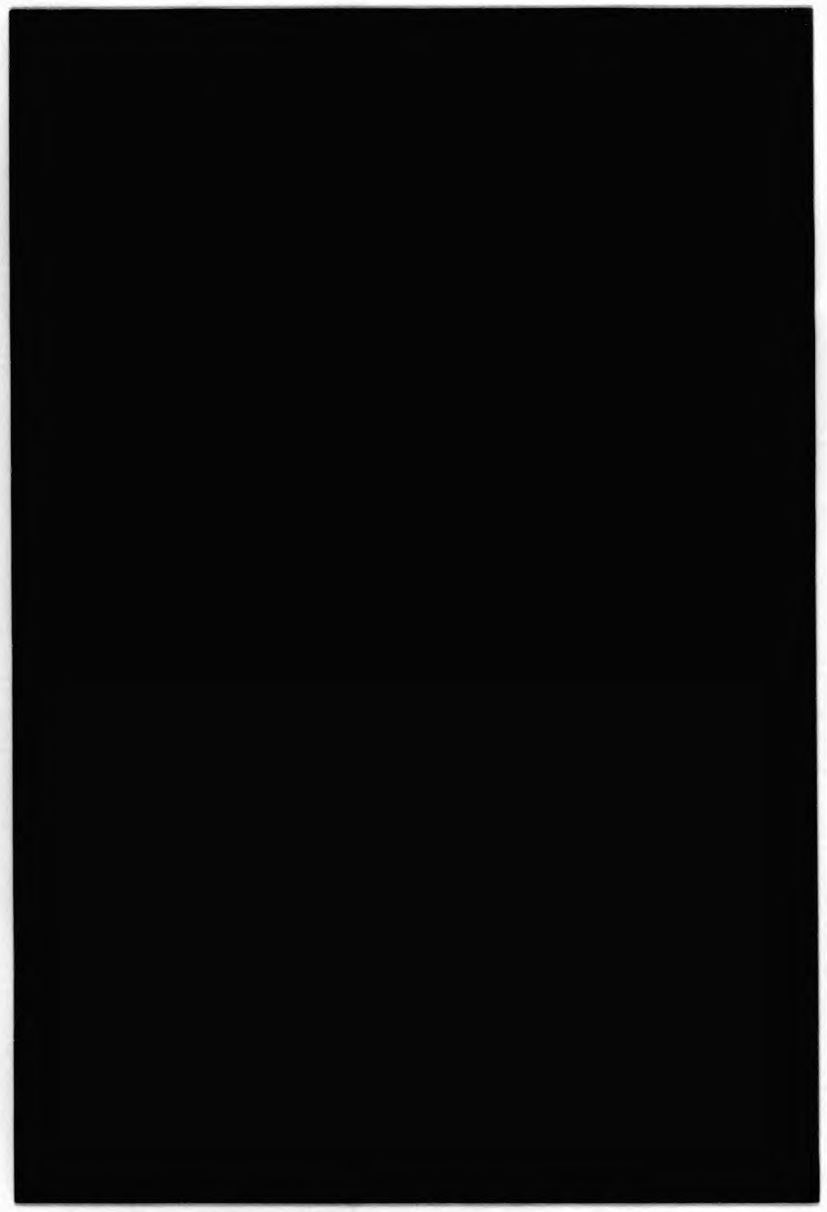
Slight to moderate mitotic activity was usually observed in the haematopoietic tissue (Table 1). There was a persistence of haemocytic infiltration, aggregation, phagocytosis, melanization and/or encapsulation/nodule formation up to 35 days in most organs, including the gills, heart, hepatopancreas, muscle, connective tissue and haemocoel (Figs. 29, 30; Tables 1, 3).

Transmission Electron Microscopy for BCG Injection

Examination of semi-thin toluidine blue-stained sections revealed slight to moderate haemocyte infiltrations, aggregations and focal to multifocal encapsulations in the muscle and gills from one hour to 32 days after BCG injection (Figs. 31, 32). More activity was observed in the gill stem than in the gill lamellae. Haemocytes were classified as granulocytes if they contained large and/or numerous intracytoplasmic granules (Figs. 31, 32, 33, 34, 35), as semi-granulocytes if they contained small and/or few granules or even one granule (Figs. 31, 32, 33) and as hyalocytes (Fig. 33) if they had no apparent granules. Semi-granulocytes and granulocytes were the predominant cell types observed in the aggregations, haemocytes with granules comprising 80-100%, compared with agranular cells comprising 0-20%

Fig. 31 An aggregation of haemocytes in the gill stem 14 days after BCG injection. Both semi-granulocytes (S) containing fewer and/or smaller intracytoplasmic granules and granulocytes (G) with more numerous and/or larger intracytoplasmic granules can be observed (toluidine blue, X1250).

Fig. 32 Aggregation and encapsulations (E) of haemocytes in the gill stem 12 hours after BCG injection. Granulocytes (G) with more numerous and/or larger granules and semi-granulocytes (S) with fewer and/or smaller granules are present. Very few granules (star) were apparent in the rather vacuolated haemocytes involved in the encapsulation reactions (E). An individual semi-granulocyte (S) with a single granule (arrow) and some of those in the encapsulation (E) have probable phagosomes (P) (toluidine blue, X1250).



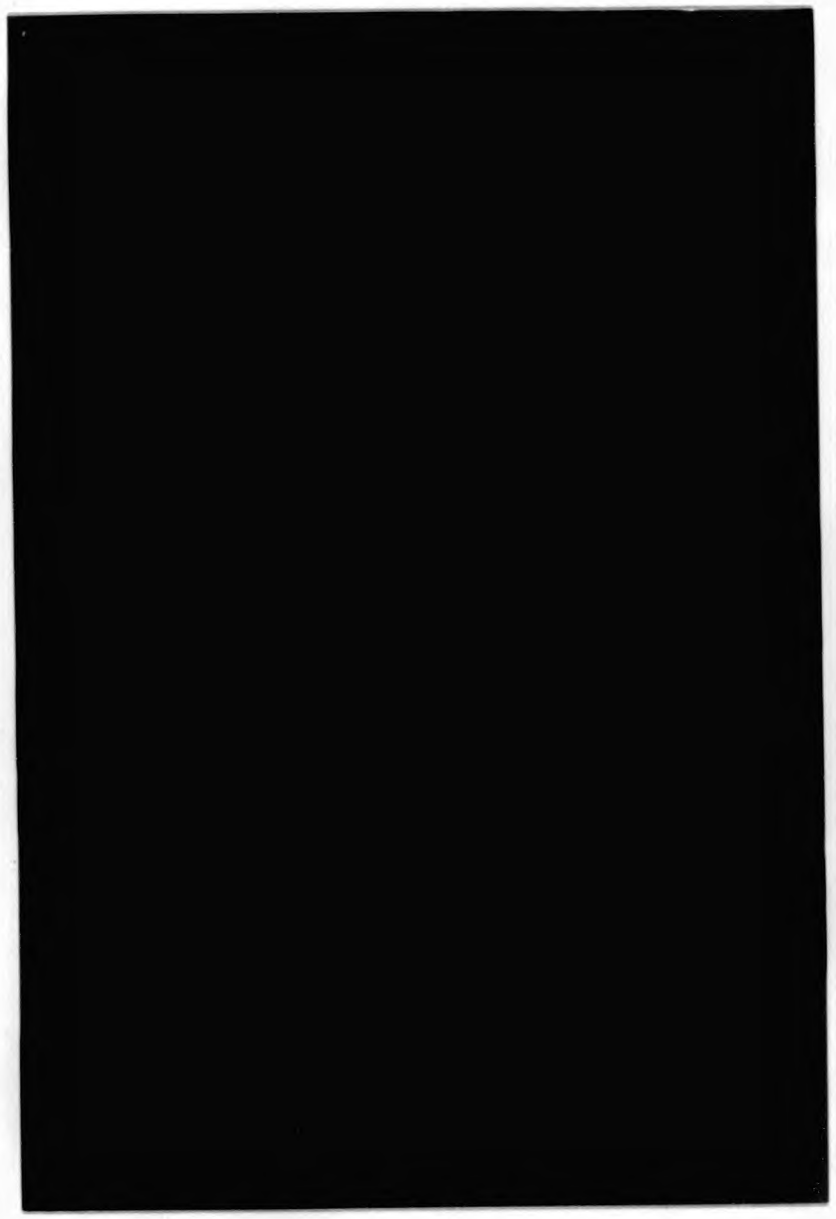


Fig. 33 An aggregation of haemocytes in the gill stem 21 days after BCG injection. A granulocyte (G) with numerous large intracytoplasmic granules (g), two semi-granulocytes (S) with fewer and smaller granules (g) and a hyalocyte with no apparent granules are present. In one of the semi-granulocytes is a form of residual body (r) or late secondary lysosome (in which digestion is nearing completion and it is loaded with undigested electron dense residues); nucleus (n), mitochondrion (m), pseudopodial extension (e) (TEM, X4500).

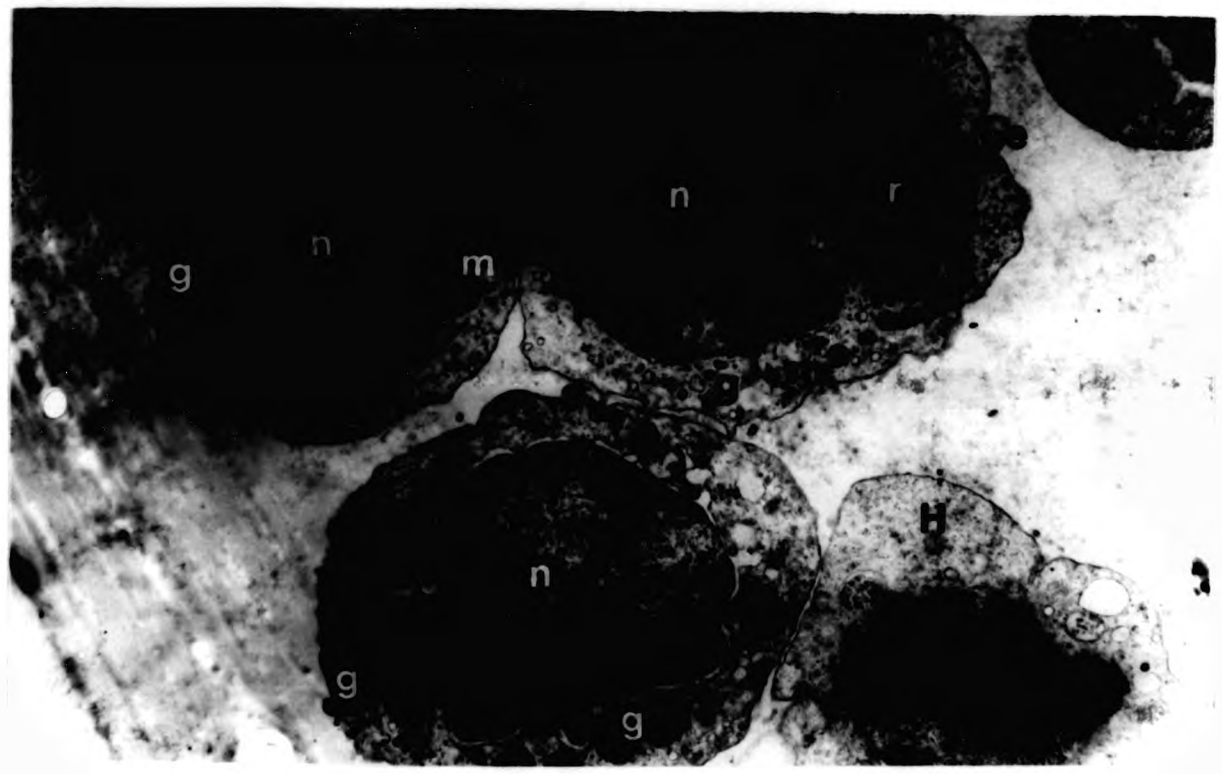
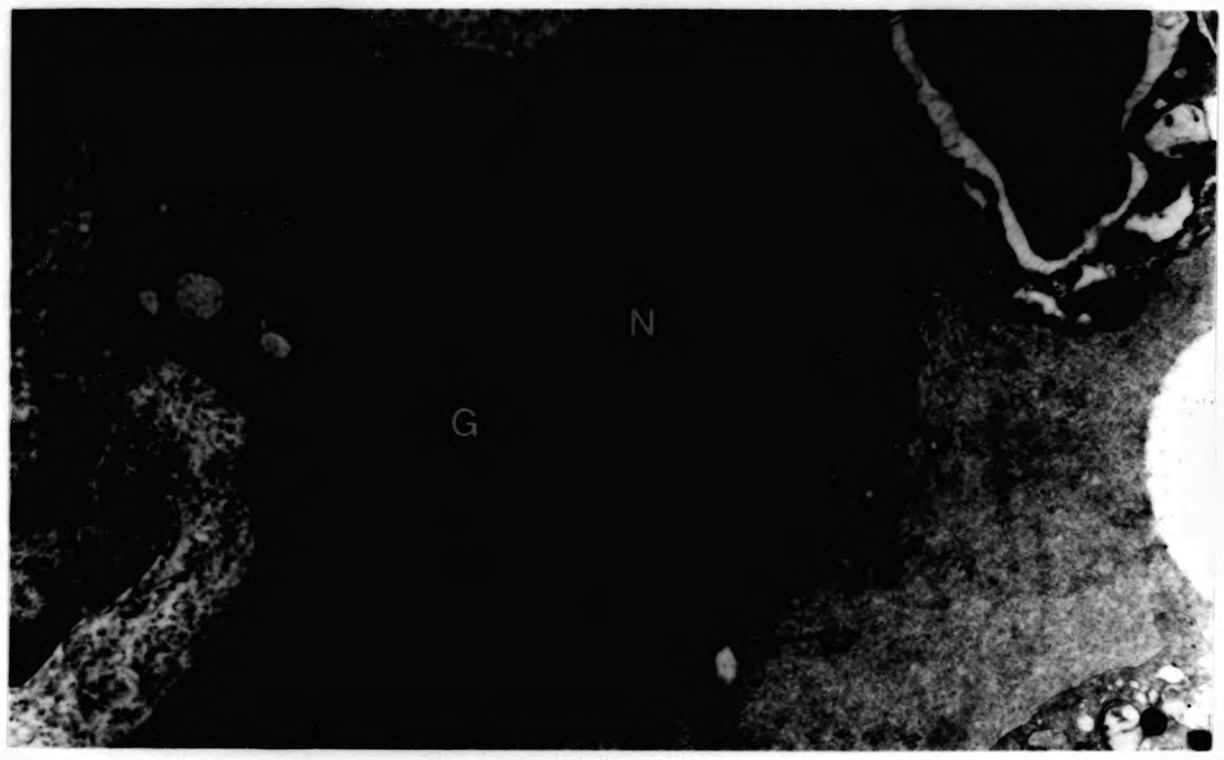

n
r
n
m
g
H
n
g
g

Fig. 34 a&b Haemocytes observed in aggregations in the gill stem:

(a) Eight hours after BCG injection, a granulocyte or semi-granulocyte with numerous but variably small to large sized spherical intracytoplasmic granules (G); nucleus (N), pseudopodial extension (E);

(b) Twenty-one days after BCG injection, a granulocyte with predominantly large intracytoplasmic granules (G); mitochondrion (M) (TEM, X6000).


N
G

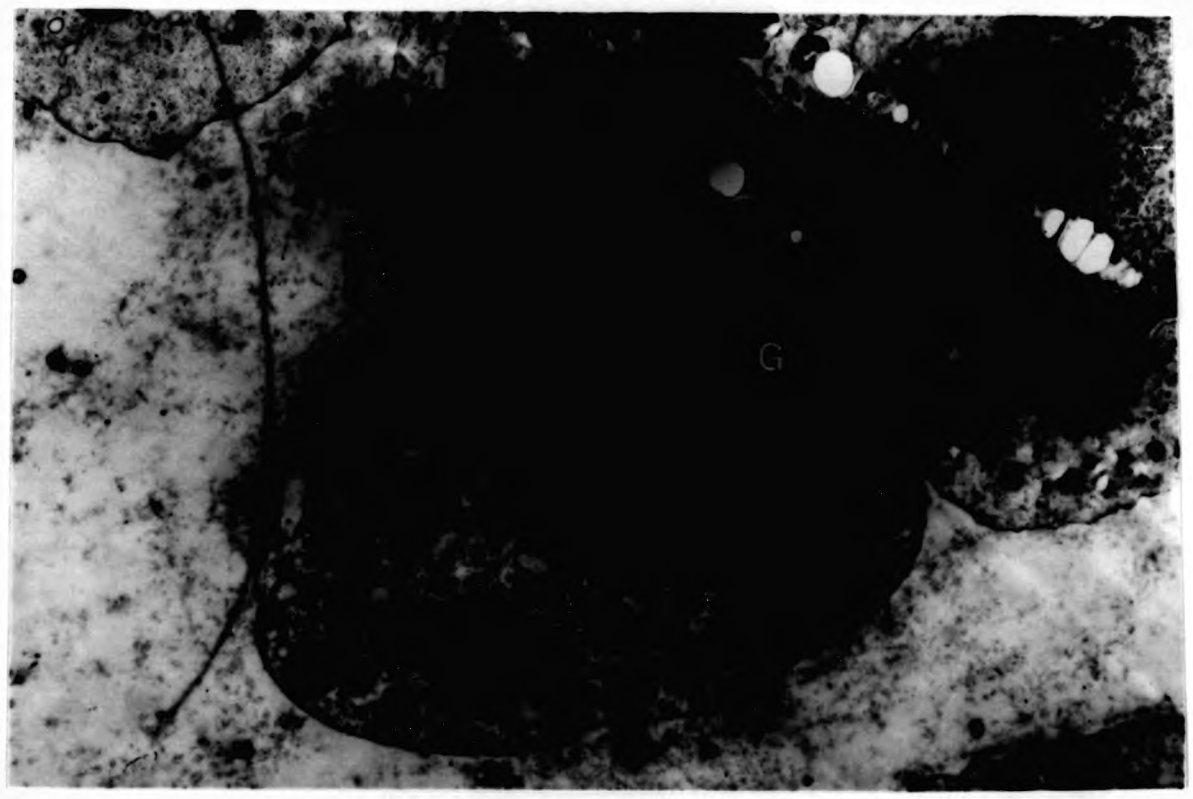
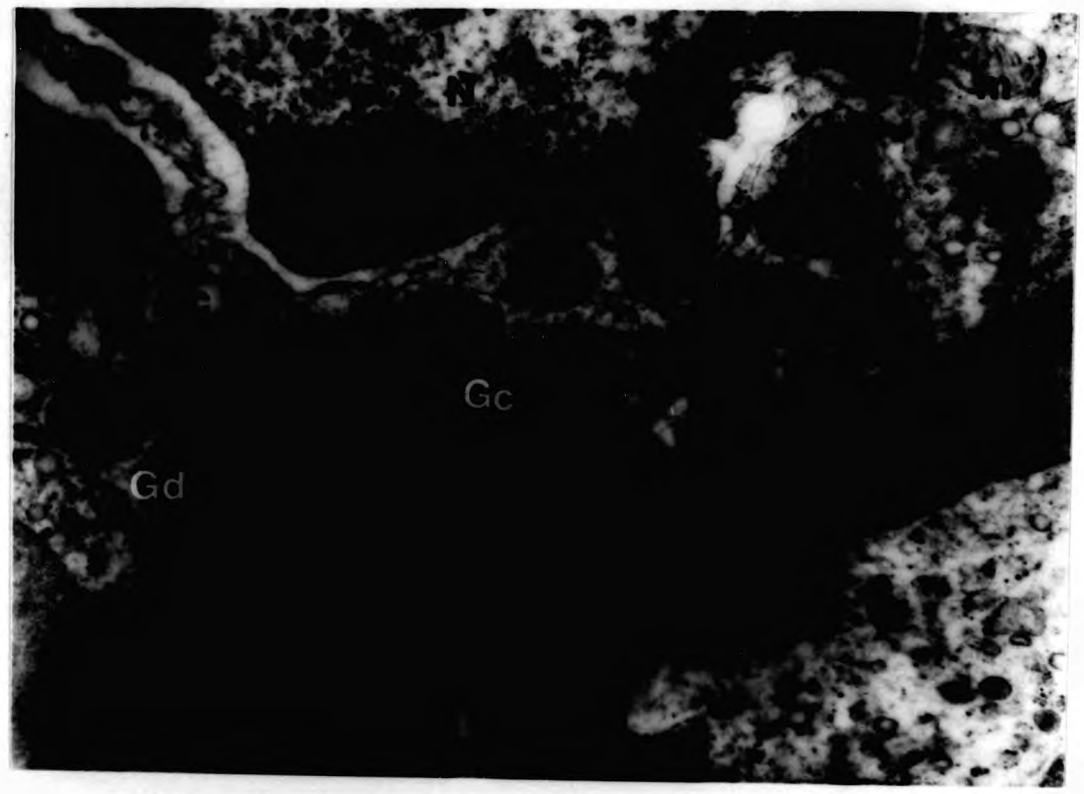

G

Fig. 35 Higher magnification of a granulocyte in the gill stem 21 days after BCG injection. The numerous intracytoplasmic granules adjacent to the nucleus (N) are of two different forms: one consists of diffuse electron-dense granules (Gd), and the other is composed of concentric bands of electron-dense material alternating with areas of lower density (Gc); Golgi complex (C), mitochondrion (m) (TEM, X15,000).

Fig. 36 In the muscle tissue (myofibre (M)) 12 hours after BCG injection, a haemocyte is present probably undergoing degeneration or "cloudy swelling", which may represent an explosive corpuscle or degranulated cell. The nucleus (N) displays chromatin clumping (e). The cytoplasm is filled with dilated vesicles (V) likely to be smooth endoplasmic reticulum. The plasma membrane (P) is noted at a point of blebbing with a vesicle (V). A phagosome or heterolysosome (h), as well as vacuoles (v) possibly the remains of granules are also present (TEM, X8750).


Gc
Gd


h
N
c
e
M
v

(based on the number of cells with or without granules per high power field).

In semi-thin and thin sections, encapsulations were composed of haemocytes with very few apparent granules and a highly vacuolated cytoplasm, some of the large vacuoles resembling heterolysosomes or phagosomes (indicating prior phagocytosis)(Fig. 32), these vacuoles may have also been the remains of previous granules (Fig. 32). A phagosome (heterolysosome) was also observed in a semi-granulocyte with one apparent granule (Fig. 32).

Ultrastructurally, most haemocytes also contained intracytoplasmic granules and were considered to be granulocytes (Figs. 33, 34, 35) or semi-granulocytes (Fig. 33), true agranular haemocytes being rarely present (Fig. 33).

At higher magnification the granules could be seen to be bound by a single membrane and consisted of two basic types: one, a diffusely dense granular type and, the other, with concentric bands of electron dense material alternating with bands of lower density (Fig. 35).

Another type of haemocyte probably undergoing degenerative changes or "cloudy swelling" was also rarely observed (Fig. 36). The nucleus of this cell had an indistinct nuclear envelope with peripheral chromatin clumping (Fig. 36). The cytoplasm demonstrated no definite organelles, rather a marked dilatation and

vesiculation of the smooth endoplasmic reticulum, a heterolysosome or phagosome, and a number of vacuoles which were possibly the remains of granules (Fig. 36). The plasma membrane also demonstrated blebbing (Fig. 36). This cell may have represented a form of effete or degranulated cell or an "explosive corpuscle". Other commonly observed organelles in the haemocytes included the nucleus, mitochondria and Golgi apparatus; pseudopodial extensions of the plasma membrane were also frequently present (Figs. 33, 34, 35).

III. 3.3. The Tissue Response to Complete Freund's Adjuvant Injection

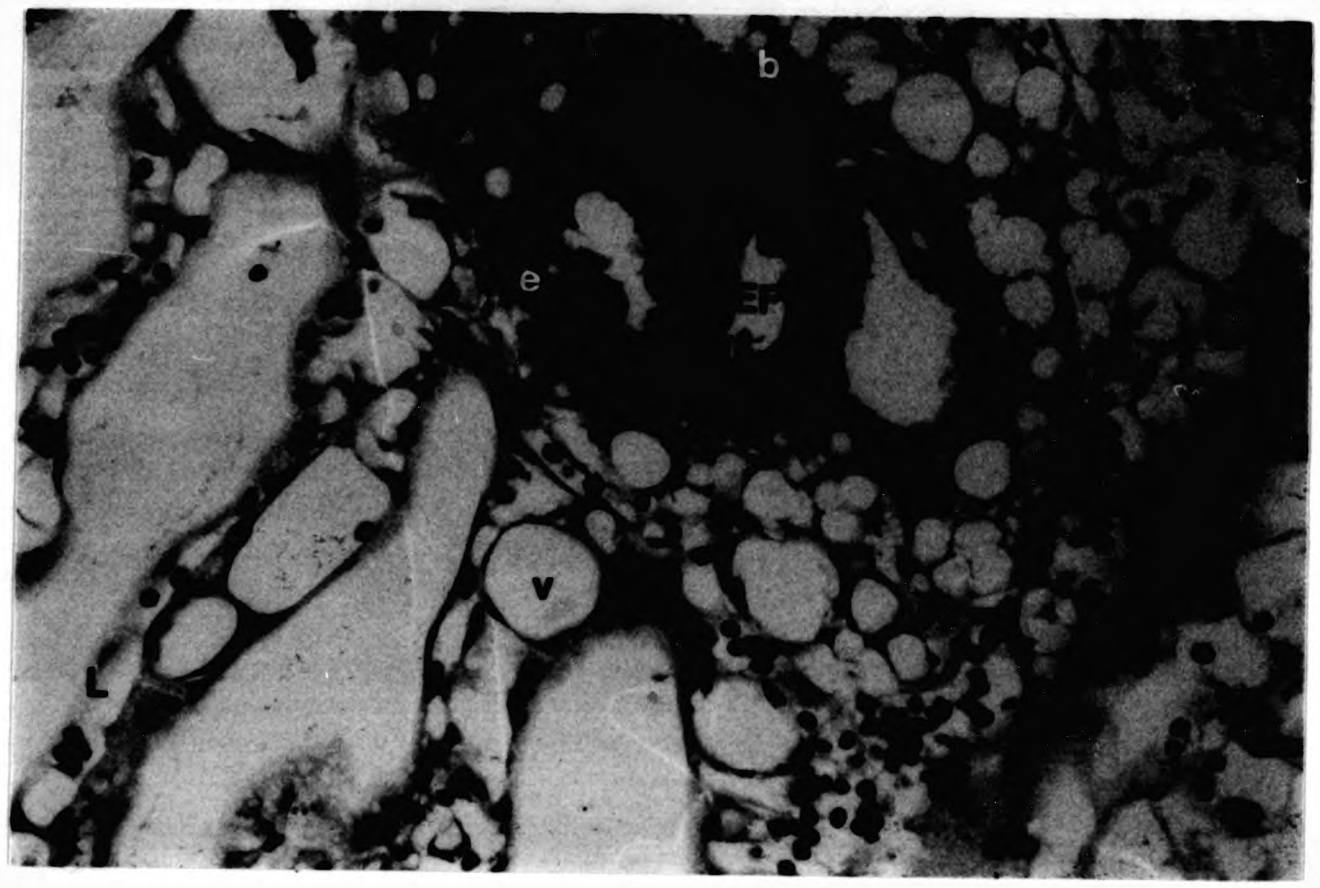
Histological Findings

The oily CFA was characterized in the tissue sections by clear vacuolation; haemocytic clear intracytoplasmic vacuolation provided evidence of phagocytosis. In the area of diffuse haemocyte activity - infiltration, aggregation and encapsulation- vacuolated networks of engulfed CFA were formed (Figs. 37, 40, 41, 42, 43). Early melanization in the tissues was also an important feature in this experiment (Tables 1, 4).

Haemocytic infiltration, aggregation and phagocytosis were observed in the hepatopancreas (Figs. 38, 39, 41; Tables 1, 4), also with necrosis in the muscle (Figs. 42, 45, 46; Tables 1, 4), nervous and connective tissues, injection site and haemocoel and with melanized encapsulations in the gills (Figs. 37, 43; Tables 1, 4) as early as 1 hour and persisted thereafter

Fig. 37 Marked diffuse haemocyte infiltration and aggregations of CFA-vacuolated phagocytic haemocytes (V) in gill lamellae (L) and gill efferent channel of the gill stem (EF), with encapsulation (e) of an area including branchial podocytes (b) suspended in the gill system. CFA injection, 7 days (HE, X250).

Fig. 38 Extensive aggregation, phagocytosis, encapsulation (E) and nodule formation involving CFA-vacuolated material (V) in the subcapsular hepatopancreatic haemal sinuses. CFA injection, 10 days (HE, X300).


b
e
v

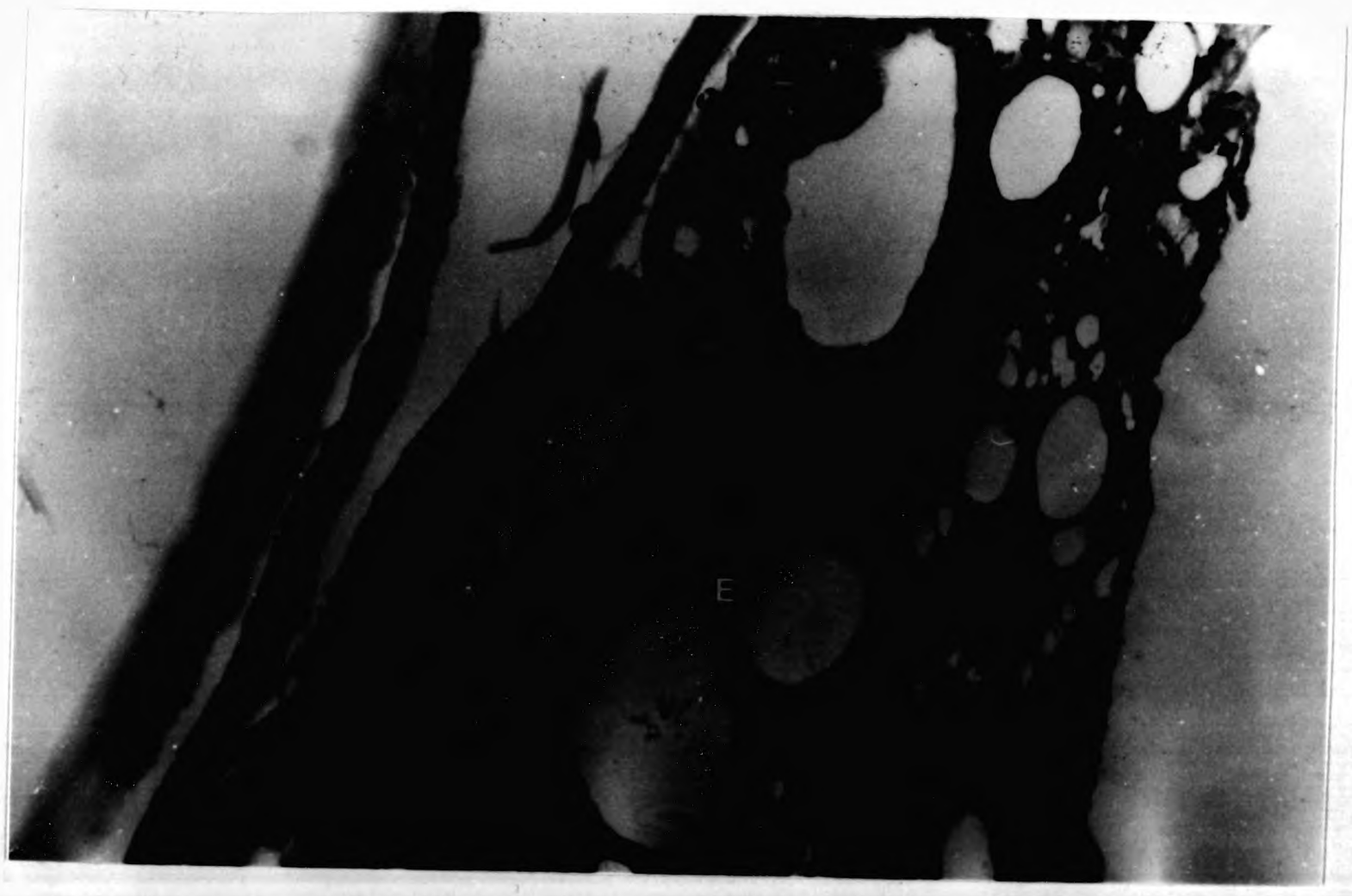
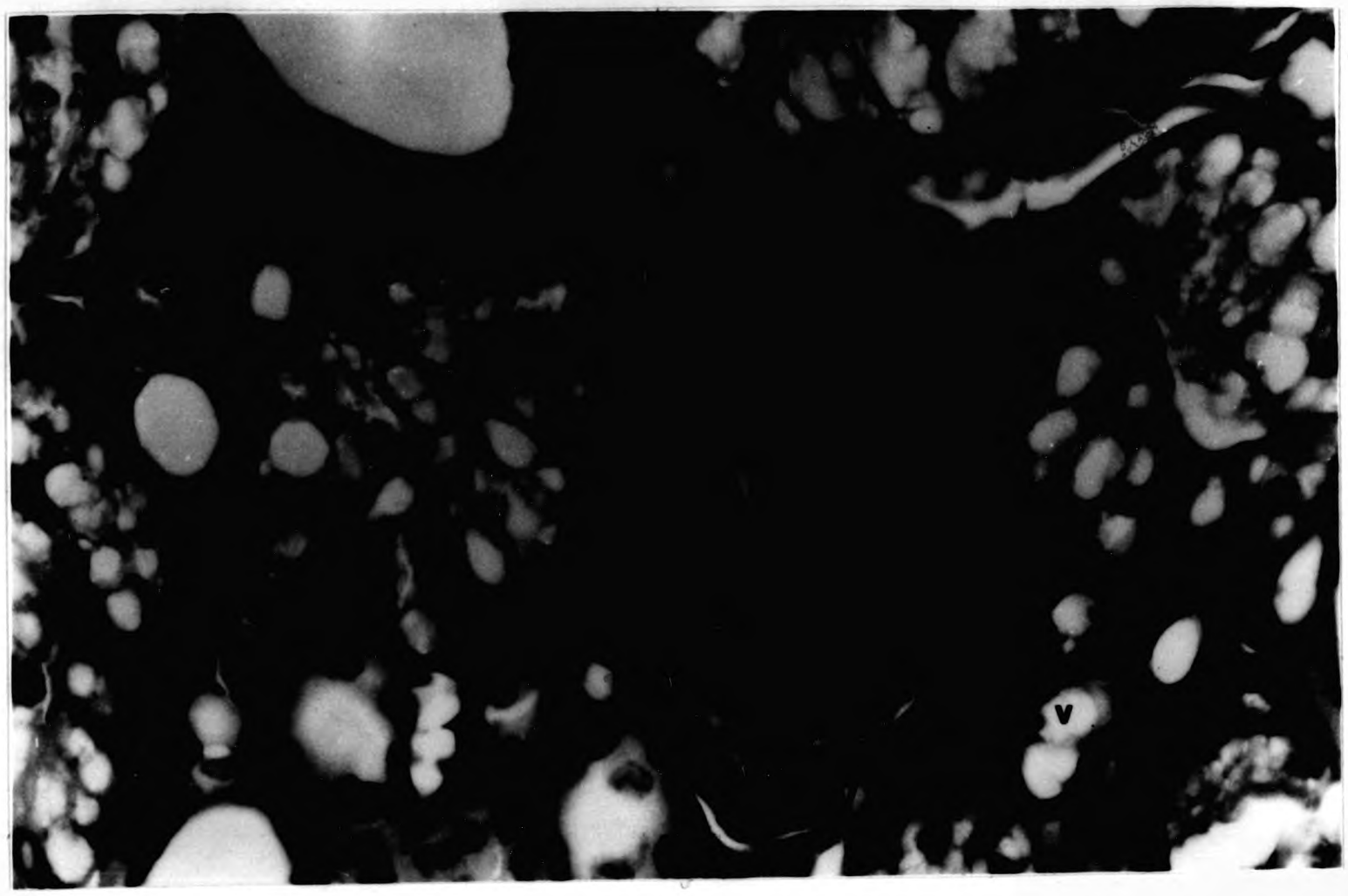

E

Fig. 39 Massive haemocytic nodule formation (n) (also of CFA vacuolated material (V)), with aggregations of CFA-vacuolated phagocytes (V) in hepatopancreatic haemal sinuses. CFA injection, 10 days (HE, X600).

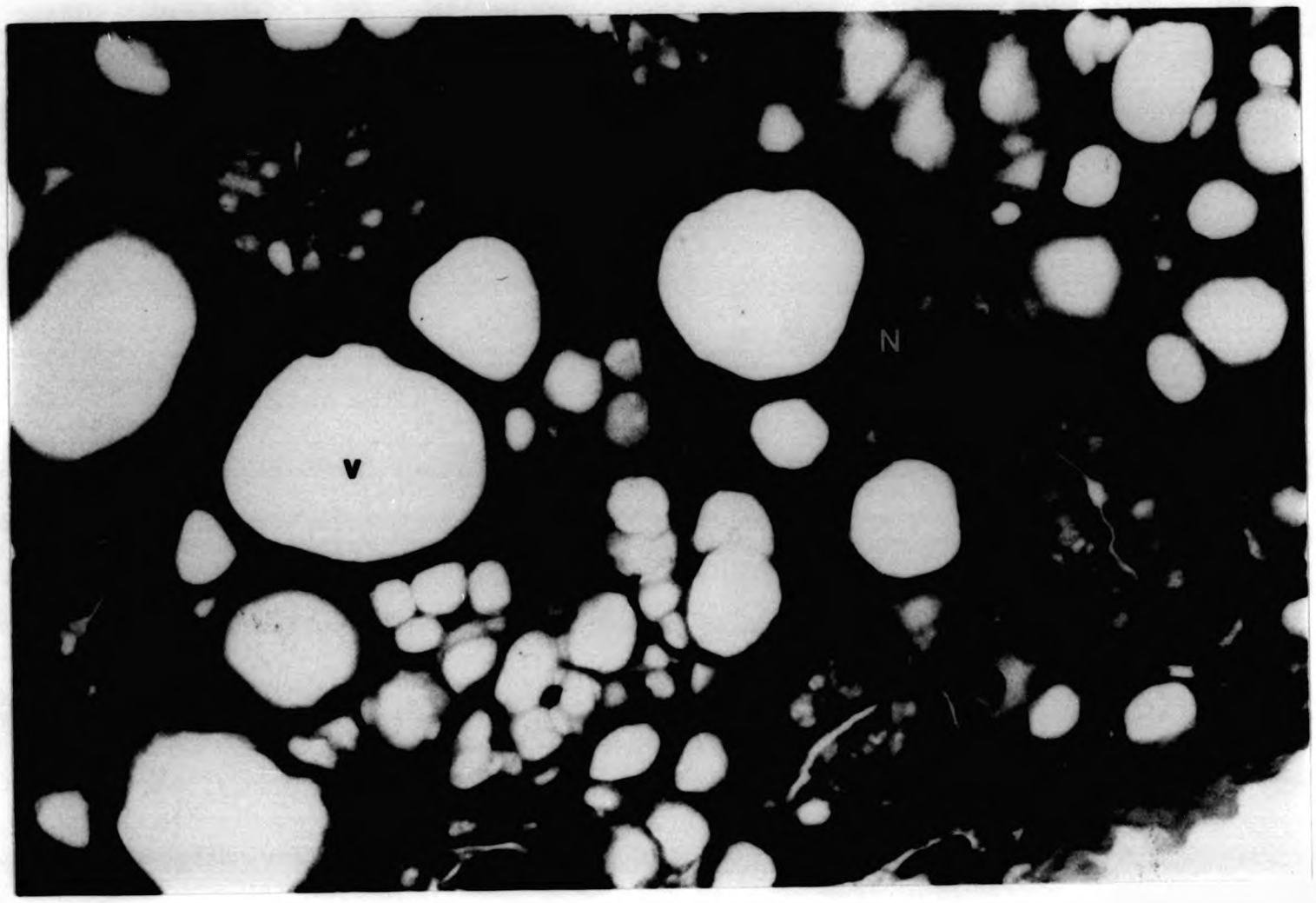
Fig. 40 Moderate multifocal to diffuse cardiac (heart)(H) endocardial trabecular aggregations of CFA-vacuolated phagocytes (V). CFA injection, 10 days (HE, X250).


V


H

Fig. 41 Extensive hepatopancreatic haemal sinus CFA-vacuolated phagocyte (V) aggregation, encapsulation and nodule formation (N). CFA injection, 14 days (HE, X600).

Fig. 42 Muscle tissue myofibrillar large aggregation of CFA- vacuolated phagocytes (V). CFA injection, 14 days (HE, X250).


N
V

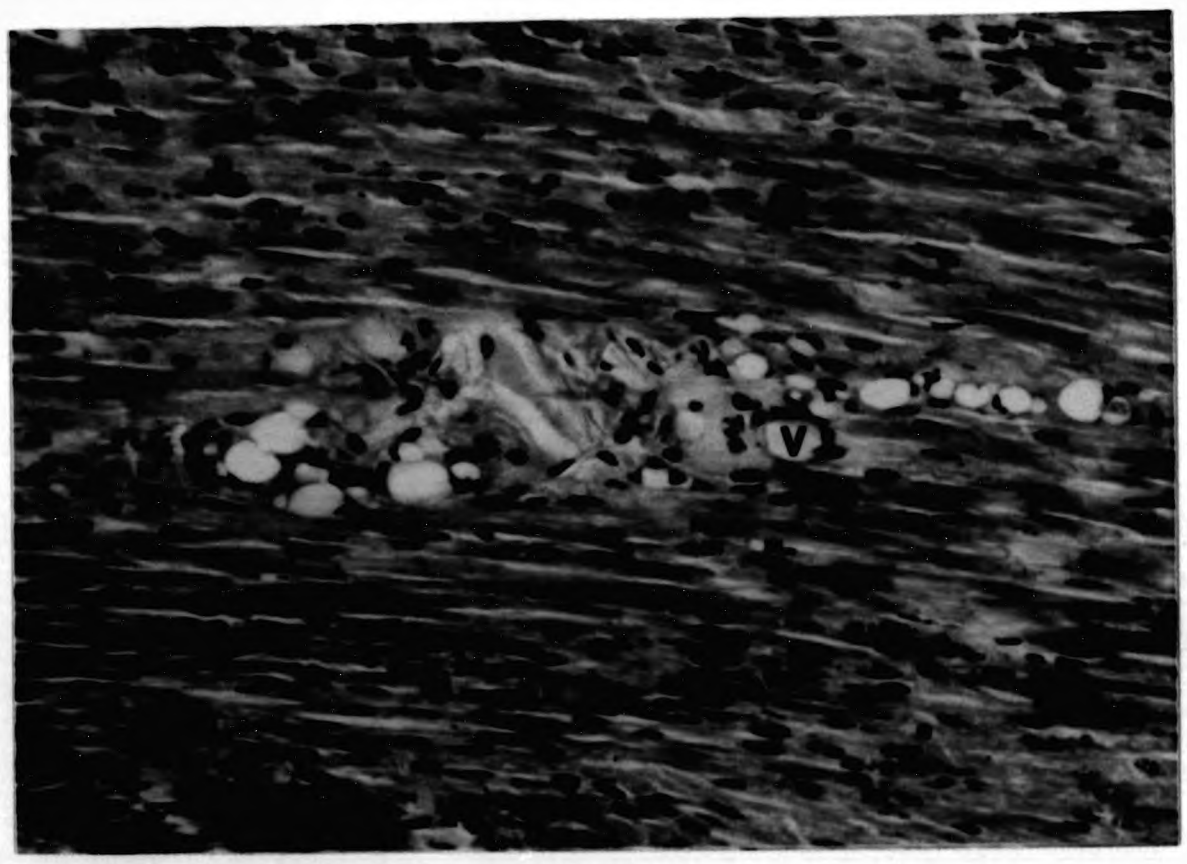
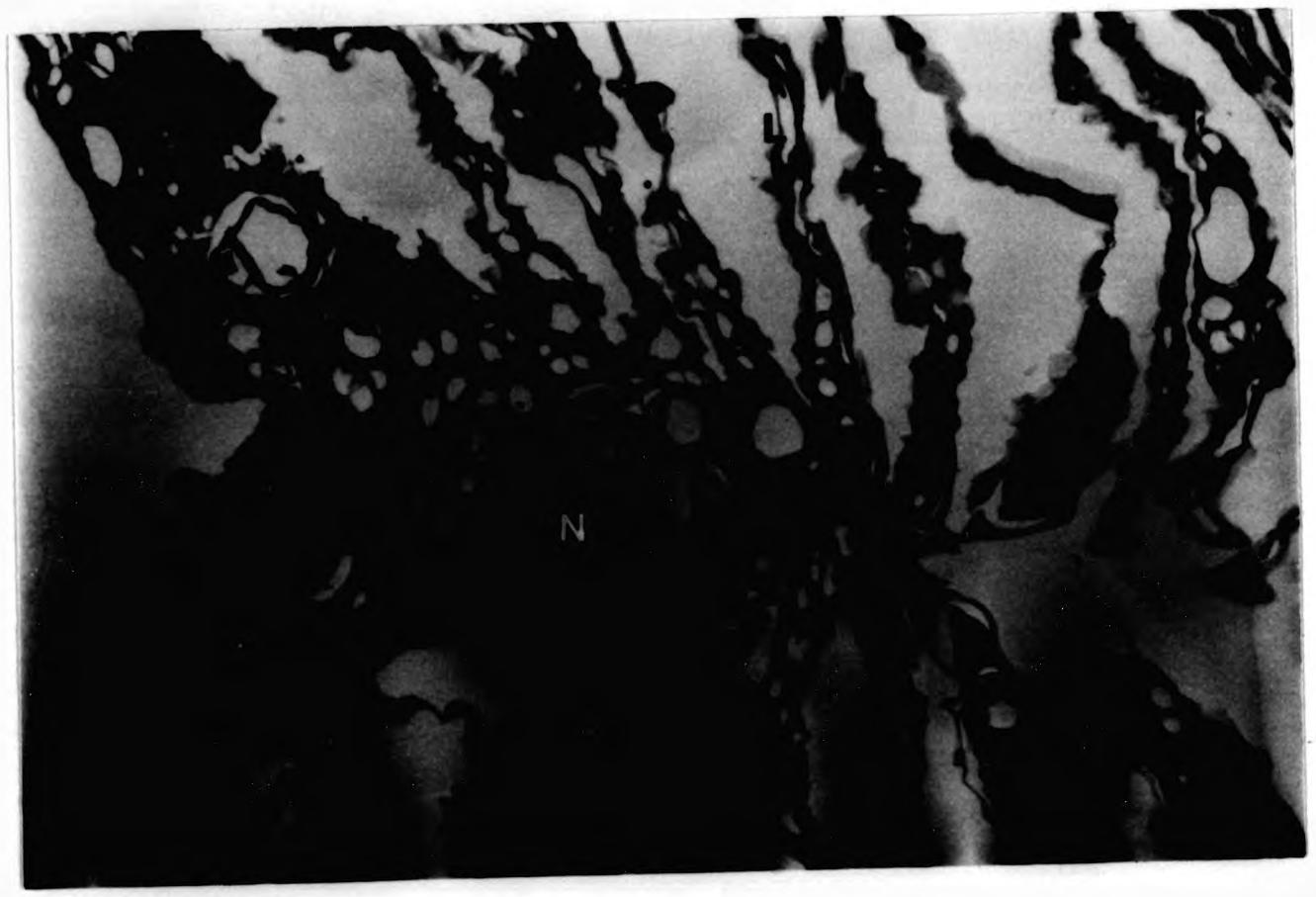

V

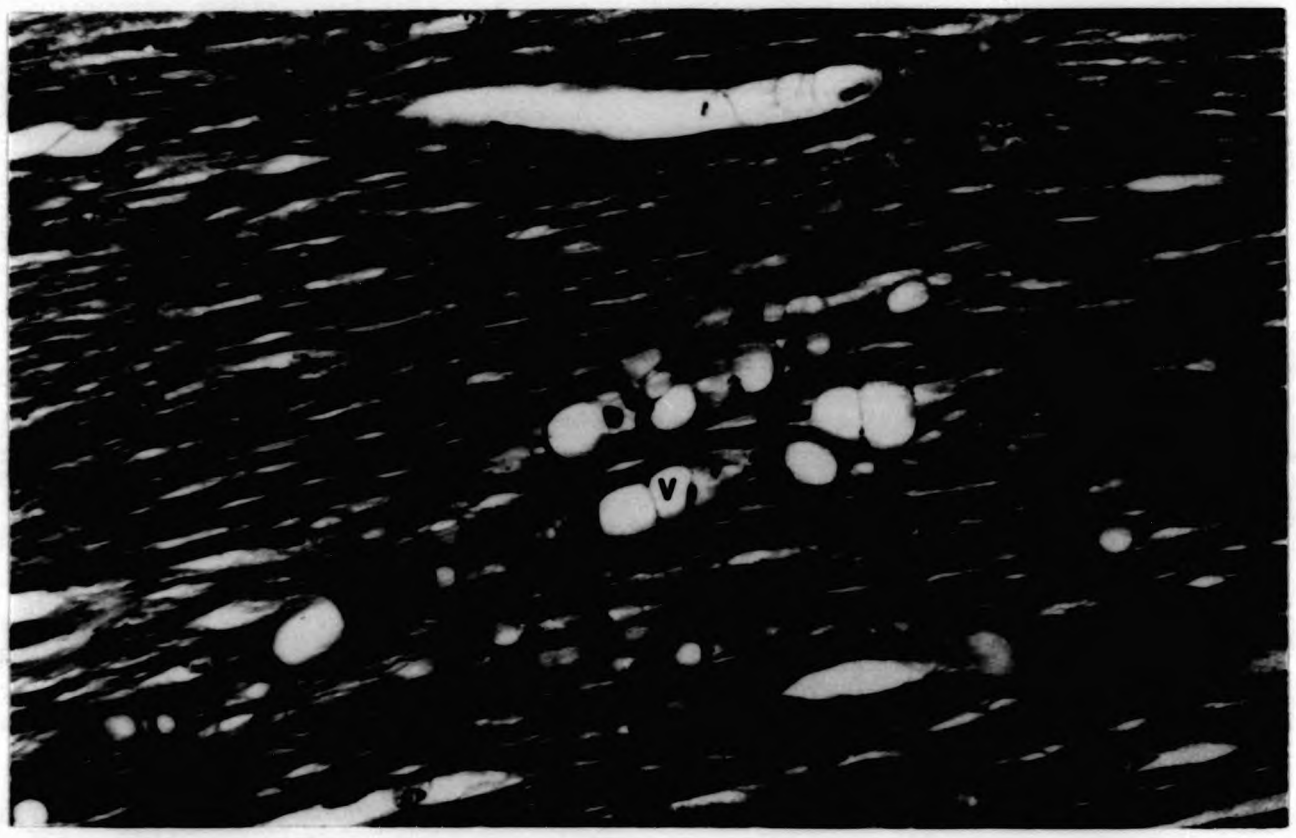
Fig. 43 Severe multifocal to diffuse gill lamellar and gill stem aggregation and encapsulation and nodule formation (N) of melanized CFA-vacuolated phagocytes (V). CFA injection, 17 days (HE, X125).

Fig. 44 Marked multifocal melanized encapsulations (E) and infiltration and aggregation of CFA-vacuolated phagocytic haemocytes (V) in haemal sinuses of antennal gland labyrinth. CFA injection, 17 days (HE, X125).


N


E

Fig. 45 a&b (detail) Multifocal to areas of diffuse muscle tissue myofibrillar haemocyte infiltration and aggregations of vacuolated haemocytes containing phagocytosed CFA (V) with fibroplasia (F). CFA injection, 17 days (HE, X250, X500).


V

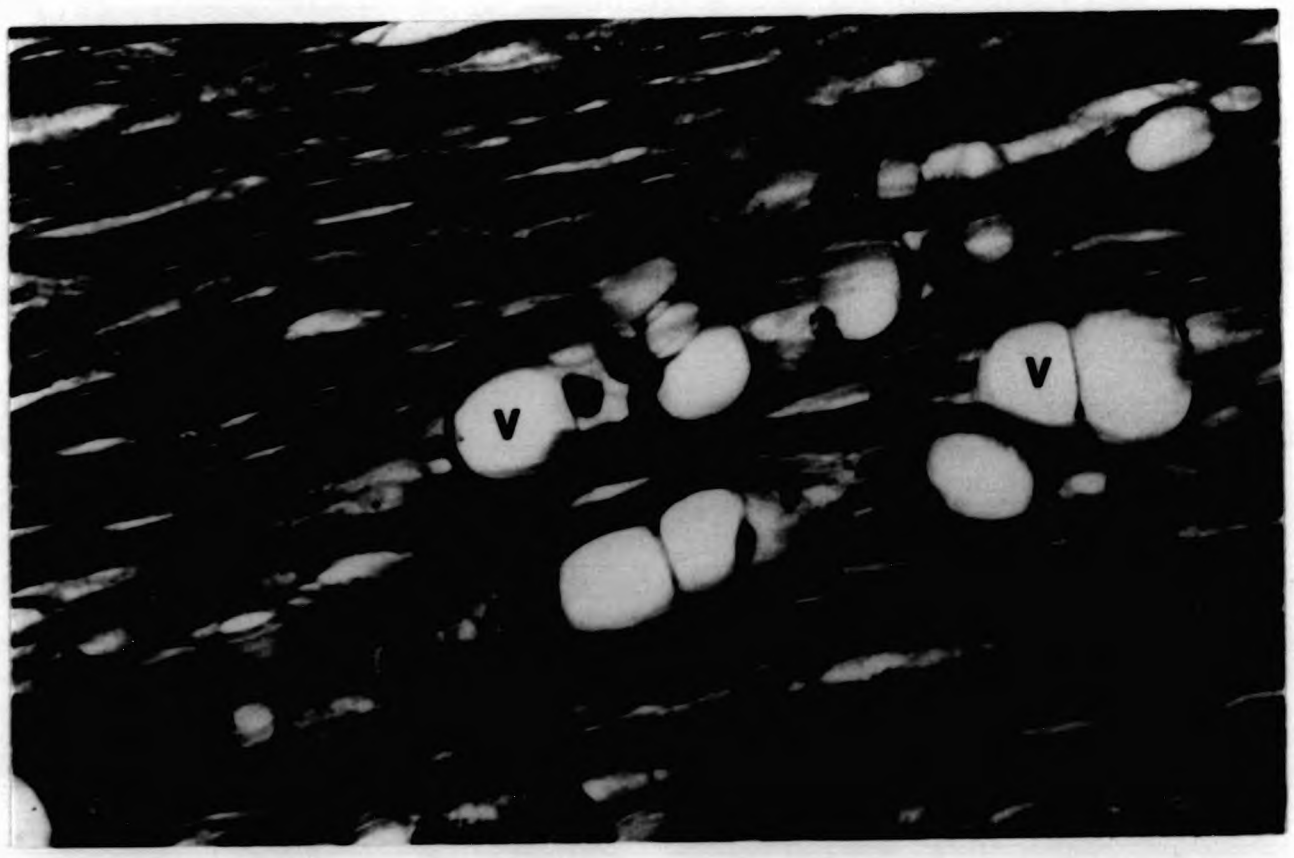

V
V

Fig. 46 Multifocal muscle tissue myofibrillar infiltration, aggregation, encapsulation and nodule formation (E) involving vacuolated CFA-containing haemocytic phagocytes (V). CFA injection, 21 days (HE, X250).


V
E

(Tables 1, 4). Infiltration occurred in the heart after 3 hours (Tables 1, 4). Encapsulations, some melanized or necrotic, were found in numerous tissues after 3 hours (Tables 1, 4) and progressively became more prominent with concomitant nodule formation and melanization and necrosis, especially in the gills, hepatopancreas, antennnal gland, muscle, connective and nerve tissues and haemocoel up to 21 days (Figs. 37, 38, 39, 41, 43, 44, 46; Tables 1, 4). Fibroplasia was observed in the four latter tissues from 7 to 21 days (Fig. 45; Tables 1, 4). From 12 hours to 10 days there was increased aggregation and phagocytosis in the heart (Fig. 40; Tables 1, 4), with encapsulation and melanization from 17 to 21 days (Tables 1, 4).

Some participation of phagocytes in capsule formation was also observed (Figs. 39, 43, 46). There was a persistence of infiltration, aggregation, phagocytosis, melanization and/or encapsulation/nodule formation in most organs up to 21 days (Fig. 46; Tables 1, 4).

III. 3.4 The Tissue Response to Auto- and Allografting

The results were similar for both experiments and are combined.

Gross Findings

Within 12 hours of grafting the cuticle into muscle there was melanization of the cuticle around the grafted cuticle ("shell insert") and the surrounding

muscle become brown in colour. After 24 hours and later, the grafted cuticle became brown in colour (Fig. 47).

Histological Findings

* 3 hours after grafting:

Slight to moderate focal, multifocal to small areas of diffuse, slightly compressed rows of infiltrating and aggregatimg haemocytes (an early multilamination or "open" encapsulation) formed around the insert and in the adjacent muscle, with slight focal myofibre necrosis (Table 1). The haemocytes had uniform basophilic nuclei, some with prominent nuclei (Fig. 48).

* 6 hours after grafting:

The haemocyte aggregations in the muscle around the insert were more organized in tighter rows suggesting early encapsulation of the insert (Table 1). The haemocyte cells and nuclei were larger and oval, rather than round, and more vesicular and displayed heterochromatin and chromatin margination, all suggesting increased activity; the eosinophilic cytoplasm was often contiguous; focal to multifocal pyknotic and karyorrhectic nuclei (of myofibres and/or haemocytes) were present. Haemocytes generally infiltrated the haemolymph spaces between the myofibrils, except in damaged areas where they infiltrated and aggregated in increased numbers; small amounts of fibrosis, with collagen formation were also present (Table 1). The haemocytes infiltrating around damaged muscle fibres were

Fig. 47 Dark brown shell autograft/insert in the muscle of the second abdominal segment after 2 weeks (arrow).

still round (unlike the more oval ones aggregating closely around the insert) and about 5 micrometres in diameter (Fig. 49); the oval ones were about 7.5 micrometres.

* 12 hours after grafting:

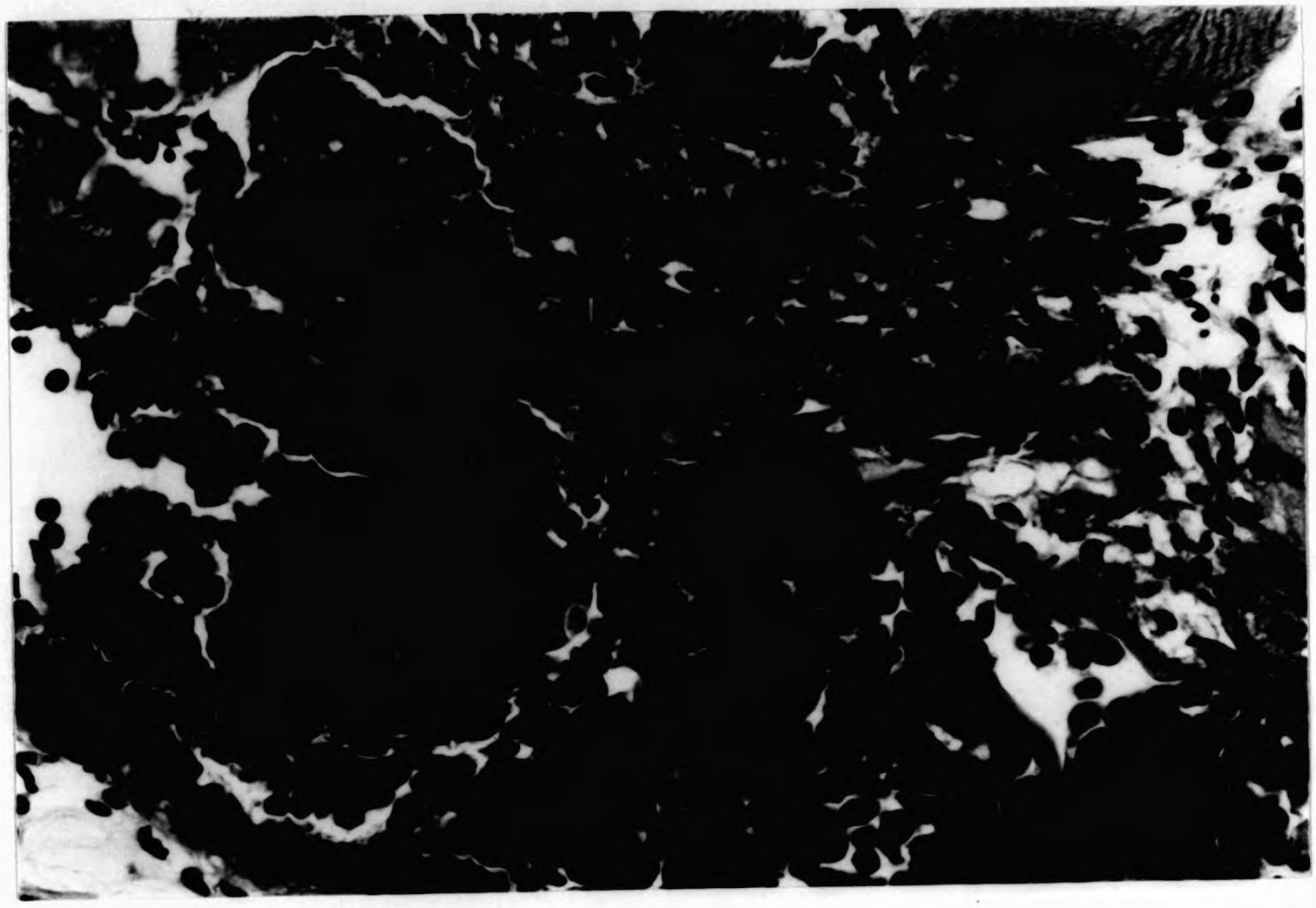
Abundant more compressed, tighter aggregations of haemocytes formed multilaminated rows, indicative of progressive encapsulation of the insert, with elaboration of collagen fibres between cells. There were focal aggregations of haemocytes in the surrounding spongy connective tissues. The encapsulating haemocytes had become more flattened, fusiform or spindle-shaped with elongated, multivesicular nuclei. Some haemocytes displayed an increasingly deep eosinophilia to light red-brown colouration indicative of early melanization; there was already a thin layer of brown melanization along the epicuticular surface of the shell insert (Table 1). Many of these haemocytes also had an effete appearance. Slight to moderate amounts of collagen fibres forming a matrix were between the haemocytes depending upon the proximity and compression of the cells. Groups of fibroblasts and fusiform haemocytes formed wavy bundles of cells.

