INTERNAL DEFENCE REACTIONS

OF

LITTORINA LITTOREA

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INTERNAL DEFENCE REACTIONS OF *LITTORINA LITTOREA*.

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ABSTRACT

The phagocytic system of the periwinkle and the *in vivo* and *in vitro* response towards foreign particles was studied by light and electron microscopy, using carmine, yeast (*S. cereviciae*), human erythrocytes, bacteria (*E. coli*) and viruses (lambda bacteriophage) as test-particles. The involvement of humoral factors was studied by haemagglutination/ haemagglutination-inhibition experiments. It was found that:

1) Injected markers are cleared by diapedesis through the epithelium covering the head, the mantle cavity and the foot (exluding the 'sole' of the foot). Emboli formed in the circulation are cleared relatively quickly by circulating haemocytes; haemocytes containing the marker may persist for longer periods in adjacent tissues but eventually find their way to diapedesis sites through circulatory routes. Clearance of carmine is not completed during the observation time (64 days), whereas yeast appears to elicit a much quicker response. Intracellular digestion may enhance the removal of injected markers in the case of digestible materials.

2) Periwinkle haemocytes form a homogenous population with respect to morphology (as determined by light and electron microscopy) and function (all haemocytes appear to be capable of migrational and phagocytic responses towards injected markers). The haemocytes are avidly phagocytic when challenged *in vivo* or *in vitro* with vertebrate RBC or yeast; around 90% of the haemocytes contain one or more particles in 30 min, and the average content of each phagocyte is 2.2 particles. Additional uptake apparently proceeds at a steady state which is considered to depend upon the rate of intracellular digestion or membrane synthesis. The correlation of *in vitro* to *in vivo* results is discussed, and lower values in the latter case are attributed to diapedesis.

3) The connective tissue of the periwinkle is composed of ground substance with fibres resembling collagen, and 5 types of cells, i.e. pore cells, calcium cells, supportive cells, granular cells and tissue-associated haemocytes. Among these, phagocytosis of injected markers, i.e. human RBC, bacteria (*E. coli*) and virus particles (lambda bacteriophage) is restricted to haemocytes, which by their morphology cannot be classified as a separate subset, and so it appears that the periwinkle does not contain 'fixed' or tissue resident phagocytes. Reports of 'fixed' phagocytes in other invertebrates are discussed, as well as functional aspects of the pore cell.

4) The haemolymph of the periwinkle contains a factor which agglutinates several types of vertebrate erythrocyte in addition to bacteria (*E. coli*) and yeast (*S. cerevisiae*). The active part of this molecule is a protein, and so it classifies as a lectin (Goldstein *et al* 1980). Agglutination of H-RBC was inhibited most strongly by L-xylose, but moderate inhibition was obtained with other pentoses (D-ribose, D-xylose, α-L-fucose). The lectin appears to function as a defence molecule, since (1) a moderate increase is found in circulating levels upon antigenic challenge (human erythrocytes), and (2) particles which are agglutinated by the lectin are phagocytosed more avidly than particles which fail to agglutinate. The 'baseline' level of the lectin is quite variable between individuals, and is not affected by age or sex; this polymorphism is believed to be genetically determined, although to some extent it may also be modified by environmental factors (starvation, antigenic challenge).

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LIST OF CONTENTS:

ABBREVIATIONS

AER = agranular endoplasmic reticulum
APC = antigen presenting cell
AS = ammonium sulphate
aSW = artificial sea water
BSA = bovine serum albumen
c4-bp = complement c4 binding protein
CMC = cell mediated cytolysis
CR (1-4) = complement receptor (1-4)
C-RBC = chicken erythrocytes
CRP = C reactive protein
CVF = cobra venom factor
DAF = decay accelerating factor
DC = dendritic cell
d.f. = degrees of freedom
EDTA = ethylene diamine tetra-acetic acid
ER = endoplasmic reticulum
FcR = Fc receptor
$Fc_\gamma R$ = receptor for IgG
fMLP-R = formyl-met-leu-phe receptor
GER = granular endoplasmic reticulum
G-RBC = gerbil erythrocytes
HAI = haemagglutination inhibition
Hcy = haemocyanin
Hg = haemoglobin
HMS = hexose monophosphate shunt
H-RBC = human erythrocytes
HRP = homologous restriction protein
Ig, IgG = immunoglobulin (G, etc).
IL = interleukin
K-cell = killer cell
kd = kilodalton
KDO = 2-keto-3-deoxyoctonate
LTB4-R = leukotriene-B4 receptor
LPS = lipopolysaccharide
MAC = membrane attack complex
MCP = membrane cofactor protein
MPO = myeloperoxidase
Mϕ = macrophage
N = number
NEM = N-ethyl-maleimide
NK, NK-cell = natural killer cell
PAF = platelet activating factor
PBS = phosphate-buffered saline
p-i = post-injection
PI = phagocytic index
PMN = polymorphonuclear granulocyte
RBC = red blood cell
R-RBC = rabbit erythrocytes
Rh^+, Rh^- = rhesus (+ or -)
SD, SE = standard deviation, standard error
SRBC = sheep erythrocytes
T_h, $T_{h/i}$ = helper, or helper/inducer T cell
T_{dh} = delayed type hypersensitivity T cell
T_c = cytolytic T cell
TCA = trichloroacetic acid
TER = transitional endoplasmic reticulum

SECTION 1: INTRODUCTION.

1.1 Historical notes.

Since prehistoric times, the human suffering caused by diseases has been a constant incitement for man to gather knowledge about illnesses and their treatments. The fight against diseases was in early times based mainly on empirical observations which only much later became explained in scientific terms; the medieval practice in oriental countries of deliberately catching smallpox from mildly infected patients (variolation) was thus based upon an observation dating back to 11th century China, that a survivor of one attack would never catch the disease again. Likewise, the observation made by Edward Jenner in 1796, that scab material from a similar but milder disease sometimes caught from cattle, cowpox, could be used to *vaccinate* people against the dreaded smallpox, was based upon the empirical observation of local old-wives tales, that cowpox offered a lifelong protection from both diseases. Important as this discovery was, leading ultimately to the worldwide eradication of smallpox (WHO 1980), it was based upon the chance finding of two closely related diseases, and about a century passed until the process involved could be explained and the principle of vaccination extended to other diseases.

The turning point came with the identification of living organisms as the causative agents of disease in 1876, which in turn was made possible by a breakthrough improvement in the design of microscopes. Dating back to around 1600, the compound microscope was in Jenner's time still plagued by chromatic aberration of the lenses, causing a rainbow of colours around the object examined; in spite of great intrinsic magnifying powers, the *effective* magnification was not much greater than that of a single lens, and the instrument could only be used to its full advantage after the advent of achromatic lenses in 1827. Until this time, claims of disease organisms, which had been repeatedly made by earlier authors could not be substantiated by microscopical examination; although Leeuwenhoek's observations in 1676 and 1683 had revealed the existence of microscopical organisms, they were barely visible in the compound microscope, and Leeuwenhoek's observations, resting on his own skill as a maker of single-lens microscopes, could barely be repeated, let alone extended for more than a century.

Table 1.1: Major events leading to the foundation of histology, microbiology & immunology.

1600-1700: MICROSCOPY (from ca. 1600); outlines of cells described in plants (Hooke, Malpighi, Grew) and in animal blood (Leeuwenhoek), including nucleus in the latter case; no clear concept formed on their nature. Leeuwenhoek describes microscopical organisms ('infusoria'; 1676, 1683).

1700-1800: INFUSORIA & ABIOGENESIS; attempts to refute the theory of spon-taneous generation (abiogenesis) with heating experiments (Joblot 1711, Spallanzani 1765, 1776) were criticized for the exclusion of air. The existence of 'infusoria' inspire the conclusion (Marten 1720) that diseases can be caused by microscopical organisms which differ with the disease. Vaccination (Jenner 1796). The concept of tissues (Bichat 1800).

1800-30: DISEASES: Various plant and animal diseases are attributed to fungi.

1830-50: ACHROMATIC LENSES (1827-30); the omnipresence of the nucleus is noted (Brown 1831); microscopical work mounts evidence for the universal occurrence of cells as basic constituents of plant and animal tissue, culminating in the CELL THEORY (Schleiden 1838, extended to animal tissue by Schwann 1839). Kölliker's textbook in histology in 1852 marks the emergence of HISTOLOGY as a separate discipline.

Improved classification of 'infusoria' (Ehrenberg 1835, Dujardin 1835-41), leaving only unicellular organisms. Attempts to refute the theory of abiogenesis (improved heating experiments; Schultze, Schwann 1836) still meet with opposition, as does also the concept of yeast as a microscopic plant, responsible for alcoholic fermentation (Schwann 1837, Kutzing 1837; Cagniard-Latour 1837). PROTOZOA are unicellular organisms (Von Siebold 1845).

1850-65: COMPARATIVE HISTOLOGY founded by Leydig's textbook on vertebrate and invertebrate histology (1857). Virchow's textbook on PATHOLOGY (1858) applies the cell concept to diseased tissues. Scultze lays down the modern view of the cell as 'a lump of nucleated protoplasm' (1861-3).

MICROBES (yeast) are alive and are responsible for the fermentation of sugars (Pasteur 1857); they arise by reproduction of pre-existing microbes (Pasteur 1860). Both findings support that microbes may also play a role in diseases.

1870-1900: Further work with the light microscope (e.g. Strasburger, Flemming) lays the foundation for CYTOLOGY which emerges into independence with O. Hertwig's textbook in 1893.

Continued work in microbiology (Pasteur, Koch) lays the foundation for the science of BACTERIOLOGY (the golden age of bacteriology 1870-90); the isolation of the anthrax bacillus (Koch 1876) marks the victory of the germ theory of diseases, thus paving the way for the study of IMMUNOLOGY, which is founded in 1884 by Metchnikoff's cell theory of immunity. The theory met with opposition from adherents of the humoral theory of immunity, notably Paul Ehrlich, after repeated observations on aspects of immunity in the absence of cells, e.g. bactericidins (Nuttall 1888), antitoxins (von Behring & Kitasato 1890) precipitins (Kraus 1897), bacteriolysins (Pfeiffer 1894), and agglutinins (Gruber & Durham 1896).

1900- Enhancement of phagocytosis by serum opsonins (Wright & Douglas 1903). Humoral reactions traced to one group of substances (antibodies) by the unitarian concept (1930). Intense work in all areas of immunology, leading to subdivisions (immunochemistry e.t.c).

The advent of metachromatic lenses in the 1830's marked the beginning of a new era in microscopy, leading to the establishment of two extremely important new branches of science, histology and microbiology (table 1.1). Both disciplines of course are rooted in earlier observations. HISTOLOGY thus benefits from the discoveries of early microscopists like Malpighi, Hooke, Leuwenhoek, Grew, Swammerdam and Lieberkuhn. But due to the limitations of early microscopes, these findings fall into the category of 'microscopical anatomy' rather than true histology, and the concept of tissues was not advanced until 1800. MICROBIOLOGY, although rooted in the study of 'infusoria' was founded through the work of Louis Pasteur (1822-95). Before his studies in 1856-60, the status of yeast was a hotly debated issue, and bacteria were largely unnoticed. Equipped with the modern compound microscope, Pasteur demonstrated, in 1857, that souring of wine was accompanied by microscopical rods and spheres that differed from the typical yeast globules; both types of fermentation could be stopped by gentle heating at the right time, and each type of fermentation could be transferred with the respective type of microbe. At this time, Pasteur believed the smaller rods and spheres to be a different type of yeast, and it now became accepted that yeast is alive, and responsible for fermentation. Pasteur also laid the foundations for the study of BACTERIOLOGY by continued studies of this kind in 20 years to follow. After moving to Paris in 1859, Pasteur went on to counter arguments that microbes could be spontaneously generated, and demonstrated in 1860 that microbes only arise by the reproduction of pre-existing microbes. This caused most philosophers to abandon the theory of abiogenesis, a hotly debated issue for centuries (table 1.1); the theory received it's final blow by Tyndall's sterilization experiments in 1877.

Pasteur used the term 'disease' to describe contamination of fermenting liquids; it had previously been shown that some plant and animal diseases were caused by fungi (table 1.1), but the theory of microbial origin of diseases was not widely supported, and the involvement of bacteria was not suspected. Pasteur's findings that a tiny parasite was the causative agent of the silkmoth disease (1865) won further support for the theory, which became fully established with the isolation of the anthrax bacillus by HH Robert Koch in 1876. The golden age of microbiology (1870-90) ensued, with most of the basic methods being developed, and over 20 important bacterial vectors of disease established.

The victory, in 1876, of the germ theory of diseases paved the way for the science of IMMUNOLOGY, the study of how life responds to the threat of invading microbes and parasites through the action of specialized cells and their products. Some observations made by early histologists had hinted at the importance of leucocytes in pathology. Histology was already in Pasteur's time an established branch of science, with textbooks in general as well as comparative aspects of the discipline (table 1.1), and both leucocytosis as a pathological phenomenon (Virchow 1858) and phagocytosis of injected dye particles (Haeckel 1862) had been described. The importance of these observations could not, however, be fully appreciated at the time; the defence value of phagocytosis only became apparent seven years after the victory of the germ theory of diseases. In a study of the starfish embryo in 1882, the Russian zoologist Elie Metchnikoff discovered that a rose thorn which he introduced into the embryo attracted an accumulation of leucocytes. The occurrence of bacteria inside leucocytes had previously been interpreted as a bacterial invasion of the cells, but Metchnikoff now went on to prove that this was due to active engulfment; in 1883 he demonstrated that the leucocytes of a transparent aquatic crustacean, *Daphnia*, could provide protection against fungal spores by attacking and destroying the pathogen. A year later he extended his observations to vertebrates, and noted that a previous encounter with the pathogen (by infection or vaccination) promotes an enhanced engulfment or phagocytosis by leucocytes, and concluded that phagocytosis is the prime method of defense. His cell theory of immunity in 1884 marks the emergence of immunology as a separate discipline. Four years earlier, Pasteur's attenuated vaccine against chicken cholera had reintroduced the subject of vaccination, followed by vaccines against anthrax (1881) and rabies, and so, about a century after Jenner's cowpox vaccination, a real attempt to explain the underlying phenomenon had been put forward, and vaccination had become a tool in the hands of the new science.

Metchnikoff's theory was criticized by those who observed defensive reactions in the absence of cells (table 1.1), suggesting immunity due to humoral factors. Proponents of this theory (especially Paul Ehrlich and other workers in Koch's laboratory) gained unexpected support from Metchnikoff's own laboratory in 1895, when Jules Bordet proved that cytolysis requires 2 factors, one thermolabile and nonspecific (complement) and the other thermostable and specific (antibody). Both factors were subsequently shown to facilitate phagocytosis, or function as opsonins (Wright and Douglas 1903), and later findings that antibodies and some

complement components are in fact produced by leucocytes further demonstrate the mutually reinforcing nature of the two theories.

As outlined above, some of the main contributions to the new science came from studies with invertebrates. Haeckel worked with snails and crayfish, and Metchnikoff's discovery of phagocytosis as a defense mechanism came from studies with the starfish and the water-flea. He was also able to explain diapedesis in vertebrates as an active process (rather than a local vascular lesion) because he had already observed the same process in invertebrates lacking definite blood vessels. Most of the interest that the new science gained however was based on the obvious implications it had for medical science, and studies on invertebrate defense mechanisms were practically abandoned during the depression and the second world war, after an inspired start in the 1890's and the first two decades of this century (for a review of early studies, see Huff 1940; Cheng & Sanders 1962; Cheng, Thakur & Rifkin 1970; Sindermann 1971).

1.2 Contemporary studies.

Studies on the internal defense of invertebrates however regained popularity in the 1960's, and have now become a well established branch of science. The current knowledge rests on studies initiated in the sixties and seventies by biologically or medically oriented scientists who apparently entered the field from two main directions; studies may accordingly be divided into:

(1) **Pathological studies**, spurred by the need to control and/or culture invertebrates of medical and economic importance. These studies were initially published mainly by periodicals concerned with either parasitology or the aquaculture of 'shellfish' (oysters, lobsters, crayfish etc.) but later acquired their own vehicle, the Journal of Insect Pathology (founded in 1959), which in 1963 changed its name to the Journal of Invertebrate Pathology, in line with the increasing volume of items dealing with invertebrates other than insects. As implied by the name, the journal deals with invertebrate pathology, but the accumulated list of contents also reveals an increasing interest in invertebrate defense reactions. The journal is currently edited by Thomas C Cheng.

(2) Studies in **comparative immunology**, spurred by curiosity about the origins and evolution of the immune system, and/or the desire to develop simple research models on biological mechanisms common to vertebrate and invertebrate animals (e.g. phagocytosis, leukocyte spreading and locomotion) in the absence of complicating factors like specific immune reactions to injected materials. These studies were initially published in various immunological and cytological journals, but acquired a vehicle in 1977, Developmental and Comparative Immunology (founding editor Edward L Cooper).

The current knowledge on invertebrate defense mechanisms also owes much to general studies on agglutinins, many of which are less concerned with the possible defensive value of these molecules than their potential in biomedical research; these studies have appeared in haematological and biochemical journals as well as in the proceedings of annual lectin meetings (Bög-Hansen 1981, 1982; Bög-Hansen *et al* 1983). A detailed review of the vast amount of findings connected with the internal defense of invertebrates would thus require good knowledge of such diverse subjects as invertebrate pathology and parasitology, comparative immunology and the study of lectins. Such a formidable task can of course not be undertaken here; at present, only a few points will be mentioned in order to highlight the areas which appeared worthy of investigation at the outset of the current project, with reference mainly to some review articles available at the time (Stauber 1962; Cheng & Sanders 1962; Cheng & Rifkin 1970; Tripp 1963, 1970, 1974, 1975; Feng 1967; Bang 1970, 1973[a,b], 1975[a,b]; Lafferty & Crichton 1973; Anderson 1975; Michelson 1975).

Compilers of the literature generally agree that the defensive response of invertebrates is mainly cellular, i.e. phagocytosis (in the case of small particles) or encapsulation (particles larger than ca. 10 μm). The mechanism underlying both responses is thought to be identical (Cheng & Rifkin 1970; Bang 1973[b]), and the first stage of encapsulation can easily be envisaged as a special case of phagocytosis, in which the large diameter of the particle precludes fusion of the pseudopods on the distal side (cf. the settling of vertebrate adherent cells on glass or plastic surfaces, which is commonly likened to phagocytosis of a particle with infinite diameter). Most claims of humoral manifestations of the defensive response have focussed on the possible involvement of agglutinins. The immobilizing effect of these molecules upon potential pathogens is an obvious advantage for the host, and

agglutinins have also commonly been implicated as opsonins (e.g. McKay & Jenkin 1970[a], Arimoto & Tripp 1977, Renwrantz & Mohr 1978). Apparently, the main questions regarding the internal defense of invertebrates thus revolve around (1) THE EXTENT AND THE CAPACITY OF THE PHAGOCYTIC SYSTEM, and (2) THE INVOLVEMENT OF AGGLUTININS IN THE DEFENSIVE RESPONSE. These questions have been accentuated by several reports on 'fixed', or tissue resident phagocytes in invertebrates (Reade 1968, Stuart 1968, Pauley & Krassner 1972, Crichton *et al* 1973, Killby *et al* 1973, Johnson *et al* 1981, Johnson 1987), and were particularly stressed by Bayne (1973[b]), after comparing blood cell counts and *in vitro* phagocytosis values to the observed rate of clearance of bacteria from the haemolymph of the pulmonate *Helix pomatia*. At the outset of the current project it appeared necessary to address at least five main topics in order to solve the main controversies connected with this research field, *viz.*:

(a) the number of haemocyte types and the relative contribution of each to the defensive response.

(b) the involvement of 'fixed', or tissue resident phagocytes.

(c) the ultimate fate of ingested markers (i.e. the relative contribution of diapedesis and intracellular digestion to the clearance process).

(d) the overall capacity of the phagocytic system (i.e. the relationship between *in vitro* phagocytosis values and *in vivo* events)

(e) the involvement of agglutinins in the defense.

The biggest gaps in our knowledge about the internal defense of invertebrates however relate to the fact that it is based on studies on only a limited number of species, belonging mainly to four classes in two phyla, i.e. bivalves and gastropods (Mollusca), and crustaceans and insects (Arthropoda). Among these, the gastropods have been less extensively studied than the other groups, with studies almost exclusively confined to pulmonates. It seemed worthwhile to extend the studies to a more primitive member of the gastropod class. The prosobranch *Littorina littorea*, being common on rocky shores all around Britain and easy to keep in tanks, was therefore chosen as a research model, investigating (a) the morphology of the haemocytes (with brief comparison to as wide a taxonomic range of gastropods as possible), (b) the phagocytic capacity of the leucocytes, (c) the path of elimination of injected material, (d) the contribution of resident connective tissue cells to the defense and (e) the involvement of serum molecules. The results will be

presented in separate sections dealing respectively with each of the above topic, and discussed briefly at the end of each section. An attempt will also be made to outline more fully the general features of invertebrate defense mechanisms at a later stage (Section 7); reference will however only be made to selected examples, as an exhaustive review of the studies made in the field is clearly outside the scope of this thesis. Let us first look at the general anatomy of the animal under study.

1.3 *Littorina littorea*.

The common, or edible periwinkle is found on most rocky shores around Britain and Ireland. Its anatomy and external features will only be briefly outlined here in connection with Fig. 1.1, paying special attention to the circulatory system; for a more detailed description on these aspects as well as on its behaviour and ecology, the reader is referred to Fretter and Graham (1962).

The most conspicuous feature of the periwinkle, like other prosobranchs, is the spirally coiled *shell*, into which the snail retracts in adverse conditions, closing the aperture with a horny plate on the posterior part of the foot, the *operculum*. In this position, all the older and smaller whorls of the shell are occupied by the soft tissues collectively termed the *visceral hump*, the tougher and bulkier anterior parts being confined to the *body whorl*. The body whorl is lined by a muscular extension from the body wall, the *mantle*, which encircles the anterior parts with the *mantle cavity* in between. A current of sea water enters the mantle cavity on the left and leaves on the right, propelled by the ciliated branchial leaflets which hang down into the mantle cavity on the left side. The right gill has been lost due to torsion. Just below the *gill* on the left, an *osphradium* tests the current for contaminations. Contaminating particles are trapped by the mucus passing over the gill from the *hypobranchial glands* on the right side, and are swept out by the exhalant current. Severe contaminations, or other adverse conditions cause the snail to contract the *columellar muscle*. This runs from the centre spine, or *columella*, of the shell to the *operculigerous disc* on the dorsal posterior surface of the foot, with some muscle strands also running to the mantle skirt. Under favourable conditions the columellar muscle is relaxed, and the head and the foot emerge from the shell, and the *mantle edge* becomes visible around the shell aperture as the snail crawls over rocks and seaweed, grazing on minute algae.

The muscular contractions of the **foot** are used for locomotion, aided by the movement of cilia, which cover the skin in general but are especially concentrated on the sole. The *head* is normally lowered to the substratum as the snail crawls along, and the *tentacles*, one on either side, move slowly to and fro, testing the substratum for food. They are tactile and olfactory, and each carries an *eye* laterally at the base, as the eyestalk has become fused to the tentacle. When food is encountered, the snail lowers the *snout* and a muscular *odontophore*, covered by a shiny cuticle and supported by a pair of *buccal cartilages*, appears from the mouth and is pressed against the substratum. A belt of cuticular material, the *radula*, bearing teeth in regular longitudinal and transverse rows, runs backwards and forwards over the odontophore, scraping food particles from the substratum with the backwards thrust. These pass between the lips into the *oral tube*, and hence through the inner lips into the *buccal cavity*. As the food is passed along the oesophagus it becomes mixed with secretory products of the paired *salivary glands* for lubrication and possibly digestion. The coiled *radular sac* lies above the oesophagus anteriorly. The smaller food particles are transferred from the *stomach* through a series of branching ducts to the lumina of the *digestive diverticula* where food is digested and absorbed. Digestion is mainly extracellular, aided by enzymes slowly dissolving from the *crystalline style* of the stomach. Waste products are passed through the *rectum*, bulging into the mantle cavity from the mantle on the right side, to the *anus* anteriorly.

The mantle arises in the region between the head-foot and the visceral hump (often referred to as the *central connective tissue*). Towards the left of this region lies the *heart*, and directly above it, the *kidney* communicates with the *pericardium* by means of a ciliated canal. It also communicates with a section of the reproductive tract and thus forms a part of the *coelom*. The left anterior wall of the kidney forms a part of the *nephridial gland*, which borders the pericardial cavity. The kidney discharges to the innermost part of the mantle cavity by a slit-like opening with conspicuous lips. Within the mantle cavity towards the right, in females, a series of glands (*capsule gland*, *albumen gland*) forms the pallial section of the *oviduct*. In males, the duct from testis opens by a pore deeply within the mantle cavity on the right of the rectum, and discharges to a ciliated groove which runs forward through a rich, glandular field, the *prostate gland*, and to the *penis*, which is posterior and ventral to the right tentacle. In females, instead of the penis, and occupying the same position, there is an unpigmented glandular tract running

down the side towards the foot, the *ovipositor*. The *gonad* is located in the visceral hump, mainly on the columellar side. The *nervous system* consists of a ring of six interlinked *ganglia* surrounding the oesophagus (cerebral, pleural and pedal pairs) and the *visceral loop*, going from these to the visceral mass, with five or six ganglia (the pleurals, a single or double visceral, and the parietals); the ganglia give off nerves to various organs.

The circulatory system is composed of arteries and veins, linked by sinuses and lacunae. Blood leaves the heart by the *bulbus arteriosus*, which separates into an anterior and a posterior aorta shortly after leaving the ventricle of the heart. The latter runs along the left border of the kidney, to which it sends a number of *renal arteries*, and then runs along the columella for most of its length, branching among the acini of the digestive gland and gonad, supplying these organs as well as the stomach and intestine by emptying the blood into the visceral haemocoel. The *anterior aorta* runs along the oesophagus, passing from the dorsal to the ventral side in the midregion. Before penetrating into the cephalopedal haemocoel it sends off a branch to the anterior part of the stomach. Below the radular sac, the anterior aorta passes ventrally through the nerve ring into the pedal haemocoel and divides into a main *pedal artery*, which branches to supply blood to the haemocoelic spaces of the foot, and a main *cephalic artery*, which enters the buccal mass to supply that organ after giving off a *radular artery* which forms a sinus around the radular sac. From these organs, the blood escapes to the cephalopedal haemocoel, and is then taken by the venous return through a short *cephalopedal vein* to the kidney. Shortly before entering the kidney on the right side, this vein unites with *visceral veins* returning blood from the visceral haemocoel. The blood is taken through numerous *renal vessels* in the walls of the kidney sac and then partly to the nephridial gland (from which it is carried by an efferent vein to the heart) but mostly by means of the *efferent renal vein* to the mantle, from which it passes along a series of vessels passing to the left between the lobes of the hypobranchial gland. "They ramify and anastomose among themselves and from this network arise the different channels passing into the ctenidial leaflets" (Fretter & Graham, p. 275). The cephalopedal and the visceral haemocoels are also connected with haemal spaces in the mantle skirt, the latter mainly through a large vessel lying around and on the right of the rectum. Blood from these spaces is gradually collected into the *afferent branchial vessels*, and after passing through spaces within the gill lamellae it reaches the auricle through the *efferent branchial vessel* in the ctenidial axis.

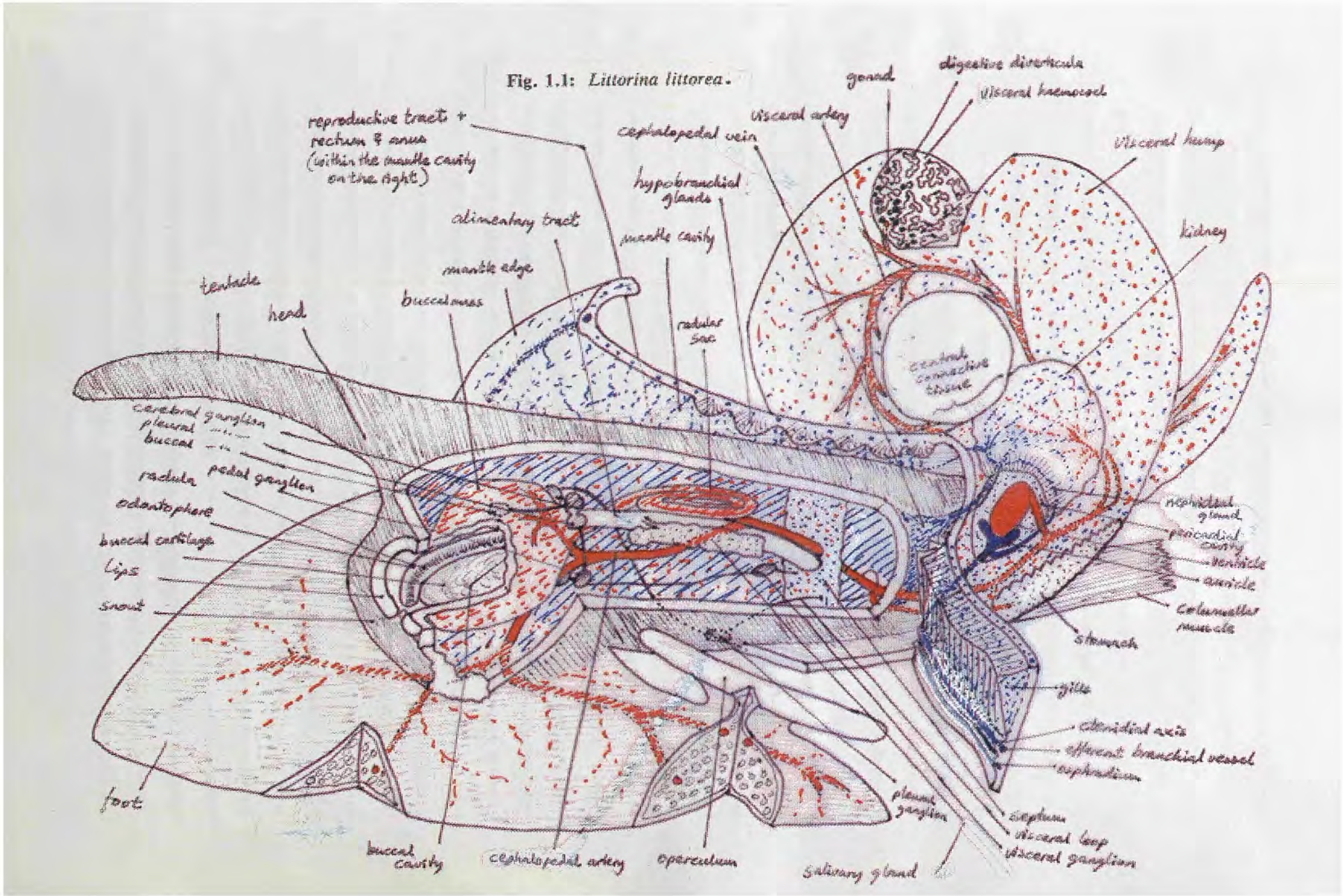


Fig. 1.1: *Littorina littorea*.

SECTION 2: HAEMOCYTES.

2.1 Introduction.

In the animal kingdom, the destruction or neutralization of invading microorganisms and metazoan parasites is brought about mainly by circulating cells and their products. These reactions are particularly well known in vertebrates, where many different types of leucocytes and their products cooperate in a highly complex fashion to eliminate effete host cells as well as foreign organisms. Invertebrates have been less well studied, but the importance of circulating cells for clearance of foreign materials has been universally stressed (e.g. Bang 1975; Anderson 1975; Ratcliffe & Rowley 1981) and is well established for molluscs (Tripp 1963, 1970; Michelson 1975; Sminia 1981). Cephalopods apparently rely on a single type of haemocytes for defence (Cowden & Curtis 1981), but morphological heterogeneity is observed in bivalves (Cheng 1981), and pulmonates (Sminia 1981). Apart from one ultrastructural observation (Yoshino 1976), prosobranch haemocytes have been studied only at the light microscopical level (George & Ferguson 1950; Brown & Brown 1965; Cheng, Thakur & Rifkin 1969; Davies & Partridge 1972), and no common agreement has been reached about the number of different cell types. As an appropriate beginning to the research into the internal defense mechanism of *Littorina littorea*, the haemocytes were studied under the optical as well as the electron microscope, and are here described for the first time. In order to gain more insight into the structural diversity of prosobranch haemocytes, and to facilitate comparison of these to other gastropod haemocytes described in the litterature, the circulating cells of the pulmonate, *Helix aspersa*, the archaeogastropods *Patella vulgata* and *Gibbula umbilicalis*, the mesogastropod *Crepidula fornicata* and the neogastropods *Nucella lapillus* and *Bucchinum undatum* were also studied in 'hanging drop' preparations, using Nomarski phase interference optics. The haemocytes of the four latter species are also described here for the first time.

2.2 Materials and methods.

ANIMALS. Periwinkles were collected at Saltdean, East-Sussex and maintained under constant aeration in recirculating artificial sea water (aSW; 1.8% salinity) at 12 °C for at least a week before use. They were fed regularly, and food remains as well as dead individuals were removed as necessary. The animals were relatively free from parasites*, and remained healthy for many months under these conditions (death rate less than 5% per month) but experiments were performed within 6 weeks of collection.

Snail food was prepared in the following manner (dr. EB Andrews, pers. comm.). 10 g complan and 10 g nettle powder were dissolved in one litre of distilled water at 60 °C, and 10 g of sodium alginate added slowly while stirring. After cooling, a thin layer was poured into a developing dish, and covered with 5% $CaCl_2$. The calcium alginate layers thus obtained were kept in tap water in a refrigerator.

Garden snails (*Helix aspersa*) were collected in a private garden in Surrey, but specimens of *Patella vulgata*, *Nucella lapillus*, *Crepidula fornicata* and *Gibbula umbilicalis* were collected at Saltdean. Specimens of *Bucchinum undatum* were collected at Plymouth (courtesy of dr. D. Herberts). The snails were used within a week after collection.

HAEMOLYMPH EXTRACTION AND TREATMENT. The limpet was sampled from the pallial vein after the method of Davies & Partridge (1972), but the other snails from the haemocoelic sinus surrounding the oesophagus (see Section 1.3). Before the snail could retract into the shell, the operculum was caught with forceps and the snail forced as much out as possible. The thumb nail of the left hand was used to secure the operculum against the shell, so that the animal could not retract while being sampled (Plate 2.1[a]). This left the right hand to manouvre the syringe, but to prevent sudden jerks of the syringe from loosening the inserted needle, a Luer 23 gauge needle was broken from its base and the blunt end connected to a plastic catheter, the other end of which was connected to the syringe by an undamaged 23 gauge needle (Plate 2.1[a]). The haemolymph was aspirated by Gillette disposable 1 ml syringes. The sampling system was sterilized with 70% methylated alcohol, and flushed at least 3 times with distilled water.

MICROSCOPY. Haemocytes and tissue sections were studied and photographed in a Zeiss photomicroscope, fitted with Nomarski interference and ordinary phase contrast optics. Cell dimensions were measured under oil immersion, against a calibrated ocular micrometer. Living haemocytes were studied in a 'hanging drop',

* Thorough microscopical examination of over 40 snails (Sections 4 & 5) only revealed one positive example.

made by inverting a coverslip containing a drop of freshly drawn blood and placing it upon a vaselin-sealed aluminium ring mounted on a slide. Haemocyte viability was tested by trypan blue exclusion (Weir 1973).

Haemocytes were vitally stained by placing a drop of blood on dry coverslips which had previously been treated with 0.05% neutral red (C.I. 50040) and/or 0.05% Janus green B (C.I. 11050) in absolute ethyl alcohol (Humason 1962). Permanent preparations were made by staining air-dried monolayers with 1% toluidine blue for 5 minutes, or with the methods of May-Grunwald/Giemsa (Culling 1974), Leishman (Drury and Wallington 1967), or Wright (Humason 1962); the latter two fix and stain in one step but staining with non-alcoholic stains was preceded by brief fixation in formaldehyde vapour.

CELL COUNTS. The effect of experimental stress upon haemocyte numbers was assessed by comparing haemocyte counts from snails at different times after inoculation or injury. Snails were injected with aSW suspensions of carmine (0.3% w/v) or *Saccharomyces cerevisiae* (7×10^8 cells/ml), while others were injected with aSW (all inocula measuring 0.5 ml) or prodded with the needle without being injected. The aSW used for injection was sterilized by filtering (Millipore, 0.2 μm pore size). Three individual blood samples were obtained from each group at 3½ and 9 hours, and from the injected groups at 1 and 20 hours, and haemocyte counts were compared with each other and with haemocyte counts from 8 untreated snails.

Haemocytes were counted in an Improved Neubauer haemacytometer (Hawksley), examining a minimum of 50 fields each measuring 0.05 mm^2. Haemocyte aggregation was prevented by 0.5% N-ethyl maleimide in aSW (NEM; Thompson *et al* 1978) to increase accuracy of haemocyte counts. Its use was restricted to cell counts, as in behavioural studies, especially those on phagocytosis (Section 4), impaired cellular adhesiveness could influence the results.

2.3 Morphology and behaviour of *Littorina* haemocytes.

With the sampling method described above, haemolymph volumes of 350 μl or even more were obtained from large animals, although most samples were in the range of 80-220 μl. The blood of the periwinkle contains ca. 5-10 million haemocytes per ml (Table 2.1), the average being 7.1 ± 1.8 millions/ml (N=8). Numbers of circulating cells apparently fluctuate in the first hour after challenge with carmine or yeast (Fig.

Fig. 2.1: L. littorea haemocyte counts

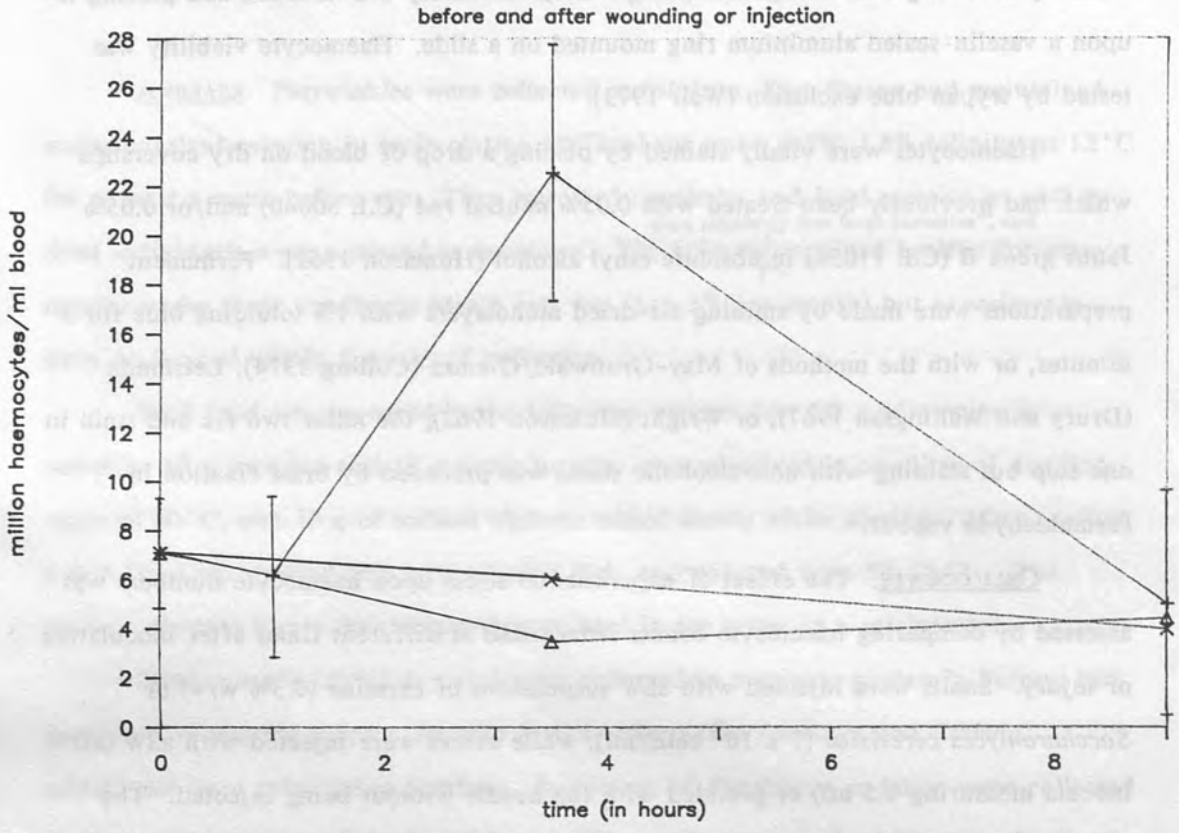


Table 2.1: Haemocyte counts and viability.

HAEMOCYTE COUNTS*								
T_0		T_1	$T_{3.5}$	T_9	T_{20}		injury	sham	test
9.6	test	9.2	17.0	3.3	5.4	T_0	7.1 ± 2.3	7.1 ± 2.3	7.1 ± 2.3
4.8		2.4	19.7	2.3	3.1				
7.4		9.6	28.5	13.0		T_1	-	-	6.2 ± 3.3
9.0		3.5	25.0	1.6					
6.1				5.0		$T_{3.5}$	6.0 ± 3.0	3.5 ± 2.1	22.5 ± 5.2**
4.7	sham		5.6	4.9					
4.5			1.4	3.6		T_9	4.0 ± 2.0	4.4 ± 0.6	5.0 ± 4.6
10.6				4.8					
	injury		4.7	6.8		T_{24}	-	-	4.3 ± 1.2
			3.1	2.3					
			10.2	3.0					

HAEMOCYTE VIABILITY in vitro**				
Incubation	viability			average ± S.D.
1 hour:	99%	95%	98%	97.3 % ± 1.7 %
2 hour:	96%	99%	96%	96.3 % ± 2.1 %
4 hour:	98%	95%	97%	96.7 % ± 1.2 %

* The table lists individual counts (to the right) as well as means and standard deviations (to the left) of haemocytes before (T_0) and after challenge (T_1-T_{24}), which involved injecting the snails with carmine- (test) or seawater (sham) or wounding them with the needle (injury).

** The table lists individual viability measurements (obtained by counting at least 200 haemocytes in trypan blue), and the calculated means and standard deviations.

2.1) but show a dramatic increase at 3.5 hours, followed by a decline to base levels at 9 hours. Injection of aSW apparently causes a transient leucopaenia, whereas injury by itself does not evoke a change in haemocyte numbers.

The haemocytes remained viable for at least 4 hours in a 'hanging drop' preparation (Table 2.1). Haemocytes suspended in a drop of freshly drawn haemolymph are approximately spherical, measuring about 6-7 μm in diameter with nuclei about 5 μm (N=10). Cells that contact while in suspension tend to adhere to each other; aggregates so formed are of course most prominent if cell contact is enhanced by agitation, but temperature also plays a role, and cooling the haemolymph suppresses the phenomenon. Suspended haemocytes have a phase-bright appearance, but on contacting the glass surface or attached cells they settle onto the glass (Plate 2.1^{b}), and then normally spread out, becoming progressively darker as they flatten against the substrate. Haemocytes usually start moving shortly after contact with the glass, in which case they do not spread out appreciably. Before contacting the glass, or shortly afterwards, they form a large, fan-shaped outgrowth at one end (Plate 2.1^{c-e}), which becomes the leading edge. The growth of this structure appears to involve a flow of cytoplasm into it, as cytoplasmic movement can sometimes be seen at its base when forming, and sometimes also just before the cell moves. This flow of cytoplasm appears to be reversible, since amoebocytes commonly change their course by retracting the leading edge and forming a new one (Plate 2.3).

The rate of amoeboid movement appears quite variable, and may perhaps depend on environmental conditions. A migration rate of 1 μm/min. appeared typical, although faster speeds were recorded. Amoeboid movement appears to be suppressed in some preparations, and instead of forming a leading edge, haemocytes remain static and flatten out, or **spread** (Plate 2.1^{f-j}). In extreme cases, spreading may progress to a stage in which cytoplasmic inclusions become very distinct, and the areas between them become confluent with the ectoplasm (Plate 2.1^{k}), suggesting that the distinction between endo- and ectoplasm may be partly artificial; the condition is however abnormal, and cell death usually ensues. Although fully spread cells were usually not observed to detach, spreading is up to a certain point reversible, and motility can be regained by forming a leading edge and retracting other cytoplasmic extensions, by which the cells reduce their spread area and round up slightly. Conversely, moving amoebocytes are often seen to pause and increase their spread area for a while, or stop completely and spread to full extent. This is especially seen upon contact between two moving cells; after a short pause, they often move a little apart, but usually remain in

contact, and thus have a very limited scope of movement (Plate 2.2[a,b]; also see Plate 2.5[a-j]). They usually stop and settle before long, and never crawl over each other. This contact inhibition is also shown by haemocyte aggregates. They spread by slow, centrifugal movement of the peripheral cells (Plate 2.2[c]) until all cells are in contact with the substrate, thus forming a monolayer. While crawling out, peripheral cells remain in contact with the aggregate, and all cells maintain contact through the whole cell body, not cytoplasmic extensions. Cells occupying the upper layers (i.e. not yet in contact with the glass) do not move or spread over their sister cells, and usually remain rounded. Monolayer formation was normally found to be a slow process, requiring more than 2 hours; monolayers were thus usually prepared by leaving the preparation for prolonged periods under humid conditions. Environmental conditions clearly affect the rate of monolayer formation; refrigeration thus reversibly suppresses all cellular activity, and is quite convenient for a 'slow motion' observation of spreading. After refrigeration, attached groups of haemocytes are seen as round cells without pseudopodial outgrowths, but warmed by the microscope illumination the peripheral cells soon form a 'halo' of ectoplasm, and shortly after, 'spikes' are seen to radiate into all directions. A lobose pseudopodium may be formed upon contact with another cell, but the cells remain static.