* 18 hours after grafting:

The changes were similar to 12 hours, but with tighter, compressed bundles forming multilaminated sheets and with focal pyknotic and karyorrhectic nuclei and focal to diffuse melanization closely encapsulating the cuticlar insert, including the setae (Fig. 50). In some

Fig. 48 Area of diffuse myofibre necrosis (S) and haemocyte infiltration and aggregation (a), with portions of predominantly oval haemocytes in early multilaminar formation (l). Autograft, 3 hours (HE, X600).

Fig. 49 Area of slight myofibre necrosis (S) with haemocyte infiltration and aggregation (a). Autograft, 6 hours (HE, X600).



areas, usually in the layers most proximal to the insert, the haemocytes were flattened and fusiform, with the cytoplasm contiguous or in such close proximity that it was difficult to discern individual cells; in other places, usually more distal to the insert, the haemocytes were more oval; there was a variable nuclear heterochromatin content, chromatin margination and cytoplasmic vacuolation. There were also slight focal haemocytes to areas of diffuse haemocytic (with round to oval nuclei) aggregations in the adjacent damaged muscle, with focal pyknotic and karyorrhectic nuclei.

* 24 hours after grafting:

The insert was encapsulated by multilaminar sheets of tightly packed, compressed fusiform haemocytes, some areas of effete melanized haemocytes, and areas with more abundant collagen between the cells (Fig. 51 a&b). The haemocytes just distal to the melanized effete ones were still intact. The layer directly in contact with the insert was melanized as was the cuticular site of shell insertion, including the epi-, exo-, endocuticle and the epidermis and subcuticular connective tissues. There was also a large focal nodule of closely packed multilaminar haemocytes containing multifocal melanized encapsulations. A few bacterial colonies probably introduced with the insert were also sequestered in melanized encapsulations, but these were not associated with the graft reaction. Adjacent muscle displayed slight

Fig. 50 Area of aggregated (a) compressed necrotic melanized haemocytes with nuclear pyknosis (Y) and karyorrhexis (K), and muscle necrosis (S) associated with cuticle and setae (t) of graft/insert (g). Autograft, 18 hours (HE, X600).

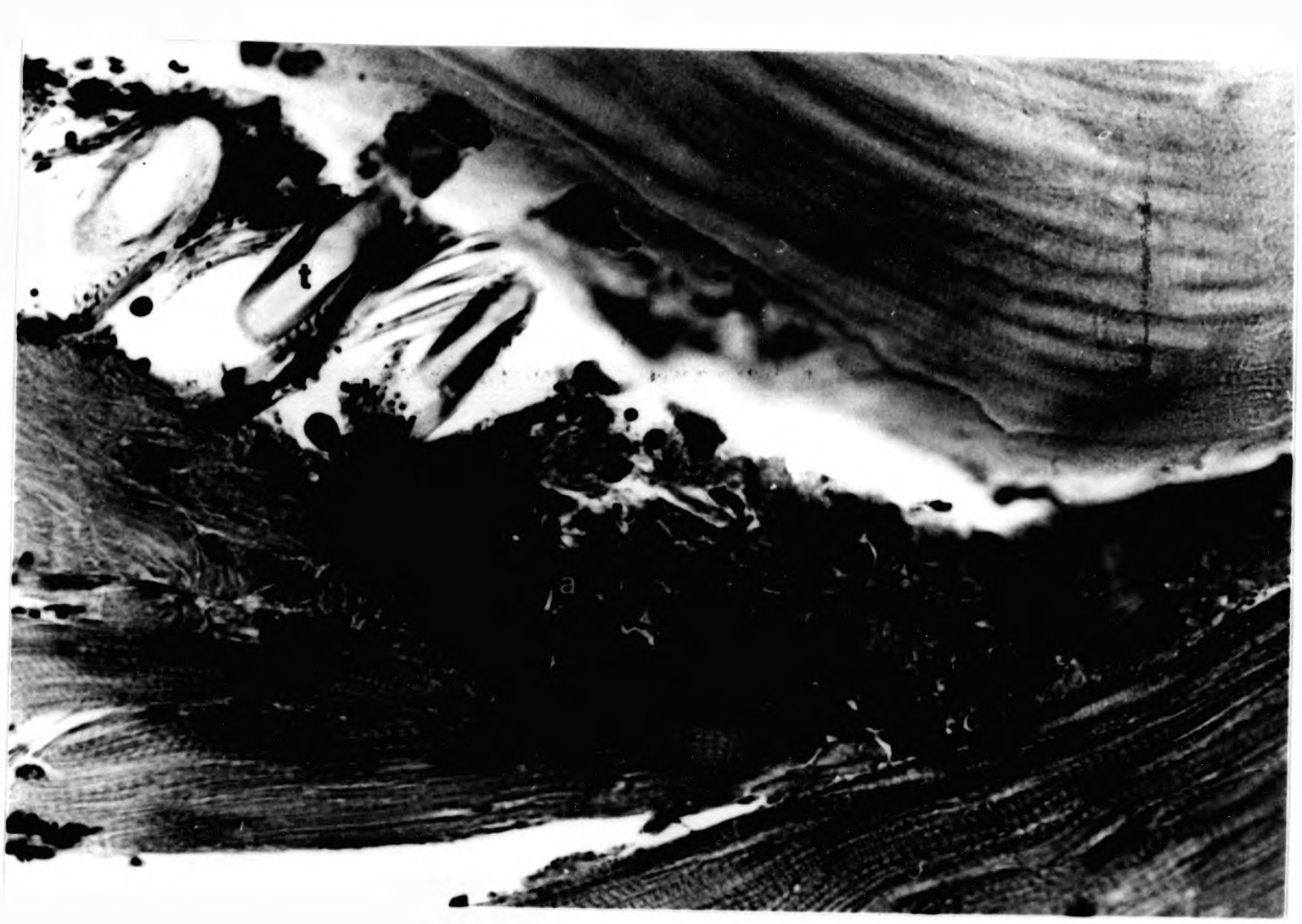
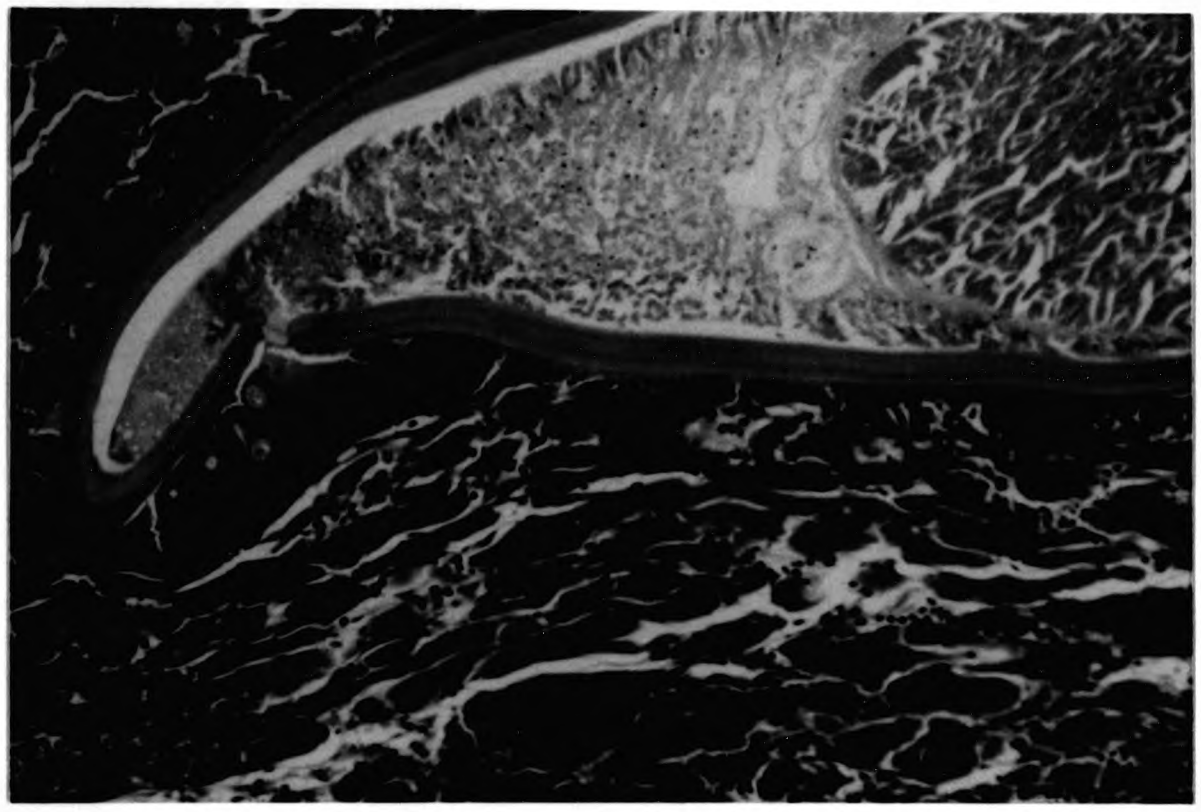


Fig. 51a Graft/insert (g) with setae encapsulated by haemocyte multilamination (l). Autograft, 24 hours (HE, x125).

Fig. 51b Encapsulation of graft/insert (g) with setae (t) by melanized compressed multilaminar oval, elongated and fusiform effete haemocytes (l). Allograft, 24 hours (HE, X600).



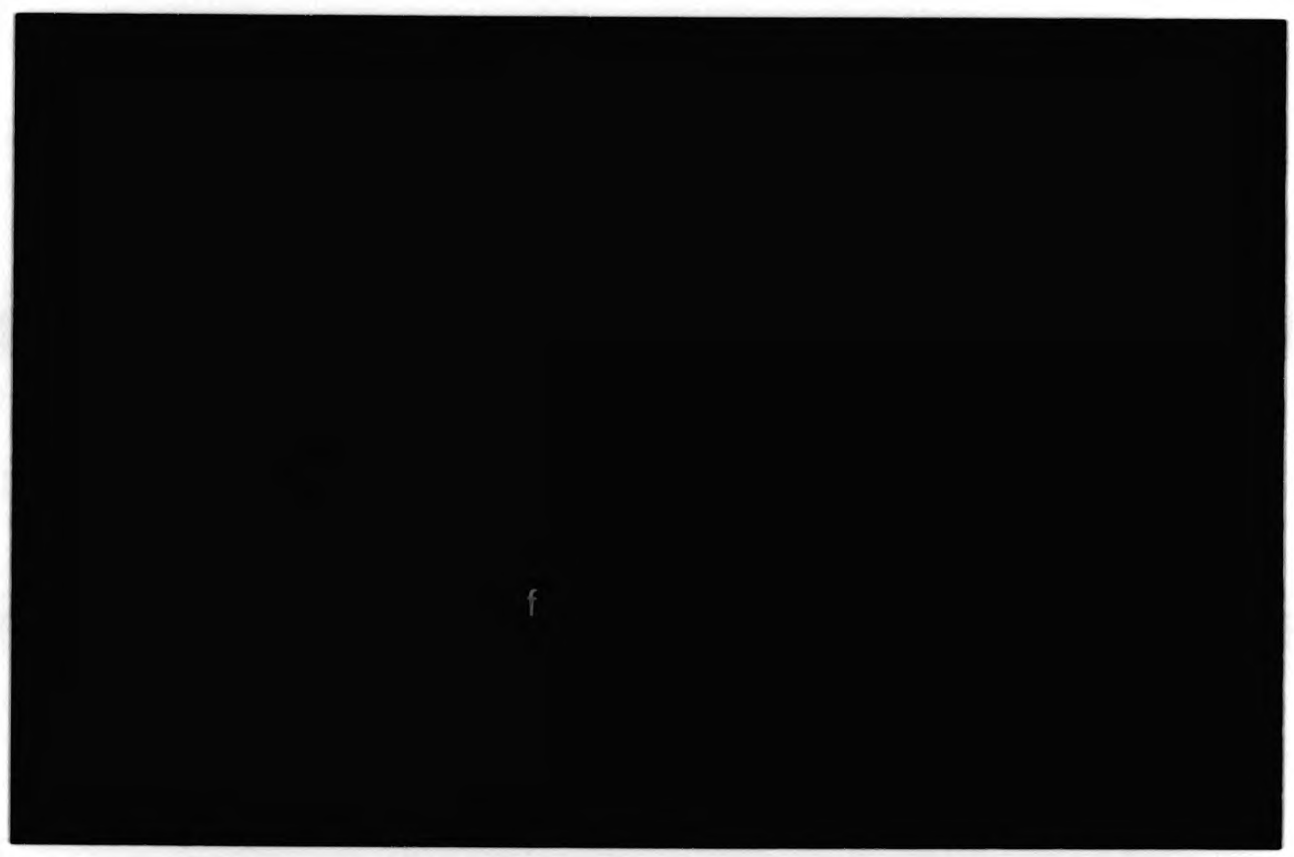

9

multifocal to moderate diffuse areas of myofibre necrosis and areas of diffuse haemocyte infiltration and aggregations.

* 26 hours after grafting:

Changes were as after 24 hours, but with a broader band of deeply brown melanized, very tightly packed multilaminar effete haemocytes encapsulating the insert (Fig. 52); one cell appeared to be on top of the other, in extremely close apposition but with increased collagen between some cells in some areas. Multifocal pyknotic and karyorrhectic nuclei were also present, especially in the tissue insert interface area. The haemocytes were more fusiform with elongated, basophilic nuclei. Haemocytes in large aggregations displayed a tendency for individual cells with discernible cell membranes to become conjoined with apparently contiguous cytoplasm. There were also focal melanized encapsulations. Damage by the insert in adjacent muscle elicited a slight to moderate haemocyte response, with slight focal fibroplasia (Fig. 52). The damaged site of insertion of the cuticle, epidermis, spongy connective tissue and muscle contained haemocyte infiltrations and aggregations and was encapsulated by compressed multilaminar sheets of haemocytes, a wide band of effete, melanized haemocytes present, especially in the endocuticle.

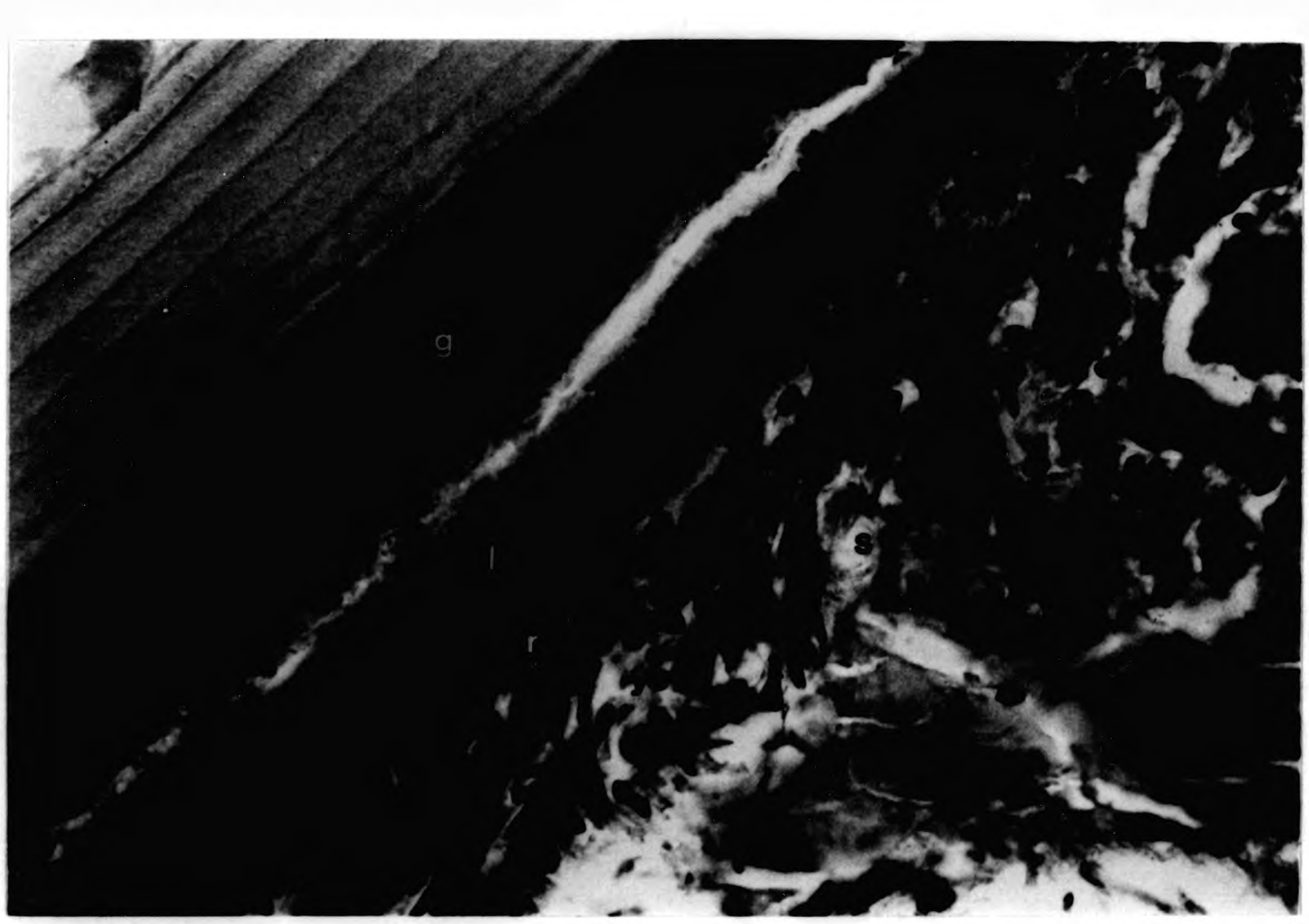
Fig. 52 Degenerating graft/insert (g) with setae encapsulated by a wide band of multilaminar effete melanized haemocytes (l), followed by an area of myofibre necrosis (S), haemocyte infiltration and aggregation (a) and slight fibroplasia (f). Allograft, 26 hours (HE, X125).



* 2 days after grafting:

The insert was encapsulated firstly by a much broader band consisting of multilaminar sheets to compressed fusiform and effete, partially melanized to melanized haemocytes, with basophilic elongated nuclei, contiguous cytoplasm and abundant collagen fibrils between adjacent cells; there was a mixture of fusiform and round/oval nuclei -a possible trend of round/oval nuclei becoming more fusiform, i.e. a transformation of typical haemocytes into fibroblast-like cells (Table 1). (Thus fibroblast and fibroblast-like cell, i.e. haemocyte, were considered to be identical in these observations). There were also multifocal pyknotic nuclei throughout the multilaminated sheets. Next, there was a band of haemocytes, forming aggregations to multilaminations, less tightly packed but in close apposition; only those adjacent to the inner appeared to be effete (Fig. 53). This zone also contained much karyorrhectic nuclear debris. The surrounding muscle contained slight to moderate infiltrations of haemocytes between and within the necrotic myofibres, with some areas of haemocytes forming multilaminar, orderly encapsulations, other more disorganized masses of cells with large round-oval nuclei with prominent heterochromatin and chromatin margination and cytoplasmic microvesiculation, the cytoplasm of cells contiguous with adjacent ones. Most haemocytes had round nuclei, but others were oval to fusiform and fibroblast-like. There was also focal pyknotic and karyorrhectic nuclei. There

Fig. 53 Detail of graft/insert cuticular surface (g) encap -sulated by a broad band of multilaminar compressed effete and melanized haemocytes (l), followed by an area of mixed round (r), oval and fusiform haemocytes, with multifocal pyknotic nuclei (y) and an area of myofibre necrosis (S) and haemocyte infiltration (i). Autograft, 48 hours (HE, X600).



was a slight proliferation of fibroblasts and collagen between the damaged muscle fibres. The site of grafting in the cuticle was well sealed by a zone of multilaminar effete melanized haemocytes. Under this, in the spongy connective tissue and muscle there was a marked diffuse aggregating network or meshwork of haemocytes, the cells exhibiting conjoined cytoplasmic strands.

* 2.5 days after grafting:

As after 2 days, some areas displayed very tightly packed disorganized haemocyte aggregations or organized haemocyte multilaminations. The insert epicuticle was encapsulated by a variably thicker, deeply brown melanized band; although acellular in appearance, the dark melanization probably masked the effete haemocyte multilaminations of earlier infiltrating cells.

* 3 days after grafting:

Similar to after 2 days, with the continued formation of a number of distinct zones. The insert was firstly encapsulated by (zone 1) a variably thick band of melanized multilaminar effete haemocytes. Another area at the apex of the insert consisted of haemocytes which varied from an organized and multilaminar encapsulation to disorganized aggregations. Next (zone 2), was a wide area of aggregating haemocytes in close apposition:- some areas had a very well organized multilaminar structure; another area was not organized in layers, but appeared to be becoming more tightly compressed, forming a network of

cytoplasmic strands (with intervening vacuolation) linking adjacent aggregating cells. Focal melanized encapsualtions and nodules; and individual and whorling nests of fibroblasts accompanied by networks of collagen fibrils interspersed between the haemocytes were also present throughout this zone. The most prominent area of fibroblast proliferation (zone 3), though, was distal to the second zone. Zone 3 also contained multifocal encapsulations and nodules with melanized centres. Haemocyte infiltration (zone 4) spread extensively into adjacent and surrounding damaged muscle tissue interspersed with areas of fibroplasia and proliferating collagenous connective tissue. Moderate multifocal karyorrhectic nuclear debris and pyknotic nuclei were also present throughout all zones of the entire inflammatory response.

* 5 days after grafting:

Zone 1 consisted of a much wider band of deeply coloured melanization. The surface of the cuticle at the site of insertion consisted of epicuticular loss or erosion/ulceration of the melanized exo- or endocuticle. There was new cuticle and epidermis formation along the insert:host tissue interface. Zones 2 and 3 now consisted of an area of marked diffuse fibroblast and collagen proliferation, with numerous individual haemocytes and haemocyte aggregations between the fibroblasts. Zone 4 in the surrounding damaged muscle also contained areas of diffuse fibroplasia interspersed with haemocytes and

haemocyte aggregations. Fibroplasia of zone 2, 3 and 4 was definitely more prominent than previously. These areas had a multivacuolar appearance due to the meshwork of interconnecting cytoplasmic strands of fibroblasts and collagen fibrils. Multifocal karyorrhectic and pyknotic nuclei were present throughout the zones, but especially prominent in zone 2 (Fig. 54 a&b).

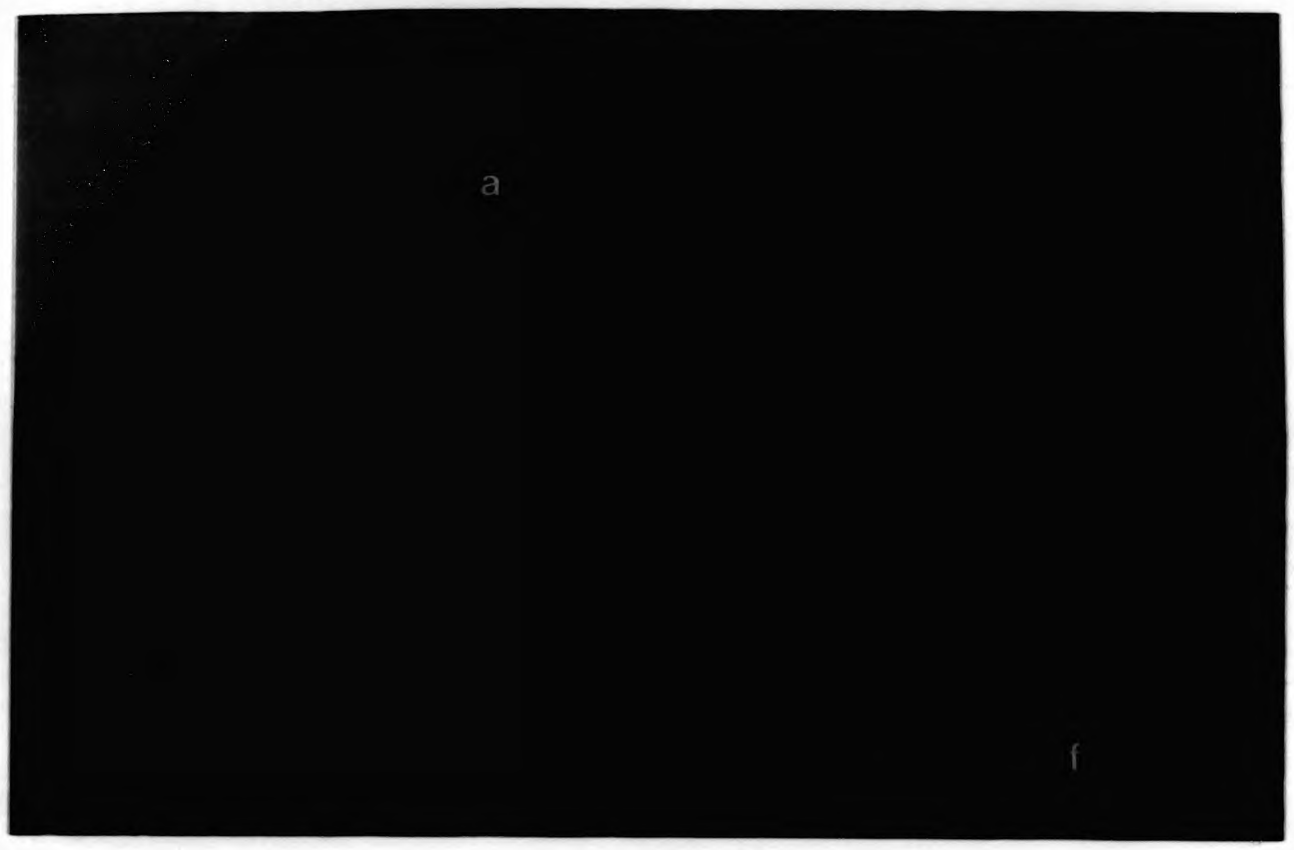
* 6 days after grafting:

Zone 1 appeared to consist of an inner area (zone 1a) of compressed multilaminar effete melanized haemocytes and an outer area (zone 1b) of a variably dense, less compressed, looser and non-melanized or partially melanized haemocyte multilamination. Zone 2, 3 and 4 were similar to those after 5 days.

* 7 days after grafting:

The insert and its cuticular setae were directly encapsulated by zone 1a, a dense, compact but variably thick melanized layer of multilaminated effete haemocytes, their nuclei still visible but not readily apparent. There were slight multifocal melanin pigment granule aggregations throughout. The cytoplasm of haemocytes was intimately associated with the melanin pigment, some encapsulating the granular masses. Focal haemolymph pooling was observed along the insert:inflammation interface. This zone was surrounded by zone 1b, consisting of more compact, non-melanized, fusiform haemocytes intermixed with aggregations of round/oval haemocytes and slight to moderate fibroplasia,

Fig. 54 a&b Band of encapsulating multilaminar effete haemocytes (l) with a large melanized nodule (n) (Fig. 54b, below), followed by layer of new cuticle (c) and epidermis (E) (Fig. 54a, above) and mixture of infiltrating (i) and aggregating fusiform, oval and round haemocytes, with nuclear karyorrhexis (K), and fibroblasts (f). Allograft, 5 days (HE, X500).


a

focal melanized encapsulations and nodules. Profuse fibroplasia with haemocyte aggregations was characteristic of zone 2, 3 and 4 (as Fig. 55).

* 9 days after grafting:

Zones 1a and 1b appeared wider. The other zones were similar to those described after 7 days, with moderate to severe diffuse myofibre necrosis in zone 4.

* 10 to 11 days after grafting:

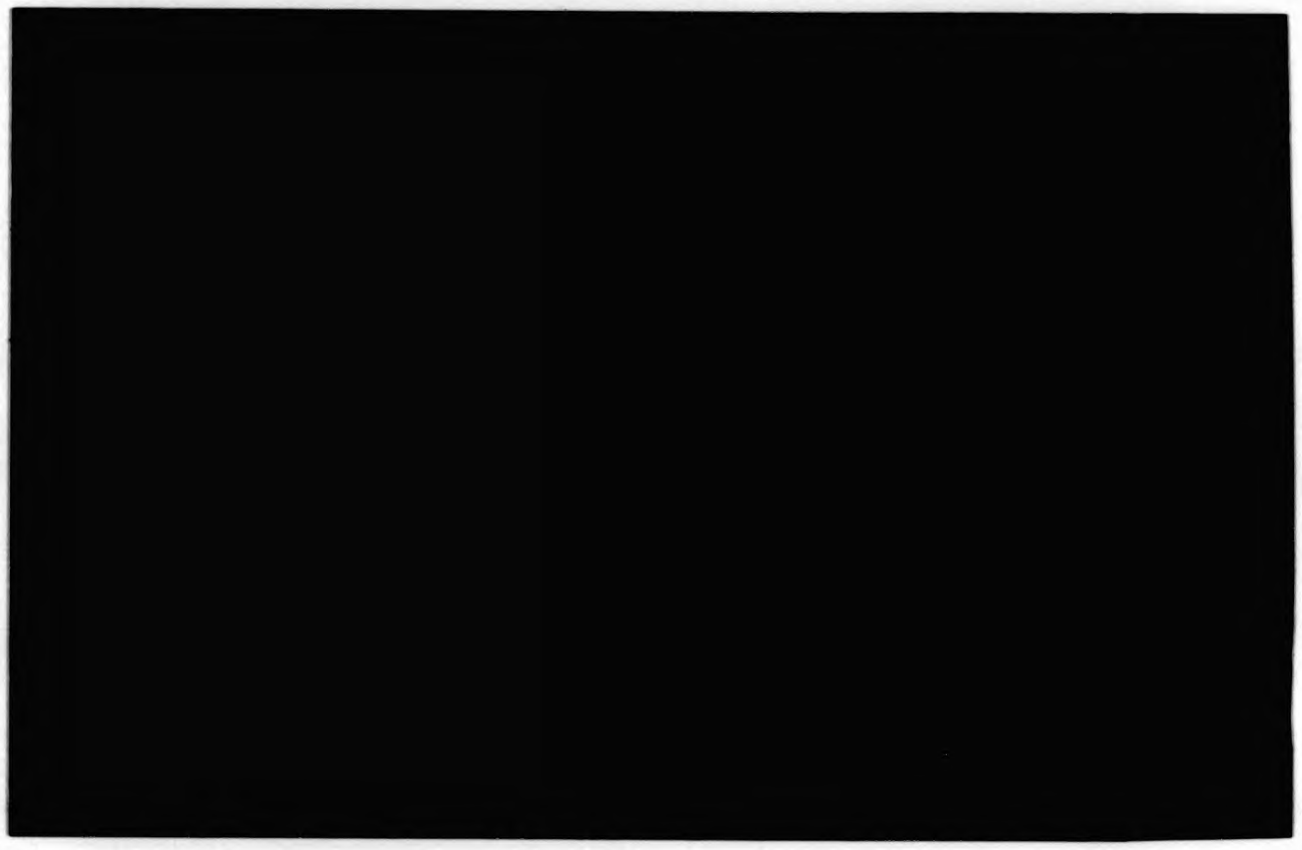
The insert was completely encapsulated by the multilaminar effete haemocytes of zone 1a. In zone 2, though, new cuticle and epidermis had formed around the insert and zone 1. Myophagia was also present in zone 4. There was prominent nuclear karyorrhexis and pyknosis throughout the zones.

* 21 days after grafting:

Zone 1 appeared similar to that described above. Zones 2, 3 and 4 were characterized by increasingly marked diffuse fibroplasia, with slight to moderate multifocal haemocyte aggregations and melanized necrotic encapsulations and nodules (Fig. 56). Large amounts of collagen were present between the fibroblasts. Moderate multifocal karyorrhectic nuclear debris and pyknotic nuclei were present throughout zones 2, 3 and 4. Myofibre necrosis and myophagia were present in zone 4, as above (Fig. 57 a&b).

Fig. 55 Graft/insert (g) has central degenerating region with haemocyte infiltration, whilst the area surrounding the new cuticular surface (c) and epidermis (E) consists of diffuse myofibre necrosis, fibroplasia (f), haemocyte infiltration and aggregations. Allograft, 21 days (HE, X125).

Fig. 56 Multifocal necrotic melanized encapsulations (e) in muscle tissue in an area of necrosis (S), haemocyte infiltration and aggregation (a) (zones 3-4). Autograft, 21 days (HE, X600).



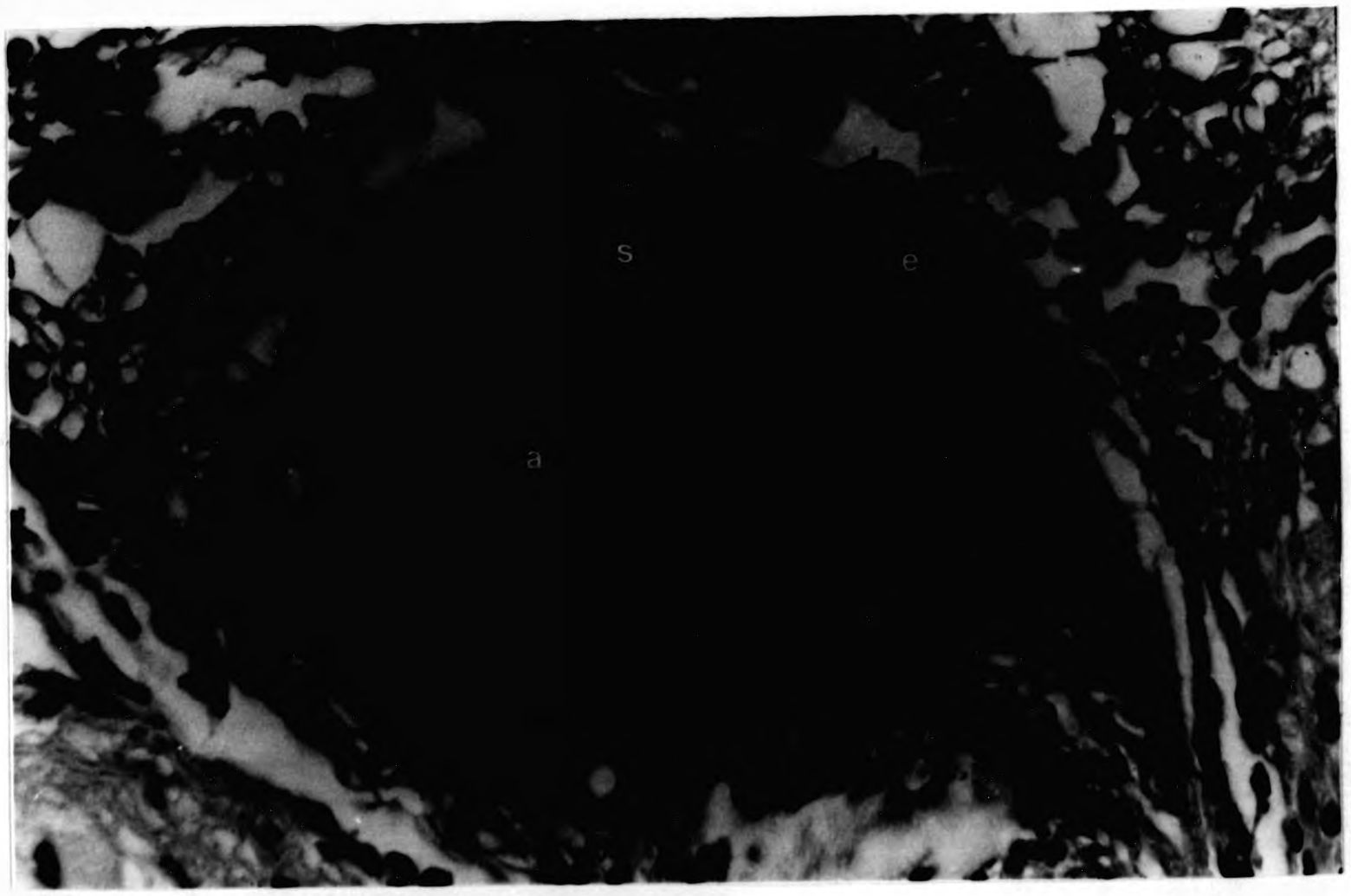
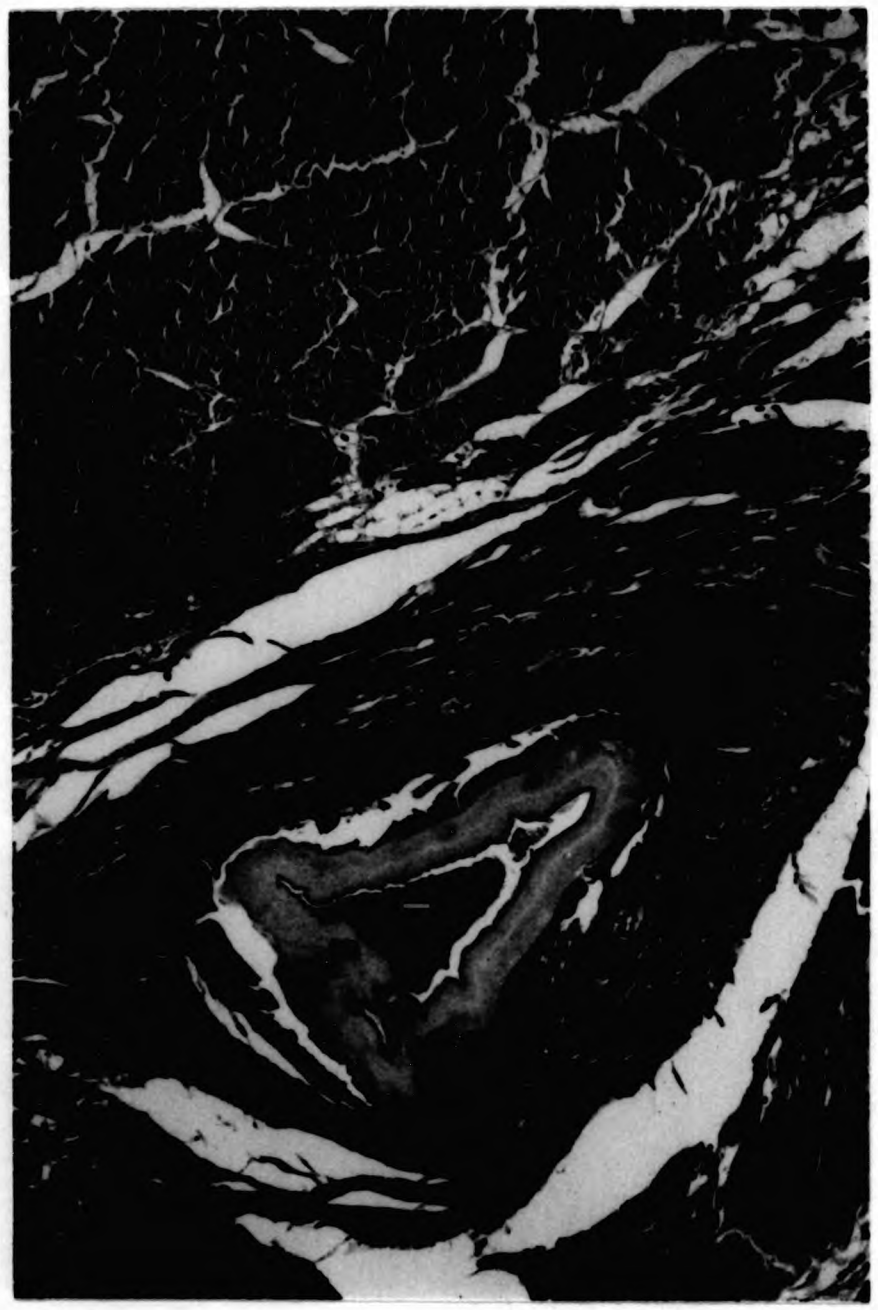

s
e
a

Fig. 57 a&b (detail) The central portion of cuticular graft/insert (g) is filled with an effete melanized haemocyte multilamination (l) (zone 1) followed by new cuticle (c) and epidermis (E), partially encapsulated; then there is an area of diffuse myofibre necrosis (S) and fibroplasia (F) mixed with haemocyte infiltration, aggregations (a), with multifocal pyknotic (y) and karyorrhectic nuclei (K) (zones 2, 3, 4). Normal muscle (M) in the surrounding area is also present (Fig. 57a, above). Autograft, 21 days (HE, X125, X300).



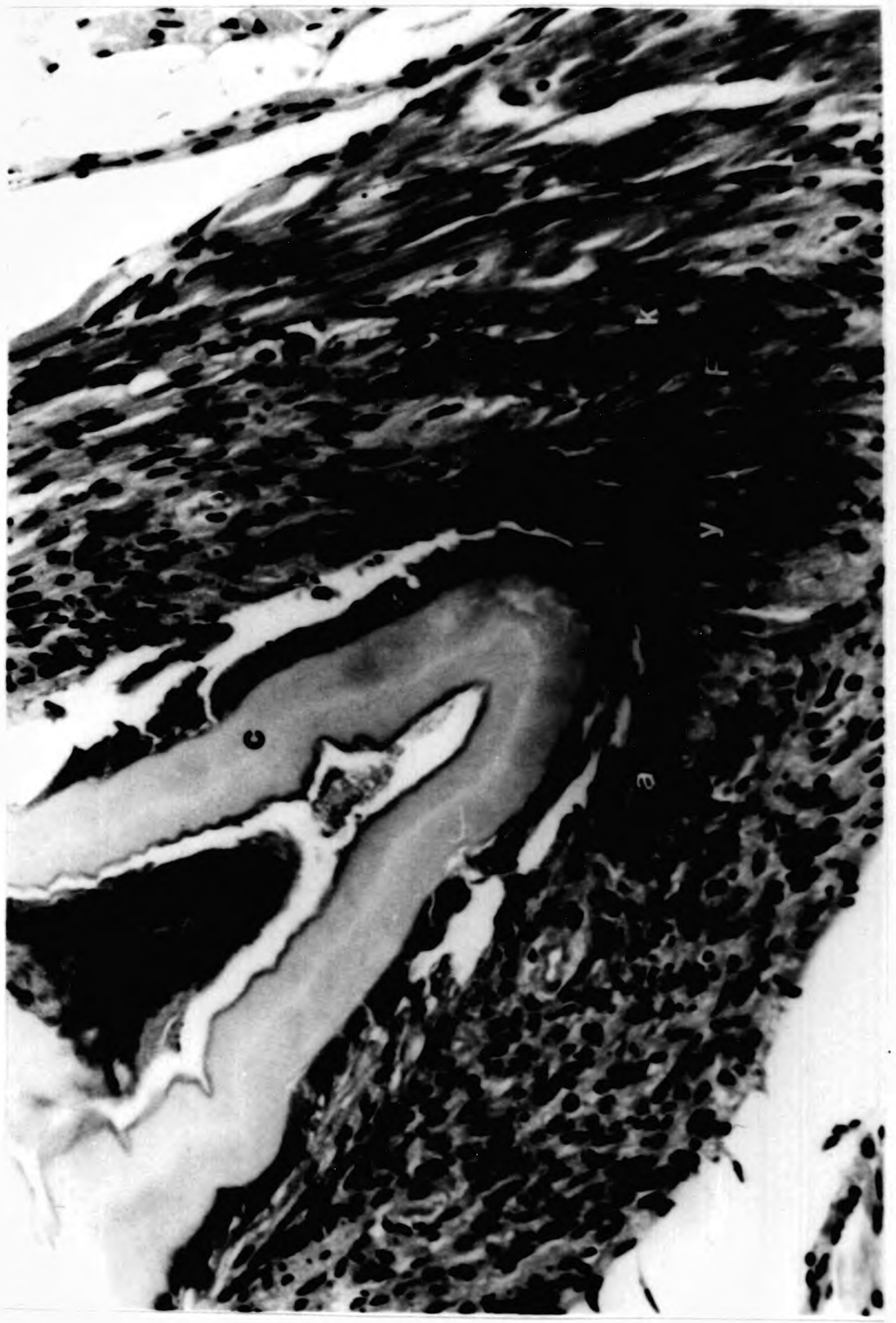
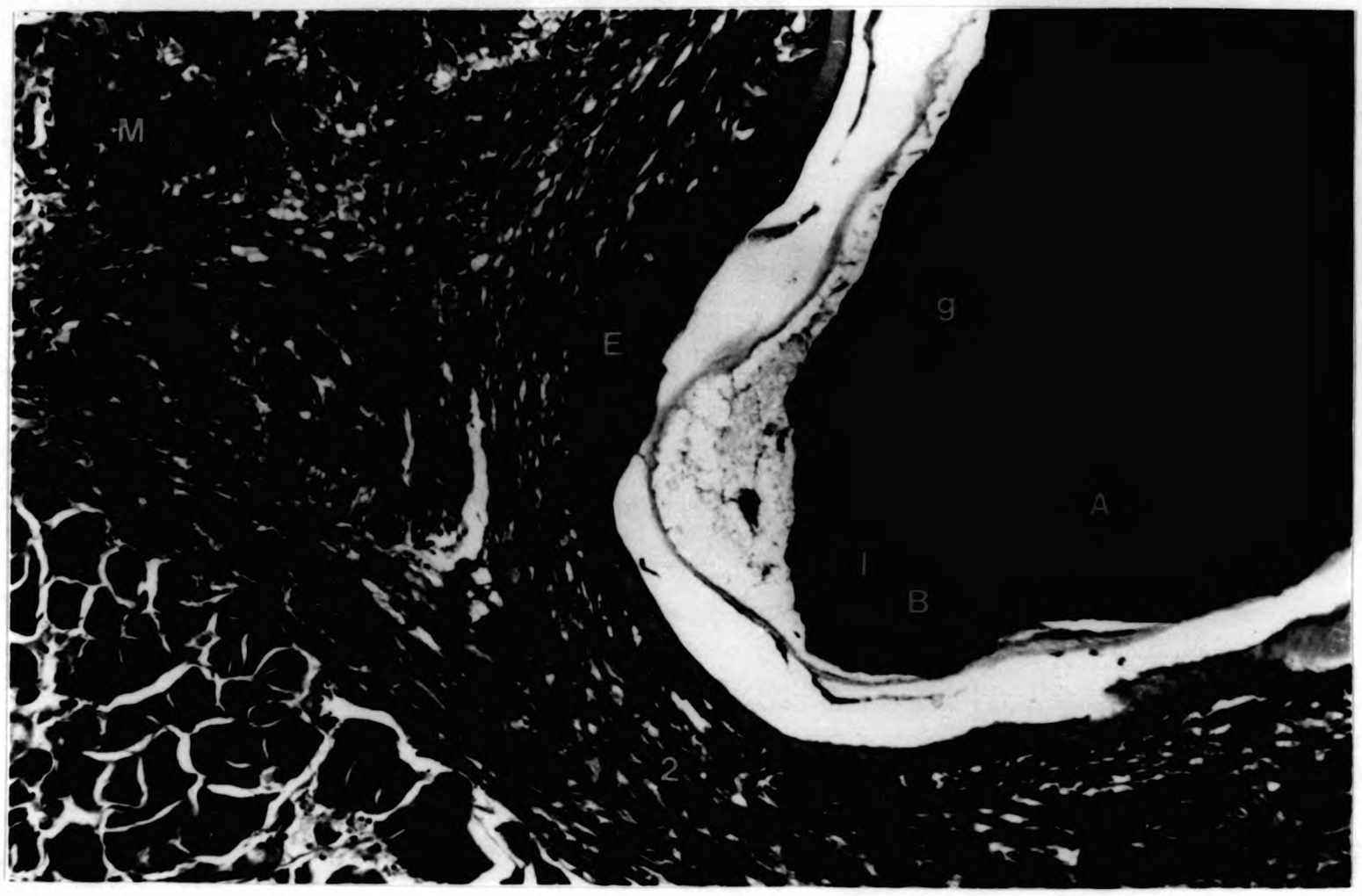


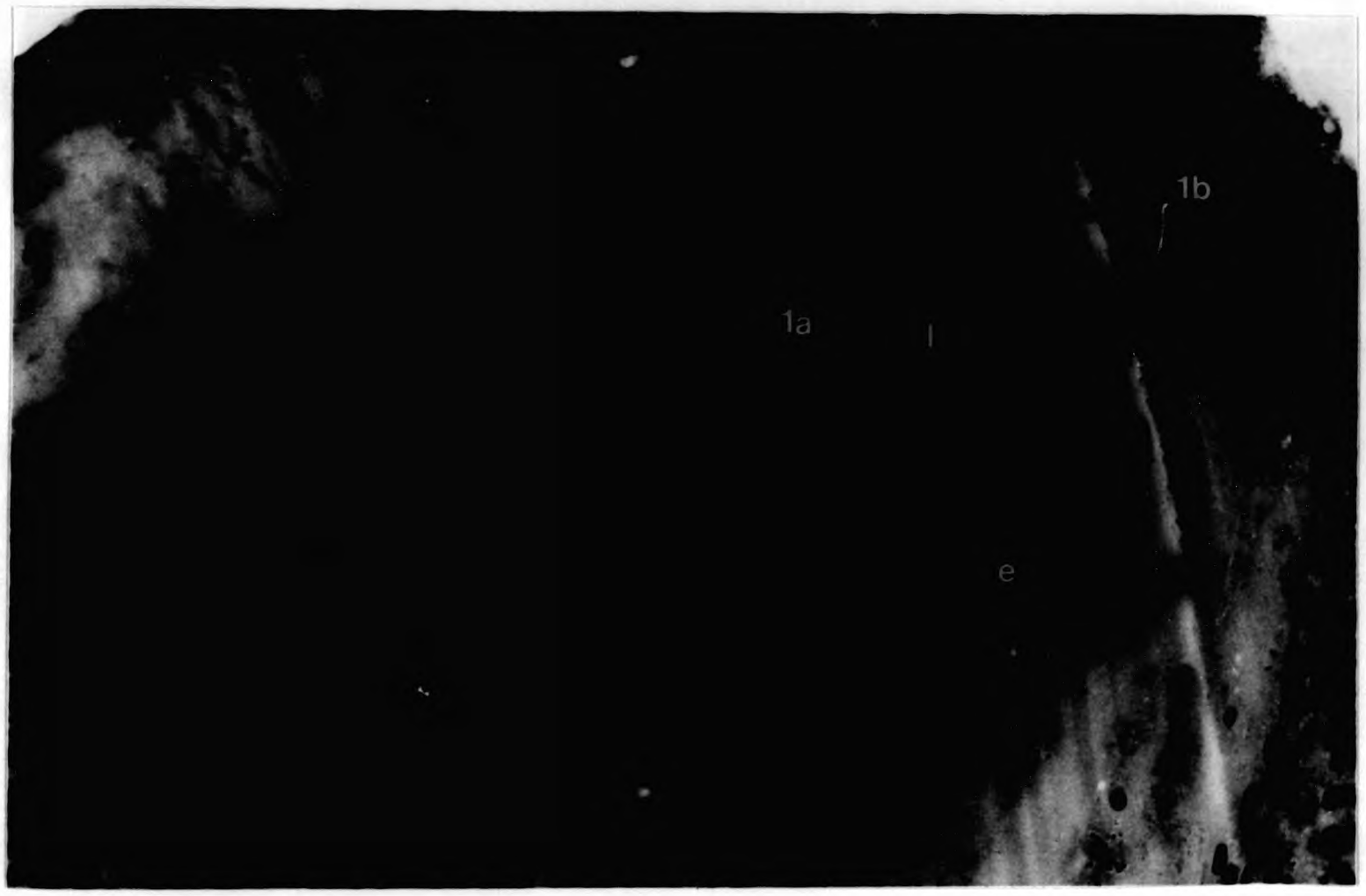
Fig. 58 a&b Original cuticular graft/insert (g) is completely encapsulated by multilaminated effete haemocytes (1)(zone 1a(A), 1b(B)), followed by new cuticle (c), epidermis (E) and spongy connective tissue. The merged zones of 2-4 (2) consist of an area of diffuse fibroplasia mixed with haemocytes and multifocal encapsulations (e) and nodules. The collagen in areas of fibroplasia (f) in zone 2, 3, 4 (Fig. 58b, below) stains green. Muscle tissue (M). Autograft, 28 days (HE, X150; Masson's trichrome, X125).


M
E
g
A
I
B
2


A
I
B
e
2
M

Fig. 59 Detail of a remnant of graft (g) encapsulated by a multilamination (1) of effete melanized haemocyte (zone 1a (1a)) with multilocular melanized encapsulations (e) surrounded by a zone 1b (1b) of partially melanized haemocytes. Autograft, 28 days (HE, X600).

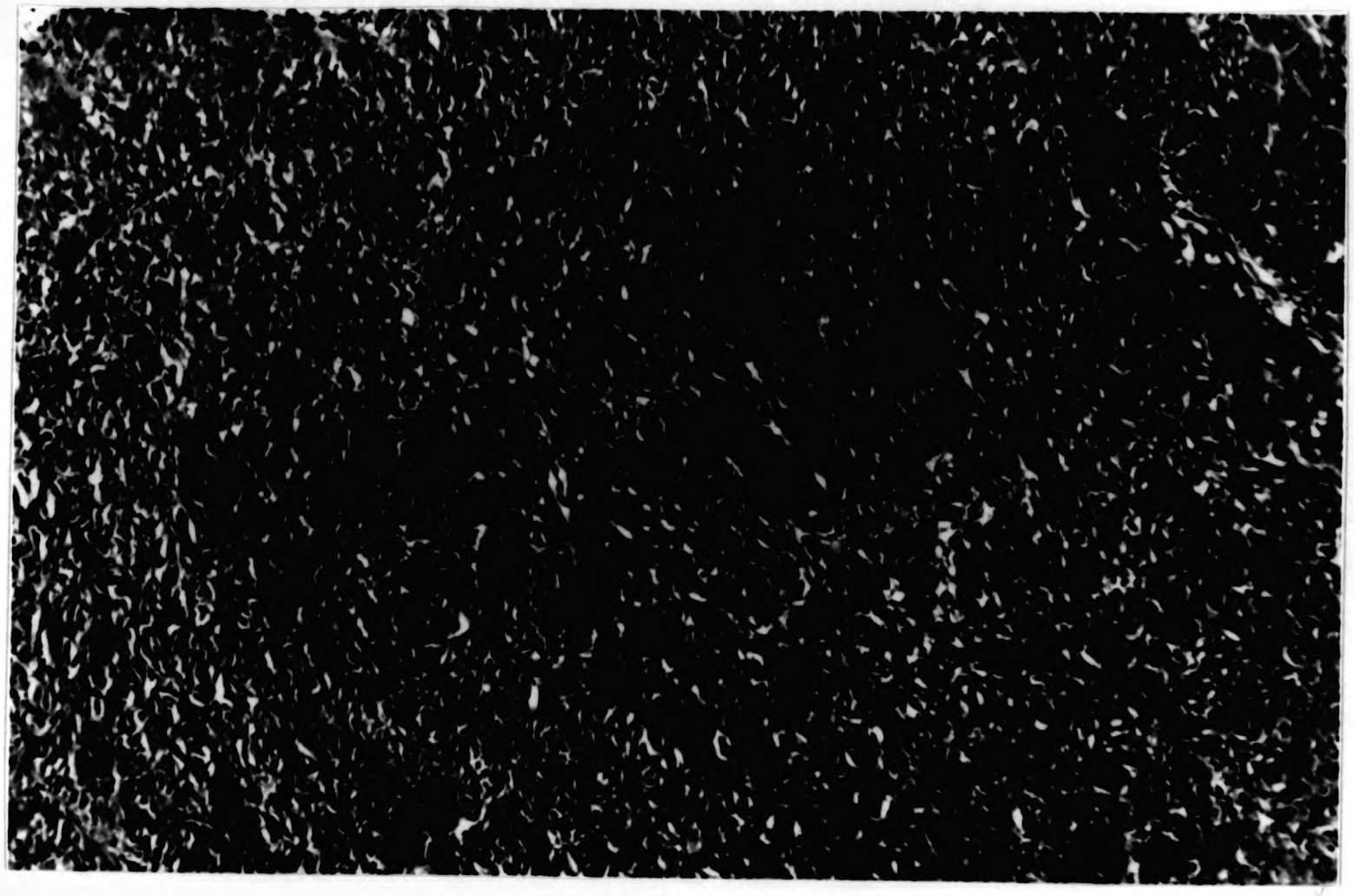
Fig. 60 Detail of new cuticle (c) and epidermis (E) and an area of diffuse fibroplasia (zone 2, 3, 4)(f) with a focal encapsulation (e). Autograft, 28 days (HE, X600).

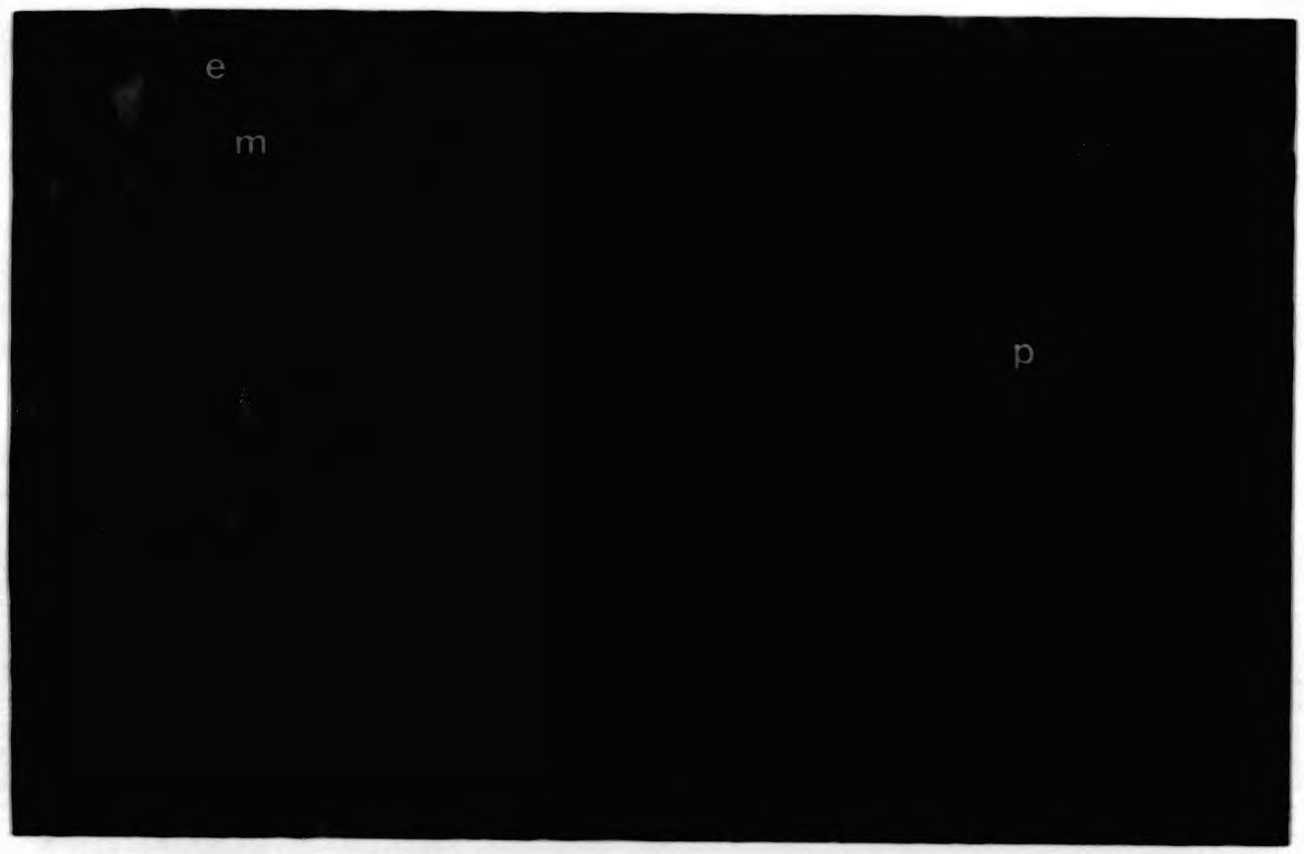

1b
1a
l
e


E
e

Fig. 61 Extensive myofibre necrosis and fibroplasia with haemocytic response in the muscle (zone 2-4) adjacent to graft. Autograft, 28 days (HE, X150).

Fig. 62 Multifocal melanized (m) phagocytes (P) and encapsulations (e) within area of diffuse myofibre fibroplasia (f) and haemocyte infiltration and aggregation (within zones 2, 3, 4). Allograft, 28 days (HE, X500).




e
m
p

* 28 days after grafting:

Results were similar to those observed after 21 days, with progressive fibroplasia (Table 1).

In summary, the remnants of the insert were encapsulated by zone 1a, a wide band of multilaminated effete melanized haemocytes. Zone 1b contained partially melanized haemocytes, some areas more compressed than others. New cuticle, epidermis and subcuticular spongy connective tissue were located between zones 1b and 2. Zones 2, 3 and 4 become increasingly merged, consisting of moderate to marked diffuse fibroplasia, the fibroblasts interspersed with haemocytes, with focal to multifocal melanized encapsulations and nodules. The chronic inflammatory fibrous connective tissue response replaced large areas of muscle in zone 4 (Figs. 58 a&b, 59, 60, 61, 62).

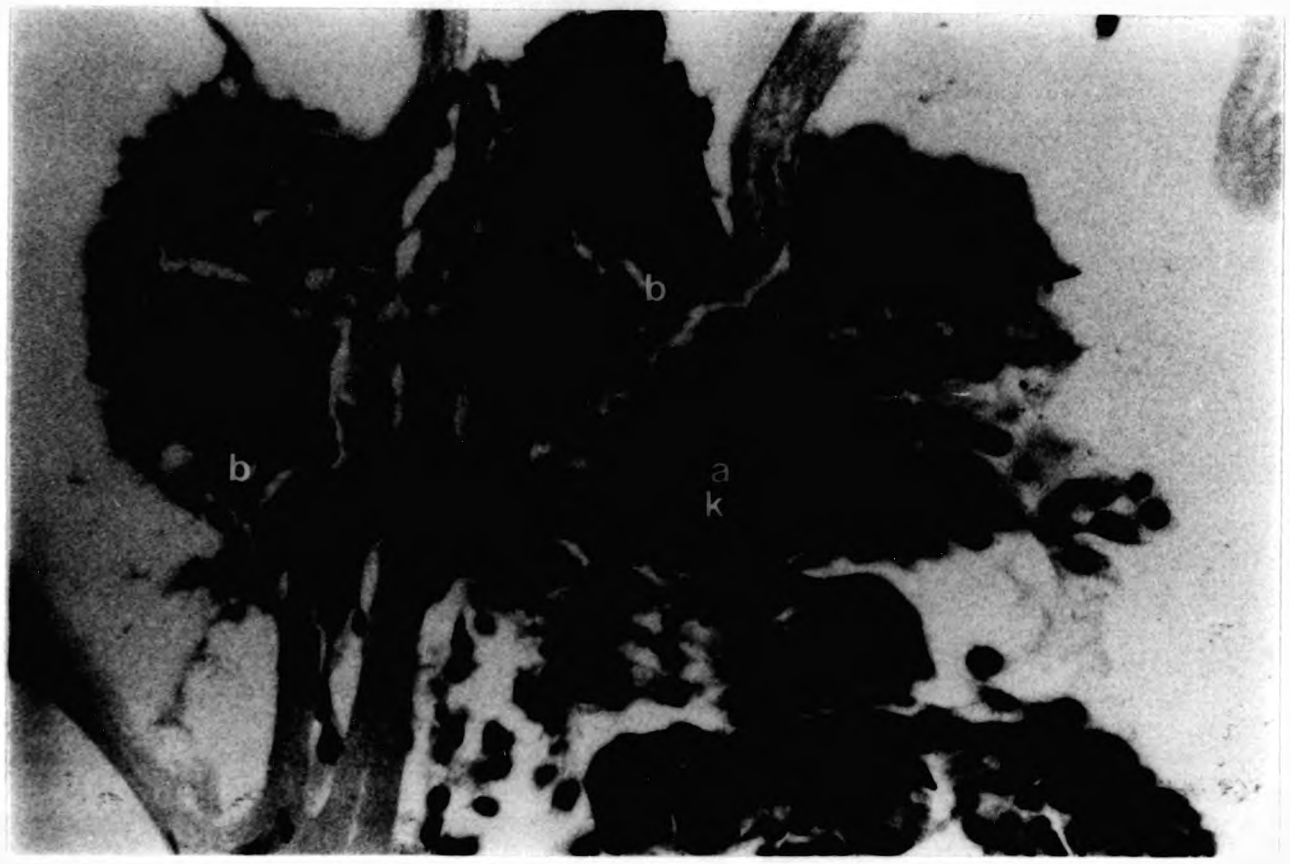
III. 3.5 The Tissue Response to Systemic and Intramuscular *Aeromonas* Injection

Histological Findings

As early as 1 hour and then continuing to the end of the experiment the haemocytic response of infiltration and aggregation was observed in numerous tissues, including the heart (Tables 1, 5); encapsulation was additionally present in the gills (as Fig. 63), muscle, connective tissues and haemocoel (as Fig. 64), also with melanization in the gills (as Fig. 63); in the hepatopancreas the haemocytic reaction also included phagocytosis (Figs. 73, 74; Tables 1, 5).

Fig. 63 Gill stem haemocytic aggregations (a) and multilocular melanized encapsulations (e) in association with branchial podocytes (b) containing engulfed material, with nuclear karyorrhexis (K). *Aeromonas hydrophila* i.m., 24 hours (HE, X500).

Fig. 64 Nodule (n) of necrotic melanized haemocytes in haemocyte aggregation (a) in dorsal haemocoel. *Aeromonas hydrophila* i.m., 24 hours (HE, X500).


b
b
a
k


n
a

Fig. 65 Peri-epicardial (heart) multilocular encapsulations (e) and nodules (n) of necrotic melanized haemocytes. *Aeromonas hydrophila* systemic, 24 hours (HE, X500).

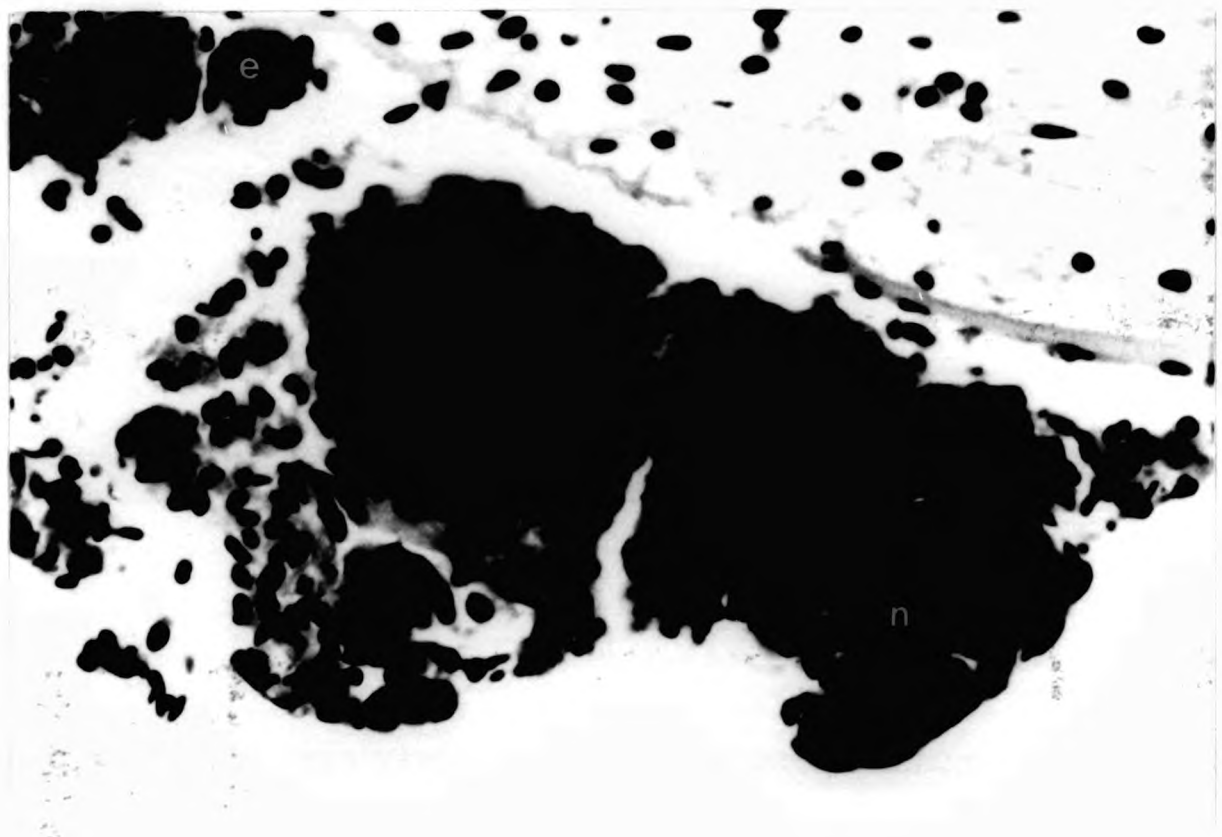

e
n

Fig. 66 a&b Small and large (heart) myo- and endocardial trabecular haemocytic aggregations (a), encapsulations (e) and nodules (n) with necrotic melanized centres. *Aeromonas hydrophila* i.m. 48 hours (HE, X250).


e
a

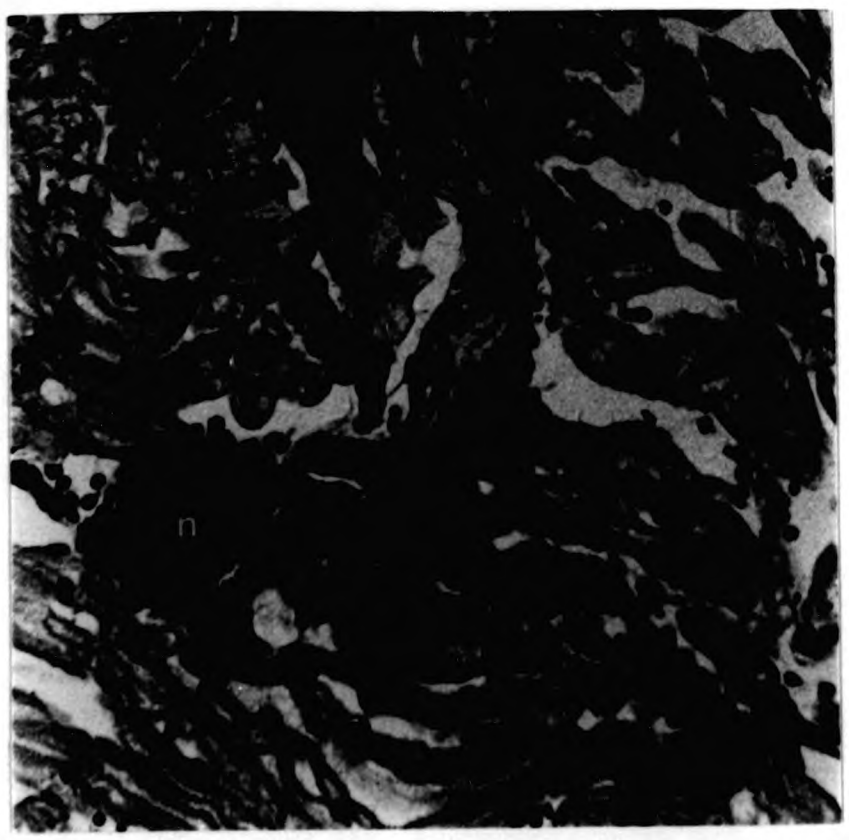
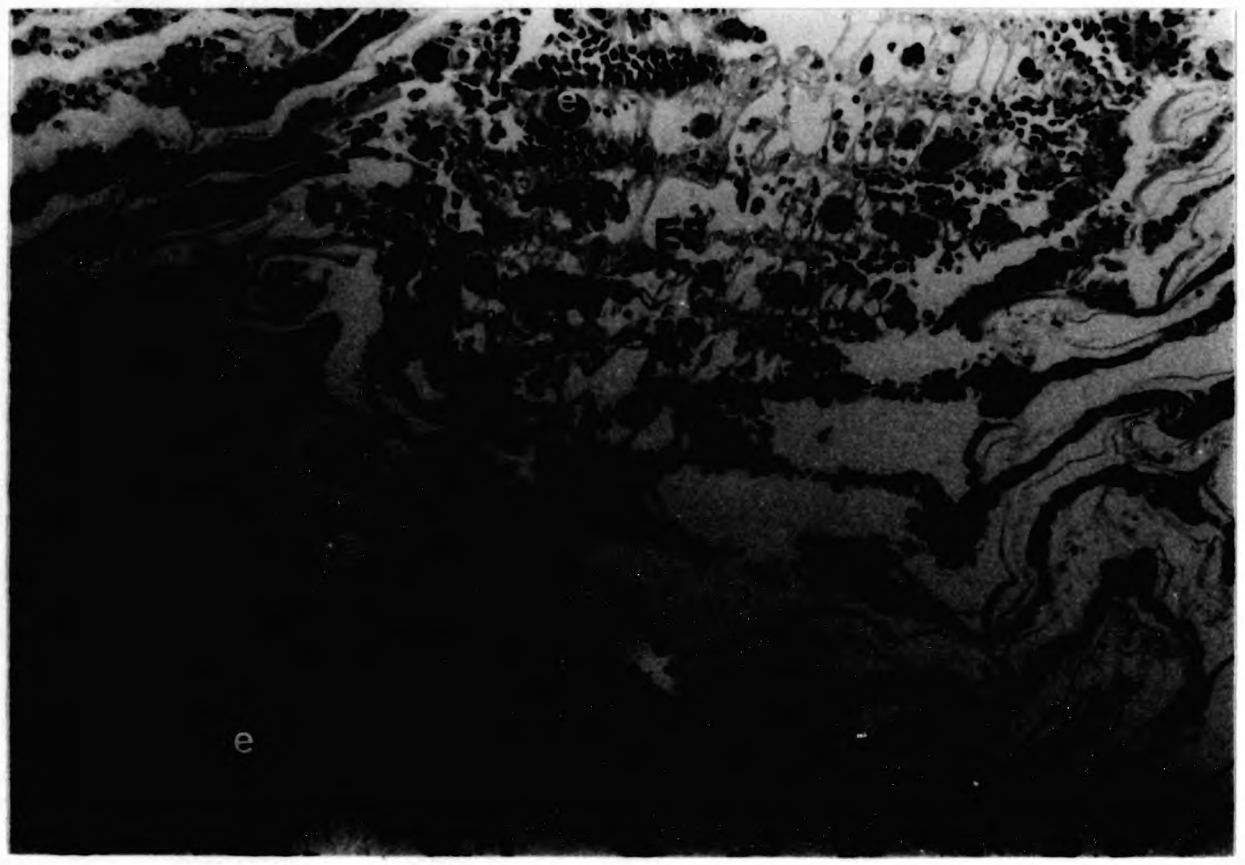

n

Fig. 67 a&b (detail) Gill stem efferent channel (EF) and gill lamellar (L) multifocal encapsulations (e) of necrotic melanized haemocytes. *Aeromonas hydrophila* systemic, 5 days (HE, X125, X250).


e

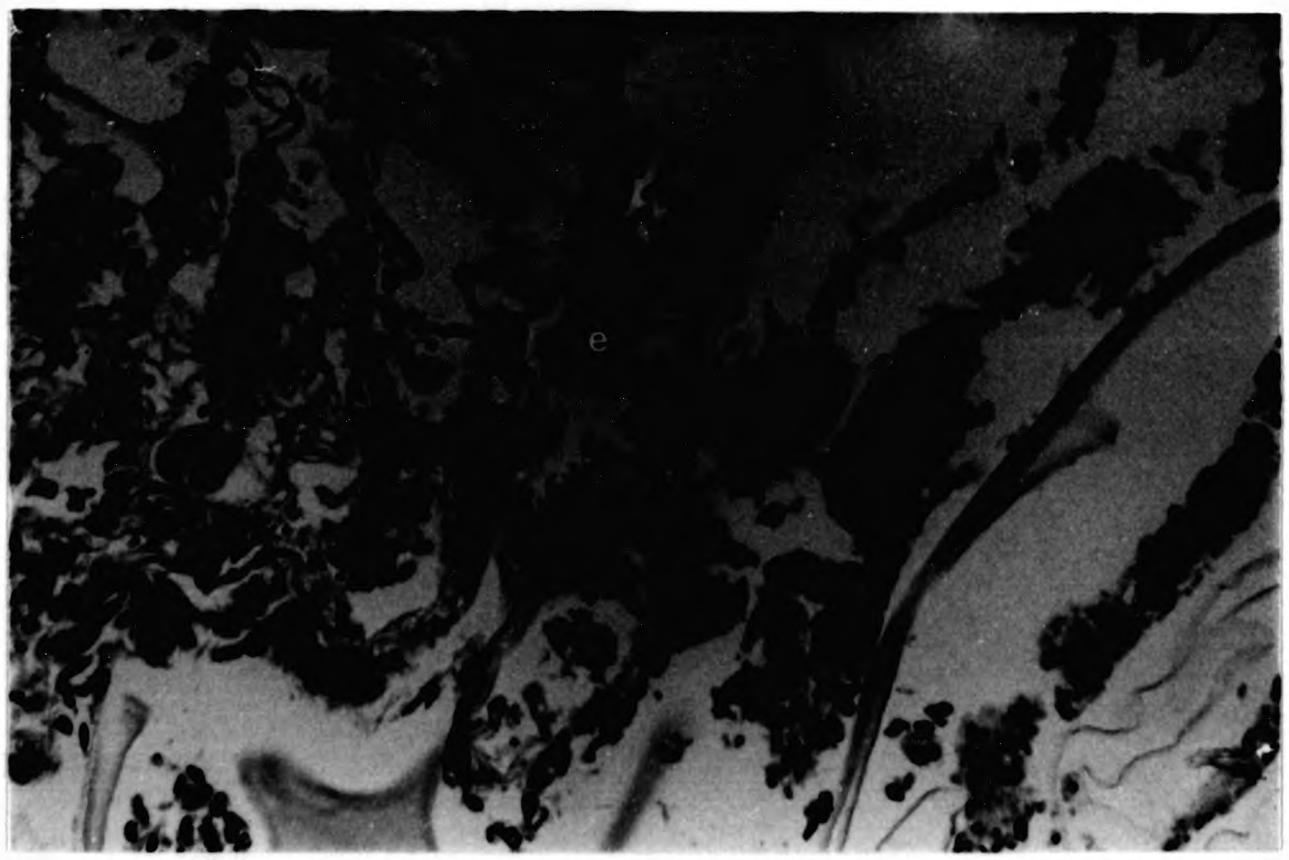
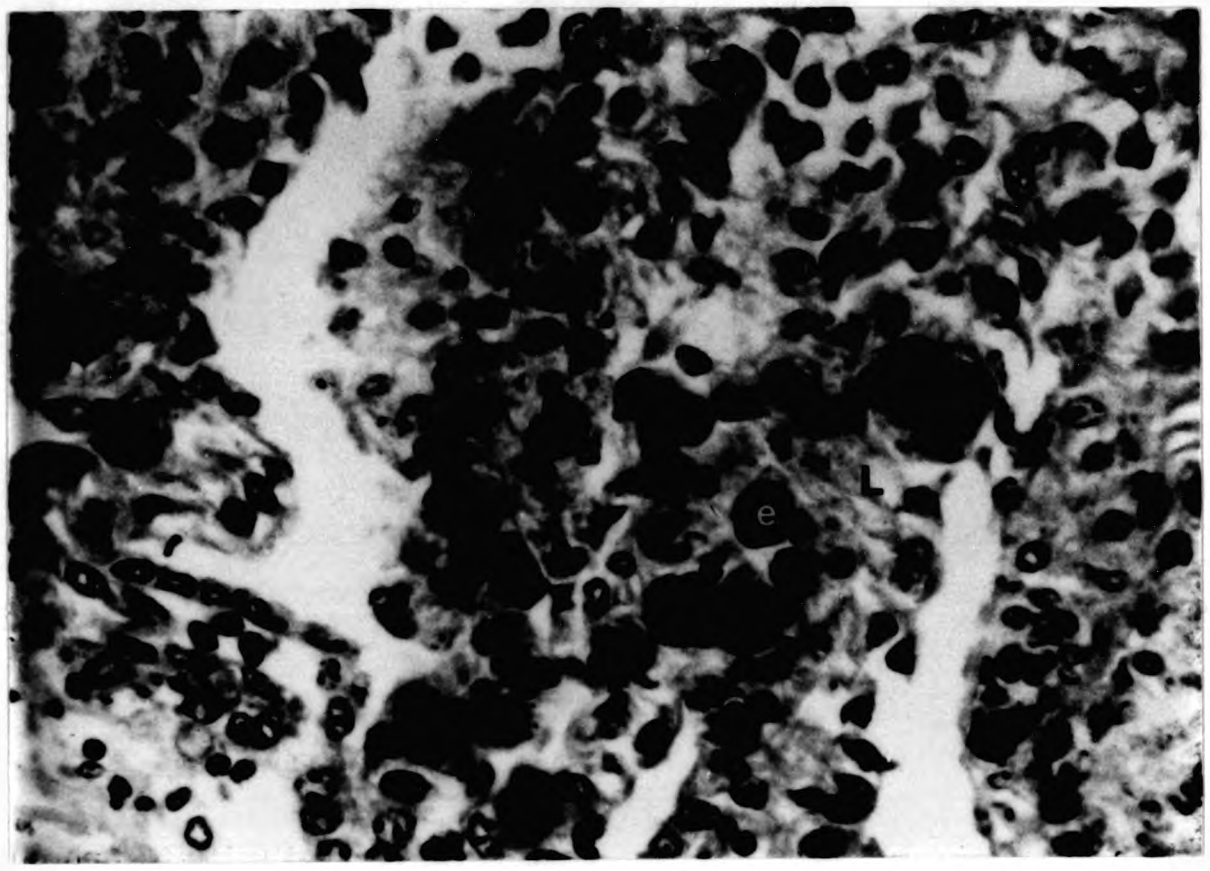

e

Fig. 68a Multifocal encapsulations (e) of necrotic melanized haemocytes in widened gill lamellae (L). *Aeromonas hydrophila* systemic, 5 days (HE, X500).

Fig. 68b Gill stem efferent channel (EF) and gill lamellar base (L) encapsulations and nodules (n) of necrotic melanized haemocytes. *Aeromonas hydrophila* systemic, 5 days (HE, X500).


e
L

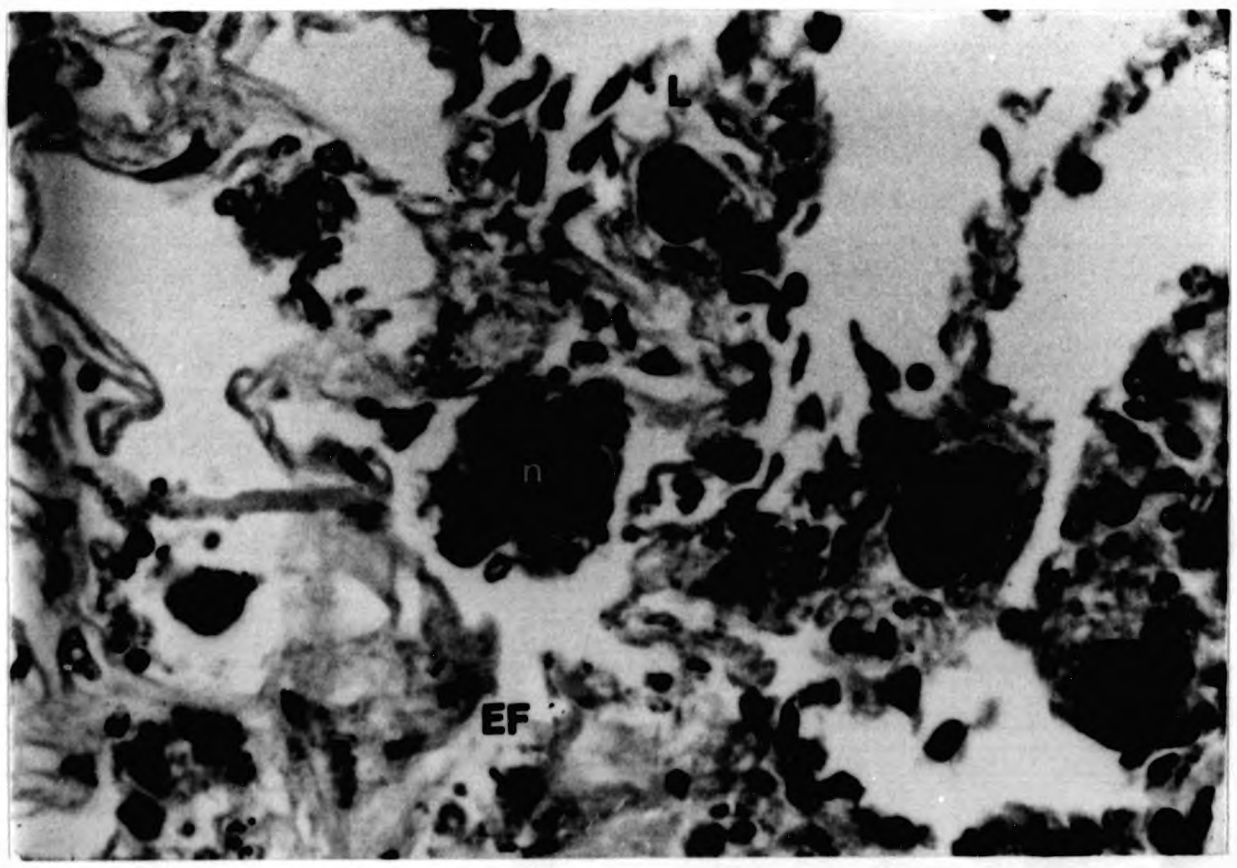

L
n
EF

Fig. 69a Diffuse (heart) epi-pericardial (P) and myo-endocardial (H) haemocyte aggregations (a) and melanized necrotic encapsulations and nodules (n). *Aeromonas hydrophila* systemic, 7 days (HE,X250).

Fig. 69b (detail) Epi-pericardial (heart) haemocyte aggregation with multilocular melanized encapsulations (e). *Aeromonas hydrophila* systemic, 7 days (HE, X250).


P
a
n

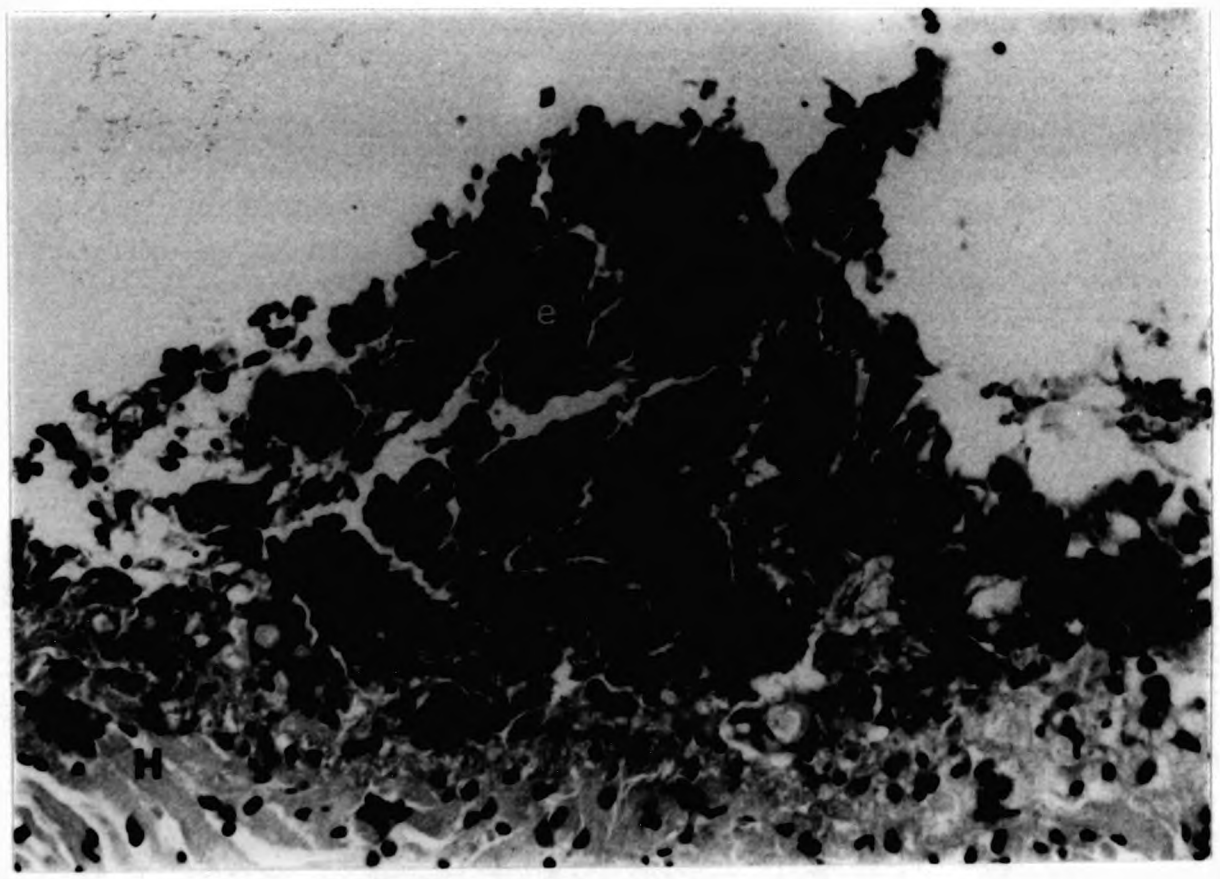

e
H

Fig. 70 a&b Multiple (heart) myo- and endocardial trabecular (H) massive nodules (n) of necrotic melanized haemocytes and aggregations. *Aeromonas hydrophila* systemic, 7 days (HE, X250, X500).


n

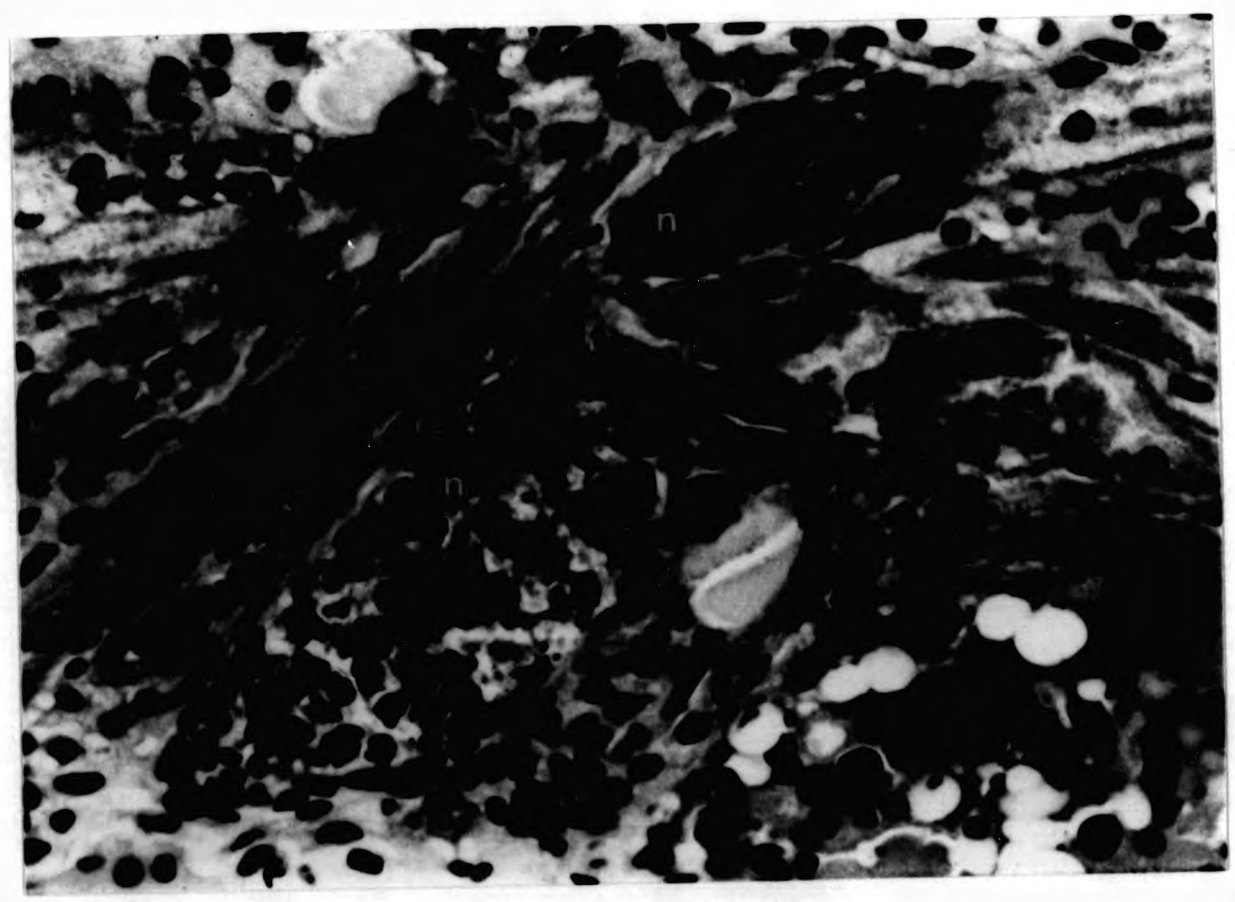
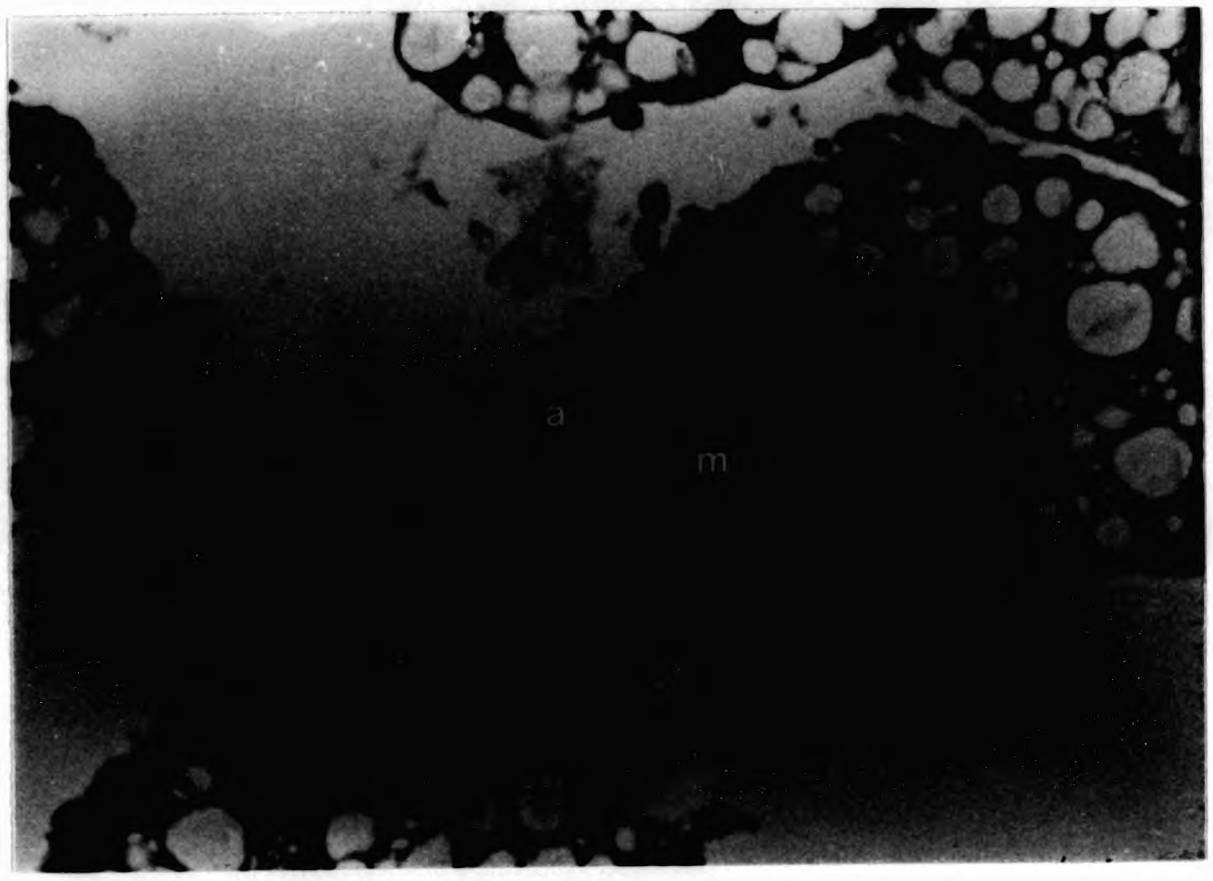

n
n

Fig. 71 Hepatopancreatic haemal sinus haemocyte aggregation (a) with central nodular melanized (m) necrotic area. *Aeromonas hydrophila* systemic, 8 days (HE, X500).

Fig. 72 Area of diffuse muscle (M) myofibre necrosis and fibroplasia (f) with haemocyte infiltration, aggregation and nodules (n) of necrotic melanized haemocytes in muscle injection site. *Aeromonas hydrophila* i.m., 8 days (HE, X250).


a
m

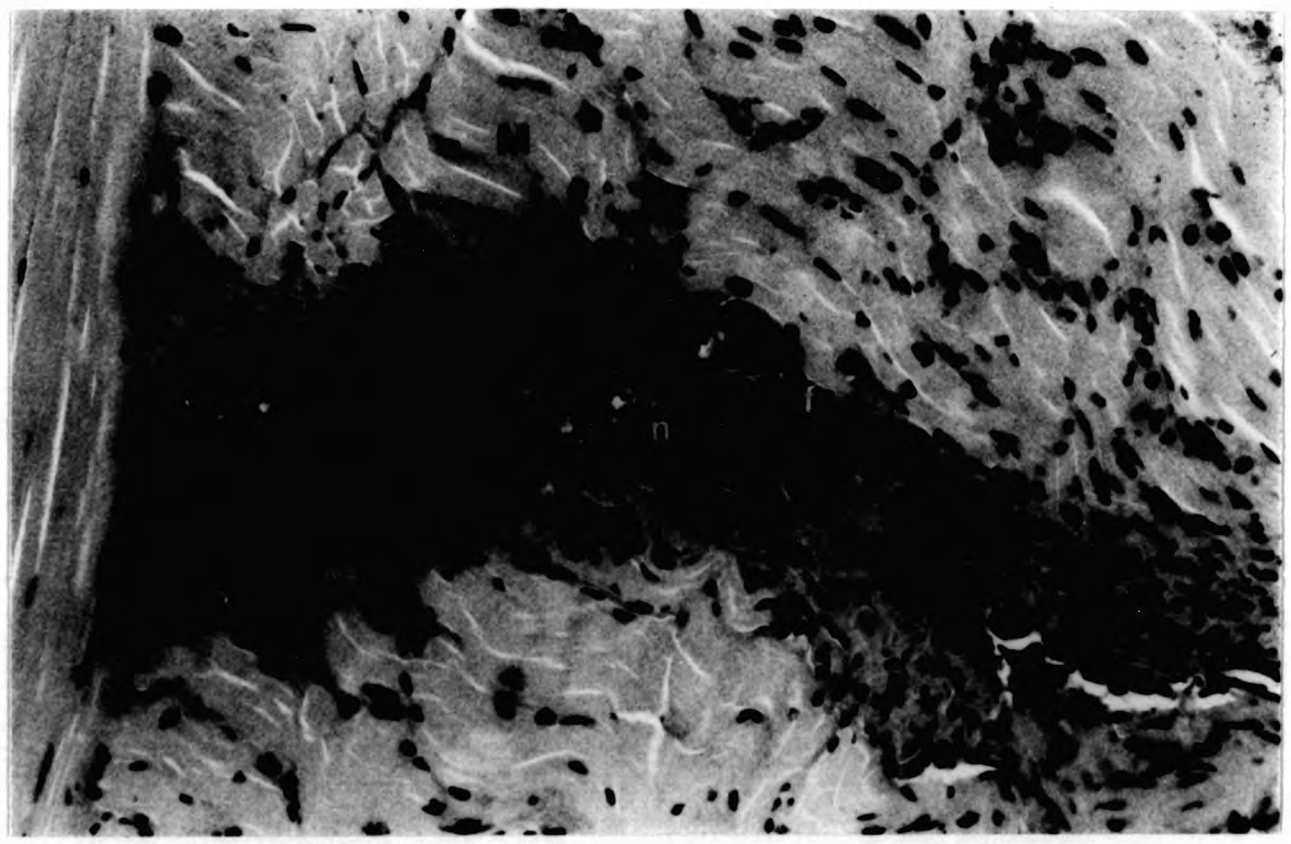
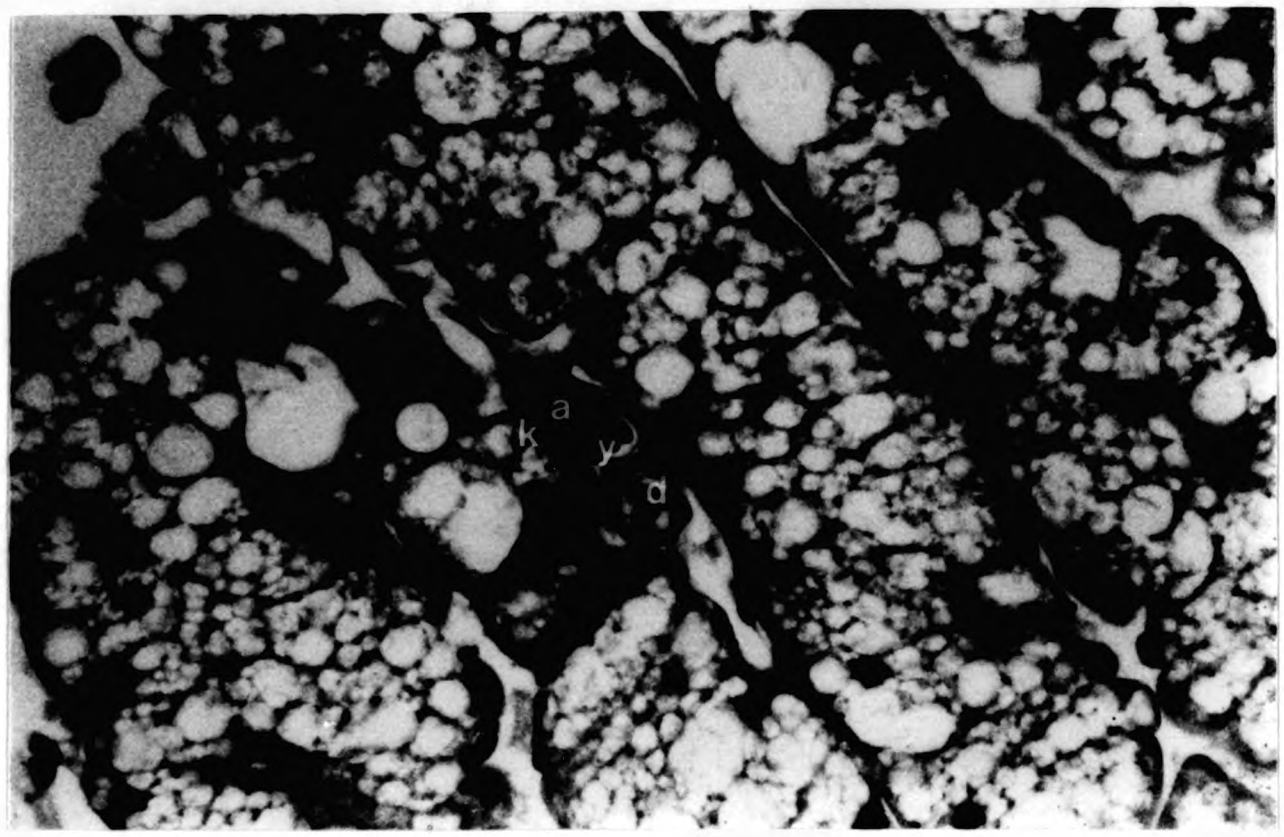

n
f

Fig. 73a Hepatopancreatic haemal sinus with haemocytic infiltration and aggregation (a) with a focus of nuclear pyknosis (y) and karyorrhexis (K) and early encapsulation of phagocyte (P), the phagosome containing degenerating material (d). *Aeromonas hydrophila* systemic, 10 days (HE, X500). Note the progression of haemocytic infiltration and aggregation with phagocytosis to the formation of encapsulations and gradually to larger nodules evident in Fig. 73 a&b, 74 & 75).

Fig. 73b Haemocytic aggregation (A) with encapsulation (e) gradually forming a nodule and undergoing melanization in hepatopancreatic haemal sinuses. *Aeromonas hydrophila* systemic, 10 days (HE, X500).



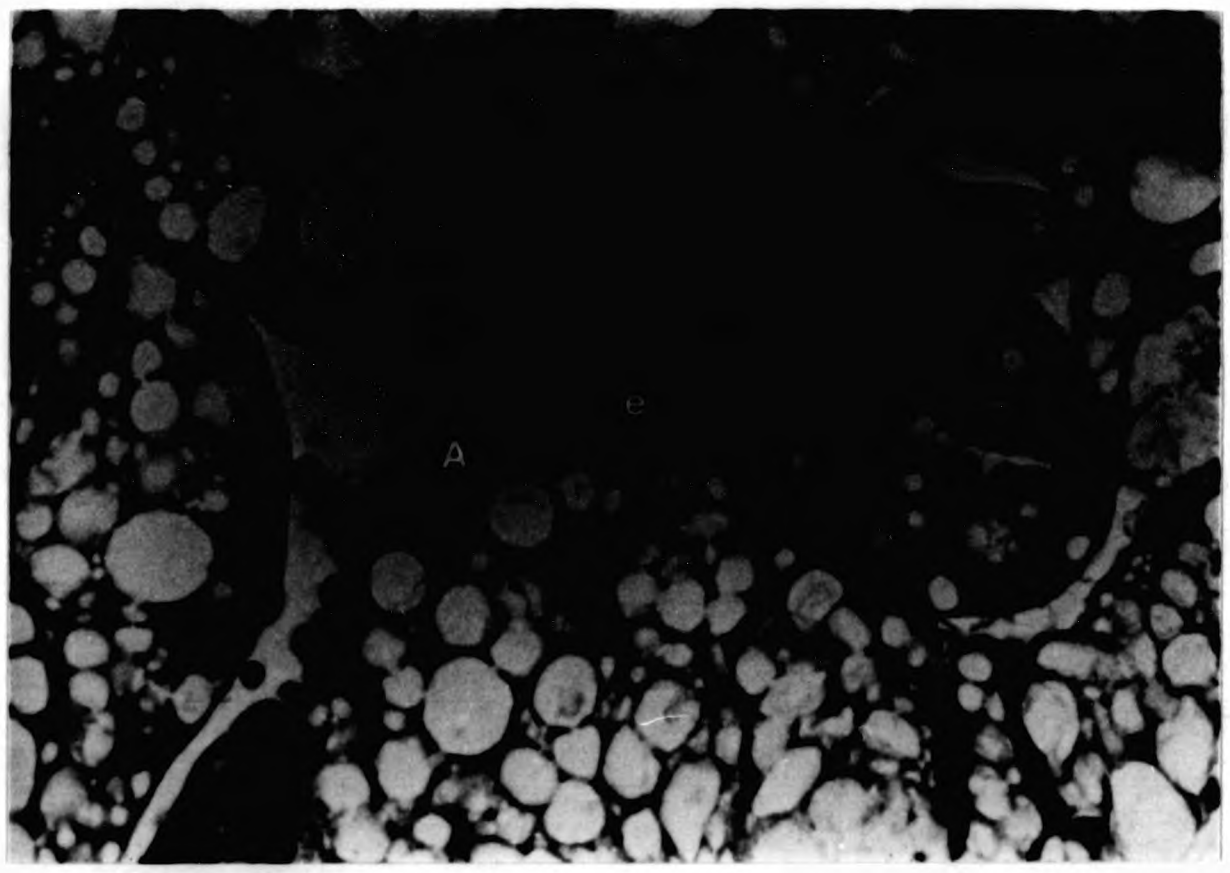
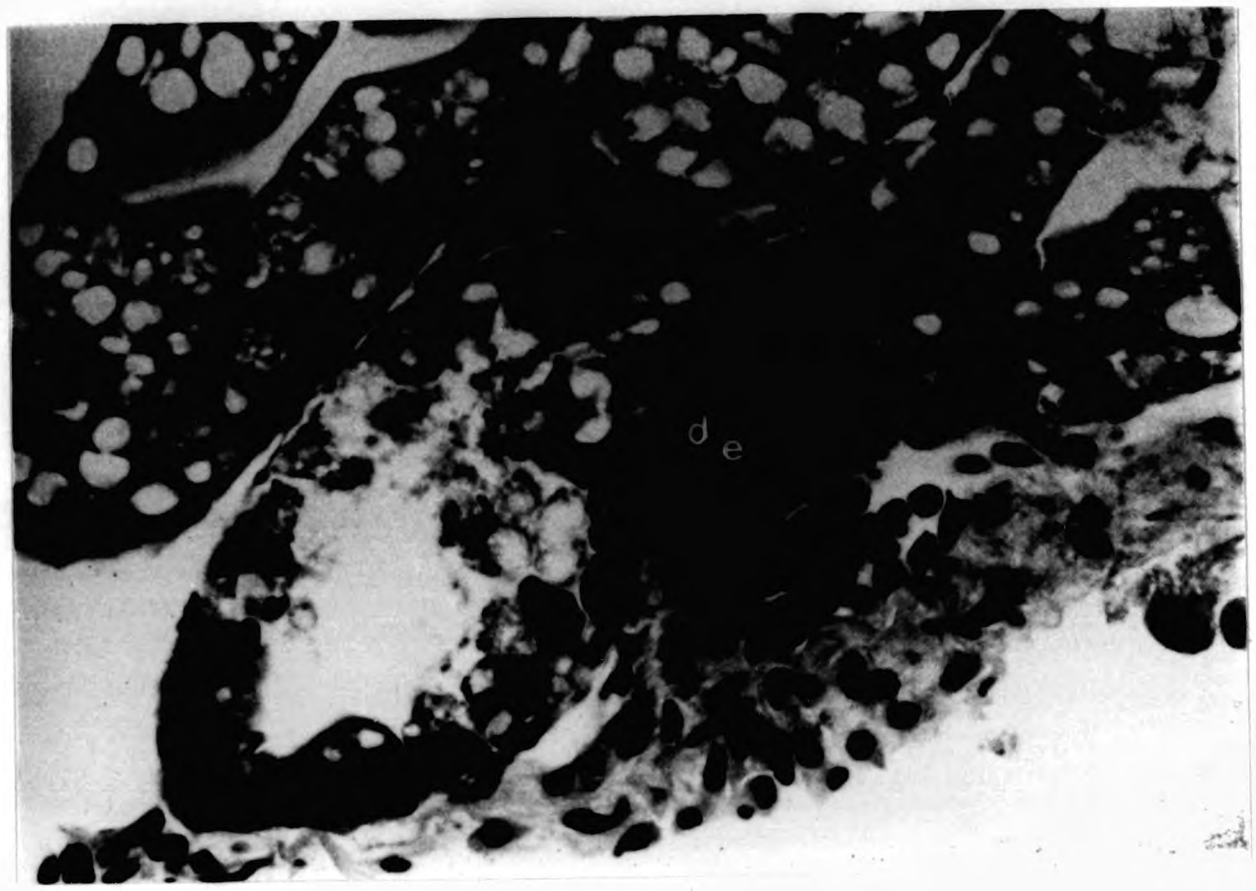


Fig. 74 Hepatopancreatic haemal sinuses with diffuse haemocytic aggregation and focal encapsulation (e) of phagocytosed melanized contents, including necrotic cellular debris (d). *Aeromonas hydrophila* systemic, 10 days (HE, X500).

Fig. 75 Melanized degenerating haemocytic nodule (n) within aggregating haemocytes in hepatopancreatic haemal sinuses. *Aeromonas hydrophila* i.m., 25 days (HE, X500).


d e

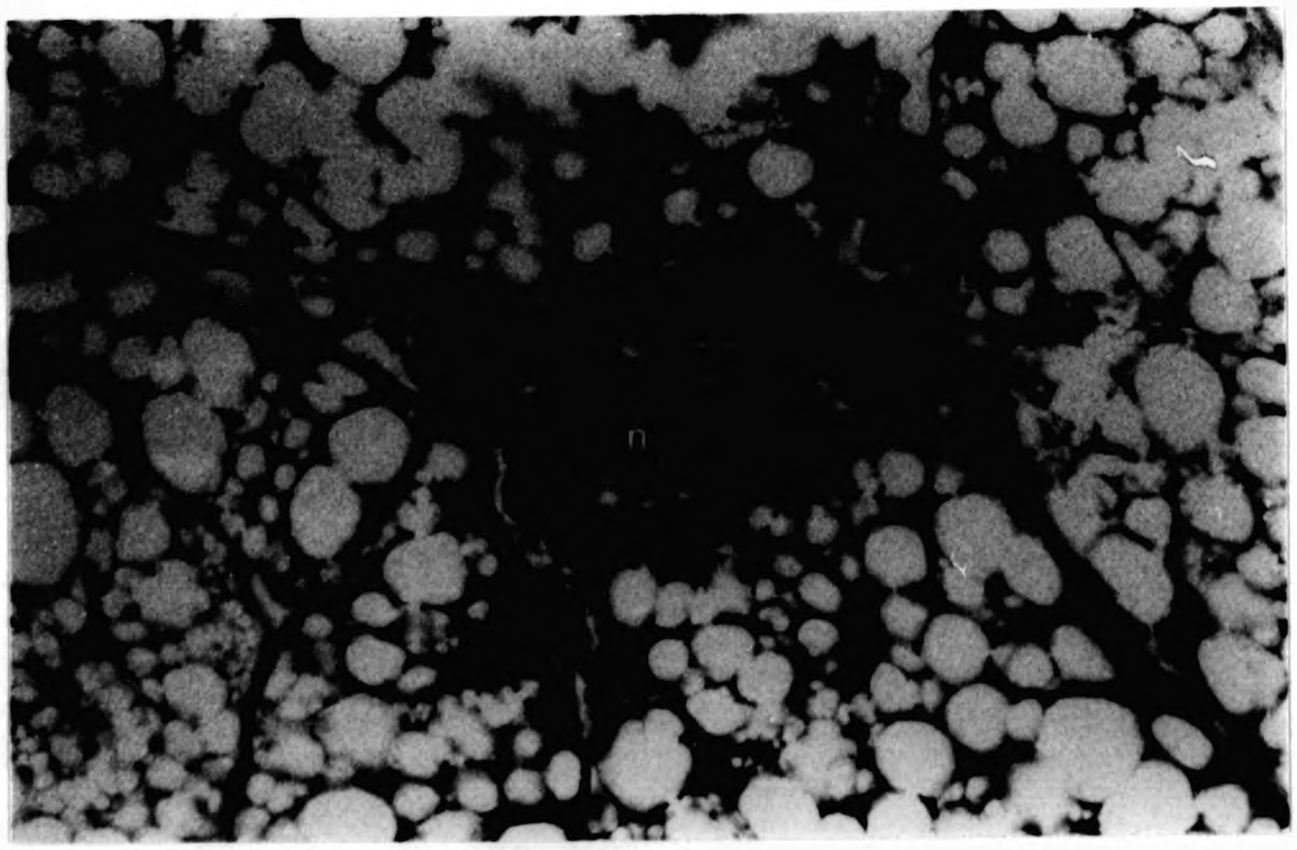
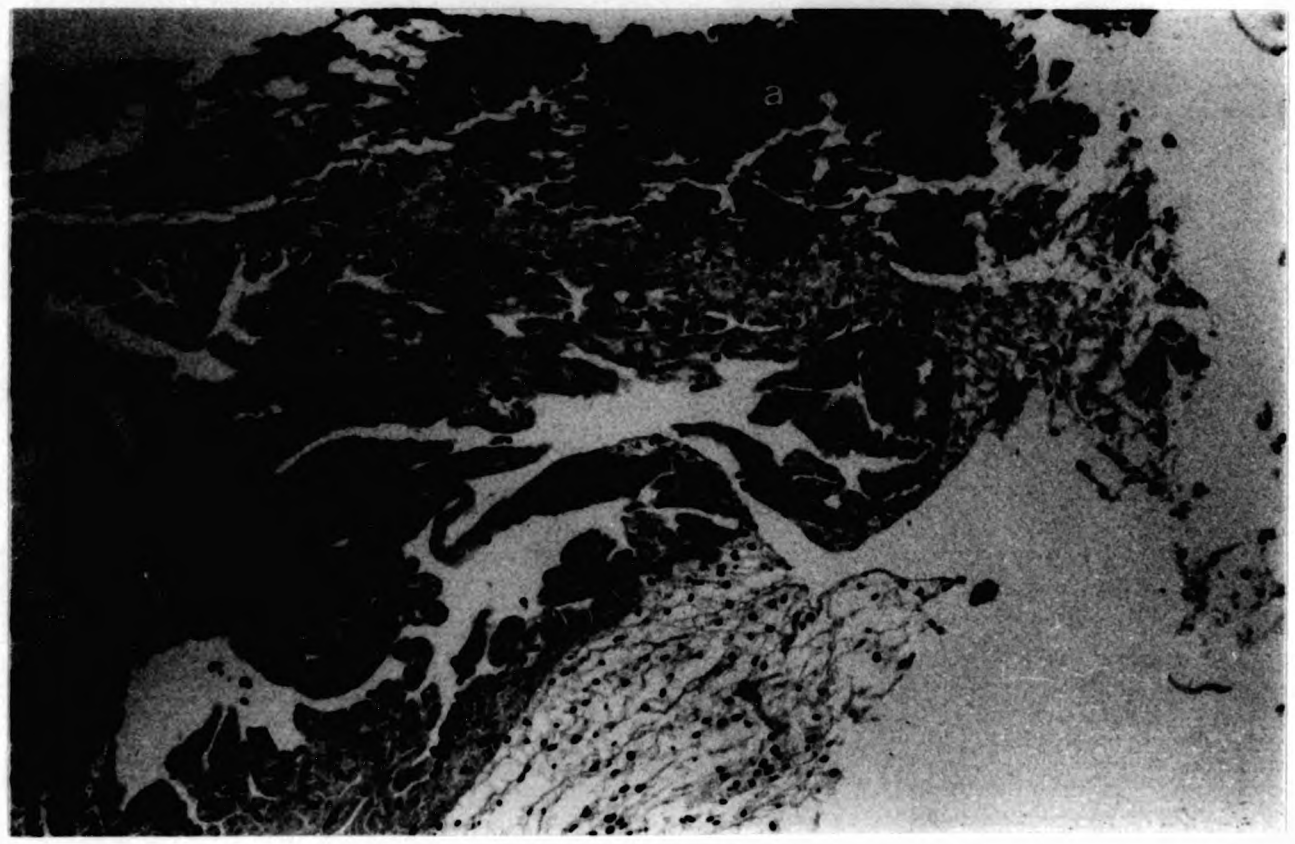


Fig. 76a Peri-epicardial (heart) (H) diffuse haemocytic aggregation (a) with multifocal encapsulations/small nodules (e) of melanized haemocytes. *Aeromonas hydrophila* systemic, 8 days (HE, X125). Note the progression of a more diffuse aggregation containing encapsulations (Fig. 76a) to the formation of discrete multifocal encapsulations/small nodules (Fig. 76b).