Spreading of course involves a decrease in focal depth, thus making cellular inclusions more discernible. The nucleus and other inclusions form the **endoplasm** centrally, but along the periphery, numerous thin, threadlike cytoplasmic extensions or **filopodia** are connected by a thin veil of inclusion-free **ectoplasm** (Plates 2.1[f-g]; 2.3). The filopodia appear to form mainly along the glass-liquid interface, although critical focussing may reveal additional veils with filopodia projecting into the medium above the cell. In aggregates, these outgrowths are only seen on surfaces not involved in mutual contacts, reflecting centrifugal movement of the peripheral cells (Plate 2.2[c]; also see Plate 2.5[k]). Solitary cells usually redistribute most of their ectoplasm to one side, which becomes the leading edge, with a few filopodia on the sides and at the rear (Plate 2.2[h], 2.3). The trailing filopodia (retraction fibrils) are usually long and thin, and have been observed to break during movement of the cell. In aggregates, loss of contact between neighbouring cells may cause one or both to redistribute its ectoplasm and change course until contact has been re-established (see Plate 2.5[a-j]). The central part of each filopodium often appears darker than the surrounding ectoplasm, and the impression may be gained that it contains an internal structure or spike, originating in the endoplasm (cf. studies on *Patella* haemocytes; Davies & Partridge 1972, Partridge &

Davies 1974). In other cases, however, its apparent flexibility made it difficult to outrule at the optical level a local thickening or even a fold of the ectoplasm membrane (Plate 2.2[d-g]). The **nucleus** is clear and without visible nucleoli (Plate 2.2[h]), but contains dark areas of chromatin. About 1-3 large, clear **vacuoles** and a variable number of smaller ones are commonly present (Plate 2.2[h], 2.3[a]), as well as 10-20 dark **granules**, that are round and regular in shape, measuring 1-2 μm in diameter. They often occur in groups of 2-5 (Plate 2.1[j,k]) and probably represent lysosomes, as they have been seen to accumulate at the periphery of vacuoles, suggesting fusion with phagosomes. They are also discernible on top of and along the periphery of RBC-containing phagosomes in phagocytosing cells (Plate 3.1[b-e]). Like lysosomes, vacuoles may move about in the cell, and small ones have been seen to coalesce; this may indicate vacuole formation by the fusion of pinocytotic vesicles. The gradual condensation of large vacuoles has also been observed in long-term observations. Vacuole contents are occasionally granular, in which case Brownian movements can clearly be seen.

Supravital staining supports the above observations on cell morphology. Neutral red (Plate 2.4[a]) leaves the cytoplasm clear, as well as some vacuoles, which are therefore believed to be primary phagosomes, but the stain is taken up by the granules (i.e. lysosomes) and some vacuoles, presumed to be secondary phagosomes, formed by the fusion of lysosomes with primary phagosomes. Janus green (Plate 2.4[c]) stains the cell body faintly violet, and later the stain accumulates into minute granules (0.5-1 μm), that are normally not discerned in unstained preparations. Electron microscope observations (Section 5.3) confirm that these are mitochondria. Small haemocytes (ca. 5 μm diam.) may contain only about 6-9 perinuclear mitochondria, with only a few lysosomes (or even none), but the average cell contains ca. 15-20 mitochondria, partly masked (and therefore difficult to count) by the vacuoles (1-3), the neutral red granules (commonly 10-20), and a number of clear vesicles. In the largest cells, 25-35 neutral red bodies may be discerned.

In sections (see Section 4) and in stained monolayers, the haemocytes are often slightly elongate, but their outlines appear mostly regular and without cytoplasmic extensions. The nucleus is often eccentric (also seen in living cells), and stains more darkly than the neutral to slightly basophil cytoplasm (Plates 2.4[b,d,e]). Cytoplasmic inclusions are normally not seen after fixing and staining. The dimensions of 8 haemocytes found in sections average 7.4 by 5.7 μm, with nuclei about 3.0 by 2.5 μm, corresponding to the dimensions of suspended cells in a 'hanging drop', whereas

spreading may increase the dimensions considerably in a two-dimensional plane; large, well spread haemocytes may thus reach dimensions of 10-15 by 15-20 μm including the leading front, although diameters of ca. 10-15 μm are more common.

2.4 Haemocytes from a few other selected gastropods.

MESOGASTROPODS. Comparison of periwinkle haemocytes to their counterparts in other gastropods reveals a striking correlation between haemocyte morphology and diversity on one hand and evolutionary relationship on the other. Haemocytes from the only other mesogastropod studied, the slipper limpet *Crepidula fornicata* (Plate 2.5) are barely distinguishable from their periwinkle counterparts, and like these, apparently form a maturation series ranging from small cells with a high nuclear-to-cytoplasmic ratio (as evidenced by their reluctance to form pseudopodial outgrowths along the substrate) to mature haemocytes resembling those already described from the periwinkle (ca. 6-7 μm in diameter, spreading to dimensions of 10-15 μm or more).

NEOGASTROPODS. The two neogastropods studied, *Bucchinum undatum* and *Nucella lapillus* exhibit more heterogeneity in their haemocyte morphology. Two morphological varieties can be distinguished, one thigmotactic with conspicuous cytoplasmic inclusions (Plate 2.6$^{a-c,l}$), the other actively amoeboid and devoid of large inclusions. This latter cell type clearly corresponds to the amoebocyte of the mesogastropods studied, and resembles it in general features, except that it is larger (diameter ca. 10-11 μm, nucleus ca. 6-7 μm). It also appears more active than its mesogastropod counterparts and does not flatten out as much on the substrate. Most or all of the ectoplasm is confined to the leading edge (Plate 2.6^{a-l}). In the endoplasm, a few vacuoles and a variable number of small granules may be discerned outside the relatively large nucleus. The thigmotactic variety appears larger than the amoebocyte (10-15 μm) although this may partly relate to the extent of spreading. It is round and regular in appearance (Plates 2.6^{a,l}), with a small and clear nucleus, and contains mainly large inclusions in *Bucchinum*, but is packed with small granules in the dogwhelk, with a strong yellowish tinge in some cells. The observations suggest maturation of both morphological forms from smaller cells with limited spreading ability, but it was not possible to resolve whether the two morphological forms represent functional subsets or merely developmental stages.

ARCHAEOGASTROPODS. The two archaeogastropods studied, the limpet *Patella vulgata* and the top shell *Gibbula umbilicalis* appear more diverse with respect to their blood cell composition than the meso- and neogastropods studied. Haemocytes of the top shell apparently form one maturating series, whereas limpet blood contains, in addition to amoebocytes, another granular variety (Davies & Partridge 1972; Cooper-Willis 1979). Top shell amoebocytes resemble their periwinkle counterpart in size and behaviour, but spread to a more irregular shape, with only a few ectoplasmic extensions around the margin that may look wide and fan-shaped (Plate 2.6^{n}) or thin and slender (Plate 2.6^{m}). Various small inclusions can be seen in the endoplasm (Plate $2.6^{n,m}$). Limpet amoebocytes resemble those of *Littorina* in most respects, but are larger (about 10 μm in diam. before settling and about 15x30 μm when spread), with a larger ectoplasmic fringe when spread. They also form ectoplasmic extensions much more readily while still in suspension.

PULMONATES. *Helix aspersa* haemocytes are larger than those of *Littorina* (Plate 2.6^{o}), or about 9-12 μm in diameter when freshly drawn, with relatively large nuclei (about 5-7 μm). When settling, they may form small 'pseudopodia' (Plate 2.6^{p}) which may persist in the fully settled cell as drawn out areas of ectoplasm (Plate 2.6^{q}). The bulky endoplasm contains various inclusions but no vacuoles, and the large nucleus is easily discerned (Plates $2.6^{o,q}$).

In spite of morphological differences, haemocytes from all gastropods studied exhibit certain behavioural characteristics in common. All haemocytes appear capable of adhering to and spreading over foreign surfaces (i.e. the coverslip), although this trait is somewhat suppressed in actively moving amoebocytes. A constant redistribution of ectoplasm between different parts of the ectoplasmic fringe is seen, and a leading edge may recede into the cell body and another form quite rapidly. Before the leading edge is formed, a flow of cytoplasm can clearly be seen at the front. The ectoplasm is mainly devoted to the foreign substrate (i.e. the coverslip), but hairlike extensions are also seen to protrude from the free surface of the cell. The uptake of medium or minute particles by the fusion of filopodia with the cell body or each other is commonly observed (Plate 2.6^{g-j}). Contact inhibition is a conspicuous feature of all haemocytes (Plate 2.5^{a-j}). Moving amoebocytes thus tend to stop and increase their spread area upon contact, and cellular extensions are limited to the areas of the cells not involved in mutual contact (Plate 2.5^{k}). In aggregates, movement is restricted to peripheral cells in contact with the substrate (Plate 2.5^{l-m}).

2.5 Discussion.

Haemocyte counts have been shown to fluctuate with temperature in gastropods (Pauley & Krassner 1971; Davies & Partridge 1972; Stumpf & Gilbertson 1978; Sminia 1981) as well as in bivalves (Feng 1965[a]). In *Biomphalaria*, there is a logarithmic increase in haemocyte counts with shell size (Stumpf & Gilbertson 1978) and observations on *Lymnaea* (Sminia 1981) support that cell counts may be age related in other gastropods as well. Middle-sized periwinkles contain around 7 x 10^6 haemocytes/ml at 12 °C, which compares well with the values given by Davies and Partridge (1972) for the limpet of 1000/μl at 5 °C and 9000/μl at 25 °C. The recorded values for pulmonates are much lower (Bayne 1974; Jeong & Heyneman 1976; Cheng & Auld 1977; Stumpf & Gilbertson 1978; Cheng & Guida 1980), commonly around 300/μl, but marine bivalves are closer to prosobranchs in this respect, with counts of about 1700/μl in the hard clam (Foley & Cheng 1974) and 700-4100/μl in *Tridacna maxima* (Reade & Reade 1976); in the oyster they are 2700/μl at 12 °C (Feng 1965[a]).

Injection of foreign markers results in an initial drop in haemocyte numbers followed by a slow return to normal values in the prosobranch *Bullia* (Brown & Brown 1965), the opisthobranch *Aplysia californica* (Pauley *et al* 1971[b]) and the pulmonates *Helix pomatia* (Bayne & Kime 1970) and *Lymnaea stagnalis* (van der Knaap, Sminia *et al* 1981). This transient leukopaenia may explain the somewhat decreased 1 hour values of the present studies, according to preliminary values from freshly injected snails, but conclusive evidence was not obtained due to difficulties connected with bleeding snails without trauma during the period in which they remain contracted. The values of Table 2.1 were obtained from relaxed snails, bled with the method described in Section 2.2, involving minimal trauma to the animal while bleeding. The increase in haemocyte numbers after inoculation of foreign particles seen in the present study has also been observed in the prosobranch *Bullia* (Brown & Brown 1965), the opisthobranch *Aplysia californica* (Pauley *et al* 1971[b]) and the pulmonates *Helix pomatia* (Bayne 1974) and *Lymnaea stagnalis* (van der Knaap, Sminia *et al* 1981), and after infection in *Biomphalaria* (Stumpf & Gilbertson 1978; Jeong *et al* 1980; Abdul-Salam *et al* 1980[a]); in *Lymnaea*, it is believed to be due to the release of cells from the connective tissue (Muller 1956; Sminia 1974, 1981). The haemocyte counts of bivalves are also known to increase after injury (Bubel *et al* 1977) and infection (Cheng 1966).

The blood of *Littorina littorea* was found to contain only one type of cell, the amoeocyte. This conclusion is also supported by electron microscope observations (Section 5) and functional studies (Section 4). Gastropod haemocytes have been studied by various workers (see review by Sminia 1981), but no common agreement has been reached on the number of different cell types. Electron microscope studies have simplified the problem, but most of the work has been done on pulmonates (Stang-Voss 1970; Fernández 1971; Sminia 1972; Harris 1975; Krupa *et al* 1977), with only two species studied among the prosobranchs, i.e. the mesogastropods *Cerithidea californica* (Yoshino 1976) and *Littorina littorea* (Section 5 of the present studies). Studies on the pulmonates indicate that two types of cells occur in the circulating fluid of planorbids (Harris 1975; Krupa *et al* 1977; Cheng & Auld 1977; Cheng & Guida 1980; Stumpf & Gilbertson 1980; LoVerde *et al* 1982; Ottaviani 1983) whereas ultrastructural reports on other pulmonates deny the presence of haemocyte subpopulations (Stang-Voss 1970; Fernández 1971; Sminia 1972, 1981; Sminia & Barendsen 1980; Sminia, van der Knaap & van Asselt 1983; Renwrantz 1979). The 'hyalinocyte' of planorbids constitutes less than 10% of the total count (Cheng & Auld 1977; Cheng & Guida 1980) and appears to differ from the amoebocyte in lacking lysosomes (i.e. acid phosphatase positive granules; Harris & Cheng 1975; Cheng & Garrabrant 1977). Among the prosobranchs, three haemocyte types have been described from neogastropods (George & Ferguson 1950), whereas two types are described in recent studies with the limpet (Davies & Partridge 1972; Cooper-Willis 1979), and the snails *Bullia* (Brown & Brown 1965), *Littorina scabra* (Cheng, Thakur & Rifkin 1970) and *Cerithidea californica* (Yoshino 1976). The present observations indicate that the exact number of haemocyte types in neogastropod blood may be a matter of interpretation, and that not all archaeo- and mesogastropods exhibit morphological heterogeneity with respect to blood cell composition. The need for careful interpretation of light microscope data is well illustrated by examples from the study of pulmonates and bivalves. Muller (1956) thus describes 4 types of cells from *Lymnaea stagnalis* blood with the light microscope, whereas ultrastructural studies do not support haemocyte subpopulations (Stang-Voss 1970; Sminia 1972). Similarly, reports on three haemocyte types in some bivalves (Foley & Cheng 1972, 1974; Narain 1973; Moore & Lowe 1977; Bayne *et al* 1979) and two in other, often closely related species (Takatsuki 1934; Nakahara *et al* 1969; Reade & Reade 1976; Cheng 1970; Ruddell 1971[a,b]) as well as larval forms (Elston 1980) may be reconciled by assuming that one of two haemocyte types is present in two developmental stages, as in fact

suggested by some authors (Cheng 1975; Cheng & Foley 1975; Moore & Lowe 1977; Cheng 1981). Much of the controversy over the number of different haemocyte types in molluscan blood may in fact result from the presence of a maturation series, recognized as one or two sub-types, depending on the author. George and Ferguson (1950) thus suggest that the larger two cell types of neogastropod blood may develop from type I amoebocytes, with interstitial stages seen, whereas in the present study, the interstitial stages were considered to preclude distinction of immature cells as a special cell type. The two forms of *Bullia* haemocytes may clearly also be looked upon as a developmental series of one type (Brown & Brown 1965). After studying 22 species from all subclasses of gastropods, Kollman (1908) concluded that all had two developmental stages of one, agranular haemocyte type. Even EM results may be open to interpretation; the three types of granules originally described from oyster amoebocytes (Feng *et al* 1971) later turned out to represent different optical views of one type (Cheng & Foley 1972). Final conclusions on the number of haemocyte types in molluscan classes and subclasses will probably not be reached until some general agreement on classification criteria exists, but pulmonates and prosobranchs are currently believed to possess one or two types (Sminia 1981; the present study), bivalves two (Cheng 1981) and cephalobranchs one type of haemocyte (Cowden & Curtis 1981).

In all studies cited above, the blood cells appear to fall into two categories: (1) granular cells, often eosinophilic, and (2) agranular or hyaline basophils. The latter may appear as one or both of two forms; a smaller, 'lymphocyte-like' cell with a relatively large nucleus, or a larger, 'macrophage-like' cell. The granules of the first group have been identified as **lysosomes** in the hard clam (Cheng & Foley 1975; Yoshino & Cheng 1976[b]) and as secondary phagosomes in the oyster (Cheng & Cali 1974); in both bivalves this cell type is correspondingly reported to be the most phagocytic one (Foley & Cheng 1975). This is also the type most active in cellular defense in planorbids (Jeong & Heyneman 1976; Anderson & Good 1976; Stumpf & Gilbertson 1980) as well as the prosobranch *Bullia* (Brown & Brown 1965); the only cell type found in the blood of the cephalopod *Eledone* (Stuart 1968) also appears to be granular. In the limpet however (Davies and Partridge 1972), and in the oysters *Pinctada radiata* (Nakahara & Bevelander 1969) and *C. gigas* (Ruddell 1971[a]), the agranular cell is the one reported as being most actively phagocytic. The phagocytic cell in *Tridacna* (Reade & Reade 1976) and in the mussel (Moore & Lowe 1977), is the less granular of the two present; the more granular variety may in the mussel function in the transport of reserve material (Moore & Lowe 1977). This clearly illustrates that

the classification of molluscan haemocytes should not be based upon morphology alone; in fact this applies to comparative haematology in general, since even in vertebrates, the granules of polymorphonuclear granulocytes may be too small in some species to be discerned by light microscopy (Welsch & Storch 1976, p. 284). Separation of haemocytes by gradient centrifugation may represent a more fruitful approach, and differences in surface characteristics, as revealed by different binding patterns of non-native lectins to different haemocyte fractions, has been used to support the presence of haemocyte subpopulations in the oyster (Renwrantz *et al* 1979, Cheng, Huang *et al* 1980). Such differences are also apparent in haemocytes of *B. glabrata* (Yoshino & Granath 1983) and *L. stagnalis* (Dikkeboom *et al* 1985), as revealed by monoclonal antibodies. However, the observations suggest that the differences may be due to maturation; it must be remembered, that subtle differences in membrane markers may characterize functional subsets in addition to blood cell types (cf. studies on mammalian lymphocytes). In general, it seems advisable to exercise caution in the classification of invertebrate haemocytes until correlation is found between structural and functional criteria.

Further studies are clearly needed before final conclusions can be reached on the structural and functional diversity of molluscan haemocytes. In the present studies, however, some advance has been made by preliminary examination of haemocytes from a number of species belonging to a relatively neglected taxonomic group, the prosobranchs, and a more detailed structural and functional study on haemocytes from one representative of this subclass, the periwinkle (cf. also Sections 4 and 5). *Littorina littorea* haemocytes appear to be smaller than their counterparts in most other molluscs except chitons (Chrichton *et al* 1973), *Tridacna* (Reade & Reade 1976), *Crepidula* and *Gibbula* (the present study), with dimensions more similar to those described from circulating stem cells of other molluscs in which larger types are also present (e.g. Moore & Lowe 1977). In other respects, they clearly resemble haemocytes of other molluscs (for further discussion on structural aspects, see Section 5.4). Functionally, the most conspicuous characteristic of periwinkle amoebocytes *in vitro*, apart from their migrating activity, is their strong thigmotactic behaviour. This is a well known feature of phagocytic cells in general, and has in studies on vertebrate adherent cells commonly been likened to phagocytosis of a particle with infinite diameter (Wright & Silverstein 1986). A relationship between spreading and phagocytosis has also been suggested for invertebrate haemocytes (Haughton 1934) and is supported by studies indicating competition between the two phenomena (e.g. Bertheussén 1981[a,b]; Rowley

1981; see Section 3.4). Extensive spreading (cf. Plate 2.2[g]) may cause the cell to extend its surface area to such dimensions as to weaken its integrity, and thus may have some analogy to the effect of overloading vertebrate PMN's with phagocytosable particles (Bridges 1981). The cytoplasmic extensions seen at the glass surface may partly be caused by surface tension, and it is difficult to assess to how much an extent they are a normal behavioural feature; in the electron microscope, the ectoplasm is seen to extend over adjacent structures but without forming long, drawn-out filopodia (Plate 5.5[a], 5.6[c]). The internal 'spike' described by Davies and Partridge (1972) may according to the present observations on periwinkle haemocytes merely represent an optical illusion brought about by a fold, or 'ruffling' (Alberts *et al* 1984, p 605) in the plasmalemma at the glass surface.

PLATES 2.1 - 2.6

Light micrographs of haemocytes from the blood of *L. littorea* (Plate 2.1-2.4), the slipper limpet *Crepidula fornicata* (Plate 2.5), the whelk, *Buccinum undatum* (Plate 2.6^{a-j}), the dogwhelk, *Nucella lapillus* (Plate 2.6^{k-l}), the top shell, *Gibbula umbilicalis* (Plate 2.6^{m-n}) and the roman snail, *Helix pomatia* (Plate 2.6^{o-q}). Bar: 10 μm.

Plate 2.1: (a) The method used for sampling blood and injecting. For further description, see text. (b-c) A haemocyte in suspension. Note the formation of pseudopodial outgrowths. (d-e) Haemocytes in migration. (f-h) A haemocyte settling on the glass (bar shown in (g)). (i-k) Spread haemocytes.

Plate 2.2: (a-b) A group of cells photographed with an interval of ca. 10 min. Note migration of the solitary cell. (c) A haemocyte aggregate settling on the glass. Note pseudopodial outgrowths from the free surface of marginal cells, indicating centrifugal movement. (d-g) A pseudopodium photographed at ca. 10-15 sec. intervals, showing redistribution of cytoplasm. (e) Two well spread haemocytes. The one on the left is moving (note leading edge at one end, and retraction fibril at the other). Cytoplasmic details (nucleus, vacuoles, granules) are clearly seen.

Plate 2.3: (a-c) A migrating haemocyte, photographed at intervals of ca. 1-2 min. (d) The same haemocyte ca. 5 min. later (note movement in relation to the fixed particle). Cytoplasmic details (nucleus, granules, vacuoles) and the shape of the ectoplasm while moving are seen to advantage in this plate.

Plate 2.4: (a-d) Haemocyte monolayers after staining with (a) neutral red, (b) Leishman, (c) Janus green and (d) Wright. (e) A section through haemocytes in a pallial blood space (iron haematoxylin + alcian blue, see Section 4). (f) A haemocyte monolayer after challenge with yeast (toluidine blue). One of the particles has been ingested (extreme left). (f-k) Wet preparations of blood sampled 1-2 hours after injecting snails with carmine (g-h) or yeast (i-k).

Plate 2.5: *Crepidula* haemocytes. (a-k) A 'tug-of-war' between two cells, photographed at intervals of ca. 10-30 sec. (l) In aggregates, pseudopodial formation is limited to the free surface of cells (contact inhibition). (m-n) Haemocytes spreading from an aggregate (interval ca. 2 min.).

Plate 2.6: (a-f) *Buccinum* amoebocyte in migration (intervals ca. 10-30 sec.). Also note stationary cell with inclusions. The microscope slide is repositioned after the third exposure. (g-j) Vesicle formation by the fusion of two pseudopods of a *Buccinum* amoebocyte (intervals ca. 10-30 sec.). (k) *Nucella* amoebocyte. (l) *Nucella* granulocyte. (m-n) *Gibbula* amoebocytes. (o) *Helix* amoebocyte in suspension. (p-q) *Helix* amoebocytes after settling.

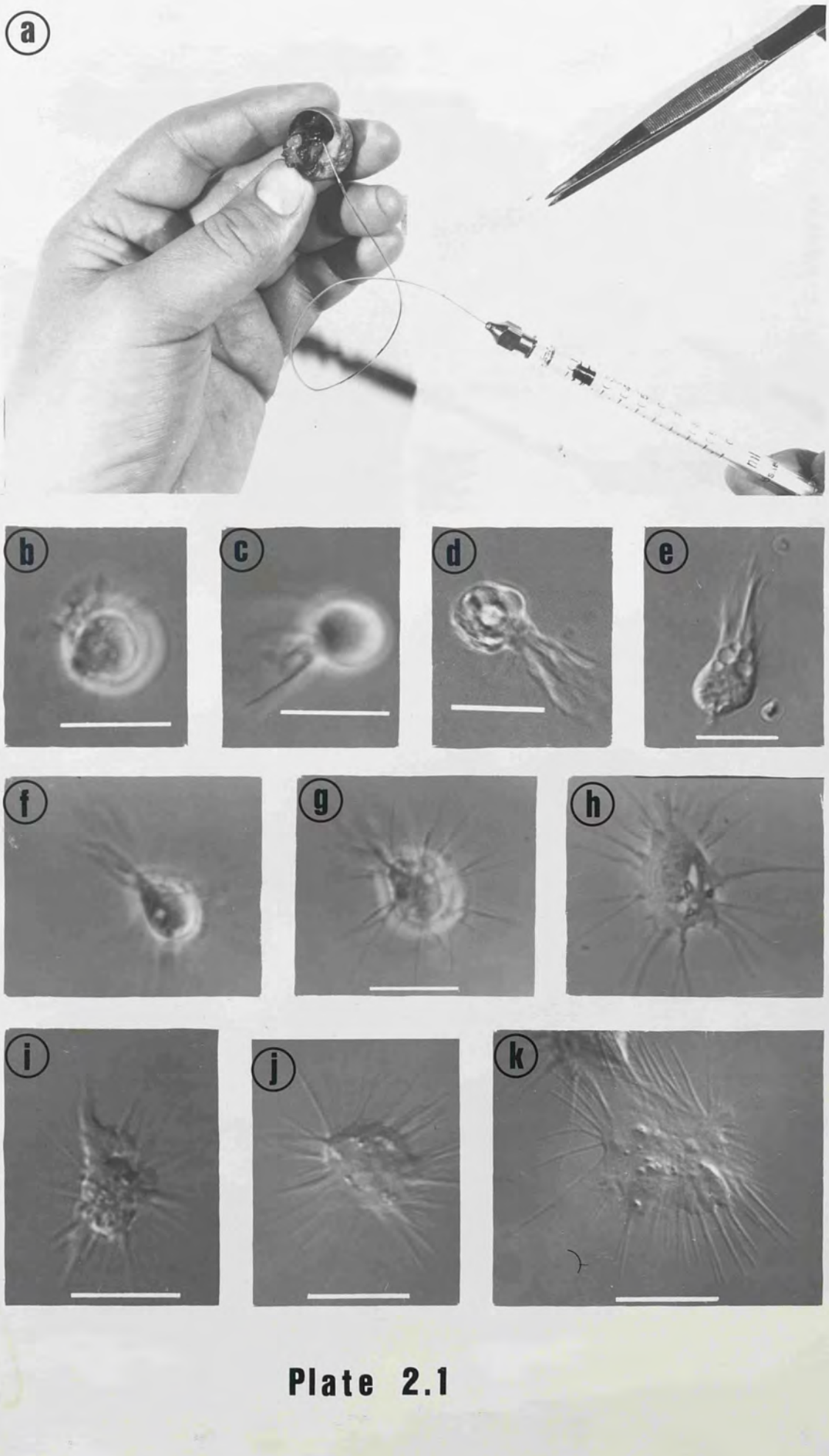


Plate 2.1

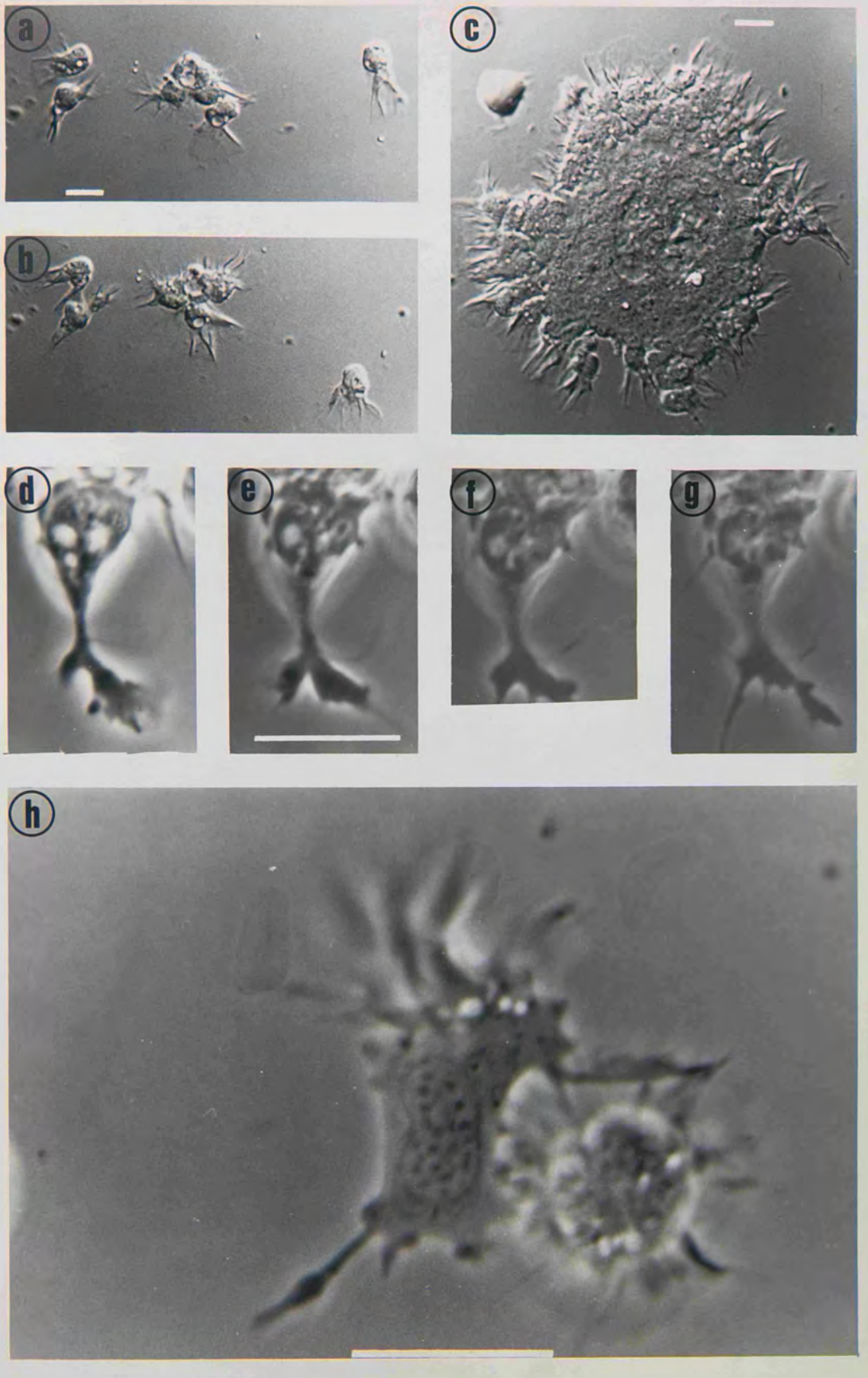


Plate 2.2

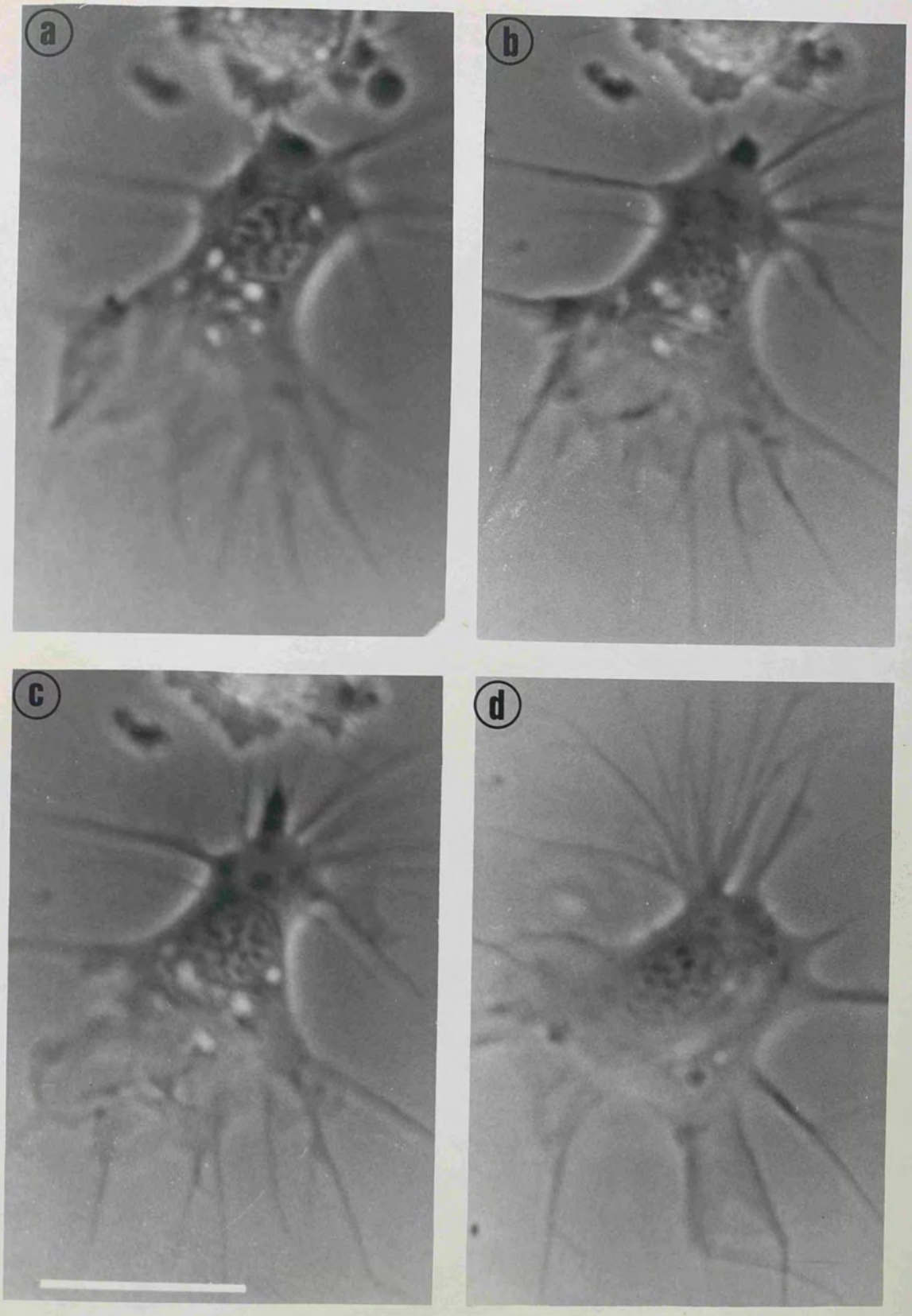


Plate 2.3

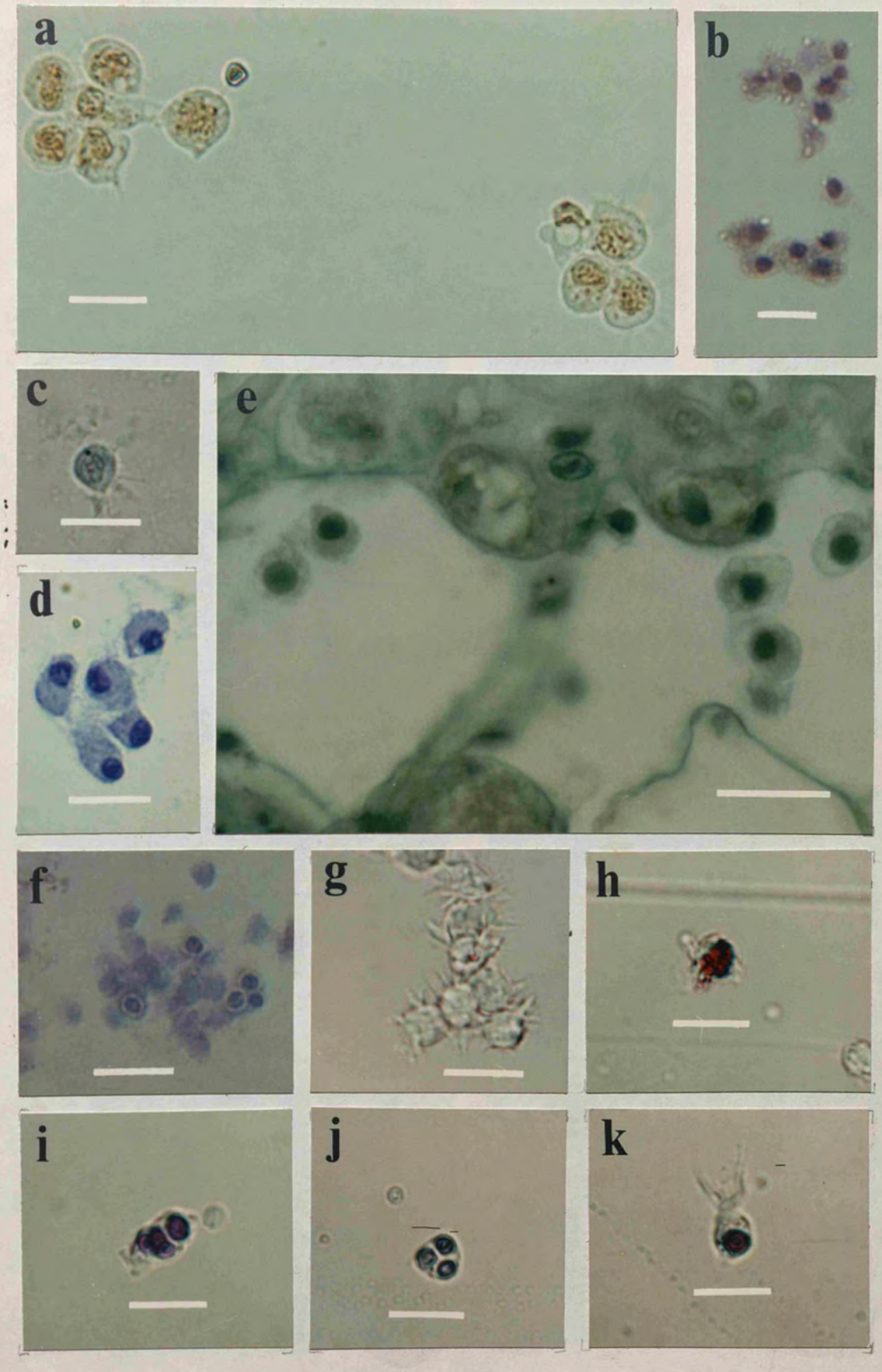


Plate 2.4

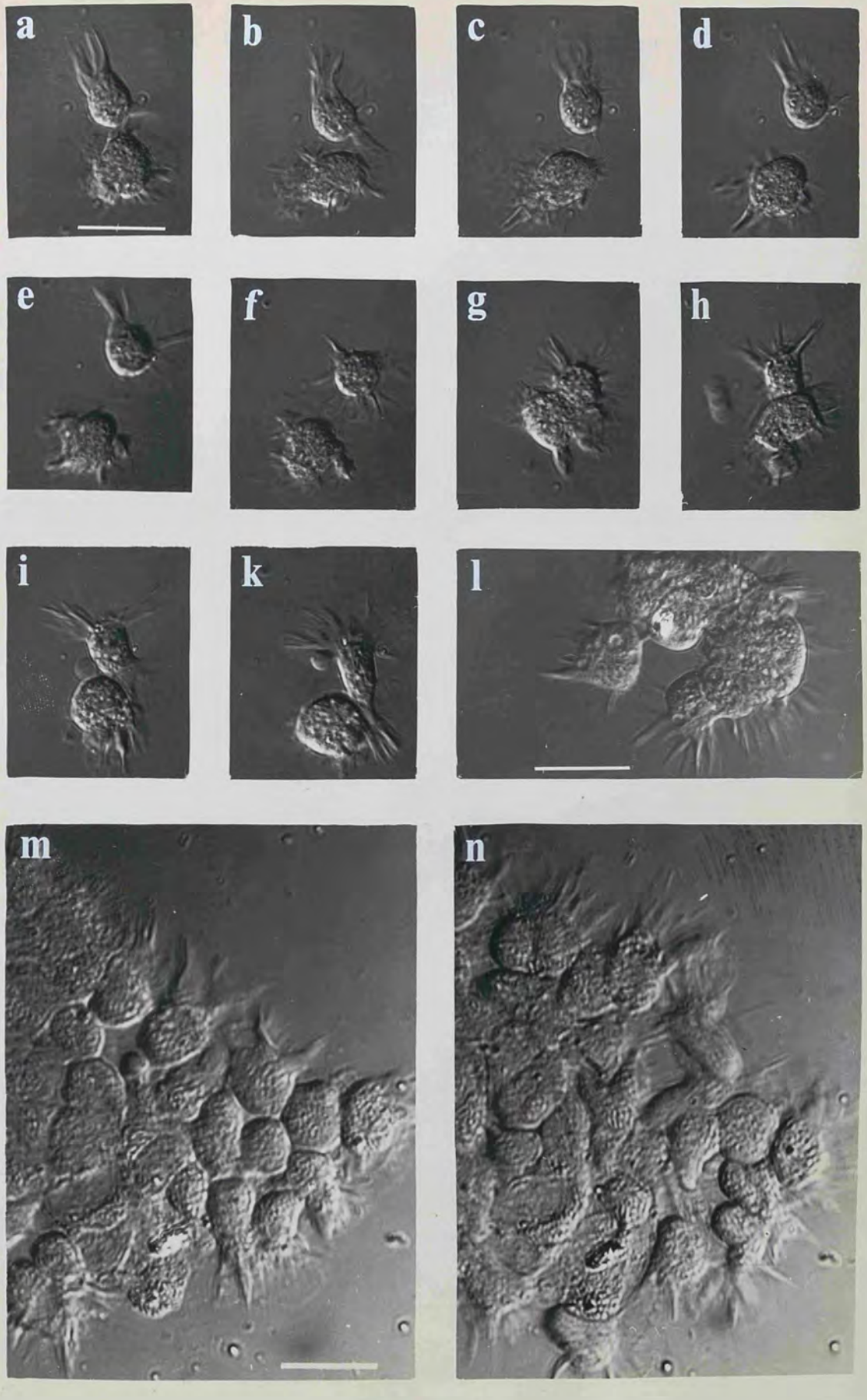


Plate 2.5

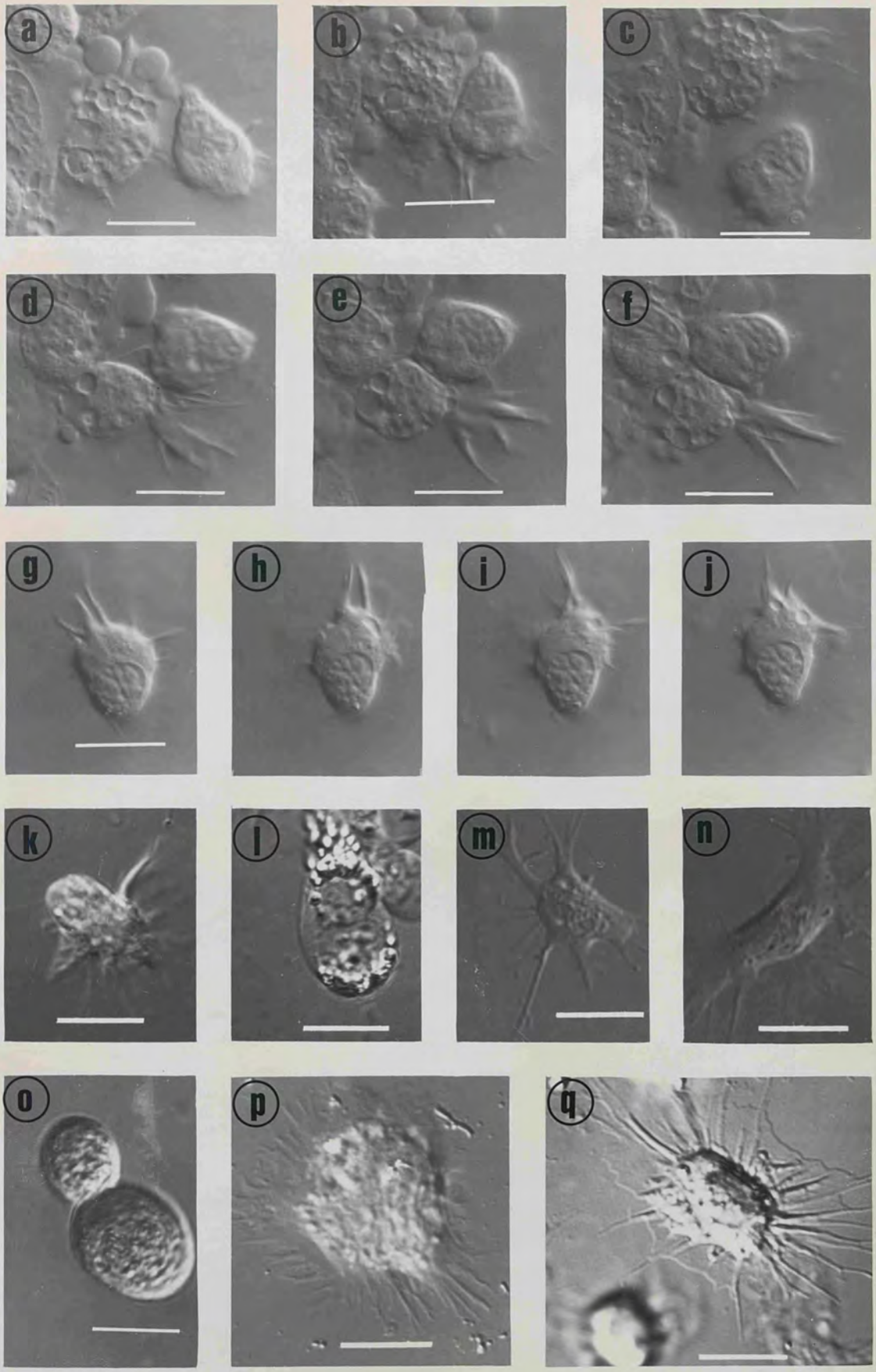


Plate 2.6

SECTION 3: PHAGOCYTOSIS.