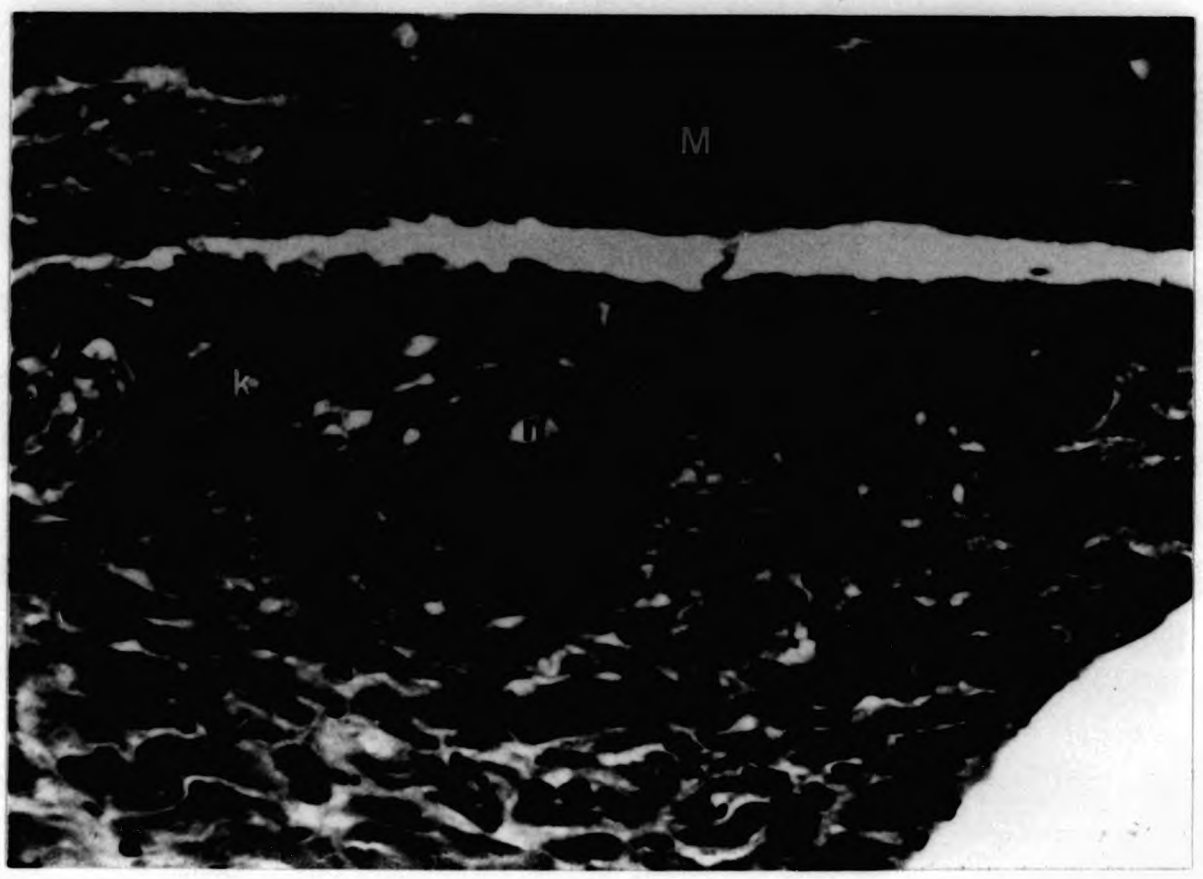
Fig. 76b Multifocal melanized peri-epicardial (P) and myo- cardial (heart)(H) haemocyte aggregations and endo- capsulations/small nodules (e). *Aeromonas hydrophila* systemic, 10 days (HE, X125).

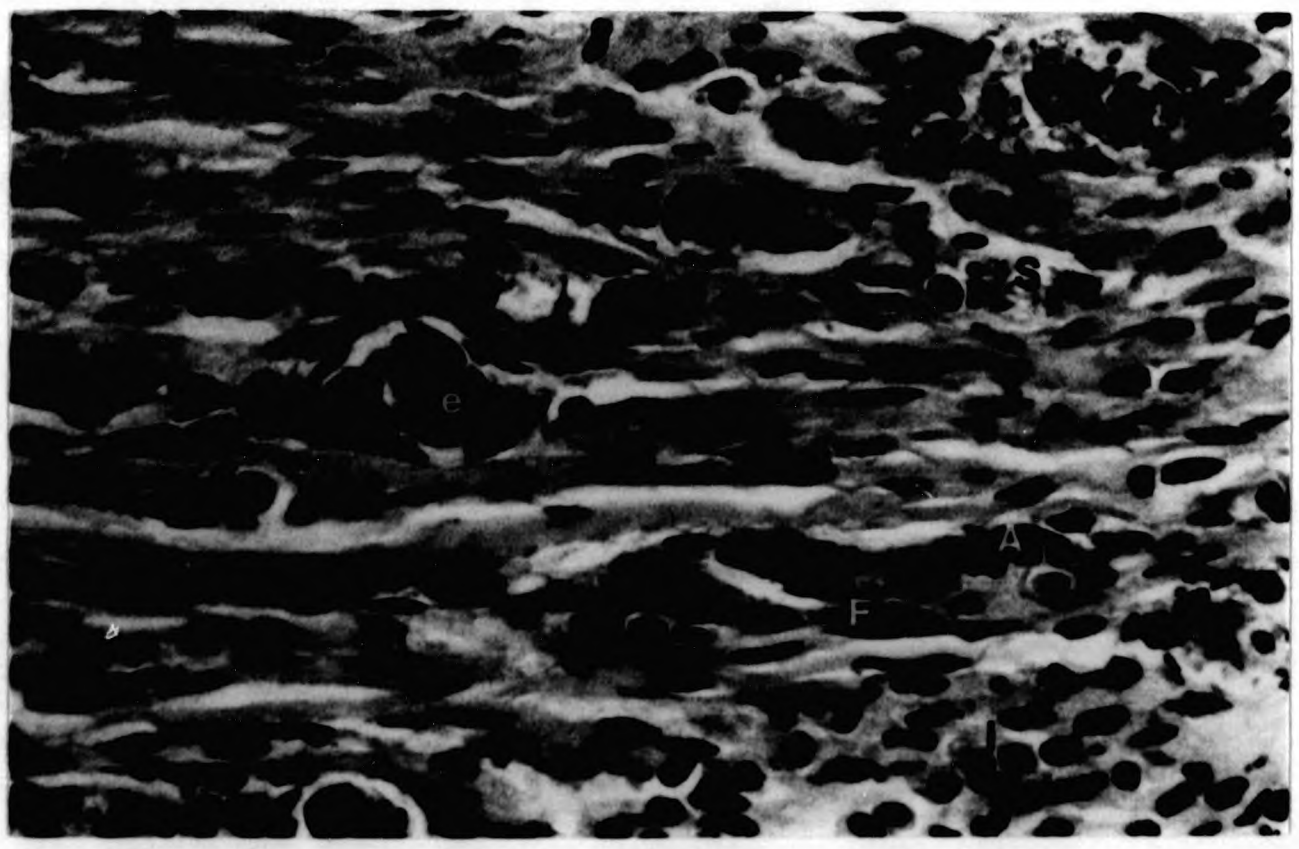

a


e

Fig. 77 In the muscle tissue (M) are multiple haemocytic nodules (n) undergoing melanization and degeneration displaying nuclear karyorrhexis (K) in an area of myofibre necrosis (S) and haemocyte infiltration and aggregation. *Aeromonas hydrophila* i.m., 10 days (HE, X500).

Fig. 78 Multifocal encapsulations (e) of necrotic melanized haemocytes are in an area of diffuse myofibre necrosis (S), fibroplasia (F), haemocyte infiltration (i) and aggregation (A) in a muscle injection site. *Aeromonas hydrophila* i.m., 15 days (HE, X500).


M
k


e
A
F

From 3 hours onwards, a reaction including melanized encapsulation was present in the antennal gland and there was a more prominent response in the gills (Figs. 63, 67, 68; Tables 1, 5), heart (Figs. 65, 66, 69, 70, 76; Tables 1, 5), hepatopancreas (Figs. 71, 73, 74, 75; Tables 1, 5), muscle (Figs. 72, 77, 78; Tables 1, 5), connective tissues (Tables 1, 5) and haemocoel (Fig. 64; Tables 1, 5), with melanized and/or necrotic aggregations, encapsulations and/or nodules. With the passage of time, there was a continuance in the strong haemocytic activity in the aforementioned organs and tissues (Tables 1, 5). Systemically injected prawns displayed an especially pronounced haemocytic response in the heart from 12 hours to 10 days (Figs. 65, 69, 70, 76; Tables 1, 5) and intramuscularly injected prawns showed a very prominent local muscle reaction, including necrotic melanized encapsulations and nodules and fibroplasia from 3 days (Figs. 72, 77, 78; Tables 1, 5). Some involvement of phagocytes in the formation of capsules was also noted (Fig. 73). A pronounced haemocytic response continued in most organs until the end of the experiment (28 days) (Tables 1, 5).

Moderate mitotic activity was present in the haemopoietic tissue from 1 hour onwards (Table 1).

III. 3.6 The Tissue Response to Systemic and Intramuscular *Vibrio* Injection

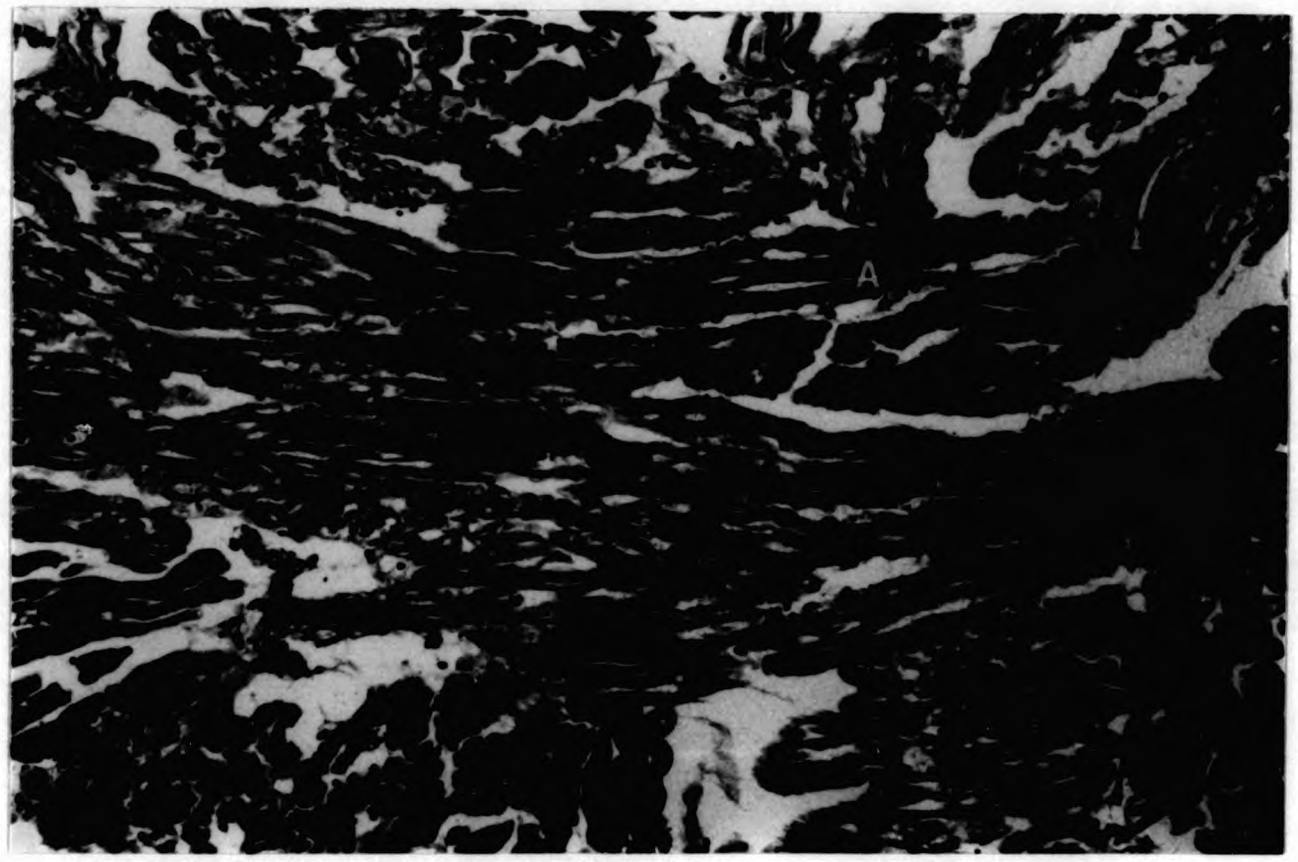
Histological Findings

As early as 30 minutes, in addition to

haemocytic infiltration and aggregation, there were melanized encapsulations and nodules in the muscle (Tables 1, 6), muscle injection site (Figs. 80, 83; Tables 1, 6), connective tissue (Tables 1, 6) and haemocoel (Tables 1, 6) and encapsulations in the gills (as Fig. 81; Tables 1, 6); aggregations were also observed in the heart (Fig. 79; Tables 1, 6). After 1 hour, encapsulations were also observed in the antennal gland and phagocytosis in the heart (Tables 1, 6). Intravascularly (within the posterior aorta) necrotic melanized encapsulation of phagocytes was also noted after 1 hour (Fig. 82). Encapsulation was also observed in the heart (see Fig. 93) after 2.5 hours (Tables 1, 6).

From 6 hours there was a further continuation of a strong tissue response of haemocytic aggregation, encapsulation and nodule formation, often of phagocytes, with melanization and/or necrosis in many tissues, including the hepatopancreas (Figs. 84, 92; Tables 1, 6), the haemocoel (Fig. 85; Tables 1, 6), the gills (Figs. 86, 90, 91; Tables 1, 6), the connective tissue (Figs. 87, 94; Tables 1, 6), the muscle (Fig. 88; Tables 1, 6) and muscle injection site (Figs. 89, 96; Tables 1, 6), heart (Fig. 93; Tables 1, 6) and antennal gland (Fig. 95; Tables 1, 6). Although encapsulation generally appeared to be more widespread, phagocytosis (Fig. 82) also occurred in numerous tissues and was particularly prominent in the hepatopancreas (Tables 1, 6). Fibroplasia was especially noted in and near the muscle

Fig. 79 a&b (detail) Multifocal (heart) myo-endocardial trabecular haemocyte aggregations (A,a). Live *Vibrio parahaemolyticus* systemic, 30 minutes (HE, X125, X250)


A

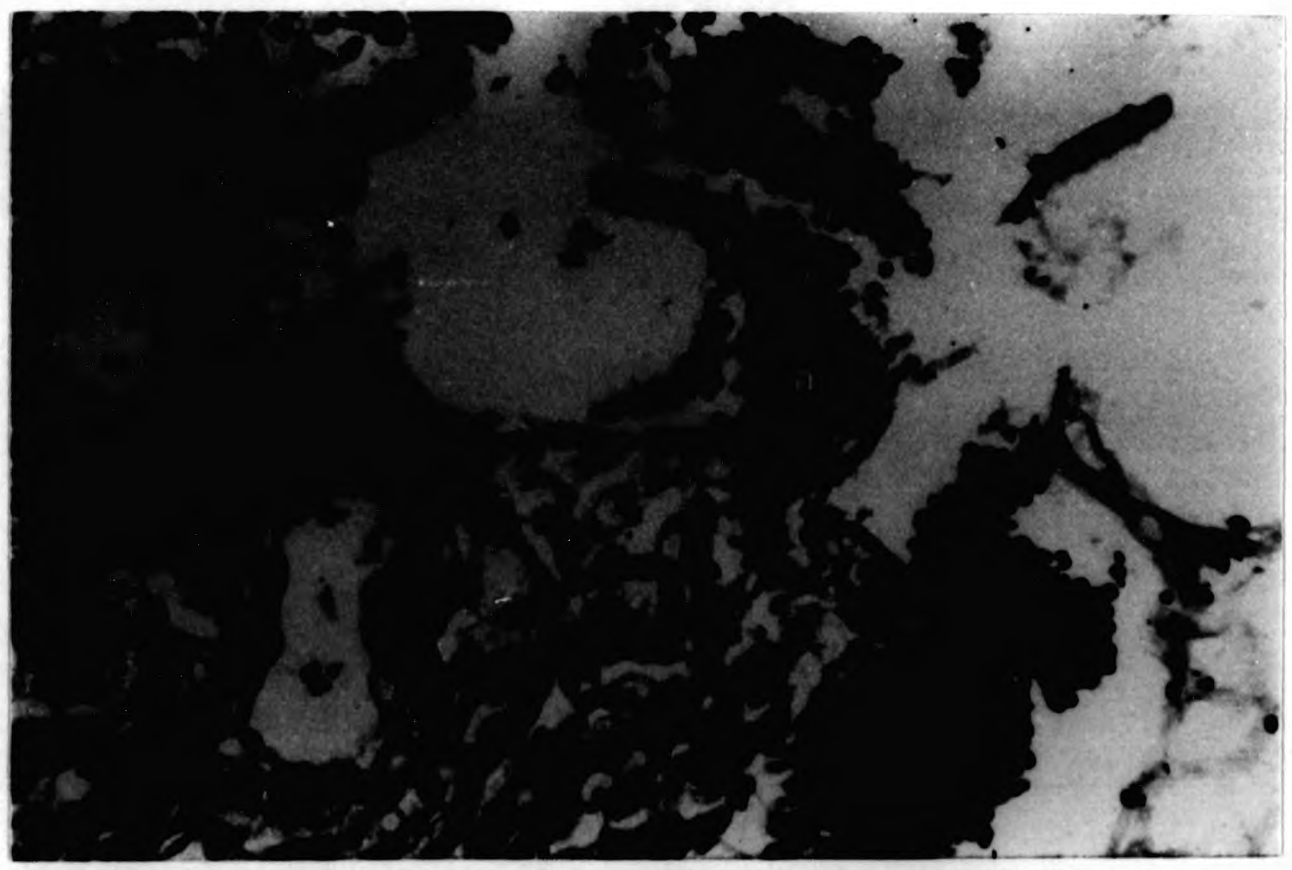


Fig. 80 Haemocytic nodule (n) with central melanization within area of diffuse haemocyte infiltration and aggregation in muscle injection site. Live *Vibrio parahaemolyticus* systemic, 30 minutes (HE, X500).

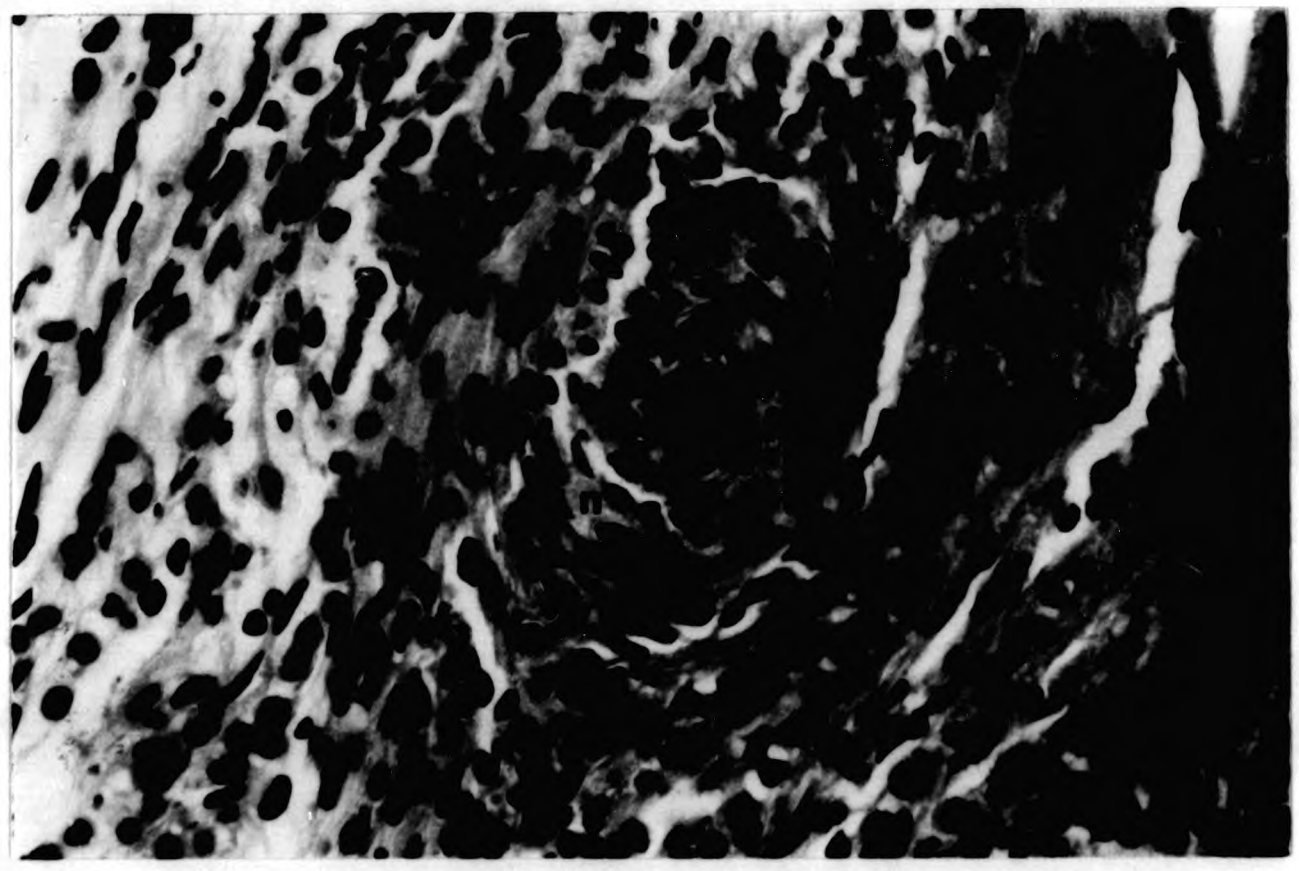
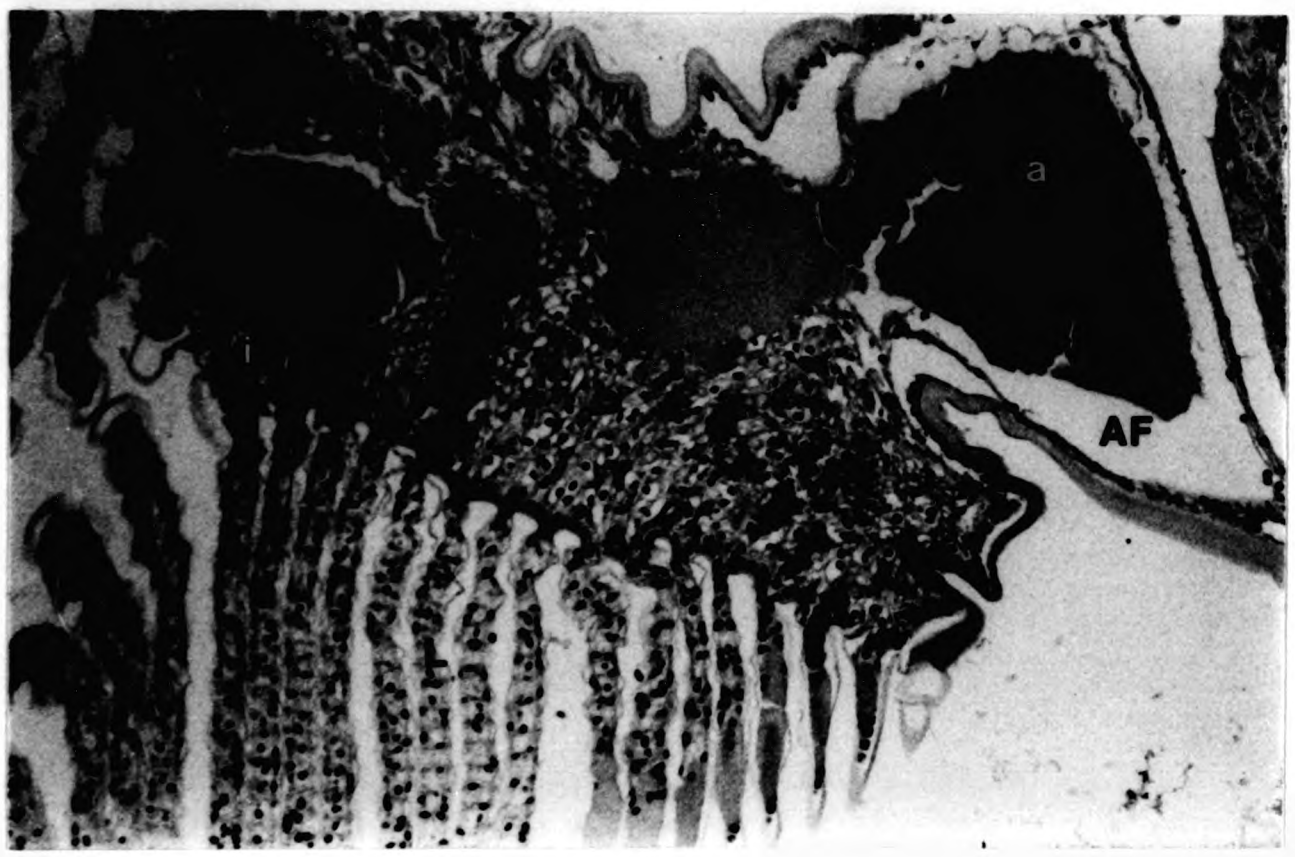


Fig. 81a Gill stem efferent channel (EF) and afferent channel (AF) and lamellar (L) haemocytic infiltration and aggregations (a). Killed *Vibrio anguillarum* i.m., 1 hour (HE, X125).

Fig. 81b Gill lamellar (L) early melanized haemocytic aggregations (a) and encapsulations (e). Killed *Vibrio anguillarum* i.m., 1 hour (HE, X500).


a
AF

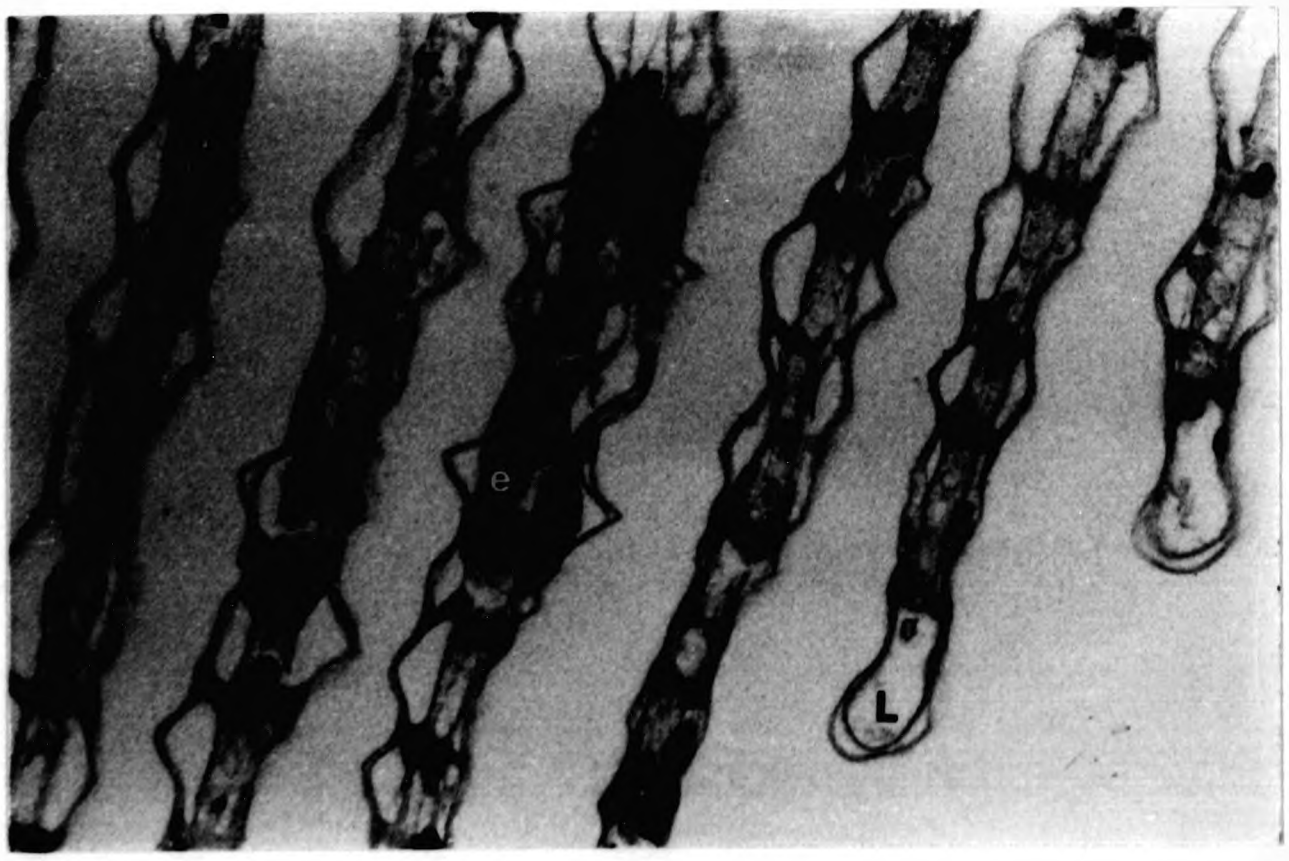

e
L

Fig. 82 Intravascular (within the posterior aorta (A)) thrombus-like nodules (T) with necrotic phagocyte centre (c) undergoing melanization. Live *Vibrio anguillarum* i.m., 1 hour (HE, X500).

Fig. 83 Encapsulation (e) of degenerating melanized haemocytes in areas of diffuse necrosis and haemocyte infiltration in muscle injection site (M). Live *Vibrio anguillarum* i.m., 1 hour (HE, bX1250).

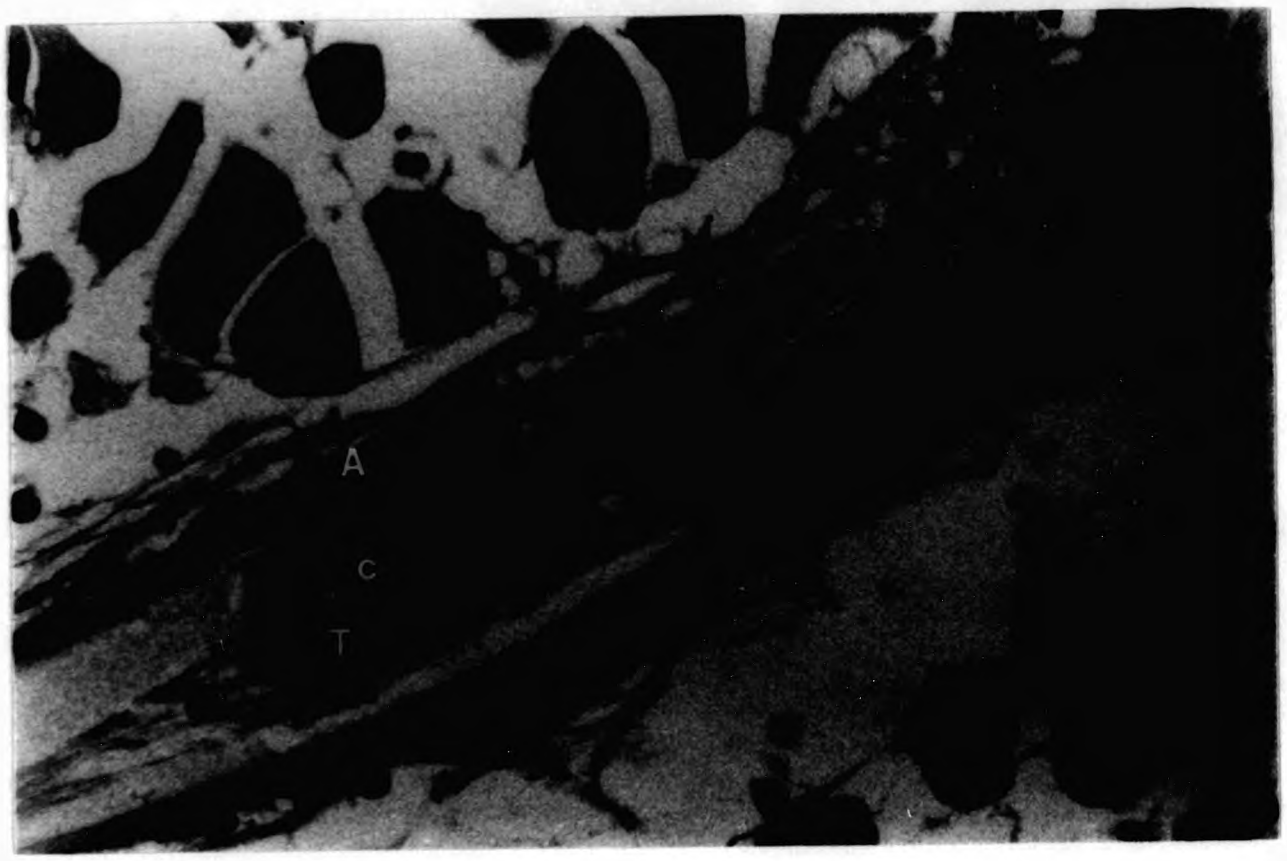
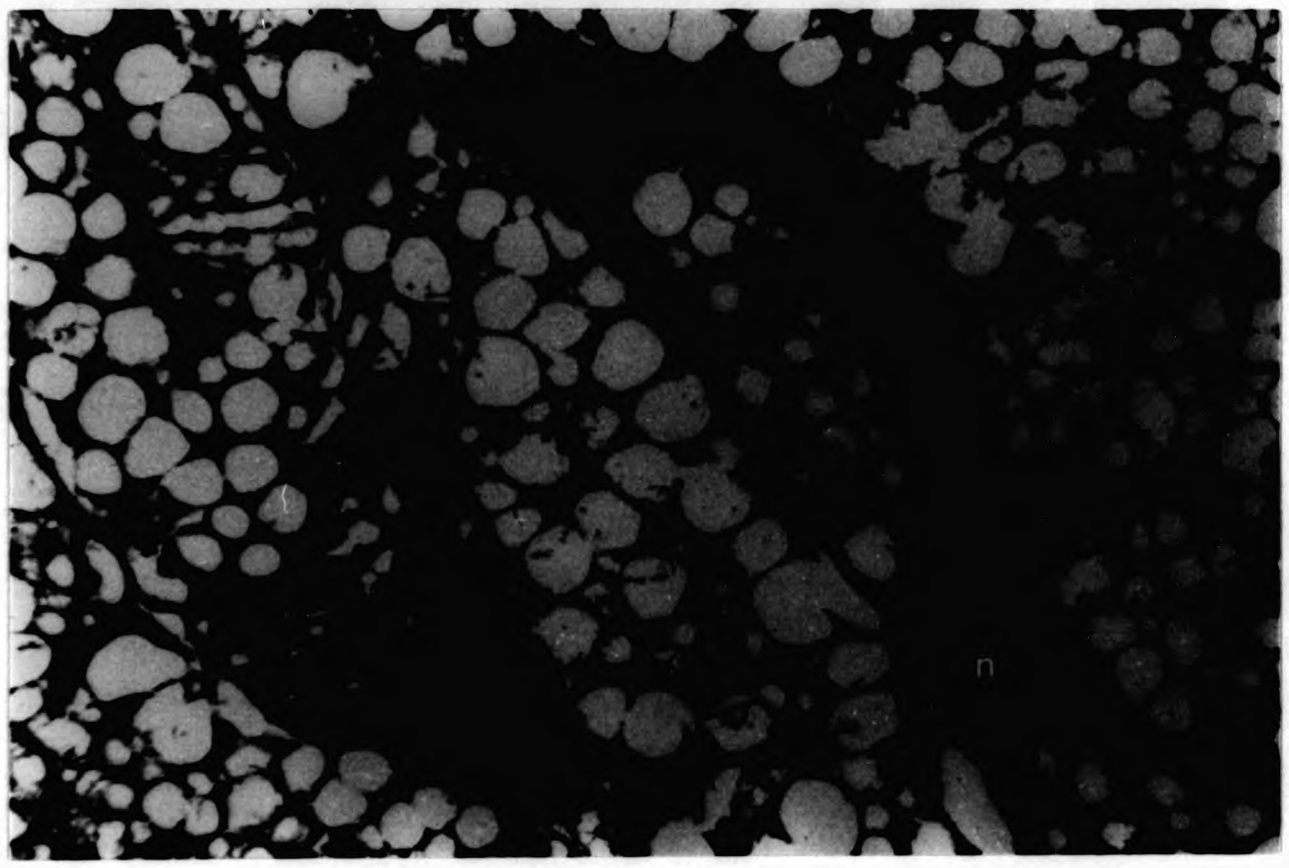


Fig. 84 Multiple melanized necrotic nodules (n) in the haemal sinuses of the hepatopancreas. Live *Vibrio parahaemolyticus* systemic, 6 hours (HE, X250).

Fig. 85 Massive haemocyte aggregation (a) with multifocal necrotic melanized encapsulations in dorsal haemocoel (H). Live *Vibrio parahaemolyticus* systemic, 6 hours (HE, X250).



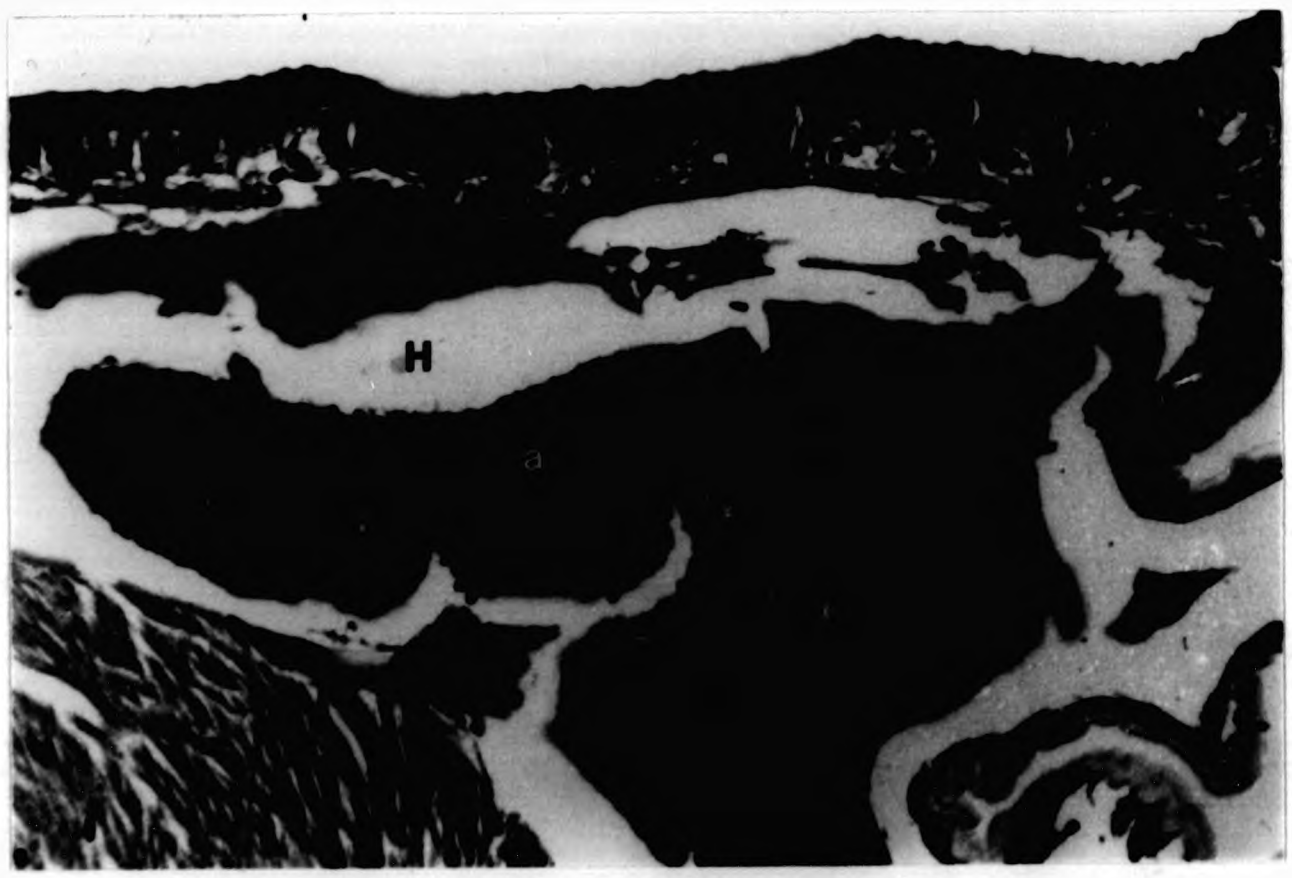
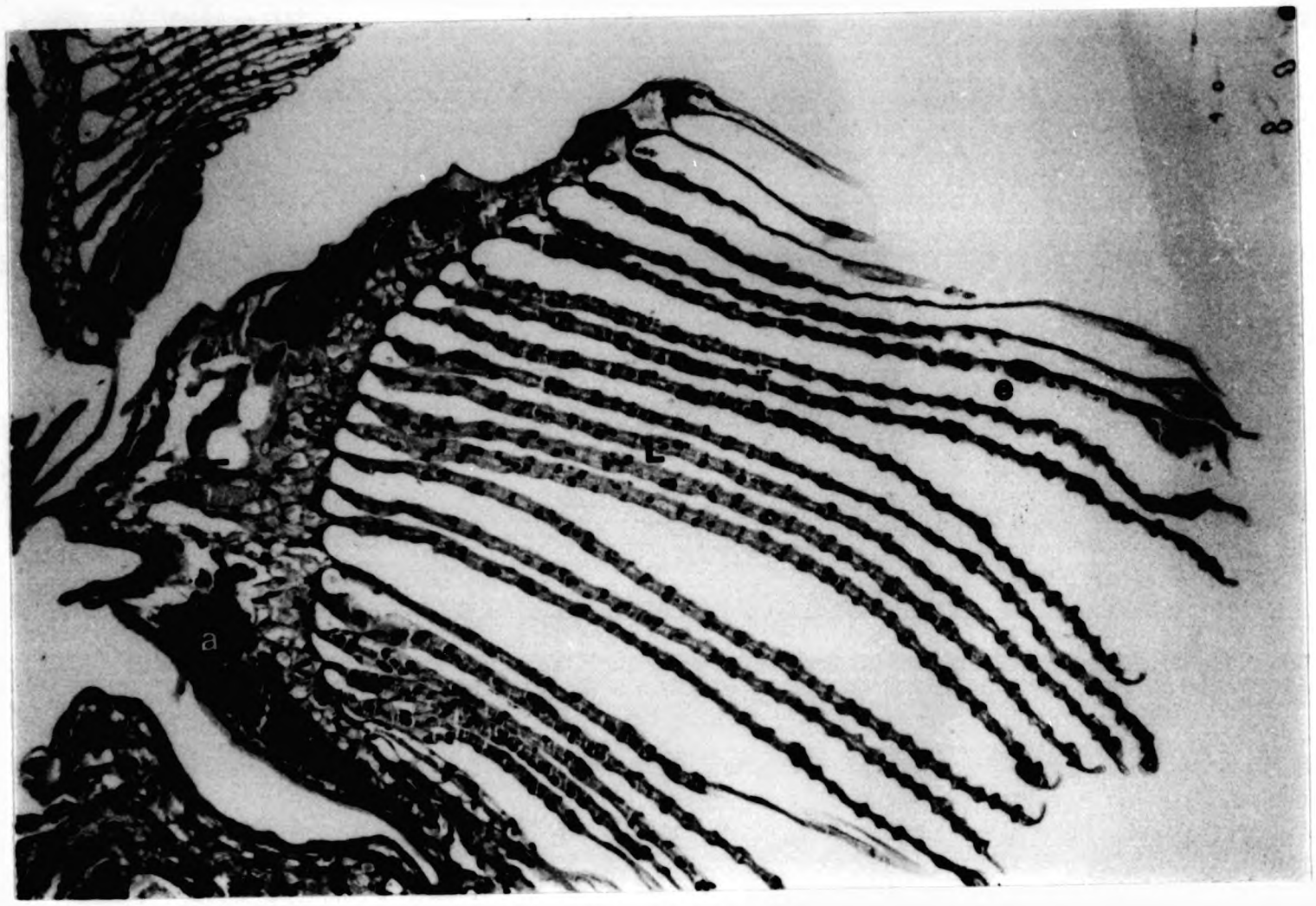

H

Fig. 86a Gill stem efferent channel (EF) and lamellar (L) melanized aggregations (a) and encapsulations (e). Killed *Vibrio anguillarum* i.m., 12 hours (HE, X150).

Fig. 86b Multifocal melanized encapsulations (e) in the gill stem (G). Tegmental gland (TE), branchial podocyte (b), lamella (L). Live *Vibrio parahaemolyticus* systemic, 12 hours (HE, X250).


a
L


G
TE
e

Fig. 87 Multifocal encapsulations (e) and nodules (n) of necrotic melanized haemocytes in subcuticular spongy connective tissue. Live *Vibrio parahaemolyticus* systemic, 12 hours (HE, x500).

Fig. 88 Large aggregation (a) of haemocytes in cephalothoracic muscle. Killed *Vibrio anguillarum* i.m., 12 hours (HE, X600).

Fig. 89 Area of diffuse myofibre necrosis (S) haemocyte infiltration, aggregations (a) and encapsulations (e) of necrotic melanized haemocytes with nuclear karyorrhexis (K) in muscle (M) injection site. Killed *Vibrio anguillarum* i.m., 2 weeks (HE, X500).

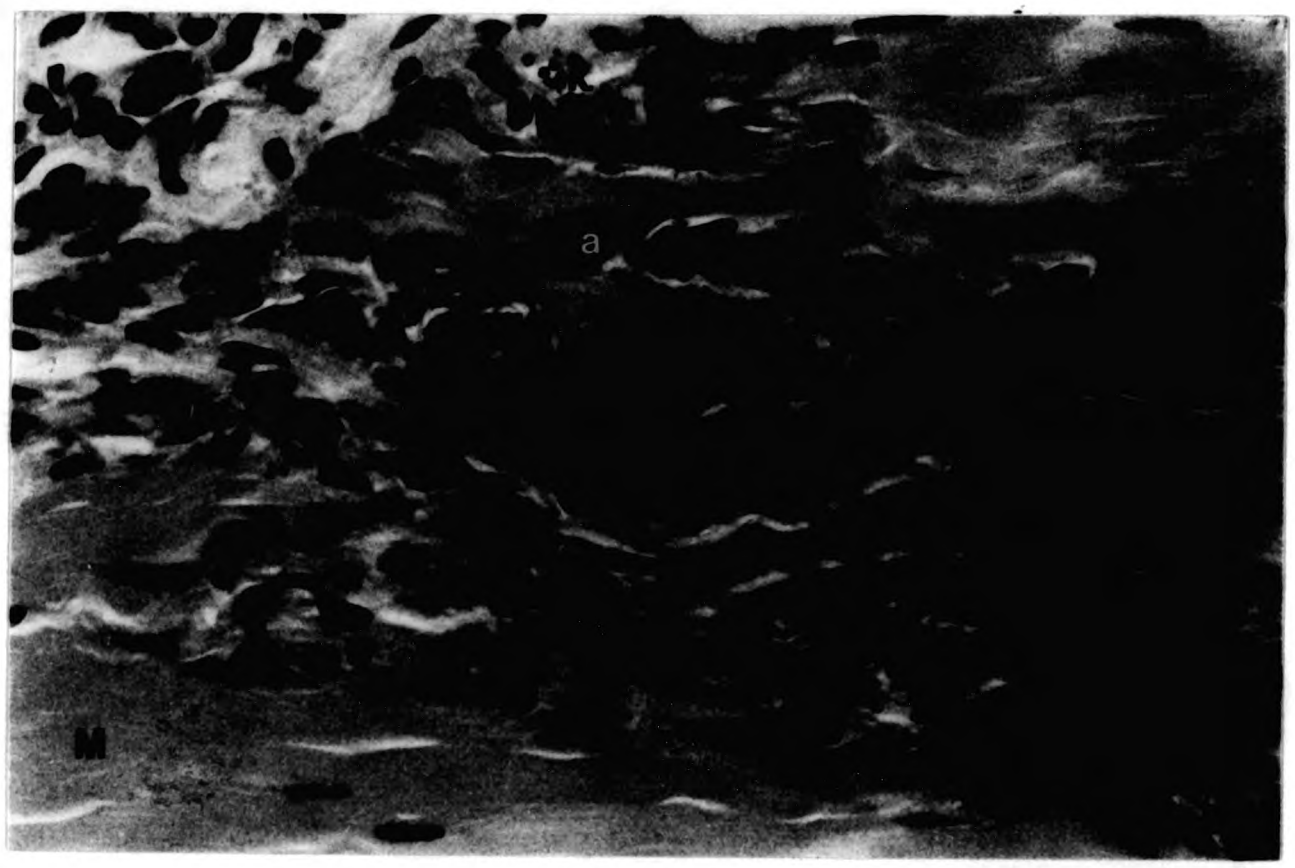
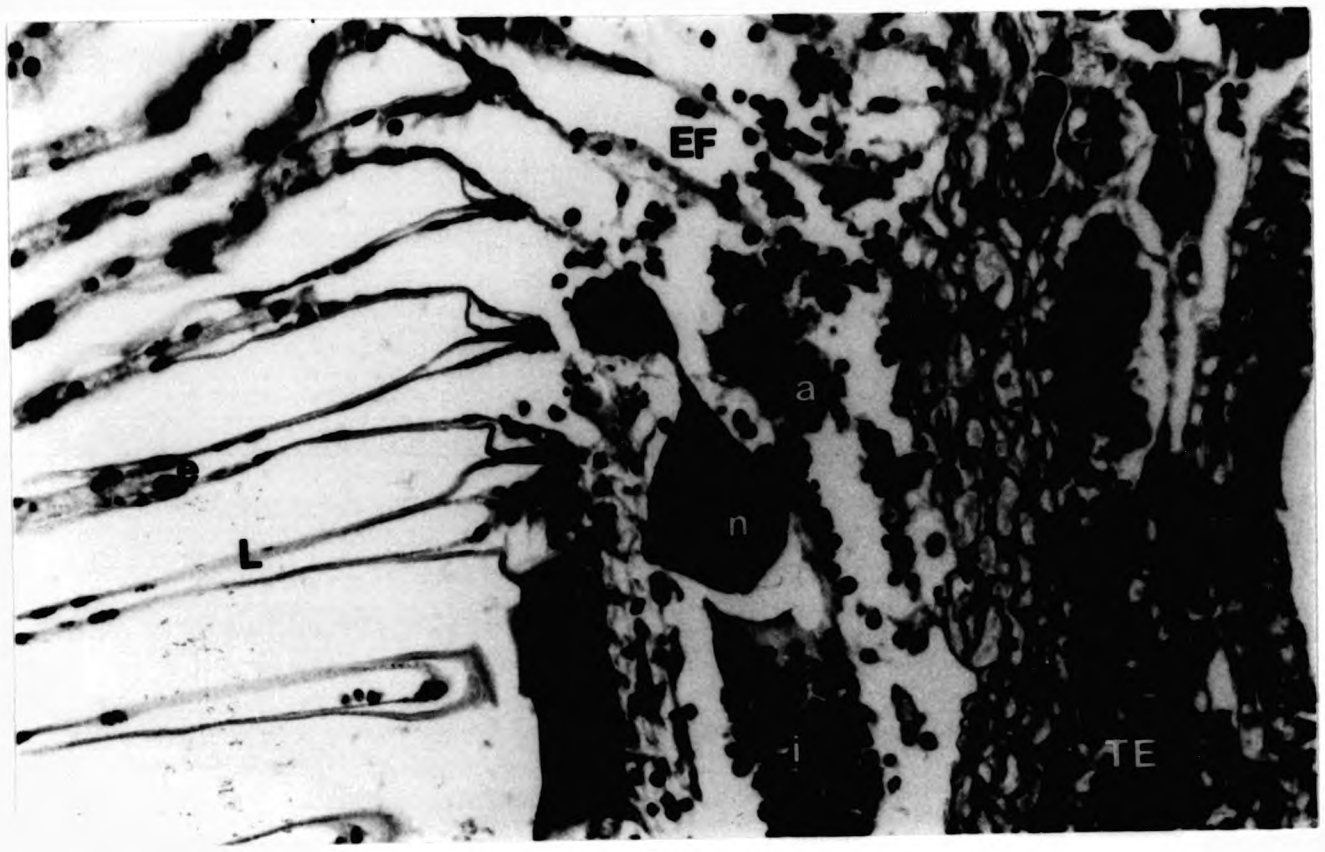

a
M

Fig. 90a Gill stem efferent channel (EF) and lamellar (L) haemocyte infiltration (i) aggregations (a), melanized encapsulations (e) and nodules (n);branchial podocyte (b), tegmental glands (TE). Killed *Vibrio anguillarum* i.m., 4 weeks (HE, X250).

Fig. 90b Gill lamellar (L) necrotic melanized encapsulation (e). Killed *Vibrio anguillarum* i.m., 4 weeks (HE, X500).


EF
a
n
L
i
TE

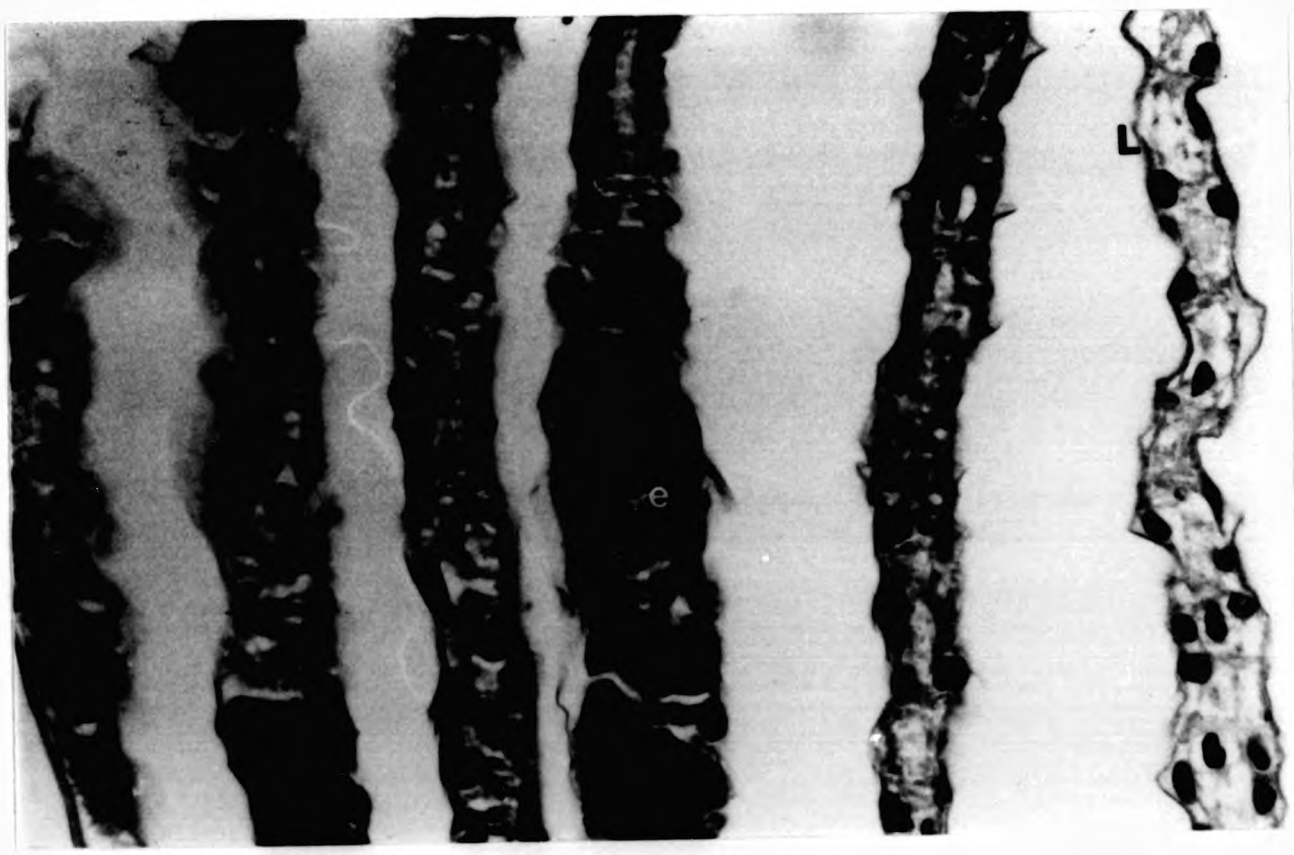

L
e

Fig. 91 Multilocular necrotic melanized gill lamellar (L) apical nodule (n). Live *Vibrio anguillarum* i.m., 4 weeks (HE, X500).

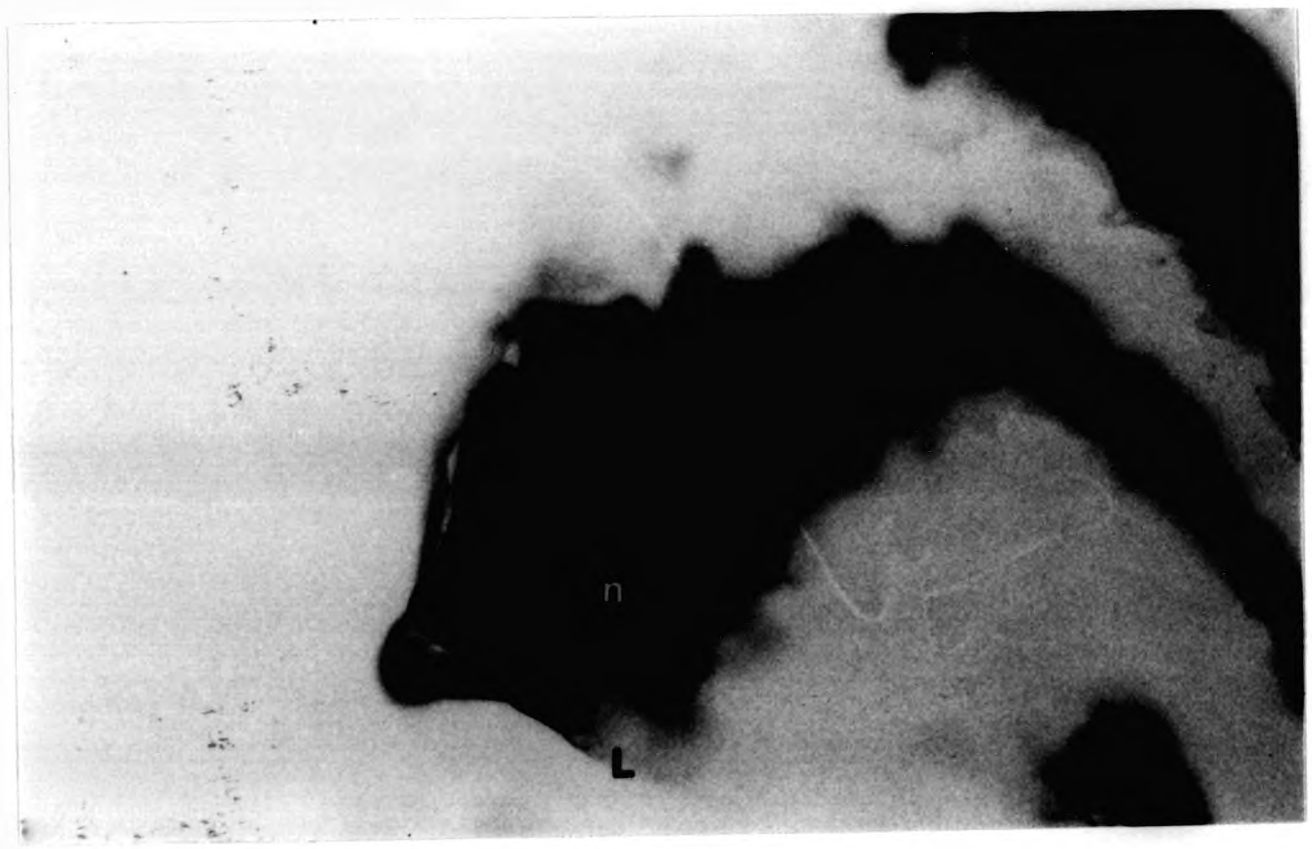

n
L

Fig. 92 Multilocular nodules (n) of necrotic melanized haemocytes with pyknotic (Y) and karyorrhectic (K) nuclei in haemocyte aggregation in hepatopancreatic haemal sinuses. Live *Vibrio anguillarum* i.m., 4 weeks (HE, X500).