3.1 Introduction.

Studies on phagocytosis have been common practice in invertebrate zoology since Metchnikov (1893) first drew attention to its importance in defense. Early studies with molluscs emphasize the nutritional rather than the defensive aspect (Cuénot 1914; Yonge 1923, 1926; Takatsuki 1934), but the value of phagocytosis for the internal defense of molluscs (Stauber 1950; Tripp 1958, 1960, 1961) as well as other invertebrates has been re-emphasized in recent decades; most collators of the literature now agree that manifestations of the internal defense against natural infections are mainly cellular, i.e. phagocytosis or, if the particle is too large for ingestion, encapsulation (e.g. Tripp 1970, Bang 1973[b]; Lafferty & Chrichton 1973). The involvement of haemocytes in these responses is known from studies on cephalopods (Stuart 1968), bivalves (Tripp 1958, 1960; Feng 1965[a,b]; Reade & Reade 1972, 1976) and gastropods (Tripp 1961; Brown & Brown 1965; Brown 1967; Arcadi 1968; Cheng, Thakur & Rifkin 1970; Pauley *et al* 1971[b]), and has lead to the general view that haemocytes function as the main effector cells in molluscan defense (Feng 1967). This view has been challenged by Bayne (1973[b], 1974), who concludes that haemocytes can not account for the rapid clearance of bacteria from the circulation of the pulmonate *Helix pomatia*, raising questions concerning the possible involvement of tissue-associated phagocytes or agglutinating substances in the response. A rapid rate of elimination or inactivation of inocula from the periwinkle, *Littorina littorea* was noted in a cursory experiment in which less than 0.1% plaque-forming activity of injected bacteriophage T2 could be recovered in 5 days. Preliminary experiments also confirmed that injected particles (carmine, yeast) were taken up by circulating cells, but in order to assess the relative importance of circulating cells in the clearance of foreign particles, a more detailed study on the effectiveness of the phagocytic response seemed appropriate. The response of circulating haemocytes towards foreign material was therefore studied *in vivo* and *in vitro*, using particles of small and uniform size, i.e. vertebrate RBC and fungal spores to enable exact quantitation of data.

3.2 Materials and methods.

In vitro PHAGOCYTOSIS. The *in vitro* experiments were designed to allow an assessment of the ability of haemocytes to ingest test-particles under optimal conditions. Samples of snail blood, containing ca. 6 x 10^6 haemocytes/ml were mixed to equal amounts of the test-particle suspension (ca. 5 x 10^7/ml) in Eppendorf tubes, and centrifuged for 1 min. at 250g. This method was found to leave the haemocytes intact, and ensure maximal haemocyte/test-particle contact; preliminary experiments with coverslip-attached haemocytes had indicated an element of competition between the flattening on glass surface and the internalization of particles. Attachment was allowed to proceed for 5 min., after which cells were resuspended by flushing the medium a few times with a Pasteur pipette. Phagocytosis was allowed to proceed for various periods, selected in accordance with previous observations on the ingestion rate of fresh HRBC, i.e. 3, 7, 12, 20, 30, 40, 60 and 90 min. Samples were stored at 4 °C after arresting phagocytosis by adding a drop of isosmolar 1% glutaraldehyde. After visual scoring of at least 100 haemocytes under the 40x objective lens, four parameters were calculated, i.e. (a) the percentage of haemocytes with one or more particles ingested, (b) the phagocytic index (PI), i.e. the total number of particles taken up by 100 haemocytes, and (c) and (d) corresponding values for the entire phagocytic process, counting the number of contacts in addition to ingested particles.

In vivo PHAGOCYTOSIS. The uptake of particles into circulating cells was assessed by visual scoring of blood sampled at different times after injecting snails with brewing yeast, *Saccharomyces cerevisiae*, which had been pre-stained with aniline-crystal violet (see below) in order to facilitate microscopical observations. 25 animals received a 0.5 ml injection in the head haemocoel (see Section 2.2), and blood was sampled in triplicates at 1, 2, 5, 12, 20 and 48 hours; the samples were mixed with a drop of isosmolar 1% glutaraldehyde and stored at 4 °C. The percentage of haemocytes with one or more particles ingested, and the phagocytic index (PI, see above) were noted after visual scoring of at least 100 haemocytes under the 40x objective lens; haemocytes with attached particles were rarely observed in these samples, and values for association were thus not calculated.

TEST-PARTICLES. Erythrocytes (type 0 Rh^+) were obtained from the authors fingers, and used fresh, or after fixation in formaldehyde (4%). Before use they were washed several times in 0.9% NaCl. Dried brewing yeast (*Saccharomyces*

cerevisiae) was obtained commercially. Before use it was fixed in 4% formaldehyde and stained by stepwise passage through aniline-crystal violet (5 min.) and Gram's iodine (3 min.), after which it was treated for 5 min. with 2% acetic acid in absolute ethanol to remove excess stain (modified after Drury & Wallington 1964, p. 331). Each step was followed by repeated washing, and the final volume, in aSW, was adjusted by optical density measurements (Section 4.2) to ca. 7 x 10^{11}/ml. The yeast was transferred between media by brief centrifugation (1000g, 5 minutes), and resuspended by shaking, and in some cases brief sonication (20-30 sec. in a Kerry KS 100 sonicator) to disperse aggregates.

MICROSCOPY. Phagocytosing haemocytes were photographed in a Zeiss photomicroscope fitted with Nomarski interference optics (*in vitro* experiments), and in a Stereoscan SP 600 scanning electron microscope; in the latter case, monolayers of haemocytes challenged *in vivo* with formalinized yeast were prepared for electron microscopy by formaldehyde fixation and dehydration by transfer through a series of upgraded ethanol, and examined after being coated with gold.

3.3 Results.

IN VITRO PHAGOCYTOSIS. Preliminary tests on haemocyte monolayers and a variety of particles (carmine, RBC, yeast) suggested that spreading on glass surfaces might interfere with the response of haemocytes towards foreign particles, as very low phagocytosis values were obtained by this method. A 5 min. incubation of haemocytes and test-particles in a pellet gave superior results, and this method was used for subsequent experiments. Aldehyde fixation was in preliminary experiments found to affect the affinity of haemocytes for the surface of RBC (compare attachment values of Table 3.1[a] to 3.1[b]) as well as the ingestion phase; attached particles were hardly ingested at all in the first 30 minutes, and very low phagocytic values were obtained at even prolonged incubation, with only 32% of haemocytes taking up particles (a total of 76) in 2 ½ hours. Fresh RBC was taken up at a much faster rate, and was therefore chosen for further observations on the phagocytic process. The results are presented in Table 3.1 and Fig. 3.1.

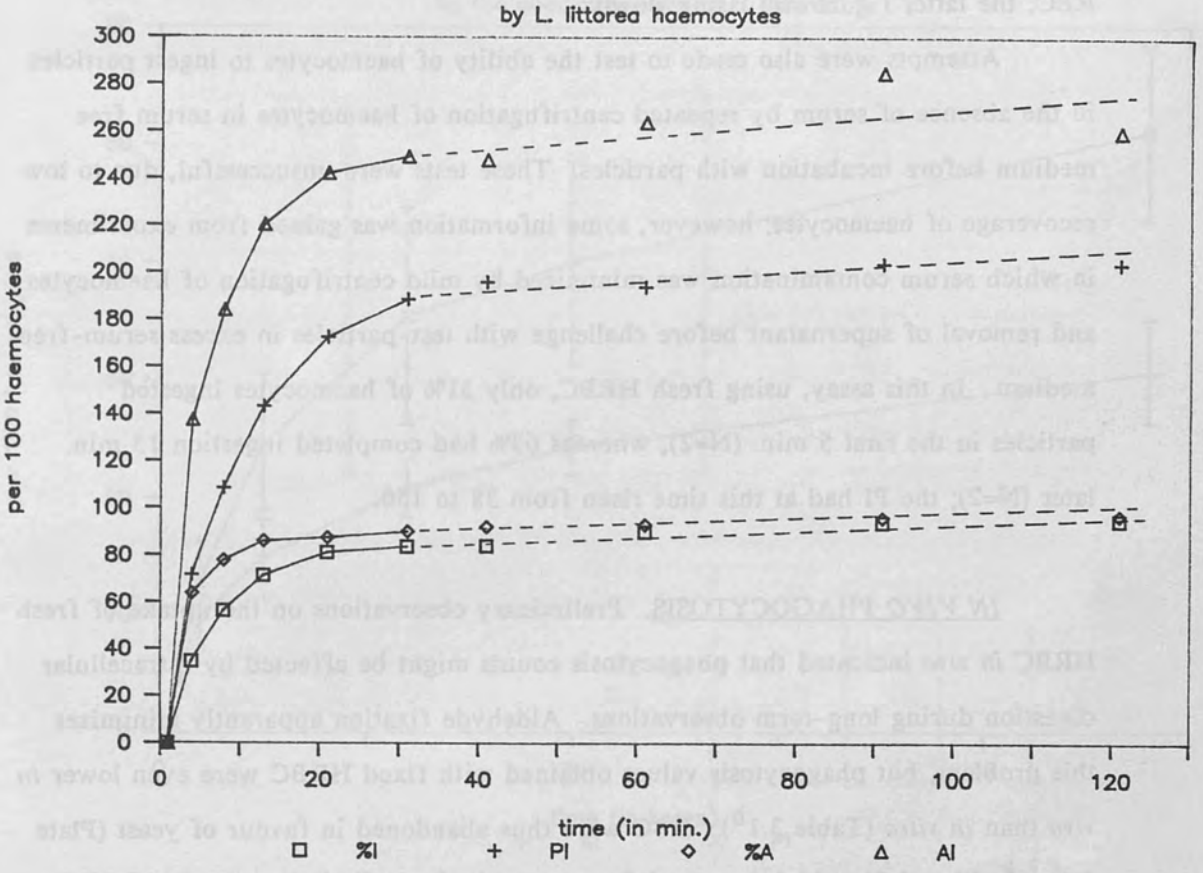
As shown, *Littorina* haemocytes are avidly phagocytic *in vitro* when tested against fresh, untreated human RBC (Fig. 3.1). Test-particles become attached either to the cell body (Plate 3.1[a,e,f]) or to ectoplasmic extensions (Plate 3.1[c,g]) and are then ingested (Plate 3.1[c,d]). When examined continually for several minutes,

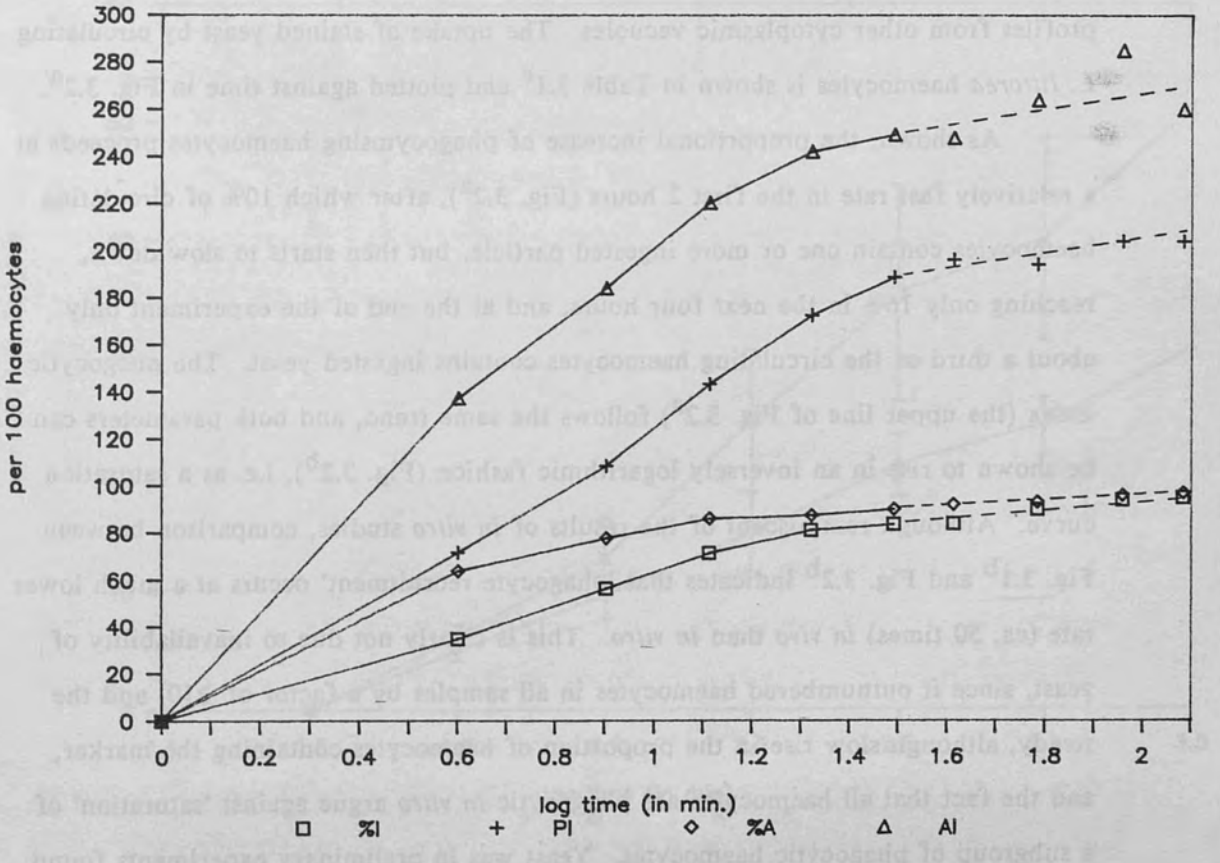
small dark granules, identical to those previously identified as lysosomes (Section 2.3) were often seen to move to the phagosome and accumulate at its periphery (Plate 3.1^{c-e}). Even at an early stage, a number of the ingested RBC were found to have lost their shape, forming round, dark vacuoles of characteristic size. Vacuoles with clear, transparent contents, recognized by their characteristic size and shape as RBC ghosts were also commonly seen, as were intermediates stages between these and the dark vacuoles. It was not possible to distinguish between intracellular digestion and extracellular haemolysis, since RBC ghosts were also observed free in the medium; however, the numbers of vacuoles appeared to increase during the experiment, indicating that at least some intracellular digestion takes place. Regardless of their digestive status, all profiles clearly resulting from the phagocytosis of erythrocytes were included in the phagocytic values calculated.

The results plotted in Fig. 3.1 support the observation made earlier (on account of morphology) that the haemocytes all belong to one population; all haemocytes appear capable of phagocytosis, and thus represent one functional group. The first collisions resulting in attachment of RBC to haemocytes probably occurred while the pellet was still forming, and the intercept of the upper line in Fig. 3.1 with the X-axis is thus probably at, or very shortly after the start of the experiment. Haemocytes continue to collide with and attach to RBC after being resuspended, although at a decreasing rate, and almost all are seen to make durable contacts in the observation time. Ingestion follows after a short lag period, and the shape of the curve (Fig. 3.1^{a}) suggests that it is limited mainly by the number of contacts made; the starting point of this phase of the phagocytic event is of course not known, and the intercept of the lower line in Fig. 3.1 with the X-axis is drawn by interpolation. An interesting fact emerging from Fig. 3.1 (compare PI to the phagocyte percentage) is that after the 30th minute, particle uptake can be accounted for almost entirely by the 'recruitment' of previously inactive haemocytes, suggesting that ingestion may not only be limited by the number of contacts made but also by other, intrinsic factors (e.g. cell volume). Numerically, this can be checked by calculating the ratio between PI and the proportion of haemocytes with ingested particles, which indicates that each active phagocyte ingests on average a maximum of 2.2 RBC, and this maximum has been reached in 30 min. A similar evaluation of the overall phagocytic process, including attachment, indicates that each active haemocyte has made durable contacts with 3 RBC on average; at the end of the observation time, almost all haemocytes are thus active in phagocytosis,

Fig. 3.1: Phagocytosis of HRBC in vitro

by L. littorea haemocytes





having ingested an average of 2.2 RBC each, and made contact with another 0.8 RBC, the latter figure still rising slowly.

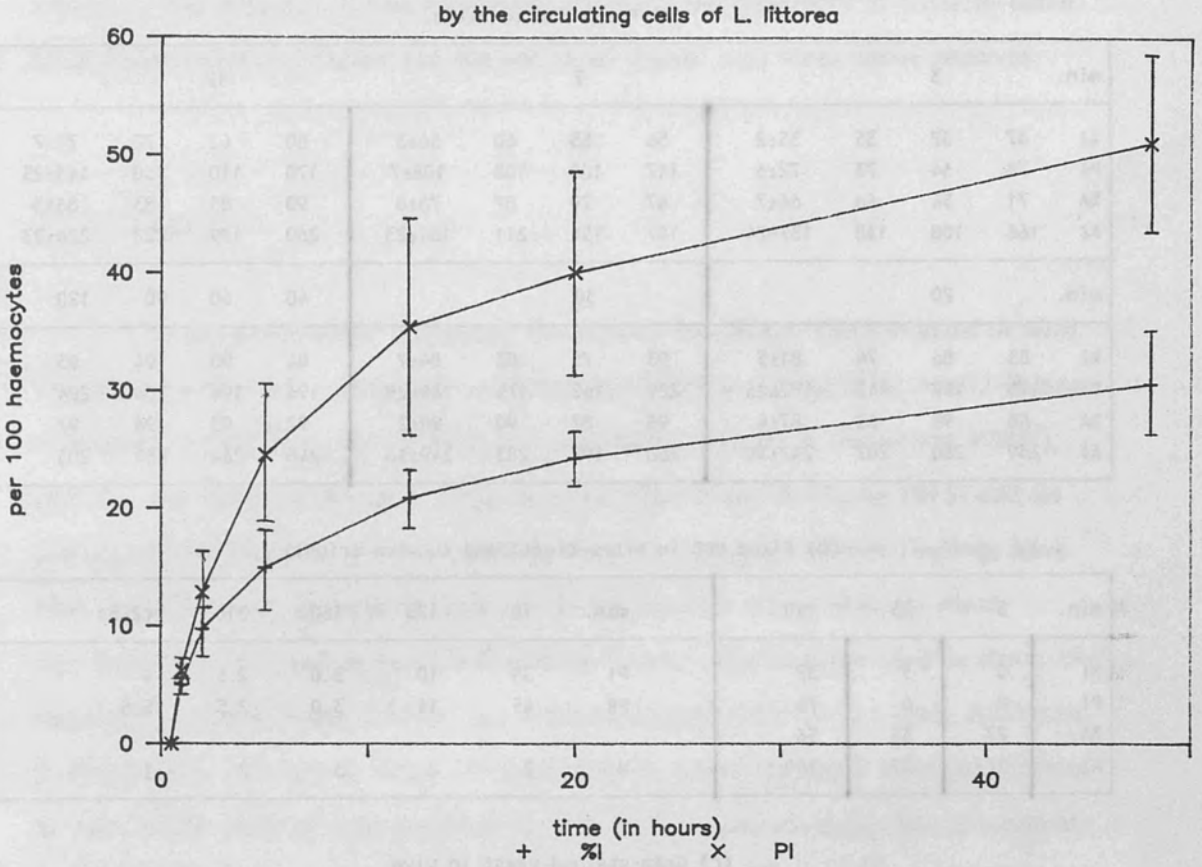
Attempts were also made to test the ability of haemocytes to ingest particles in the absence of serum by repeated centrifugation of haemocytes in serum free medium before incubation with particles. These tests were unsuccessful, due to low recoverage of haemocytes; however, some information was gained from experiments in which serum contamination was minimized by mild centrifugation of haemocytes and removal of supernatant before challenge with test-particles in excess serum-free medium. In this assay, using fresh HRBC, only 31% of haemocytes ingested particles in the first 5 min. (N=2), whereas 63% had completed ingestion 15 min. later (N=2); the PI had at this time risen from 38 to 150.

IN VIVO PHAGOCYTOSIS. Preliminary observations on the uptake of fresh HRBC *in vivo* indicated that phagocytosis counts might be affected by intracellular digestion during long-term observations. Aldehyde fixation apparently minimizes this problem, but phagocytosis values obtained with fixed HRBC were even lower *in vivo* than *in vitro* (Table 3.1[b]). RBC were thus abandoned in favour of yeast (Plate 2.4[f,i-k]; Plate 3.2), which gave satisfactory results in preliminary tests and had the extra advantage of being stainable, which facilitates the distinction of digestive profiles from other cytoplasmic vacuoles. The uptake of stained yeast by circulating *L. littorea* haemocytes is shown in Table 3.1[c] and plotted against time in Fig. 3.2[a].

As shown, the proportional increase of phagocytosing haemocytes proceeds at a relatively fast rate in the first 2 hours (Fig. 3.2[a]), after which 10% of circulating haemocytes contain one or more ingested particle, but then starts to slow down, reaching only 16% in the next four hours, and at the end of the experiment only about a third of the circulating haemocytes contains ingested yeast. The phagocytic index (the upper line of Fig. 3.2[a]) follows the same trend, and both parameters can be shown to rise in an inversely logarithmic fashion (Fig. 3.2[b]), i.e. as a saturation curve. Although reminiscent of the results of *in vitro* studies, comparison between Fig. 3.1[b] and Fig. 3.2[b] indicates that 'phagocyte recruitment' occurs at a much lower rate (ca. 50 times) *in vivo* than *in vitro*. This is clearly not due to inavailability of yeast, since it outnumbered haemocytes in all samples by a factor of ≥ 10, and the steady, although slow rise in the proportion of haemocytes containing the marker, and the fact that all haemocytes are phagocytic *in vitro* argue against 'saturation' of a subgroup of phagocytic haemocytes. Yeast was in preliminary experiments found

Fig. 3.2: Phagocytosis of yeast

by the circulating cells of L. littorea



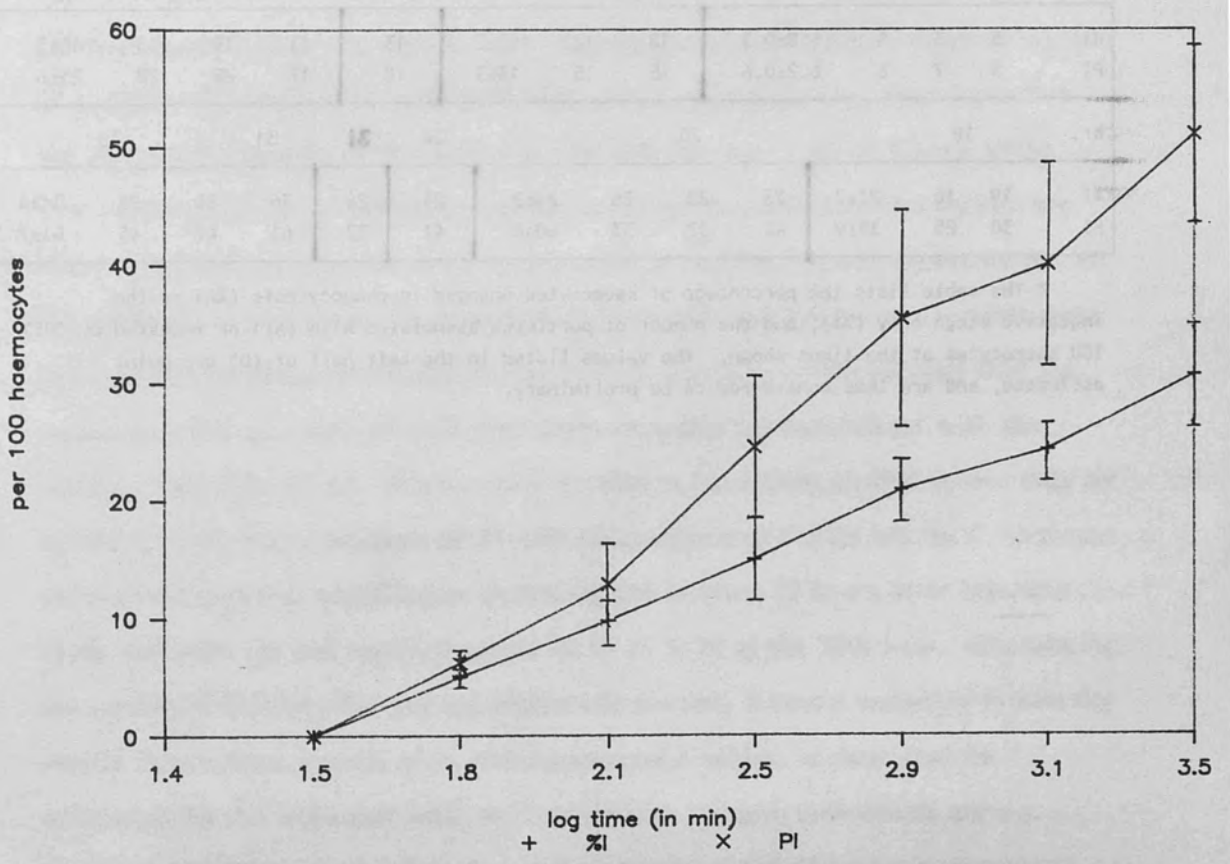


Table 3.1: Phagocytosis by L. littorea haemocytes.*

(a) Fresh RBC in vitro

min.	3				7				12			
%I	37	32	35	35±2	56	53	60	56±3	80	62	72	71±7
PI	78	64	73	72±6	117	100	108	108±7	170	110	150	143±25
%A	71	54	66	64±7	67	79	87	78±8	90	85	83	86±3
AI	166	108	138	137±24	187	154	211	184±23	260	179	222	220±23
min.	20				30				40	60	90	120
%I	83	86	74	81±5	93	75	83	84±7	84	90	94	95
PI	178	197	142	172±23	229	162	175	189±29	196	194	204	204
%A	86	93	83	87±4	93	87	90	90±2	92	93	96	97
AI	239	280	207	242±30	268	197	283	249±38	248	264	285	203

(b) Fixed RBC in vitro (left) and in vivo (right)

min.	5	25	180
%I	0	3	32
PI	0	6	76
%A	27	34	56
AI	27	37	100

min.	10	120	180	210	(24h)
PI	35	10	3.0	2.5	4
PR	45	14	3.0	2.5	5.5
N	2	2	3	2	1

(c) Gram-stained yeast in vivo

hr.	1				2				3		5			
%I	5	3	5	5.0±0.3	12	12	10±2		13		11	19	15	15±3
PI	5	7	6	6.2±0.6	15	15	13±3		18		17	29	28	25±6
hr.	12				20				24	31			48	
%I	19	18	21±2		23	22	26	24±2	21	26	36	30	25	31±4
PI	30	25	35±9		44	32	33	40±8	41	32	61	47	45	51±7

* The table lists the percentage of haemocytes engaged in phagocytosis (%A) or the ingestive stage only (%I), and the number of particles associated with (AI) or ingested by (PI) 100 haemocytes at the times shown. The values listed in the left half of (b) are point estimates, and are thus considered to be preliminary.

to be ingested at a rapid rate *in vitro*, and it seems likely that the observed effect is caused by the dynamic *in vivo* conditions, allowing the departure of particle-laden haemocytes from circulation and the influx of 'fresh' ones from tissue reserves.

3.5 Discussion.

The phagocytosis of molluscan haemocytes has mostly been studied *in vitro*. The phagocytosis rate varies with temperature (Foley & Cheng 1975; Abdul-Salaam & Michelson 1980[b]) as well as time and pH (Abdul-Salaam & Michelson 1980[b]), and also the test-particle used (Tripp & Kent 1967; Foley & Cheng 1975) and its pre-treatment (Jeong & Heyneman 1976; the present study). These findings have been confirmed for gastropods (van der Knaap, Sminia *et al* 1983), in which starvation is also found to be an influencing factor. The medium used is also important, since humoral factors may enhance phagocytosis (Tripp 1966; Anderson & Good 1976; Arimoto & Tripp 1977) or even be necessary for it to occur (Prowse & Tait 1969). Also of importance is the cell surface area available for attachment (Rabinovitch 1967), which in turn depends on the cell size, and whether or not it is spread on glass (see below). The final limiting factor in the uptake of foreign particles appears to be the total volume of phagocytic cells (Munthe-Kaas 1976), and in animals with haemocyte subpopulations, phagocytic values may thus depend on the phagocytic capacity of the different subtypes (see e.g. Foley & Cheng 1975). The values given for *in vitro* phagocytosis in studies on molluscan haemocytes are thus not directly comparable to those presented in Section 3.3, and especially not to the values of *in vivo* phagocytosis as they are not obtained using a stable population. Most studies on phagocytosis also do not provide kinetic evaluations, and thus the values recorded generally provide only point estimates for comparison with the curve in figs. 3.1 and 3.2. Phagocytosis appears to have been studied *in vivo* only by Stauber (1950), whose estimate of 15-19% phagocytosis of Indian ink by *C. virginica* haemocytes found in blood spaces in histological sections 22 hours after injection agree well with the one reported herein of PI 24 ± 10 at the 20th hour. Considering the variety of factors affecting the phagocytic process, it seems necessary to exercise care in interpreting records of *in vitro* phagocytosis values, as these may be influenced by the technique used; moderately high phagocytosis counts are e.g. quoted in studies on the bivalves *Crassostrea virginica* and *Mercenaria mercenaria*

(Tripp 1966; Tripp & Kent 1967; Foley & Cheng 1975), the pulmonate *Lymnaea stagnalis* (van der Knaap, Sminia *et al* 1983) and the opisthobranch, *Aplysia californica* (Pauley *et al* 1971[b]), but the values quoted in studies on other molluscs (e.g. Anderson & Good 1976; Jeong & Heyneman 1976; Cooper-Willis 1979; Abdul-Salaam & Michelson 1980[a,b]) and many other invertebrates (McKay & Jenkin 1970[a]; Paterson & Stewart 1974; Smith & Ratcliffe 1978) are commonly quite low, and in marked contrast with the high values obtained *in vivo* (e.g. Smith & Ratcliffe 1980[b]). It seems likely that the quoted figures to some extent reflect techniqual problems, related e.g. to the spreading of haemocytes on foreign surfaces.

In the present study on *Littorina littorea* haemocytes, a comparison between the uptake rate of fresh, untreated HRBC *in vitro* at 8 min. and fixed HRBC *in vivo* at 10 min. (Table 3.1[b], 3.2[a]) indicates that *in vivo* conditions can be mimicked by providing close contact between haemocytes and test-particles in the absence of competing surfaces, whereas haemocyte monolayers gave unsatisfactory results, apparently due to an element of competition between test-particles and the coverslip surface for haemocyte surface determinants or internal volume. This phenomenon has also been noted in a study on echinoid hemocytes (Bertheussen 1981[a,b]) but may have been overlooked in other studies on invertebrate haemocytes (Bang 1975), indicating the need for careful interpretation of *in vitro* phagocytosis values. In vertebrate immunology, the spreading of adherent cells onto surfaces such as glass, plastic or fibres is commonly known as 'abortive' or 'frustrated' phagocytosis, and a similar analogy has been drawn for snail haemocytes (Haughton 1938). Studies on haemocyte reactions against implants of broken glass coverslips (Anderson 1971) and other material of abiotic and biotic origin (e.g. Tripp 1961; Sminia *et al* 1974; Harris & Cheng 1975) indicate that this analogy can be extended to encapsulation reactions as well; the basic reaction of invertebrate haemocytes towards surfaces recognized as foreign thus appears to be phagocytosis, but a surface too large for ingestion may be encapsulated (Tripp 1963, 1970), and *in vitro* this may be seen as 'spreading' on coverslips. Whether or not the overall similarity of these responses has a common molecular explanation (cf. Wright & Silverstein 1986) can only be revealed by studies on the surface receptors of invertebrate phagocytes.

The *in vitro* experiments clearly demonstrate the ability of *Littorina littorea* haemocytes to attach to and ingest foreign particles under favourable conditions, and strongly support the observation made in Section 2, that they form a homogenous population. The fraction of hemocytes that had neither ingested nor attached to

fresh RBC in one hour (Table 3.1, Fig. 3.1) was morphologically indistinguishable from active haemocytes and may correspond to cells occupying the centre of small aggregates seen in the preparations, as well as non-viable cells (Table 2.1) and perhaps immature cells. The close, prolonged contact between haemocytes and test-particles and the absence of competing surfaces provide optimal conditions for phagocytosis, as shown by the high association values at even 3 minutes; however, attachment clearly proceeds after resuspension, although at a decreased rate which probably reflects haemocyte adhesion to the sides of the Eppendorf tube. The possibility of haemocyte activation by mutual contact should not be overlooked; aggregation was commonly observed to accompany phagocytosis *in vivo* (see Section 4.3), and also appeared to enhance phagocytosis in coverslip preparations. Electron microscope observations also suggest that haemocytes increase their surface area after challenge with foreign particles or mutual contact (Plate 5.6^{c}, 5.8^{a}). Fresh RBC are ingested shortly after attachment (Fig. 3.1), and some intracellular digestion appeared to take place in the first two hours, although exact quantification could not be achieved. The observations suggest that *Littorina* haemocytes are unable to ingest more than ca. 2.2 HRBC on average, due to intrinsic factors such as availability of membrane determinants or size of the phagocytes. Cell volume is known to limit ingestion by human (Bridges 1981) and invertebrate leucocytes (Hostetter & Cooper 1972; Wago & Ichikawa 1979[c]); in fact, studies on phagocytosis in rat Kupffer cells (Munthe-Kaas 1976) indicate that the final limiting factor in the uptake of foreign particles is the total volume of phagocytic cells. A maximal endocytic volume of 2.2 RBC pr. haemocyte is compatible with light and electron microscope observations of RBC ingestion (Plate 3.1^{b-e}, $5.9^{b,d}$); the reluctance of spread haemocytes to phagocytose (see above) may thus relate to the relatively small size of periwinkle haemocytes. The slow increase in numbers of attached particles after ingestion has reached a maximum however suggests that phagocytosis is also affected by lack of cell surface area or membrane determinants for RBC.

A corollary of the above consideration is that the rate of ingestion by individual haemocytes may in the long run be influenced by the rate of intracellular digestion, which in turn appears to be affected by the nature of the particle; digestion of fresh RBC thus appeared to be relatively rapid, whereas yeast appears more resistant, probably due to the rigid cell wall. In estimating the overall phagocytic capacity of circulating cells in general however, it must be remembered that haemocytes have the ability to leave and enter the circulation, which causes

fluctuations in the size and composition of that part of the haemocyte population that circulates with the blood. Histological studies (Section 4) confirm that injected materials (carmine and yeast) are taken up by haemocytes which become associated with renal and pallial blood vessels and sinuses and later leave by diapedesis in the mantle and other exposed organs. Diapedesis is clearly offset by the recruitment of fresh haemocyte supplies from tissue reserves, as shown by total haemocyte counts (Section 2.3) and the shape of the curve in Fig. 3.2 thus probably reflects the balance between phagocytes leaving circulation in diapedesis sites and those entering it from tissue reserves (compare also the uptake of fixed RBC *in vivo* and *in vitro*). This provides a likely explanation to the slow rise in PI, which after the 6th hour appears to be limited by the same factor as the proportional increase in phagocytosing cells. With only 1.7 yeast cells taken up on average by each active phagocyte, the PI does not seem to be limited by the internal volume of haemocytes, and reduced chances of multiple phagocytosis by individual haemocytes because of margination and diapedesis, induced by the phagocytic event, appears a plausible solution. It thus seems likely that the slow rate of 'phagocyte recruitment' *in vivo* to a large extent reflects a slow rate of haemocyte 'renewal' at the injection site, which in turn depends on haemocyte diapedesis and mobilization of tissue reserves, haemocyte multiplication and the circulatory rate. None of these factors are operative *in vitro*, in which a stable population of haemocytes is used, and the rate of haemocyte recruitment appears to be increased by a factor of ca. 50.

The nature of the test-particle is clearly important in determining the response, as high phagocytosis counts were readily obtained in 30 minutes with fresh human RBC *in vitro*, whereas the response towards glutaraldehyde-fixed RBC was relatively moderate even in 2½ hours. It seems pertinent in this respect, that aldehyde fixation was also found to abolish the affinity of the serum lectin for human RBC (Section 6). Studies with crayfish (McKay *et al* 1969; McKay & Jenkin 1970[a]) and pulmonates (van der Knaap, Sminia *et al* 1983; van der Knaap, Boots, Sminia 1983) demonstrate that serum lectins may serve as opsonins as well as cytophilic receptors for foreignness, and opsonic properties of other invertebrate sera have also been traced to agglutinins or lectins (Bang 1962, 1967; Cornick & Stewart 1968[a,b]; Pauley *et al* 1971[a,b]; Arimoto & Tripp 1977; Pistole & Britko 1978; Harm & Renwrantz 1980). Taken together, the present data on the agglutinability and phagocytosis of fresh and aldehyde-treated RBC as well as the decreased phagocytosis of fresh HRBC under conditions where serum contamination was

minimized supports that in *Littorina*, as in other invertebrates, adsorbtion of lectin onto the foreign surface may be important in determining the response. These results are in agreement with the finding (Section 6) that injection of HRBC causes a moderate increase in lectin titers, suggesting a role in defense by this molecule. The marked effect of aldehyde fixation of the HRBC on the ingestive phase suggests that the phagocytic stimulus depends in part on the density on the foreign particle of surface determinants recognized by the haemocyte. The recognition and ingestion of RBC by mouse macrophages is also affected by aldehyde fixation, after which the RBC are recognized only by the "foreign surface receptor" (Rabinovitch 1967). Further experiments are needed to confirm the opsonic involvement of the serum lectin of *Littorina littorea* in the phagocytic process, and to resolve whether different rates of ingestion are mediated by different receptors.

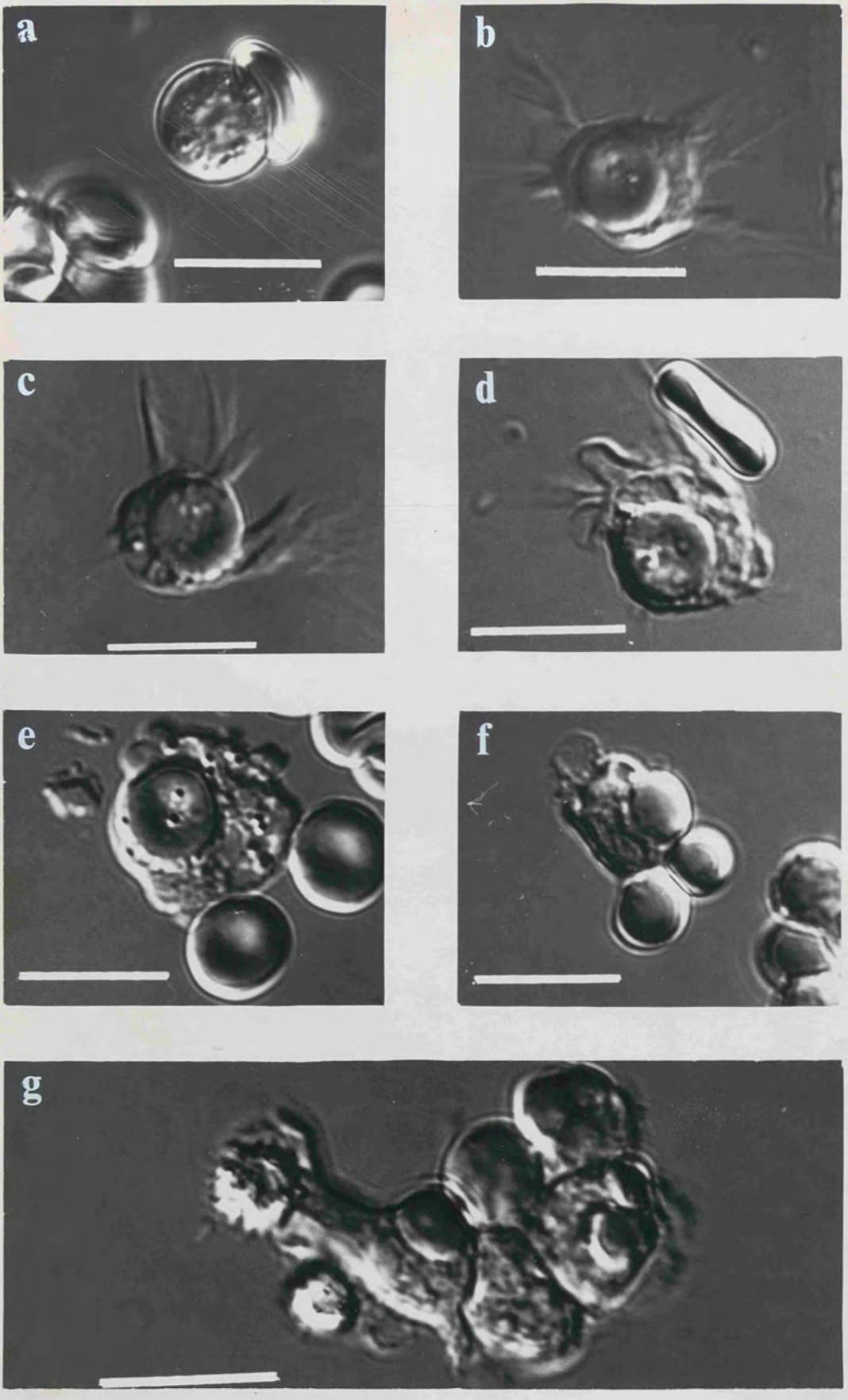
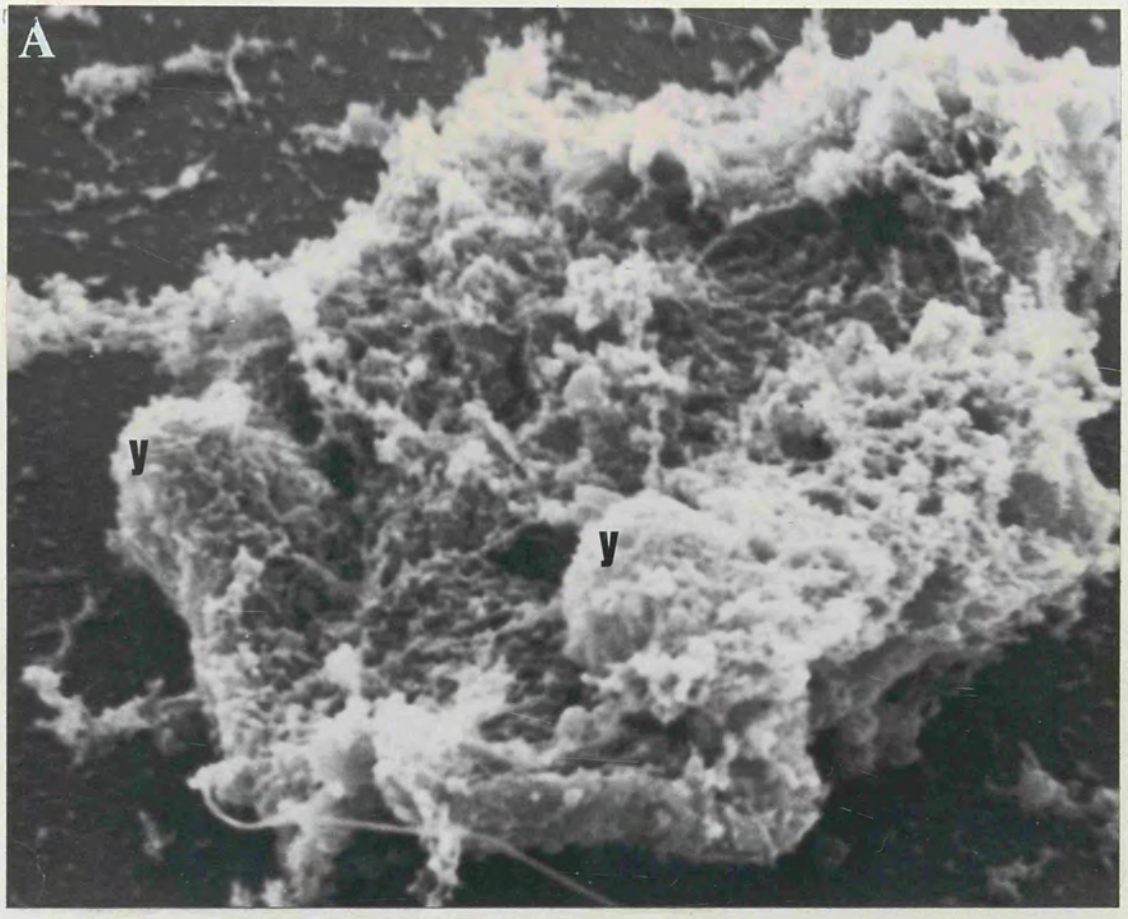


Plate 3.1

PLATES 3.1-3.2

Plate 3.1: Phagocytosing *Littorina* haemocytes after challenge with formaldehyde-fixed human (a-e) or rabbit (f-g) erythrocytes *in vivo*. The blood samples were drawn into a syringe containing glutaraldehyde (f-g) or photographed after a short incubation on glass coverslips (a-e). Note spreading in (b) and (c); association of small granules (lysosomes) with the phagosome in (b-e), filopodial attachment of HRBC in (d) and pseudopodial attachment of RRBC in (g). Bar: 10 μm.