Fig. 93 Heart (H) endocardial trabecular encapsulation (e) of necrotic melanized (m) haemocytes. Killed *Vibrio anguillarum* i.m., 4 weeks (HE, X1250).


y
y

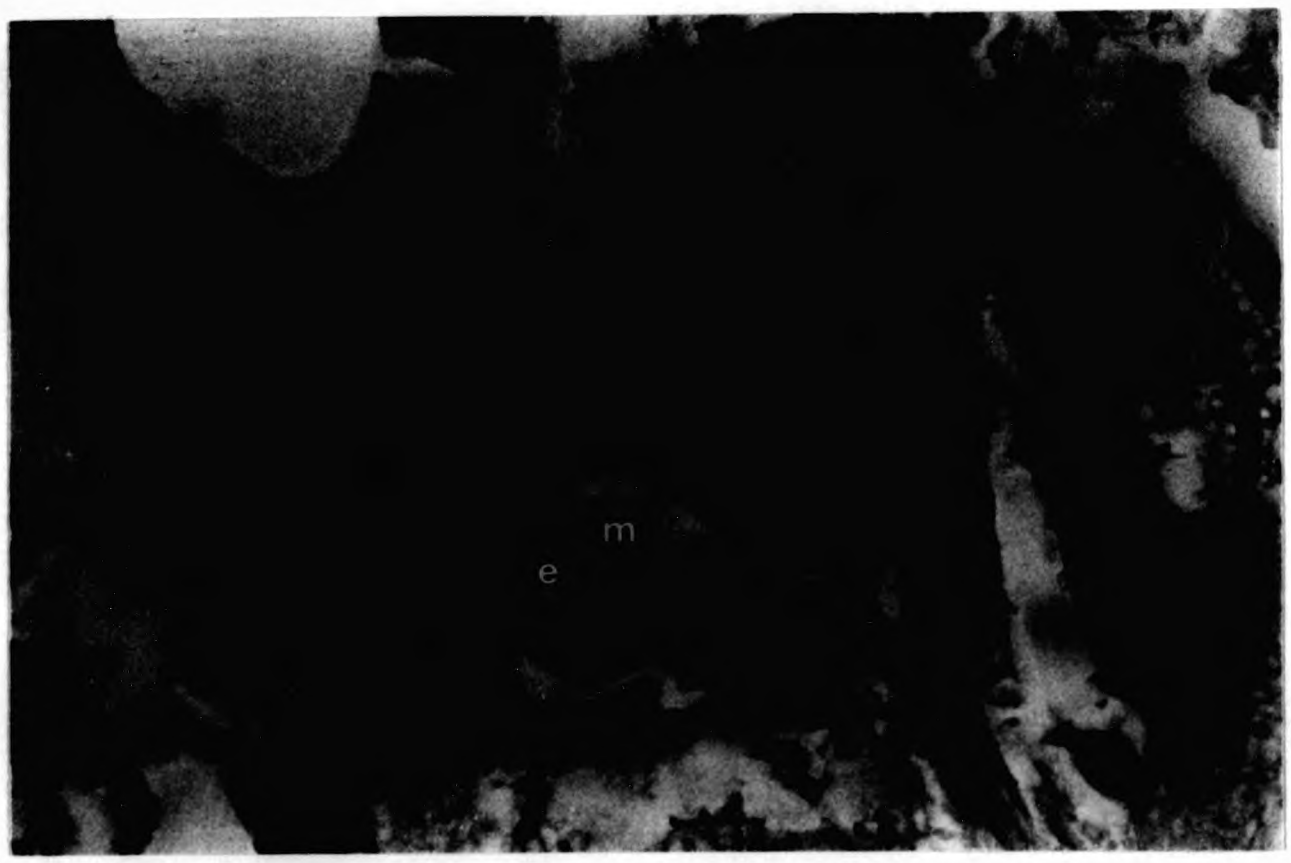

m
e

Fig. 94a Connective tissues in vicinity of muscle injection site with an area of diffuse haemocyte infiltration and aggregations and multifocal melanized encapsulations (E) and nodules (n) and fibroplasia (f). Live *Vibrio anguillarum* i.m., 4 weeks (HE, X250).

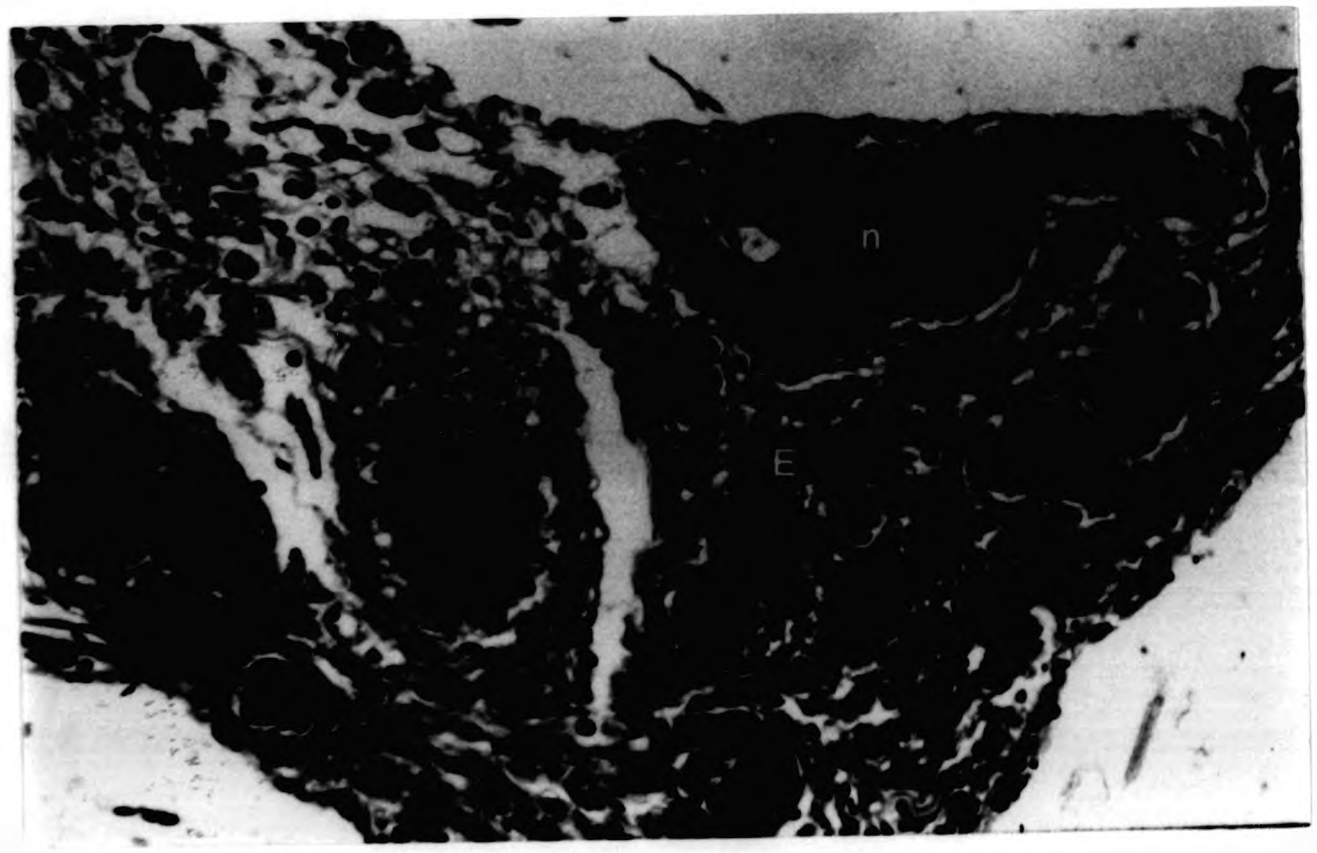
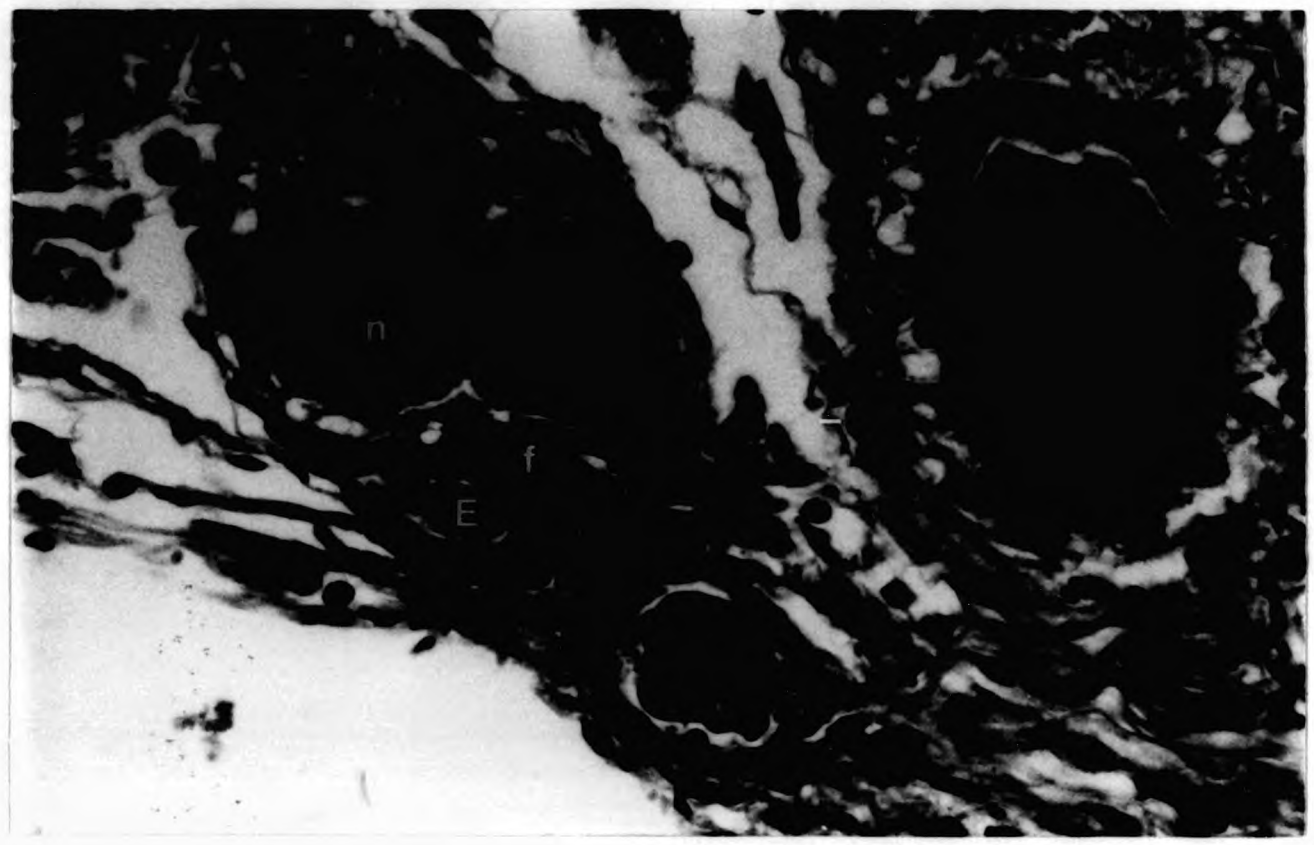

n
E

Fig. 94 b&c (detail of 94a) Connective tissue near muscle injection site showing encapsulations (e,E) and multilocular nodules (n) of melanized effete haemocytes within an area of diffuse fibroplasia (f). Live *Vibrio anguillarum* i.m., 4 weeks (HE, X500).


n
f
E

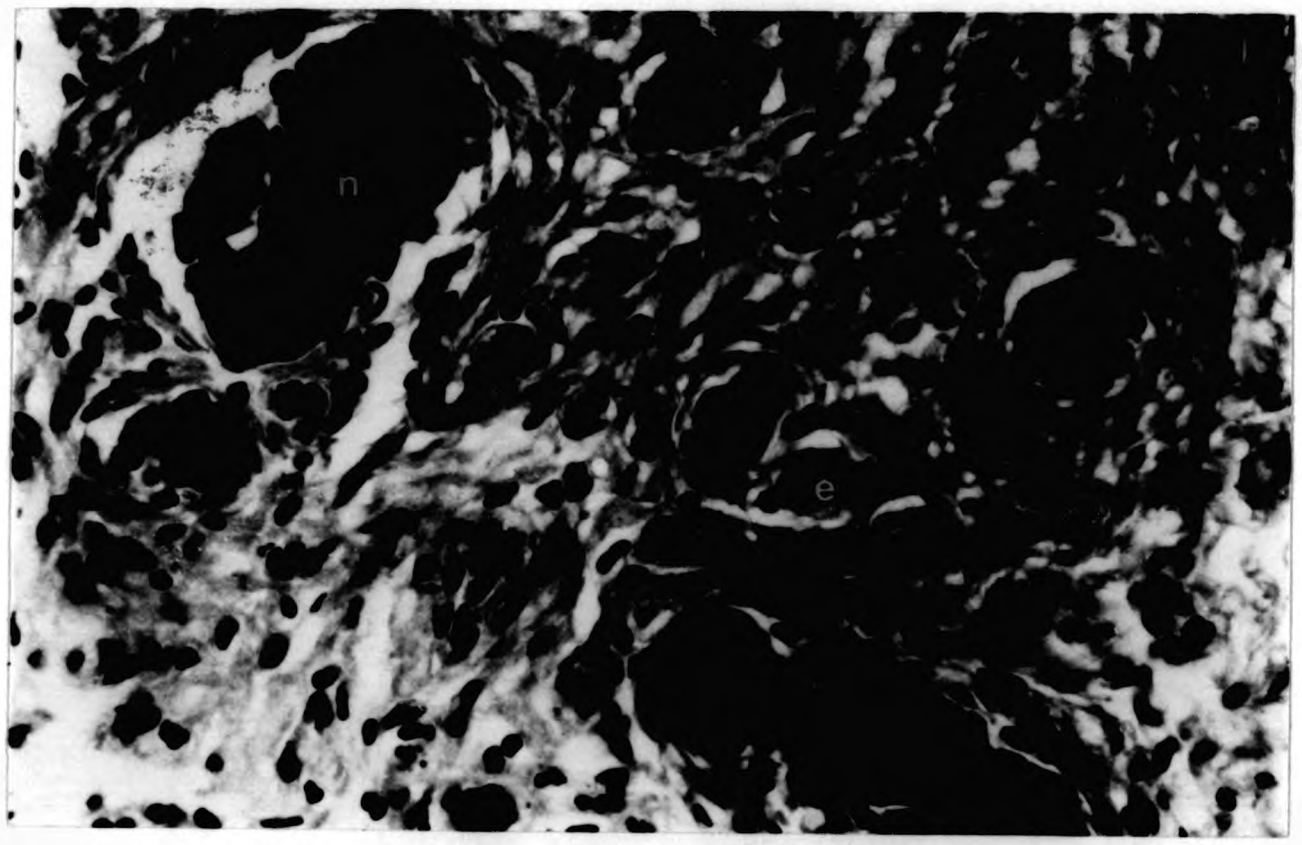
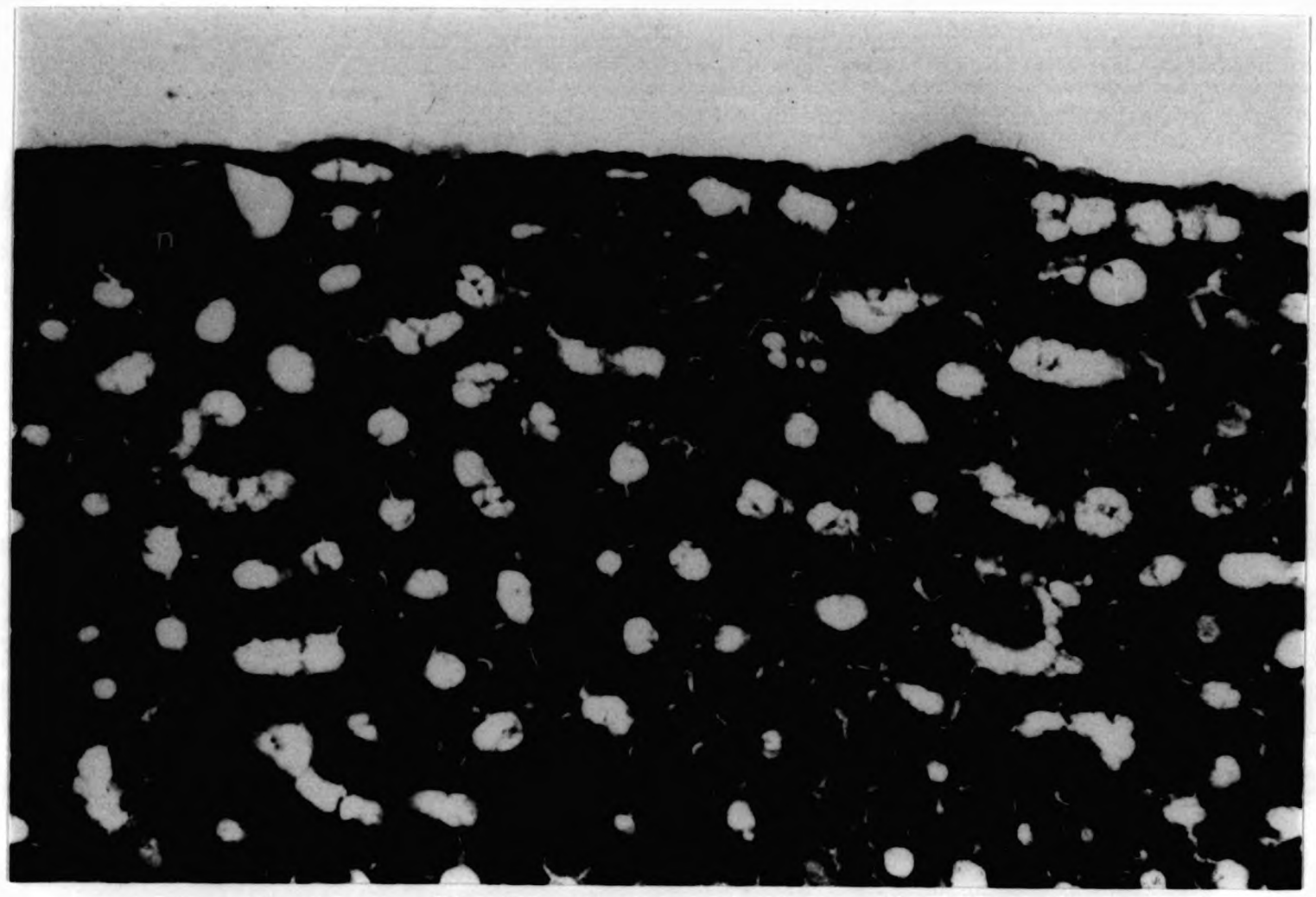

n
e

Fig. 95 a&b (detail) Necrotic melanized haemocytic nodules in the haemolymph sinuses of the tubular antennal gland labyrinth. Live *Vibrio anguillarum* i.m., 4 weeks (HE, X300, X600).



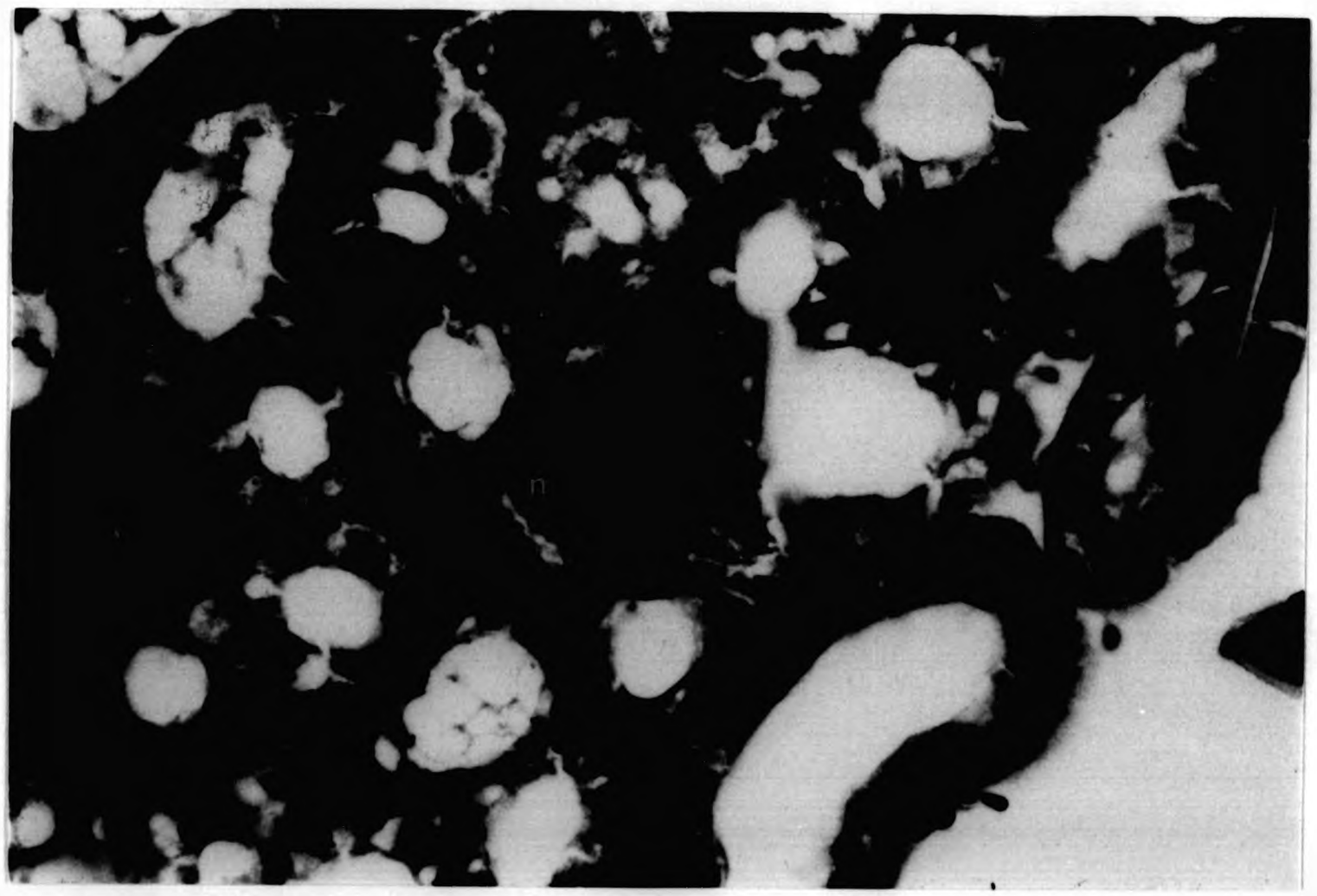


Fig. 96 Area of marked diffuse myofibre necrosis (n) and fibroplasia (f) with necrotic melanized haemocyte aggregations, encapsulations and nodules (N) in muscle injection site (M). Live *Vibrio anguillarum* i.m., 4 weeks (HE, X300).


N

injection site (Figs. 94, 96; Tables 1, 6) from 2 days onwards. Massive melanization of nodules was observed in the muscle, connective tissue and muscle injection site from 14 days (Figs. 94, 96; Tables 1, 6). Haematopoiesis (Table 1) was apparently markedly increased after 6 hours. There was a persistence of the haemocytic response in most tissues up to 28 days (Figs. 90, 91, 92, 93, 94, 95, 96; Tables 1, 6).

III. 3.7 The Tissue Response to Cuticular Traumatization

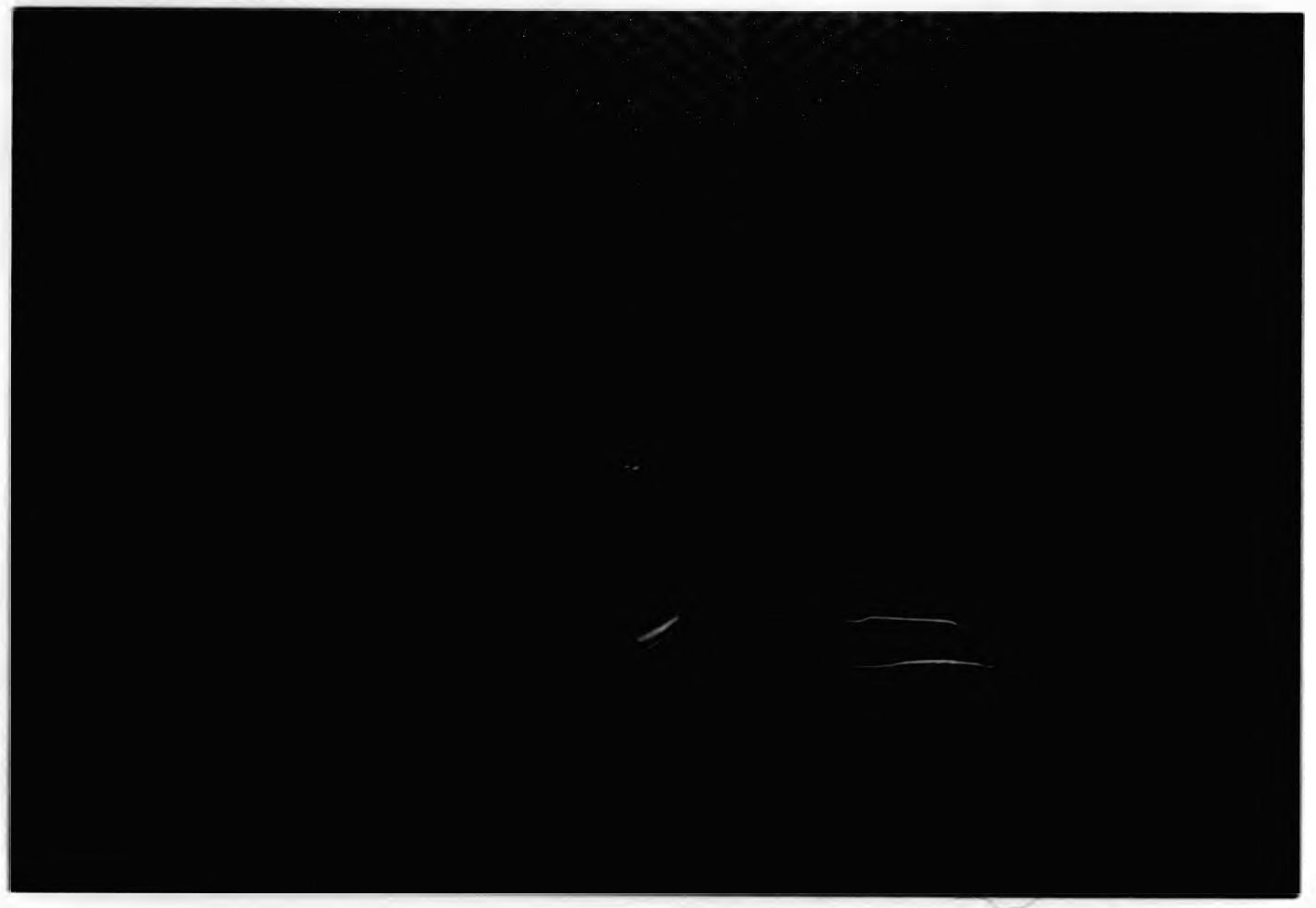
Gross Findings

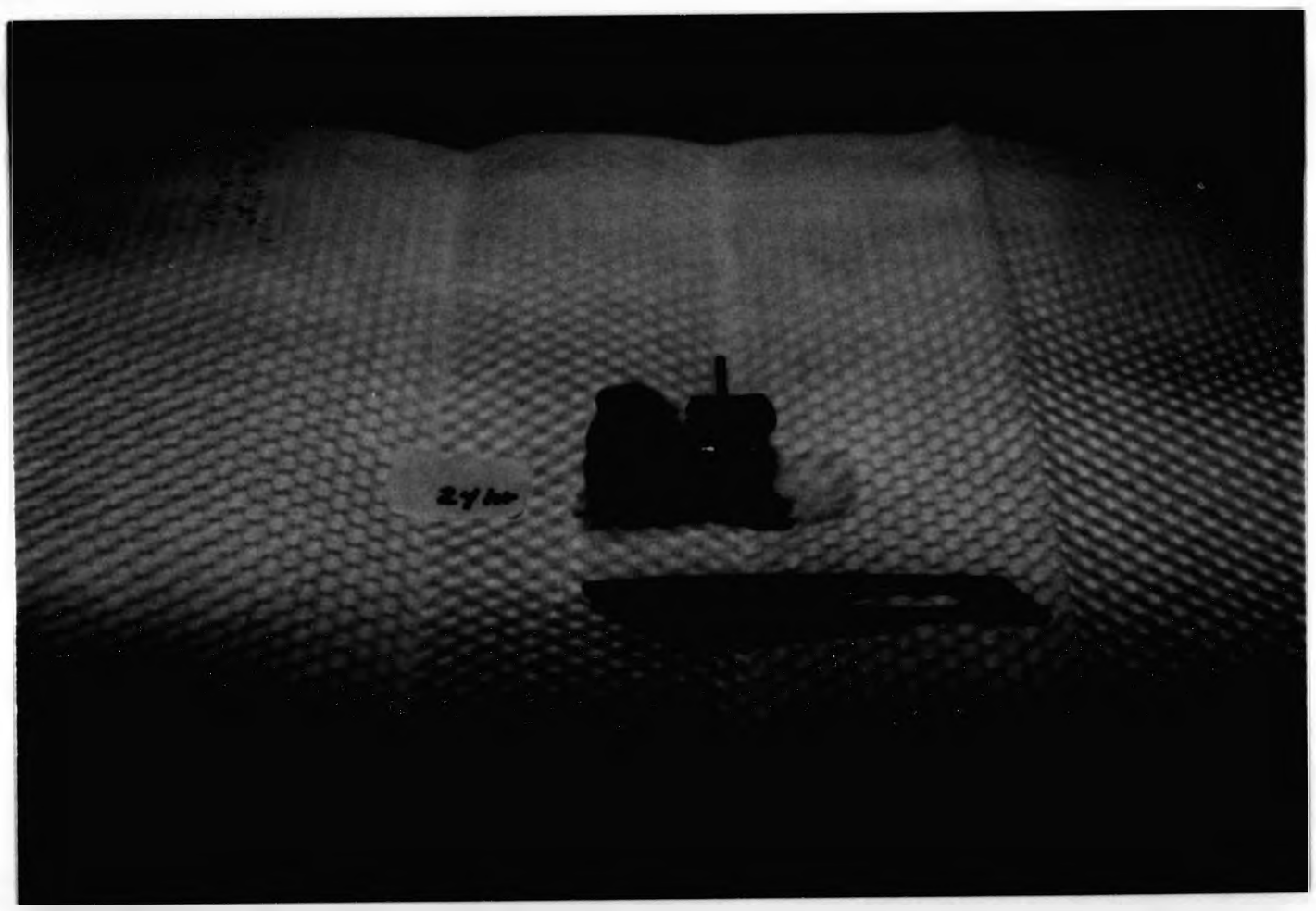
Twenty-four hours after abrasion of the cuticle, slight to moderate brown-black discolouration (i.e. melanization) was observed in the injured area of the prawns moderately (group 2) and severely (group 3) abraded (Fig. 97 a&b). Controls (group 1) and slightly abraded prawns (group 2) showed no change (Fig. 98 a&b). After 2 to 3 days, cuticular melanization was present in prawns of all groups except the controls, with group 2 showing slight (Fig. 99 a&b), and groups 3 and 4 showing moderate to marked melanization (Fig. 100 a&b). After 6 to 8 days and onwards until moulting, groups 3 and 4 displayed marked melanization (Fig. 101) with group 2 showing slight melanization (Fig. 102) and the controls appearing unaffected (as Figs. 8, 98a).

Histological Findings

Note:- All findings refer to the cuticular region which

Fig. 97 a&b Cuticular trauma, traumatized second abdominal segments of groups 3 (moderate, above) and 4 (severe, below), 24 hours: slight and moderate melanization (arrow), respectively.




24 hr

includes: epicuticle, exocuticle (pre-ecdysial procuticle), endocuticle (post-ecdysial procuticle), epidermis, subcuticular spongy connective tissue and muscle.

Controls (group 1) showed no abnormalities throughout the experiment.

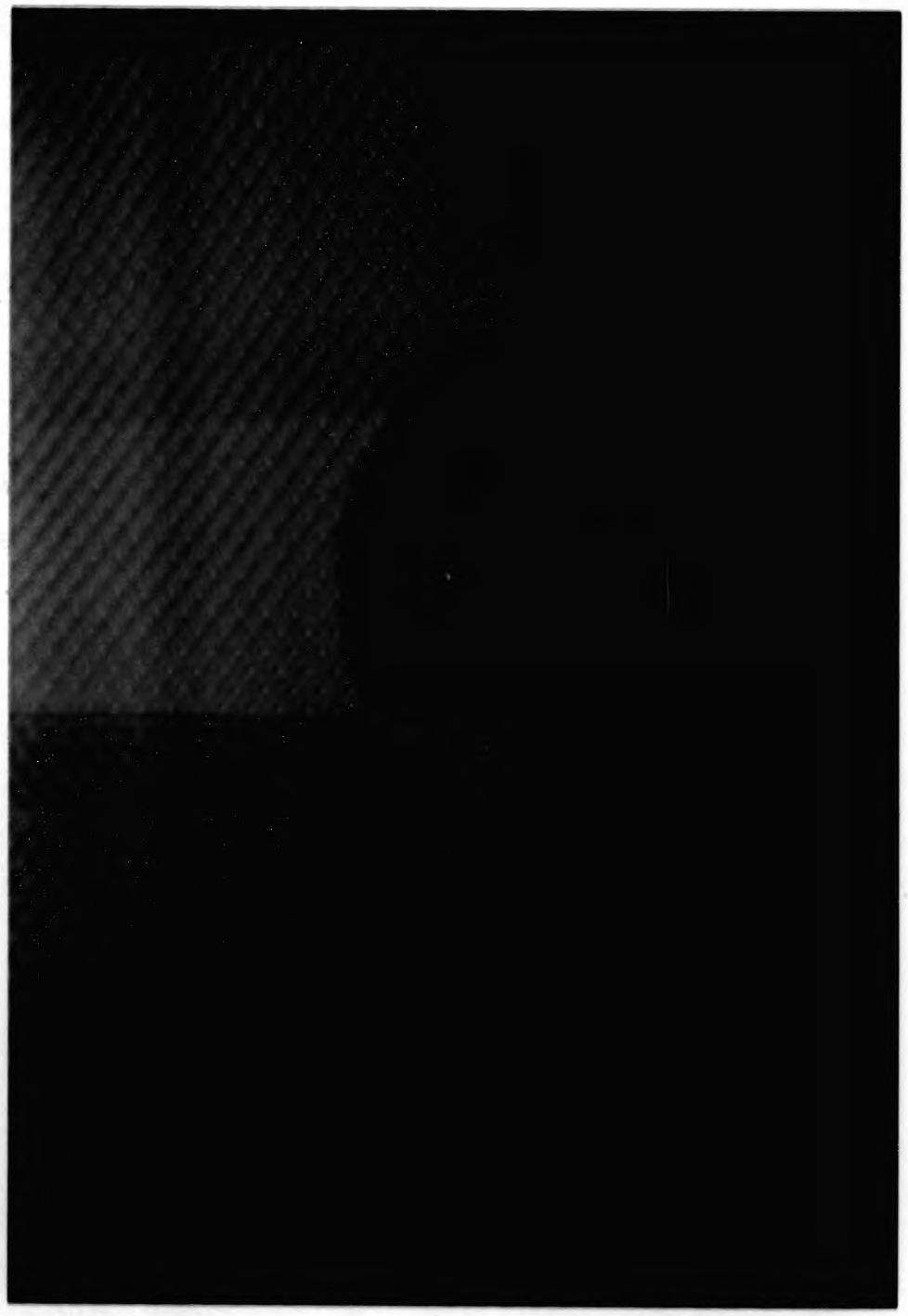
* 24 hours after traumatization:

Group 2: There was an area of slight epi- to exocuticular erosion/ulceration, with haemocyte infiltration and partial melanization; slight haemocyte infiltrations were present in the muscle (Table 1).

Group 3: An area of moderate epi- to endocuticular ulceration was observed, with the intact edges of lesion limited by multilaminar sheets of partially melanized to melanized haemocytes, difffuse haemocyte infiltration in the surrounding areas.

Group 4: Severe damage to all cuticular layers, epidermis, subcuticular spongy connective tissue and areas of muscle was noted. Intact edges of the lesion from the epicuticle to the spongy connective tissue layer were rounded off by a multilaminar partially melanized to melanized "open" haemocyte encapsulation. A large nodular area on the damaged surface appeared to be a haemolymph clot (coagulation) with haemocyte infiltration and aggregations. In the inner portions of the wound edges, haemocyte infiltration and aggregation were also observed. Below the clot the entire area was encapsulated

Fig. 98 a&b Cuticular trauma, group 1 (control, above) and 2 (slight, below), 24 hours: no apparent changes.



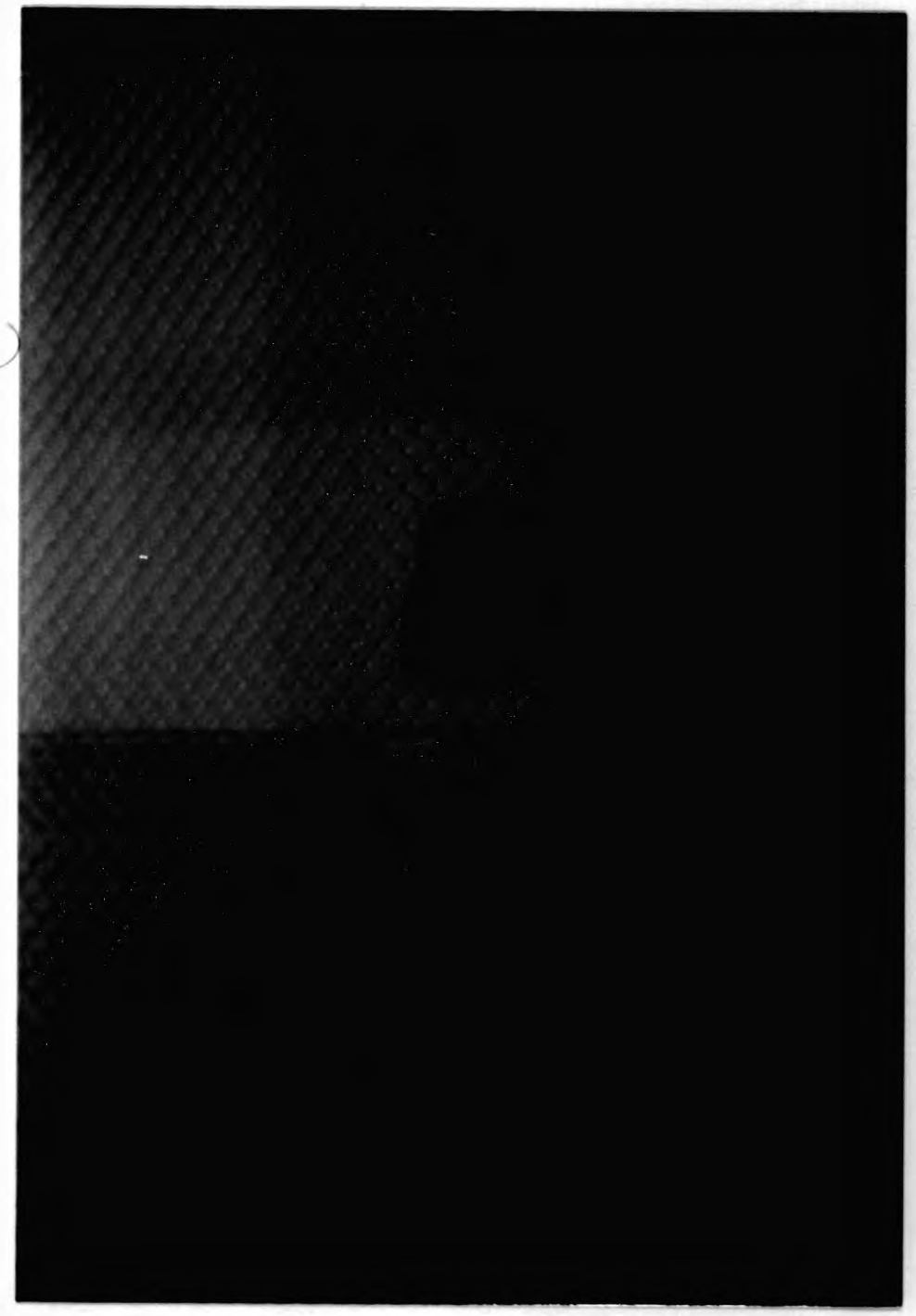
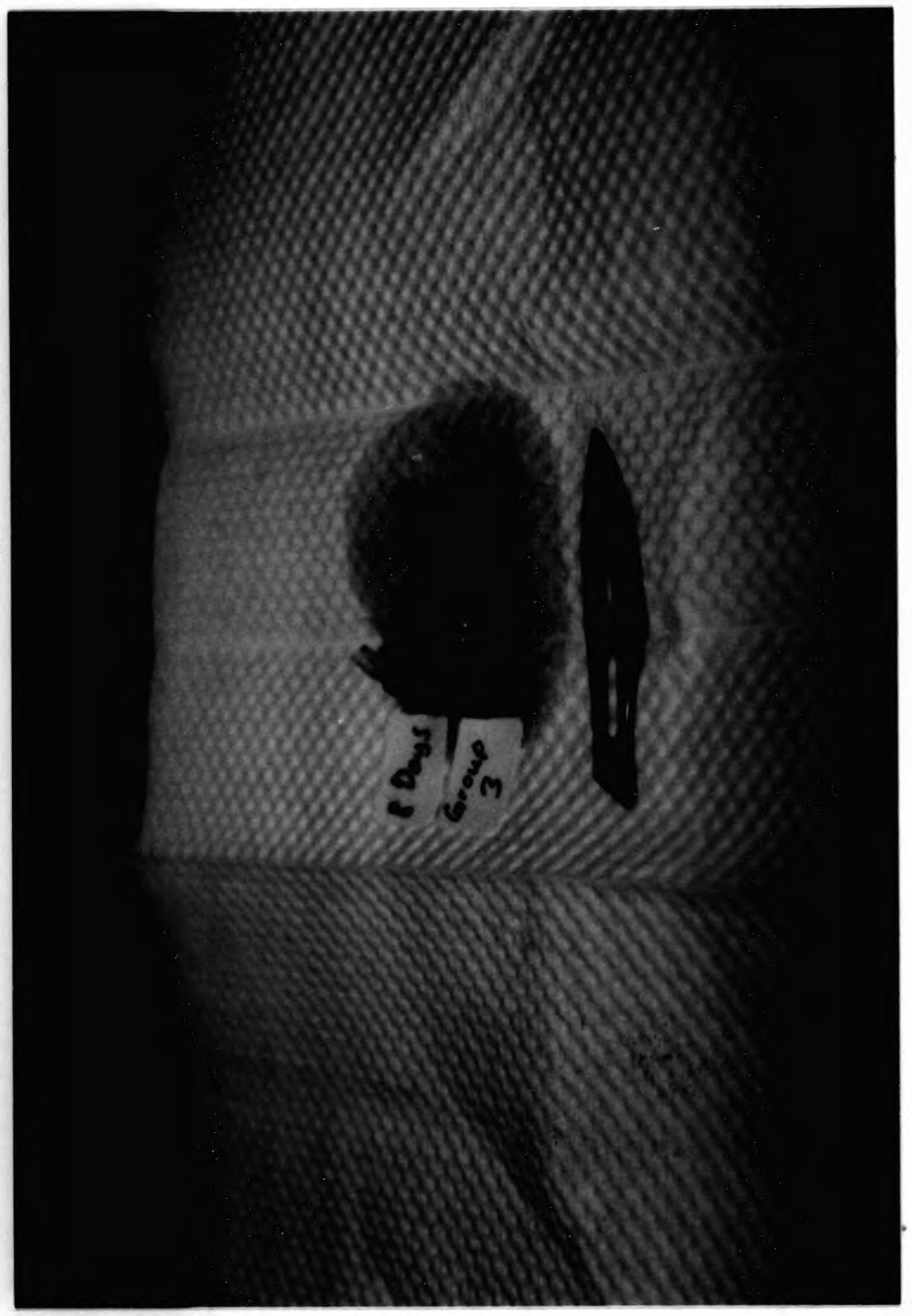


Fig. 99 a&b Cuticular trauma, group 1 (control, above) and 2 (slight, below), 72 hours: no changes and slight melanization (arrow), respectively.

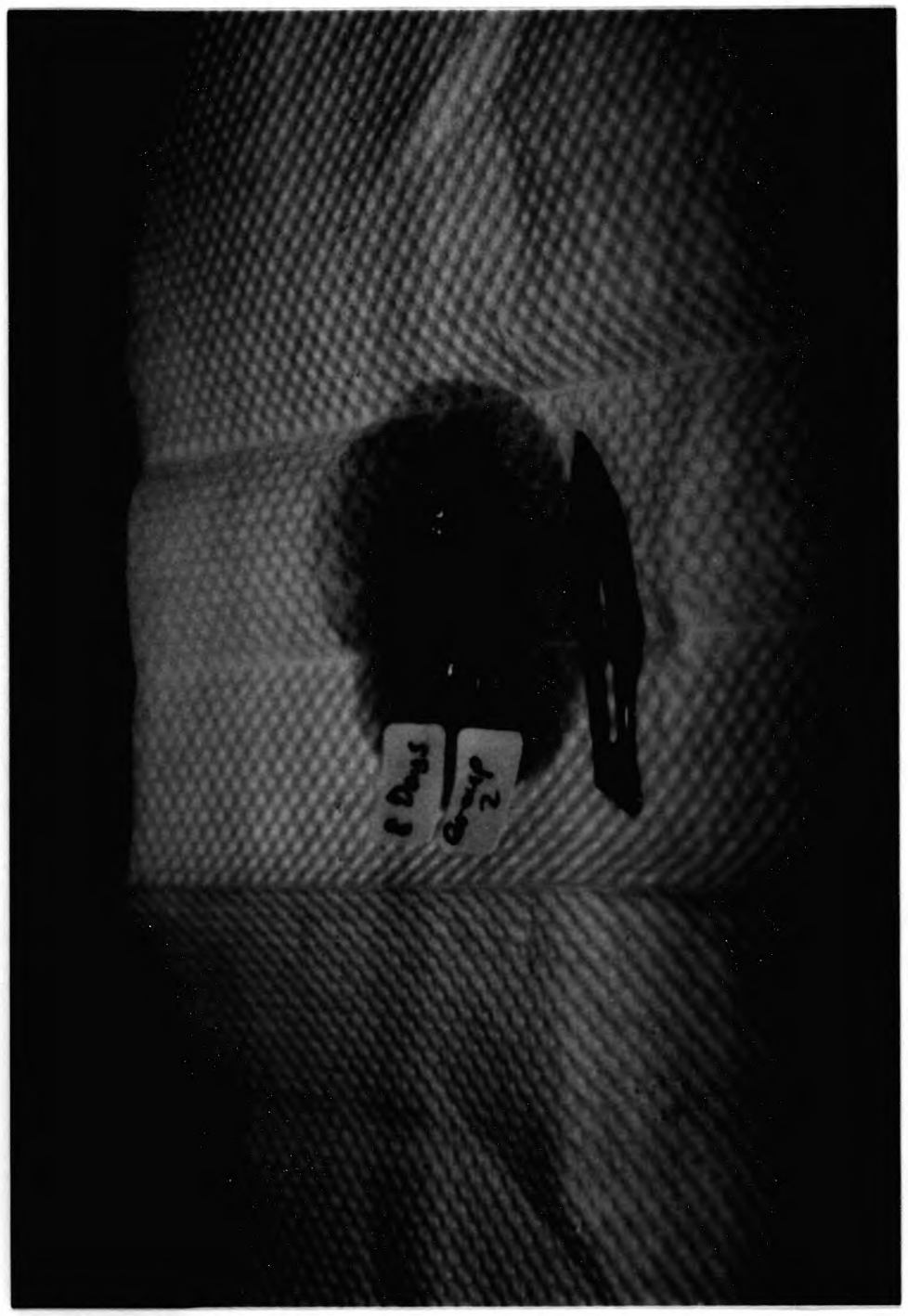
Fig. 100 a&b cuticular trauma, groups 3 (moderate) and 4 (severe), 72 hours: slight to moderate to marked melanization of the traumatized cuticle (arrow).

Fig. 101 a&b Cuticular trauma, groups 3 (moderate) and 4 (severe), 8 days: marked melanization of the injured cuticle (arrow).


5 Days
Group
3


5 Days
Group
4

Fig. 102 Cuticular trauma, group 2 (slight), 8 days: minimal melanization of the abraded surface (arrow).


I Dance
2
Group

by multilaminar partially melanized to melanized haemocytes, most of them effete. Below this, in the region of the subcuticular spongy connective tissue and muscle was a focally melanized area of haemocyte aggregation. The muscle below the lesion was partly involved in a massive multilamilar encapsulation, the upper parts consisting of compressed, effete haemocytes, with discrete haemocytes infiltrating the deeper area. Other areas of muscle showed slight to moderate diffuse haemocyte infiltration and aggregations (Fig. 103).

* 2 days after traumatization:

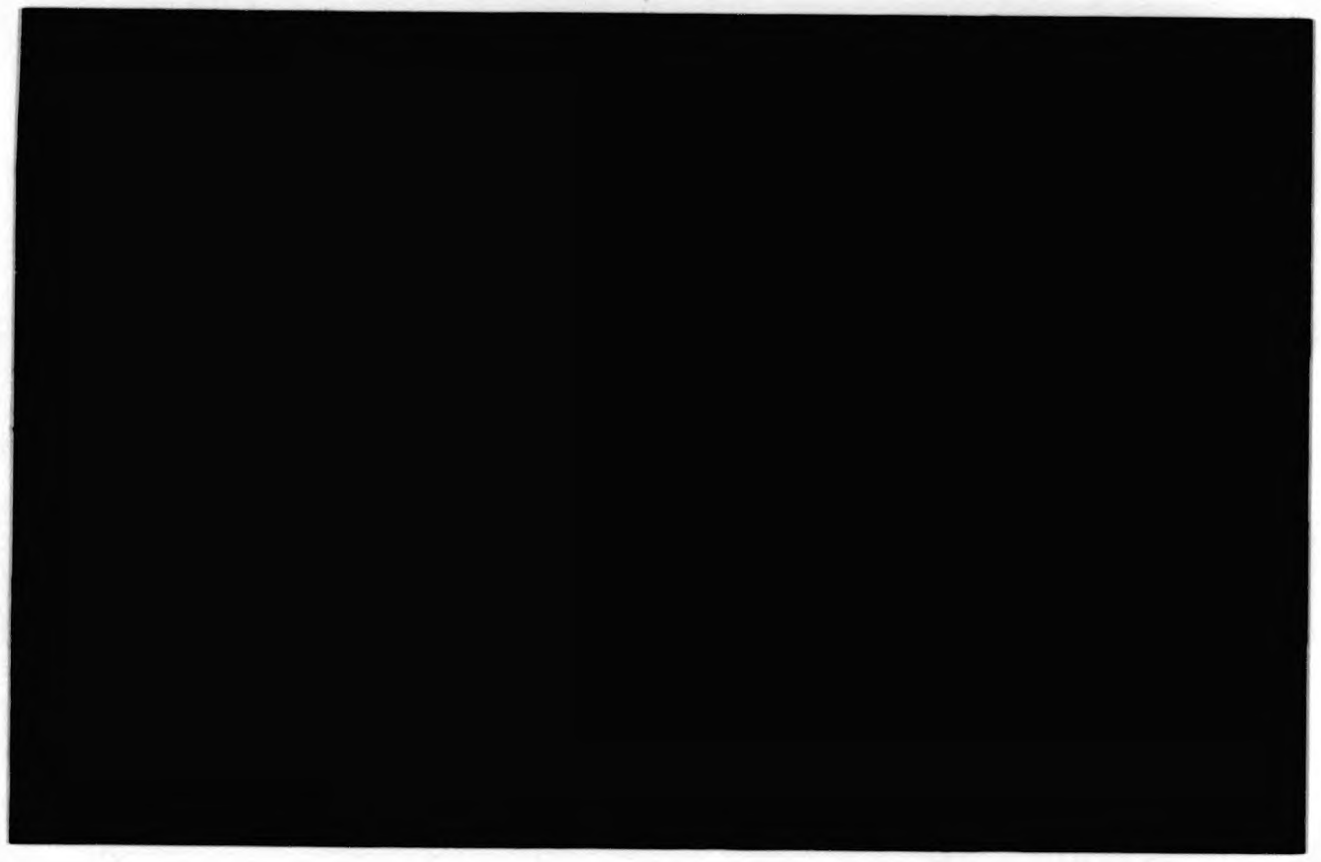
Group 2: There was melanization of the endocuticle, with haemocyte infiltration and aggregation and partially melanized to melanized multilaminar encapsulation in the exo- to endocuticle.

Group 3: Findings were similar to group 4 (below) but with only slight changes in the epidermis and muscle.

Group 4: There was melanization of the entire endocuticular wound surface, consisting of a multilaminar open haemocyte encapsulation. Multifocal karyorrhectic and pyknotic nuclei and focal melanized encapsulations were present throughout the extensive area of haemocyte infiltration, aggregation and fibroplasia in the epidermis, spongy connective tissue and muscle (Fig. 104; Table 1).

Fig. 103 Cuticular trauma, group 2 (severe), 24 hours: cuticular ulceration displaying diffuse haemolymph/haemocyte clot (o), a melanized multilaminar effete haemocyte encapsulation (l) encompassing an area of cuticle, epdermis and spongy connective tissue and muscle (M), with a myofibre haemocytic infiltration (i) response (HE, X125).

Fig. 104 Cuticular trauma, group 4 (severe), 2 days: the damaged cuticle (C) is replaced by multilaminar melanized effete haemocyte "open" encapsulation (l); the epidrmis (E), spongy connective tissue and muscle demonstrate haemocyte infiltration (i) and aggregation (a), with nuclear pyknosis (P) and karyorrhexis (K)(HE, X500).



* 3 days after traumatization:

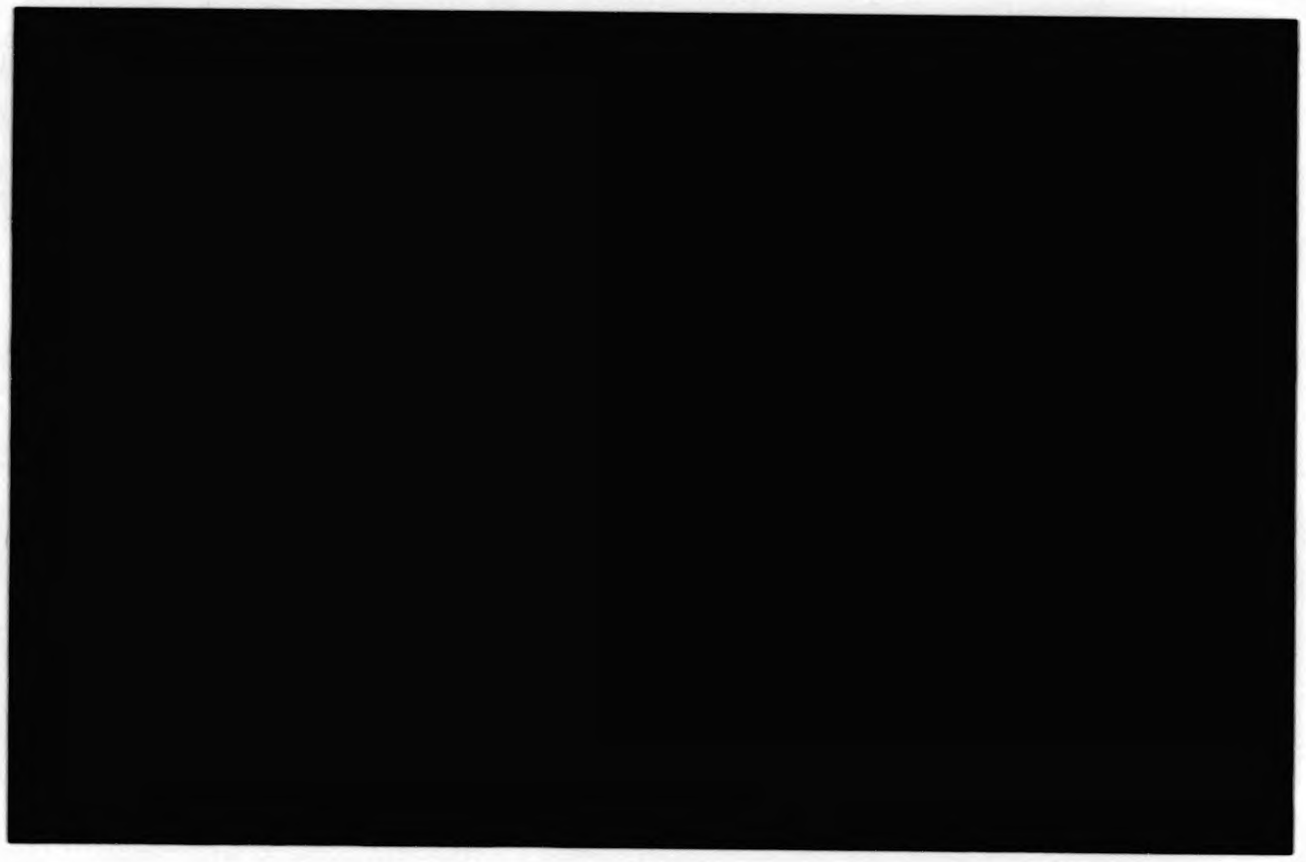
Group 2: Melanization of the encapsulation of the lesion surface was more complete, which consisted of effete haemocyte multilaminations.

Group 3: Ulceration was present to the deep endocuticle. The endocuticular lesion and epidermis consisted of a melanized multilaminar haemocyte encapsulation, with some bacterial accumulation at the surface. Haemocytes had also infiltrated the spongy connective tissue and upper myofibres (Fig. 105).

Group 4: There was severe cuticular ulceration, with loss of the epi- to exo- and/or endocuticle. The entire surface of the exocuticular or endocuticular lesion was encapsulated by multilaminar haemocytes, with variable partial melanization to melanization. the upper layers consisted of effete, melanized haemocytes; in the deeper layers the haemocytes had intact nuclei, contiguous cytoplasm and were partially melanized. There was also encapsulation and melanization in parts of the remaining epi-, exo- and endocuticle and epidermis. The haemolymph clot was more organized. Some areas of necrotic epidermis displayed diffuse haemocyte infiltration and aggregation, with extension into the subcuticular spongy connective tissue and muscle, which also demonstrated focal necrosis. Fibroplasia in the epidermis, spongy connective tissue and muscle was more extensive than previously. Multifocal to areas of diffuse haemocyte karyorrhexis and pyknosis were observed

Fig. 105 Cuticular trauma, group 3 (moderate), 3 days: damaged cuticle displaying endocuticular effete multilaminar haemocytic melanization (l), with spongy connective tissue haemocyte infiltration (i). Cuticle (c), epidermis (E), muscle (M). (HE, X125).

Fig. 106 Cuticular trauma, group 4 (severe), 3 days: the cuticular ulcer is delimited by an encapsulation of multilaminar melanized effete haemocytes (l), whilst the central area consists of an organizing haemolymph/haemocytic coagulation (o) (HE, X250).



throughout the areas of haemocyte infiltration and in the focal melanized encapsulations (Fig. 106).

* 6 days after traumatization:

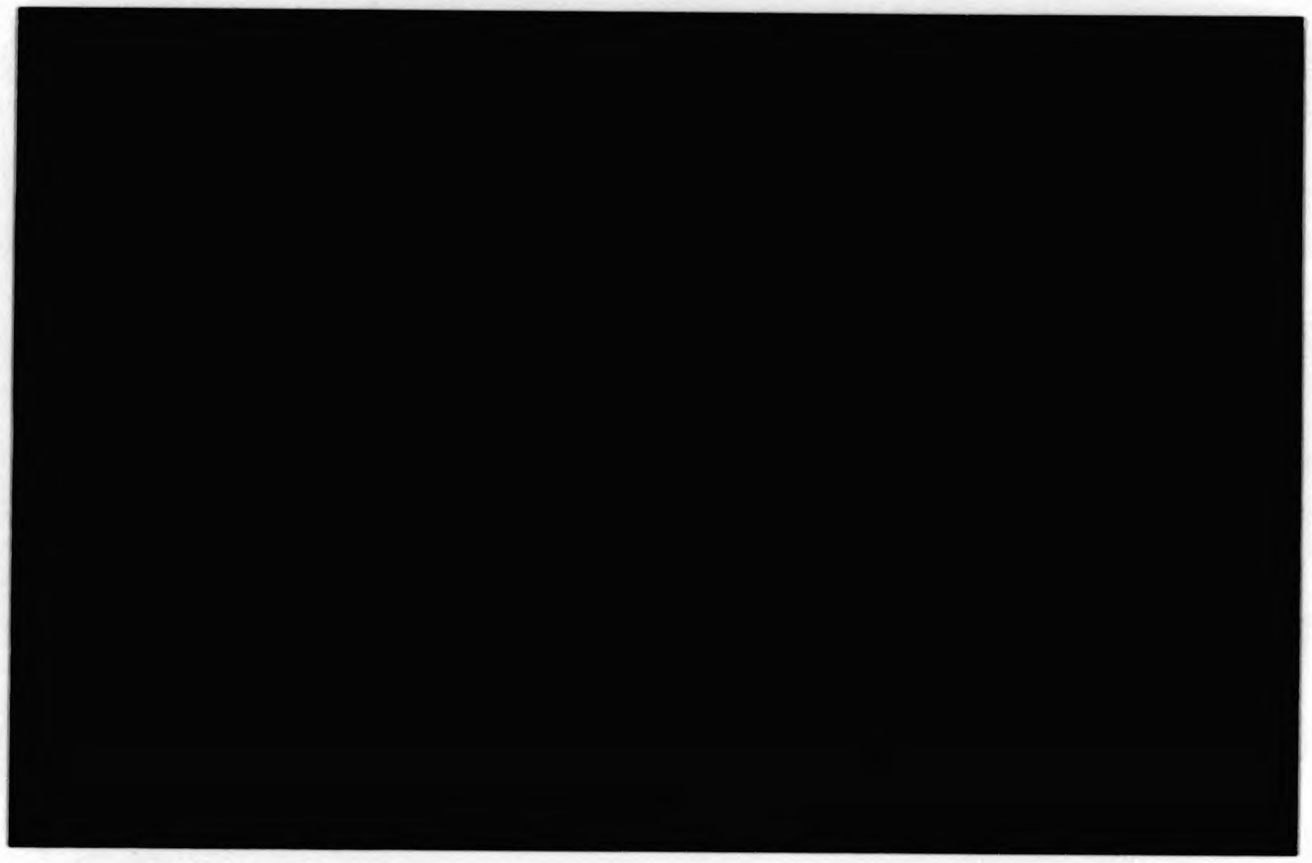
Group 2: A layer of melanized effete haemocytes was present on the abraded cuticular surface, in the lower third of the endocuticle and/or in the spongy connective tissue or muscle (Fig. 107).