Plate 3.2: A SEM study of *Littorina* haemocytes after challenge with *S. cerevisiae*. (a) Haemocyte with 2 ingested particles (y). (b) A pseudopodium encircling yeast. Bar: 2 μm.



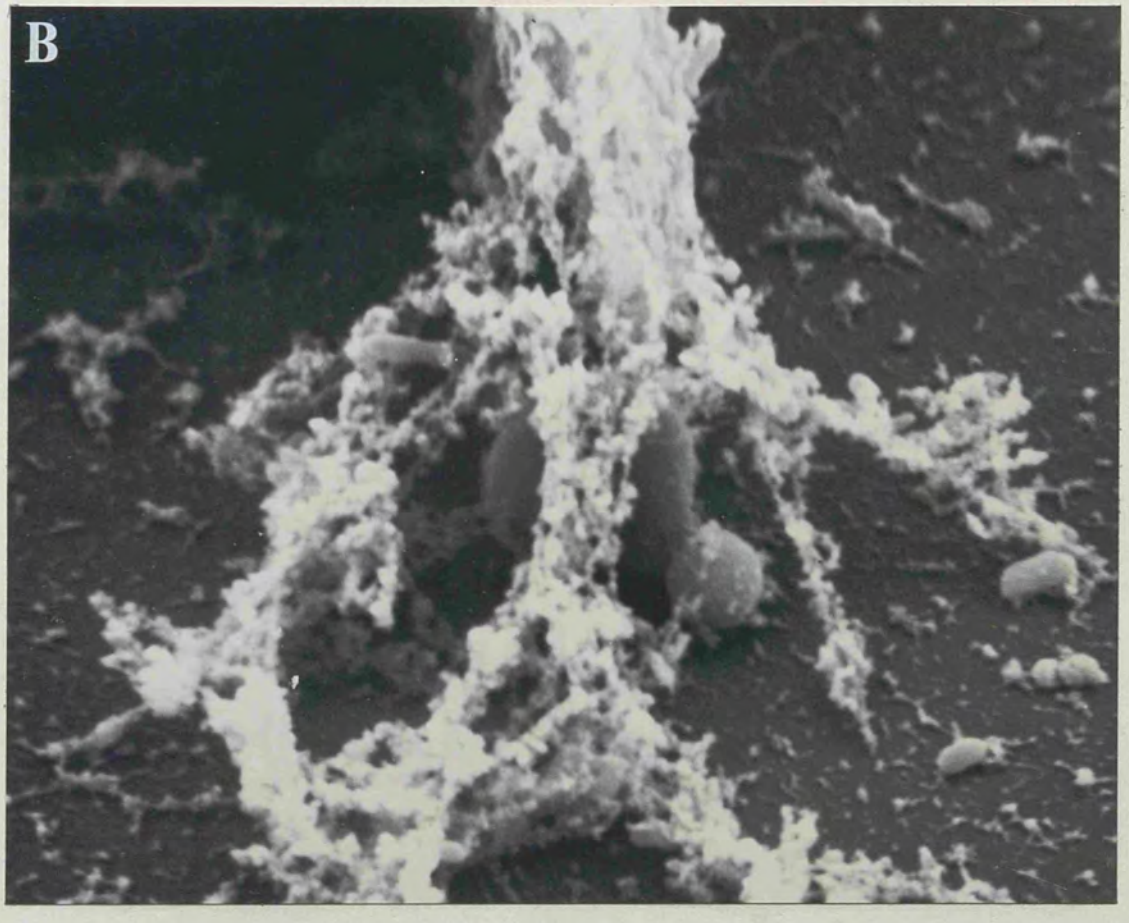


Plate 3.2

SECTION 4: THE CLEARANCE OF INJECTED PARTICLES.

4.1 Introduction.

Since phagocytosis by molluscan haemocytes was first observed by Haeckel in 1862, and its value for defense against pathogens was first shown by Metchnikov in 1884-92, this phenomenon has received good attention from workers on molluscan physiology (e.g. Kollman 1908; Cuénot 1914). Early studies on the fate of injected markers in molluscs focussed on their immediate clearance from the circulation, but in more recent studies, the attention has turned to the ultimate fate of foreign materials in the molluscan body (Stauber 1950; Tripp 1958, 1960, 1961). Studies on bivalves demonstrate that inert materials are ingested by haemocytes which then migrate out of the body via epithelial surfaces (Stauber 1950; Reade & Reade 1972) and that this response is augmented by intracellular digestion in the case of degradable materials (Tripp 1958, 1960). These studies have been extended to other invertebrates (Brown & Winterbottom 1969; Brown & Davies 1971; Reinisch & Bang 1971; Fontaine & Lightner 1974), including cephalopods (Stuart 1968), pulmonates (Tripp 1961; Brown 1967; Reade 1968; Arcadi 1968), opisthobranchs (Pauley & Krassner 1972) and prosobranchs (Brown & Brown 1965; Cheng, Thakur & Rifkin 1969). In the latter case, the results appear to be conflicting, unless one postulates that different elimination pathways are used by haemocytes in different genera of prosobranchs, which seems rather unlikely. These results are further complicated by claims of 'fixed' phagocytes in various molluscs (Reade 1968; Stuart 1968; Pauley & Krassner 1972; Crichton *et al* 1973; Killby *et al* 1973), and the suggestion, by Bayne (1973[b]) that phagocytosis by circulating haemocytes can not account for the rapid clearance of injected bacteria observed in the pulmonate *Helix pomatia*. To throw more light on the subject, the fate of particulate matter (carmine) injected into *Littorina littorea* was followed for up to 64 days in histological sections, and the fate of injected yeast was checked at 5 hours and at 5 days for brief comparison of the times needed for eliminating different particles.

4.2 Materials and methods.

EXPERIMENTS. 40 periwinkles were injected into the haemocoel with 0.1 ml of a sterile suspension of carmine (0.3%) in aSW. The snails were kept along with uninjected controls at 10-12 °C in 1-2 dm^3 pyrex jars, changing aSW twice a week, and sacrificed, in groups of three, at 0, 2, 4 and 16 hours, and at 2, 4, 8, 16, 32, and 64 days after injection. For comparison, four additional snails were injected with 1 ml of fixed yeast cells (7 x 10^8/ml), and sacrificed, in pairs, at 5 hours and 5 days. Animals were fixed and processed for light microscopy, and the resulting slides compared within and between sampling times, noting the changes in histology and test-particle distribution as the experiment proceeded.

Examination of the freshly injected snails gave an indication of the initial distribution of the inoculum, but to confirm this, and to determine changes brought about by different injection conditions, snails were examined macroscopically while being injected, having had either the whole shell or only the body whorl removed (the shell was broken in a vice after securing the snail to the shell as described in Section 2.2). Also, two snails injected with gram-stained yeast (Section 3.2) were sacrificed at 4 hours and the optical density of selected body parts monitored at 594 nm after extraction with acid alcohol (1% HCl in ethanol; Humason 1962). The wavelength was selected after recording the absorbtion spectrum of extracts of the inoculum in a PYE Unicam SP8-100 spectrophotometer.

TEST PARTICLES. BDH carmine (C.I. 75470) was washed at least once by filtration and prepared as a 0.3% suspension in sterile aSW shortly before injection. The aSW was from the sea-water system; prior to use it was sterilized by passing through a membrane filter, pore size 0.2 μm (Sartorius). Dried brewing yeast, *Saccharomyces cerevisiae* was obtained commercially (CWE). Prior to use, it was fixed overnight in 4% formaldehyde, washed several times, harvested by brief centrifugation (1000g for 5 minutes), and its final volume adjusted to contain approximately 7 x 10^8 cells per μl of aSW. Some inocula were stained by a modified Gram-Twort method (Drury & Wallington 1967; see Section 3.2) and adjusted by optical density measurements (see above) to ca. 7 x 10^{11}/ml.

INJECTIONS. All injections were made into the haemocoelic sinus surrounding the oesophagus (refer to Section 1.3), using the same apparatus and

experiments, animals had to be anaesthetized, but when sufficient expertice had been gained with the injection method this was abandoned. The best anaesthetizing agent found was magnesium dichloride, which was made up as a 3% solution in hypotonic aSW (a 3:1 solution of aSW and tap water); after relaxing snails in this medium for 6-12 hours the concentration was slowly increased to 7% (modified after Dales 1969), which left the snails fully narcotized in an hour or two.

HISTOLOGY. For histological study of sections, animals were fixed in Stieve's fixative (Humason 1962) for 16-18 hours, removing the shell and the operculum after the first hour. Mercury deposits were washed out with Lugol's iodine (1% iodine in 70% alcohol) for 6-9 hours, adding iodine as needed. Specimens were sometimes kept for a few days in 70% alcohol before they were dehydrated in alcohols (90% and absolute), cleared in xylene or chloroform and embedded in fibrowax after infiltrating at 60 centigrades. Each of the above steps involved three 20 min. washes, except clearing in chloroform, in which case specimens were left overnight and then briefly rinsed in fresh chloroform. Sections were cut on a Beck sledge microtome set at 5-7 μm. Specimens were sectioned from anterior to posterior, and from each animal, 16-18 sections (or series of sections) spaced at regular intervals were mounted on slides, previously coated with glycerin-albumen. The sections were treated with 0.5% iodine in 70% alcohol followed by 0.75% sodium thiosulphate in 10% alcohol (Carleton and Drury 1957) in order to remove the last traces of mercury deposits, stained with Heidenhain's iron haematoxylin (Pantin 1948) and alcian blue (Pearse 1960) (carmine clearance) or with the methods of Gram-Twort (Drury and Wallington 1967) or Glynn (Humason 1962) for bacteria in sections (yeast clearance), and finally mounted in DPX and overlaid with a coverslip.

4.3 Results.

(A) INOCULUM DISTRIBUTION. As soon as they were released after injection, animals withdrew into their shells. These forceful body contractions helped to distribute the inoculum, as evidenced by the immediate reddening of distant body parts of 'half-exposed' snails as soon as they were released, and the relatively lighter colouring of corresponding organs in 'naked' snails that were fully contracted when injected. Colour changes in the anterior body parts were

completely masked by the pigmented epithelium, although a deep-lying pinkish colour at the margin at the sole of the foot indirectly betrayed the subepitheliar carmine depositions seen in histological sections; posteriorly, the greatest share was deposited in the **kidney**, with large patches or even the whole organ staining brilliantly red. The dye reached the **mantle** in amounts that made much of its circulation, esp. the pallial vein, traceable through its colouring. Other organs did not stain as intensely, although patches of dark colour were sometimes seen in the **gills** and in the **posterior aorta** and its branches. The distribution was varible between individuals, with different amounts settling out in the kidney, and no visible colouring of subcutaneous layers of the foot in some animals.

Histological examination on specimens sacrificed on the first day confirm the above findings, and also reveal that considerable amounts of carmine remain for several hours in the cephalic haemocoel (Plate 4.1[a]). The main blood vessels are virtually free from carmine even at 2 hours, and apart from individual differences, the anatomical pattern of dye distribution remains essentially unchanged in the first day. The dye seems to be quickly deposited in tissue lacunae, and subsequent changes in distribution are slow and gradual, apparently being brought about mainly or wholly by the migration of phagocytes.

The anatomical and histological pattern of carmine distribution will be described with reference to the 4th hour sample. The largest concentrations are found in the **head haemocoel**, especially near the injection site (Plate 4.1[a]), and in blood vessels of the **kidney** (Plate 4.1[c]) and the **mantle** (Plate 4.1[b]). The **gills** (Plate 4.1[d]), the **nephridial gland** and the **heart** (Plate 4.2[a,b]) receive much less material. Most of the dye reaching arterial blood has been distributed to the **foot** (Plate 4.2[c]), where its distribution and abundance is highly variable between individuals, probably depending on blood flow to the area at injection time, which in turn is affected by foot movement. Most of the dye is found in the subcutaneous area, which is more vascular than deeper layers (Plate 4.2[c]). The same applies to the body wall surrounding the alimentary tract, although with less abundant dye deposits. It is quite sparse in the **visceral haemocoel**, where it is mainly deposited near the aorta branches, e.g. adjacent to the stomach (Plate 4.2[d]) or even 'trapped' in the vessel walls; the latter is somewhat reminiscent of the deposition of the pigment in the walls of the **anterior aorta**, indicating that its occurrence in this tissue (and elsewhere in the head haemocoel and related tissues) is not all due to its proximity to the injection site but may partly result from recirculation of the dye.

Other tissues (e.g. hypobranchial gland, columellar muscle, reproductive organs) were literally free of marker.

Preliminary observation on the distribution of gram-stained yeast in different body parts of two snails at 4 hours p-i indicate minor differences in the share of the inoculum received by organs supplied by renal and arterial blood (pallial comples ca. 15%, foot ca. 15% and visceral hump ca. 11%), but different amounts were retained in the head haemocoel (55% resp. 35%) of these snails, apparently depending on contraction of the animal when released after injection; the remainder (5% resp. 25%) was associated with the kidney.

(B) CLEARANCE. As can be inferred from the above description, inoculum distribution is very uneven initially, both anatomically (with dye abundance inversely related to the distance, in terms of blood flow, from the injection site), and on the tissue level (deposits being most prominent near afferent blood vessels). Enough material was however found in each tissue for the major clearance steps to be assessed through the examination of dye ingestion and the subsequent anatomical redistribution of the material.

Phagocytosis of injected materials was not found to be prominent in the first day, except in kidney and mantle blood vessels, where the marker was found almost exclusively in association with haemocytes. Carmine-laden haemocyte aggregates are seen in the main blood vessels of the **mantle** shortly after injection (2-4 hours; Plate 4.3[a,b]), and the dye has been extensively phagocytosed by circulating haemocytes in the **kidney** (Plate 4.3[c,d]); these are quite numerous in injected as well as uninjected animals, filling many small blood vessels almost to occlusion, and lining the inside of larger vessels. The marker seems to accumulate into kidney sinuses with time, and roughly equal amounts are found in blood vessels and sinuses at 16 hours. At this time, increasing amounts of dye-laden haemocytes are found in mantle blood vessels and surrounding tissue, and diapedesis through covering epithelia is commonly observed. The dye also appears to be largely intracellular in the **gills** in the first day (Plate 4.3[e]), although scarce and dispersed. The observations suggest clearance of anterior haemocoels to the kidney and the mantle, with some loss of marker to the gills. The subsequent events in the clearance process in these and other tissues will now be described separately for each part of the body.

KIDNEY: The marker seems to be disappearing from kidney sinuses in the 2nd and especially the 4th day, and is now found mostly in blood vessels again (Plate 4.4[a]), in associatation with haemocytes (4.3[f]). No carmine is ever found in the renal epithelium, and the occurrence of carmine-laden haemocytes in the nephridial gland and organs supplied by arterial blood (see below) supports clearance by haemocytes leaving through the circulatory route. Dye concentrations continue to decrease at all later stages (Plate 4.4[b,c]) and very little is found at the end of the experiment (Plate 4.4d).

MANTLE: Dye-laden haemocyte aggregates continue to arrive in blood vessels in increasing amounts in the 2nd and the 4th day (Plate 4.5[a,b]) and are seen in diapedesis through the epitheliar layer at all sampling times after 4h; the process continues throughout the experiment (Plate 4.5[c,d]; 4.6[a-f]). Haemocytes migrate through the upper as well as the lower epithelium, and are in maximal numbers at 16h and 2d.

GILLS, NEPHRIDIAL GLAND & HEART: Carmine is at all times dispersed and rather scarce in these organs. Phagocytosis is only observed in blood vessels in the first day, indicating that it may have taken place outside these organs (i.e. in the mantle and the kidney). At 4 days most dye particles have been phagocytosed in all organs. A few haemocyte aggregates with ingested dye are found in blood spaces of the gills from the 4th day onwards (Plate 4.7[a,b]) but no signs of diapedesis are ever found. Haemocyte aggregates are also found in the nephridial gland at 4 days (Plate 4.7[c]), but are only exceptionally seen in the heart (Plate 4.7[e,f]). Carmine is never found in epitheliar layers of these organs, nor in fact outside blood vessels except in a few random cases (Plate 4.7[d-f]).

HEAD: The main clearance of carmine seems to occur around the 4th day, when haemocytes are seen to dominate carmine clusters and most of the dye appears to be intracellular (Plate 4.8[a]). Haemocytes are no longer prominent at 8 or 16 days, and carmine is much less concentrated, especially at 16 days when the dye has become scarce even at the injection wound (Plate 4.8[b]). Carmine concentrations continue to drop at 32 days and 64 days (Plate 4.8[c,d]), and what little remains at the end of the experiment is mainly found trapped in haemocoelic septa or in the aorta walls.

FOOT: Although much of the deposited dye still seems extracellular in the 4th day, many carmine-laden haemocytes are seen, especially in and around blood lacunae (Plate 4.9[b]). These have multiplied in the 8th day, when phagocytes are seen to arrive in groups in blood channels and migrate towards the epithelium (Plate 4.9[a]). Carmine particles are even found above the basement membrane, although infrequently. Diapedesis is much more pronounced in the 16th and the 32nd day (Plate 4.9[c-e]), with more occurrences of dye particles in the epitheliar layer, but phagocyte arrivals seem diminishing at the last sampling time (Plate 4.9[f]).

VISCERAL HUMP: The marker appears to be mainly extracellular in the first 2 days, but groups of dye-laden haemocytes are seen in the 4th day (Plate 4.10[a]), especially near aorta branches. These are still common in the 8th day (Plate 4.10[b]) but reduce in numbers at later times (Plate 4.10[c-f]). The arrival of carmine-laden haemocytes through blood-vessels (Plate 4.10[d]) is clearly offset by phagocytes leaving the area; carmine was however never found in visceral or reproductive epithelia, except for a single occurrence in the 16th day of 3 grains in the rectal epithelium. Towards the end of the experiment carmine concentrations seem to decrease slowly, and phagocytosis appears complete (Plate 4.10[e-f]).

PHAGOCYTOSIS: Only haemocytes were positively identified as being phagocytic (Plate 4.11[a-e]). Histological sections of animals injected with *Saccharomyces cerevisiae* 5 hours and 5 days before fixation demonstrate a much faster clearance rate for this organism (Plate 4.11[h]), but the anatomical and histological distribution indicates a similar elimination route. In addition to diapedesis however, intracellular digestion apparently contributes to the inoculum clearance, as profiles of yeast were observed which appeared distorted and bleached in colour, as if half-digested (Plate 4.11[f-h]).

4.4 Discussion.

The initial distribution of the inoculum reflects the course of venous return from the cephalic haemocoel. The bulk of the dye is taken to the **kidney**, where the afferent renal vein branches, "breaking down into vessels of almost capillary dimensions" (Fretter and Graham 1962, p. 275). The dye is partly deposited in the kidney sinuses, with the remainder passing partly through the **nephridial gland** but

mostly by means of the **efferent renal vein** (refer to Section 1.3) through the mantle, hypobranchial gland and gills before reaching the heart. Traces of dye are left in these organs, mainly the **mantle**. A small fraction of venous blood from the anterior haemocoels bypasses the kidney, and some of the dye therefore makes its way straight to the mantle and onwards. Carmine reaching arterial blood becomes distributed mainly to the **foot**, due to blood redistribution into it after the shock of injection has passed; some of it is however found in and near aorta branches in the **visceral hump** and the **head**. Observations on the distribution of gram-stained yeast indicate that the amounts per tissue weight retained by the kidney and the mantle are ca. 5-30 times resp. 4-5 times that of the foot and the visceral hump.

The high phagocytosis counts in **kidney** blood vessels shortly after injection point to rapid uptake of the marker by circulating haemocytes, either before entering the kidney (i.e. in the cephalic haemocoel or the afferent renal vein) or locally, in which case the narrow diameter of renal blood vessels and their high content of haemocytes may provide important 'filtering' conditions. At any rate, the retention of carmine-laden haemocytes in kidney blood vessels during the first 4 hours is likely to be due mainly to this 'filtering' effect. Haemocytes are however commonly seen in close association to blood vessel walls, even in large, empty vessels, indicating that margination also has a part to play in the delayed clearance of the kidney; this may in fact be followed by migration of phagocytes into renal sinuses, as indicated by the increase of marker in these locations between 4 and 16 hours p-i. The renal blood vessels are however also cleared via the circulatory route; the phagocyte groups arriving in **mantle** blood vessels at 4 hours p-i thus clearly originate in the kidney, and the dramatic increase in haemocytes migrating from pallial blood vessels towards epithelia at 16 hours is concomitant with clearance of the renal blood vessels. Carmine is voided to the exterior by diapedesis through epithelia on both surfaces of the mantle, the peak of which appears to coincide with the peak of clearance from the kidney, between 1 and 4 days p-i.

Carmine escaping phagocytosis at the time of injection apparently settles out in blood sinuses in various organs (kidney, mantle, foot and digestive gland) and may even become embedded in muscle or connective tissue. In this situation it apparently does not elicit a quick cellular response, since phagocytosis was hardly observed outside renal and pallial blood vessels in the first day. The **kidney sinuses** however provide an exception, and this is thought to be related to the interconnecting web of haemocytes normally found in these sinuses (see Section 5), providing

optimal conditions for trapping foreign materials. In spite of this, and the transient influx of phagocytes clearing renal vessels in the first day, carmine is never found in the renal epithelium, and the disappearance of marker in the 2nd and the 4th day was concomitant with the arrival of phagocyte groups in tissues supplied by renal and arterial blood, suggesting clearance via the circulatory route. This applies to the **cephalic** and the **visceral haemocoel** as well; phagocytes clearing these from the 4th day onwards apparently leave by venous return since with one exception, the marker was never found in visceral, reprocuctive or renal epithelia, and phagocytes migrating through the body wall of the head-foot region apparently arrive by arterial blood and not by migration from the cephalic haemocoel. No signs of diapedesis were found in the **gills** or the **nephridial gland** either, and the phagocytes seen to escape pallial blood vessels for the gills probably continue circulating until reaching diapedesis sites in the mantle and the foot. This is supported by the groups of phagocytes occasionally seen in branches of the posterior aorta, and may in fact partly explain the inability of the animal to rid itself of the marker during the observation time. Although clearance is not completed in 64 days, carmine is much reduced in all tissues, and the peak of clearance of tissues other than the kidney appears to occur concomitantly with increased diapedesis through epithelia covering the head-foot in the 16th-32nd day. At the end of the observation period, some carmine is still found embedded in tissues (e.g. 'trapped' in haemocoelic septa and aorta walls) and clearance is thus a rather slow process, with traces of dye even persisting for two months; the process probably continues for a long period of time with the curve of elimination gradually flattening out, as Stauber (1950) proposes for the oyster.

Studies on bivalves have shown that in animals belonging to this class, haemocytes containing injected dyes will leave the body mainly through epithelia of the digestive organs, but to a lesser extent through the external body surface (Stauber 1950; Reade & Reade 1972). Haemocytes however also accumulate on exterior surfaces by diapedesis under normal circumstances (Galtsoff 1964), and are normally abundant in epithelia of digestive organs in connection with the role they play in digestion in this class (Yonge 1926). It therefore seems apparent, that the migratory route of haemocytes eliminating injected dyes is directly related to the normal activity of the haemocytes, and that in this respect the dye may act simply as a cytological marker. Once ingested, the test particle may however be eliminated with different speeds depending on the test particle used, and if digestible, it may

be degraded intracellularly rather than voided out through epithelia (Tripp 1961). In experiments of this kind, the principal question to ask is clearly: to what extent does the injected material elicit a cellular response when ingested. Inert materials are likely to act mostly as cytological markers; clearance of Indian ink injected into the oyster (*Crassostrea virginica*), reported to take more than 42 days (with a peak on or about the 33d day; Stauber 1950) can hardly take place much faster than normal diapedesis, and this probably also holds true for the elimination of carmine from the periwinkle in the present study, which was found to take more than 64 days with a peak on or around the 16th day. Preliminary results with yeast, *Saccharomyces cereviciae* indicate that this organism, being far more immunogenic than the dye, is eliminated much faster, although the route of elimination appears to be largely the same. The faster clearance reate of this organism appears at least partly to be due to a larger contribution of intracellular digestion to the elimination process.

PLATES 4.1-4.11

Tissue clearance of markers injected into the cephalic haemocoel of *Littorina littorea*. The markers used were carmine (Plates 4.1-10, Plate 4.11^{a-e}) and yeast, *Saccharomyces cerevisiae* (Plate 4.11^{f-h}). Bar: 50 μm, or as indicated (in μm).

Plate 4.1-4.2: Distribution of the inoculum at 4 hours p-i. (a) Injection site (head); (b) mantle; (c) kidney; (d) gills; (e) heart; (f) nephridial gland; (g) foot; (h) digestive gland.

Plate 4.3: Phagocytosis. (a) A group of haemocytes with ingested particles in a pallial blood vessel at 4 hours p-i; (b) overall view of the same; (c) and (d) overall view of the clearance of blood vessels in the kidney at 4 hours p-i; (e) haemocytes with ingested particles in the gill connective tissue at 4 hours p-i; (f) haemocytes infiltrating carmine deposits in the kidney, 4 days p-i.

Plate 4.4: Clearance of carmine deposits in the kidney at (a) 4, (b) 8, (c) 16 and (d) 64 days p-i. Insert (a): departure of particle-laden haemocytes in blood vessels at 4 days.

Plate 4.5: Carmine clearance in the mantle at (a) 4, (b) 8, (c) 16 and (d) 32 days p-i. At all times, particle-laden haemocytes are seen to arrive in groups in blood vessels.

Plate 4.6: (a) Carmine clearance in the mantle at 8 days p-i; (b-f) diapedesis of particle-laden haemocytes in the mantle at 16 (b,d,f) and 64 days p-i (d,e). Haemocyte aggregates are still arriving in mantle blood vessels at 64 days p-i (f).

Plate 4.7: Carmine clearance in the gills at 4 (a) and 8 days p-i (b), the nephridial gland at 4 (c) and 32 days p-i (d) and the heart at 16 (e) and 32 days p-i. Note association of carmine with circulating groups of haemocytes.

Plate 4.8: Carmine clearance in the head at 4 (a), 16 (b), 32 (c) and 64 days p-i (d). Note clearance of the injection wound (compare (b) to Plate 4.1^{a}).

Plate 4.9: Carmine clearance in the foot at 8 (a), 4 (b), 16 (c,d), 32 (e) and 64 days p-i (f). Diapedesis of particle-laden haemocytes can be seen at all stages (arrows).

Plate 4.10: Carmine clearance in the digestive gland at 4 (a), 8 (b), 16 (c), 32 (d,e) and 64 days p-i (f). Note arrival of particle-laden haemocytes in a blood vessel in (d), also seen outside blood vessels as cell aggregates (a,b,c,f).

Plate 4.11: (a-e) Particle-laden haemocytes migrating through the connective tissue of the mantle and the foot. (f-h) Clearance of *Saccharomyces cerevisiae* in the foot at 5 hours p-i. Note diapedesis through the epithelium (arrow).