Group 3: Ulceration partly involved the endocuticle, with melanization of the surface. The complete edges of the lesion were limited by effete melanized multilaminar harmocytes. New undamaged cuticle was present under the damaged layer.

Group 4: Ulceration involved the epi- and the upper portion of the endocuticle which were all melanized (Fig. 108). Bacteria were present on the inner epicuticular edges of the lesion. There was a diffuse area of damaged epidermis, spongy connective tissue and muscle which displayed a marked diffuse haemocyte infiltration, aggregation, with multifocal melanized encapsulations and fibroblast proliferation. Moderate to marked karyorrhectic haemocytic nuclear debris and pyknotic haemocytes were present (Fig. 109 a&b). A massive nodule composed of compressed, multilaminar, effete, diffuse haemocyte infiltration was observed. The nodule emanated from a thickened malformed portion of the newly formed underlying cuticle. The inflammatory response also extended into the new epidermis through the

Fig. 107 Cuticular trauma, group 2 (slight), 6 days: area of melanization (m) of the abraded cuticular (c) surface and focal aggregation (a) in the spongy connective tissue. Epidermis (E) (HE, X125).

Fig. 108 Cuticular trauma, group 4 (severe), 6 days: the cuticular (c) ulcer has been replaced by an encapsulation of multilaminar effete melanized haemocytes (l), with an extensive haemocytic reaction (i) in the spongy connective tissue and muscle (HE, X125).



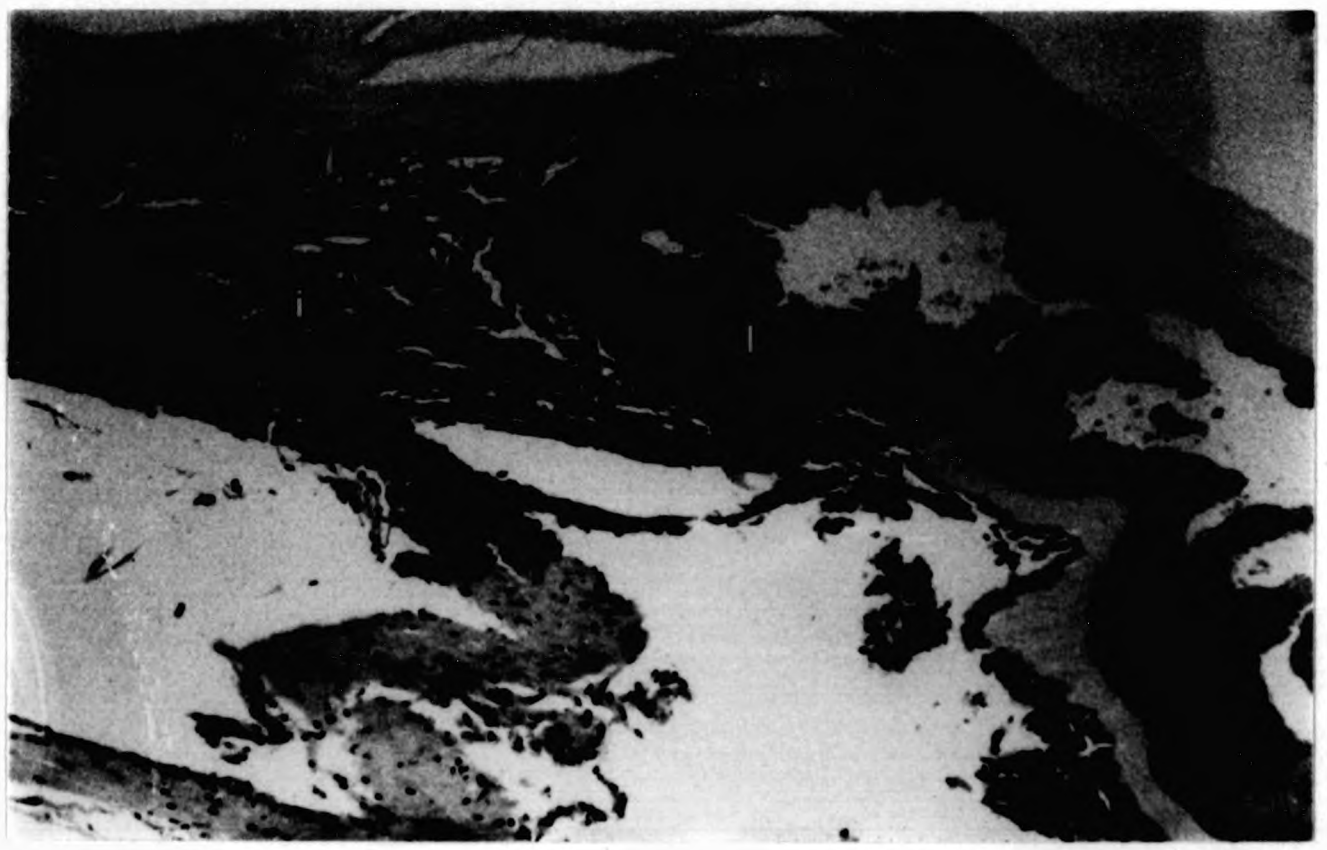
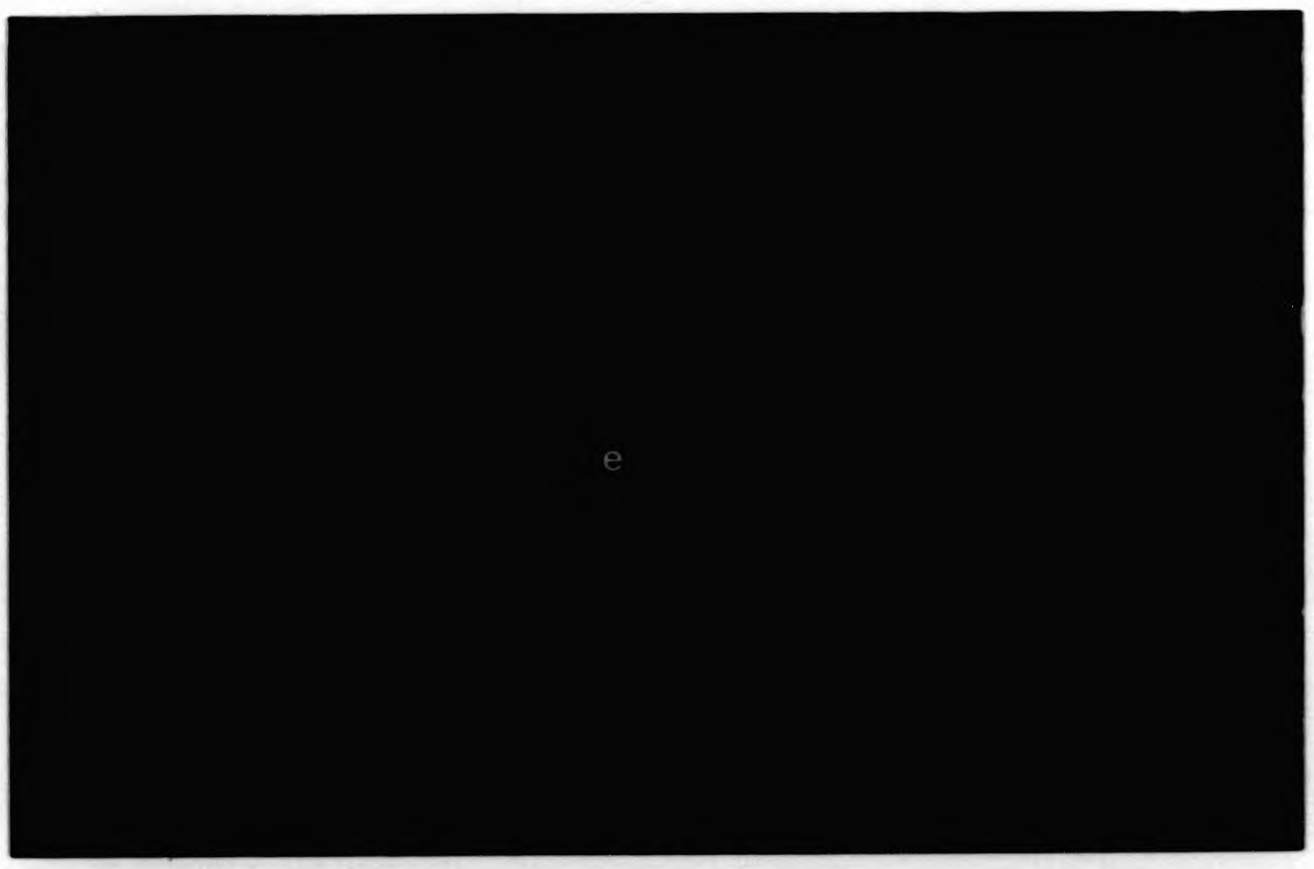


Fig. 109a Cuticular trauma, group 4 (severe), 6 days: massive haemocytic response (i) in subcuticular spongy connective tissue and muscle cuticle (C). (HE, x50).

Fig. 109b Cuticular trauma, group 4 (severe), 6 days: multifocal melanized necrotic encapsulations (e) in an area of diffuse muscle necrosis, haemocyte infiltration, aggregation and fibroplasia (HE, X500).



cuticular layers where there were areas of multilaminar effete melanized (epi- to exocuticle) haemocytes.

* 8 days after traumatization:

Group 2: An area of exo- to endocuticular melanized effete haemocyte multilamination was present.

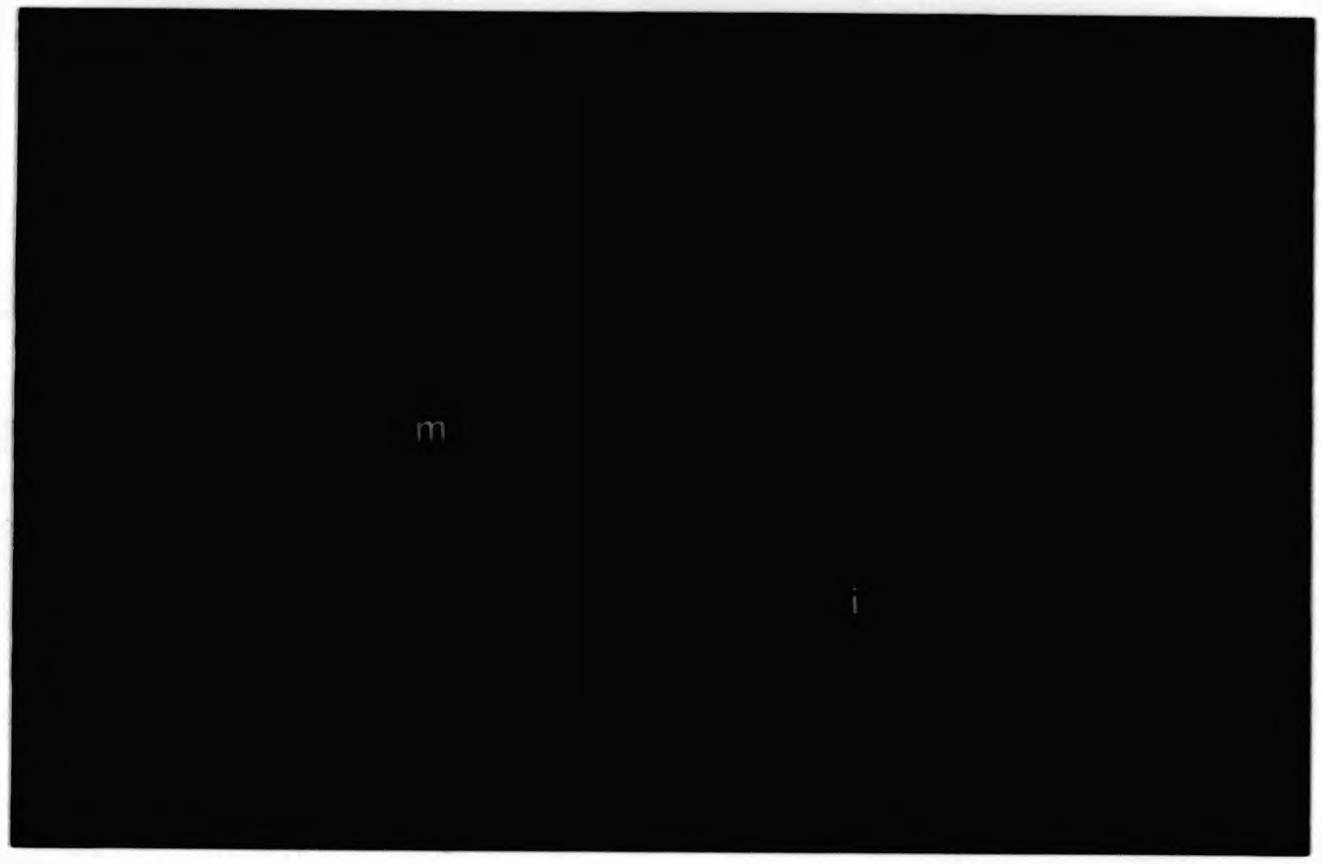
Group 3: Similar to after 6 days, with more prominent melanization.

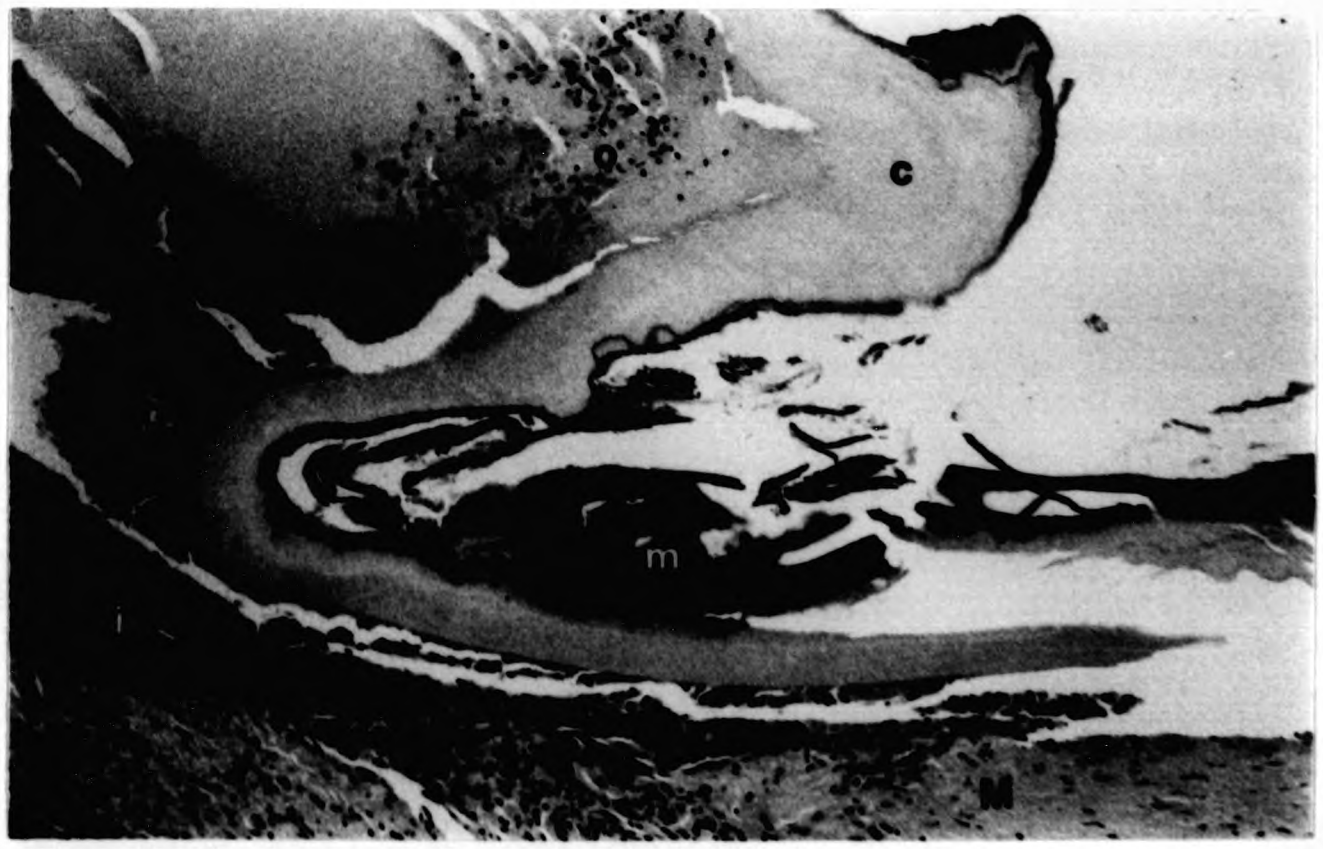
Group 4: The edges of the lesion were very well encapsulated and melanized from the epicuticle to the spongy connective tissue. Slight detrital and bacterial fouling was present on the surface of the lesion. In the melanized exposed surface and the exo- to upper endocuticular portion of the lesion effete haemocyte nuclei were discernible only with difficulty; some deeper portions from the membranous layer and epidermis to the spongy connective tissue and muscle consisted of multilaminar effete haemocytes; beneath these areas, into the deep muscle, were extensive haemocyte infiltrations and aggregations, with multifocal melanized encapsulations and marked fibroblast proliferation. The haemolymph clot under the damaged area of the lesion contained extensive haemocyte infiltration, numerous aggregations of effete haemocytes and diffuse fibroplasia (Fig. 110 a&b).

* 10 days after traumatization:

Group 2: The edges and irregular surface of the

Fig. 110 a&b Cuticular trauma, group 4 (severe), 8 days: marked raised lesion exhibiting variably thick melanized (m) surface, organizing haemolymph/ haemocyte clot (o) and extensive underlying tissue damage and haemocytic reaction (i). Cuticle (C), muscle (M) (HE, X125).


m
i


o
c
m
M

lesion from the epi- to endocuticle were encapsulated by multilaminar melanized haemocytes. there was slight detrital and *Epistylis* fouling of the surface of the lesion. The endocuticle contained multilocular encapsulations and nodules with melanized centres. The subcuticular connective tissue and muscle had moderate diffuse areas of haemocyte infiltration and multifocal haemocyte aggregations.

Group 3: There was an ulceration which had removed the epicuticle and most of the exocuticle. The entire exocuticular surface and the irregular epi- and exocuticular edges of the lesion were infiltrated by multilaminar effete melanized haemocytes. The rest of the exocuticle contained slight multifocal to diffuse melanized haemocytes. One thickened area of the exocuticle displaced the endocuticle downward.

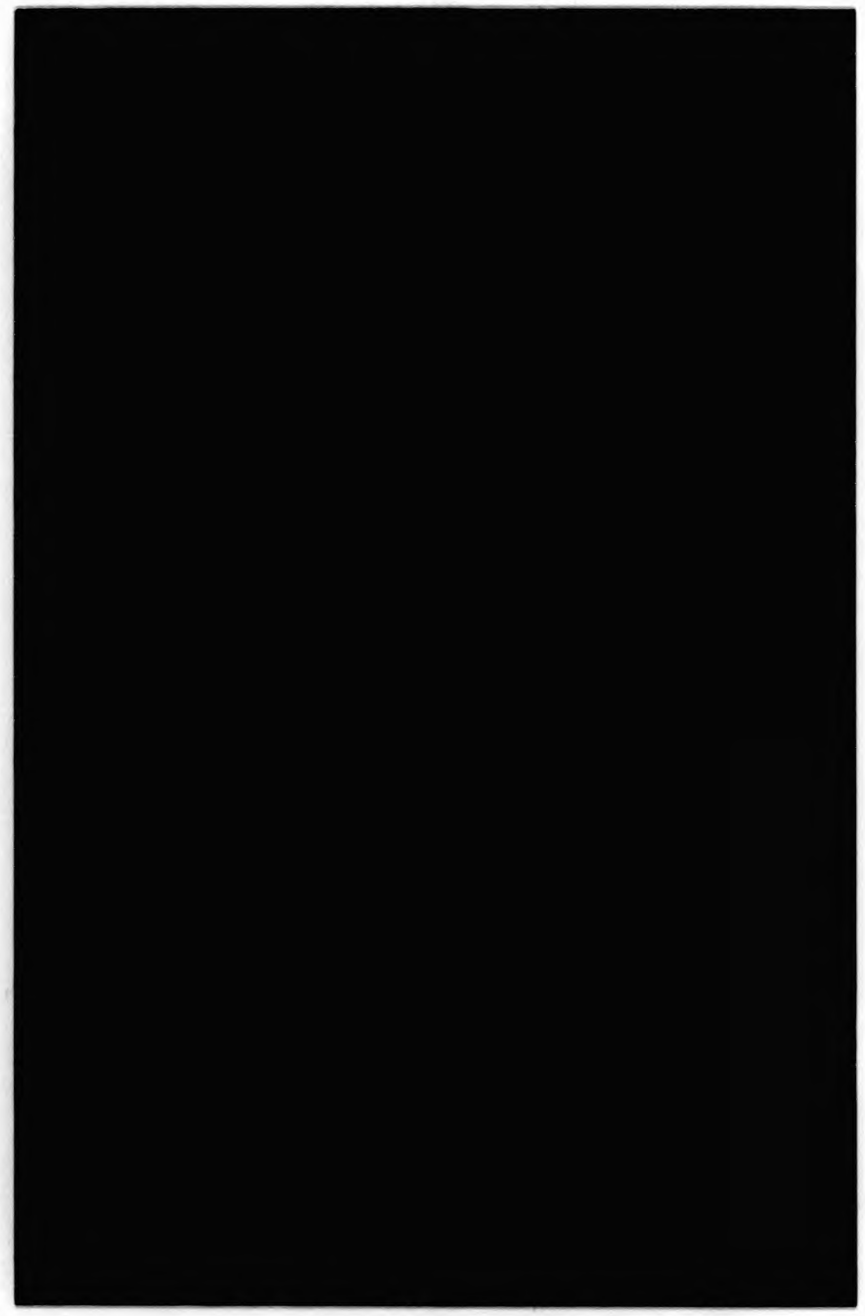
Group 4: Marked ulceration almost completely removed the entire endocuticle. On top of this were split sheets of melanized cuticle, some displaying distinct haemocyte multilaminations. The surface of the lesion had a layer of detrital and bacterial fouling. The epidermis, subcuticular spongy connective tissue and muscle where necrotic, with a marked diffuse haemocyte infiltration, fibroplasia and multifocal melanized encapsulations (Fig. 111).

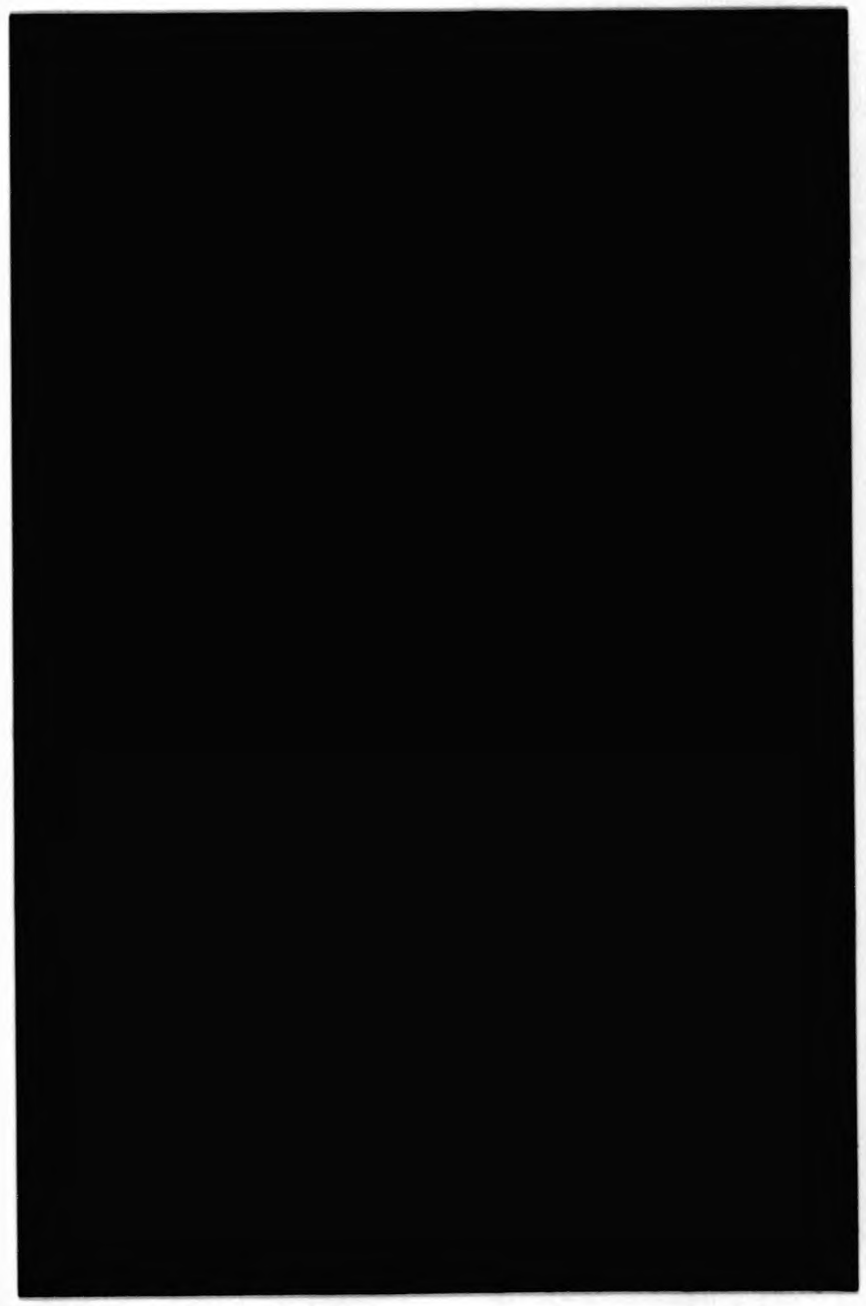
* 14 days after traumatization:

Group 2: The irregular, eroded surface of the

Fig. 111 Cuticular trauma, group 4 (severe), 10 days: area of extensive myofibre necrosis, haemocyte infiltration, aggregation, multifocal melanized encapsulations and fibroplasia. Muscle (M) (HE, X50).

Fig. 112 Cuticular trauma, group 2 (slight), 14 days: surface and deep (epi-, exo- and endocuticular) melanization (m) of irregularly thickened cuticle (C) (HE, X50).





epi- and exocuticle showed varying degrees of melanization which also extended to deeper portions of the thickened exo-endocuticle with a haemocytic response (Fig.112).

Group 3: Extensive loss of cuticle was observed from the epi- to endocuticle. The edges and upper layers of the lesion surface were encapsulated by multilaminar effete melanized haemocytes. An area from the epidermis, spongy connective tissue to the deep muscle consisted of diffuse haemocyte infiltration, aggregations and fibroplasia, with focal melanized encapsulations.

* 21 days after traumatization:

Groups 2 and 3: Prawns had moulted successfully. No discernible changes were noted.

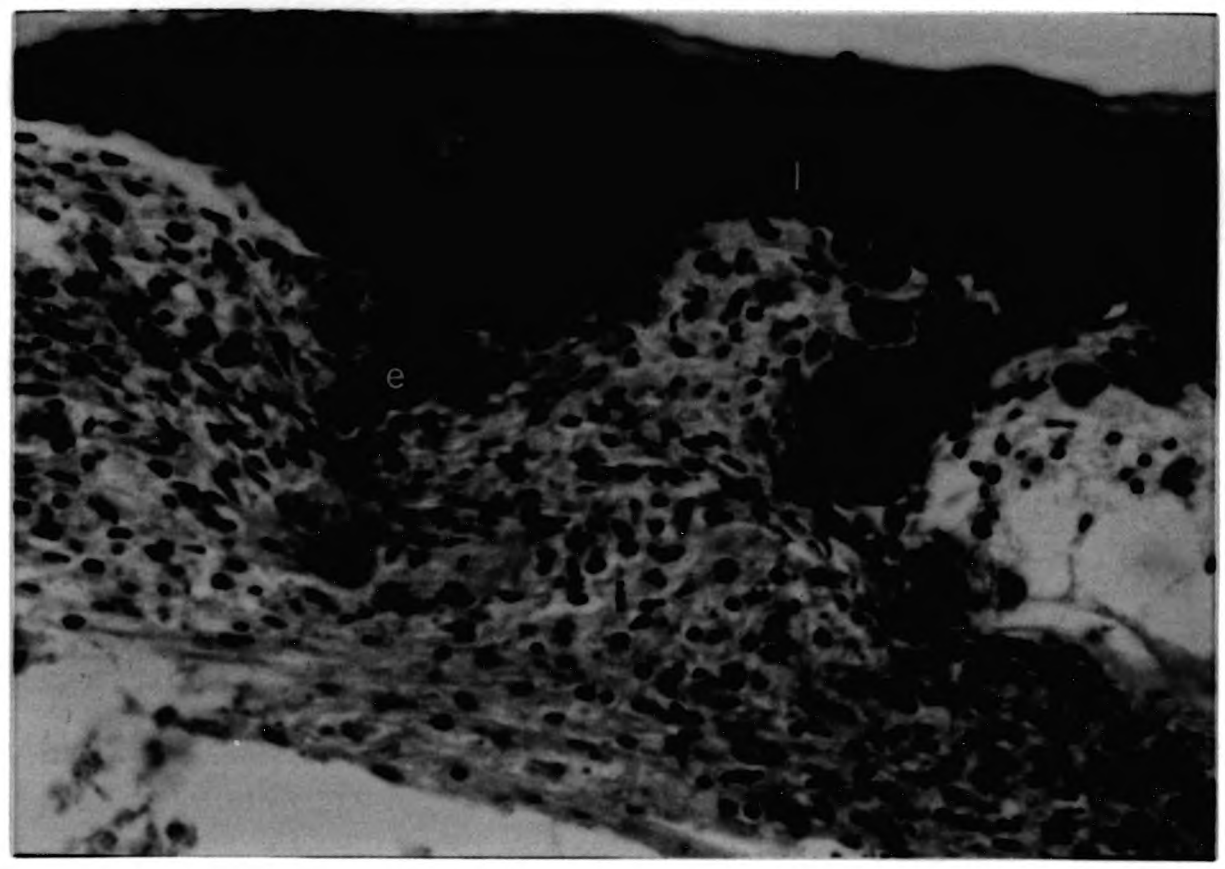
Group 4: Changes were similar to after 14 days, with a diffuse haemocyte infiltration and aggregations and fibroplasia throughout the epidermis, spongy connective tissue and muscle.

* 28 days after traumatization:

Groups 2 and 3: Prawns had moulted successfully, without any discernible changes.

Group 4: The edges and the entire exposed irregular surface of the lesion-variable due to the depth of the ulceration from the epi- to endocuticle and membranous layer- contained compressed, multilaminar effete melanized haemocytes. Areas of damaged exo- to

Fig. 113 Natural cuticular (cuticle (C)) lesion features characteristic encapsulation by multilaminar, effete melanized haemocytes (l), with an underlying area of tissue damage and an associated haemocytic response including infiltration (i), aggregation and encapsulation (e) (HE, X250).


l
e

endocuticle appeared thicker. Very slight detrital fouling was present on the surface of the lesion. There were areas of slight to moderate diffuse haemocyte infiltrations, aggregations interspersed with areas of fibroblast proliferation throughout the epidermis, spongy connective tissue and muscle. Some extremely deep areas of damaged muscle also had areas of diffuse haemocyte infiltration, aggregation and fibroplasia with pockets of haemocyte infiltration and melanized and non-melanized encapsulations (Table 1).

A natural lesion (chronicity unknown) was also observed on an experimentally unabraded area of the shell. It was very similar to the experimental lesions, with extensive melanized multilaminar haemocyte encapsulation of the lesion surface and edges and underlying diffuse haemocyte infiltration and aggregation. Fibroblast proliferation and haemocyte infiltration extended from the epidermis deep into the muscle (Fig. 113).

Scanning Electron Microscopy for Cuticular Traumatization

Scanning electron microscopy corroborated the varying degrees of cuticular damage present which were observed histologically throughout the experimental sampling period. At 2 days, specimens of the slight degree of trauma displayed minimal damage at low magnification (Fig. 114), though at higher magnifications (Fig. 115 a&b) more pronouced epi- and exocuticular

damage were apparent. The moderate degree at 2 days was characterized by deeper cuticular erosions at low power (Fig. 116a) but appeared similar to slight degree at higher magnification (Fig. 116). For the marked degree, severe ulceration at 6 days was depicted by deep pitting and ridges (Fig. 117 a&b). At high power prominent surface irregularities were observed (Fig. 118 a&b). For all samples, areas of undamaged cuticle had a regular structure resembling overlapping scales (Fig. 119 a&b). At lower power, cuticular pores were also apparent (Fig. 120). At 21 and 28 days, the cuticle of slight and moderate degrees appeared more regular (Fig. 121) or, if moulted, as the normal specimen (Fig. 120). The cuticle of the marked degree displayed prominent ridges and crevices indicative of ulceration at lower magnifications (Figs. 122, 123, 124 a&b) and irregularities at higher magnifications (Figs. 122 b&c, 123 b&c, 124 c) at 2, 3 and 4 weeks although organization of the lesion appeared to progress with its chronicity.

Fig. 114 SEM, group 2, slight cuticular trauma, 2 days: at low magnification the abraded area of cuticle (A) and surrounding uninjured normal surface (B) with regular cuticular pores (C) is apparent (X40).

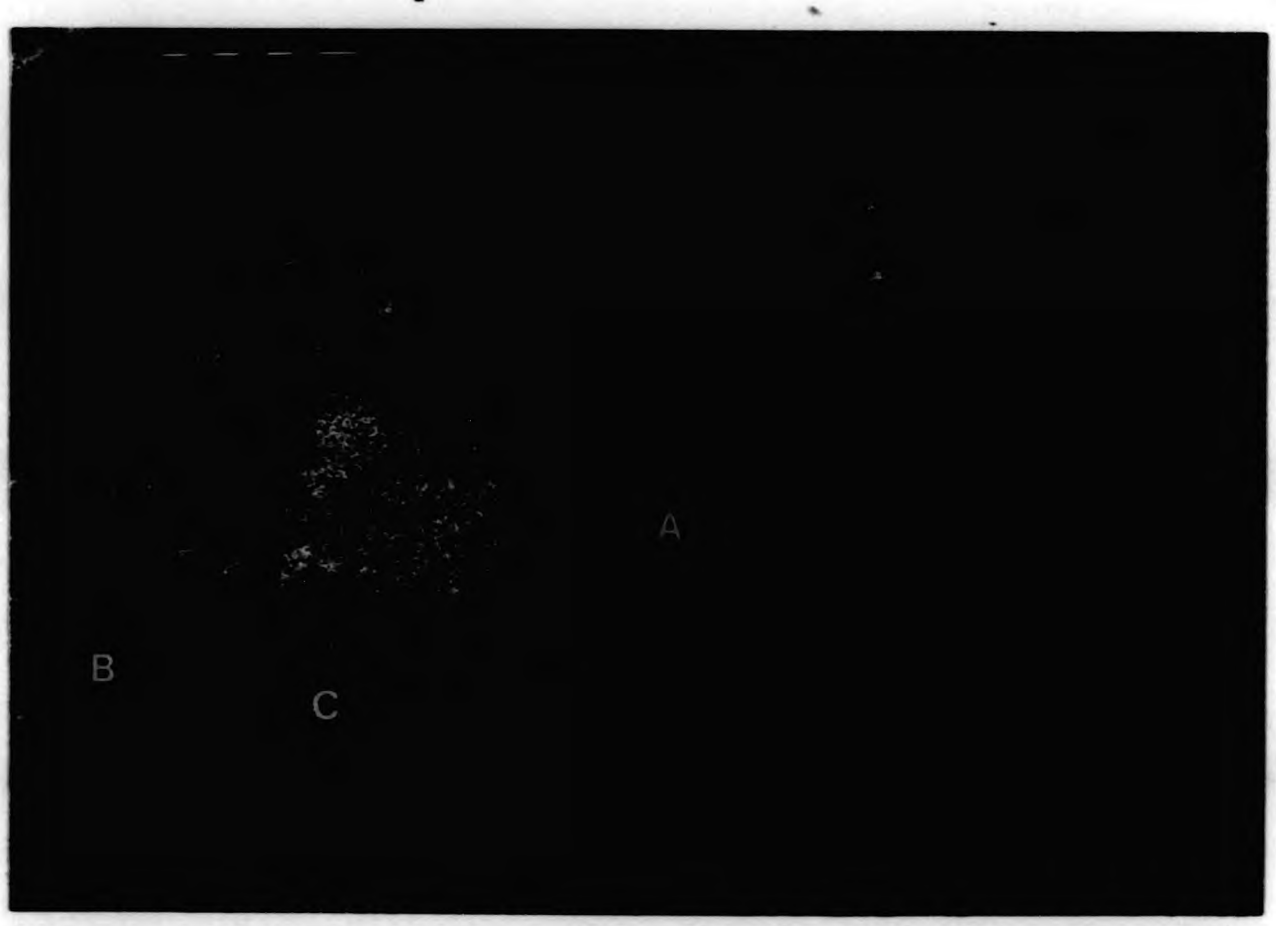

A
B
C

Fig. 115 a&b SEM, group 2, slight cuticular trauma, 2 days: at higher magnification epi- and exocuticular injury is present (X300, X2000).

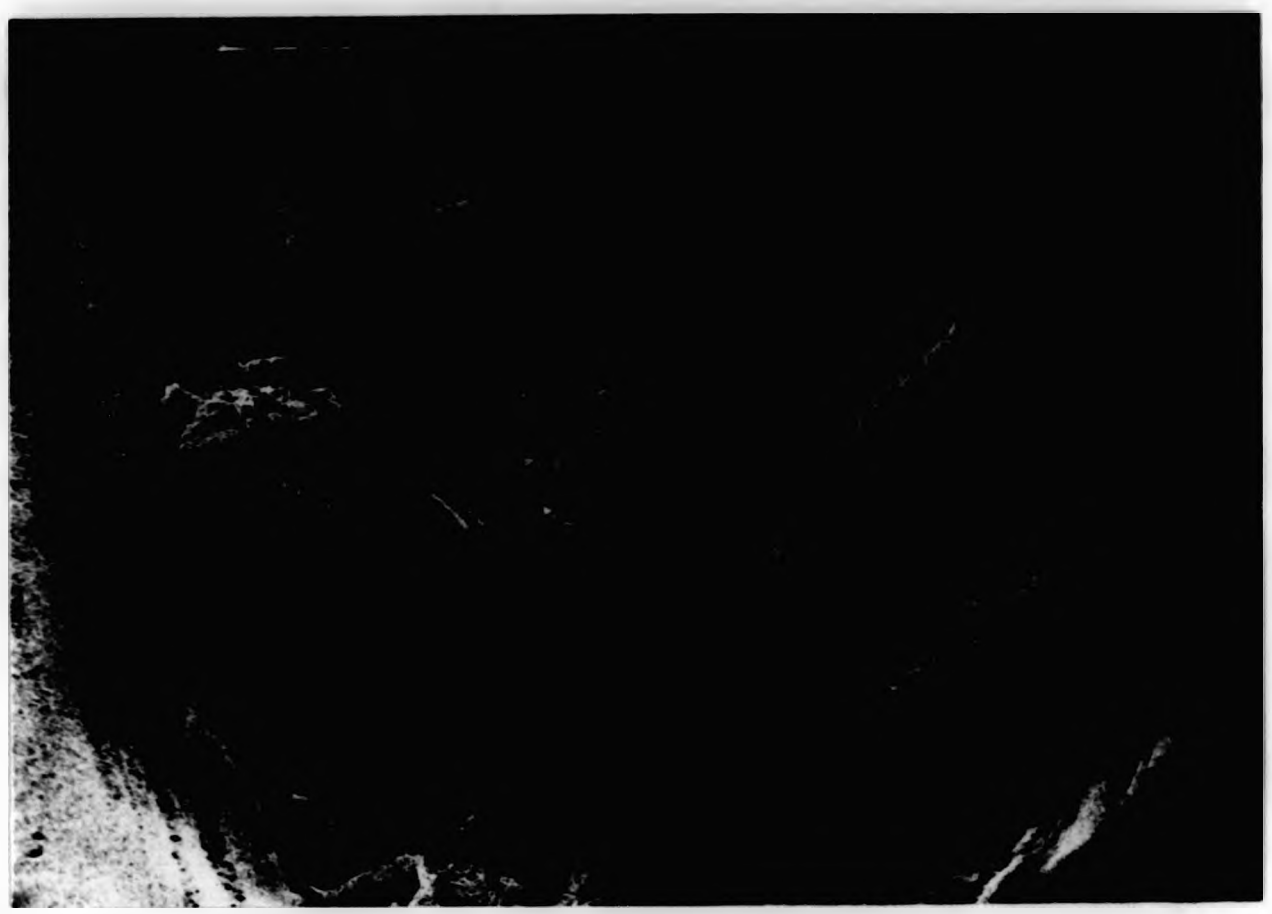


Fig. 116a SEM, group 3, moderate cuticular trauma, 2 days: the uneven, traumatized cuticle (A) adjacent to the normal undamaged cuticle (B) (X50).

Fig. 116b SEM, group 3, moderate cuticular trauma, 2 days: at higher magnification unusually shaped formations are present in the irregular, damaged area (X500).

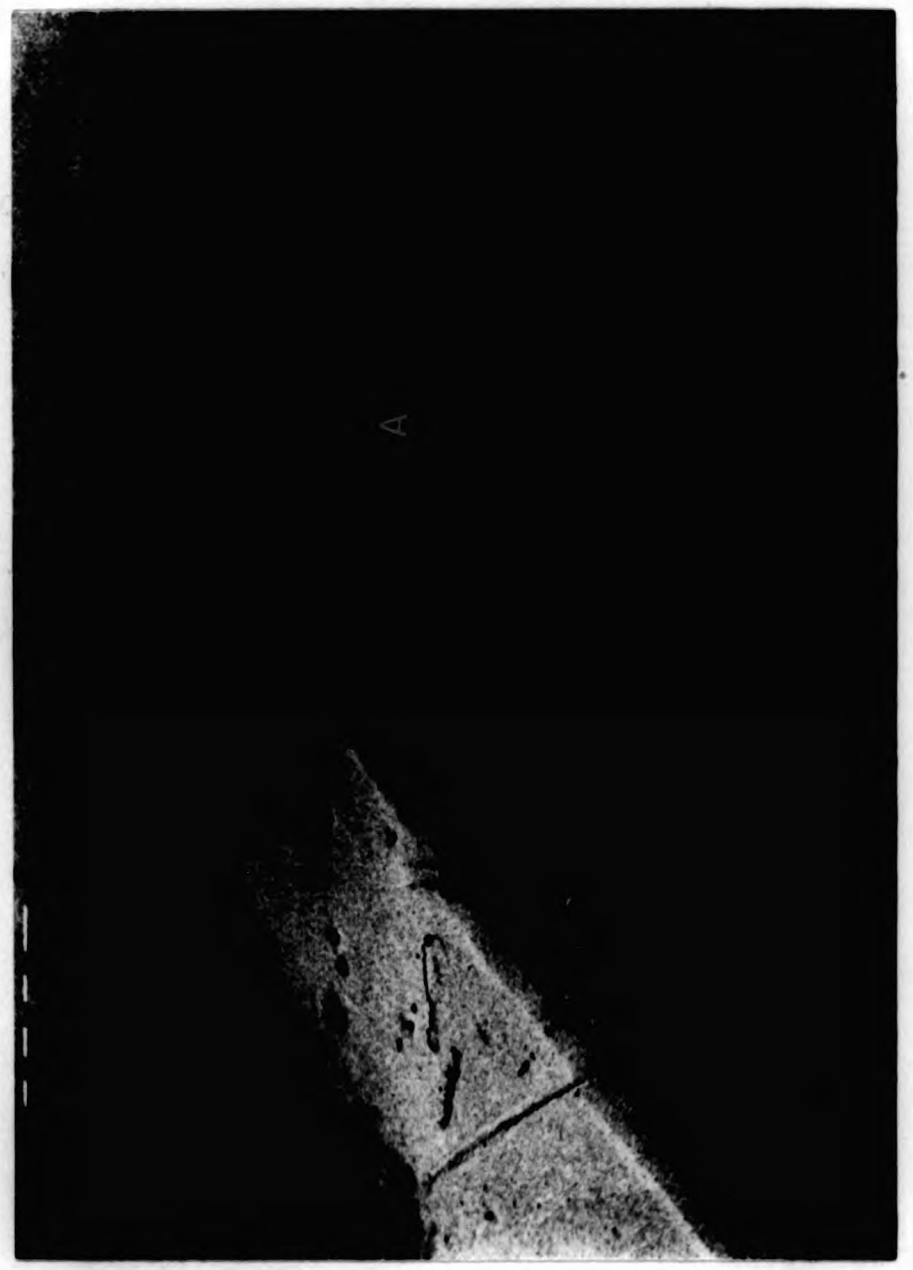


Fig. 117 a&b SEM, group 4, severe cuticular trauma, 6 days: the lesion consists of gully- and ridge-like cuticular malformations (X50, X100).

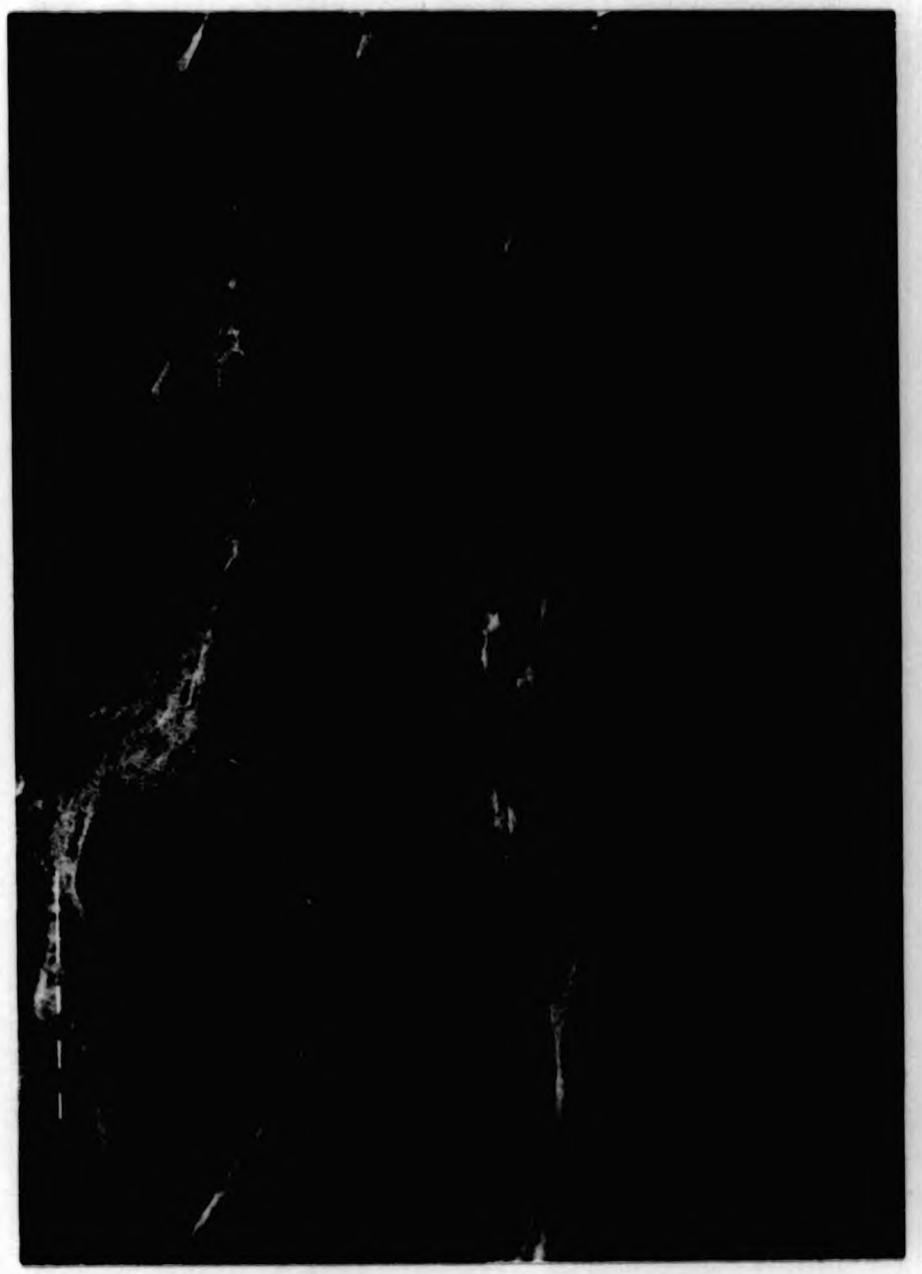
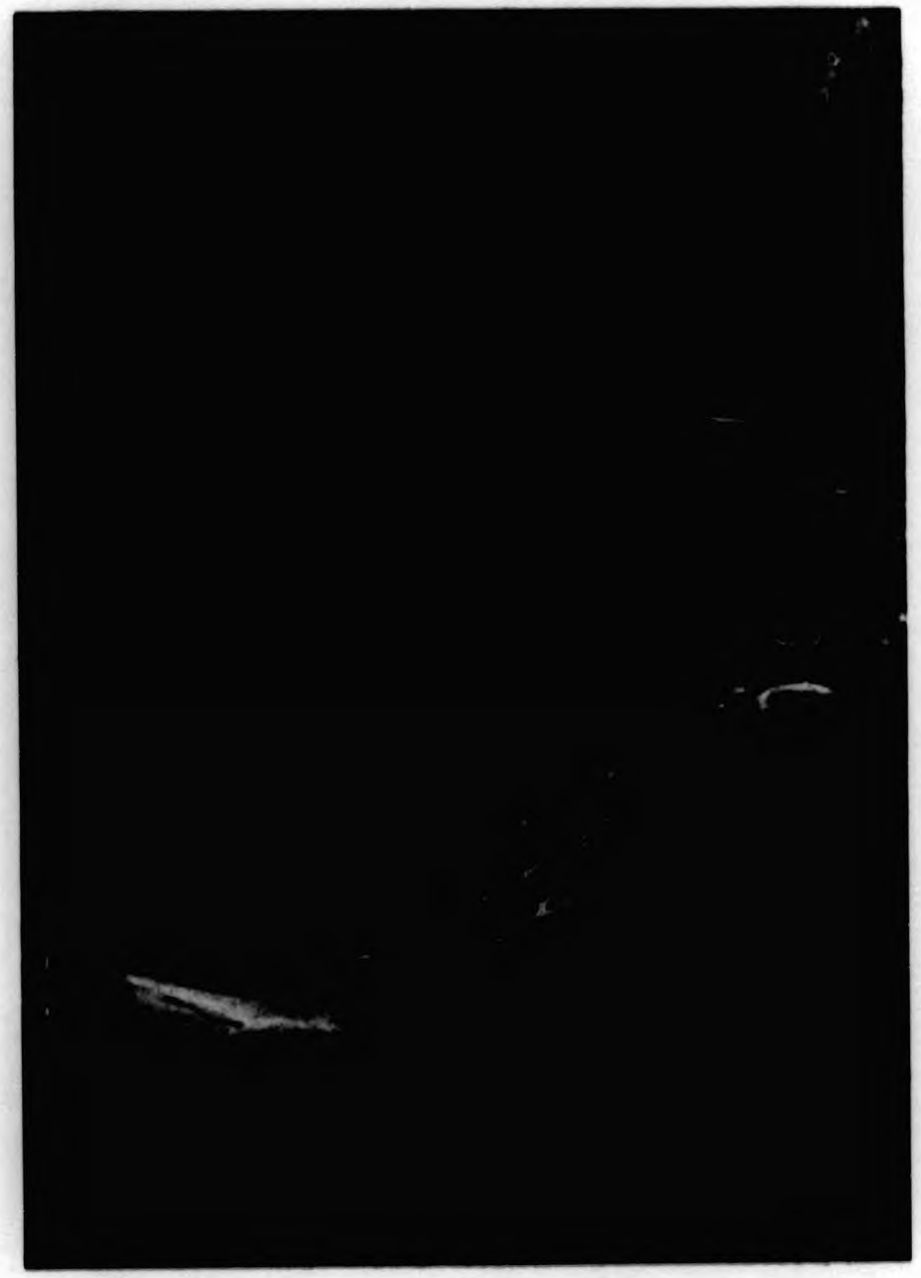


Fig. 118 a&b SEM, group 4, severe cuticular trauma, 6 days: marked cuticular irregularities are apparent (X300, X900).



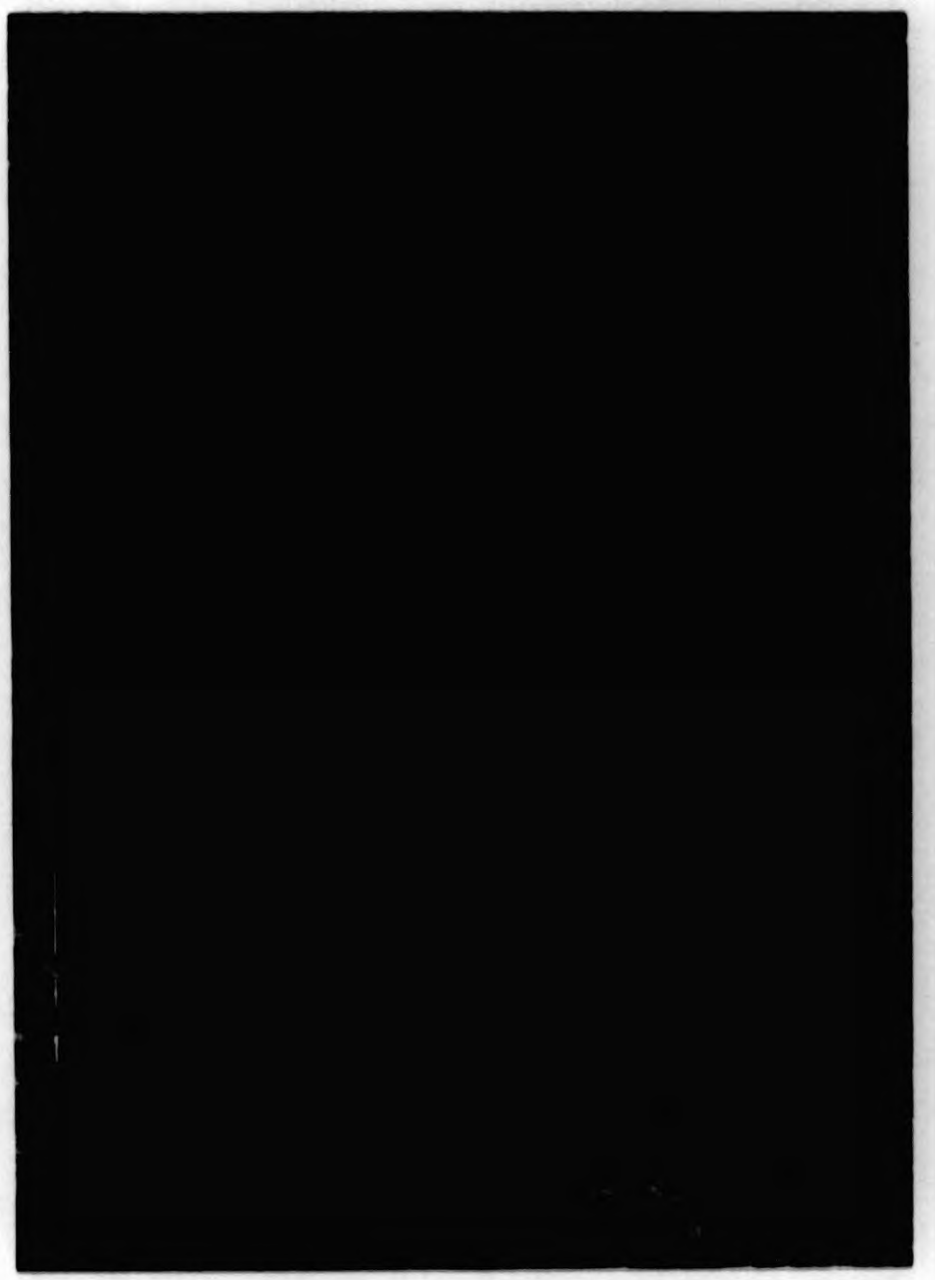
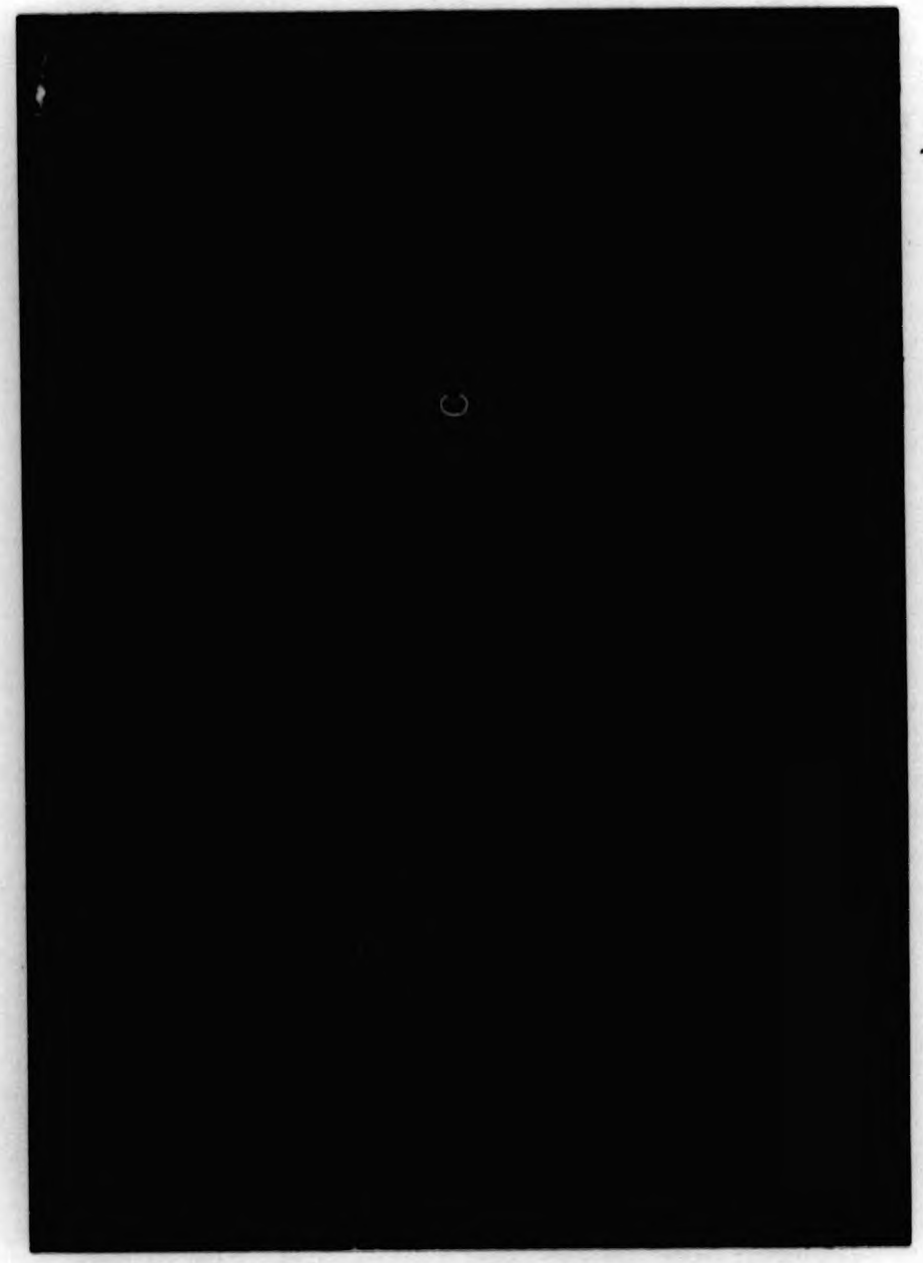


Fig. 119 a&b SEM, group 4, severe cuticular trauma, 28 days: an area of undamaged cuticle exhibits a regular surface resembling interlocking scales or shingles (i.e. roof tiles); focal bacilli (B) are present (X600, X1700).