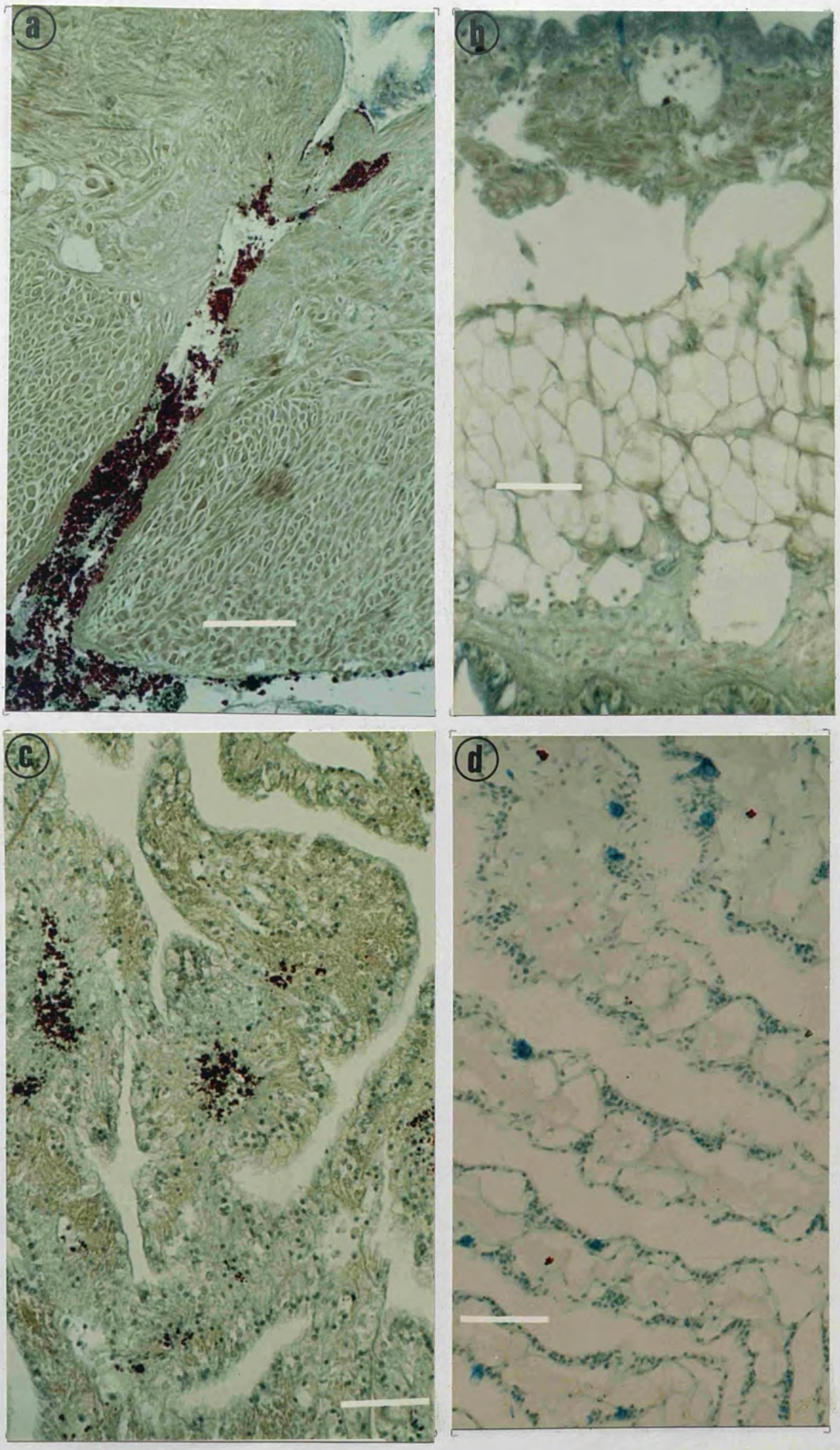


Plate 4.1

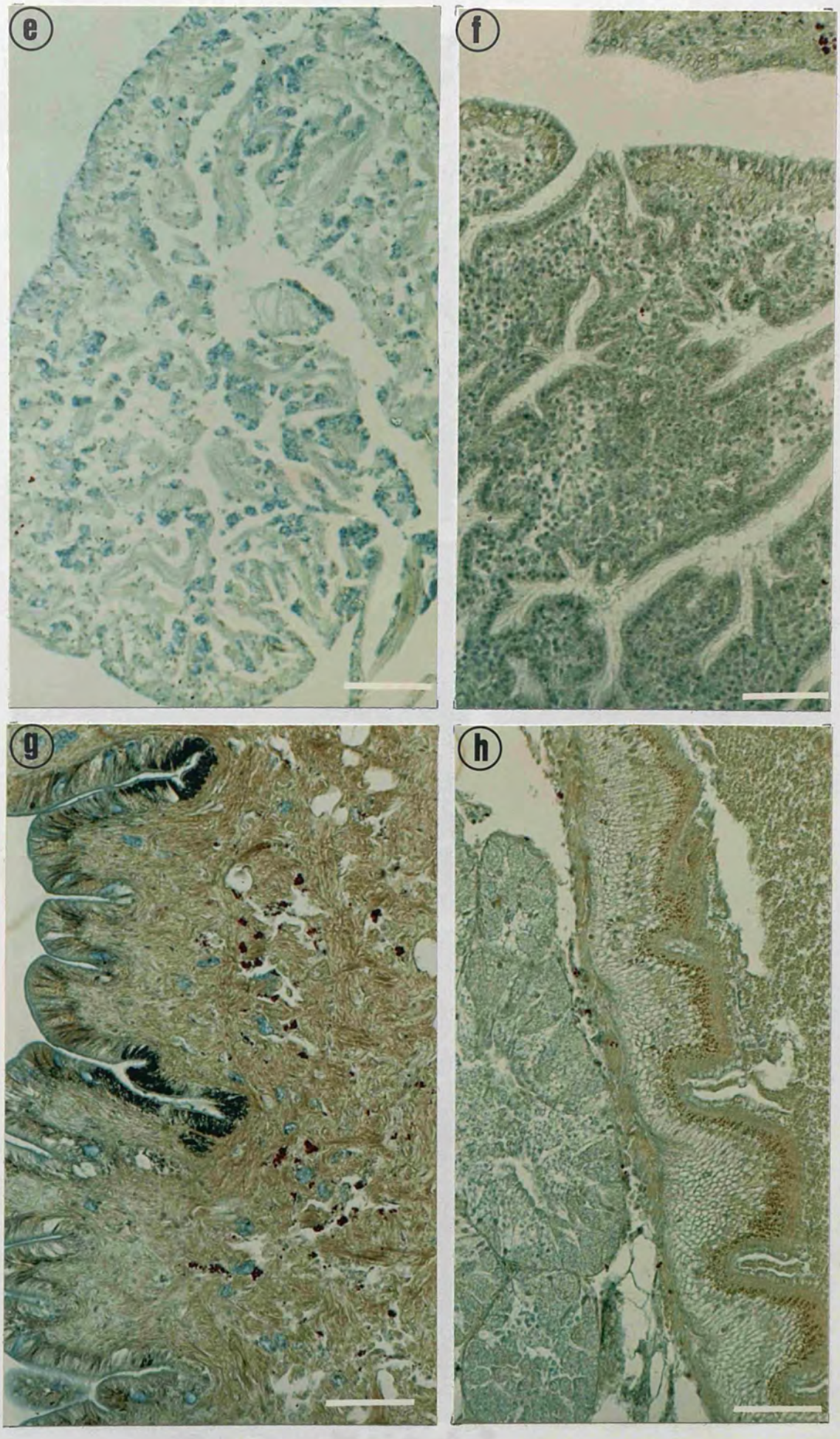


Plate 4.2

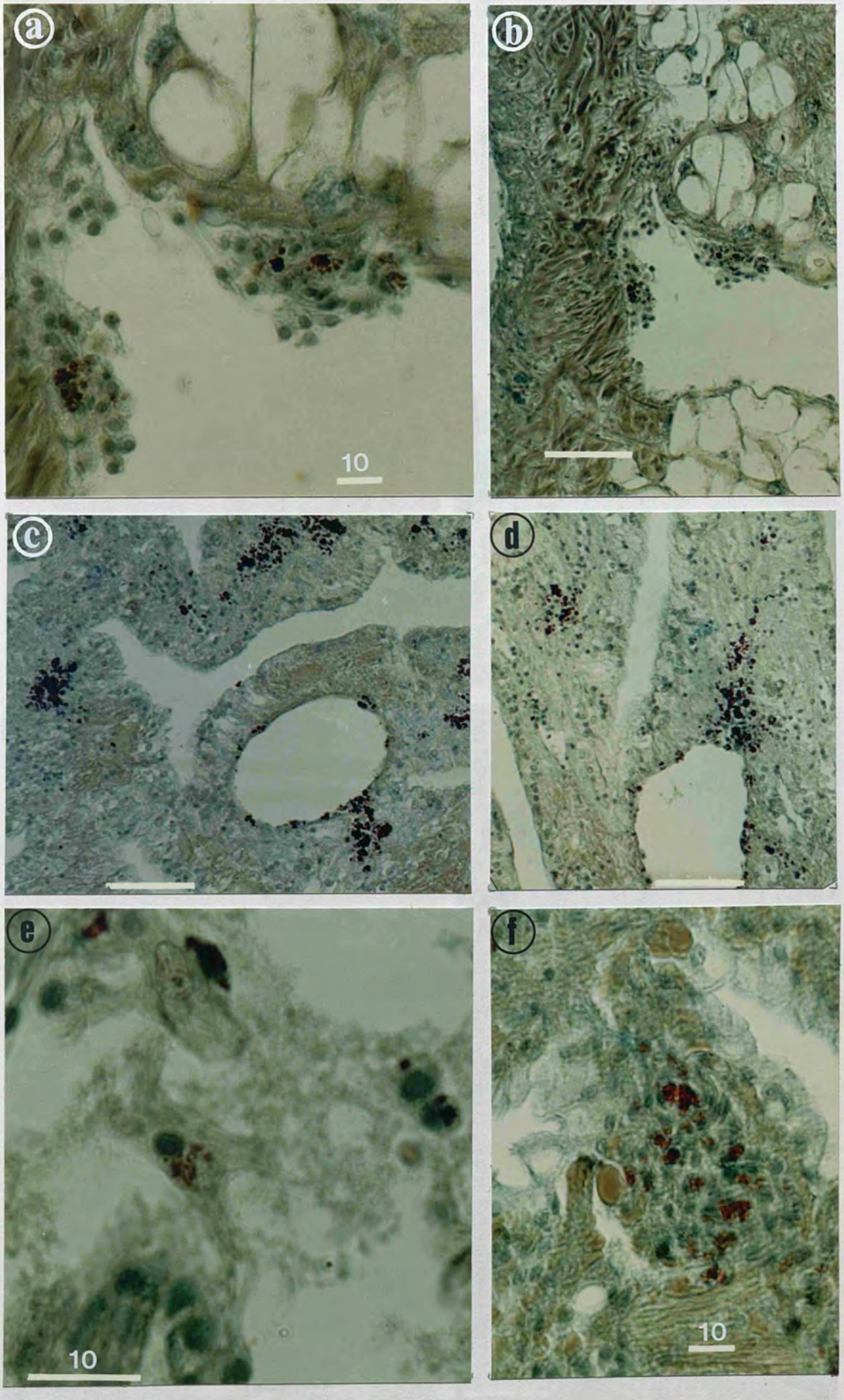


Plate 4.3

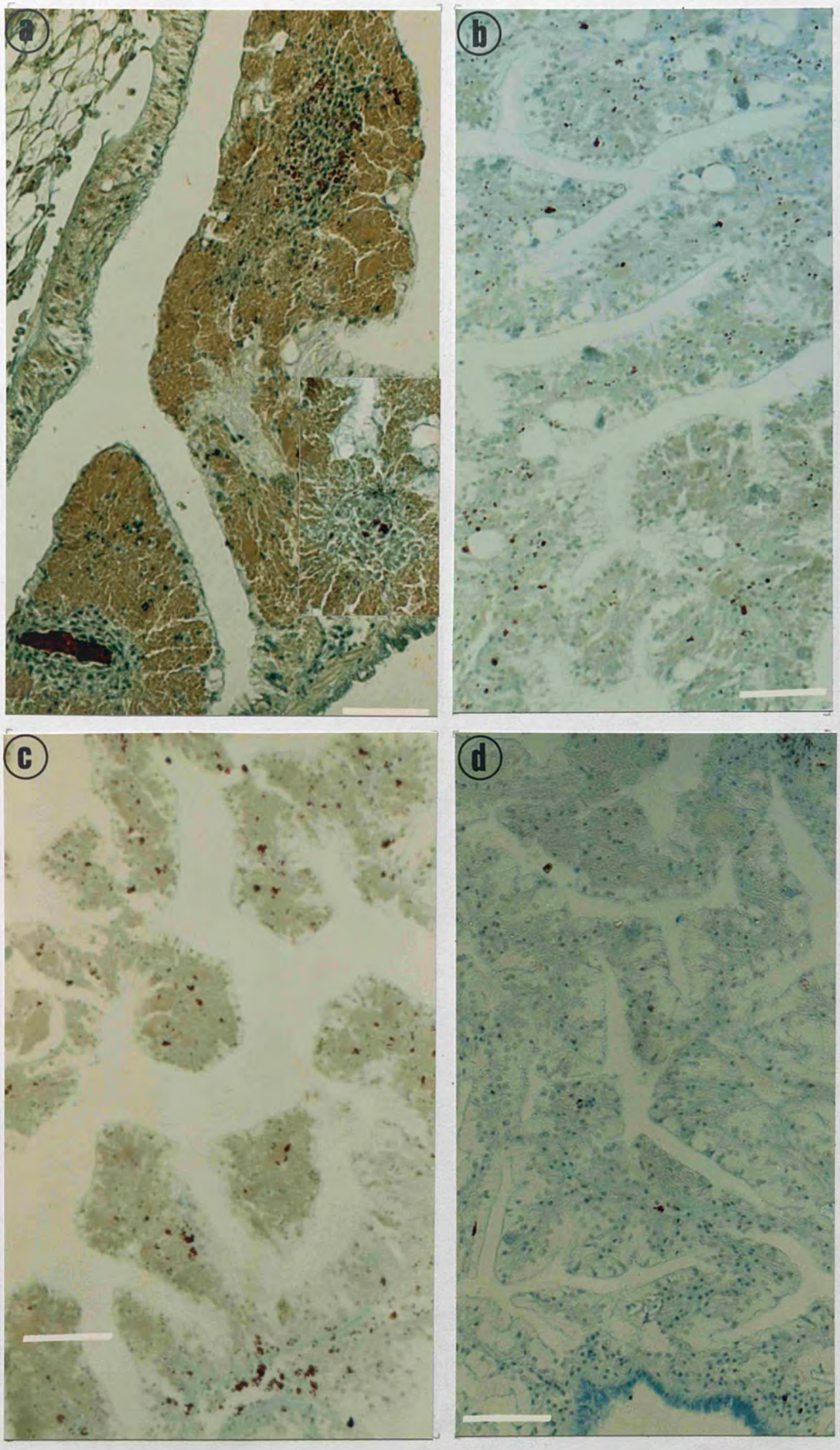


Plate 4.4

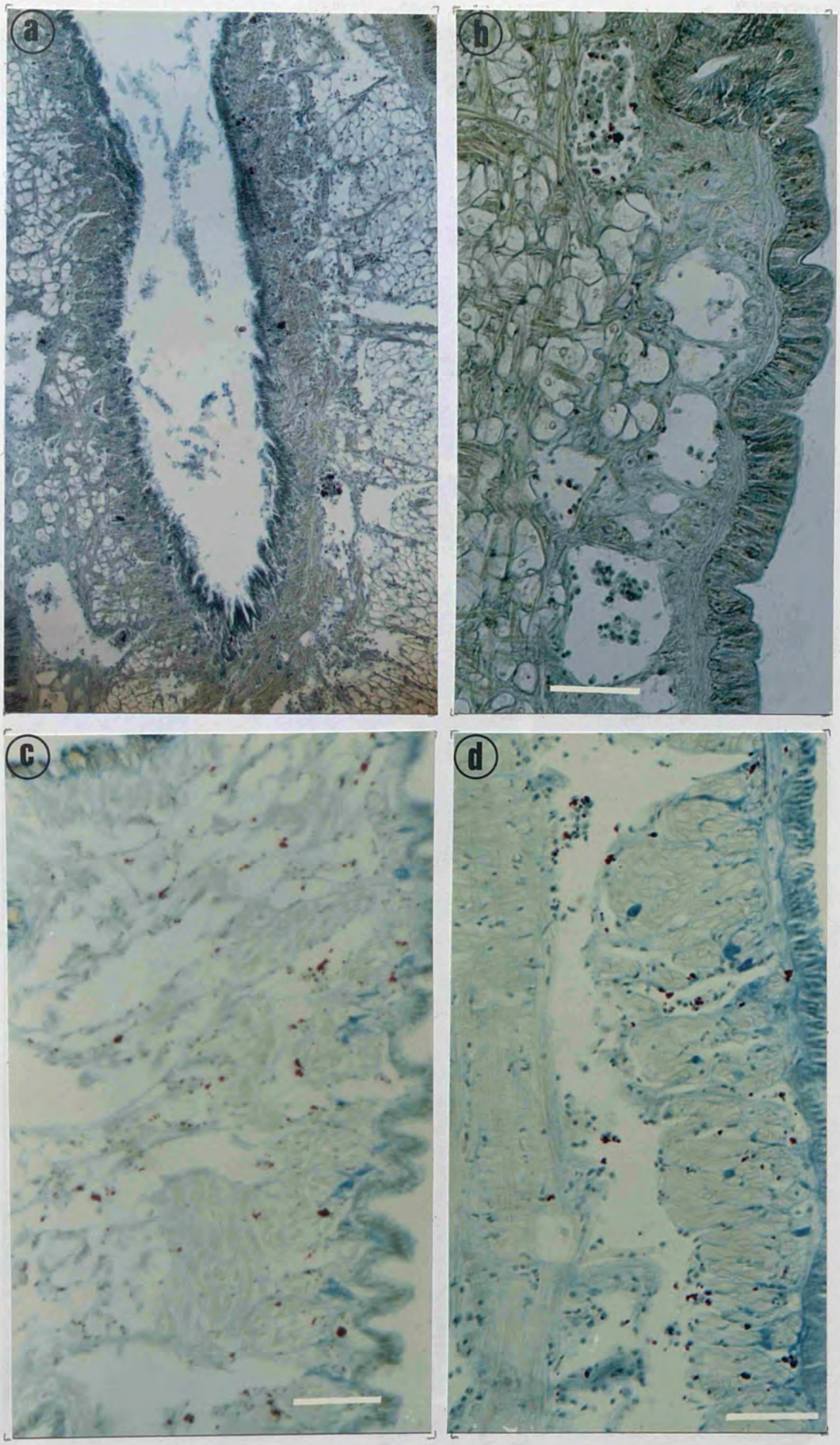


Plate 4.5

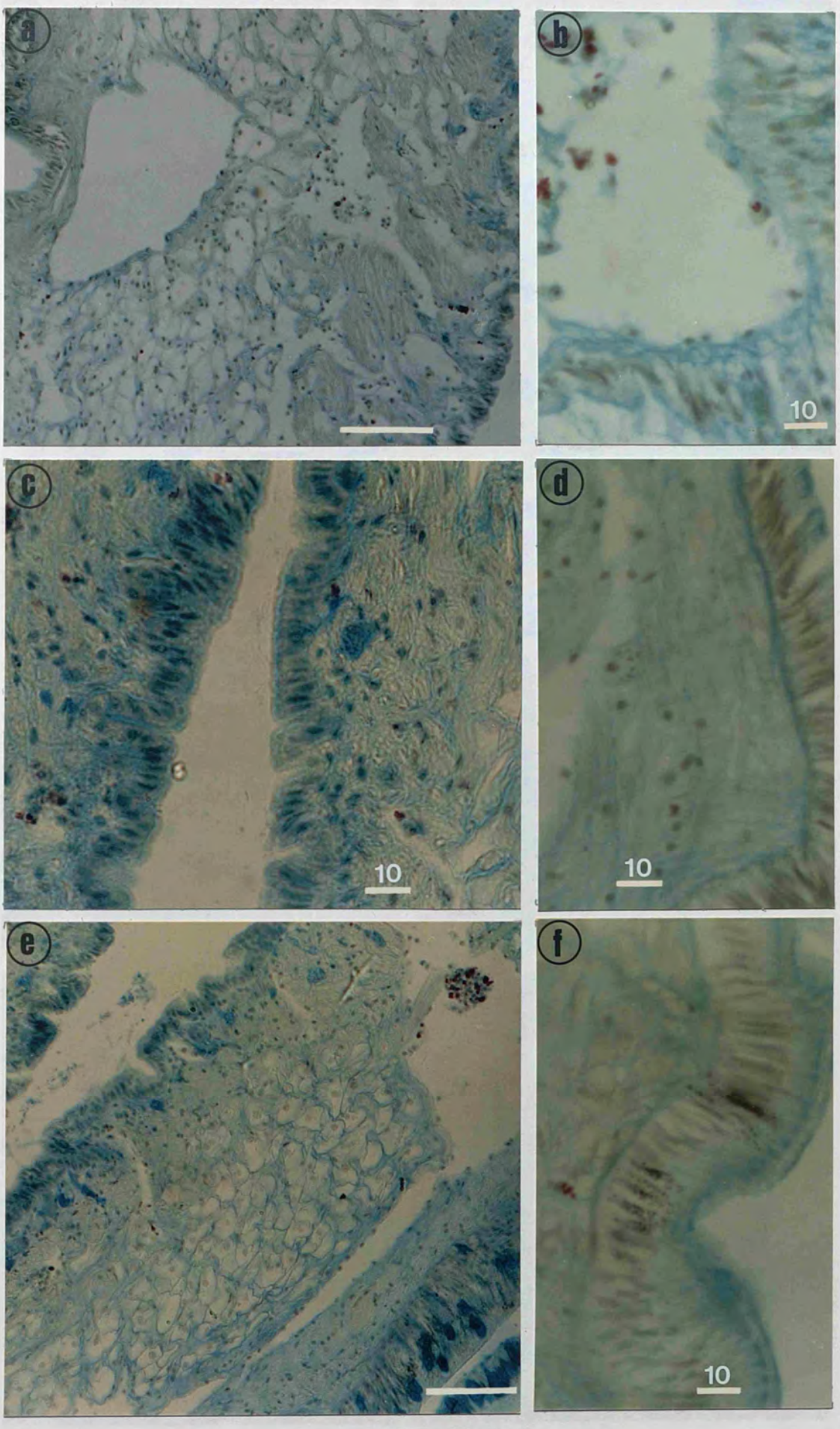


Plate 4.6

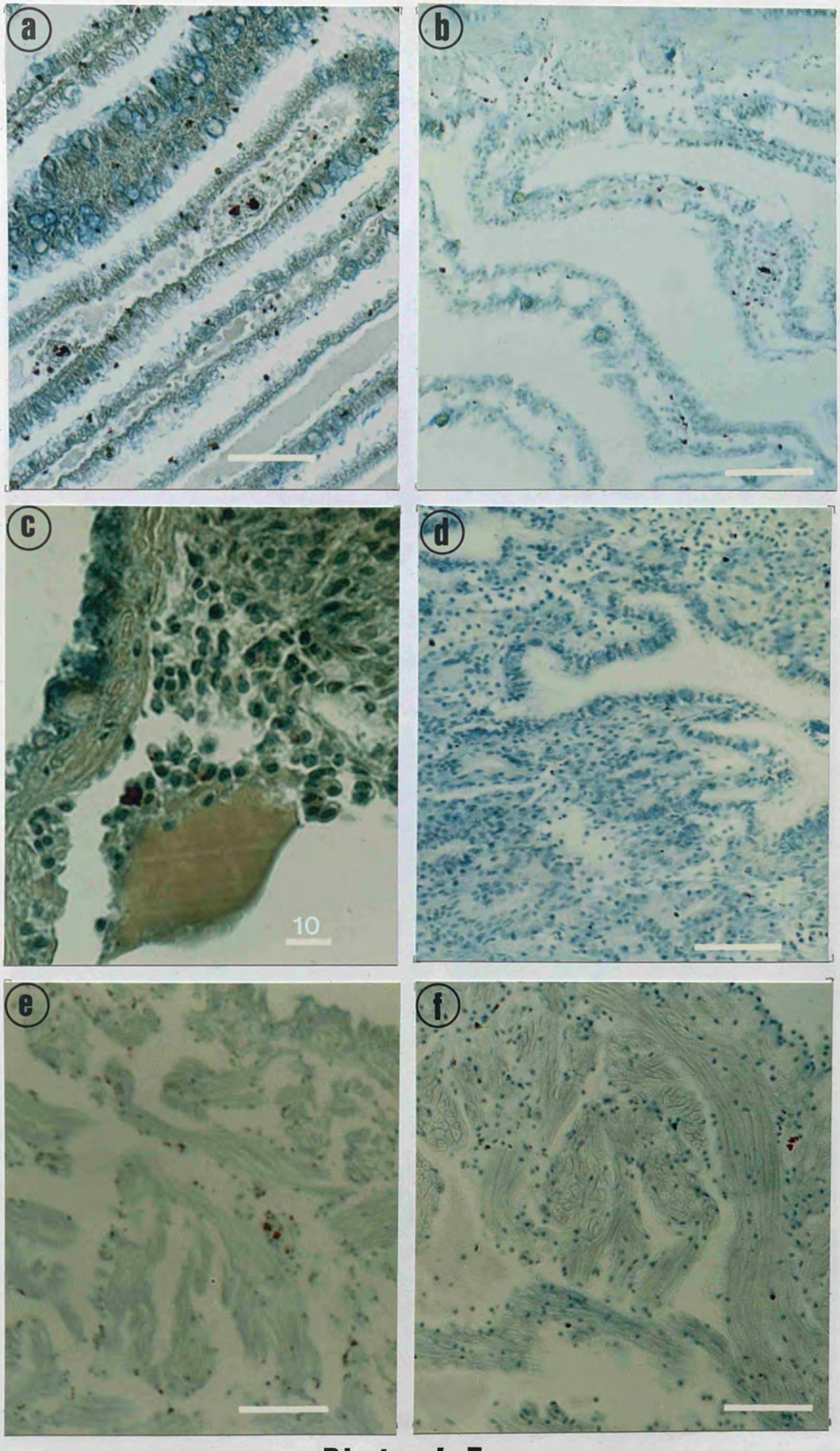


Plate 4.7

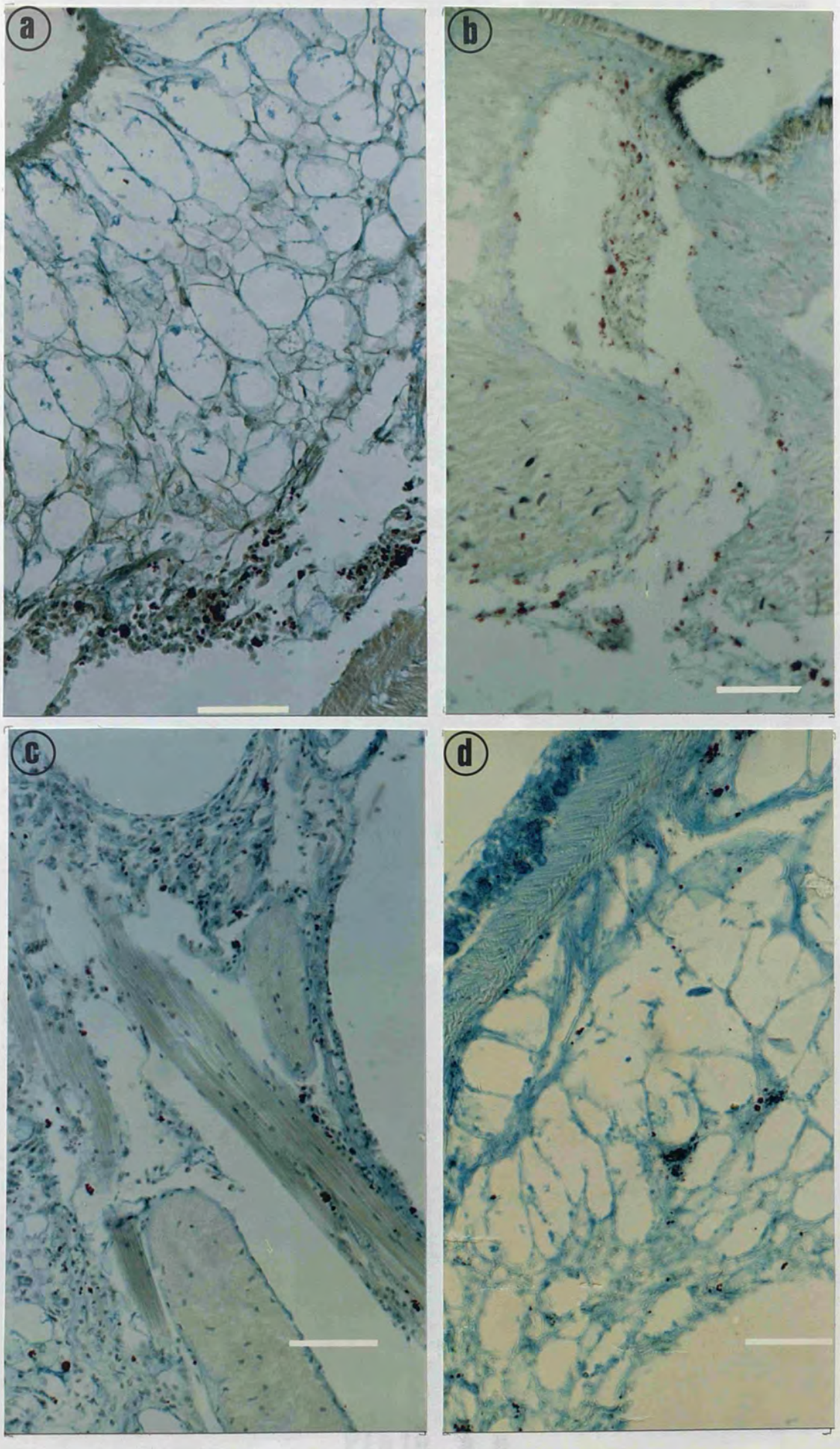


Plate 4.8

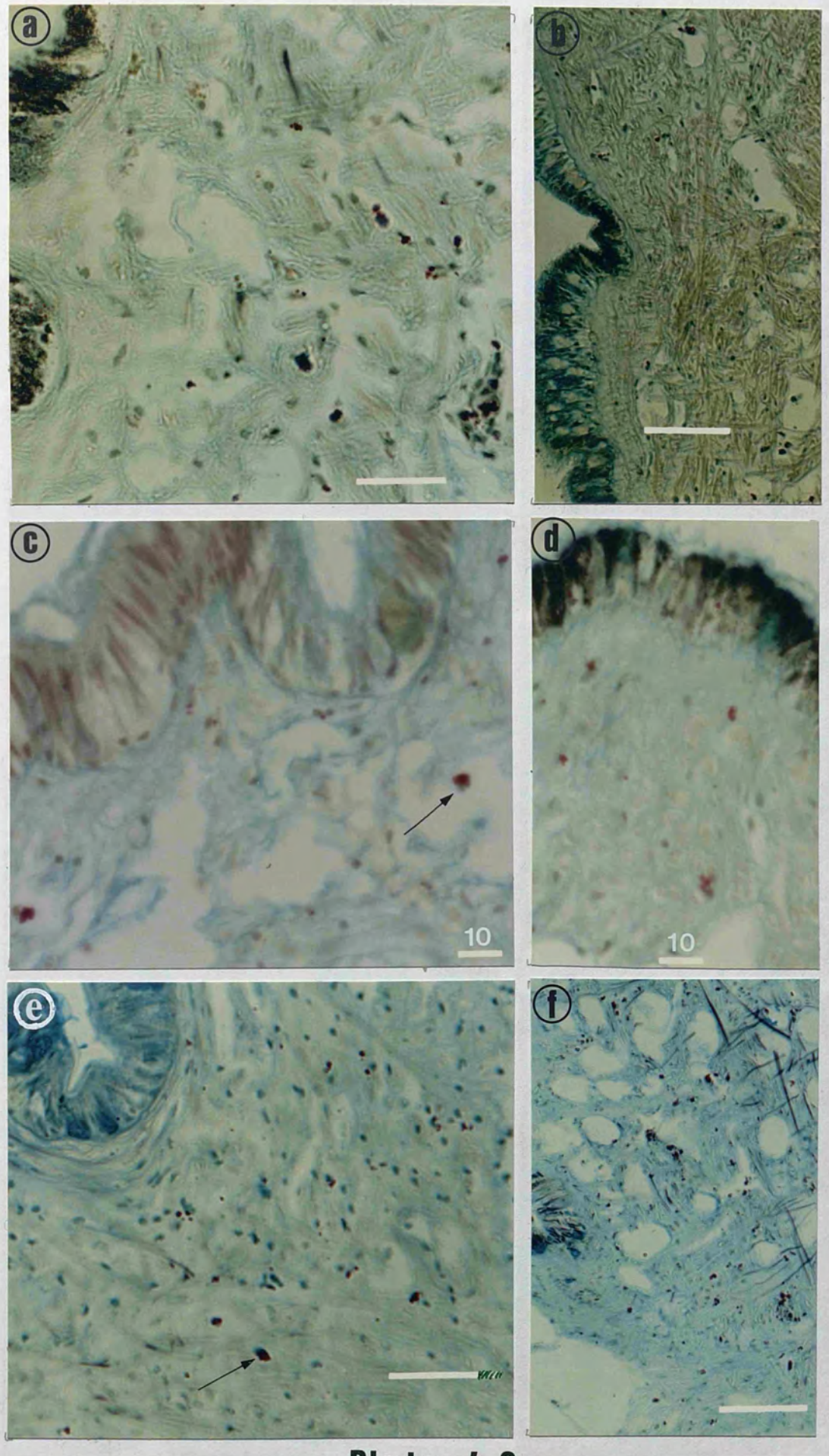


Plate 4.9

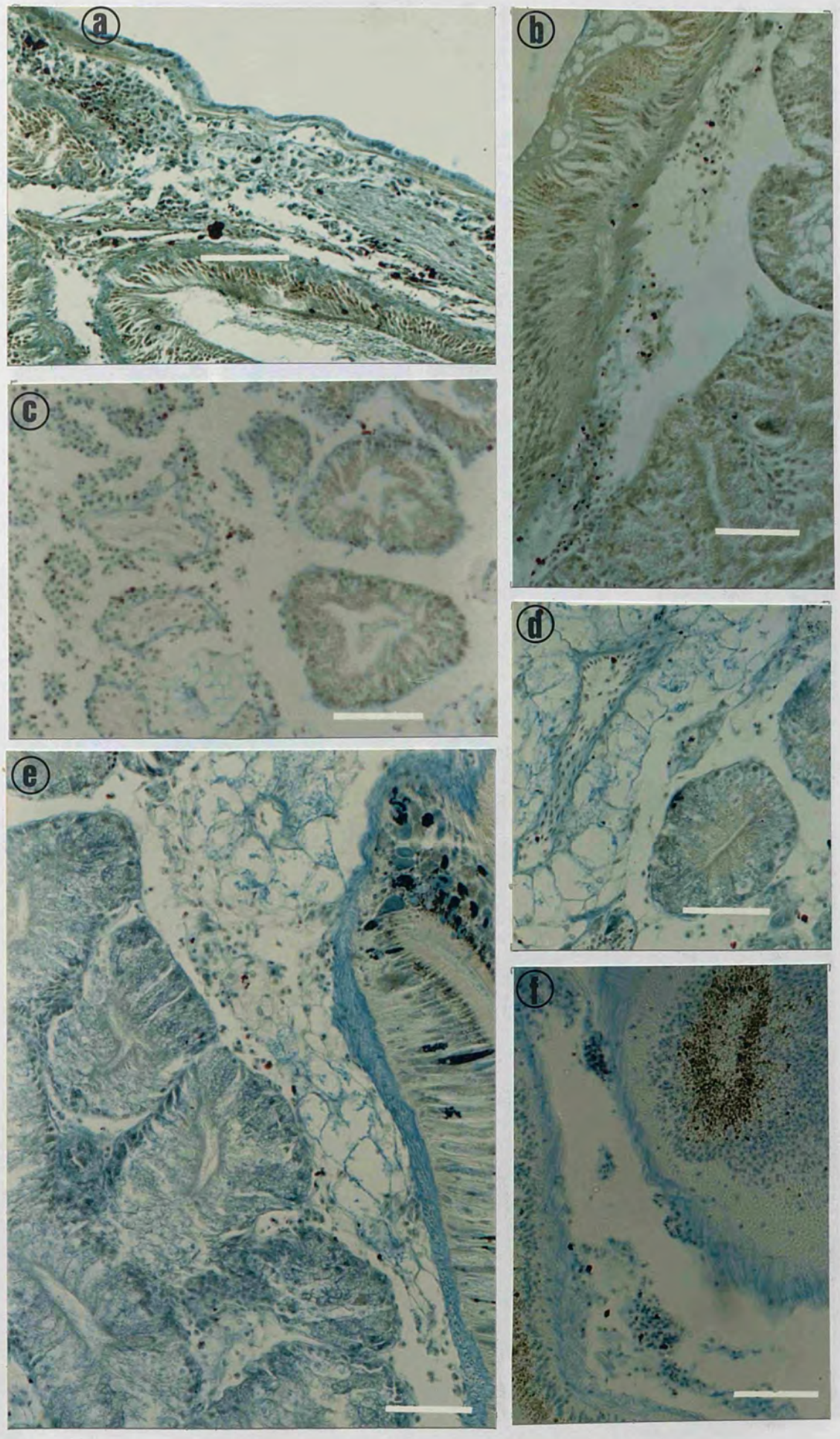


Plate 4.10

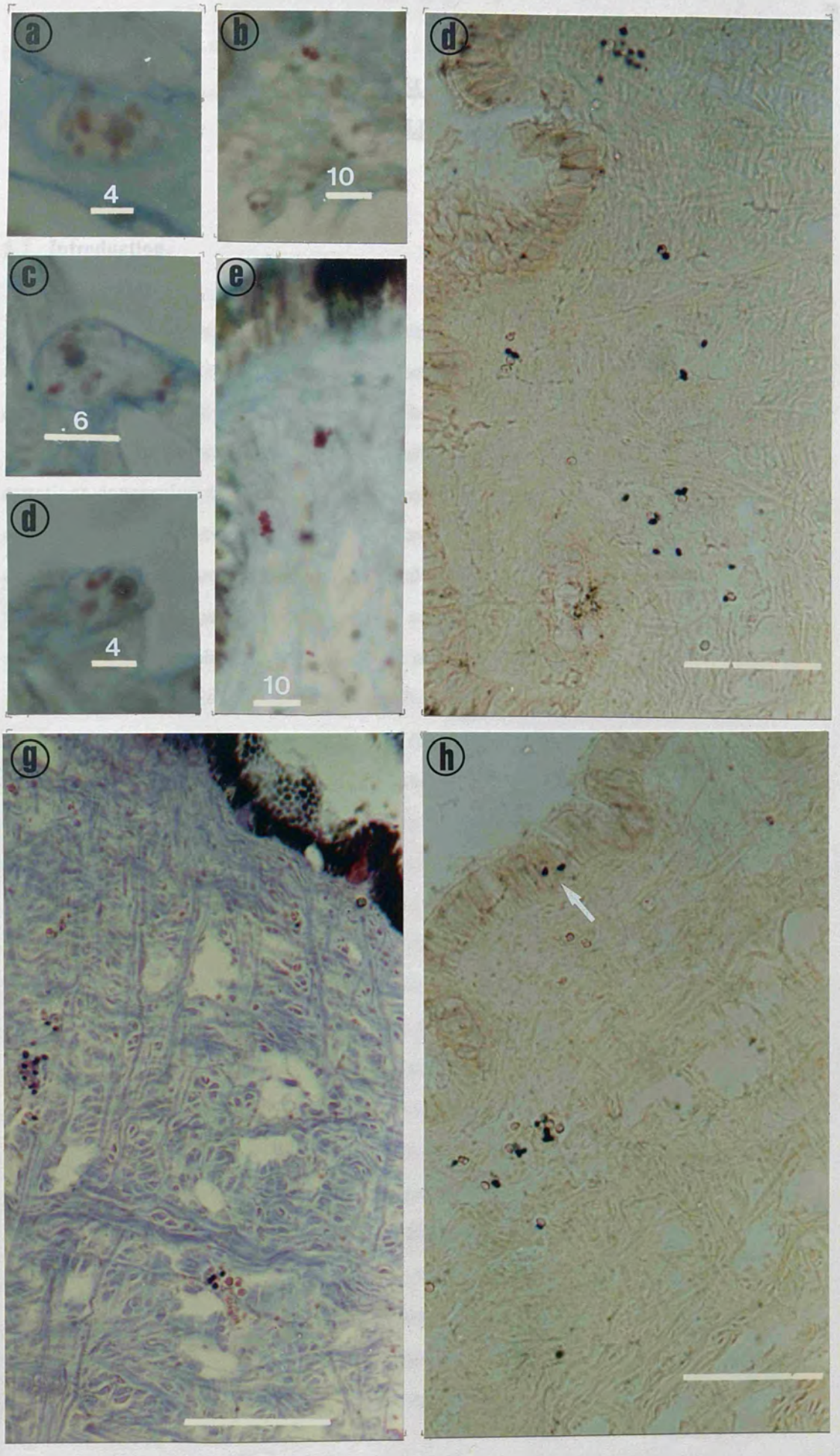


Plate 4.11

SECTION 5: CONNECTIVE TISSUE CELLS: ULTRASTRUCTURE AND ROLE IN CLEARANCE.

5.1 Introduction.

An overwhelming majority of the studies hitherto made into the defence system of invertebrates emphasizes the importance of circulating cells in the elimination of foreign particles and effete host cells (Ratcliffe & Rowley 1981). The rapid clearance of injected markers into *Helix pomatia* has nevertheless raised questions concerning the possible involvement of tissue-associated phagocytes or agglutinating substances in the response (Bayne 1973[b], 1974). Histological studies on chitons, cephalopods, opisthobranchs and pulmonates have also prompted suggestions that the connective tissue may contain fixed cells which assist in the cellular defence by phagocytosing injected material (Reade 1968, Stuart 1968, Pauley & Krassner 1972, Crichton *et al* 1973, Killby *et al* 1973, Sminia, van der Knaap & Kroese 1979). Most of these studies were carried out with the light microscope, and did not present clear evidence that the connective tissue phagocytes are distinct from wandering or settled haemocytes; the ability of haemocytes to leave the circulation and wander freely through the connective tissue or even settle for shorter or longer periods between the connective tissue cells (Stang-Voss 1970, Fernández 1971) underlines the need for careful interpretation of light microscope data. Ultrastructural studies on *Lymnaea stagnalis* (Sminia 1972, Sminia, van der Knaap & Kroese 1979) however support claims of fixed phagocytes in pulmonates, and raise the question of the involvement of fixed connective tissue cells in more primitive gastropods. The structure and function of prosobranch connective tissue has not yet been subject to a detailed study with the electron microscope, and the diversity of cell types and the contribution of each to defence against pathogens is not known. In order to establish whether the fixed phagocytes of *Lymnaea* also occur in more primitive gastropods, and to extend the studies already made on the cellular defence of *Littorina littorea*, the composition of the connective tissue was examined under the electron microscope, and experiments were performed in order to establish the phagocytic properties of connective tissue cells.

5.2 Materials and methods.

The ultrastructure of *Littorina littorea* connective tissue was studied in three untreated animals, which were sacrificed 1-2 weeks after collection, and selected parts of the body fixed for electron microscopy.* These included the heart, the kidney, the nephridial gland, the mantle, the foot, the tentacle, the ovotestis/digestive gland, and a branch of the posterior aorta. The tissues were excised and immediately placed in a fixative while, with the aid of a single-edge razor blade and a dissecting microscope, they were carefully diced into blocks or slices not exceeding 1 mm in thickness. They were then fixed for an additional hour in the same fixative (see Fahrenbach 1970), consisting of 1% glutaraldehyde, 3.5% formalin, 3.5% sucrose and 0.8% NaCl in a 0.1 M phosphate buffer (Glauert 1972) at pH 7.4. The tissue blocks were washed for an hour in buffered 8% sucrose (Fahrenbach 1970), and post-fixed in phosphate-buffered 1% osmium tetroxide for one hour. They were then stained *en bloc* with 0.5% uranyl acetate for one hour (Farquhar & Palade 1965) after being washed for one hour in 0.1 M acetate buffer (pH 7.4). The tissues were dehydrated in a closely graded series of ethyl alcohols (30%, 50%, 70%, 95% and absolute) and propylene oxide (two washes), allowing 15 min. for each washing. Fixation, dehydration and infiltration were carried out on a TAAB rotation table. The tissues were embedded in TAAB (after 16-18 hours infiltration in TAAB diluted to 50% with propylene oxide and 6-9 hours in undiluted TAAB) or in Spurr's low viscosity resin (Spurr 1969). In the latter case, propylene oxide washes were omitted, and tissues were infiltrated for one hour in an equal mixture of the resin and absolute ethanol, followed by at least 8 hours in the undiluted resin. Both resin types were polymerized at 60 °C.

The ultrastructure of circulating cells was also studied in sections made from haemocyte pellets. The haemocytes were harvested from whole blood by gentle centrifugation (250g for 3 min.), and fixed for 2 + 10 min. in a mixture (1:2) of 2.5% glutaraldehyde and 1% osmium tetroxide (Hirsch & Fedorko 1968), using 0.1 M cacodylate as a buffer. After treatment with uranyl acetate (0.25% in 0.1 M acetate buffer), the cells were processed according to the above schedule, using mild centrifugation between steps to prevent dispersion of the cells.

In order to establish the phagocytic properties of connective tissue cells, 8 animals were injected in the cephalic haemocoel with 0.5 ml. of sterile aSW containing 5×10^7 HRBC-0, 1×10^9 E.coli and 5×10^{12} T4 bacteriophage. The

* An additional snail, originally fixed and processed was abandoned as it turned out to be parasitized.

RBC had been previously fixed in 4% formalin. The animals were sacrificed in pairs at 30 min., 2 hours, 6 hours and 60 hours after injection, and tissues fixed and processed as described above. A part of each tissue was treated for the demonstration of lysosomes and/or peroxisomes at the ultrastructural level. The method of Graham & Karnovsky (1966) was used for the ultrastructural demonstration of peroxidase. Small cubes or slices of tissues were fixed and washed as described above, but before post-fixation, they were incubated for 10 min. at room temperature in a saturated solution of 3,3'-diaminobenzidine (DAB) in 0.05 M Tris-HCl buffer pH 7.6, containing 0.01 % hydrogen peroxide, and washed in 3 changes of buffer. Control tissues were subsequently treated with 3 mM potassium ferricyanide and washed 3 times before post-fixation. The method of Seeman & and Palade (1967) was used for the ultrastructural demonstration of acid phosphatase. The tissue slices were fixed in 2% glutaraldehyde for 30 min., and after washing for 1 hour in 3 changes of phosphate buffer, they were incubated in freshly prepared acid phosphatase medium at room temperature for 1 hour with continuous shaking. They were then postfixed and dehydrated as described above. The acid phosphatase medium contained 10 mM Na-glycerophosphate and 4 mM $Pb(NO_3)_2$ in 50 mM Na-acetate buffer (pH 5.0). Small pieces of tissue serving as controls were incubated in a medium that either lacked the substrate or contained 5 mM NaF.

Sections were cut to a thickness of 60-90 nm, as judged by refraction colours (silver or silvery-gold), using freshly made glass knives. The sections were mounted on uncoated copper or copper-palladium grids (200 mesh), and stained for 20-30 min. with uranyl acetate (Dawes 1971) and for 8-12 min. with lead citrate (Watson 1958). Spurr's resin sections required the longer staining times. The sections were examined and photographed in a Zeiss 109 electron microscope operated at 80 kV.

5.3 The ultrastructure of fixed connective tissue cells.

Apart from wandering haemocytes, or fibrocytes (described in section 5.4), four different cell types were distinguished in the connective tissue of the periwinkle; pore cells, calcium cells, supportive cells and granular cells. The pore cell has commonly been implicated in phagocytosis of extraneous materials, and was therefore studied in some detail, but as shown in the following section, no evidence

was found of involvement in the clearance process by this or other fixed connective tissue cells.

PORE CELLS are irregularly round or oblong in shape (Plates 5.1-5.3), measuring up to ca 20 μm (average section area 8 by 13 μm, N=23) with an oval nucleus of ca. 2.5 by 4.2 μm (N=13). Their main characteristic is a specialization of the plasmalemma; around the outline, it forms numerous invaginations bridged by cytoplasmic bars originating in the border of the invagination. In transverse sections the outline thus seems broken by **pores**, occurring mostly in groups over a common extracellular cisterna or invagination (Plate 5.1[a,b,e]), but tangential sections show the 'pores' to be slit-like in 3-dimensional structure (Plate 5.1[d]). The slit follows a winding course between 3-10 (or more) cytoplasmic bars arising alternately on either side (Plate 5.1[d,f]); the bars are usually straight but occasionally bent or angular (Plate 5.1[d]), and are 22-23 nm thick and ca. 70-76 nm wide. Most appear to be connected only at the end (Plate 5.1[g]), but along some of their length a cytoplasmic extension may also connect them to the bottom of the invagination (Plate 5.1[h]); adjacent pores, belonging to the same slit system may therefore in transverse sections appear to be totally separate (compare Plate 5.1[b] and 5.1[f]). The slit measures ca. 20 nm across and is bridged by a double diaphragm originating in the plasmalemma bordering the slit (Plate 5.1[c,d]). Its structure appears to correspond to the description given by Boer and Sminia (1976) for *Lymnaea* pore cells, i.e. with teeth of opposite rows facing each other, and teeth of the upper row set directly above the ones of the lower. An electron-dense material of ca. 15-22 nm thickness is associated with the other (i.e. cytoplasmic) side of the plasmalemma bordering the slit (Plate 5.1[b,c]). Pore cells are surrounded by a basement membrane, which is separated from the plasmalemma by an electron-lucent space of ca. 20-100 nm (Plate 5.1[b]; 5.3[b,c]).

Apart from this surface specialization, individual pore cells may take on widely different appearances, which apparently correlate with cyclic activity of protein-synthesis and release (compare Plate 5.1 & 5.2). The pore cells of any given tissue appear to be more or less synchronized, and in the tissue blocks selected for the present study, pore cells were clearly engaged in protein synthesis in the heart and the digestive gland, with GER cisternae being the most prominent organelle (Plate 5.2), but in the foot, most pore cells were highly vacuolated (Plate 5.1). Both types were seen in the mantle in tissue blocks fixed from two different animals, and

the observations suggest that the vesicular appearance corresponds to a nutrient uptake/product release phase in the cell cycle. Let us now look in more detail at the cytoplasmic features of pore cells.

Protein synthesizing pore cells typically contain whorls of dilated GER cisternae with oblong or dumb-bell shaped mitochondria at the periphery (Plate 5.2[a,c,d]) and a prominent dictyosome containing electron-lucent and more osmiophil material in separate compartments (Plate 5.2[d]). Glycogen, mostly present as β-particles (Plate 5.2[a,b]; cf. Revel *et al* 1960) occupies large areas in the cytoplasm; being soluble in aqueous solutions, however, it is conspicuous by its absence in material receiving routine fixation, leaving areas of leached appearance, interspersed with AER cisternae (Plate 5.2[d,e]; 5.3[a]). At its maturing face, the dictyosome gives rise to a number of small vesicles containing homogenous, moderately electron-dense material (Plate 5.2[d]); these apparently form a series of prosecretion granules, but profiles suggesting material release were not observed. The cisternae of the slit system may contain material of similar appearance to that inside the granules (Plate 5.2[b]), but the apparent uptake of this material by coated vesicles argues against it being a secretory product. The material inside the GER appears to be amorphous or finely fibrillar, and is apparently passed to the AER through the transitional ER (Plate 5.2[b,e,f]). It was not possible to identify haemocyanin (Hcy) molecules in the endoplasmic reticulum or other organelles normally associated with secretion, although Hcy was sometimes seen in slit system cisternae and vacuoles (Plate 5.3[a]). The appearance of the cell corresponds to a role in protein synthesis and secretion (Alberts *et al* 1983) but profiles indicating secretion were not seen; conversely, the vesicular variety of pore cell appeared to be active in either exo- or endocytosis or both, suggesting cyclic activity of protein synthesis and release. In vesicular pore cells, the GER is absent, or reduced to a few cisternae around the nucleus or near mitochondria, which are usually found in groups near the nucleus (Plate 5.1[a]). A juxtanuclear dictyosome is usually also present, containing electron-dense material which buds off at the ends as 0.1 - 0.3 μm vesicles. The main part of the cytoplasm is occupied by vacuoles and vesicles of varying size and appearance (Plate 5.1[a,e]) and granules of 0.5 - 1.5 μm diameter resembling those described from protein syntesizing pore cells. The vacuoles are widely interconnected, and apparently communicate also with granules on one hand and the slit system on the other (Plate 5.1[e,i]). Profiles of the AER often feature prominently in vesicular pore cells, and

may in tangential sections sometimes be seen in close connection to the slit system. The AER tubuli appear to be especially associated with the borders of the slits at the points where the cytoplasmic bars originate (Plate 5.3[c-e]). This close association of slit system cisternae and profiles of the AER is in fact seen also in protein synthesizing pore cells (Plate 5.2[e,f]; 5.3[b]).

CALCIUM CELLS (Plate 5.4[a-e]) are very large and conspicuous cells which occur throughout the connective tissue but are especially common in the walls of large blood vessels. They appear to be round or oval, measuring ca. 15-25 μm across. The nucleus measures ca. 2.4 by 4.3 μm (N=10), and has a thin rim of condensed chromatin at the edge and a few small clusters scattered in the nucleoplasm in connection with the nucleoli (Plate 5.4[d]). A few electron-dense granules of ca. 0.5 - 1 μm, identified with the acid phosphatase test as lysosomes are usually found near the nucleus, but the rest of the cytoplasm, apart from a thin rim at the periphery is more or less filled with an amorphous, electron-lucent material which in developing cells appears to accumulate by the fusion of vacuoles and disintegration of their limiting membranes (Plate 5.4[b]). These in turn are formed by the fusion and condensation of smaller vesicles (Plate 5.4[a,c]) which apparently develop from the Golgi system of young cells (Plate 5.4[a]). Developing calcium cells usually also contain a number of mitochondria (Plate 5.4[a]) which are spherical or rod-shaped and normally rather electron-dense. The nature of the storage product of these cells was not tested histochemically, but they are here presumed to be calcium cells on account of their similarity to such cells described from the pulmonate *Lymnaea stagnalis* (Sminia, de With *et al* 1977). Concretions, presumed by analogy to *Lymnaea* cells to represent $CaCO_3$ were observed (Plate 5.4[e]), but most cells appeared to be in stage *d* of Sminia's development diagram (Sminia, de With *et al* 1977, p. 205).

GRANULAR CELLS (Plate 5.3[g-i]) are found mainly in the mantle. They are elongate or irregular in shape and rather large, measuring ca. 5-10 x 15-20 μm. The nucleus is commonly polymorphic, and measures ca. 2-3 x 3-6 μm. It is relatively poor in chromatin but contains a prominent nucleolus. The cytoplasm is filled with GER and granules of ca. 0.2 - 0.5 μm diameter, containing material which ranges from rather low to medium electron-density (Plate 5.3[g,h]). These appear to form from Golgi vesicles, considered to be prosecretion granules. They later grow and

become less electron-dense, and eventually appear to release their contents into the extracellular matrix by fusing with the plasmalemma. The material is presumably proteinaceous, derived from the GER; in less mature cells, the GER often appears dilated with material of low electron-density (Plate 5.3[i]).

SUPPORTIVE CELLS (Plate 5.3[f]) are found in low numbers in the mantle and the foot, but are quite common in the gills, where they constitute the main part of the connective tissue, along with their products. They are filled with large secretory vacuoles containing finely fibrillar or granular material, and the remaining cytoplasm is largely confined to the nuclear area, apart from thin rims between some of the vacuoles; the outline of the cell is for this reason very indistinct. The vacuolar material did not seem to occur 'free' in the extracellular matrix, and therefore appears to be released in its membrane-bound form with mero- or holocrine secretion, unless the cells are syncytial and retain the vacuoles within the syncytium. The nature of the material is unknown, but may, with respect to the situation of the cell within the gill filament, serve a supportive function. The vacuoles appear to form by the fusion of small vesicles from the Golgi system. The cytoplasm also contains cisternae of the GER in continuity with the outer nuclear membrane. In immature cells, the cytoplasm is less displaced by vacuoles and the cells are much smaller, with a distinct periphery.

Pigment cells, or melanocytes appear to be absent from the connective tissue of the periwinkle, unlike pulmonates (Wondrak 1969, Wolburg-Buchholz 1972[a], Sminia 1972). The only profiles observed with superficial resemblance to pigment cells were, on closer examination, determined to be sagittal sections of **smooth muscle** fibres. The nucleus of large myofibres (e.g. in the mantle, tentacle or foot) is usually eccentric, protruding from the filaments into the surrounding space along with much of the non-filamentous sarcoplasm (Plate 5.4[f]); sagittal sections through this area but not including the filaments may thus be mistaken for a new type of connective tissue cell. Small myofibres, occurring e.g. in the gills and the nephridial gland (Plate 5.4[g]) also require close consideration, due to their irregular shape during relaxation, and variable content of myofilaments.

The connective tissue is also traversed by nerves, consisting of nerve cell fibres accompanied by glial cells, and blood spaces of various dimensions. In the

material studied, the latter appeared mostly as empty, unlined gaps in the connective tissue, although haemocytes were commonly seen clinging to the inside of the channels (Plate 5.4[h]).

5.4 Ultrastructure of the haemocytes, with special reference to phagocytosis.

The electron microscope does not reveal any significant ultrastructural differences between individual blood cells of the periwinkle, and they are therefore considered to belong to a single population. Circulating hamocytes are round to oval in shape with a central nucleus (Plate 5.5) and quite small, measuring on average 5.1 x 6.5 μm (N = 20). The nucleus is ovoid and relatively large, 2.7 x 3.8 μm (N = 22), with a variable number of nucleoli (Plate 5.5[a,b]; 5.6[a,c]) and moderate amounts of chromatin condensed at the edge and in dispersed clusters centrally (Plate 5.5; 5.6). Cells with bilobed nuclei are occasionally seen, giving the impression, in some sections, that the cells are binucleate (Plate 5.6[c]). The main characteristic of the haemocytes is the ability to alter their shape according to conditions and the formation of pseudopodia of various sizes and shapes (Plate 5.5[a]; 5.6[c]; 5.8[b]). Most of these are long and slender, measuring ca. 0.1 - 0.2 μm in width and up to 2 μm in length; they may fuse with the plasmalemma or each other (Plate 5.5[a]), forming vesicles and vacuoles of varying sizes which become transferred into the cytoplasm (cf. Plate 2.6[g-j]) . The vacuoles form a condensating series, as shown by the increasing density of their haemocyanin (Hcy) contents (Plate 5.6[b]; 5.8[b]). The vacuolar system also receives material from pinocytotic vesicles. Apart from vacuoles, rod-shaped mitochondria are the most conspicuous cytoplasmic constituent; they measure about 0.1 - 0.2 μm in diameter and 1-1.5 μm in length (Plates 5.5, 5.6[a]) and contain short, numerous cristae in an osmiophil matrix. Cisternae of the GER are found superficially (Plate 5.5[b]) or surrounding the nucleus and other organelles centrally (Plate 5.6[c]). The AER is commonly present in the form of horse-shoe shaped vesicles, which, when cut through both 'ends' may form profiles resembling autophagosomes (Plate 5.5[b], 5.6[b]). Most cell sections include a juxtanuclear dictyosome (Plate 5.5[a,b]; 5.8[b]); its convex part is usually dilated and contains material of low electron-density, the concave part being more osmiophil. A number of electron-dense vesicles of ca. 0.1 μm diameter bud off from the bulb-shaped ends of the Golgi and become transferred into the cytoplasm (Plate 5.5[a,b]). The granules contain acid phosphatase and thus classify as primary lysosomes; tests for peroxidase

were negative or weakly positive, and should be repeated using a stronger reaction mixture or prolonged incubation. Larger granules, measuring 0.3 - 0.6 μm in diameter probably represent residual bodies (compare Plate 5.6^{c}, insert, to Plate 5.6^{b}). Some cell sections include a centriole (Plate 5.6^{a}) but the present study did not reveal whether or not haemocytes are able to divide while in circulation. Glycogen is not preserved by routine methods, and is conspicuous by its absence as electron-lucid areas in Plate 5.5^{b}, 5.6^{a} and 5.7^{b}.

Haemocytes also have the ability to wander through the connective tissue, where they may be seen as variably elongated, fibroblast-like cells. The outline is commonly irregular, following the contours of neighbouring cells (Plate 5.7), giving the impression that the cell shape is governed mainly by the available space. This impression is in fact gained also from circulating cells (Plate 5.5^{a}; 5.6^{c}; 5.8^{a}), and apart from a more slender shape, wandering or settled haemocytes retain all characteristics of the circulating form (compare Plate 5.7^{a} to 5.5^{a} and 5.6^{c}), including overall dimensions and nuclear-to-cytoplasmic ratio. The cytoplasmic composition of Golgi, mitochondria, lysosomes and ER shows strong similarity to that of circulating cells, and vacuoles containing Hcy further support that these cells have gone through a circulating stage. The phagocytic capacity of the cell is also demonstrated by the common occurrence of residual bodies. The present study did not clarify whether these cells are capable of re-entering the circulation or whether they represent a fully differentiated type, but profiles were observed which suggest that outside circulation, they function as fibrocytes. As shown in Plate 5.7^{a} (insert), extracellular filaments with the banding characteristic for collagen may be seen in close association with the plasmalemma.

Although all of the injected markers, i.e. RBC, bacteria and viruses were frequently seen in close proximity to fixed connective tissue cells, only haemocytes were seen to attach to and ingest the particles (Plate 5.8). As already shown by light microscopy (Section 4), injected markers and circulating cells were most abundant in the kidney in the initial stages after injection, and this organ was thus selected for demonstration of the ultrastructural features of the phagocytic process, which looked similar in all tissues.

At all times, haemocytes can be found that do not contain an ingested particle in the plane of sectioning (Plate 5.8 a). These are commonly quite irregular in shape, suggesting activation in response to the presence of foreign particles; their

overall similarity to phagocytizing cells argues against their classification as a separate subset, although of course they may represent young, or immature cells.

Viruses were often seen close to the surface of the haemocytes, possibly attached to the cell coat (Plate 5.8[b,d]) but were not normally seen in phagocytic vacuoles except along with particles of the other two types (Plate 5.8[d]). Bacteria on the other hand were avidly phagocytosed, even in groups of 5-10 per phagosome (Plate 5.8[c,e]), with total numbers per sectioned area commonly exceeding 20. Profiles of the atttachment stage were rarely seen; the ingestion appears to proceed much quicker than with RBC (see below). Phagocytosed particles were first seen 30 min. after injection (Plate 5.8[b,c]), and throughout the experiment, profiles of partly degraded as well as freshly ingested bacteria were observed.

Phagocytosis of RBC proceeded at a slower rate, and was first seen at 30 min. (Plate 5.9[b-d]), although many haemocytes were seen with attached particles in freshly injected samples (Plate 5.9[a]). The cells become progressively more elongated as they spread out over the surface of the RBC (Plate 5.9[a]), eventually forming cytoplasmic extensions on either side (i.e. on all sides in 3-D) which envelope the particle as they meet (Plate 5.8[e], 5.9[b-d]). The observations suggest that fusion of the pseudopods may not be completed in 30 min. (Plate 5.9[b]), although the phagocytic vacuole, or heterophagosome often appeared to have lost all connection with the outside (Plate 5.9[c,d]). Multiple phagocytosis was commonly observed, but haemocytes were never seen to contain more than 3 RBC in the section area (Plate 5.9[c]). At the 6th hour, many of the RBC appeared to be losing their shape due to intracellular digestion Plate 5.9[e]), although phagocytosis still continued, as seen by freshly attached or ingested particles (Plate 5.8[e]). The digestion appeared to follow the fusion between RBC-containing heterophagosomes and small cytoplasmic vacuoles; these are identical to the ones found in the Golgi region, and clearly represent primary lysosomes. Throughout the experiment, several degradation stages of RBC and bacteria were observed.

5.5 Discussion.

Most of the available information on the connective tissue of gastropods appears to be incidental to work focussing on certain organs or organ systems, in particular the nervous system (e.g. Fernández 1966; Sanchis & Zambrano 1969;

Rogers 1969; Pentreath & Cottrell 1970; Dyer & Cowden 1973; see also Curtis & Cowden 1979), and only two species of pulmonates, i.e. *Arion hortensis* (Wondrak 1969) and *Lymnaea stagnalis* (Sminia 1972; Sminia, de With *et al* 1977; Sminia, van der Knaap & Kroese 1979) appear to have received a detailed overall examination with respect to the general composition of the connective tissue. A comparison between these studies and the current one, with support from other studies containing information on prosobranch connective tissue (Delhaye 1974, Martoja *et al* 1980) indicates that some differences may occur between pulmonate and prosobranch connective tissue. The most striking of these is the absence of **pigment cells** (=melanin cells) from the connective tissue of the periwinkle. Although searched for, no equivalents to these cells were found; their evolution could conceivably relate to the exposure of land-living snails to direct sunlight. The **'supportive cells'** of *Littorina* appear to have no counterpart in the pulmonates; this may well be linked with the disappearance of the gills in latter. These cells display a curiously ill-defined outline, and may in fact be syncytial; more work will be needed in order to clarify this point, and to establish the nature of the material inside its vesicles. **Pore cells, haemocytes** and **fibroblasts** are universally present, although whether or not the latter are to be looked at as a special cell type is open to interpretation (see below). **Calcium cells** were not described from *Arion*, but may have been overlooked; they were not included in the original report on *Lymnaea* connective tissue (Sminia 1972) but reported separately at a later stage (Sminia, de With *et al* 1977). These cells are especially abundant in the walls of large blood vessels, and appear to be quite similar in gastropods belonging to different orders (Sminia, de With *et al* 1977; Curtis & Cowden 1979). **Granular cells** also occur in the connective tissue of all 3 species, but the granular cells of *Littorina* clearly differ from those of the pulmonates. The developmental stages of *Lymnaea* calcium cells (Sminia, de With *et al* 1977) appear to bear some resemblance to the cells originally designated 'granular cell' (Sminia 1972), and whether or not the latter is still to be regarded as a special cell type may need more clarification. In the periwinkle, granular cells are clearly distinct from developing calcium cells, but their function, and phylogenetical relationship to the granular cells of *Arion* and *Lymnaea* remains to be decided. A brief account of cell types in *Cepaea nemoralis* connective tissue includes pore cells, melanocytes, fibrocytes and granular cells (Wolburg-Buchholz 1972[a]), but the latter 3 are not described and thus do not provide comparison to their counterparts in other pulmonates.

Of the cell types residing in the connective tissue of the periwinkle, only pore cells and haemocytes have been widely described from other molluscs. Although the former were not found to contribute to the cellular defence of the periwinkle, they merit some attention because of frequent claims of their phagocytic powers (e.g. Wolburg-Buchholz 1973). **Pore cells** have been described from 15 species of pulmonates and 2-3 species of opisthobranchs (Table 5.1); although they are known to occur in prosobranchs as well (Wondrak 1969), no detailed study has hitherto been made on pore cells from animals belonging to this order. This cell type is also known to occur in bivalves (Cheng & Rifkin 1970, Ruddell & Wellings 1971) and corresponding cells are found in arthropods, where they are termed pericardial cells (Wigglesworth 1970) or nephrocytes (Smith & Ratcliffe 1981; Crossley 1983). A number of different functions have been assigned to the pore cell, including collagen synthesis (Nicaise *et al* 1966, Plummer 1966, Newman *et al* 1968, Baleydier *et al* 1969), the storage of nutrients for use during hibernation (Fernández 1966, Sanchis & Zambrano 1969, Rogers 1969, Curtis & Cowden 1979; Dyer & Cowden 1973), uptake and digestion of exogenous materials (Buchholz *et al* 1971; Wolburg-Buchholz 1972[a,b], 1973; Wolburg-Buchholz & Nolte 1973) and blood

TABLE 5.1 : Observations on pore cells in molluscan connective tissue.

	OPISTHOBRANCHS:
Glossodoris	Nicaise et al 1966, Baleydier 1969
	PULMONATES:
Achatina	Plummer 1966, Skelding & Newell 1975
Arion	Skelding & Newell 1975, Wondrak 1969
Limax	Curtis & Cowden 1979
Cepaea	Buchholz et al 1971; Wohlburg-Buchholz 1972[a,b], 1973; Wohlburg-Bucholz et al 1973
Helix	Pentreath & Cottrell 1970; Fernandez 1966; Newman et al 1968; Sanchis & Zambrano 1969; Rogers 1969; Nicholas 1973; Sminia & van Dalen 1977
Planorbarius	Sminia et al 1972
Biomphalaria	"
Lymnaea	Stang-Voss 1970; Stang-Voss & Staubesand 1971; Sminia 1972, Boer & Sminia 1976
Triodopsis	Dyer & Cowden 1973
Ferrissia	Richardot 1975
	BIVALVES:
Crassostrea	Cheng & Rifkin 1970; Ruddell & Wellings 1971.

pigment synthesis (Sminia *et al* 1972; Sminia & Boer 1973; Sminia & Vlugt-van Dalen 1977). The first supposition seems very unlikely, as the connective tissue of gastropods is shown to contain typical fibroblasts (Plummer 1966, Wondrak 1969, Sminia 1972); later studies have also shown that the plasma membrane thickening at the slit border is associated with a diaphragm bridging the slit, indicating an anchoring role (Boer *et al* 1976), rather than being indicative of tropocollagen secretion (Plummer 1966). Logically, one would expect the laying down of collagen to require cell mobility. The accumulation of glycogen by pore cells has been mentioned by many authors working on Stylommatophorans (Fernandez 1966, Rogers 1969, Curtis & Cowden 1979), but is not emphasized in studies on other molluscs (e.g. Ruddell & Wellings 1971, Sminia 1972, Killby *et al* 1973, the present study). A role in nutrient storage and metabolism may be an original one, but it is logical to assume that this function is especially important in hibernating snails. The only decisive evidence on pore cell function supports a role in the synthesis of blood proteins (Sminia *et al* 1972; Sminia & Boer 1973; Sminia & Vlugt-van Dalen 1977). Molecules and crystals of haemocyanin (Hcy) or haemoglobin (Hg) have been observed within the GER of a number of different pulmonates (Hg in *Planorbarius corneus* and *Biomphalaria glabrata*, Hcy in *Lymnaea stagnalis* and *Helix aspersa*; Sminia *et al* 1972; Sminia & Vlugt-van Dalen 1977) and cytochemical evidence and the use of an analytical electron microscope reveals intracellular copper or iron in pore cells of the freshwater species (Sminia *et al* 1972). The accumulation of copper by pore cells of *Littorina* has been interpreted as the uptake and degradation of Hcy (Martoja *et al* 1980). Hcy molecules are not invariably found in protein synthesizing and secreting organelles of pore cells (Skelding & Newell 1975, Curtis & Cowden 1979, the present study), but combined with the results of Sminia and his associates, the finding of haemocyanin molecules in slit system cisternae and intracellular vacuoles of pore cells from other pulmonates (Skelding & Newell 1975, Curtis & Cowden 1979) and a representative of the prosobranchs in the present study (see also Martoja 1980), supports the general conclusion that blood protein metabolism is the main function of gastropod pore cells. Perhaps this definition can in fact be widened to encompass the processing and remodelling of tissue fluid proteins and nutrients in general (cf. vertebrate hepatocytes), as suggested by the dual role played by *Helix* pore cells; convincing evidence has been produced to support a function in glycogen storage (Fernandez 1966, Rogers 1969) as well as haemocyanin-synthesis (Sminia & van Dalen 1977) in stylommatophoran pulmonates.

Although the possibility of some degree of functional differences between pore cells from different tissues can not be outruled, the different appearance, in the present study, of pore cells in different tissues is believed to be due mainly to cyclic activity. The groups of mitochondria seen in various locations in vacuolar pore cells of the foot thus do not appear to have any obvious function and may be relics of a producing part of the cycle, the GER having been remodelled to form a part of the vacuolar system. Morphological changes in pore cells have before been suggested in connection with cell maturation (Plummer 1966); pore cells of newly hatched animals were found to contain extensive GER in the cytoplasm, vesicles and vacuoles becoming predominant at a later stage. In the present study, both aspects of pore cell activity were seen in the same animal, indicating cyclic activity rather than differences in connection with maturation. The temporal separation of nutrient uptake and product release may help to avoid the uptake and digestion of freshly released products; the uptake of abiotic material (Buchholz *et al* 1971) suggests that endocytosis may occur without the involvement of specific receptors. Cyclic activity may explain the failure of previous studies to observe dictyosome involvement in the exocytosis of pore cell products (e.g. Sminia 1972, Dyer & Cowden 1973, Skelding & Newell 1975) although the suggestion that the slit system is directly (Sminia 1977) or indirectly (Plummer 1966) connected to the ER receives some support from micrographs in this (Plate $5.2^{e,f}$; $5.3^{b,d,e}$) and previous studies, demonstrating close association between the slit system and profiles of the GER (Skelding & Newell 1975) or the AER (Plummer 1966, Rogers 1969; the present study). The direct passage of materials from the AER to the outside has never been demonstrated, and secretion of material by fusion of prosecretory vesicles with vacuolar extensions from the slit system cisternae as indicated by Plate $5.1^{e,i}$ seems more probable, but the present study does not support any final conclusions on the mechanism of product release; in future studies on the remarkable surface specialization of the pore cell, attention should be given to its relationship to the exocytic as well as the endocytic process, and the possibility of cyclic activity investigated further.

Relative to claims of an endocytic function for pore cells (Buchholz *et al* 1971; Wolburg-Buchholz $1972^{a,b}$, 1973; Wolburg-Buchholz & Nolte 1973), it seems very likely that they can be explained in terms of nutrient uptake, i.e. the uptake of blood pigment for recycling. These claims have risen mainly in connection with studies on the fate of injected markers (e.g. Wolburg-Buchholz 1973), but the

present study suggests that the uptake and digestion of endogenous material is a function normally assumed by the cell. It seems pertinent that none of the injected markers was taken up by the pore cell, and a close look at the published claims of a role in clearance by these cells often reveals failure to distinguish between phago- and pinocytosis. In fact, only materials with particle size < 20 nm are taken up by *Lymnaea* pore cells (Boer & Sminia 1976); this correlates well with the dimensions of the slit system diaphragm, suggesting a role in ultrafiltration of fluids to be pinocytosed. The endocytic capacity of pore cells thus seems restricted to pinocytosis. This appears to exclude pore cells from the uptake of pathogens, most of which far exceed 20 nm (excepting only the smallest viruses), and in the light of the above considerations, and the failure of pore cells in the present study to ingest viruses of ca. 60-70 nm. dimensions, it seems reasonable to conclude that pore cells do not contribute to the clearance of pathogens.

Of the cell types under observation in the present studies, only **haemocytes** were shown to contribute directly to the defence against potential pathogens by phagocytosis and intracellular digestion of the unwanted material. This response may be augmented by diapedesis of phagocytes through epithelial borders, as shown in Section 4. Particles become ingested by the formation of cytoplasmic outgrowths around their periphery, which fuse on the distal side. Profiles of the phagocytic process (Plate $5.8^{c,e}$; 5.9^{a-d}) imply close association between the plasmalemma and the RBC surface, reminiscent of the 'zipper' mechanism of mammalian phagocytes ingesting IgG-coated particles (Griffin *et al* 1975, 1976). Bacteria, on the other hand, occur in groups in phagosomes (Plate $5.8^{c,d}$) and thus apparently do not require circumferential attachment for ingestion, suggesting similarity to the ingestion of complement-coated particles by vertebrate macrophages (Kaplan 1977). The observations support the suggestion made earlier (Section 4) that the cell volume limits the uptake of RBC to an average of 2-3 per haemocyte, but the ingestion of smaller particles like bacteria appears less limited by this factor and it may not be important *in vivo*. After internalization of the marker, digestion ensues by fusion of the phagosome with small vesicles, apparently formed by the dictyosome, which clearly represent primary lysosomes. The ultrastructural observations confirm the point made earlier (Section 4.3) that fixed RBC are taken up and digested at a relatively slow rate, and profiles of intracellular digestion were first seen at 6 hours; the lack of material between 30 min. and 6 hours precludes any final comments on

the onset of digestion but the observations suggest that it is not very pronounced at 6 hours. Slow digestion of markers has been attributed to glutaraldehyde fixation in studies on annelids (Braunbeck & Dales 1984); in the present study, it may be affected also by the apparent failure of serum lectins to bind to RBC's after fixation (cf. Section 6). Haemocytes containing half-digested markers look healthy, and are probably able to re-enter the circulation and repeat the process or continue with other functions.

Morphological and functional aspects of gastropod haemocytes have been recently reviewed in detail (Sminia 1981), and a comparison of *Littorina* haemocytes to those of other gastropods indicates many common features. The haemocytes of the periwinkle form a homogenous population of small, extremely pleomorphic cells, which may be found in the connective tissue as well as in the vessels. They clearly correspond to **amoebocytes** of other gastropods (cf. Sminia 1981), with hyalinocytes absent; this contrasts with the picture from the only other prosobranch studied ultrastructurally with respect to blood cell types, *Cerithidea californica* (Yoshino 1976). *Littorina* haemocytes are highly phagocytic (cf. also Sections 3 & 4) and commence digestion of the endocytosed material in a matter of hours; their migrating activity is demonstrated by the presence of particle-laden phagocytes in all tissues at all times; although some of the material may have been ingested *in situ*, there is little doubt that much of it has been phagocytosed in circulation and carried to the observation site (cf. also Section 4).

Migrating haemocytes are identified by their similarity to the circulating form in size, cytoplasmic composition and nuclear-to-cytoplasmic ratio. The migrating activity of molluscan haemocytes is well established (Yonge 1923, Galtsoff 1964, Fernandez 1971, Sminia 1981) and in *Lymnaea*, 'the amoebocytes by grouping together are able to form tissues, this being a reversible process' (Stang-Voss 1970). In *Littorina*, the tissue-associated haemocytes appear to be capable of collagen secretion, and thus may be regarded as fibrocytes, but their overall similarity to the circulating cells and the frequent observation of intermediate stages speaks against their separation as a special cell type. The ability of haemocytes to produce collagen under some circumstances has been noted in studies on pulmonates (e.g. Sminia *et al* 1973), and haemocytes have been implicated as fibroblast precursor cells in bivalves (Rifkin *et al* 1969). There is thus considerable support to the suggestion (Wagge 1955) that molluscan fibroblasts and amoebocytes may be different functional stages of the same cell. Final conclusions can however only be made after more detailed

studies on collagen synthesis, using radioactive collagen precursors, and on cell dynamics and proliferation in different species of molluscs.

The present studies indicate that haemocytes may settle in the connective tissue, but that they do not give rise to a separate cell population of fixed phagocytes. This agrees with previous, light microscope observations on prosobranch gastropods (Brown & Brown 1965, Cheng, Thakur & Rifkin 1970) and bivalves (Stauber 1950, Tripp 1958, Reade & Reade 1972); animals belonging to these taxa appear to rely exclusively on haemocytes for clearance of potential pathogens. Fixed phagocytes have been reported in studies on the chiton *Liolophura gaimardi* (Chrichton *et al* 1973, Killby *et al* 1973), the cephalopod *Eledone cirrosa* (Stuart 1968), the opisthobranch *Aplysia californica* (Pauley & Krassner 1972), and the pulmonates *Helix pomatia* (Reade 1968) and *Lymnaea stagnalis* (Sminia, van der Knaap & Kroese 1979). Only the first and the last of the cited studies however used the electron microscope, and, significantly, all but the last failed to distinguish between pinocytosis and phagocytosis. Critical examination of the above reports suggests that the cells referred to as 'fixed' may be identical to tissue-associated haemocytes in the chiton and the octopus, and to pore cells in the sea hare and *Helix*; ultrastructural studies on the endocytosis of injected markers in the latter species (Buchholz *et al* 1971) did not confirm the presence of fixed phagocytes other than the pore cell with its limited endocytic powers (see above discussion). The close association of this cell type to blood vessels and sinuses is a feature shared by the cells referred to by Reade (1968) as fixed phagocytes. The fixed phagocytes of *Lymnaea* (Sminia, van der Knaap & Kroese 1979) differ from the circulating haemocytes in lacking intracellular peroxidase, and being highly irregular in outline, suggesting intimate association with the ground substance. Both features could of course be of secondary nature, and the uptake, by these cells of immobile markers of large particle size in fact implies previous contact with circulating fluid, i.e. differentiation from a mobile stem cell. 'Fixed' phagocytes may be a special adaptation, by higher gastropods, to conditions met in the course of their evolution, but their overall similarity to haemocytes suggests a common origin, and they may conceivably represent a functional subset. Their absence from the connective tissue of the periwinkle underlines the need of additional studies for final conclusions to be drawn on the phylogenetic distribution of fixed phagocytes in gastropod connective tissue and their relationship to circulating haemocytes.

PLATES 5.1 - 5.9

Ultrastructural aspects of *L. littorea* haemocytes and connective tissue cells. Abbreviations: N=nucleus, nu=nucleolus, M=mitochondria, V=vacuoles, v=vesicles, G=granules, D=dictyosome, L=lysosomes, S=slit system, C=slit system cisternae, T=tongue bar, bl=basal lamina, gl=glycogen (partly dissolved), MF=myofilaments, P=pseudopods, B=bacteria, RBC=red blood cells. Bar: 1 μm, or as indicated (in μm).

Plate 5.1: Pore cells in connective tissue of the foot, apparently engaged in nutrient uptake or product release. (a) Overall view. (b-c) Enlarged view of the slit system. Note diaphragm (arrow), and dense material (*) associated with the cytoplasmic side of the plasmalemma. (d) A grazing section of the slit system. (e) The vacuolar system and its relationship to granules. (f) A grazing section of the slit system. (g) A tongue bar in LS. (h) A tongue bar in LS, showing confluence with cisternal bottom. (i) Cytoplasmic vacuoles and their relationship to extracellular cisternae.

Plate 5.2: Protein synthesizing pore cells from the heart (a-c) and the posterior aorta (d-f). (a) Overall view. (b) Enlarged view of the boxed area in (a), showing the relationship between the GER and the AER. (c) Enlarged view of the GER (another cell). (d) Overall view of a cell with a well developed dictyosome and prosecretion granules. (e, f) The formation of AER from the transitory ER. Note double nature of slit system. Z=prosecretion granules, TER=transitory ER.

Plate 5.3: Pore cells (a-e), supportive cells (f) and granular cells (g-i). (a) Haemocyanin (*) in a slit system cisterna. (b) Possible relationship between AER and slit system cisternae (arrow). (c) Vesicular profiles connecting to the exterior through a slit system cisterna. (d-e) Tubular profiles of the AER (arrrows) in close connection with slit system tongue bars (seen sagittally in d). (f) a supportive cell from the foot. (g) Overall look of a granular cell. (h) Enlarged view of granules. (i) An immature granular cell.

Plate 5.4: Calcium cells (a-e), muscle cells (f-g) and blood vessel wall (h). (a) Immature calcium cell. (b) Calcium cell in maturation. (c) Vesicles of a maturing calcium cell. (d) Overall view of a calcium cell (PC=pore cell). (e) Calcium concretions (*). (f) LS of a muscle cell from foot. (g) LS of a muscle cell from gill. (h) A marginating haemocyte (H). Note non-cellular nature of vessel wall.

Plate 5.5 & 5.6: Circulating haemocytes. Note vesicle formation by the fusion of pseudopods (5.5^a), ectoplasmic processes extending over neighbouring cells (5.5^a, 5.6^c), centriole (5.6^a, arrow), well developed AER with horseshoe-shaped vesicles (5.6^b, *), vacuoles with Hcy (5.6^b), and irregular shape of nucleus (5.6^c; 5.6^c insert).

Plate 5.7: Migrating or tissue resident amoebocytes. Note the close relationship of the cell in (a) with ground substance fibrils resembling collagen (insert), and binucleate appearance of the cell in (b).

Plate 5.8: Phagocytic amoebocytes. (a) Irregularly shaped cell, 6 hours p-i. (b-c) Amoebocytes with ingested bacteria, 30 min. p-i. The bacteria are enclosed singly or in groups in endocytotic vacuoles (ev). Note ectoplasmic outgrowth in (b). (d) Ingested bacteria, 6 h p-i. (e) Haemocyte with a phagocytosed bacterium and an attached RBC 6 h p-i. Note viruses associated with the cell coat (*).

Plate 5.9: Phagocytic amoebocytes. (a) Haemocyte attaching 3 RBC immediately following ingestion. (b) Haemocyte with ingested RBC 30 min. p-i. Note confluence of phagosome with the plasmalemma. (c) Haemocyte with 2 ingested and 1 half-ingested RBC 30 min. p-i. (d) Haemocyte with an ingested RBC 30 min p-i. (e) Haemocyte with 2 RBC showing signs of degradation 6 h p-i.

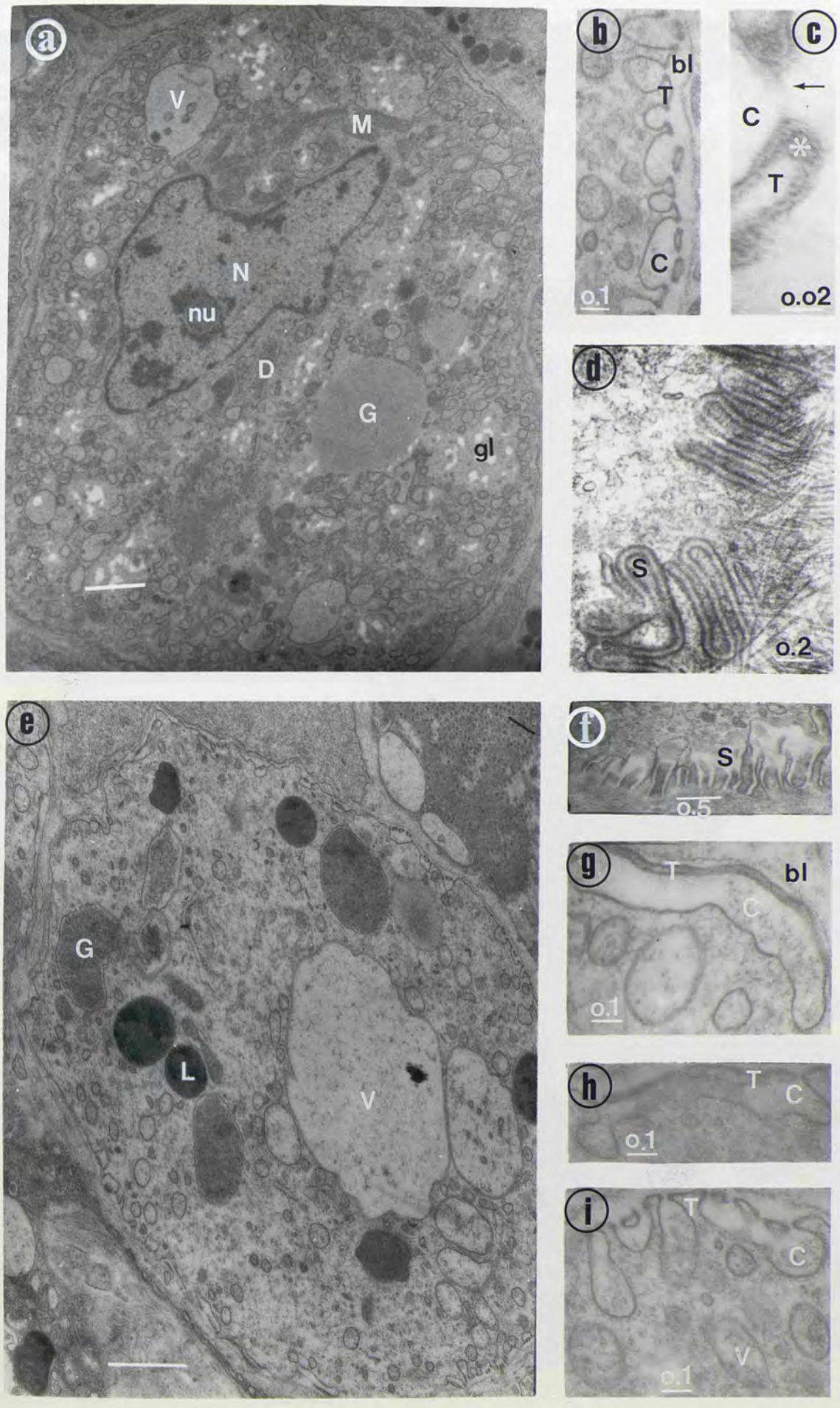

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Plate 5.1

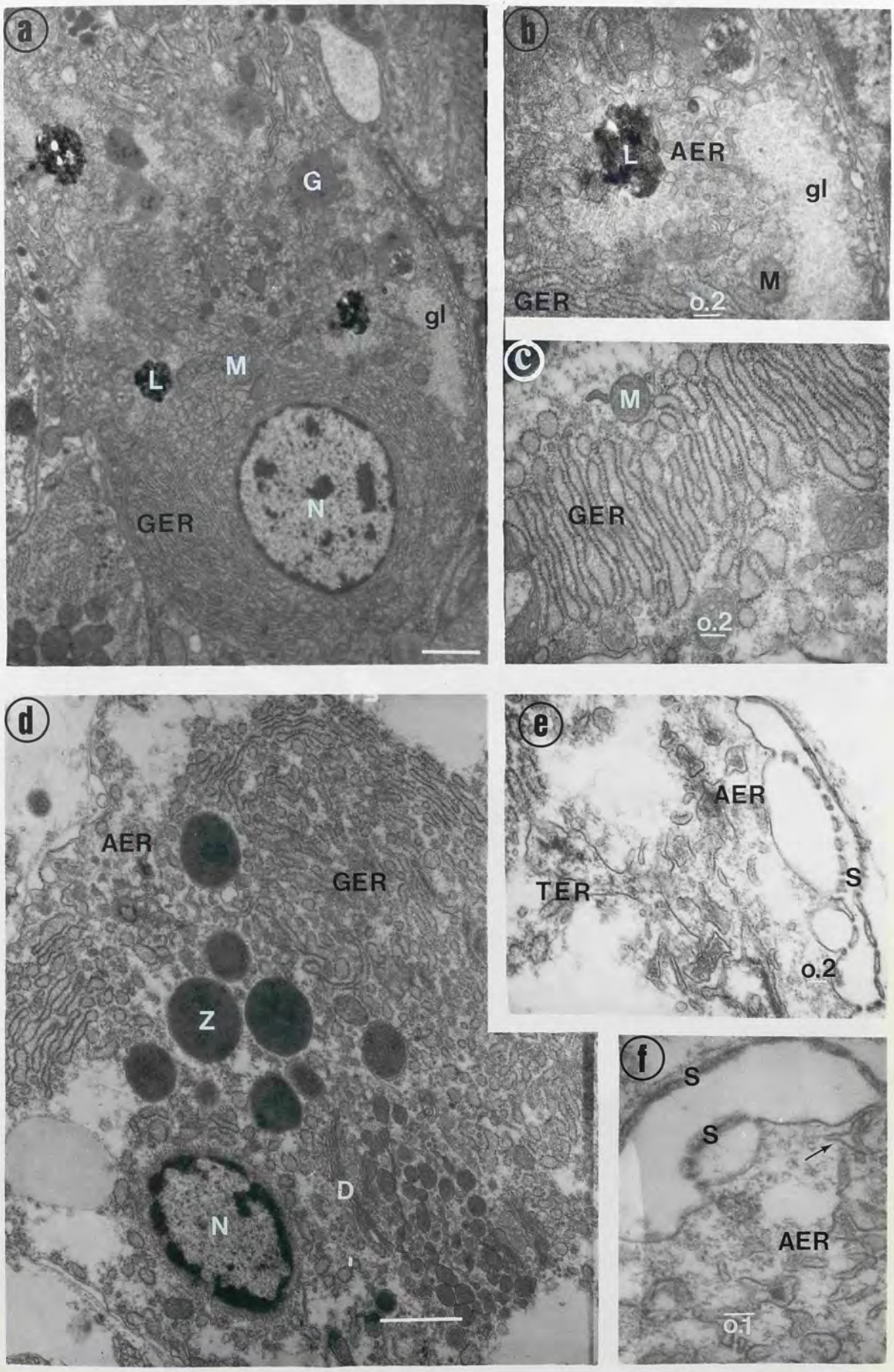
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Plate 5.2

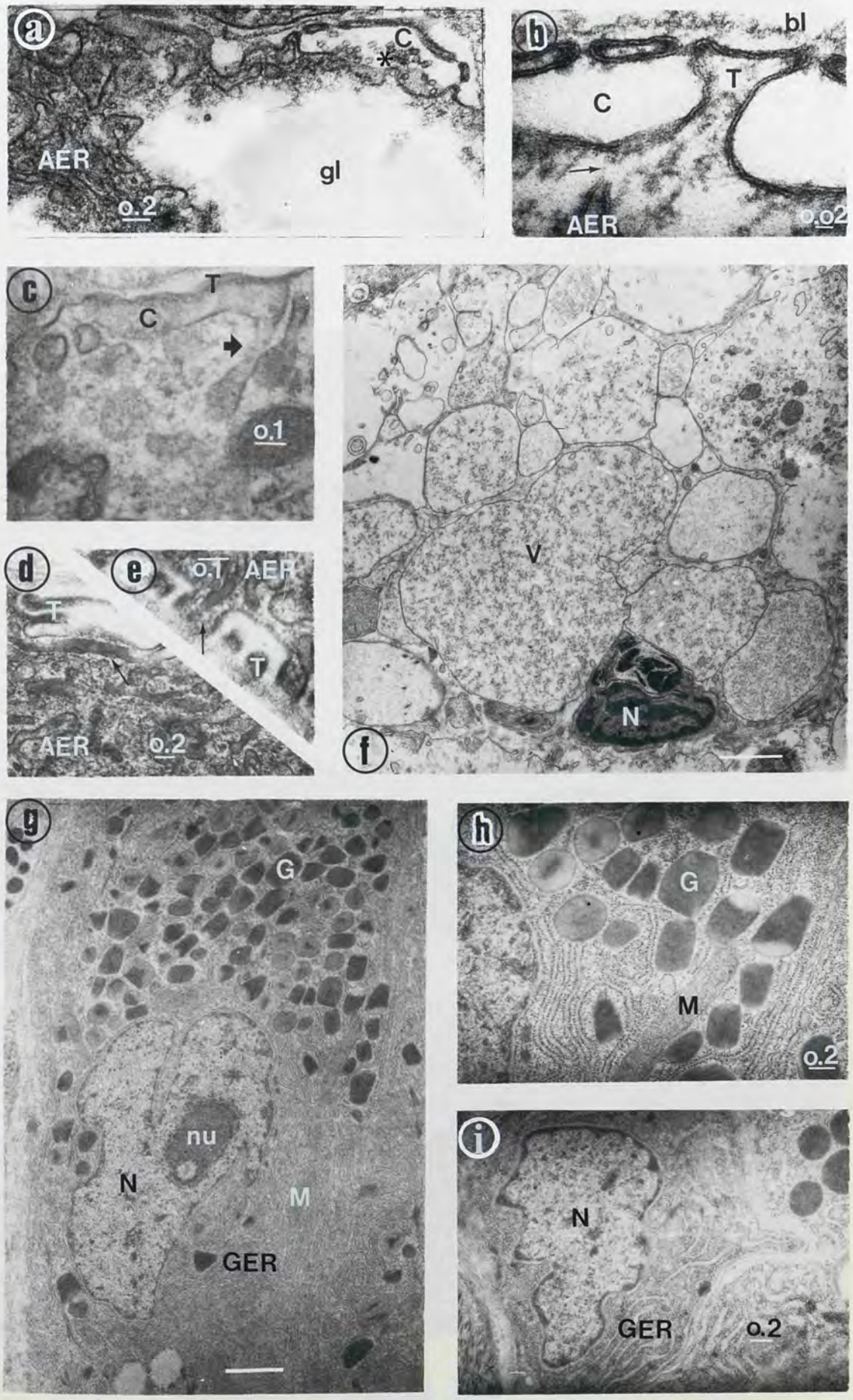
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Plate 5.3

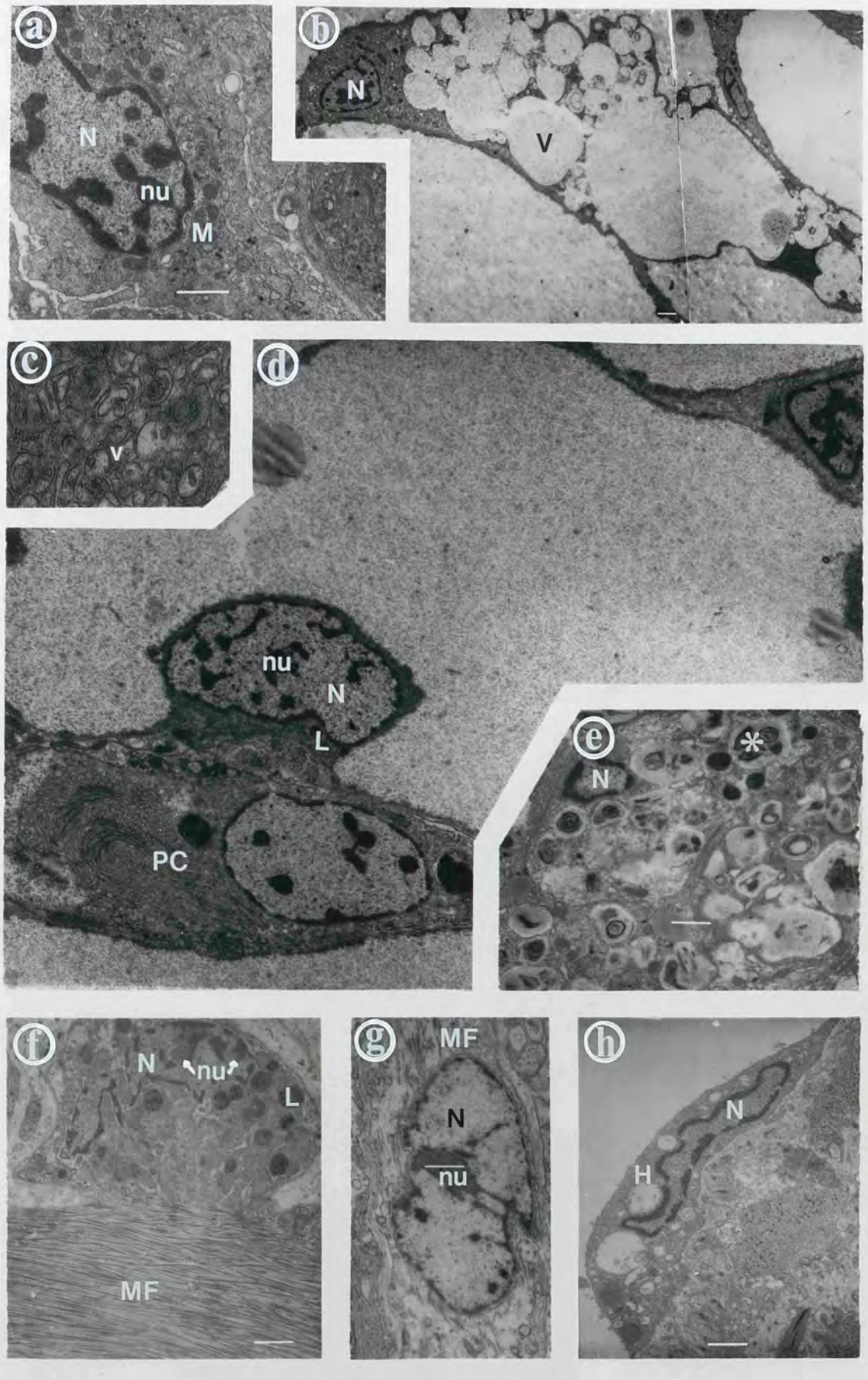
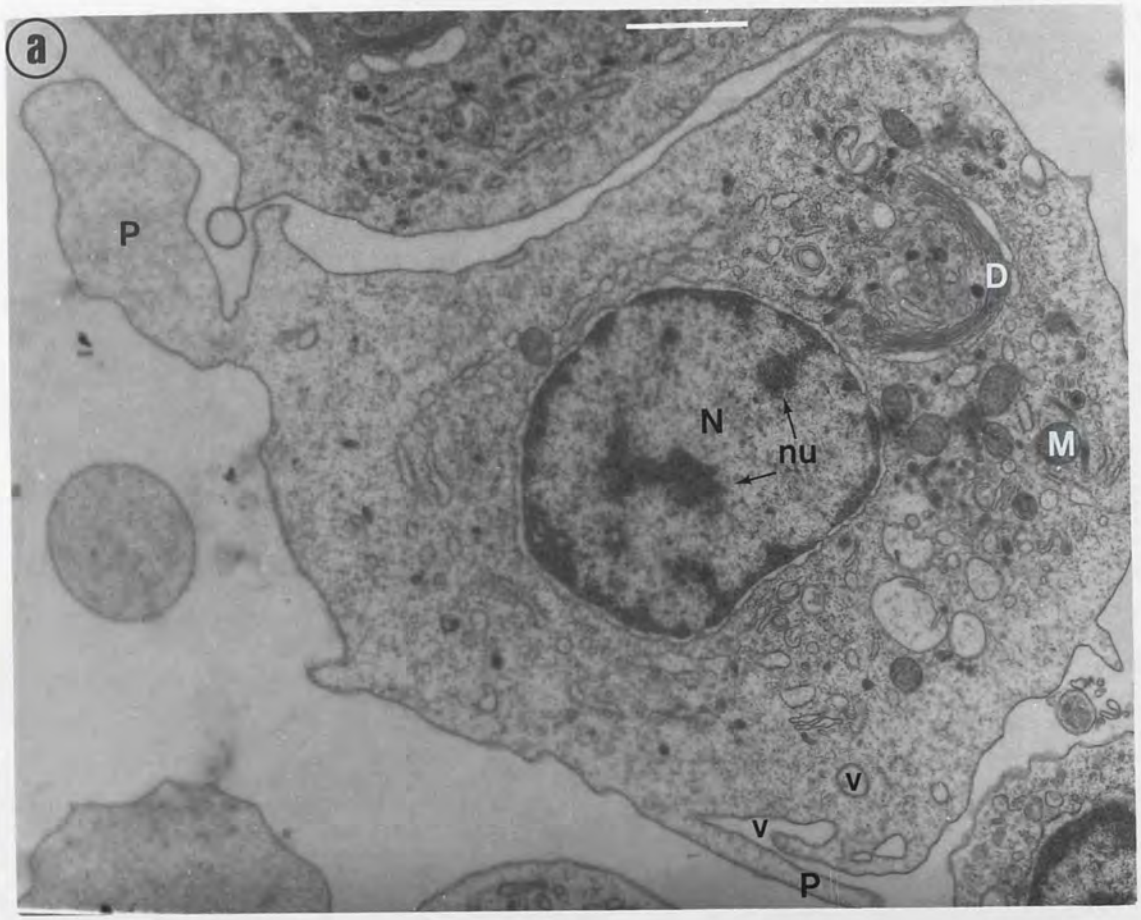