Fig. 120 SEM, group 3, moderate cuticular trauma, 21 days: the surface of the damaged cuticle showing diffuse irregularities, although less remarkable than previously (X900).

Fig. 121 SEM, group 3, moderate cuticular trauma, 21 days: the surface of the damaged cuticle showing diffuse irregularities, although less remarkable than previously (X900).



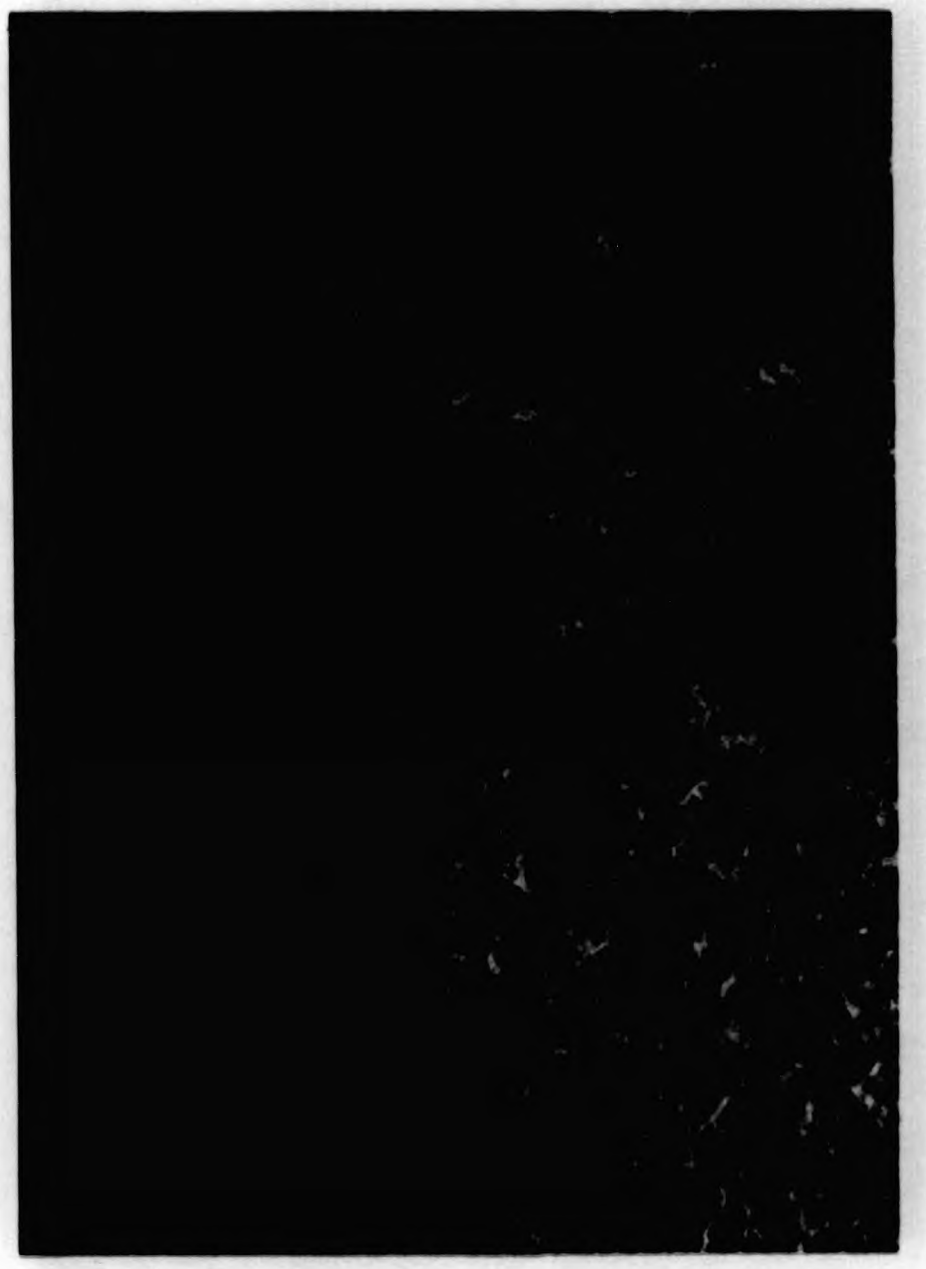


Fig. 122a SEM, group 4, severe cuticular trauma, 14 days: the extensive area of cuticular ulceration consists of numerous cervices and large ridges (X30).

Fig. 122 b&c SEM, group 4, severe cuticular trauma, 14 days: a variety of irregularities is present in the injured cuticle (X600, X600).

Fig. 123a SEM, group 4, severe cuticular trauma, 21 days: prominent area of damaged cuticle with ridges, pits and fissures demonstrating some organization compared with the earlier lesion (X40).

Fig. 123 b&c SEM, group 4, severe cuticular trauma, 21 days: conspicuous cuticular defects are evident (X300, X1250).

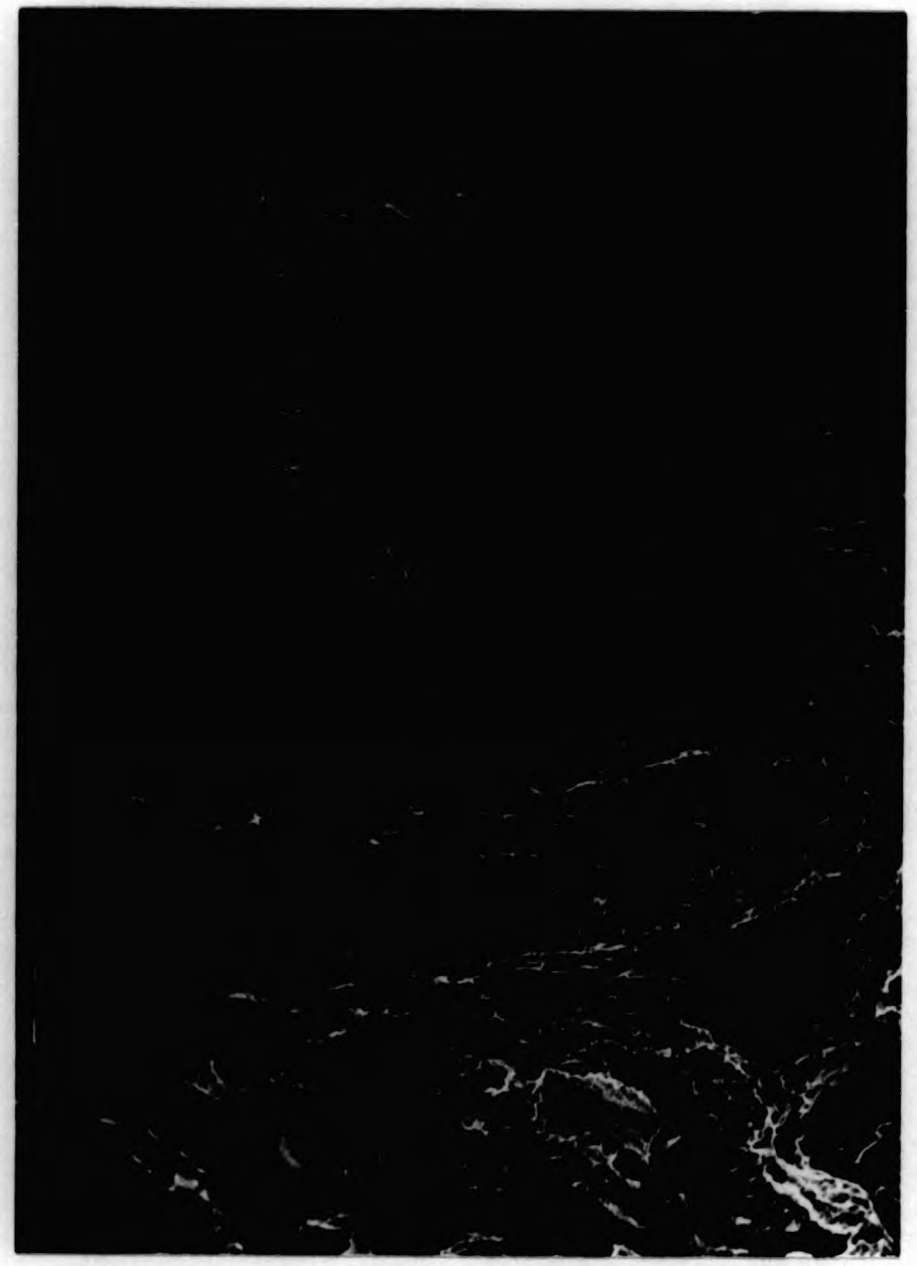


Fig. 124 a&b SEM, group 4, severe cuticular trauma, 28 days: although notable cuticular abnormalities are apparent, the lesion (A) is noticeably more organized than previously (X30, X85).

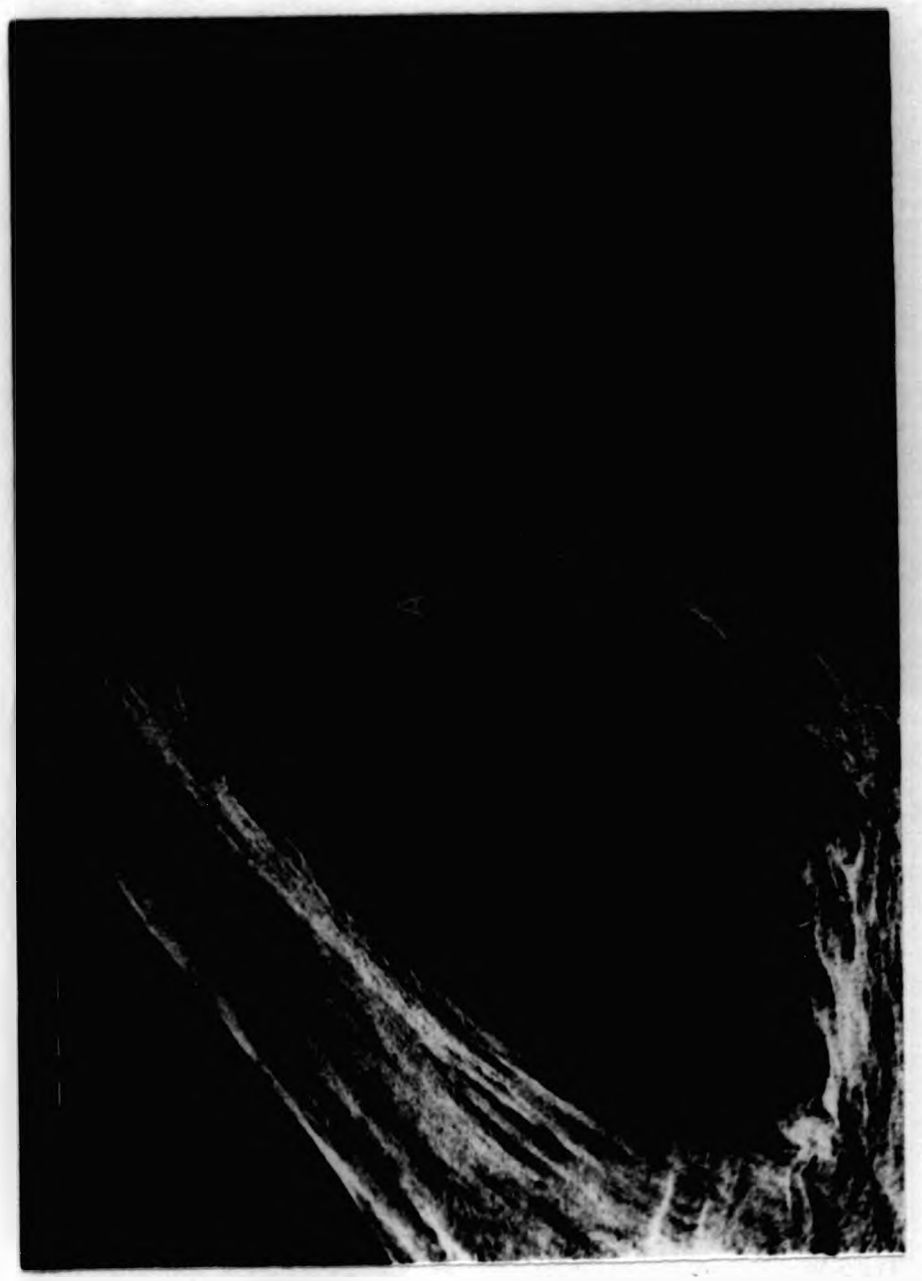


Fig. 124c SEM, group 4, severe cuticular trauma, 28 days: raised nodule-like formation in damaged area of cuticle (X3000).

CHAPTER IV: Discussion

General Considerations on the Tissue Response in Natural Disease and Resulting from Experimentally Induced Insults

The investigations demonstrated that *Macrobrachium* consistently mounts a similar generalized tissue defence response in both infectious and noninfectious natural disease conditions (viz. *Aeromonas hydrophila*, *Citrobacter freundii*, trematode metacercaria, systemic parasite infections and larval bacterial necrosis; and, IMN and hard water shell disease, respectively) and resulting from a variety of experimentally induced tissue insults (including injection of foreign biological materials - viz. live and/or heat-killed Gram-negative bacteria, *Vibrio* and *Aeromonas* spp., and the acid fast bacterium, *Mycobacterium tuberculosis* - as a live attenuated vaccine, BCG, and heat killed (also with paraffin oil and mannide monoalate) as CFA; and injection of a foreign non-biological material - viz. alcian blue pigment; as well as grafting and traumatization).

The tissue responses to injury and injected foreign material were found to be both rapid in onset and enduring, consisting of haemocytic infiltration, aggregation, coagulation, phagocytosis, encapsulation, nodule formation, melanization and fibroplasia, not only localized at or in the vicinity of the site of immediate damage or injection but also in certain target organs, especially the gills, the hepatopancreas and the heart. Tissue defence reactions were additionally observed in the antennal gland, which has also been reported by

Johnson (1987) and in other tissues and organs. Considering all tissues, though, the gills represented the site of the most prominent haemocytic tissue responses, as also found by other researchers (Smith & Ratcliffe 1976). As the gills provide the most permeable part of the crustacean integument, it is considered that microbial invasion and subsequent confinement before infection can become established elsewhere in the body, is most likely to occur here (Smith & Ratcliffe 1976). This emphasizes the importance that maintaining healthy gills could have in preventing subsequent generalized infections in crustaceans. In addition to the gill-haemolymph route of infection, the oral-hepatopancreas route may also be important in shrimps and prawns as has been observed in *Penaeus monodon* (Nash unpublished observations 1989-90).

The findings concerning the nature of the basic tissue response in *Macrobrachium* are similar to those determined experimentally in other crustaceans, such as the lobster (Cornick & Stewart 1968), the crayfish (McKay & Jenkin 1970b), the penaeid shrimp (Fontaine & Lightner 1974, 1975) and in crabs (Johnson 1976a, 1980; Smith & Ratcliffe 1976, 1980a,b). Comparable tissue defence responses in natural disease as were found in these investigations have also been described in other reports of disease conditions in *Macrobrachium* (Brock 1988) and in other crustaceans, including penaeids (Lightner 1988) and crabs (Johnson 1976a). Comparing *Macrobrachium* prawns with penaeid shrimp, though, one important difference is

the presence of the Oka or lymphoid organ in penaeids (Oka 1969) which is absent in *Macrobrachium*. In both natural and experimental infections with *Vibrio* spp. the Oka organ has been found to be one of the earliest sites for a prominent haemocytic tissue inflammatory response as well as Oka organ hyperplasia (Nash unpublished reports 1989-90).

The generalized tissue reaction involving haemocytes in *Macrobrachium* is, indeed, similar to that observed in the other invertebrates, such as insects (Salt cited by Lightner 1974) and molluscs (Sparks 1985).

The histopathological results from the experimentally induced tissue insults assist in providing an understanding of the nature, degree, chronicity, sequence and sites of lesions due to natural injury or infection. For example, the presence of fibroplasia in a natural lesion associated with *Aeromonas hydrophila* would indicate that the tissue damage due to infection was of approximately three days duration. Thus, similar information can be obtained considering many aspects of the other haemocytic tissue reactions. Comparing the findings in experimentally induced shell damage with natural shell disease it would also be possible to make a better estimation of the initial severity of trauma resulting in a certain degree of shell injury and the chronicity of the lesion.

Following from this, observations of the haemocytic tissue reactions (ie. infiltration,

aggregation, encapsulation, nodule formation and melanization) in the gills, hepatopancreas or whole larval prawns, as well as the haemolymph, both histologically and even more readily in fresh preparations (as the encapsulations described in the case of larval bacterial necrosis in Chapter II, aided by phase contrast microscopy or special staining methods), have been shown in the most recent experience of the author to be extremely useful for monitoring the health or identifying the presence of disease in cultured shrimps or prawns. If an abnormal condition such as an infection, reflected by the haemocytic response and/or an infectious agent, can be detected before acute clinical morbidities and mortalities occur, it will be possible to more rapidly undertake measures to alleviate the situations, such as improvement of suboptimal environmental conditions (eg. by water exchange, management procedures) or carry out the requisite treatment with better possibility of a favourable outcome.

The above results are now used in conjunction with microbiology, clinical history, pond, feeding and other management data to obtain the full picture of the disease problem so that valid recommendations for disease treatment and prevention can be made.

At this point it may be expedient to briefly review the haemocytic tissue reactions that have been described by the author and in the literature in the

natural diseases of *Macrobrachium* under culture conditions and, when appropriate, make some comparisons with those observed resulting from experimentally induced results.

Shell disease is characterized by "open" melanized multilaminar haemocytic encapsulation of the damaged cuticle and subcuticular tissues (reported by the author in *Citrobacter* & *Aeromonas* infections and by Brock 1988). In the experimental study of cuticular traumatization (as well as grafting) "open" melanized haemocytic multilaminations were commonly observed. In natural disease, haemocytic infiltration, aggregation, phagocytosis and/or encapsulations and nodules may by widespread locally and/or systemic if secondary microbial infection occurs (Brock 1988). Similar widespread changes have been reported in bacterial necrosis (Aquacop 1977a) and by the author in larval bacterial necrosis.

Citrobacter and *Aeromonas* infections were described by the author as eliciting a tissue response of haemocytic infiltration, aggregation, encapsulation, nodule formation and/or phagocytosis especially in the gills, heart, hepatopancreas and muscle as well as in other tissues similar to results in experimental injection of the Gram-negative bacteria.

Systemic parasitic infection in *M. lanchesteri* was associated with haemocytic infiltration, aggregation and melanization, as described in these investigations.

In IMN, haemocytic infiltration, aggregation, encapsulation and myophagia may be present as were reported here (and in Nash *et al*. 1987).

Nodular encapsulating, haemocytic lesions are characteristic of mycobacterial infection in *M. rosenbergii* (Brock *et al*. 1986). In the experimental studies using preparations containing forms of *Mycobacterium* (i.e. BCG and CFA) the participation of phagocytes in the formation of capsules was also an important feature in addition to encapsulation, whilst in the natural infection, the encapsulation of bacteria alone appeared to dominate (Brock *et al*. 1986).

Hard water shell disease was associated with body and gill cuticular haemocytic infiltration, aggregation, encapsulation and melanization as was reported here. In black nodule disease, haemocytic infiltration, aggregation, melanization and nodules were found in the cuticular epidermis (Delves-Broughton & Poupard 1976).

The midgut mucosa, dorsal caecum and hindgut gland displayed haemocytic infiltration in haemocytic enteritis (Brock 1983).

Melanized haemocytic nodules in the muscle, ovary, heart and antennal gland may be present in white prawn disease (Brock 1983).

Haemocytic infiltration was observed in white syndrome (Delves-Broughton & Poupard 1976).

In trematode metacercarial infection, haemocytic infiltration and encapsulation and fibroplasia were reported by the author here and in Nash (1989).

Precipitating chemicals and nitrogenous wastes, heavy metals and other biocides most commonly cause haemocytic infiltration, aggregation and/or encapsulation and melanization of the gills and branchiostegites (Doughtie & Rao 1983; Ghate 1984).

Haemocytes

In the histological studies, mixed populations of representative cells of all the three main haemocyte types (viz. hyalocytes, semi-granulocytes and granulocytes) were considered to have participated in the inflammatory reactions constituting the tissue defence response of *Macrobrachium rosenbergii*. Hyalocytes and semi-granulocytes (containing small and/or few granules) appeared to predominate. Typical granulocytes with prominent eosinophilic granules discernible histologically were not recognized. The cells which were possibly granulocytes usually had smaller weakly eosinophilic granules rather than large strongly eosinophilic ones. In addition, cells with very eosinophilic cytoplasm often appeared to be agranular. Thus, in many cases it was not possible to definitively categorize the haemocytes into specific types. Variability in staining characteristics between individual specimens may have also complicated the matter.

Based on the TEM investigations carried out here it was considered that the granulocytes (with larger and/or more numerous granules) and semi-granulocytes (with smaller and/or fewer granules) dominated in the tissue defence response; true agranular haemocytes were rarely observed. Concerning the nature of the granules present, the findings here concurred with those of Martin and Graves (1985) who described two granule types, one with granules of punctate areas of increased electron density and another with concentric bands of electron-dense material alternating with bands of lower electron density (Fig. 35).

As mentioned previously, based on ultrastructural studies of circulating haemocytes, Tsing *et al.* (1989) found only granular haemocytes (viz. "small granule haemocytes", "large granule haemocytes" and large granule haemocytes" with "large and dense inclusions, very irregular in shape") and an absence of true agranular haemocytes in *Macrobrachium rosenbergii* and recommended that these two terms (i.e. hyaline or agranular haemocytes) describing haemocyte morphology should be reserved for use "only in light microscopical studies where cells of this type appear little contrasted and seem devoid of granular inclusions". Considering both the frequent observation of apparently agranular cells in the histological studies and the results of the TEM study in which agranular cells were, at most, rare, it seems reasonable to follow the above advice of Tsing *et al.* (1989), or perhaps use the terms "apparent agranular

haemocyte" or "apparent hyalocyte" for descriptions in histological studies.

Blewett & Eble (1979) also found no true "hyaline" or agranular cells in *Macrobrachium* although their "Cytotype I" contained relatively few granules. In the ridgeback prawn, *Sicyonia ingentis*, TEM revealed that cells classified as agranular by light microscopy frequently contained one or two small granules making them accurately classifiable only by using TEM (Martin *et al.* 1987). Cells with one or two granules were similarly observed in these TEM investigations. As previously noted, though, the presence of hyaline or agranular cells has been widely described in crustacea (Bauchau & de Brouwer 1972; Bauchau & Mengeot 1978; Bauchau 1981).

Additional evidence that semi-granulocytes and/or granulocytes probably played a large role in the reactions is that the end product of the proPO cascade, melanin (Smith & Söderhäll 1986), was observed in varying degrees in all the studies.

Frequently observed in the haemocytic reactions in these studies were haemocytic changes described as "nuclear karyorrhexis", karyorrhectic nuclei", etc.. These terms denoted basophilic nuclear fragments aggregated or scattered in varying degrees in or from apparently necrotic, degenerating or otherwise altered haemocytes. Considering both their morphology and strategic location at site of self and foreign body interfaces, these cells may have often represented the

exocytotic or degranulating form of semi-granulocytes which also become lytic when degranulation occurs (Smith & Söderhäll 1986). Also described in the histological studies were abundant "effete" haemocytes at the sites of injury or inflammation. These are considered to be another and/or later form of exocytotic haemocyte. Blewett & Eble (1979) state and illustrate that degranulated cells exhibit nuclei filled with fluid and chromatin fused into several (viz. 3 - 4) large masses, with much cytoplasmic vacuolation. Bauchau (1981) described degranulated granulocytes as having "degenerating nuclei" and depicts them as cells with a thin rim of cytoplasm around a degenerating nucleus resembling the effete haemocytes observed in the experiments. Thus the degranulated cells do share morphological similarities with the haemocytes undergoing "nuclear karyorrhexis" in these studies.

In the final stage of the "explosive corpuscle", which is probably the degranulating haemocyte, only the nucleus and a thin rim of cytoplasm remain (Blewett & Eble 1979). Tsing *et al.* (1989) have also indicated that they found another cell type, the "haemocytes after natural lysis", rounded cells with their cytoplasm only present as a thin rim all around an empty nucleus, only at the level of injury or at blood removal. Both these descriptions are highly suggestive of the "effete" haemocytes also observed in abundance associated with the inflammatory reactions in these studies.

The TEM study identified a probable degenerating haemocyte (Fig. 36) very similar to that described by Hearing and Vernick (1967), with the features of granular disintegration and bleb formation. The particular haemocytic tissue reactions will now be examined in turn, in light of the experimental findings.

Haemocyte Infiltration and Aggregation

Similar to the penaeid shrimp (Fontaine & Lightner 1974), the first observed event of the internal defence system in response to injury, injection of a variety of foreign materials or grafting in *Macrobrachium rosenbergii* was haemocytic infiltration accompanied or soon followed by haemocytic aggregation and/or phagocytosis both locally in the site of damage (which was the abdominal muscle in these experiments) and in specific target organs distant to the site (Table 1). Results were similar to those of other researchers with heat-killed bacteria (Smith & Ratcliffe 1980b) and live bacteria (White cited by Smith & Ratcliffe 1980b). As mentioned above, the gills, hepatopancreas and heart were the most frequently observed sites of increased haemocytic activity; the antennal gland, connective tissues, neurilemma and haemocoel were also common sites for aggregation and other haemocytic reactions. Results for earliest observed tissue/organ haemocytic response localization in the experiments are summarized in Table 1. It should also be noted that the haemocytic responses including aggregation persisted up to 21 to 42 days post-

treatment (depending upon the experiment and the latest sampling time) and probably would have been observed to persist even longer had time permitted longer experiments (Table 1).

Haemocytic aggregation or clumping is considered to be a significant component of the crustacean cellular defence system, probably initiated by the presence of bacteria (Smith & Ratcliffe 1976) and other foreign materials, and as is phagocytosis, mediated by the presence of readily lysable cells containing refractile granules (Smith & Ratcliffe 1976). The response is extremely effective in quickly immobilizing bacteria and preventing the spread of infection (Smith & Ratcliffe 1980b) and also facilitates the degeneration and ultimate disposal of the material from the body (Smith & Ratcliffe 1980a). It is considered that clumping as an adjunct to phagocytosis in the rapid removal of test particles from the circulation, may occur by the haemocytes clumping around the foreign substance and then become deposited in specific body organs (Smith & Ratcliffe 1980a). The clumping of haemocytes around injected materials is in itself considered to indicate the participation of humoral factors (Fontaine & Lightner 1974). A cell adhesive protein has been reported to be released along with opsonins upon degranulation of the semi-granular and granular cells (Söderhäll 1986). This results in the haemocytes becoming sticky and adherent (Smith & Ratcliffe 1980b). (See also "Note" page 27).

Phagocytosis

It has been reported that crustacean haemocytes are capable of recognizing and engulfing a wide variety of biotic and abiotic particles (Smith & Söderhäll 1986) and the results of this study support this finding. As mentioned previously, it is considered that "phagocytosis, augmented by humoral factors of low specificity, constitutes the basic mechanism of internal protection in the large Crustacea" (Sindermann 1971, 1979). The results of these experiments indicate that phagocytosis, occurring soon after or with infiltration and aggregation of haemocytes, at least constitutes one of the most important defence mechanisms, but in conjunction with encapsulation and nodule formation, which were often observed simultaneously and sometimes independently of and/or prior to phagocytosis, as for example, with the BCG experiment, and, with tissue variability, in the CFA and the *Vibrio* and *Aeromonas* injections (see Table 1). Phagocytosis appeared to be the first and most common reaction in many organs in the alcian blue experiment (Table 1). Phagocytosis was also observed prior to encapsulation in the hepatopancreas in the BCG, CFA, *Aeromonas* and *Vibrio* experiments; and in the heart in the BCG, CFA and *Vibrio* experiments (Table 1). One of the most commonly observed reactions in the experiments was primary phagocytosis of foreign material, then melanization of the cells and/or contents followed by encapsulation with successive layers of haemocytes. It has also been found in *Carcinus maenus* that although

phagocytosis of injected bacteria or yeast cells occurs, encapsulation and nodule formation are most common reactions (Smith & Ratcliffe 1980a,b; Bauchau & Mars cited by Bauchau 1981). As noted previously, Brock *et al.* (1986) also described that in *Mycobacterium* infections in *M. rosenbergii* encapsulation of bacteria, rather than phagocytosis, was the primary haemocytic tissue response. Encapsulation and nodule formation were common in all the experiments, but especially seemed to predominate in the *Vibrio* and *Aeromonas* experiments.

It has been reported that there is initial phagocytosis of foreign material followed by accumulation of the haemocytes in capillary networks of the haemocoel (Smith & Ratcliffe 1980a).

Apparent hyaline cells were considered to be the primary ones involved in phagocytosis in *M. rosenbergii* based on the histological studies and this is reputed to be one of their basic properties (Smith & Söderhäll 1986). Semi-granular cells, which are phagocytic in some species only, apparently lose this ability upon degranulation (Smith & Söderhäll cited by Smith & Söderhäll 1986). In semi-thin sections in the TEM study, though, phagosomes were observed in haemocytes considered to be semi-granulocytes (Fig. 32). In addition, as melanization of phagocytic haemocytes and their contents was commonly observed in the injection experiments, it is regarded that in *M. rosenbergii* semi-granulocytes also play an important role in phagocytosis. Granular cells

are considered to be non-phagocytic (Smith & Söderhäll 1986) or at least less active in phagocytosis (Bauchau 1981).

It is thought that the granular cells and, to a lesser extent, the semi-granular cells of crustaceans upon degranulation contribute the signals comprising opsonic factors which stimulate greater phagocytic activity of the hyaline cells (Söderhäll & Smith 1986a).

Johnson (1987) described three types of phagocytic cells in decapod crustaceans: phagocytic reserve cells, weakly phagocytic fixed cells in the heart of penaeid and palaemonid shrimp, fixed phagocytes in the hepatopancreatic haemal sinuses (not in penaeids) and haemocytes, which are the only free cells responsible for clearance of foreign particles. The latter are said to phagocytose particles wherever they meet them, but are especially likely to do so where haemocyte:particle contact is most frequent, as in narrow haemal spaces, or in spaces with reduced hydrostatic pressure where haemolymph flow is slowed. This includes the haemal sinuses of the gills, antennal gland and possibly also the hepatopancreas and heart (Johnson 1987). The branchial podocytes are considered to be pinocytotic rather than phagocytic (Johnson 1987). In these experiments phagocytic activity was most prominent in the organs mentioned above and it is considered that the free haemocytes played a significant role in the engulfment of foreign material. Prior to the injection of foreign

substances, free haemocytes were frequently observed in the haemal spaces of the gills, hepatopancreas, heart and antennal gland, but their presence and activity increased markedly following injection, suggesting migration and localiżation in these sites possibly due to reasons mentioned above. Fixed phagocytes are apparently derived from circulating hyaline haemocytes which subsequently settle on the exterior of the hepatopancreatic arterioles (Johnson 1987). Thus, the prominently phagocytic haemocytes noted in the hepatopancreas in the experiments may have represented this group of cells, in addition to haemocytes. Concurring with the descriptions of Johnson (1987) was the hypertrophy and presence of large cytoplasmic vacuoles (also observed by Smith & Ratcliffe (1978)) with acidic contents in these phagocytes as they became activated. These changes are due to the acquisition of material from outside the cell. Bauchau (1981) reports that once contact with the foreign material has been established, the haemocyte membrane may invaginate, elongated pseudopodia or large cytoplasmic veils are produced to surround particles or droplets of haemolymph and give rise to phagosomes. In the experiments involving histological studies including alcian blue, BCG, CFA, *Aeromonas* and *Vibrio* injection) large vacuolated cells resembling these, apparently containing phagocytosed antigenic material and/or the eosinophilic haemolymph were seen especially in the hepatopancreas but also in the heart and other organs and tissues. It was considered unlikely in these cases that

the phagocytes were previously exocytotic semi-granulocytes or granulocytes (Stagner 1979) as concurrent cytolysis (Smith & Söderhäll 1986) was not present. Smith and Ratcliffe (1978) also reported that degranulation of haemocytes during phagocytosis was not apparent. Fixed phagocytes in the hepatopancreas may also sequester large amounts of particulate matter, not only through phagocytosis but by trapping and retaining vast numbers of particles within a net- or sieve-like layer of granular material which surrounds the free surface of the fixed phagocyte (Johnson 1987). It was also found that the phagocytic reserve cells of the heart which may also have participated did appear to be more weakly phagocytic (Johnson 1987) than haemocytes in the gills and hepatopancreas. Generally, but with some variability, phagocytosis was less pronounced and/or occurred later in the heart than in the gills or hepatopancreas.

The intracardiac, phagocytic fixed cells of the shrimp heart are provisionally considered to be phagocytic reserve cells (Johnson 1987). "Fixed phagocytes" may also represent sedentary haemocytes and/or representatives of a population of weakly phagocytic fixed cells known to occur in the heart of penaeid and palaemonid shrimp (Johnson 1987). That fixed phagocytes may have been active in the heart may be supported by the less remarkable phagocytosis in this organ as mentioned above, as the fixed phagocytes must wait until the circulation brings foreign particles into their vicinity, whilst on the other hand, the greater

number of extremely mobile haemocytes can respond immediately wherever foreign material is introduced into the haemocoel (Johnson 1987). It is considered that the free haemocytes were most active in phagocytosis in these experiments and these are considered to provide an adequate first line of defence against microbial invasion (Smith & Ratcliffe 1978).

Phagocytic reserve cells have been found to be minimally involved in the clearance of injected carmine and carbon, but not in the clearance of naturally occurring particles such as viruses or bacteria (Johnson 1987). This may again indicate that a form of haemocyte was actively phagocytic in the *Aeromonas* and *Vibrio* injection experiments.

Haemocytic phagocytosis of necrotic muscle tissue or myophagia which was frequently observed in the experiments and in the investigation of IMN (also in Nash *et al.* 1987) has also been reported by Hoarau (1979).

Encapsulation and Nodule Formation

Encapsulations and the larger nodules of haemocytes (generally sequestering larger foreign bodies and parasites (Bauchau 1981), are, as mentioned previously, considered to be the main adjunct to phagocytosis (which serves to dispose of smaller foreign particles (Bauchau 1981) in crustaceans and other arthropods (Smith & Söderhäll 1986) and this was with variability also found to be the case in these

experiments. Although encapsulation apparently generally predominated in the *Vibrio* and *Aeromonas* experiments, phagocytosis was one of the earliest reactions in the hepatopancreas for both experiments and also in the heart in the *Vibrio* experiment(Table 1). Encapsulation and nodule formation reactions are progressive structural developments closely following aggregation (and may occur concomitant with, after or preferentially to phagocytosis (Smith & Ratcliffe 1980a,b; Brock *et al*. 1986) as mentioned above in bacterial infections) in which the rather disorganized arrangement of haemocytes becomes more organized (note Figs. 73a&b, 74, 75 and 76a&b). The reaction was found to be generally similar whatever the nature of the foreign material, injected or inserted (such as in grafting) or the insult (such as in wounding). The intensity of the reaction was found to vary, though, with the degree of trauma or damage. In the process there is initial aggregation of the haemocytes and around the foreign material or sealing off the damaged area (as in wound healing), with the subsequent formation of concentric or multilaminar haemocytic capsules or sheets (eg. the so-called "open encapsulations" of Fontaine (1971) and Fontaine and Dyjak (1973)) and the gradual flattening of cells to a fusiform shape in the inner layers. Similar changes were observed in the grafting and cuticular trauma experiments. In a marked or chronic reaction there is the development of larger granuloma-like nodules. As the modified macrophage or epithelioid cell is not strictly present in prawns,

the term "granuloma" is better reserved for the chronic inflammatory response in vertebrates. These changes are accompanied in most crustaceans, including *Macrobrachium* and penaeids, by the deposition of melanin either on the surface of the foreign material or within the haemocyte matrix (Bauchau 1981; Smith & Söderhäll 1986).

It was observed that free foreign material (possibly too large for phagocytosis), haemocytes containing phagocytosed material and/or necrotic cellular debris including haemocytes, all often melanized, are encapsulated both at the site of injection or insertion or trauma and in tissues and organs of haemocyte localization, persisting up to 42 days after treatment (Table 1). Semi-granular cells, exclusively, or all cell types acting in concert, are reported to form capsules and nodules (Smith & Söderhäll 1986). Semi-granular cells were also considered to have participated in encapsulations seen in semi-thin sections in the TEM study of BCG injection (Fig. 32). Key processes during their development are attachment and spreading of haemocytes on the foreign material or cellular complex (Smith & Söderhäll 1986). A cell adhesive protein has been found to be generated (Johansson & Söderhall 1985) and this factor formed when the proPO system is activated as a response to, for example, microbial infection, participates in cell adhesion and spreading during encapsulation of foreign objects and nodule formation in crustacean host defence (Johansson & Söderhäll 1986).

The encapsulation of necrotic phagocytes containing carmine has been noted by Fontaine & Lightner (1975) who also reported that none of the cells actively engaged in the process of encapsulating the necrotic phagocytes were observed to contain carmine particles. Encapsulation of necrotic alcian blue-containing phagocytes was also observed in the alcian blue experiments, but haemocytes forming the capsules were occasionally seen to contain alcian blue pigment. Necrotic cellular debris was also seen within heterophagosomes of encapsulation haemocytes in the *Aeromonas* experiment. Additionally, there were some cases in the CFA experiment of individual or aggregations of phagocytes apparently fused with an enlarging encapsulation or nodule leading to multilocular forms. In the TEM study of BCG injection, phagosomes may have been present in semi-granulocytes involved in encapsulation observed in semi-thin sections (Fig. 32). Generally, though, based on the histological studies, non-phagocytic cells appeared to predominantly form the multiple layers of encapsulations and nodules.

Bauchau (1981) described an ultrastructural study of capsules formed in *C. maenas* by implanting nylon threads or pieces of human hair into the hemocoel, the changes in which parallel those observed histologically in the cuticle:muscle insertion carried out in the grafting experiments. As the shell, a foreign material albeit host tissue, was introduced into an unnatural site in the body, it can be understood why it was recognized

as non-self and a typical melanized encapsulation (also related to physical presence of the shell and injury to the muscle tissue) inflammatory response occurred. Common features of the haemocytic tissue response observed in these studies included: early aggregation, later formation of concentric layers of cells, inner layers of flattened cells which adhere to the implant surface, necrosis and melanization of the inner layer ("innermost flattened cells become necrotic and small beads of melanin are deposited principally along the cell membranes and around nuclei"). Bauchau (1981) also observed all three basic haemocyte types in the middle layers of the capsule present as flattened, interdigitation cells (described in these experiments as being held together by cytoplasmic strands) sometimes held together by a dense collagen-like substance. Collagen formation was noted histologically in this study after six hours (Table 1). Hoarau (1979) described encapsulation haemocytes as arranged in a "stratified pseudo-epithelium" by the formation of various types of junctions including membrane infoldings, accumulation of dense material in the intercellular spaces, zonula occludens, macula adherens and septate junctions. No necrotic cells were observed after five hours in the middle layer (Bauchau 1981) but in these experiments multifocal necrotic haemocytes were present in this part of the capsule after 26 hours. Bauchau (1981) also described spherical haemocytes, as were in this study, located in the outer region of the capsule. Fontaine & Lightner (1975) made similar observations concerning encapsulation in penaeids.

Variably increased vacuolation was observed in haemocytes participating in the encapsulation reaction. Hoarau (1979) reports an increase in the number of autophagic vacuoles, myelin spirals and multivesicular bodies in these cells.

In the later stages of encapsulation the inner layers are composed of a persistent wide band of multilaminated effete melanized haemocytes. Fontaine & Lightner (1975) describe these cells as lysed and eventually not recognizable and as forming thick brown leathery capsules which are not resorbed. This was also a prominent feature in the cuticular trauma and grafting experiments.

In association with the haemocytic reaction, new cuticle formation reported to be secreted by regenerating epidermal cells (Fontaine & Lightner 1973; Bauchau 1981) was also observed in the wounding and grafting experiments. The other haemocytic tissue reactions in these experiments were also similar to those observed in the process of wound repair in penaeid shrimp (Fontaine & Lightner 1973). In addition, as in the wounding experiment, the defensive processes were apparently active not only in the immediate wounded area but also in the adjacent intact cuticular area (Nyhlén & Unestam 1980).

As found by Smith & Ratcliffe (1980b) haemocytic responses were similar for studies utilizing heat-killed or live bacteria. The systemic injection route elicited

a more pronounced reaction in the heart in the *Aeromonas* experiment.

Fibroplasia

Fibroplasia, fibrosis or fibroblast proliferation with the elaboration of collagen was observed to play a significant role in the chronic inflammatory response in all the experiments as can be seen at a glance in Table 1, commencing variably as early as six hours or two to seven days and persisting up to 28 days in the trauma and grafting experiments (Table 1). Similar results concerning fibrosis in wound repair in crustaceans have been confirmed by Fontaine & Lightner (1975) and Bauchau (1981). The characteristics of the dense fibrous tissue as being well organized, stable and not resorbed reported by Fontaine & Dyjak (1973) were also noted in the trauma and grafting experiments and in the muscle injection sites of the other experiments. It is also said to remain as a "permanent scar" (Fontaine & Dyjak 1973; Fontaine & Lightner 1975).

The gradual transformation of typical round haemocytes to fusiform fibroblast-like cells was noted particularly in the grafting experiment (eg. at 2 days after grafting). The elaboration of collagen by these cells confirmed by Masson trichrome staining was also observed. The production of collagen by haemocytes has also been reported by Hubert *et al.* (1976), in this case by granulocytes.

Coagulation

Haemolymph coagulation or clotting was observed to occur particularly in response to traumatization in an attempt to seal off the damaged cuticular ulcer. The prior or concomitant infiltration and aggregation of haemocytes was observed and has also been described by Bauchau (1981) as a "two-step reaction" of cell aggregation and plasma gelation with agglutination of adjacent haemocytes, especially hyaline and semi-granulocytes, producing an extensive cellular network by means of numerous elongated pseudopodia adhering to whatever cell they come in contact with. Gelation of plasma occurs simultaneously with the release of material contained in large vacuoles within these cells, the granules dissolving slowly, either within vacuoles or *in situ* and their contents are expelled into the plasma causing a more extensive coagulation. The cytoplasm of the haemocytes is finally reduced to a thin rim around an enlarged, degenerating nucleus (Bauchau 1981). The large number of effete haemocytes observed in the area of coagulation would support the role of these cells in this process.

The rapid occurrence of coagulation occluding the open cuticular wounds thereby preventing bleeding and the further intrusion of micro-organisms (Bauchau 1981) was observed in the wounding experiments. The value of the process in crustacean defence is evidenced by the complete absence of any local or systemic infections in

the wounding experiments although agglomerations of bacteria were present on the surface of the lesion. The sealing of the damaged cuticle by multilaminated sheets of melanized haemocytes was also considered to prevent infection. The healing of shell lesions in crustaceans without infection has also been reported by Sindermann (1971). Preliminary experiments involving cuticular traumatization and bacterial application also demonstrated that in healthy, unstressed animals under conditions of good water quality, infection did not occur. These findings corroborate those of Fisher *et al.* (1976) who found that excised appendages, carapace abrasion and heavy bacterial inoculation did not increase susceptibility to a chitinolytic bacterium associated with shell disease. It was only under stress conditions such as consumption of a long-term synthetic diet and a short-term synthetic diet concomitant with malachite green treatment that the susceptibility to shell disease was greatest.

Clotting in Decapoda has been reviewed by Durliat (1985). Coagulogen in the haemocytes can be converted to a gel by a serine protease pro-clotting enzyme which can be activated either by lipopolysaccharides or 1,3 B-D-glucans. The proPO system of semi-granular and granular cell is also activated and phenoloxidase attaches to foreign surfaces of haemocyte lysates (Söderhäll & Smith 1983).

Concerning the role of bacteria in the initiation

of disease in marine invertebrates, bacteria are unable to penetrate unabraded exoskeleton and generally cause disease only under condition of injury or stress (Davidson 1986). It is considered that, rather, disease can be caused only if stress and/or injury are extremely severe or a combination of stress, injury and/or suboptimal environmental conditions exists. In the wounding experiment, although injury was present the animals were otherwise unstressed and water quality conditions were good. Prawns of groups 2 and 3 respectively moulted without any residual lesions by 21 days. The haemocytic tissue reaction was indicative of a progressive healing response in all groups. On the other hand, the natural shell disease lesions associated with *Citrobacter* and *Aeromonas* described in this study had both localized and systemic infections and tissue changes. Stress was considered to play an important role in the disease condition. Such was also considered to be the situation in which shell disease lesions were found to be associated with severe local, adjacent and distant undercutting and systemic bacterial infections in *Penaeus monodon* broodstock (Nash unpublished report 1986). Primary shell injury was caused by trauma associated with shrimp interactions, handling or mussel encrustation on the tank. Infections may have developed in the animals because they were stressed by multiple spawning, overcrowding and suboptimal water quality with heavy bacterial loading. Thus, the prawn culturist can be advised to avoid stressors such as overcrowding,

suboptimal pond and water conditions and inadequate diet as a possible means of disease prevention.

Melanization

Melanization, generally demonstrated grossly from 12 to 24 hours and histologically from 30 minutes to two days (refer to Table 1) after treatment in all the experiments, was shown to be an important component of the tissue defence response in *Macrobrachium rosenbergii* as in most other crustaceans. The relatively late observation time for melanization in the alcian blue experiment may have been due to the fact that it was masked by the blue pigment (note Fig. 24). Its presence was recognizable histologically as red-to-brown-to-black pigment deposits (histochemically demonstrated by Lightner & Redman (1977) to indeed be melanin in and in association with haemocytes involved in a wide range of activities including phagocytosis, aggregation, encapsulation (also in wound repair and shell insertion), nodule formation and necrotic, degenerating and effete states. As the presence of melanin provides evidence of the participation of the proPO system, in association with increased haemocytic activity, it also indicates the cooperation of the cellular and humoral defence systems in *Macrobrachium*. That melanization occurred also confirms that semi-granular and/or granular cells in which the proPO system resides (Smith & Söderhäll 1986) participated in the haemocytic tissue defence reactions, as mentioned previously.

Haematopoiesis

It has been demonstrated that with the attachment of haemocytes to capsules or nodules there is a corresponding decline in the number of circulating cells in the haemolymph (Smith & Ratcliffe 1980a). This is only temporarily detrimental to the host as mobilization or haematopoiesis of new haemocytes returns the circulating cell count to normal within 48 hours (Ratcliffe, White, Rowley & Walters cited by Smith & Söderhäll 1986). Increased compensatory haematopoiesis in comparison to the controls in the haematopoietic nodules was observed as early as one hour after injection in the *Aeromonas* experiment and six hours in the *Vibrio* experiment; in the other experiments variable moderately active haematopoiesis was observed, but this was not demonstrably different from the controls (Table 1).

Elimination of Foreign Material

As the gills were found to be the earliest and/or primary sites of haemocytic tissue activity associated with foreign material, it is concluded, concurring with Fontaine & Lightner (1973, 1975), that the gills are probably one of the primary sites of elimination, which is considered to occur with the moulting of the gill cuticle.

As mentioned previously, Smith and Ratcliffe (1981) determined that the branchial podocytes could remove haemocyte-derived cellular debris. Branchial

podocytic activity was identified in all the injection experiments, the vacuolated podocytes were suspended by connective tissue strands in irregular rows and often formed diffuse networks in the efferent gill stem. Within the small to large, single to multiple podocytic vacuoles engulfed material was variably observed; this consisted of small amounts of eosinophilic to brown granular (most commonly in the *Vibrio* and *Aeromonas* experiments) to larger amounts (often leading to replete cytoplasmic vacuoles) of homogeneous eosinophilic material, with or without clear to pink vacuolation and apparent basophilic karyorrhectic nuclear debris (most frequently in the BCG experiment). In the alcian blue injection experiments, alcian blue pigment was also observed within the podocytic vacuoles from 24 hours to 42 days. As the amount of engulfed material within the podocytes appeared to be variably related to the animal's pathological condition, it was thought that the condition of the cells must also be related to stage of the moult cycle, premoult podocytes probably containing a greater amount of material. Drach (cited by Johnson 1980) concluded that the foreign material, once taken up, is retained in the podocytes during an entire intermoult cycle. Haemocytic activity was also high in the gill stem in the vicinity of the podocytes, and could provide an opportunity of increased uptake of material by the podocytes.

Although haemocytic tissue activity associated with foreign material was often observed in appendage

connective tissues near the cuticular setae, elimination of foreign material through cuticular pores at the bases of setae (Fontaine & Lightner 1973, 1975) was not definitely recognized, though this is considered highly probable.

Conclusions

In these studies of the tissue response, *Macrobrachium rosenbergii* has been found to be similar to other crustaceans in possessing a variety of defence strategies which effectively protect the host against disease or parasitization (Smith & Söderhäll 1986). More studies of the internal defence mechanisms, and their roles in natural and experimentally-induced diseases in *Marcrobrachium rosenbergii*, prawns, shrimps and other crustaceans are required in the future as their culture becomes increasingly important throughout many regions of the world and disease control becomes more difficult. The potential use of knowledge of those responses in the development of disease control programmes has been described and more possibilities must be explored.

TABLES

TABLES

List of Abbreviations Used in Tables 2-6

a = haemocyte aggregation

e = encapsulation or haemocyte multilamination

f = fibroplasia

i = haemocyte infiltration

im = intramuscular injection only

m = melanization involving haemocytic response

mi = mitosis, haematopoietic

n = nodule formation

ne = necrosis involving haemocytic reaction

N = within normal limits

p = phagocytosis, haemocytic

pi = free alcian blue pigment

s = systemic injection only

--> = description as previously

DEGREE OF RESPONSE

0 = nil

1 = minimal

2 = slight

3 = moderate

4 = marked

5 = extensive

Table 1. Summary of the earliest and persistence of tissue/organ localization of hemocytic reactions observed histologically.

	Alcian blue	BCG	CFA	Grafting	Aeromonas	Vibrio	Trauma
Infiltration/ Aggregation	immediate esp. gills; also heart, hepatopancreas followed by connective tissues	1 hr gills 3 hr hepatopancreas muscle, hemocoel, connective tissues (CT) 24 hr antennal gland	1 hr gills, hepatopancreas 3 hr heart	3 hr	1 hr gills, heart hepatopancreas, muscle, CT, hemocoel	30 min gills, heart, hepatopancreas	24 hour
Phagocytosis	5 min gills, hepatopancreas 15 min - 1 hr heart 8 hr antennal gland	1 hr hepatopancreas 6 hr gills 3 da heart	1 hr gills, hepatopancreas		1 hr hepatopancreas 3 hr gills	30 min hepatopancreas 1 hr heart	
Encapsulation/ Nodule Foraation	2 hr gills 6 hr hemocoel 12 hr muscle, CT 24 hr hepatopancreas	1 hr gills 6 hr hepatopancreas, muscle, hemocoel, CT	1 hr gills, muscle, hemocoel CT, neurilemma 3 da hematopoietic tissue 5 da hepatopancreas	6 hr (open)	1 hr gills 3 hr heart 6 hr antennal gland 3 da hepatopancreas	30 min gills, muscle 6 hr hepatopancreas, heart 12 hr antennal gland	24 hr

Fibroplasia	4 & 5 da muscle injection site	3 da muscle, CT hemocoel	7 da muscle	6 hr muscle	3 da muscle	2 da muscle	2 da
Melanization	2 da muscle injection site 3 da gills	6 hr hepatopancreas, cuticle, muscle 12 hr gills	1 hr gills (early) 3 hr gills (definitive) 24 hr muscle	12 hr early 12 hr complete	1 hr gills 3 hr heart, antennal gland, muscle, CT, hemocoel	30 min muscle 6 hr heart 12 hr gills, hepatopancreas, antennal gland	24 hr
Persistence of Reaction	-persistence of phagocytosis, aggregation & encapsulation up to 42 days in gills, hepato-pancreas, heart, antennal gland, CT, etc.	-persistence up to 35 da of infiltration, aggregation, phagocytosis & encapsulation in gills, heart, hepatopancreas, muscle, CT, hemocoel	-persistence up to 21 da in most tissues	-persistence of diffuse reaction up to 28 da after grafting and traumatization	-persistence up to 28 da in most tissues	-persistence up to 28 da in both killed & live in gills etc.	-persistence of diffuse reaction up to 28 da after grafting and traumatization
Hematopoiesis	-moderate hematopoiesis	-slight to moderate hematopoiesis	-slight to moderate hematopoiesis		-moderate hematopoiesis	-moderate to extensive hematopoiesis	

Table 2. Hemocytic Response in Alcian Blue Dye Injection

Time / Organ/Tissue	0	5 min	15 min	1 hr	2 hr	4 hr	6 hr	8 hr	12 hr	24 hr	2 da	3 da	4-5 da	6-7 da	14 da	21 da	42 da
gills	pi, a 3-4	pi 3-5 p 3	pi 3-4 p, a 4	pi 3 p,a 4-5	--> p,e	pi 2-3 p 4-5	pi 0 p 4 a 2	pi 3 a,p 2-3	p 3	i,a,e,p 2-3	-->	pi 1 -->	-->	pi 1 -->	pi 0-1 -->	i,a,p,e 2-3	-->
heart	pi, a 1-2	--> 2-3	--> 3	pi,a,p 3-4	-->	p 3	-->	-->	p 2	-->	-->	-->	-->	--> p 2-3	-->	-->	a,p,e 3
hepatopancreas	pi, a 1-2	pi, p 2-3	pi,p,a 3-4	pi p,a 4	-->	-->	a, p 2-3	-->	-->	p 3 p,e,ne 2	-->	--> ne 3-4	-->	i, a, p 3-4	-->	i,a e,ne 3	-->
antennal gland	pi 1-3	-	-	-	-	-->	pi 0	p 2	p 3	p 2-3	-->	-	-	i a p 2-3	-	i p 3	--> 2-3
muscle injection site	-	-	pi, a 3-4	-	-	-	pi, a 3-4	pi 2-3	i a p 2-3	pi 2 m,p,e 2	--> m	-	--> e,ne,f	-	e m 3	-	-->
muscle/ connective tissue	pi 1-2	pi, a 2-3	-	pi, a 2-3	-->	-->	-	a,e 2-3	a e n 2-3	a,p 2-4 pi 2-3	-->	--> e,p	-	i,a p e, m 2-3	-->	-->	--> ne 3
nerve/ganglion	pi 2	-->	-->	-->	-->	-->	pi 0	a,p	-	p 2	-->	-	-	i a p 2-3	-->	-->	-->
hematopoietic tissue	-	pi, i 2	-	-->	-->	-	-	p 2 ai 2-3	p 2-3	p 2 ai 2-3	p 2-3	-	-	-	-	-->	--> ai 3
subcuticular epithelium	-	pi 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	p 2
eye	-	pi 2	-	-	-	-->	pi 0	-	p e 3	-	-	-	-	-	-	-	-
stomach/ midgut/ hindgut	-	pi 2	-	-	-	-	-	n,p 2-3	-	-	i,a,p 3	e p n 2-3 e	-	-	-	-->	-->
ovary	-	-	pi 2	-	-	-->	-	-	-	-	-	p 2	-	-	-	-	-
hemocoel	-	-	-	-	-	-	a,p e,ne 3	-	a e n 2-3	a,p 2-4	-->	-->	-->	i a p 2-3	-->	-->	-->

Table 3. Hemocytic Response in BCG Injection

Time / Organ/Tissue	1 hr	4 hr	6hr	12 hr	24 hr	2 da	3 da	4 da
gills	i,a,e 2	-->	i,a,e,p 2-3	e,p,e, ne 3	-->	-->	-->	-->
heart	-	N	-->	-->	-->	p 2-3	p 2-3 a 2	-->
hepatopancreas	-	i,a,p 2-3	i,a,e,p, ne,m 3	-->	--> a 4	-->	-->	-->
antennal gland	-	N	-->	-->	i,a 2-3	i,a,e, m 2-3	-->	-->
muscle injection site	-	-	i,a,ne, m 3	-	-->	-->	-->	-->
muscle/ connective tissue	-	i,a 2	i,a,e 2-3	-->	-->	i,a,e,n, m,ne,f 3	-->	-->
hematopoietic tissue	-	N	-->	-->	-->	-	-->	-->
hemocoel	-	i,a 2	-	-->	-->	i,a,e,n, m,ne,f 3	-->	-->

Time / Organ/Tissue	5 da	6 da	7 da	10 da	14 da	17 da	21 da	31-35 da
gills	-->	-->	-->	-->	-->	-->	--> 2-3	--> 3
heart	i,a,p 2-3	-->	-->	i,a,p, ne 2-3	i,a,p,e, ne,m 3	-->	--> 2-3	--> 3
hepatopancreas	-->	-->	--> 3-4	-->	a,p,e, m 2-3	-->	--> 2-3	-->
antennal gland	-->	-->	p,e 2	-->	-->	--> ne 2-3	-	-
muscle injection site	-->	-->	--> 3-4	--> f 3	--> f 4	--> n 3-4	-	--> n,ne 2-3
muscle/ connective tissue	-->	i,a,e 2-3	-->	-->	-->	-->	-	i,a,e, ne
hematopoietic tissue	-->	-->	-->	p,ne mi 2-3	-->	-->	N	-
hemocoel	-->	i,a,e 2-3	-->	-->	-->	-->	-	i,a,e ne

Table 4. Hemocytic Response in CFA Injection

Time / Organ/Tissue	1 hr	3 hr	4 hr	6hr	12 hr	24 hr	2 da	3 da
gills	i,a,p,e m 2-3	-->	-->	-->	-->	--> 3-4	-->	-->
heart	N	i 2	-->	-->	a 2	p 2	-->	-->
hepatopancreas	--> 2-3	-->	-->	-->	-->	--> a 3-4	-->	-->
antennal gland	N	-	-->	-->	-->	-->	-->	-->
cuticular injection site	i,a,p ne 3-4	--> e,m 3-4	-->	-->	-->	-->	--> e,m 4	-->
muscle/ connective tissue	i,a,p, ne 3-4	--> e 3-4	-->	-->	-->	-->	--> e,m 3-4	-->
nerve/ ganglion	i,a,p,ne 3-4	--> e,ne 3-4	-->	-->	-->	-->	--> e,m, 3-4	-->
hematopoietic tissue	-	-	-	-	-	-	-	i,p,e 2-3
hemocoel	i,a,p,ne 3-4	--> e 3-4	-->	-->	-->	-->	--> e,m 3-4	-->
subcuticular epidermis	i,p 2	-	-->	-->	-->	-->	-->	-->
ovary	-	-	-	-	-	-	-	-

Time / Organ/Tissue	5 da	7 da	8 da	10 da	12 da	14 da	17 da	21 da
gills	-->	--> e,n,m 4-5	-->	-->	--> ne 3	-->	--> n 2-3	--> n,m 4
heart	-->	a,p 3	-->	--> 3-4	-->	-->	i,a,p, e 3	--> e,m 2-4
hepatopancreas	i,a,e 3-4	--> n,m 3-4	-->	i,a,p,e n 3	-->	--> 4-5	-->	-->
antennal gland	-->	--> i,p 2-3	-->	-->	-->	--> e 4	p,e,m 4	-->
cuticular injection site	-->	-->	-->	-->	-->	-->	-->	-->
muscle/ connective tissue	-->	--> e,n,m,f4	--> e,n, m,ne,f4	-->	-->	i,a,p,e,n m,ne 5	-->	--> f 4
nerve/ ganglion	-->	-	i,p 2-3	p,e 2	-->	i,a,p,e, n,m,ne 5	-->	--> f 4
hematopoietic tissue	-->	-	-->	-->	-->	-->	--> m2,mi2-4	-->
hemocoel	-->	-	--> e,m,ne 4	-->	-->	i,a,p,e, n,m,ne 5	-->	--> f 4
subcuticular epidermis	-->	-->	-->	-->	-->	-->	-->	-->
ovary	-	-	-	-	-	-	-	i,a,e 2-3

Table 5. Hemocytic Response in Aeromonas Injection

Time / Organ/Tissue	1 hr	3 hr	6hr	12 hr	18 hr	24 hr
gills	i,a,e m 2-3	-->e,n,p, m,ne 2-3	-->	-->	-->	-->e,n,m, ne 3-4
heart	i,a 2-3	i,a,e, m, 2-4	--> n 2-4	-->s-i,a, e,n,m, ne 2-5	-->	-->s-i,a, e,p,n,m, ne 2-5
hepatopancreas	i,a,p 2-3	-->	-->	--> 2	-->	-->
antennal gland	-	i,a,e, m 2-3	-->	-->	-->	-->
muscle injection site	-	-	i,a,e, m3	-->	-->	-->
muscle/ connective tissue	i,a,e 2-3	-->e,n m,ne 2-3	-->	-->	-->	--> 2-4
nerve/ ganglion	-	-	-	-	-->	i,a,e,m, ne 2-3
hematopoietic tissue	mi 2-3	-->	-->	-->	-->	-->
hemocoel	i,a,e 2-3	-->e,n, m,ne 2-3	-->	-->	--> 2-4	--> 2-4
ovary	-	-	-	-	-	-

Time / Organ/Tissue	2 da	3 da	4 da	5-6 da	7 da	10 da	15-28 da
gills	-->p,e,n, m,ne 3-4	-->	-->	-->	-->	-->	-->
heart	-	-->a,p, e,m,ne3-4	-->	-->	-->s-i,a, e,n,m, ne 5	-->	-->
hepatopancreas	-->	--> s-e 2-3	a,p,e,m, ne 2-3	-->	--> n,ne,m 3	i,a,p,e,n m,ne 3-4	-->
antennal gland	-	-->	a,p,e,m, ne 2-3	-->	-->	-->	-->
muscle injection site	-	--> im-i,a,e, e,n,m,ne f 3-5	--> 4-5	-->	-->	-->	-->
muscle/ connective tissue	-	-->	a,p,e,m, ne 2-3	-->	-->	-->	-->
nerve/ ganglion	-	-->	a,p,e,m, ne 2-3	-->	-->	-->	-->
hematopoietic tissue	-->	-->	--> mi 3-4	-->	-->	-->	-->
hemocoel	-	-->	a,p,e, m.ne 2-3	-->	-->	-->	-->
ovary	-	-	-	-	-	i,n,ne 2	-->

Table 6. Hemocytic Response in Vibrio Injection

Time / Organ/Tissue	30 min	45 min	1 hr	2.5 hr	6hr	12 hr	18 hr	24 hr
gills	i,a,e 2-3	-->	-->	-->	--> e,m 2	-->e,n,m, ne 3-4	-->	-->e,n,m ne 4
heart	a 2-4	-->	i,a,p 2-4	--> e 2	--> e,m 3-4	-->	-->	-->
hepatopancreas	i,a,p 2-3	-->	-->	-->	-->e,n, ne 3-4	-->e,n,m ne 3-4	-->	--> p3
antennal gland	-	a 2-3	--> e 2	-->	-	--> e,m 3-4	-->	-->
muscle injection site	i,a,e, n,m 4-5	-->	-->e,p, m,ne 4-5	-->	-->	-->e,n, m,ne 4-5	-->	-->
muscle/ connective tissue	i,a,e, n,m 4-5	-->	-->	--> ne	--> 3-5	-->e,n, m,ne 4-5	-->	-->
nerve/ ganglion	i,a,e,n, m 4-5	-->	-	-	-	-	-->	-->
hematopoietic tissue	N	-->	-	-	mi 4-5	mi 3	-->	-->
hemocoel	i,a,e, n,m 4-5	-->	-->	--> ne	--> 3-5	-->e,n, m,ne 3-4	-->	-->
aorta	-	-	a,p,en, ne 3	-	-	-	-	-

Time / Organ/Tissue	2 da	3 da	4 da	5 da	7 da	10 da	14 da	21 da	28 da
gills	i,a,e, m 2-3	-->	-->e,n, m 3-4	-->	-->e,n,p, m 3-4	-->	-->	-->	i,a,e,n m,ne 3-4
heart	i,a,e, m 2-4	-->	-->	-->p,e, m 2-3	-->	-->	-->	-->	i,a,e,n, m,ne 3
hepatopancreas	i,a,p,e, n,m 3-4	-->	-->	-->	-->	--> ne 3-4	-->	-->	-->
antennal gland	-	-->	N	-->	-	i 2	i,a,e, m 2-3	-->	i,a,e,m, ne 2-3
muscle injection site	--> f 4	-->	-->	-->	--> f 4	-->	--> m 4-5	-->	-->
muscle/ connective tissue	-->	-->	-->e,n m,ne 4	-->	-->e,n, p,m,ne	-->	--> m 4-5	-->	-->
nerve/ ganglion	-	-->	-->	-->	-->e,n p,m,ne 3-4	-->	-->	-->	-->
hematopoietic tissue	-	mi 2-3	-->	-->	-	-->	-->	-->	-->
hemocoel	-->	-->	-->	-->	-->e,n,p, m,ne 3-4	-->	--> m 4-5	-->	-->
aorta	-	-	-	-	-	-	-	-	-

REFERENCES

Akita G., Nakamura R., Brock J., Miyamoto G., Fujimoto M., Oishi F., Onizuka D. & Sumikawa D. (1981) Epizootiologic study of mid-cycle disease of larval *Macrobrachium rosenbergii*. Journal of the World Mariculture Society 12, 223-230.