Plate 5.4



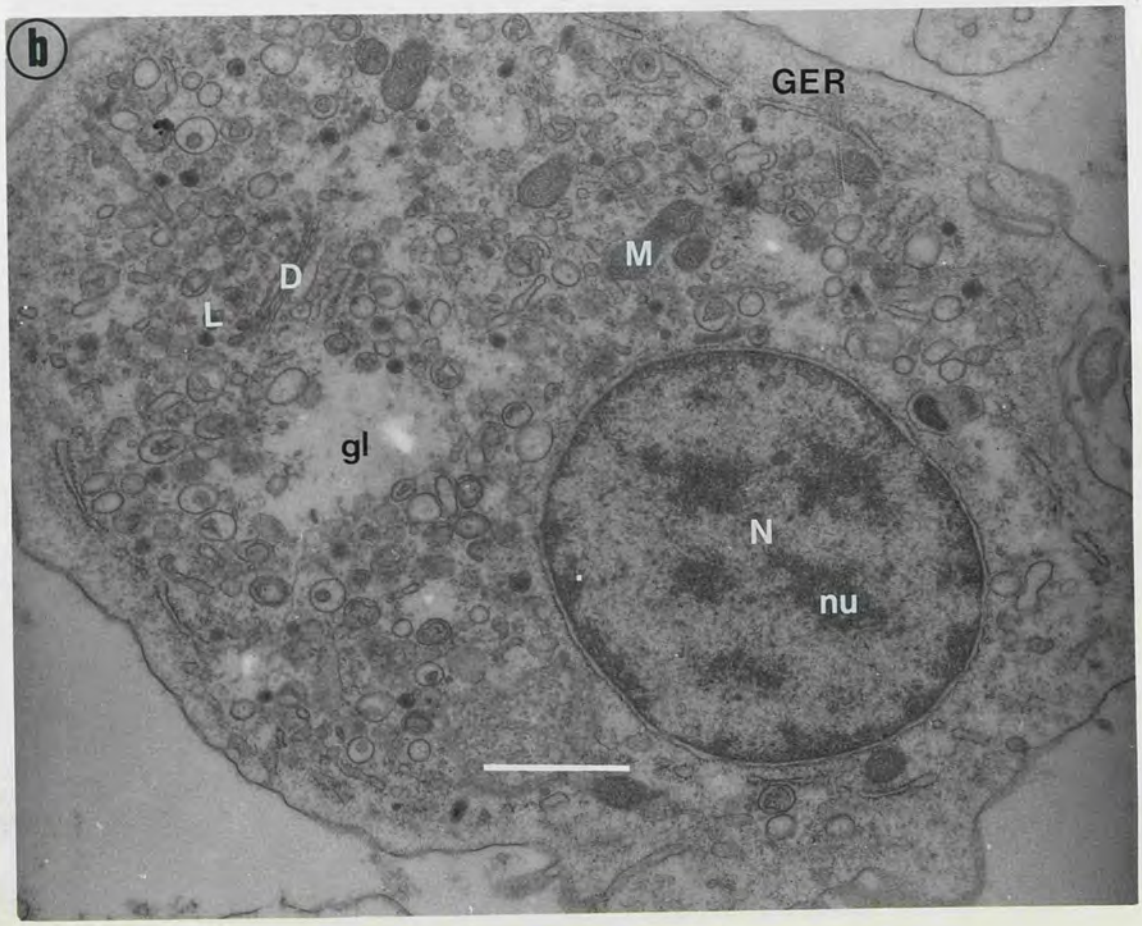


Plate 5.5

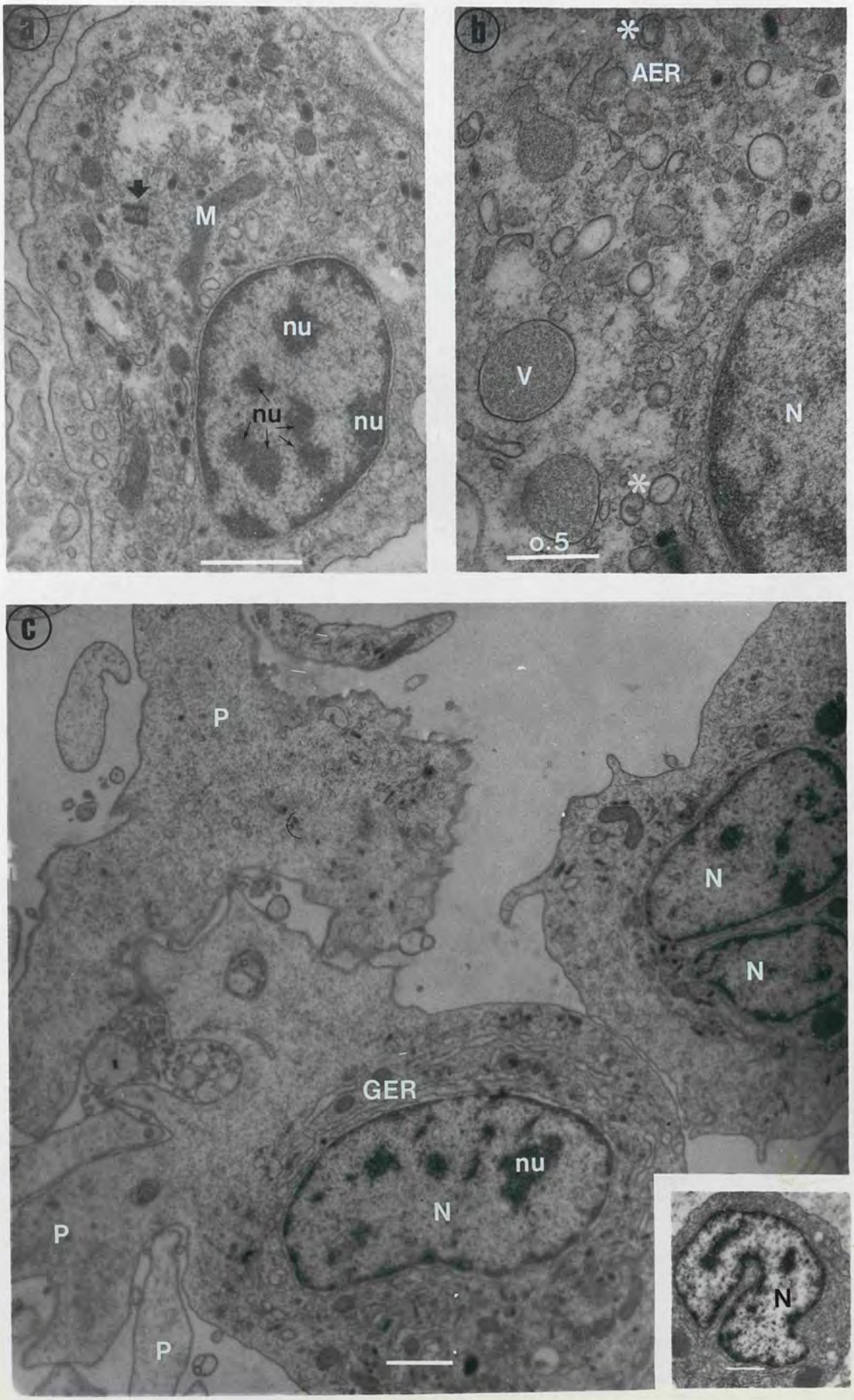


Plate 5.6

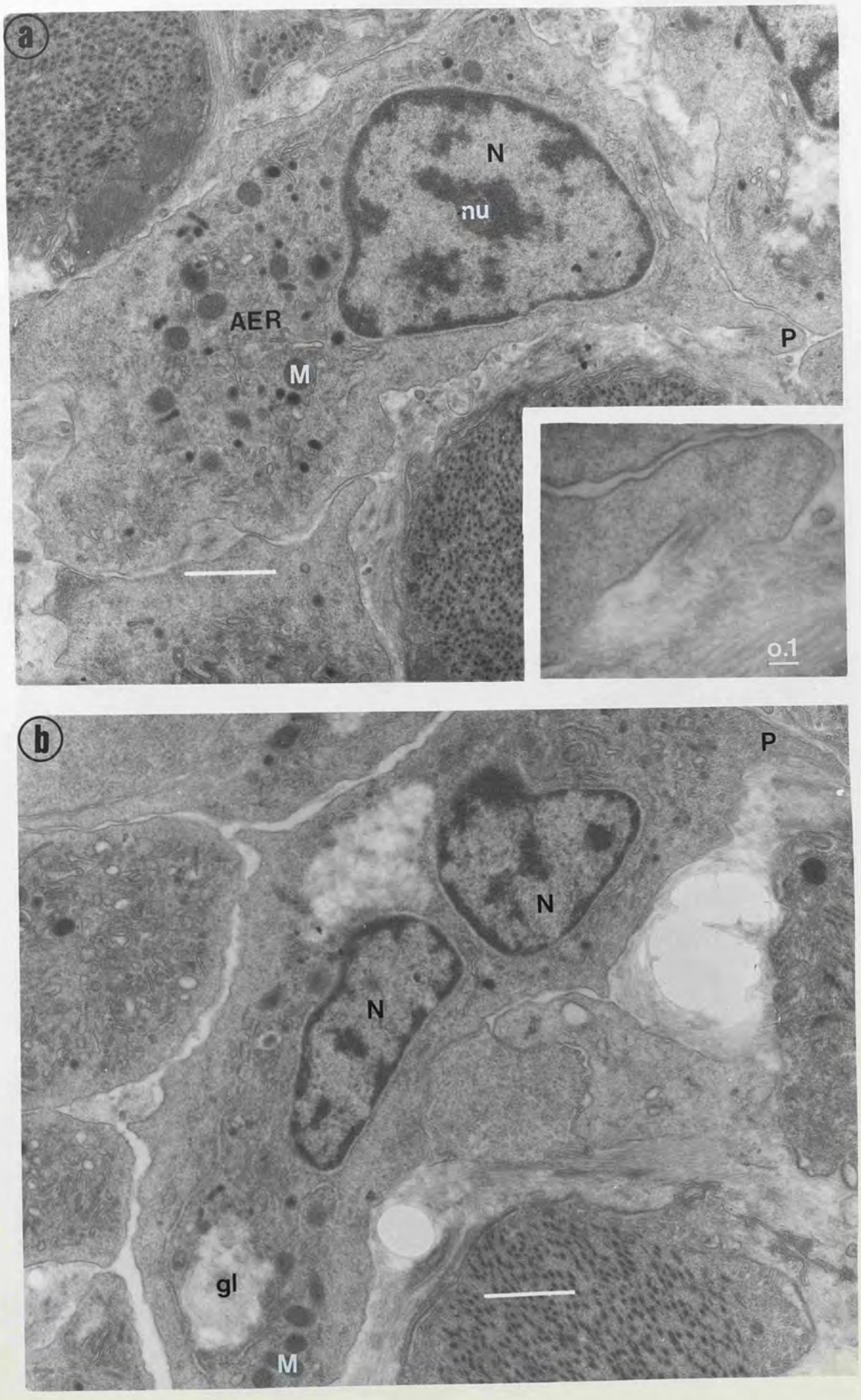


Plate 5.7

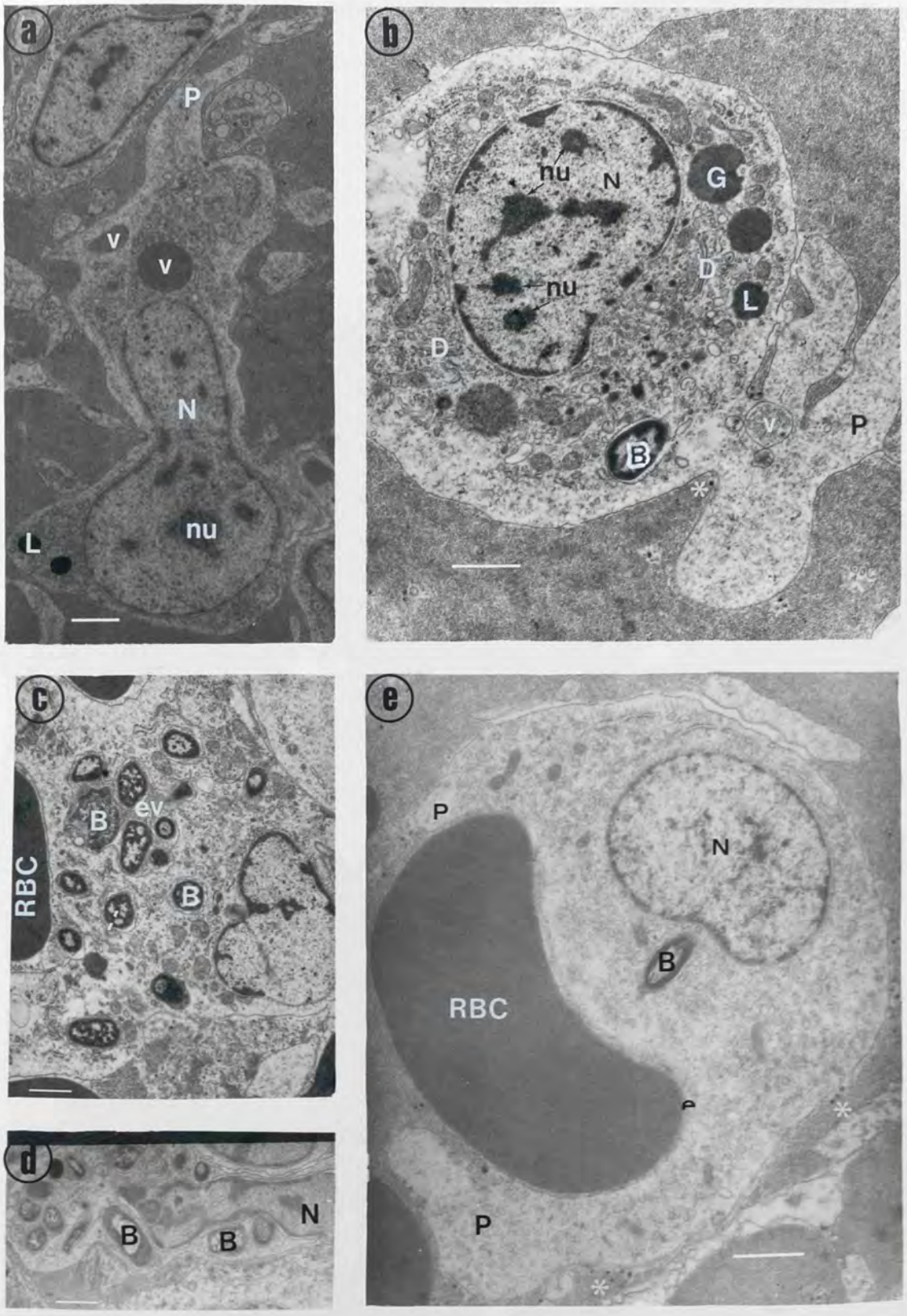


Plate 5.8

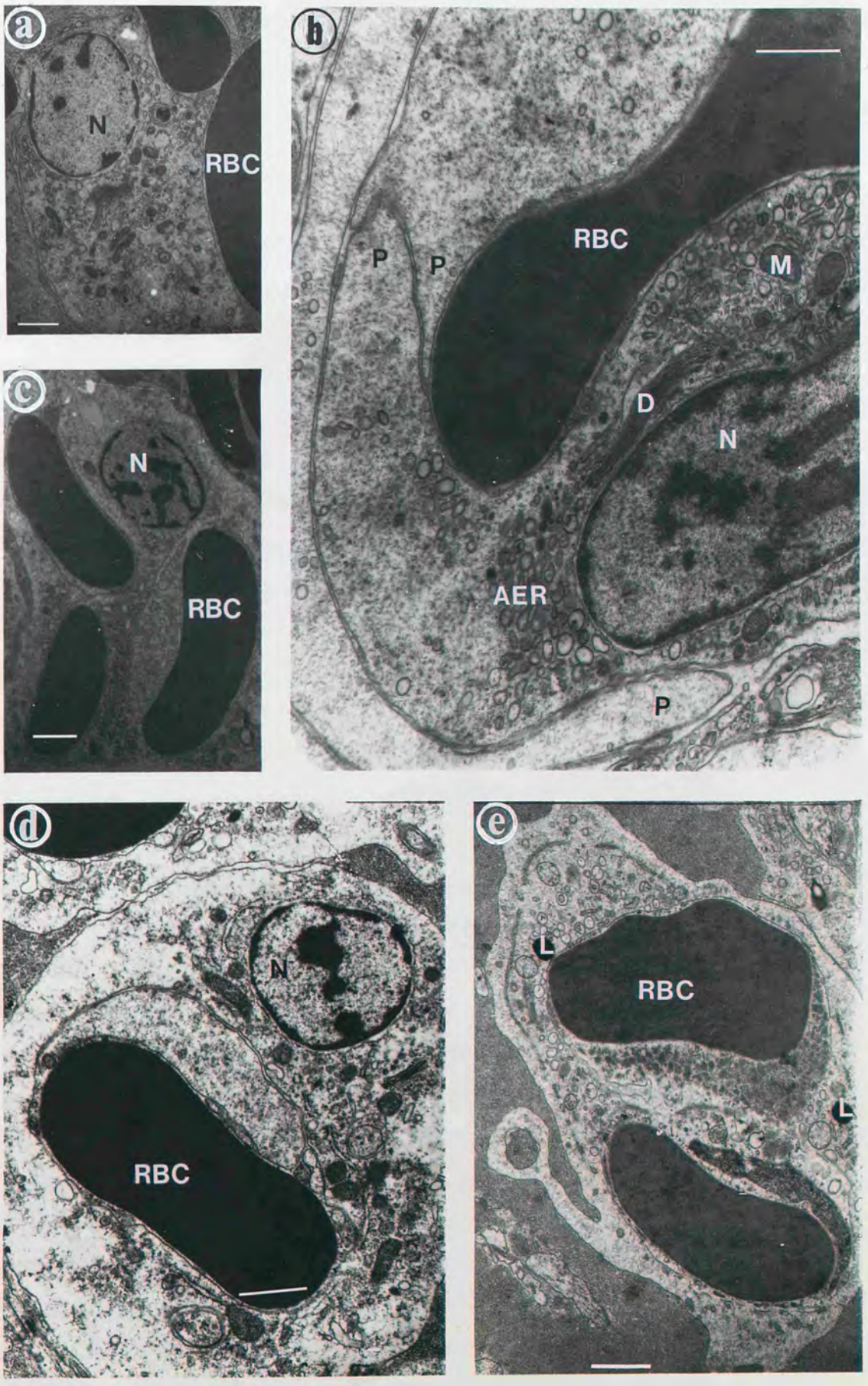


Plate 5.9

SECTION 6: SERUM REACTIONS TO FOREIGN PARTICLES.

6.1 Introduction.

Since an agglutinating factor was first reported from the blood of the horseshoe crab (Noguchi 1903), many invertebrates have been shown to contain substances in their circulating fluid that will interact with foreign cells or molecules (Huff 1940; Tyler 1946; Triplett *et al* 1958; Cohen *et al* 1962; Cheng & Sanders 1962; Pauley 1974[b]). The presence of these substances is most easily demonstrated through functional studies, and in parallel with early studies in vertebrate immunology, factors found in the circulating fluid in invertebrates were for many decades grouped according to their action, e.g. antitoxins, precipitins, agglutinins, opsonins, lysins etc. The pitfalls of such terminology are obvious from the history of vertebrate immunology, which has traced a range of different functions to relatively few factors; immunoglobulins may thus effectuate all of the functions listed above except lysis, which is brought about by the complement cascade. In the last two decades, much more effort has been devoted to the biochemical and physiological characterization of the molecules behind these functions, paying special attention to **agglutinins** (Pemberton 1971, 1974; Pauley 1974[a,b]; Gold & Balding 1975; Yeaton 1981[a,b]) which are more easily detected and more universally occurring than other factors in invertebrate sera. Agglutinins have turned out to belong to a class of proteins known as **lectins** (Goldstein *et al* 1980), and the current information suggests that these molecules are of value to the defense system not only by immobilizing bacteria, viruses, fungi, sperm and parasites by agglutination (Tyler & Metz 1945; Tyler 1946; Bang 1962; Pauley *et al* 1971[a]; Pistole 1976, 1978; Bretting *et al* 1978; Gilbride & Pistole 1979; Huang *et al* 1981) but are also commonly (perhaps even universally) responsible for the **opsonizing** effects observed (McKay & Jenkin 1970[a], Tyson & Jenkin 1973, 1974[a]; Arimoto & Tripp 1977; Renwrantz & Mohr 1978; van der Knaap, Barendsen *et al* 1981; van der Knaap, Doderer *et al* 1982; van der Knaap, Sminia *et al* 1983). As a part of the current research into the internal defense of the periwinkle, attempts were made to detect lytic or agglutinating properties in the circulating fluid, and to characterize the factors present. This section reports on the results of studies made into the nature and specificity of the factors detected, and the relationship between lectin titers and a number of physiological parameters.

6.2 Materials and methods.

SERUM. Sera were collected by centrifugation (5 min. at 1250g) of freshly aspirated samples of snail blood (for sampling method refer to Section 2). The ability of periwinkle serum to agglutinate vertebrate erythrocytes (Table 6.1) was in preliminary tests shown to be unaffected by repeated freezing and thawing (Table 6.2), and in subsequent experiments, sera were collected at convenience and stored at -20°C until titration could be carried out.

TEST-PARTICLES. Chicken and sheep erythrocytes (C/S-RBC) were obtained commercially, as were also the rabbit (R-) RBC used in the early agglutination experiments. At later stages, a rabbit was acquired from which blood samples were taken by a syringe from the ear veins. Gerbil (G-) RBC were supplied by the Department of Zoology at Bedford College. Human (H-) RBC included types ARh^- (subgroup unknown), BRh^+, $0Rh^-$ and $0Rh^+$, all given by single donors. The RBC were kept in Alsever's solution for periods up to 2 weeks, except rabbit RBC which did not keep well and were used on the day of sampling. Before use, the RBC were washed at least 3 times in 0.9% NaCl and their concentration adjusted to 2% (ca. 1 x 10^8/ml). Formalinized cells (treated with 4% formalin for 2-12 hours) were kept at 4°C and washed several times in 0.9% NaCl before use. Brewer's yeast (*S. cerevisiae*) was obtained commercially. Bacteria (*Micrococcus luteus*, *Staphylococcus aureus*, *S. albus* and *Escherischia coli*) were supplied by the Department of Zoology at Bedford College. The bacteria were cultured from laboratory strains of commercially obtainable types. They were washed several times in 0.9% NaCl before use, and the density adjusted to ca. 5 x 10^8/ml with the aid of a nephelometer. The yeast concentration was monitored with a haemocytometer, and adjusted to correspond to ca. 1 x 10^8/ml.

HAEMAGGLUTINATION TESTS. Agglutination was demonstrated by slide tests, haematocrit tube tests or microtiter. Microtitrations were performed by serially diluting 40 µl of snail serum with equal amounts of snail saline or aSW in plastic U-wells. In some cases, sera were previously diluted 1:4 in a microsyringe (Hamilton) in order to minimize serum wastage. All other dilutions and volume measurements were done with adjustable or fixed delivery micropipettes (Oxford). Heat-inactivated sera or wells containing only the diluent served as a negative control. The experiment was initiated by adding to each well a drop of the suspended test-particle, using a Pasteur pipette, and the plate was left at 20° and read after 2 hours. The Pasteur pipettes had

previously been shown to deliver drops of uniform size (40 μl). The starting dilutions of sera were thus 1:4 or 1:16, and the final concentration of the test-particle suspension ca. 5 x 10^7/ml (RBC, yeast) or 2 x 10^8/ml (bacteria). Bacterial agglutination was confirmed by microscopical examination on microscope slides.

Sera are commonly positive in tests with rabbit RBC (Table 6.2), but reactivity with human RBC was seen only in a minority of samples. Before being titrated against R-RBC, sera were therefore routinely screened for reactivity against H-RBC-0Rh$^+$ in haematocrit tube tests (Dales 1982) in order to minimize serum wastage. A sub-sample (minimum 5 μl) was drawn into a 10 μl micropipette, followed by a small volume of packed RBC, and after inverting the micropipette, the RBC were watched under a magnifying glass as they slid down the side of the tube, which was placed at an angle of ca. 45° into a lump of 'Plasticene'. Positive samples, i.e. those forming an interrupted line of RBC were assayed for agglutinin titers by microtitration. Haematocrit tube tests were also chosen in the initial experiments, while establishing the presence of agglutinin(s)in the serum, as the volumes did not normally suffice for multiple titrations. Haematocrit tube tests are not suitable for detecting R-RBC activity, as a prozone effect is occasionally observed with this test-particle.

CROSS-ADSORBANCE TESTS. In these tests, sera were adsorbed by one test-particle and then tested against others. Two methods were used; the simpler involved using the adsorbed supernatant from microtiter test U-wells. The fluid from each well was divided into two wells, and tested against a heterologous particle as well as the original, which served as a control. The results were corrected for the additional dilution caused by the second titration. In other cases, an equal volume of packed test-particles and undiluted serum was mixed and allowed to stand for 1 hour, followed by centrifugation (1250g, 5 min.). The supernatant was then titrated against a second particle, with the original as a control.

PHYSICOCHEMICAL TESTS. The tolerance of the agglutinin(s) to freezing was examined by titrating sera shortly after collection, and again after 1-12 weeks at -20°C. Some sera were repeatedly frozen and thawed before the second titration. The tolerance to heat was tested by incubating subsamples from pooled sera for 30 or 60 min. at 60°C, 70°C or 80°C in constant temperature cabinets, and at 100°C by immersion in boiling water. Coagulates which commonly formed at the higher temperatures were removed by centrifugation (1250g, 5 min.). The sera were titrated against R-RBC with an untreated subsample as control. Subsamples from pooled sera were also treated with protein-precipitating chemicals and the fate of the agglutinating

activity followed. After an hours incubation in ammonium sulphate (30% and 50% final concentration), trichloroacetic acid (TCA, 10%) or ethanol (50%), the samples were centrifuged (1250g, 5 min.) and pellets and supernatants were dialysed separately for 3 days against several changes of distilled water. The dialysis membranes were washed with distilled water, and the washings collected into Beem capsules and lyophilized. The subsamples were made up to their original volume in DW, and titrated against R-RBC, using an untreated but dialysed and lyophilized subsample as control.

SPECIFICITY. The inhibitory effect of 35 carbohydrates, listed in Table 6.5 on the agglutination of R-RBC was tested in 4 separate haemagglutination-inhibition experiments. In preparation for these tests, serum samples with known titers were pooled to make up a lectin source with a predicted final titer of 64. In the first experiment, stock solutions of carbohydrates in PBS (mono- and disaccharides 0.2 M, polysaccharides 1% w/v) were serially diluted in microtiter U-wells, and an equal aliquot (40 μl) of a 1:4 dilution of the serum pool added. In the second experiment, the serum pool was serially diluted in bulk to give final dilutions of 1:16 to 1:256, and then aliquoted into the titer plates, and an equal amount (40 μl) of undiluted carbohydrate stock solution added to each well of the corresponding row. The remaining two experiments were performed in the same manner but with a previous 2-fold resp. 8-fold dilution of the carbohydrate stock solution. Control rows received PBS instead of the carbohydrate solution. After an hours incubation at room temperature, the experiments were initiated by adding 40 μl of a 2% R-RBC suspension to each well, and the results were recorded after an additional hour.

THE PHYSIOLOGICAL CAUSE OF LECTIN FLUCTUATIONS: (I) CONGENITAL FACTORS. In order to gain information on the physiological significance of lectin fluctuations, sera were collected individually from at least 30 snails in each of the four seasons, keeping a record of the size and the sex of the animals. The sera were titrated at least twice, and titers compared between the sexes and between age groups as well as between seasons. Some of the samples were large enough to also allow for serum protein determination. The methods used for determination of these parameters are as follows: (a) the **sex** was determined by visual examination of the external sexual organs (see Section 1.3). (b) The **age** was indirectly estimated from the size by measuring the body whorl diameter against a ruler; sera were denoted with - (small, <16 mm) or + (large, ≥16 mm) according to the size of the animal in relation to a previously calculated mean diameter (16 mm, N=50). (c) **Seasonal changes** in the physiology of the animal (e.g. caused by the breeding cycle) were assessed by

comparing titers of individuals sampled in different seasons, i.e. winter (40), spring (50) summer (55) and autumn (79). (d) The **serum protein** concentration was measured with the Folin phenol method (Lowry *et al* 1951). Specifically, 10 μl subsamples were brought to 100 μl with distilled water and allowed to react for 10 min. with 1 ml of a freshly prepared mixture of 2% copper sulphate (1:50), 4% sodium potassium tartrate (1:50) and 3% sodium carbonate in 0.2 N NaOH (48:50), and for 30 min. with 50 μl of Folin and Ciocalteu's phenol reagent. The absorbtion was measured at 640 nm, using aSW as buffer blank, and compared to a standard curve made up from known concentrations of BSA and calibrated at 280 nm.

THE PHYSIOLOGICAL CAUSE OF LECTIN FLUCTUATIONS: (II) ACQUIRED FACTORS. The effect of certain stress conditions (starving, haemorrhage, inoculation) was checked in separate experiments. (a) The effect of **starving** was checked by comparing titers of starved animals to those of controls. (b) **Serum level stability** and the effects of bleeding was checked by comparing titers at different times after bleeding to base values obtained from the same animals at the start of the experiment. The fact that animals can be repeatedly bled with the method described in Section 2.2 with minimal damage other than loss of blood was used with advantage in this experiment and the next one. The snails were identified by labels marked with permanent ink on the dry shell at the start of the experiment. (c) Finally, an attempt was made to correlate titers to previous exposure to foreign particles. H-RBC-0Rh$^+$ were preferred to R-RBC as test particles, as it was thought that using the latter might cause massive agglutination and sequestration of the inoculum at the site of injection; previous titrations (Tables 6.1, 6.3 & 6.6) implied that H-RBC are poorer in lectin binding sites than R-RBC. A total of 25 snails were injected, and additional 15 animals, serving as control received a sham-injection of sterile aSW; all injections were made into the cephalopedal sinus and measured 200 μl. A 200 μl serum sample was aspirated from each snail immediately before the inoculation, and again 4, 8 or 12 days later. The sample size and timing was selected in accordance with results from the bleeding experiment (see above), ensuring minimal lectin fluctuations due to experimental handling *per se*. The samples were titrated against heterologous (R-RBC) as well as homologous particles, and compared by statistical analysis.

LYSIN TESTS. Preliminary lysin tests were done simply by noting whether lysis occurred in the microtiter plates at prolonged storage (24 h/4 °C). Results were considered positive if the fluid above the RBC pellet in the test well became coloured while the fluid in the control well remained clear.

6.3 Physicochemical characterization of the agglutinin.

PRACTICAL CONSIDERATIONS. No lytic factors were detected, but periwinkle serum contains a factor which agglutinates bacteria (*E. coli*), yeast (*S. cerevisiae*) and several different types of vertebrate RBC, including rabbit and gerbil RBC, as well as human RBC of all types tested (Tables 6.1-6.3). Good inter-assay reproducibility and high individual variation (Tables 6.1-6.2) clearly demonstrates that the observed activity is due to a serum agglutinin and not an artefact; this is confirmed by the loss of activity upon boiling. For practical reasons, experiments were conducted in order to assess the stability of the responsible factor(s) upon storage, and the binding capacity of the test-particles after fixation. The results, as shown in Table 6.1 & 6.2, indicate that lectin binding sites on the rabbit RBC become masked or distorted by formalin fixation, but agglutinin tolerance to freezing and thawing is clearly demonstrated (Table 6.2). Sera were kept at -20°C for up to 2 months without apparent loss of titer even after repeated freezing and thawing. These results have later been confirmed and extended to at least 6 months (results not shown).

TABLE 6.1: The reaction of *L. littorea* sera to foreign particles.

	R - RBC	G - RBC	H-A$^-$ RBC	H-O$^-$ RBC	H-O$^+$ RBC	H-B$^+$ RBC	S - RBC	titers A-	titers O+	titers G
1	**	*	**	*	*	-	-	ND	512	8/16
2	**	-	*	+	-	-	-	≤4	>8	≤4
3	**	+	*	-	-	-	-	8/16	ND	≤4
4	*	-	+	-	-	-	-	ND	ND	ND
5	**	**	**	**	**	*+	-	ND	ND	128
6	**	**	**	**	**	*	-	ND	ND	32/64
7	**	+	*	+	-	+	-	ND	ND	≤4
8	**	+	*	+	-	-	-	4/8	ND	≤4
9	**	**	**	**	*+	*	-	ND	ND	32/64
10	**	*	**	*+	*+	*	+	16	ND	32
11	**	*	**	*	+	*	-	ND	ND	4/8
12	**	+	*	+	+	+	+	ND	ND	≤4
13	*	*	*	+	+	-	-	≤4	ND	≤4
14	**	+	*	+	+	-	-	≤4	ND	≤4

Agglutination was scored by the haematocrit tube method described in section 7.2, using rabbit (R-), gerbil (G-), sheep (S-) or human (H-) erythrocytes (RBC) of the types indicated ($^+$ and $^-$ denotes Rh reactivity). Samples are recorded as negative (-), or positive with increasing intensity (+ , * , *+ , **). Formalinized rabbit and sheep RBC always failed to agglutinate. Preliminary tests with 10 additional sera revealed that E. coli but not M. luteus, St. aureus and St. albus were agglutinated. Only a few titrations could be carried out, due to the small size of the samples (ND = not done).

CROSS-ADSORPTION. Preliminary cross-adsorption tests using supernatants from microtitration wells (Table 6.3[a]) demonstrate that adsorption with either of two RBC types (R-RBC and HRBC-0^+) is equally effective in reducing the agglutinating power for each of the two erythrocyte types tested. These results were confirmed and extended in experiments with whole, untreated sera (Table 6.3[b,c]); the agglutinating activity was found to be mutually cross-reactive between all RBC-types tested, as well as between *E. coli* and *S. cerevisiae*, and yeast adsorption appears to remove the agglutinating power for R-RBC. The results suggest that adsorption with one test-particle is sufficient to remove all agglutinating activity of the serum, which implies that all of the test-particles so far tested are agglutinated by one and the same molecular species. In subsequent studies, R-RBC were commonly chosen as representative test-particles, with H-RBC-0^+ as comparison. The particles were selected on account of their reactivity, availability and 'readability' in titration experiments.

TABLE 6.2: Tolerance of *L. littorea* agglutinin to freezing & thawing.

I: R-RBC: Repeated freezing and thawing (1 weeks storage).
II: R-RBC: Prolonged storage (2-3 months).
III: H-RBC-0^+: (a) Repeated freezing and thawing (1 month). (b) Prolonged storage (2-3 months).

I.	control*	test		control	test		control	test
1-5)	<4	<4	11)	16/32	32	17)	128	128
6)	4	4	12)	32	32/64	18)	128	128
7)	8/16	8/16	13)	32/64	32/64	19)	128	128
8)	8/16	8/16	14)	64	64	20)	128/256	128
9)	16	16	15)	64/128	64/128	21)	256	128/256
10)	16/32	16/32	16)	64/128	128	22)	256/512	256/512

II.	control	test		control	test		control	test
1)	<4	<4	5)	16/32	16/32	9)	32/64	64
2)	<4	<4	6)	16/32	32	10)	64/128	128
3)	<4	<4	7)	32	32	11)	256	256/512
4)	16/32	16	8)	32/64	32/64	12)	512	256/512

III.	control	freeze-thaw	storage
1)	64/128	64/128	
2)	64/128	64/128	
3)	64	64	64
4)	256	128/256	128/256

* Agglutinating titers are recorded as the reciprocal of the highest dilution which shows a positive reaction, before (control) and after treatment (test). All samples were labelled and read 'blind'.

PHYSICOCHEMICAL TESTS. The titrations recorded in Table 6.1, in which boiled sera served as a control, demonstrated the loss of activity upon boiling; experiments were now performed in order to further investigate the molecular identity of the agglutinin. Sera were subjected to denaturing temperatures (60 °C, 70 °C or 80 °C for 30 or 60 min) or reacted with AS (30% & 50%), TCA (10%) or ethanol (50%) and the fate of the agglutinin followed with R-RBC titration before and after treatment. As shown in Table 6.4, the agglutinin is denaturated by heating for 30 min. at 70 °C, with reduced titers at 60 °C, and is precipitated out of solution with AS, TCA and ethanol. It seems clear that a protein forms the active part of the agglutinin, and the molecule thus classifies as a **lectin** (Goldstein *et al* 1980).

Table 6.3: Cross-adsorption results.

(A) serum no.	(1)	(2)	(3)	(4)
a) R-RBC	32	64	8/16	32/64
b) R-RBC	8	8/16	2/4	<4
b) HRBC-O$^+$	8	8	2/4	<4
a) HRBC-O$^+$	16/32	32/64	8	16/32
b) HRBC-O$^+$	4/8	4/8	2	<4
b) R-RBC	4/8	<8	2	<4

serum no.	(5)	(6)	(7)	(8)
a) R-RBC	16/32	32	64/128	128
b) HRBC-O$^+$	-	-	-	-

(9)		
	a) HRBC-O$^-$	8
	b) R-RBC	-
	a) HRBC-O$^+$	16
	b) HRBC-O$^+$	-
	b) R-RBC	-
	a) G-RBC	16
	b) G-RBC	-
	b) R-RBC	-

(10)		
	a) E. coli	16/32
	b) S. cerev.	-
	c) R-RBC	-
	a) R-RBC	64/128
	b) S. cerev.	-
	c) E. coli	-

(B) serum no.	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)
a) S. cerev.	2/4	8	4/8	4				
b) E. coli	-	-	-	-				
a) E. coli	64	128	64	32	64	8	8	8
b) S. cerev.	-	-	-	-	-	-	-	-
a) R-RBC	8	16	32	16	64	4	8	16/32
b) S. cerev.	-	-	-	-	-	-	-	

The values represent agglutinating titers (a) before and (b) after adsorbtion with homologous or heterologous test-particles of the types indicated. HRBC = human RBC (of the blood group indicated; $^+$ and $^-$ denotes Rhesus reactivity); G-RBC = gerbil RBC; R-RBC = rabbit RBC. In experiment A, samples 1-4 were tested by using the supernatant from wells in microtiter assays, but subsequent tests (A 5-10; B) involved a repeated 10 min. incubation of packed test-particles (1/10 vol.) in undiluted sera.

SPECIFICITY. The wide variety of test-particles agglutinated by the lectin indicates that its specificity is directed against a determinant commonly found on cell surfaces. An attempt was made to establish the identity of this binding site in haemagglutination-inhibition (HAI) experiments, using a range of mono-, di- and polysaccharides listed in Table 6.5. As shown, the best inhibitors of the R-RBC-lectin interaction are the pentoses L-xylose, D-ribose, D-xylose and α-L-fucose, in that order. None of the macromolecules tested appeared to be effective in blocking the reaction of the lectin with the RBC surface determinant; the results with mucin appear controversial and need confirmation. The results imply that the lectin reacts strongly with terminal pentoses, and more strongly with the L- than the D- configuration, suggesting that further calibration of the lectin specificity might be gained by including L-ribose and related sugars in future tests.

Table 6.4: Physicochemical characterization of the agglutinin.

I. TEMPERATURE-TOLERANCE.
II. ACTIVITY OF THE PROTEIN FRACTION OF THE SERUM.

I. (a) treatment*	8°C/48 h	20°C/16 h		20°C/48 h			
control	32/64	256	64	256	128	64	64/128
test	16	64	16	8	8	16	4
(b) treatment	contr.	80°/60	80°/30	70°/60	70°/30		60°/30
1)	16	-	-	-	-		ND
2)	8	-	-	-	-		ND
3)	8	ND	ND	ND	-		ND
4)	32	ND	ND	ND	ND		8
5)	32/64	ND	ND	ND	ND		8
6)	256/512	ND	ND	ND	ND		32
7)	512	ND	ND	ND	ND		-

II. fraction	titer
control	16
50% AS precipitate	16
" supernatant	-
30 % AS prec.	16
" sn.	-
50% TCA prec.	16
30% TCA prec.	8/16
10% TCA prec.	8
50% ethanol prec.	16

* I(a) records titers of individual samples before (first row) and after treatment (second row); I(b) records the titers of individual samples before (control) and after treatment. II records titers of fractions before (control) and after chemical treatment (see text)

TABLE 6.5: The binding specificity of *L. littorea* lectin.

Experiment no:	1	2	3	4	
Sugar conc. (mM):	25-200	50	25	6.25	inh.conc in mM (%)*
SUGAR\CONTROL**	64	64	64	64	
L-xylose	3	3	(2)	2	6.25
mucin	(lysis)			(2)	(1/20)
D-ribose	3	3	2	2-3	6.25
D-xylose	>3	2	2	0	25
Sucrose	3		2-3	2-3	10-50
α-L-fucose	3	1?	2	1	25-100
α-D-fructose	2-3	(0-1)	(1)	2-3	25-100
Maltose	3		1-2	1	25-50
L-glucose	>3		1	0	50?
L-arabinose	3	2	0	0	50
L-mannose	2	2	(1)	1	25-50
sorbitol	0		(1)	1-2	?
D-arabinose	3	1-2	1	0	50
N-ac-D-gluc.NH	3	1-2	1	0	50
M-gluco-pyr.	3	1	1	0	100?
1-M-β-D-gal.pyr.	(3?)	1	1-2	0-1	25-100
M-α-D-gal.pyr.	>3	(1)	1-2	0	25-100
N-ac-D-gal.NH	2-3		1	0-1	50-100
D-galactose	3	1	1	0	>100
D-glucose	2	(0)	0-1	1	50-200
D-mannose	2	(0)	(1)	0-1	50-200
D-gal.NH	2		1	0-1	50-200
heparin	3		0	0	(1/4-1)
rhamnose			(1)	0	>25
chitin	(?)			0-1	(>1/20)
α-lactose	0			0	>200
trehalose	0-1		(>1)	0-1	>200
dextran	0			0	>200
dextran sulph	1		(0)	0	>200
N-ac-neur.acid	(lysis)		0	0	>200
D-gluc.NH	1	0	0	0	>200
D-mann.NH	0	0		0	>200
D-glucuron.ac	0			0	>200
glycogen	0			0	>200
cellulose	0			0	>200

* The lowest carbohydrate concentration, in mM (or %) giving at least a twofold titer decrease.

** ROW: The titer of the lectin pool in the absence of inhibitory compounds. COLUMN: Inhibitory compounds. The results of experiment 1 are given as the number of twofold dilutions of carbohydrates which had an observable inhibitory effect on agglutination. The results of experiment 2-4 are given as the number of twofold titer decreases observed in the presence of each particular carbohydrate (or other compound) at the concentration shown. For further description of methods, see text.

6.4 Correlation to physiological parameters.

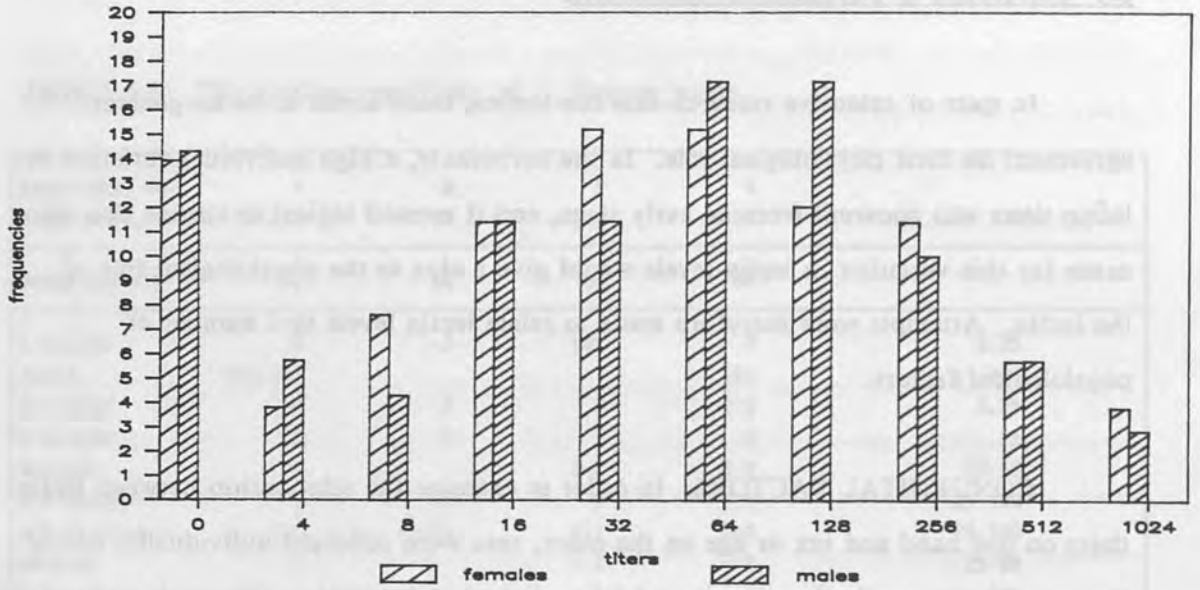
In spite of intensive research into the lectins, there seems to be no general agreement on their physiological role. In the periwinkle, a high individual variation in lectin titers was apparent from an early stage, and it seemed logical to assume that the cause for this variation in lectin levels would give a clue to the physiological role of the lectin. Attempts were therefore made to relate lectin levels to a number of physiological factors.

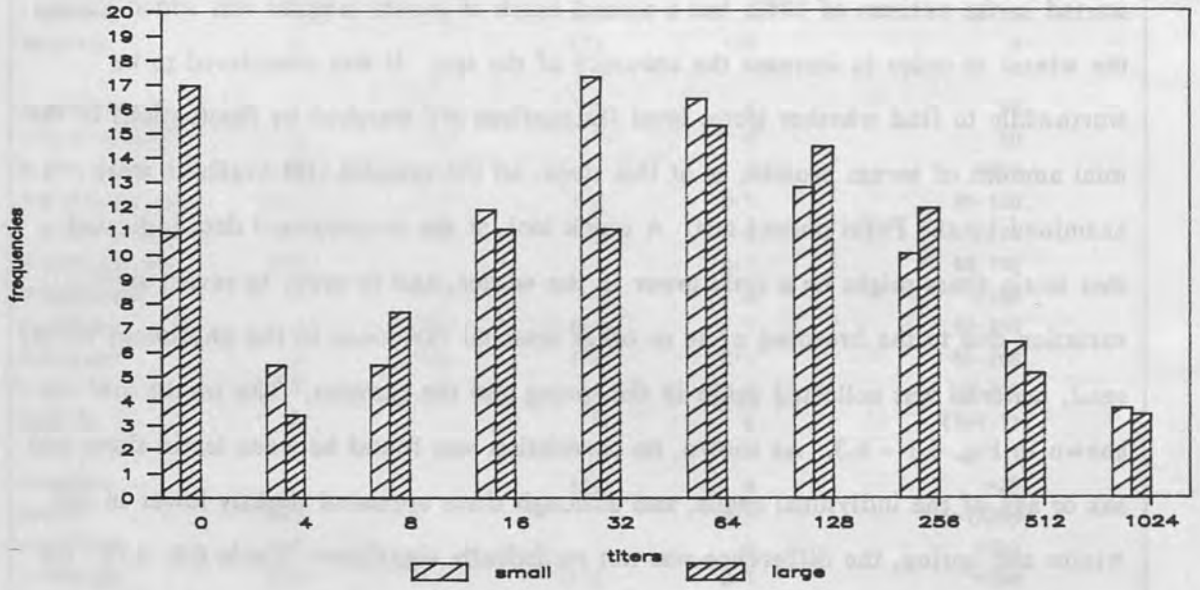
CONGENITAL FACTORS. In order to examine the relationship between lectin titers on one hand and sex or age on the other, sera were collected individually, noting the sex and the age (indirectly assessed from size) of the animals. This work was started in the autumn of 1982, but a second batch of serum samples was added during the winter in order to increase the accuracy of the test. It was considered to be worthwhile to find whether lectin level fluctuations are matched by fluctuations in the total amount of serum protein, so at this stage, all the samples still available were examined by the Folin phenol test. A quick look at the accumulated data indicated that lectin titers might be a little lower in the winter, and in order to reveal any variation due to the breeding cycle or other seasonal variations in the physiology of the snail, material was collected again in the spring and the summer. The results are shown in Fig. 6.1 - 6.3. As shown, no correlation was found between lectin titers and sex or age of the individual snails, and although titers appeared slightly lower in the winter and spring, the difference was not statistically significant (Table 6.6, 6.7). An attempt to correlate lectin titers to total serum protein was also unsuccessful (Fig. 6.3).