Akiyama D.M., Brock J.A. & Haley S.R. (1982) Idiopathic muscle necrosis in the cultured freshwater prawn *Macrobrachium rosenbergii*. Veterinary Medicine/Small Animal Clinician, 1119-1121.

Anderson I.G., Law A.T., Shariff M. & Nash G. (1990) A parvo-like virus in the giant freshwater prawn, *Macrobrachium rosenbergii*. Journal of Invertebrate Pathology 55, 447-449.

Aquacop (1977a) Observations on diseases of crustacean cultures in Polynesia. Proceedings of the World Mariculture Society 8, 685-703.

Aquacop (1977b) *Macrobrachium rosenbergii* (de Man) Culture in Polynesia: progress in developing a mass intensive larval rearing technique in clear water. Proceedings of the World Mariculture Society 8, 311-326.

Aquacop (1979a) *Macrobrachium rosenbergii* culture in Polynesia: pH control in experimental pond waters by phytoplankton limitation with an algicide. Proceedings of the World Mariculture Society 10, 392-402

Aquacop (1979b) About the concept of crowding disease and sanitary lot in modern intensive aquaculture: a short note.Proceedings of the World Mariculture Society 10, 551-533.

Armstrong D.A., Stephenson M.J. & Knight A.W. (1976) Acute toxicity of nitrite to larvae of the giant Malaysian prawn, *Macrobrachium rosenbergii*. Aquaculture 9, 39-46.

Bailey-Brock J.H. & Hayward P.J. (1984) A freshwater bryzoan, *Hyalinella vaihiriae* Hastings (1929), from Hawaiian prawn ponds. Pacific Science 38, 199-204.

Bang F.B. (1970) Disease mechanisms in crustacean and marine arthropods. In: Symposium on Diseases of Fishes & Shellfishes (ed. by S.F. Snieszko), pp. 383-404. American Fisheries Society Special Publication 5., Washington, D.C.

Bang F.B. (1983) Crustacean disease reponses. In The Biology of Crustacea Vol. 6 Pathobiology (ed. by A.J. Provenzano Jr.), pp. 113-153. Academic Press, N.Y.

Baross J.A., Tester P.A. & Morita R.Y. (1978) Incidence, microscopy and etiology of exoskeleton lesions in the tanner crab, *Chionoecetes tanneri*. Journal of the Fisheries Research Board of Canada 35, 1141-1149.

Bauchau A.B. (1981) Crustaceans. In: Invertebrate Blood Cells Volume 2 (ed. by N.A. Ratcliffe and A.F. Rowley), pp. 385-420. Academic Press, London.

Bauchau A.G. & de Brouwer M.B. (1972) Ultrastructure des hémocytes d'*Eriocheir sinensis*, crustacé décapode brachyoure. Journal de Microscopie 15, 171-180.

Bauchau A.G. & de Brouwer M.B. (1974) Étude ultrastructurale de la coagulation de l'hémolymphe chez les crustacés. Journal de Microscopie 19, 37-46.

Bauchau A.G. & Mengeot J.C. (1978) Structure et fonction des hémocytes chez les crustacés. Archives Zoologiques d' expèrimentation genérale 19, 227-248.

Bauchau A.G. & Plaquet J.C. (1973) Variation du nombre des hémocytes chez les crustacés branchyomes. Crustaceana 24, 215-223.

Bellon-Humbert C. (1983) *Fecampia erythrocephala* Giard (*Turbellaria neorhabdocoela*), a parasite of the prawn *Palaemon serratus* Pennant: the adult phase. Aquaculture 31,117-140.

Bland C.E., Ruch D.G., Salser B.R. & Lightner D.V. (1976) Chemical control of *Lagenidium*, a fungal pathogen of marine crustacea. Proceedings of the World Mariculture Society 7, 445-472.

Blewett C. & Eble A.F.(1979) Cytology and cytochemistry of haemocytes from the freshwater prawn, *Macrobrachium rosenbergii*. In: Proceedings of the Second Biennial Crustacean Health Workshop (compiled by D.H. Lewis & J.K. Leong),pp. 38-54. TAMU-SG-79-114, College Station, Texas.

Blogoslawski W.J., Stewart M.E. & Rhodes E.W. (1978) Bacterial disinfection in the shellfish hatchery disease control. Proceedings of the World Mariculture Society 9, 589-602.

Bloom W. & Fawcett D.W. (1968) A Textbook of Histology 9th Edition. W.B. Saunders Co., Philadelphia. 858 pp.

Bodammer J.E. (1978) Cytological observations on the blood and haemopoietic tissue in the crab, *Callinectes sapidus* I. The fine structure of haemocytes from intermolt animals. Cell and Tissue Research 187, 79-96.

Bowser P.R. & Rosemark R. (1981) Mortalities of cultured lobsters, *Homarus*, associated with a molt death syndrome. Aquaculture 23, 11-18.

Brisson S. (1985) Gas-bubble disease observed in pink shrimps, *Penaeus brasiliensis* and *Penaeus paulensis*. Aquaculture 47, 97-
99.

Brock J.A. (1983) Diseases (infectious and non-infectious), metazoan parasites, predators and public health considerations in *Macrobrachium* culture and fisheries. In: CRC Handbook of Mariculture Vol. 1 Crustacean Aquaculture (ed. by J.P. McVey) pp. 329-370. CRC Press Inc., Boca Raton.

Brock J.A. (1988) Diseases and husbandry problems of cultured *Macrobrachium rosenbergii*. In: Disease Diagnosis and Control in North American Marine Aquaculture. (ed. by C.J. Sindermann and D.V. Lightner), pp. 134-180. Elsevier, Amsterdam.

Brock J.A. Nakagawa L.K. & Shimojo R.J. (1986) Infection of a cultured freshwater prawn, *Macrobrachium rosenbergii* de Man (Crustacea: Decapoda), by *Mycobacterium* spp., Runyon Group II. Journal of Fish Diseases 9, 319-324.

Brown J.H., Robertson D.A. & Wootten R. (1985) Report on an Aquaculture Study Mission to Ecuador on Behalf of Overseas Development Administration July 2-22, 1985. Institute of Aquaculture, University of Stirling, 65 pp.

Burns C.D., Berrigan M.E. & Henderson G.E. (1979) Fusarium sp. infections in the freshwater prawn, *Macrobrachium rosenbergii* (de Man). Aquaculture 16, 193-198.

Castille Jr., F.L. & Lawrence A.L. (1981) The effects of EDTA (ethylenedinitrotetraacetic acid) on the survival and development of shrimp nauplii (*Penaeus stylirostris* Stimpson) and the interactions of EDTA with the toxicities of cadmium, calcium and phenol. Journal of the World Mariculture Society 12, 292-304.

Cohen D., Finkel A. & Sussman M. (1976) On the role of algae in larviculture of *Macrobrachium rosenbergii*. Aquaculture 8, 199-207.

Cipriani G.R., Wheeler R.S. & Sizemore R.D. (1980) Characterization of brown spot disease of Gulf Coast shrimp. Journal of Invertebrate Pathology 36, 255-263.

Cook D.W. & Lofton S.R. (1973) Chitinoclastic bacteria associated with shell disease in *Penaeus* shrimp and the blue crab (*Callinectes sapidus*). Journal of Wildlife Diseases 9, 154-159.

Cornick J.W. & Stewart J.E. (1968) Interaction of the pathogens, *Gaffkya homari*, with natural defense mechanisms of *Homarus americanus*. Journal of the Fisheries Research Board of Canada 25, 695-709.

Cornick J.W. & Stewart J.E. (1973) Partial characterization of a natural agglutinin in the hemolymph of the lobster, *Homarus americanus*. Journal of Invertebrate Pathology 21, 255-262.

Cornick J.W. & Stewart J.E. (1978) Lobster (*Homarus americanus*) haemocytes: classification, differential counts and associated agglutinin activity. Journal of Invertebrate Pathology 31, 194-203.

Couch J.A. (1977) Ultrastructural study of lesions in gills of a marine shrimp exposed to cadmium. Journal of Invertebrate Pathology 29, 267-288.

Cripps M.C. & Nakamura R.M. (1979) Inhibition of growth of *Macrobrachium rosenbergii* by calcium carbonate water hardness. Proceedings of the World Mariculture Society 10, 575-580.

Cuénot L. (1891) Études sur le sang et les glandes lymphatiques dans la série animale. Archives de Zoologiques Experimentation Générale, (Serie 2) 9, 13-90.

Davidson E.W. (1986) Bacterial diseases of terrestrial and marine invertebrates. In: Fundamental and Applied Aspects of Invertebrate Pathology (ed. by R.A. Samson, J.M. Vlak & D. Peters), P. 353. Foundation of the Fourth International Colloquium of Invertebrate Pathology, Wageningen, The Netherlands.

Delves-Broughton J. (1974) Preliminary investigations into the suitability of a new chemotherapeutic, furanace, for the treatment of infectious prawn diseases. Aquaculture 3, 175-185.

Delves-Broughton J. & Poupard C.W. (1976) Disease problems of prawns in recirculation systems in the U.K.. Aquaculture 7, 201-217.

Dennell R. (1947) The occurrence and significance of phenolic hardening in the newly formed cuticle of Crustacea Decapoda. Proceedings of the Royal Society of London 134, 485-503.

Doughtie D.G. & Rao K.R. (1983) Ultrastructural and histological study of degenerative changes in the antennal gland, hepatopancreas and midgut of grass shrimp exposed to two dithiocarbonate biocides. Journal of Invertebrate Pathology 71, 281-300.

Dugan C.C., Hagood R.W. & Frakes T.A. (1975) Development of Spawning and Mass Larval Rearing Techniques for Brackish - Freshwater Shrimps of the genus *Macrobrachium* (Decapods, Palaemonidae). Florida Marine Research Publication No. 12. Florida Department of Natural Resources, St. Petersburg, Florida 28 pp.

Durliat M. (1985) Clotting processes in Crustacea Decapoda. Biological Review 60, 473-498.

El-Gamal A.A., Alderman D.J., Rodgers C.J., Polglase J.L. and Macintosh D. (1986) A scanning electron microscope study of oxolinic acid treatment of burn spot lesions of *Macrobrachium rosenbergii*. Aquaculture 52, 157-171.

Fieber L.A. & Lutz P.L. (1984) Calcium requirements for molting in *Macrobrachium rosenbergii*. Journal of the World Mariculture Society 13, 21-27.

Fisher W.S. (1977) Epibiotic microbial infestations of cultured crustaceans. Proceedings of the World Mariculture Society 8, 673-684.

Fisher W.S., Rosemark T.R. & Nilson E.H. (1976) The susceptibility of cultured American lobster to a chitinolytic bacterium. Proceedings of the World Mariculture Society 7, 511-520.

Fontaine C.T. (1971) Exoskeletal intrusions: a wound repair process in penaeid shrimp. Journal of Invertebrate Pathology 18, 301-303.

Fontaine C.T. (1975) Observations on the wound repair processes in the freshwater crayfish *Procambarus* sp.. Journal of Invertebrate Pathology 25, 391-393.

Fontaine C.T., Bruss R.G., Sanderson I.A. & Lightner D.V. (1975) Histopathological response to turpentine in the white shrimp, *Penaeus setiferus*. Journal of Invertebrate Pathology 25, 321-330.

Fontaine C.T. & Dyjak R.C. (1973) The development of scar tissue in the brown shrimp, *Penaeus aztecus*, after wounding with the Petersen disk tag. Journal of Invertebrate Pathology 22, 476-477.

Fontaine C.T. & Lightner D.V. (1973) Observations on the process of wound repair in penaeid shrimp. Journal of Invertebrate Pathology 22, 23-33.

Fontaine C.T. & Lightner D.V. (1974) Observations on the phagocytosis and elimination of carmine particles injected into the abdominal musculature of the white shrimp, *Penaeus setiferus*. Journal of Invertebrate Pathology 24, 141-148.

Fontaine C.T. & Lightner D.V. (1975) Cellular response to injury in penaeid shrimp. Marine Fisheries Review 37,4-10.

Forster J.R.M. & Beard T.W. (1974) Experiments to assess the suitability of nine species of prawns for intensive cultivation. Aquaculture 3, 355-368.

Frerichs G.N. (1984) Isolation and Identification of Fish Bacterial Pathogens. Institute of Aquaculture, University of Stirling, Scotland, 47 pp.

Ghate H.V. (1984) Gill melanization and heavy metals in freshwater prawns. Indian Journal of Fisheries 31, 389-393.

Golterman H.L., Clymo R.S. & Ohnstad M.A.M. (1978) Methods for Physical and Chemical Analysis of Freshwaters. IBP Handbook No. 8. Blackwell Scientific Publications, London, 231 pp.

Goodwin H.L. & Hanson J.A. (1975) The Aquaculture of Freshwater Prawns/*Macrobrachium* sp., NOAA-75100801. NTIS NO. PB-246-657. 95 pp.

Green J.P., Richards T.L. & Singh T. (1977) A massive kill of pond-reared *Macrobrachium rosenbergii*. Aquaculture 11, 263-272.

Haeckel E. (1857) Über die Gewebe des Flusskrebes. Müller's Archiv der Anatomische Physiologie , 469-568.

Hall T.J. (1979) Ectocommensals of the freshwater shrimp, *Macrobrachium rosenbergii*, in culture facilities at Homestead, Florida. In: Proceedings of the Second Biennial Crustacean Health Workshop (compiled by D.H. Lewis & J.K. Leong), pp. 214-219. TAMU- SG-79-114. College Station, Texas.

Harrison K.E. & Lutz P.L. (1980) Studies on the ontogenesis of osmoregulation in *Macrobrachium rosenbergii* with application for shipping postlarvae. Proceedings of the World Mariculture Society 11, 181-182.

Hearing V. & Vernick S.H. (1967) Fine structure of the blood cells of the lobster, *Homarus americanus*. Chesapeake Science 8, 170-186.

Hoarau F. (1976) Ultrastructure des hemocytes de l'oniscoide *Helleria brevicornis* Ebner (Crustace Isopode). Journale de Microscopie de Biologie Cellulaire 27, 47-52.

Hoarau F. (1979) Comportement des hemocytes apres amputation d'un pereopide chez *Helleria brevicornis* Ebner (Crustace Oniscoide). Societe Zoologique de France 104, 167-178.

Hose J.E., Lightner D.V., Redman R.M. & Danald D.A. (1984) Observations on the pathogenesis of the imperfect fungus, *Fusarium solani* in the California brown shrimp, *Penaeus californiensis*. Journal of Invertebrate Pathology 44, 292-303.

Hose J.E., Martin G.G., Nguyen V.A., Lucas J. & Rosenstein T. (1987) Cytochemical features of shrimp haemocytes. Biological Bulletin 173, 178-187.

Huang M.T.F., Eble A.F. & Hammen C.S. (1981) Immune response of the prawn, *Macrobrachium rosenbergii*, to bacterial infection. Journal of Invertebrate Pathology 38, 213-219.

Hubert M., Chassard-Bouchard C. & Bocquet-Védrine J. (1976) Cytologie aspects ultrastructuraux des hémocytes de *Carcinus maenas* L. (Crustacé Décapode), parasité par *Sacculina carcini* Thompson (Crustacé Cirripède): activité réactionelle, genèse de collagène. Comptes Rendues de l'Académie des Sciences de Paris 283,789-792.

Johansson M.W. & Söderhäll K. (1985) Exocytosis of the prophenoloxidase activating system from crayfish haemocytes. Journal of Comparative Physiology B, 156, 175-181.

Johansson M.W. & Söderhäll K. (1986) Cell adhesion of crayfish granular haemocytes. In: Fundamentals and Applied Aspects of Invertebrate Pathology (ed. By R.A. Samson, J.M. Vlak & D. Peters), p. 464. Foundation of the Fourth International Colloquium of Invertebrate Pathology, Wageningen, The Netherlands.

Johnson P.T. (1976a) Bacterial infection in the blue crab, *Callinectes sapidus*: Course of infection and histopathology. Journal of Invertebrate Pathology 28, 25-36.

Johnson P.T. (1976b) Gas-bubble disease in the blue crab, *Callinectes sapidus*. Journal of Invertebrate Pathology 27, 247-253.

Johnson P.T. (1987) A review of fixed phagocytic and pinocytotic cells of decapod crustaceans with remarks on haemocytes. Developmental and Comparative Immunology 11, 679-704.

Johnson P.T. (1980) Histology of the Blue Crab, *Callinectes sapidus*. A Model for the Decapoda. Praeger, New York. 440 pp.

Johnson S.K. (1978) Handbook of Crawfish and Freshwater Shrimp Diseases. TAMU-SG-77-605, College Station, Texas. 20 pp.

Johnson S.K. (1982) Diseases of *Macrobrachium*. In: Giant Prawn Farming. Developments in Aquaculture and Fisheries Science 10. (ed. by M.B. New), pp. 269-277. Elsevier, Amsterdam.

Johnston M.A. & Davies P.S. (1972) Carbohydrates of the hepatopancreas and blood tissues of *Carcinus*. Comp. Biochem. Physiol. 41B, 433-443.

Johnston M.A., Elder H.Y. & Davies P.S. (1973) Cytology of *Carcinus* haemocytes and their function in carbohydrate metabloism. Comp. Biochem. Physiol. 46, 569-581.

Jones T.C. & Hunt R.D. (1983) The musculoskeletal system. In: Veterinary Pathology, pp. 1135-1207. Lea & Febiger, Philadelphia.

Joseph J.D. (1977) Assessment of the nutritional role of algae in the culture of larval prawns (*Macrobrachium rosenbergii*). Proceedings of the World Mariculture Society 8, 853-861.

Lakshmi G.J., Vendataramiah A. & Howse H.D. (1978) Effect of salinity and temperature changes on spontaneous muscle necrosis in *Penaeus aztecus* Ives. Aquaculture 13, 35-43.

Lawrence A.L., Fox J. & Castille Jr. F.L. (1981) Decreased toxicity of copper and manganese ions to shrimp nauplii (*Penaeus stylirostris* Stimpson) in the presence of EDTA. Journal of the World Mariculture Society 12, 271-280.

Lewis D.H. & Lawrence A.L. (1983) Immunoprophylaxis to *Vibrio sp.* in pond reared shrimp. In: Proceedings of the 1st International Conference on Warm Water Aquaculture -Crustacea. Brigham Young University, Hawaii Campus, Feb. 9-11, 1983. (ed. by G.L. Rogers, R. Day & A. Lim), pp. 304-307. Brigham Young University Hawaii Campus Office of Continuing Education, Honolulu.

Licop M.S.R. (1988) Sodium-EDTA effects on survival and metamorphosis of *Penaeus monodon* larvae. Aquaculture 74, 239-247.

Lightner D.V. (1988) Diseases of cultured penaeid shrimp and prawns. In: Disease Diagnosis and Control in North American Marine Aquaculture (ed. by C.J. Sindermann and D.V. Lightner), pp. 8-133. Elsevier, Amsterdam.

Lightner D.V., Colvin L.B., Brand C. & Danald D.V. (1977) Black death, a disease syndrome of penaeid shrimp related to a dietary deficiency of ascorbic acid. Proceedings of the World Mariculture Society 8, 611-623.

Lightner D.V., Danald D.A., Redman R.M., Brand C., Salser B.R. & Rerpieta J. (1978) Suspected blue-green algal poisoning in the blue shrimp. Proceedings of the World Mariculture Society 9, 447-458.

Lightner D.V., Hunter B., Magarelli, Jr. P.C. & Colvin L.B. 91979) Ascorbic acid: nutritional requirement and role in wound repair in penaeid shrimp. Proceedings of the World Mariculture Society 10, 513-528.

Lightner D.V. & Redman R. (1977) Histochemical demonstration of melanin in cellular inflammatory processes of penaeid shrimp. Journal of Invertebrate Pathology 30, 298-320.

Lightner D.V., Redman R.M., Price R.L. & Wiseman M.O. (1982) Histopathology of aflatoxicosis in the marine shrimp, *Penaeus stylirostris & P. vannamei*. Journal of Invertebrate Pathology 40, 279-291.

Lightner D.V., Salser B.R. & Wheeler R.S. (1974) Gas-bubble disease in the brown shrimp (*Penaeus aztecus*). Aquaculture 4, 81-84.

Luna L.G. (1968) Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. 3rd Edition. McGraw-Hill Book Company, New York, 258 pp.

Maddox M.B. & Manzi J.J. (1976) The effects of algal supplements on static system culture of *Macrobrachium rosenbergii* (de Man) larvae. Proceedings of the World Mariculture Society 7, 677-698.

Mangum C.P., McMahon B.R., de Fur P.L. & Wheatley M.G. (1985) Gas exchange, acid-base balance, and the oxygen supply to the tissues during a molt of the blue crab *Callinectes sapidus*. Journal of Crustacean Biology 5, 188-206.

Manzi J.J., Maddox M.B. & Sandifer P.A. (1977) Algal supplement enhancement of *Macrobrachium rosenbergii* (de Man) larviculture. Proceedings of the World Mariculture Society 8, 207-223.

Martin G.G. & Graves B.L. (1985) Fine structure and classification of shrimp haemocytes. Journal of Morhology 185, 339-348.

Martin G.G., Hose J.E. & Kim J.J. (1987) Structure of haematopotietic nodules in the ridgeback prawn, *Sicyonia igentis*: light and electron microscopic observations. Journal of Morphology 192, 193-204.

Martinez L.E., Molinares A.M., Villanueva J. & Conroy D.A. (1982) Preliminary observations on the application of nifurpirinol for the control of potential disease problems in *Macrobrachium acanthurus*. In: Giant Prawn Farming (ed. by M.B. New), pp. 285-291. Elsevier, Amsterdam.

McKay D. & Jenkin C.R. (1969) Immunity in the Invertebrates. II Adaptive immunity in the crayfish (*Parachaeraps bicarinatus*). Immunology 17, 127-137.

McKay D. & Jenkin C.R. (1970a) Immunity in the invertebrates. The role of serum factors in phagocytosis of erythrocytes by haemocytes of the freshwater crayfish (Parachaeraps bicarinatus). Australian Journal of Experimental Biology and Medical Science 48, 139-150.

McKay D. & Jenkin C.R. (1970b) Immunity in the invertebrates. The fate and distribution of bacteria in normal and immunised crayfish (Parachaeraps bicarinatus).

Australian Journal of Experimental Biology and Medical Science 48, 599-607.

McKay D. & Jenkin C.R. (1970c) Immunity in the invertebrates. Correlation of the phagocytic activity of haemocytes with resistance to infection in the crayfish (Parachaeraps bicarinatus). Australian Journal of Experimental Biology and Medical Science 48, 609-617.

McKay D. & Jenkin C.R. & Rowley D. (1969) Immunity in the invertebrates. I. Studies on the naturally occurring haemagglutinins in the fluid from invertebrates. Australian Journal of Experimental Biology and Medical Science 47, 125-134.

Menasveta P. (1980) Effect of ozone treatment on the survival of prawn larvae (*Macrobrachium rosenbergii* de Man) reared in a closed, recirculating water system. Proceedings of the World Mariculture Society 11, 73-78.

Metchnikoff E. (1893) Lectures on the comparative pathology of inflammation. Delivered at the Pasteur Institute in 1891. Kegan, Paul, Trench, Trubner and Co. Ltd., London. (Republished 1968 by Dover Publications, Inc., New York).

Miles A.A. & Misra S.S. (1938) The estimation of the bactericidal power of the blood. Journal of Hygiene 38, 732-749.

Mix M.C. & Sparks A.K. (1980a) Tanner crab *Chionocetes bairdi* Rathburn haemocyte classification and an evaluation of using differential counts to measure infection with fungal disease. Journal of Fish Diseases 3, 285-293.

Mix M.C. & Sparks A.K. (1980b) Haemocyte classification and differential counts in the dungeness crab, *Cancer magister*. Journal of Invertebrate Pathology 35, 134-143.

Mürer E.H., Levin J. & Holme R. (1975) Isolation and studies of the granules of the amoebocytes of *Limulus polyphemus*, the horseshoe crab. Journal of Cell Physiology 86, 533-542.

Nash G. (1983) Studies on Flavobacterial Infection in Oreochromis mossambicus and Salmo gairdneri M.Sc Thesis, University of Stirling, 168 pp.

Nash G. (1989) Trematode metacercarial infection of cultured giant freshwater prawns, *Macrobrachium rosenbergii*. Journal of Invertebrate Pathology 53, 124-127.

Nash G. & Brown J.H. (1988) Pathology associated with culture in hard water in *Macrobrachium rosenbergii* de Man. Institute of Aquaculture - EEC Report, 10 pp.

Nash G., Chinabut S & Limsuwan C. (1987) Idiopathic muscle necrosis in the freshwater prawn, *Macrobrachium rosenbergii* de Man, cultured in Thailand. Journal of Fish Diseases 10, 109-120.

New M.B. & Singholka S. (1985) Freshwater Prawn Farming. a Manual for the culture of *Macrobrachium rosenbergii*. FAO Fisheries Technical Paper 225. Revision 1/FIRI/T225, FAO, Rome, 118 pp.

Nilson E.H., Fisher W.S. & Shleser R.A. (1975) Filamentous infestations observed on eggs and larvae of cultured crustaceans. Proceedings of the World Mariculture Society 6, 367-375.

Nyhlén L. & Unestam T. (1980) Wound reactions and *Aphanomyces astaci* growth in crayfish cuticle. Journal of Invertebrate Pathology 36, 187-197.

Oka M. (1969) Studies on *Penaeus orientalis* KISHINOUYE-VIII. Structure of the newly found lymphoid organ. Bullentin of the Japanese Society of Scientific Fisheries 35, 245-250.

Paterson W.D. & Stewart J.E. (1974) *In vitro* phagocytosis by haemocytes of the American lobster (*Homarus americanus*). Journal of Fisheries Research Board of Canada 31, 1051-1056.

Paterson W.D., Stewart J.E. & Zwicker B.M. (1976) Phagocytosis as a cellular immune response mechanism in the American lobster, *Homarus americanus*. Journal of Invertebrate Pathology 27, 95-104.

Peebles B. (1978) Molting and mortality in *Macrobrachium rosenbergii*. Proceedings of the World Mariculture Society 9, 39-46.

Peebles J.B. (1980) Competition and habitat partitioning by the giant freshwater prawn *Macrobrachium rosenbergii* (de Man) (Decapoda, Palaemonidae). Crustaceana 38, 49-54.

Piyan B.T., Law A.T. & Cheah S.H. (1985) Toxic levels of mercury for sequential larval stages of *Macrobrachium rosenbergii* (de Man). Aquaculture 46, 353-359.

Rabin H. (1970) Haemocytes, haemolymph and defense reactions in crustaceans. J. Res. 7, 195-207.

Rigdon R.H. & Baxter K.N. (1970) Spontaneous necrosis in muscles of brown shrimp, *Penaeus aztecus* Ives. Transactions of the American Fisheries Society 3, 583-587.

Rittenburg J.H. & Bayer R.C. (1980) Lobster gaffkemia vaccine, United States patent, 4, 214, 108, July 29, 1980.

Robbins S.L. Cotran R.S. & Kumar V. (1984) Pathologic Basis of Disease 3rd Edition. W.B. Saunders Co., Philadelphia. 1467 pp.

Roberts R.J. (1975) Melanin-containing cells of teleost fish and their relation to disease. In: The Pathology of Fishes (ed. by W.E. Ribelin & G. Migaki), pp. 399-428. University of Wisconsin Press, madison.

Roegge M.A., Rutledge W.P. & Guest W.C. (1979) Chemical control of *Zoothamnium* on larval *Macrobrachium acanthurus*. In: Proceedings of the Second Biennial Crustacean Health Workshop. (compiled by D.H. Lewis & J.K. Leong), pp. 295-299. TAMU-SG-117, College Station, Texas.

Rosemark R., Bowser P.R. & Baum N. (1980) Histological observations of the hepatopancreas in juvenile lobsters subjected to dietary stress. Proceedings of the World Mariculture Society 11, 471-478.

Rosen B. (1970) Shell disease of aquatic crustaceans. In: A Symposium on Diseases of Fishes and Shellfishes (ed. by S.F. Snieszko), pp. 409-415. Special Publication No. 5, American Fisheries Society, Washington, D.C.

Sandifer P.A. & Smith T.I.J. (1975) Effects of population density on growth and survival of *Macrobrachium rosenbergii* reared in recirculating water management systems. Proceedings of the World Mariculture Society 6, 43-53.

Sandifer P.A. & Smith T.I.J. (1985) Freshwater prawns. In: Crustacean and Mollusk Aquaculture in the United States (ed. by J.V. Huner & E.E. Brown), pp. 63-125. AVI Publishing Co. Inc., Westport.

Sandifer P.A., Smith T.I.J. & Calder D.R. (1974) Hydrozoans as pests in closed-system culture of larval decapod crustaceans. Aquaculture 4, 55-59.

Sarojini R. & Reddy C.V.R. (1984) Effect of sevimol on the moulting of the freshwater prawn, *Macrobrachium kistinensis*. Rev. Roum. Morphol. Embryol. Physiol., Physiologie 21, 111-116.

Sarver D, Malecha S.R. & Onizuka D.R. (1982) Possible sources of variability in stocking mortality in post larval *Macrobrachium rosenbergii*. In: Giant Prawn Farming. (ed. by M.B. New), pp. 99-113. Elsevier, Amsterdam.

Schapiro H.C. (1975) Immunity in decapod crustaceans. American Zoologist 15, 13-20.

Schapiro H.C., Steenbergen J.F. & Fitzgerald A.A. (1977) Haemocytes and phagocytosis in the American lobster,

Homarus americanus. In: Comparative Pathobiology Vol. 3. Invertebrate Immune Responses. (ed. by L.A. Bulla Jr. & T.C. Cheng), pp. 127-133. Plenum Press, New York.

Schnick R.A., Meyer F.P., Marking L.L., Bills T.D. & Chandler J.H. (1979) Candidate chemical for crustacean culture. In: Proceedings of the Second Biennial Crustacean Health Workshop (compiled by D.H. Lewis & J.K. Leong), pp. 245-294. TAMU-SG-79-114, College Station, Texas.

Segal E. & Roe A. (1975) Growth and behaviour of post juvenile *Macrobrachium rosenbergii* (be Man) in close confinement. Proceedings of the World Mariculture Society 6, 67-88.

Sindermann C.J. (1971) Internal defenses of *Crustacea*: a review. Fisheries Bulletin 69, 455-489.

Sindermann C.J. (1977a) Disease and disease control in *Macrobrachium* culture. In: Freshwater Prawn Farming in the Western Hemisphere (ed. by J.A. Hanson & H.L. Goodwin), pp. 210-219. Dowden, Hutchinson & Ross, Stroudsburg, Pa.

Sindermann C.J. (1977b) Fresh-water shrimp diseases. In: Disease Diagnosis and Control in North American Marine Aquaculture (ed. by C.J. Sindermann), pp. 78-97. Elsevier, Amsterdam. 329 pp.

Sindermann C.J. (1979) Epizootics in crustacean populations. In: Proceedings of the Second Biennial Crustacean Health Workshop (compiled by D.H. Lewis & J.K. Leong), pp. 1-37. TAMU-SG-79-114, College Station, Texas.

Singholka S., New M.B. & Vorasayan P. (1980) The status of *Macrobrachium* farming in Thailand. Proceedings of the World Mariculture Society 11, 60-72.

Smith R.H. & Pistole T.G. (1985) Bactericidal activity, of granules isolated from amoebocytes of the horseshoe crab, *Limulus polyphemus*. Journal of Invertebrate Pathology 45, 272-275.

Smith T.I.J. & Sandifer P.A. (1975) Increased production of tank-reared *Macrobrachium rosenbergii* through use of artificial substrates. Proceedings of the World Mariculture Society 6, 55-66.

Smith T.I.J., Sandifer P.A., Jenkins W.E., Stokes A.D. & Murray G. (1982) Pond rearing trials with Malaysian prawns, *Macrobrachium rosenbergii*, by private growers in South Carolins, 1981. Journal of the World Mariculture Society 13, 41-55.

Smith T.I.J., Sandifer P.A., Manzi J.A. (1979) Epibionts of pond-reared adult Malaysian prawns, *Macrobrachium rosenbergii* (de Man), in South Carolina. Aquaculture 16, 299-308.

Smith V.J. & Ratcliffe N.A. (1976) Defensive reactions of the shore crab, *Carcinus maenas*, to foreign particles. In: Proceedings of the First International Colloquium on Invertebrate Pathology (ed. by T.A. Angus, P. Faulkener & A. Rosenfield), pp. 312-313. Kingston, Canada, Queens University.

Smith V.J. & Ratcliffe N.A. (1978) Host defence reactions of the shore crab, *Carcinus maenas* (L) *in vitro*. Journal of the Marine Biological Association of Great Britain 58,

Smith V.J. & Ratcliffe N.A. (1980a) Host defence reactions of the shore crab, *Carcinus maenas* (L): Clearance and distribution of injected test particles. Journal of the Marine Biological Association of Great Britain 60, 89-102.

Smith V.J. & Ratcliffe N.A. (1980b) Cellular defense reactions of the shore crab, *Carcinus maenas*: *in vivo* haemocytic and histopathological responses to injected bacteria. Journal of Invertebrate Pathology 35, 65-74.

Smith V.J. & Ratcliffe N.A. (1981) Pathological changes in the nephrocytes of the shore crab, *Carcinus maenas*, following injection of bacteria. Journal of Invertebrate Pathology 38, 113-121.

Smith V.J. & Söderhäll K. (1986) Cellular immune mechanisms in the Crustacea. Symposium of the Zoological Society of London 56, 59-79.

Söderhäll K. (1982) Prophenoloxidase activating system and melanization -a recognition mechanism of arthropods? A review. Developmental and Comparative Immunology 6, 601-611.

Söderhäll K. (1986) The cellular immune system in crustaceans. In: Fundamental and Applied Aspects of Invertebrate Pathology (ed. by R.A. Aamson, J.M. Vlak & Peters), pp. 417-420. Foundation of the Fourth International Colloquium of Invertebrate Pathology, Wageningen, The Netherlands.

Söderhäll K. & Ajaxon R. (1982) Effect of quinones and melanin on mycelial growth of *Aphanomyces* spp. and extracellular protease of *Aphanomyces astaci*, a parasite on crayfish. Journal of Invertebrate Pathology 39, 105-109.

Söderhäll K. & Smith V.J. (1983) The prophenoloxidase activating system -a complement-like pathway in arthropods? In: Infection Processes of Fungi (ed. by J. Aist & D.W. Roberts), pp. 160-167. Rockefeller Foundation, New York.

Söderhäll K. & Smith V.J. (1986a) The prophenoloxidase activating system: the biochemistry of its activation and role in arthropod cellular immunity, with special reference to crustaceans. In: Immunity to Invertebrates (ed. by M. Brehelin), pp. 208-223. Springer-Verlag, Berlin.

Söderhäll K. & Smith V.J. (1986b) Prophenoloxidase-activaing cascade as a recognition system in arthropods. In: Hemocytic and Humoral Immunity in Arthropods (ed. by A.P. Gupta), pp. 251-285. John Wiley & Sons, Inc., New York.

Söderhäll K., Smith V.J. & Johansson M.W. (1986) Exocytosis and uptake of bacteria by isolated haemocyte populations of two crustaceans: evidence for cellular co-operation in the defence reactions of arthropods. Cell and Tissue Research 245, 43-49.

Söderhäll K. & Unestam T. (1975) Properties of extracellular enzymes from *Aphanomyces astaci* and their relevance in the penetration process of crayfish cuticle. Physiol. Plant 35, 140-146.

Söderhäll K., Vey A. & Ramstedt M. (1984) Haemocyte lysate enhancement of fungal spore encapsulaton by crayfish haemocytes. Developmental and Comparative Immunology 8, 23-29.

Solangi M.A. & Lightner D.V. (1976) Cellular inflammatory response of *Penaeus aztecus* and *P. setiferus* to the pathogenic fungus, *Fusarium sp.*, isolated from the California brown shrimp, *P. californiensis*. Journal of Invertebrate Pathology 27, 77-86.

Sparks A.K. (1985) Synopsis of Invertebrate Pathology Exclusive of Insects. Elsevier, Amsterdam. 423 pp.

Sparks A.K. & Fontaine C.T. (1973) Host response in the white shrimp, *Penaeus setiferus*,to infection by the larval trypanorhynicd cestode, *Prochristianella penaei*. Journal of Invertebrate Pathology 22, 213-219.

Spotts D.G. & Lutz P.L. (1981) L-lactic acid accumulation during activity stress in *Macrobrachium rosenbergii* and *Penaeus duorarum*. Journal of the World Mariculture Society 12, 244-279.

Stagner J.I. (1979) A system of phagocytes in *Limulus polyphemus* -a primary defense mechanism. In: Proceedings of the Second Biennial Crustacean Health Workshop (compiled by D.H. Lewis & J.K. Leong), pp. 159-177. TAMU-SG-79-114, College Station, Texas.

Stagner J.I. & Redmond J.R. (1975). The immunological mechanisms of the horseshoe crab, *Limulus polyphemus*. Marine Fisheries Review 37, 11-19.

Stang-Voss C. (1971) Zur Ultrastruktur der Blutzellen wirbelloser Tiere V. Uber die Hamocyten von *Astacus astacus* (L). (Crustacea). Zeitschrift fur die Zellforschung 122, 68-75.

Steenbergen J.F., Steenbergen M. & Schapiro H.C. (1978) Effects of temperature on phagocytosis in *Homarus americanus*. Aquaculture 14, 23-30.

Stewart J.E. & Foley D.M. (1969) A precipitation reaction of the haemolymph of the lobster, *Homarus americanus*. Journal of the Fisheries Research Board of Canada 26, 1392-1397.

Stewart J.E. & Zwicker B.M. (1972) Natural and induced bactericidal activities of the lobster, *Homarus americanus*: products of haemocyte-plasma interaction. Canadian Journal of Microbiology 18, 1499-1509.

Summer S.E. & Eversole A.G. (1978) Effects of water-borne mirex on the survival and production of *Macrobrachium rosenbergii* (de Man). Proceedings of the World Mariculture Society 9, 47-54.

Takahashi Y., Itami T., Nakagawa A., Nishimura H. & Abe T. (1985) Therapeutic effects of oxytetracycline trial tablets against vibriosis in cultured kuruma prawns *Penaeus japonicus* Bate. Bulletin of the Japanese Society of Scientific Fisheries 51, 1639-1643.

Tareen I.U. (1982) Control of diseases in the cultured population of penaeid shrimp, *Penaeus semisulcatus* (de Haan). Journal of the World Mariculture Society 13, 157-161.

Taylor R.L. (1969) Suggested role for the polyphenol-phenoloxidase system in invertebrates. Journal of Invertebrate Pathology 14, 427-428.

Terazaki M., Tharnbuppa P. & Nakayama Y. (1980) Eradication of predatory fishes in shrimp farms by utilization of Thai tea seed. Aquaculture 19, 235-242.

Tsing A., Arcier J.M. & Brehelin M. (1989) Haemocytes of penaeid and palaemonid shrimps: morphology, cytochemistry and haemograns. Journal of Invertebrate Pathology 53, 64-77.

Tunsutapanich A. (1982) The eradication of crabs in *Macrobrachium* ponds. In: Giant Prawn Farming. Developments in Aquaculture and Fisheries Science, 10. (ed. by M.B. New), pp. 279-284. Elsevier, Amsterdam.

Tyson C.J. & Jenkin C.R. (1974) Phagocytosis of bacteria *in vivo* by haemocytes from the crayfish (*Parachaeraps bicarinatus*). Australian Journal of Experimental Biology and Medical Sciences 52, 341-348.

Unestam T. & Beskow S. (1976) Phenol oxidase in crayfish blood: activation by and attachment on cells of other organisms. Journal of Invertebrate Pathology 27, 297-305.

Unestam T. & Nylund J.-E. (1972) Blood reactons *in vitro* in crayfish against a fungal parasite, *Aphanomyces*. Journal of Invertebrate Pathology 19, 94-106.

Vasta G.R., Warr G.W. & Marchalonis J.J. (1983) Serological characterization of humoral lectins from the freshwater prawn *Macrobrachium rosenbergii*. Developmental and Comparative Immunology 7, 13-20.

Venkataramiah A. (1971a) "Necrosis" in shrimp. FAO Aquaculture Bulletin 3(3), 11.

Venkataramiah A. (1971b) "Necrosis" in shrimp. FAO Aquaculture Bulletin 4(1), 14.

Wickens J.F. (1972) The food value of brine shrimp, *Artemia salina* (L.) to larvae of the prawn, *Palaemon serratus* Pennant. Journal of Experimental Marine Biology and Ecology 10, 151-170.

Wickens J.F. (1976) The tolerance of warm-water prawns to recirculated water. Aquaculture 9, 19-37.

Williams A.J. & Lutz P.L. (1975a) The role of the haemolymph in the carbohydrate metabolism of *Carcinus maenus*. Journal of the Marine Biological Association of Great Britain 55, 667-670.

Williams A.J. & Lutz P.L. (1975b) Blood cell types in *Carcinus maenus*. Journal of the Marine Biological Association of Great Britain 55, 671-674.

Willis S.A. & Berrigan M.E. (1977) Effects of stocking size and density on growth and survival of *Macrobrachium rosenbergii* (de Man) in ponds. Proceedings of the World Mariculture Society 8, 251-264.

APPENDICES

APPENDIX I Published Papers

(a) Nash G. (1989). Trematode Metacercarial Infection of Cultured Giant Freshwater Prawns, *Macrobrachium rosenbergii* . J. Invertebr. Pathol. 53,124-127.

(b) Nash G., Chinabut, S. and Limsuwan, C. (1987). Idiopathic Muscle Necrosis in the Freshwater Prawn,*Macrobrachium rosenbergii* de Man, Cultured in Thailand. J. Fish Dis. 10,109-120.

PUBLISHED PAPERS NOT FILMED FOR COPYRIGHT REASONS

APPENDIX I

APPENDIX II

Health Management of *Macrobrachium rosenbergii*

The Concept of "Sanitary Lot Technique" (Aquacop 1979b)

The goal in health management of *Macrobrachium rosenbergii* is prevention of disease by providing optimum environmental, nutritional and husbandry conditions, thereby minimizing stress. Prevention by these means should prove more economical and successful than attempting to control disease outbreaks.

Cultured aquatic animals are intensively maintained in comparison to those in the wild. In *Macrobrachium* culture, the sequence of events leading to development of disease, usually involves facultative, opportunistic organisms proliferating under poor culture conditions and infecting a stressed and weakened or injured host.

To prevent the development of culture-related disease ("crowding disease"), Aquacop (1979b) advocated the establishment of the "sanitary lot technique" in order to limit the spread of potential pathogens and the adaptation or selection of virulent strains. The principle is to establish the cultured population into "sanitary lots", groups of animals of the same age, segregating them in time and space and isolating them from other groups, thus limiting the risk of the spread of infection. The "vectors of contamination" (water, workers, materials, predators and the animals themselves) must be controlled continuously to prevent vertical and

horizontal transmission of pathogens. This concept was developed under intensive rearing conditions, and thus, is most readily applicable to the hatchery and nursery phases of *Macrobrachium* culture, but some aspects of the practical sanitary measures which they envisaged can be applied to more extensive and pond conditions. The potential disease risks and practical preventive measures should also be considered during the planning and construction of aquaculture facilities.

A Summary of Factors to Consider in Health Management of Established Facilities

(1) Water quality and general environment

(a) Important water quality parameters include: DO, CO_2, temperatures, pH, salinity, water hardness ($CaCO_3$, CA), nitrogenous compounds (especially NH_3, NO_2^-), microbial levels. Hydrogen sulphide should be absent (New & Singholka 1985). Levels should be regularly monitored and controlled when required.

(b) Proper use of algal culture may be an adjunct to water quality. Levels of algal in greenwater cultures should be controlled. The success of greenwater in larviculture depends upon a balance between the uptake of ammonia by the algae and the production of ammonia by the *Artemia*, grazing upon it, and by the *Macrobrachium* larvae. This develops into a more delicate balance as the more algae which are consumed by the *Artemia*, the more ammonia is produced and fewer algae remain to assimilate it, leading to a marked accumulation of toxic ammonia.

Constant exchange of water and reintroduction of algae are therefore required (Fujimura cited by Cohen *et al.* 1976; Cohen *et al.* 1976).

Covers may be used to reduce light and limit algal burdens in the hatchery (Johnson 1982).

Avoid algal blooms and die-offs in ponds.

(c) Presence of pollutants, contaminants, heavy metals, herbicides or pesticides should be avoided. Ethylenedinitro -tetraacetic acid (EDTA) has been recommended to increase hatching rates and survival of penaeid shrimp larvae by decreasing the toxicities of heavy metals (eg. cadmium, copper and manganese) through chelation. For long term use 0.03 mM (10 mg/l) is recommended to avoid any detrimental effects of chronic exposure (Castille & Lawrence 1981; Lawrence, Fox & Castille Jr. 1981). In another study, use of the disodium salt of EDTA (Na-EDTA) at 5.0 and 10.0 ppm greatly improved the survival of *P. monodon* larvae (Licop 1988).

(d) Organic matter levels should be controlled. Limit accumulation of organic debris, decompossing wasted food, dead algae and *Artemia* and exuvia by daily cleaning and siphoning of tanks; carry out flushing when required.

(e) Maintain adequate water exchange and aeration. Increase flow rate and aeration when indicated. For the simple hatchery there is no substitute for frequent water exchange (New & Singholka 1985).

(f) Use mechanical filtration to clean water and prevent the introduction of harmful organisms and material into the system (predatory or competitive fish, hydrozoans, micro-organisms, debris) (Johnson 1982).

(g) Establish biofiltration units apart from actual rearing area to localize the biofiltration away from the animals, which may serve as substrates for epibionts (Johnson 1982).

(h) Ultraviolet lighting and ozonation have been used successfully in some culture systems. Menasveta (1980) used ozone treatment in a closed recirculating system and found that it significantly improved the survival of *Macrobrachium* larvae. Water quality was improved by the acceleration of nitrogenous wastes into stable forms and the removal of dissolved organics. it had a prophylactic effect, killing microbes (more rapidly than chlorine) but leaving sufficient bacteria for efficient biological filtration. Blogoslawski, Stewart & Rhodes (1978) caution that ozonation can lead to genetic defects but found it useful at low concentrations. Ultraviolet (UV) lighting has effects similar to microfiltration, killing microbes in incoming water, but permitting their remains to serve as substrates for the growth of other bacteria. The most beneficial effects is in the exclusion of problem-causing agents from the system (Johnson 1982). Blogoslawski *et al.* (1978) found it useful as a disinfectant.

Costs and technical difficulties may outweigh

the benefit derived from use of these elaborate systems (Johnson 1982).

(i) Ion-exchange filtration may be useful for removal of toxic wastes in hatcheries for recirculated or incoming (new) water. Its use, though, may adversely affect water quality and nutrient decomposition by upsetting bacterial interactions (Johnson 1982).

(j) Chlorination of water has also been recommended (Aquacop 1977a). Water is chlorinated (about 1.5 ppm) and dechlorinated by intensive bubbling (24-48 hr) and natural illumination.

(k) Ensure proper level of lighting. Too much can cause distress in prawns (Fujimura & Okamoto cited by Johnson 1982) and with dietary imbalance may lead to chromatophore abnormalities in larval culture or post-transport (Johnson 1982).

(2) Hygiene and Sanitation

(a) Attempt to implement those facets of the "sanitary lot technique" that are practical in the given culture system and situation. Each tank should have its own portable equipment such as nets, siphon tubes, buckets, filters, air stones etc.. Submersible pumps should only be used in water storage or mixing tanks not in the larval tanks themselves since they could be a potential source of disease tranfer (New & Singholka 1985).

(b) Use routine preventive sanitary measures such as cleaning, disinfection and drying of tanks, pipes, equipment, clothes, footwear, floor, walls etc..

Avoid horizontal transmission of infection by disinfecting nets and equipment after use and use between tanks. Disinfect and dry tanks between cycles.

Recommended tank disinfection techniques include:

(1) scrape the tank and treat with 1.5 ppm chlorine (60 ppm) "chlorox" solution or 6 ppm commercial bleach powder for one day, and rinse/flush again before use;

(2) scrape the tank, spray with 250 ppm formalin solution, expose to sunlight for one day, flush/rinse and re-use (New & Singholka 1985).

A solution of potassium permanganate at pH 3 has been recommended to sterilize equipment in the hatchery between each larval cycle (New & Singholka 1985).

(c) Restrict the entry into facilities, especially for those people who may have recently come from another farm. Implement disinfection of footwear at the entry to facilities.

(d) After a disease outbreak, tank special precautions to sterilize all tanks and equipment to prevent the re-introduction of the pathogen into future

culture operations (Blogoslawski *et al*. 1978).

(e) Preliminary disinfection of *Artemia* cysts using sodium hypochlorite has been recommended. *Artemia* cysts are usually covered with fungal spores and bacteria and the indigestible cyst can also cause blockage of the intestine of larvae. The best method to prevent this is by decysting the *Artemia*. If this is not practical or possible, preliminary disinfection can be done using sodium hypochlorite. Newly hatched *Artemia* can be disinfected by giving them an osmotic shock in freshwater, but this is unlikely to kill many bacteria or fungal spores (Brown, Robertson & Wootten 1985).

(3) Stocking Density

(a) Avoid overcrowding, paying attention to water quality, survival, growth, competition and cannibalism. Reduce population density when necessary.

(b) Continually selectively harvest larger prawns.

(c) Provide artificial habitats maximizing the "edge effect".

(4) Handling

(a) Avoid indiscriminate handling.

(b) Avoid physical damage to prawns during handling.

(5) Predators and Competitors

(a) Prevent their entry into system (eg. netting

over ponds for birds, screening water inflow for fish);

(b) Eradicate when necessary.

(6) Nutrition

(a) Pay attention to proper level of feeding as well as quality (nutritional balance, freedom from contamination, length of storage); use good quality *Artemia*. Good nutrition is vital not only for survival and growth but also for disease resistance, tissue healing, epicuticle formation and integrity and exoskeleton formation and moulting. Prolonged underfeeding has been reported as a common cause of depressed growth rate, low prawn survival and, hence, low yields from production ponds in Hawaii (Brock 1988).

(b) Consider the use of nutritional supplements (lecithin, soybean curd, protein, vitamins etc.);

(c) Use algal culture (greenwater) to advantage.

(7) Quarantine and Stock Movement

(a) Quarantine newly arrived prawns to verify health status; at the same time undertake microbiological, parasitological and histological monitoring of representative animals from each group.

(b) Avoid indiscriminate stock movement.

(c) Ship or export only animals verified to be disease-free.

(8) Diagnostic Procedures

(a) Carry out routine monitoring of water quality, feed samples and prawns by direct observation and using laboratory procedures (as mentioned in 7 (a)).

(9) Chemotherapy

(a) Use when other preventive and prophylactic husbandry measures fail.

(b) Use to treat specific disease problems which have been diagnosed. Avoid indiscriminate use which could lead to selection for resistant, virulent strains or biofilter die-offs (Nilson *et al.* 1975; Johnson 1982) and have been known to result in the proliferation of fungi (Nilson *et al.* 1975).

(c) Avoid prophylactic and widespread use of antibiotics. Use only to treat specific bacterial problems, preferably after isolation (culture) and determination of sensitivity and resistance.

(d) When possible and practical, test drug at given dosage level on a few animals before treating an entire tank, to determine efficacy and toxicity (safety).

(General references: Aquacop 1977 a,b 1979 b; Sindermann 1977 a,b; Johnson 1982; Brock 1983, 1988; Sandifer & Smith 1985).

Summary of Chemicals and Drugs Used for Treatment of Diseases in *Macrobrachium*

Disease Problem	Drug/Chemical and Treatment Duration	Reference
Epistylis sp.	- Acetic acid 2 ppt/1 min dip (use on larvae) recommended as the treatment of choice, repeated treatments may be required.	Sindermann (1977b)
	- Sulfaquinine effective but expensive; the dosage used was not reported	Sindermann (1977b)
	Note:- Formalin was reported to cause motalities, moulting deformities and cessation of feeding, but the dosage used was not reported.	Sindermann (1977b)
Zoothamnuim sp.	- Formalin 20 ppm/24 hr effective and tolerated (used on larvae)	Roegge *et al.* (1979)
Epistylis sp. or *Zoothamnium* sp.	- Formalin at 25-125 ppm for 12 hr as a standing bath for larvae or adults.	Brock (1988)
Suctorians	- Formalin 200 ppm for 30 min/da (used on larvae). - Copper sulfate 0.4 ppm/6 hr/da - Malachite green 0.2 ppm/30 min/da	Goodwin & Hanson (1975); Fujimura cited by Sindermann (1977b)
Leucothrix sp	- Furanace 1 ppm/1 hr (used on larvae). Note:- The toxicity of furanace has been tested in *M. rosenbergii* (3-5 gm in weight) Furanace at 20 mg/l for 20 min as a short exposure bath was determined to be the limit for safe treatment; - a constant bath of 2 mg/l was also considered adequate.	Le Bitoux cited by Sindermann (1977b) Delves-Broughton (1974)
	- Nifurpirinol (furanace) was also well tolerated by *M. acanthurus* at concentrations of o.05-2.0 ppm as a permanent bath over	Martinez Molinares, Villaneueva & Conroy (1982)

	a 96 hr period of exposure and was recommended as having potential in the treatment of bacterial infections.	
	- In larvae with *Leucothrix* fouling copper sulfate at 0.05 mg Cu/l for 12 hr as a standing bath	Brock (1988)
Leucothrix sp. in a mixed bacterial infection in "Bacterial necrosis" of larvae	- bipenicillin - streptomycin 2 IU/ml (2 ppm) gave the best results. - also effective: tetracycline chlorhydrate (1 ppm) erythromycin phosphate (0.65-1 ppm) furanace (0.1 ppm)	Aquacop (1977a)
Necrosis of appendages larvae and postlarvae	- bipenicillin - streptomycin in 2 IU/ml (2 ppm) - erythromycin phosphate (1 ppm); - tetracycline chlorhydrate (1 ppm); - sulfamethazine (3 ppm); - furanace (0.1 ppm) - all were preventive or curative; erythromycin phosphate at 1 ppm was used from the second day of zoea one and repeated every other day until the postlarval stage.	Aquacop (1977a)
"Black nodule"	- furanace at 0.09 mg/l arrested the disease	Delves-Broughton & Poupard (1976)
Black spot (shell disease) associated with *Aeromonas hydrophila* infection	- Oxolinic acid 10 mg/l hr as a static bath arrested the disease	El-Gamal *et al.* (1986)
Vibriosis	- Oxytetracycline tablets administered at 50 or 100 mg/kg b.w./day for 4-6 days had excellent therapeutic effects in *Penaeus japonicus* infected with *Vibrio* sp.	Takahashi, Itami, Nakagawa, Nishimura & Abe (1985)
Fungal infections	- salinity alterations 20 ppt 15-30 min static bath	Dugan *et al.* (1975); Goodwin & Hanson(1975)

Lagenidium sp.	- trifuralin (Treflan, Lilly) fungistatic at 1 ppm, fungicidal at 3-5 ppm Note:- tested in infected penaeids.	Bland, Ruch, Salser & Lightner (1976)
Lagenidium sp. *Sirolpidium* sp.	- effective against zoospores and non-toxic to larvae; - in larval rearing tanks at 10-100 ppb Note:- used in infected penaeids	Aquacop (1977a) Le Bitoux cited by Lightner (1988)
	- malachite green 0.01 ppm	Tareen (1982)
Hydrozoan medusae (*Moerisia lyonsi*, *Stylactis arge*, *Clytia gracilis*)	- formalin 250 ppm for 1 hr static bath (used with larvae) - nigrosin 100 mg/l and concentrated seawater	Sandifer *et al.* (1974) Choa & Liao cited by Johnson (1982)
Air breathing insects	- diesel fuel and motor oil mixed 10:1 poured over the water surface as repeated treatments.	Johnson (1982)
Phytoplankton algal blooms (*chlorella* sp.)	- Clarosan (algicide) 0.02 mg/l	Aquacop (1979a)

Note: Information concerning treatments used in *Penaeus* sp. and other crustaceans are contained in the following references:

Aquacop 1977a; Sindermann 1977b; Schnick, Meyer, Marking, Bills & Chandler 1979; Tareen 1982; Brock 1988; Lightner 1988.