ACQUIRED FACTORS. Experiments were then performed in order to assess the effects of environmental stress upon lectin titers. In the first experiment, snails were randomly divided into two groups, one receiving food and the other not; sera were collected 1 - 3 months after the start of the experiment and titers compared between the groups with the Chi sq. test.* In the second experiment, snails were individually bled at the start of the experiment and at 1, 2, 4, 6-7, 10 and 30 days thereafter; some were also bled at 4 months. The results are presented in Table 6.6, and graphically in Fig. 6.5. As shown, lectin titers vary considerably with the condition of the animal. Starvation causes a marked reduction in lectin levels; the difference in titers between starved snails and those receiving food is significant at the

* The experiment was performed in the spring, and starved snails (series S) were thus compared to snails collected in that season (series C of Table 6.6).

Fig. 6.1: Sex & age vs. lectin titers





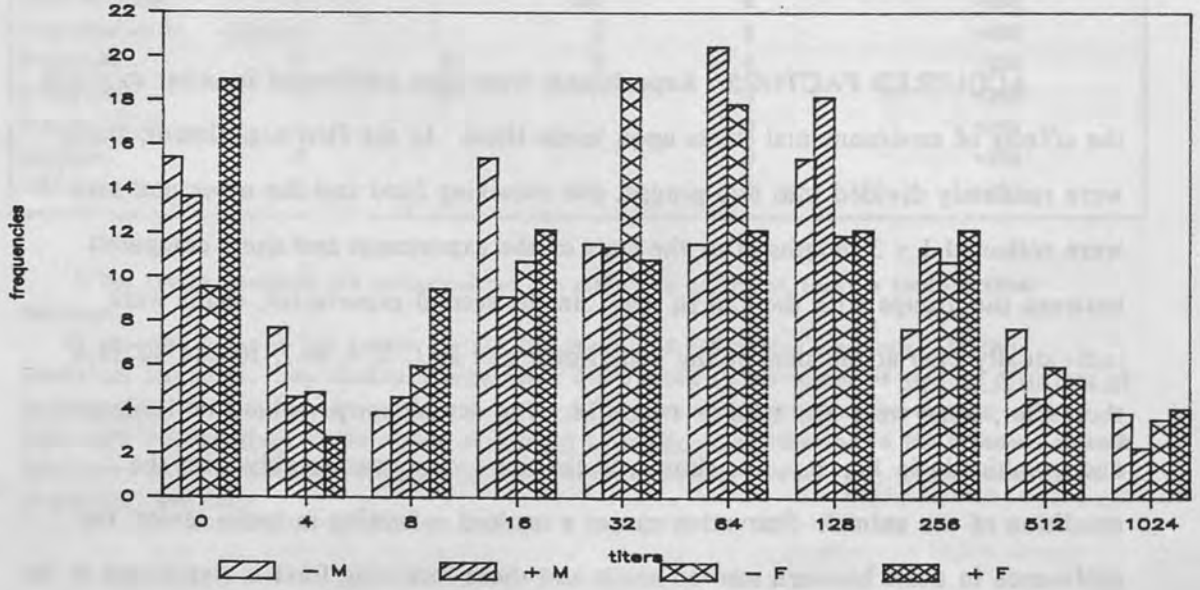


Fig. 6.2: Seasonal titer variations

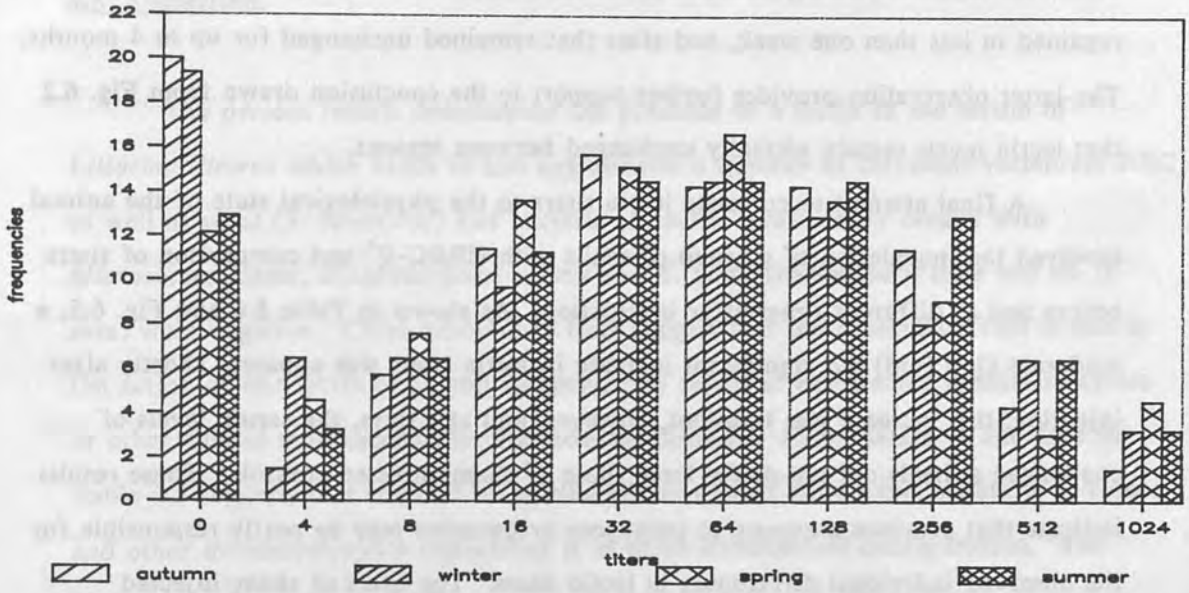


Fig. 6.3: Serum protein levels vs. lectin titers

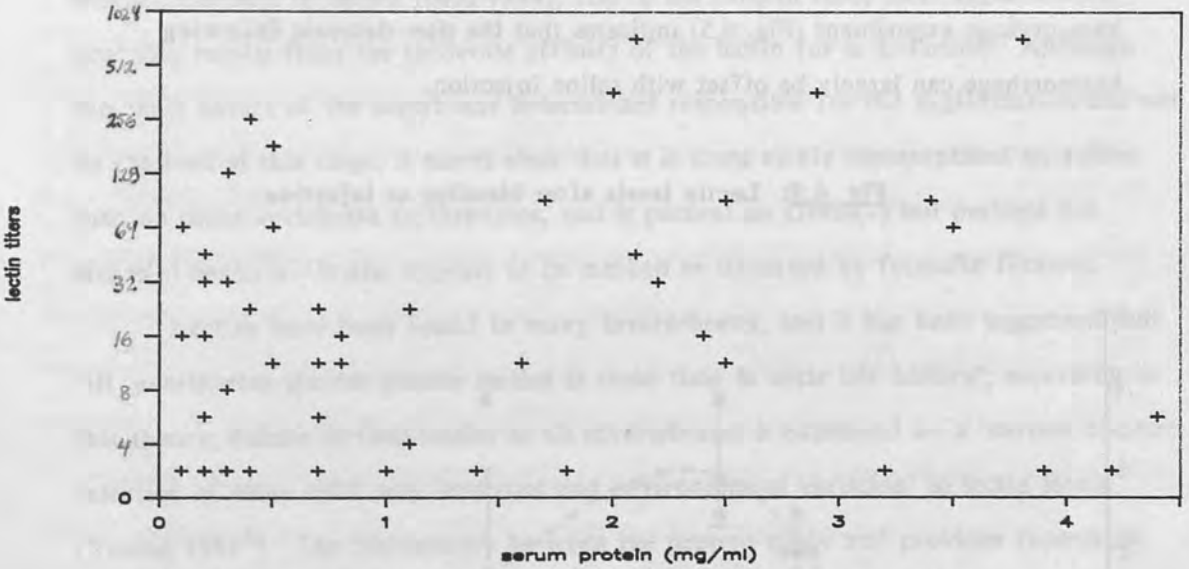
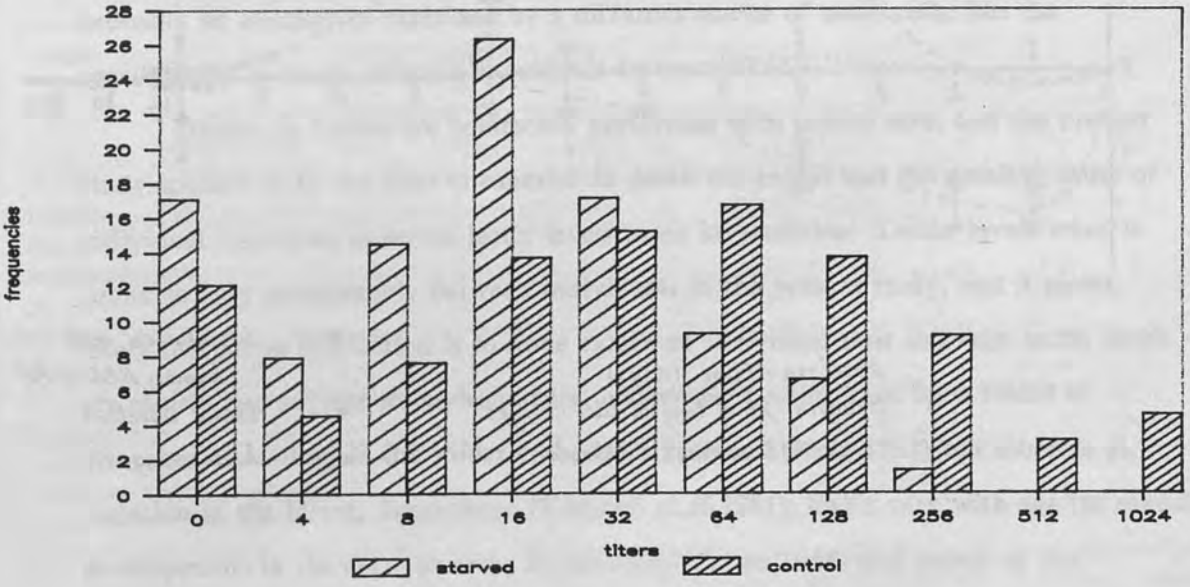


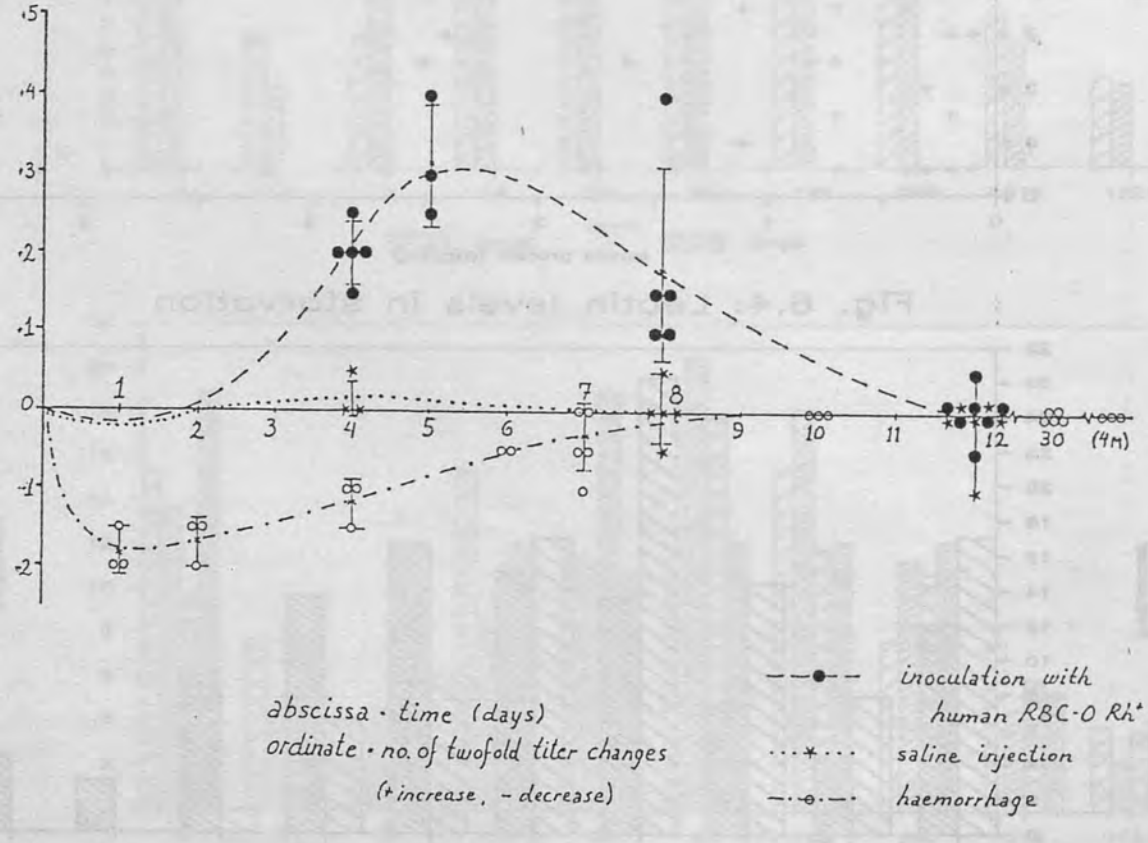
Fig. 6.4: Lectin levels in starvation



1% level. Titers invariably dropped after bleeding (Table 6.6, Fig. 6.5), but were regained in less than one week, and after that remained unchanged for up to 4 months. The latter observation provides further support to the conclusion drawn from Fig. 6.2 that lectin levels remain virtually unchanged between seasons.

A final attempt to correlate lectin titers to the physiological state of the animal involved the inoculation of a group of snails with HRBC-0^+ and comparison of titers before and at different times after inoculation. As shown in Table 6.6 and Fig. 6.5, a moderate (2-4 fold) but significant increase in lectin titers was apparent shortly after injection; this increase was transient, however, and at 8 days, the serum levels of challenged animals did not differ from those of sham-injected controls. These results indicate that previous exposure to pathogens or parasites may be partly responsible for the observed individual differences in lectin titers. The titers of sham-injected controls are unchanged at 4 days p-i, and comparison with the values obtained in the haemorrhage experiment (Fig. 6.5) indicates that the titer decrease following haemorrhage can largely be offset with saline injection.

Fig. 6.5: Lectin levels after bleeding or injection.



6.5 Discussion.

The present results demonstrate the presence of a lectin in the serum of *Littorina littorea* which binds to and agglutinates a number of different vertebrate RBC as well as yeast (*S. cerevisiae*) and bacteria (*E. coli*). Preliminary results with *Micrococcus luteus*, *Staphylococcus aureus* and *S. albus* (haematocrit tube test on 10 sera) were negative. Cross-adsorbance tests suggest that the observed effect is due to the action of one lectin only, and its specificity seems to be directed against L-xylose or other similar monosaccharide in a terminal position. Examination of the data in Table 6.5 suggests that it could be worthwhile to extend these experiments to L-ribose and other monosaccharides resembling it in three-dimensional configuration. The glycocalyx of vertebrate RBC contains only seven of the ca. 100 known monosaccharides in nature (Bird 1974), and in the present case, their agglutination probably results from the moderate affinity of the lectin for α-L-fucose. Although the exact nature of the membrane determinant responsible for the agglutination can not be resolved at this stage, it seems clear that it is more richly representated on rabbit than on other vertebrate erythrocytes, and is present on Gram(-) but perhaps not Gram(+) bacteria. It also appears to be masked or distorted by formalin fixation.

Lectins have been found in many invertebrates, and it has been suggested that 'all invertebrate species possess lectins at some time in their life history'; according to this theory, failure to find lectins in all invertebrates is explained by a 'narrow biased selection of assay cells' and 'temporal and environmental variation' in lectin levels (Yeaton 1981[a]). The discrepancy between the present study and previous reports on the absence of agglutinins in *Littorina littorea* (Boyd *et al* 1966; Pemberton 1974) can probably be adequately explained by a different choice of assay cells, but the possibility of a strain variation should not be overlooked.

Studies on lectins are commonly performed with pooled sera, and the present study appears to be the first to examine in detail the extent and the possible cause of individual variations in serum lectin levels in an invertebrate. Lectin levels were found to vary considerably between individuals in the present study, and it seems likely that lectin production is to some extent an individual trait although lectin levels are clearly also subject to environmental influence. Lectins have been found to disappear at molting in the spider crab, *Maia squinada* (Bang 1967) but increase at pupation in the insect, *Sarcophaga* (Komano *et al* 1981); levels vary with age (or sexual development) in the coconut crab, *Birgus latro* (Cohen 1968) and season in the

Table 6.6: Serum lectin levels in *L. littorea*.

no.	titer	*	protein	no.	titer	*	protein	no.	titer	*	no.	titer	*
a-1				b-1	<4	+ f	0.1	c-1	<4	+ m	d-1	<4	+ m
to	<4			b-2	<4	- f	0.3	c-2	<4	- f	d-2	<4	- m
a-5				b-3	<4	+ f	1.0	c-3	<4	- m	d-3	<4	+ f
a-6	8/16			b-4	<4	+ f	3.2	c-4	<4	- f	d-4	≤4	- f
a-7	8/16			b-5	<4	+ f	4.2	c-5	4	- m	d-5	≤4	- f
a-8	16/32			b-6	<4	+ f	3.9	c-6	4/8	+ m	d-6	8	- f
a-9	16/32			b-7	4	- f	1.1	c-7	8/16	+ f	d-7	8/16	+ f
a-10	32			b-8	4/8	+ f	4.4	c-8	8/16	- f	d-8	8/16	- f
a-11	32/64			b-9	4/8	+ m	0.7	c-9	8/16	- m	d-9	16	- f
a-12	32/64			b-10	8/16	- f	1.6	c-10	8/16	+ f	d-10	16/32	- m
a-13	64/128			b-11	8/16	+ f	0.8	c-11	16	- f	d-11	16/32	- f
a-14	128			b-12	16	+ m	0.2	c-12	16	- m	d-12	16/32	- f
a-15	128			b-13	16/32	+ f	1.1	c-13	16/32	+ m	d-13	32	- f
a-16	256			b-14	16/32	+ m	0.7	c-14	32	- f	d-14	32	- f
a-17	<4	+ m	1.4	b-15	32	- m	0.3	c-15	32	+ f	d-15	32/64	- f
a-18	<4	+ f	0.4	b-16	32	- f	2.2	c-16	32	- f	d-16	32/64	- f
a-19	4/8	+ f		b-17	32	- f	0.2	c-17	32/64	+ m	d-17	32/64	- f
a-20	8/16	+ f	0.5	b-18	64	- f	0.1	c-18	32/64	+ f	d-18	64/128	- f
a-21	16	+ f	0.8	b-19	64	- f		c-19	32/64	+ m	d-19	64/128	+ m
a-22	16/32	+ f	0.4	b-20	64/128	+ m	2.5	c-20	64	+ m	d-20	64/128	- f
a-23	32	+ f		b-21	64/128	+ f	1.7	c-21	64	- f	d-21	64/128	+ f
a-24	32/64	+ f	0.2	b-22	64/128	+ f	3.4	c-22	64/128	+ m	d-22	64/128	- m
a-25	32/64	+ m		b-23	64/128	+ f		c-23	64/128	- m	d-23	128/256	+ m
a-26	64	- f		b-24	64/128	- m		c-24	64/128	+ m	d-24	128/256	- f
a-27	64	+ f	0.5	b-25	128/256	+ m	0.5	c-25	64/128	- f	d-25	128/256	- f
a-28	64/128	+ f		b-26	128/256	- f		c-26	128/256	- f	d-26	128/256	- f
a-29	128	+ f	0.3	b-27	128/256	+ f	0.5	c-27	128/256	+ m	d-27	256	+ f
a-30	128/256	- m		b-28	256/512	+ m		c-28	128/256	- f	d-28	256/512	- f
a-31	128/256	+ m		b-29	256/512	+ f		c-29	128/256	- f	d-29	256/512	- m
a-32	256	+ f	0.4	b-30	512/1024	+ f	3.8	c-30	128/256	+ f	d-30	512	- f
a-33	256/512	+ f	2.0	b-31	512/1024	- f		c-31	256/512	- f	d-31	1024	+ f
a-34	256/512	+ f	2.9					c-32	512/1024	- m			
a-35	512/1024	+ m	2.1					c-33	1024	- f			

starvation				bleeding			sham-inj.			RBC-injection		
s-1	<4	s-21	16	B1-a	16/32	4/8	W4-a	2	2/4	R4-a	128/256	512
s-2	<4	s-22	16	B1-b	64/128	16/32	W4-b	16/32	16/32	R4-b	512/1024	2048/4096
s-3	<4	s-23	16	B1-c	128/256	64	W4-c	4/8	4/8	R4-c	128	512/1024
s-4	<4	s-24	16/32	B2-a	16/32	4/8	W8-a	256/512	256/512	R4-d	16	64
s-5	<4	s-25	16/32	B2-b	32	8/16	W8-b	32/64	32/64	R4-e	512/1024	2048/4096
s-6	<4	s-26	16/32	B2-c	64/128	32	W8-c	64/128	64/128	R5-a	2/4	16/32
s-7	≤4	s-27	16/32	B4-a	32/64	16/32	W8-d	32/64	32	R5-b	16	256
s-8	4	s-28	32	B4-b	32/64	16/32	W8-e	32/64	64	R5-c	16/32	128
s-9	4/8	s-29	32	B4-c	64/128	32	W12-a	16/32	16/32	R8-a	128	256
s-10	4/8	s-30	32/64	B6-a	4/8	4	W12-b	16/32	8/16	R8-b	64	128/256
s-11	4/8	s-31	32/64	B6-b	8/16	8	W12-c	<4	<4	R8-c	128	256/512
s-12	8	s-32	32/64	B7-a	4/8	2/4	W12-d	64/128	64/128	R8-d	32/64	64/128
s-13	8	s-33	64	B7-b	32	32	W12-e	4/8	4/8	R8-e	128/256	2048/4096
s-14	8/16	s-34	64/128	B7-c	64	32/64	W12-f	4/8	4/8	R12-a	128	64/128
s-15	8/16	s-35	64/128	B7-d	64/128	64				R12-b	64/128	64/128
s-16	8/16	s-36	128	B7-e	256	256				R12-c	64/128	64/128
s-17	8/16	s-37	128/256	B10-a	8/16	8/16				R12-d	128/256	128/256
s-18	16			B10-b	8/16	8/16				R12-e	16/32	32
s-19	16			B10-c	64/128	64/128				R12-f	>1024	>1024
s-20	16									R12-g	<4	<4

* size and sex (see text). Series (a) was sampled in autumn, (b) in winter, (c) and (s) in spring, and (d) in summer. Series (B) was bled, (W) was sham-injected and (R) injected with HRBC at the time indicated.

echinoderm *Anthocidaris crassispina* (Ryoyama 1974). Neither age nor season was found to affect lectin levels in the periwinkle in the present study, and lectin titers did not correlate with levels of total serum protein; the latter of course reflects primarily the haemocyanin concentration, and the lectin thus appears to be distinct from the Hcy molecule. A contrasting example may be found in the horseshoe crab, *Limulus polyphemus* (Gilbride & Pistole 1981) and possibly also in the squid (Stuart 1968). The fact that periwinkle lectin levels vary independent of the sex is hardly surprising. Apart from one isolated report on the blue crab, *Callinectes sapidus* (Pauley 1974[a]), the only sex-specific lectins so far recorded from invertebrates are the **protectins** of pulmonates (Uhlenbruck *et al* 1972). These lectins are produced by the female reproductive tract and are structurally distinct from the circulating ones (Uhlenbruck *et al* 1972); they probably serve to protect the eggs against microbial attack (WJ Kuhns 1974). These molecules are apparently absent from the periwinkle; the titers of 4 albumen gland homogenates were similar to serum levels of the individuals tested, and the range of agglutinated test particles also appeared similar. Protectins do not appear to have been found in other prosobranchs, and although the lack of research in this area precludes any final conclusions to be made, it seems quite possible that these molecules in fact evolved as a response to the new conditions met by the pulmonate ancestral stock at their immigration into freshwater. Soil bacteria are less likely to put evolutional pressure on animals spawning in exposed places on the rocky shore than e.g. animals laying their eggs on the bottom of stagnant water; the survival of a littoral species is more likely to depend on factors such as exposure and predation than microbial threat to eggs.

Table 6.7: Correlation between lectin titers and physiological factors.

Chi sq. comparison:		d.f.	Chi sq.		d.f.	Chi sq.
a:b*	autumn/winter	7	2.50	male/female	7	2.61
b:c	winter/spring	8	2.19	small/large	7	3.58
c:d	spring/summer	7	1.04	females ("/")	7	5.31
d:a	summer/autumn	7	2.02	males ("/")	7	2.17
bc:ad	wint+spr/sum+aut	7	2.18	large (m/fem)	7	3.11
s:c	starved/control	7	16.8***	small ("/")	7	2.58

Regression output: serum protein levels vs. lectin titers					
constant	2.80	no. of obs.	50	X coefficient	0.29
SE of Y est.	2.41	d.f.	48	SE of coefficient	0.27
R squared	0.02				

* see Table 6.6 (series a,b,c etc).

The survival of the individual however may depend on its ability to resist microbial or parasite invasion, and the results of the induction experiment strongly support a protective role for the lectin found in periwinkle blood. Although moderate when compared to original titers, the increase in lectin titers may not all be apparent, as the lectin fraction adsorbed onto RBC membranes at injection can not be evaluated. The functional importance of the lectin may be implied by the chance observation, in one blood sample, of a group of flagellates which appeared to stick to each other by their flagellae. Agglutination of ciliates appears to be an important protective measure in the spider crab, *Maia squinado*, (Bang 1962, 1967). It seems likely that protection is offered not only by agglutination and immobilization of the invaders but also by opsonization. Although not conclusively demonstrated, an opsonizing role for the periwinkle lectin is implied by experiments with phagocytosis of RBC *in vitro*, under circumstances where serum contamination was minimized (Section 3.3), and the fact that formalinized R-RBC differ from fresh ones not only in their failure to become agglutinated by the lectin but also in their slow uptake rate by periwinkle haemocytes is strongly supportive of an opsonizing role for the lectin. The common observation of opsonizing effects of invertebrate sera (e.g. Tripp 1966; Stuart 1968; Prowse & Tait 1969; Pistole & Britko 1978; Sminia, van der Knaap & Edelenbosch 1979) has often been associated with the ability of these sera to agglutinate test-particles (Tripp 1966; Tripp & Kent 1967). Indirect evidence for this supposition comes from studies indicating correlation between agglutinability and clearance from circulation (Bang 1962, 1967; Cornick & Stewart 1968[a,b]; Pauley *et al* 1971[a,b]; Miller *et al* 1972; Huang *et al* 1981) and from experiments revealing similarities in carbohydrate specificity (Harm & Renwrantz 1980) or test-particle affinity of the two principles, the latter being shown by simultaneous removal of both properties by one adsorption (McKay *et al* 1969; Arimoto & Tripp 1977) or by similar *in vitro* adsorbtion spectra (Pistole & Britko 1978). A sharp decrease in haemocyte numbers and lectin titers concomitant with test-particle clearance has been interpreted as evidence for opsonic involvement of lectins (McKay *et al* 1969; Pauley *et al* 1971[b]; Wright 1971); it may be worth noting that incubation in serum (Tyson & Jenkin 1973) or purified albumen gland lectin (Renwrantz & Mohr 1978) may correct for the opsonin depletion when injections are made with short interval. Direct evidence of opsonization by serum lectins has only been obtained for the crayfish, *Cherax destructor* (McKay & Jenkin 1970[a]), the pulmonate, *Lymnaea stagnalis* (van der Knaap, Barendsen *et al* 1981; van der Knaap, Doderer *et al* 1982; van der Knaap, Sminia *et al* 1983) and the bivalves *Crassostrea*

gigas (Hardy *et al* 1977[a]) and *Mytilus edulis* (Renwrantz & Stahmer 1984). Apparently, the opsonin can be reversibly bound to the phagocyte surface even in the absence of test-particles and thus function as a soluble cytophilic recognition molecule (Tyson & Jenkin 1974[a]; van der Knaap, Boots & Sminia 1983). Available information thus supports that lectins may at least partly account for the opsonizing properties of invertebrate sera. There are a few reports of opsonisation in the absence of recognizable agglutinating activity (e.g. Stuart 1968) but it is virtually impossible to affirm that lectins really are absent in these cases; the assumption by Anderson & Good (1976) that *Otala lactea* lacks serum agglutinins is thus based upon the reported absence of agglutinins against human RBC (Boyd *et al* 1966), whereas opsonins were only detected using yeast as test-particle (Anderson & Good 1976). The strongest evidence in favour of non-agglutinating opsonins comes from a study on the lobster (Goldenberg & Greenberg 1983), in which the opsonizing and agglutinating activity of serum are traced to two distinct (although related) molecules of >300.000 MW; the large size of both molecules argues against one being a subcomponent of the other. Incidentally, since available data indicates that multiple lectins occur in invertebrates (Tyler & Metz 1945; McKay *et al* 1969; Jenkin & Rowley 1970; Pauley 1974[a]; Hardy *et al* 1977[b], 1978; Bretting et al 1976, 1978; Vretblad *et al* 1979; Yeaton 1981[b]; Boswell & Bayne 1984) including, in fact, the lobster (Cornick & Stewart 1968[a]; Hall & Rowlands 1974[a,b]), claims of non-agglutinating opsonins require careful interpretation; a particular test particle-lectin combination may thus be unsuitable for demonstration of agglutination although opsonization is achieved. A final conclusion on the nature of the opsonin should only be made after tests against a wide range of test-particles.

The available information thus indicates that lectins may be universally present in all invertebrates, and their common function as opsonins is also widely supported. Much less information has been gathered on the mode of opsonization by invertebrate lectins. Their ability to bind to haemocyte surfaces in the absence of test-particles (Tyson & Jenkin 1974[a]; van der Knaap, Boots & Sminia 1983) suggests that the reaction is specific, i.e. that opsonization is not brought about simply by a change in particle hydrophobicity or wettability. However, agglutination of test-particles but not of own cells indicates a higher affinity for foreign surfaces than for internal determinants, suggesting that lectins become adsorbed onto the foreign surface before interacting with phagocytes. This contention has been strongly supported by by experiments with ascidians (Coombe, Ey & Jenkin 1984), and the underlying mechanism may be seen as either (1) an increase in lectin affinity for carbohydrate

determinants on haemocytes, brought about by the increased density of lectins on the foreign surface, or (2) a conformational change in the lectin, allowing for its recognition by a specific receptor on the phagocyte membrane (Renwrantz 1986). Further studies are warranted for elucidation of the mode of action of invertebrate lectins in the opsonizing event.

The correlation found between individual lectin levels of *Littorina littorea* and the nutritional state of the animal is hardly surprising; the immediate threat of starvation is likely to affect the metabolism of all substances which can be used as fuel and are of no other immediate value to the survival of the animal. Under normal circumstances however, the serum levels of the lectin are kept remarkably stable, although at individually different levels. Lectin base levels apparently do not affect the survival chances of the individual (as shown by the comparison between different age groups), and it can thus be argued that a temporary decrease in lectin base levels, resulting from starvation is no threat to survival as long as the ability to respond to antigen challenge is not affected. Additional work is required to ascertain this point for the animal under study.

To conclude, the individual difference in lectin titers may partly be due to individual differences in food availability and exposure to pathogens (although not statistically significant, the slight increase in lectin titers in the summer, hinted at in Fig. 6.2 may thus be real, reflecting more favourable food conditions and possibly higher microbial attack in the summer). The present data however suggests that individual titers remain quite stable when followed for up to 4 months (Fig. 6.5), suggesting that in spite of minor fluctuations due to environmental challenge, the normal base levels of individuals are determined by the genotype. Genetic variability in lectin levels has been experimentally proved in a distant relative, the pond snail, *Lymnaea stagnalis* (van der Knaap, Doderer *et al* 1982). The unusual specificity of the periwinkle lectin as well as the apparent relationship between antigen challenge and serum levels make it an interesting topic for further research.

SECTION 7: GENERAL DISCUSSION.

The results of the present investigation into the defense system of the prosobranch gastropod *Littorina littorea* can be summarized as follows:

1) Injected markers are cleared by diapedesis through the epithelium covering the head, the mantle cavity and the foot (exluding the 'sole' of the foot). Emboli formed in the circulation are cleared relatively quickly by circulating haemocytes; haemocytes containing the marker may persist for longer periods in adjacent tissues but eventually find their way to diapedesis sites through circulatory routes. Clearance of carmine is not completed during the observation time (64 days), whereas yeast appears to elicit a much quicker response. Intracellular digestion may enhance the removal of injected markers in the case of digestible materials.

2) Periwinkle haemocytes form a homogenous population with respect to morphology (as determined by light and electron microscopy) and function (all haemocytes appear to be capable of migrational and phagocytic responses towards injected markers). The haemocytes are avidly phagocytic when challenged *in vivo* or *in vitro* with vertebrate RBC or yeast; around 90% of the haemocytes contain one or more particles in 30 min, and the average content of each phagocyte is 2.2 particles. Saturation of the phagocytic capacity of haemocytes appears to be reached when 2.2 particles have been ingested, and it appears that additional uptake proceeds at a steady state which is considered to depend upon the rate of intracellular digestion or membrane synthesis. The correlation of *in vitro* to *in vivo* results is discussed, and lower values in the latter case are attributed to diapedesis.

3) The connective tissue of the periwinkle is composed of ground substance with fibres resembling collagen, and five types of cells, i.e. pore cells, calcium cells, supportive cells, and granular cells in addition to migrating or resident haemocytes. Two types of muscle cells are present, i.e. smooth and obliquely striated. Phagocytosis of injected markers, i.e. human RBC, bacteria (*E. coli*) and virus particles (lambda bacteriophage) is restricted to haemocytes, which by their morphology cannot be classified as a separate subset, and so it appears that the periwinkle does not contain 'fixed' or tissue resident phagocytes. Reports of 'fixed' phagocytes in other invertebrates are discussed, as well as functional aspects of the pore cell.

4) The haemolymph of the periwinkle contains a factor which agglutinates several

types of vertebrate erythrocyte in addition to bacteria (*E. coli*) and yeast (*S. cerevisiae*). The active part of this molecule is a protein, and so it classifies as a lectin (Goldstein *et al* 1980). Agglutination of H-RBC was inhibited most strongly by L-xylose, but moderate inhibition was obtained with other pentoses (D-ribose, D-xylose, α-L-fucose). A possible role of the lectin as an opsonin is discussed in connection with the finding that particles which are agglutinated by the lectin appear to be phagocytosed more avidly than particles which fail to agglutinate. A moderate increase is found in circulating lectin levels upon antigenic challenge (human erythrocytes), pointing to a role in defense. The 'baseline' level of the lectin is quite variable between individuals, and is not affected by age or sex; this polymorphism is believed to be genetically determined, although to some extent it may also be modified by environmental factors (starvation, antigenic challenge).

The results of the current work thus emphasize the importance of haemocytes in the defense against invading organisms, and strongly imply the lectin as a co-factor in the phagocytic response. A similar dependance on circulating haemocytes and opsonic factors appears to be typical for the invertebrates in general, and in order to highlight the main features of this system, it appears useful to present a more detailed analysis of the events occurring during phagocytosis. To facilitate comparison of the periwinkle defense system to that of other invertebrates, it is necessary also to discuss the main deviations from this general pattern, including the lack of demonstrable opsonic activity in some invertebrates and the possible involvement of fixed phagocytes or lytic molecules in others. Before turning our attention to the internal defense system of invertebrates, let us however first look at what is known about the contribution of extrinsic factors to the overall defense.

7.1 Extrinsic factors contributing to resistance.

Epithelial tissues form the first line of defense against microbial attack in all multicellular animals. The opposite demands on these tissues to form an effective barrier between the internal and the external environment, and at the same time, to allow for gaseous exchange and the transport of metabolites, is in all but the most primitive phyla solved by reserving a special, invaginated part of the surface for functions requiring thin surfaces. In invertebrates, the external epithelium may be covered by a tough cuticulum or shell, or remain uncovered. Remarkably little has been written on the defensive value

of covering structures or in fact the surface barrier in general, but the importance of the crustacean integument for resistance is indicated by studies on fungal infections in crayfish (Unestam & Nyhlén 1974), ciliate infection in crabs (Bang *et al* 1972; Armstrong *et al* 1981) and bacterial infection in lobsters. Apart from a bacterial skin infection (Hess 1937), lobsters are known to be subject to only one disease, 'Gaffkaemia', caused by *Aerococcus viridans* var. *homari*, which appears to be transmitted only after rupture of the integument (Stewart, Dockrill & Cornick 1969). The effectiveness of the molluscan shell as a barrier may also be shown, although rather indirectly, by the ability of prosobranchs, so long as the shell is intact, to survive a journey through a fish's intestine (Norton 1988). Shell repair may thus be seen as a contributing factor in the resistance to pathogens as well as to other threats to survival, and the role of haemocytes in this function (Wagge 1951; Kapur & Gupta 1970; Abolins-Krogis 1972; Bubel *et al* 1977) and in wound healing (Armstrong *et al* 1971; Sminia, Pietersma & Scheerboom 1973) further emphasizes the importance of these cells in defence. The operculum forms an effective seal against unfavourable conditions, but other protective measures must take over as it is opened when feeding, and in fact be continually active in less well covered invertebrates (even some which are protected by a shell; pulmonates e.g. are without an operculum). The available evidence suggests that mucociliary mechanisms may be important for protection of uncovered surfaces, including respiratory epithelia. The body surface mucus of molluscs has thus been shown to contain a varitety of antimicrobial substances, e.g. lysozyme in the oyster, *Crassostrea virginica* (McDade & Tripp 1967[c]), antibacterial activity in the slug, *Achatina fulica* (Iguchi *et al* 1982), lectin-like activity in the pulmonate, *Helix aspersa* (Fountain 1985) and the cephalopod, *Loligo vulgaris* (Marthy 1974) and a precipitating substance in the prosobranch, *Littorina angulifera* (AC Smith 1984, 1986). These substances may offer some protection to microbial attack, and there are indications that parasites may be trapped (Loker 1978) or fail to attach (Kinoti 1971), but invertebrates are probably more susceptible to parasite invasion via epidermal surfaces than vertebrates, as they generally lack stratified epithelia (Welsch & Storch 1976, p. 52). It seems likely that susceptibility to different pathogens may be modified also by environmental influence; adaptation of the external surface for gaseous exchange by earthworms may thus perhaps reflect a relative shift in challenge from metazoan parasites towards more easily contained soil bacteria.

The internal, or mucosal epithelium is generally thinner than external barriers,

allowing for metabolic exchange, and is thus used as infection route by a great number of vertebrate pathogens (cold, influenza). This probably applies to invertebrates as well, but studies on the route of infection, or the effectiveness of the internal epithelium and accessory factors (enzymes, pH extremes, resident bacterial flora, antimicrobial molecules etc.) as a barrier to infection are few and far between, insects receiving the lion's share of the interest (see Maramorosch & Shope 1975, pp 3-114). A virus-inactivating protein has been found in the digestive juice of the silkworm, *Bombyx mori* (Uchida *et al* 1984) and earlier studies (see Huff 1940) indicate the presence, in insect gut, of powerful bactericidal principles. The significance of pH extremes may be shown by the fact that in lobster gastric fluid, lytic proteins with functional resemblance to lysozyme are of minor importance, and the prime antibacterial agent is the acidic environment, a function of gastric proteins; the importance of this factor may be seen by the fact that it forms an adequate barrier for transmission of *A. viridans* by the oral route (Stewart, Dockrill & Cornick 1969). It would seem worthwhile to search for accessory factors in all invertebrate phyla showing capacity for modulating the contents of the gut with enzyme secretion and extracellular digestion.

Apart from external and internal epithelia and associated factors, other non-specific defense adaptations may also contribute to resistance. In starfish cells, cholesterol has thus been replaced with a different type of steroid, allowing the simultaneous evolution of special saponins which interact with cholesterol. Echinoderms lacking lytic saponins on the other hand possess cholesterol in their membranes (Mackie et al 1977).

7.2. Internal defense: general features.

All animals have the means to resist or combat infection by pathogens, yet may at some stage harbour symbiotic or parasitic agents. This apparent paradox is conditioned by the fact that propagation of the species only requires that a minimal number of individuals remain viable and fertile for each generation time. Mild parasitism may be tolerated as long as chances of survival from more fierce attacks are optimized by (a) the combination of geographical dispersion, high numbers of offspring and short generation times ('biological avoidance') and (b) special defense adaptations. All metazoan animals apparently rely upon a combination of both factors, with a relative shift from the former

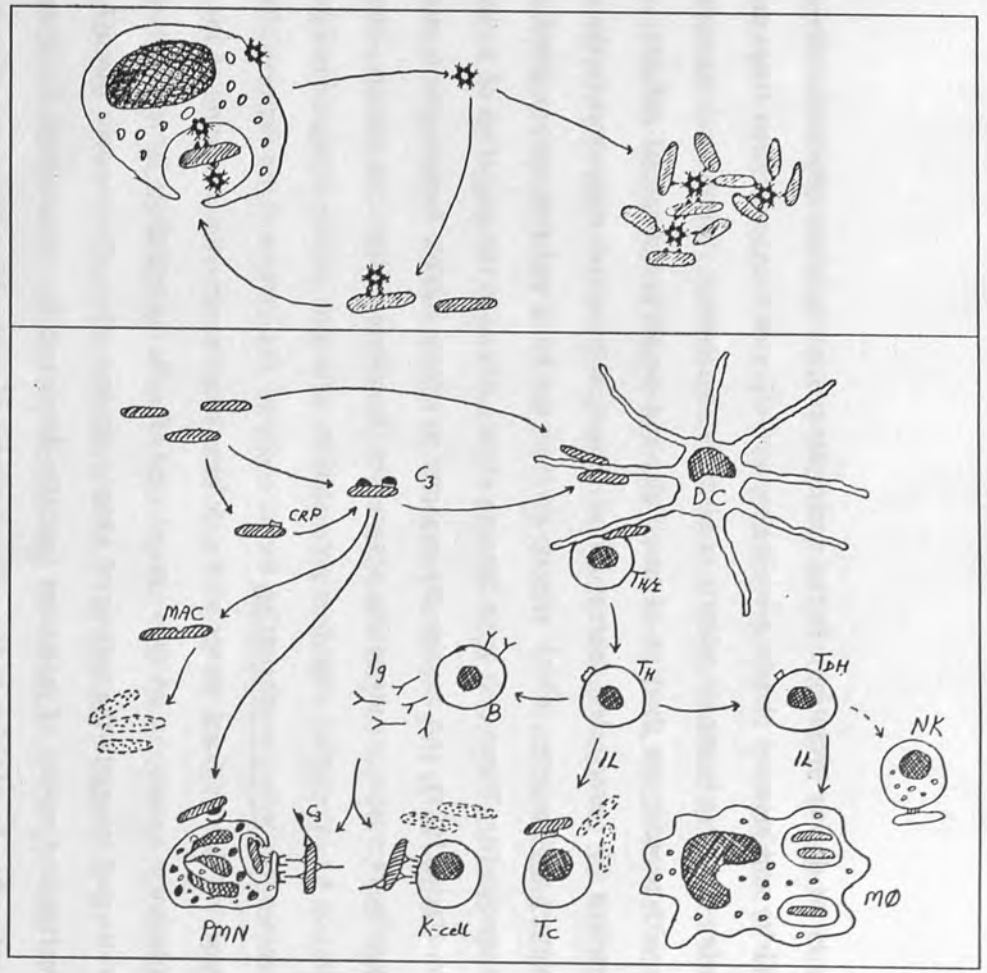
to the latter during evolution. In the deuterostomes, an immune system comprising specificity and memory clearly precedes the evolution of long generation times and low offspring numbers (compare teleosts to birds and mammals). Primitive deuterostomes share with protostomes the lack of specificity and memory, but special defence adaptations are nevertheless present in even the most primitive metazoans in the form of phagocytic amoebocytes, which migrate through the body and remove unwanted materials by phagocytosis. These cells later become closely linked to the evolution of a circulatory system (Dales 1981), and acquire the capacity to release soluble factors which assist in the defence by recruiting or attracting phagocytes (leucocytosis-inducing factors, chemotactic factors) or by interacting with foreign materials, acting as soluble recognition factors for phagocytes (opsonins) or disrupting the integrity of the invader (lytic factors). The emphasis on this humoral aspect of the defense characterizes the evolution of the vertebrate immune system, in which a special type of cell, the lymphocyte, has evolved to supervise and integrate the actions of phagocytes and other effector cells through the production and release of molecules specially developed for this purpose, i.e. lymphokines and antibody. In addition to the original phases of **recognition** and **destruction/inactivation**, clonal expansion of T- and B-lymphocytes and secretion of their soluble products thus offers the possibility of an intermediate phase of **amplification** (fig. 7.1) as well as conferring both **specificity** and **memory** upon the response. Lymphocytes and their products have been extensively searched in invertebrates, but the overwhelming evidence, coming from studies on specificity and memory as well as more direct biochemical and histological analysis (see below) speaks for their absence in non-chordate phyla. Phagocytes thus form the cornerstone of the defense system of invertebrates, although humoral factors also exist. The main factors implicated are lectins, but the presence in higher invertebrate phyla of lytic substances also merits some consideration, as it might point to the evolution of more complex, interacting humoral systems like complement. For convenience, some theoretical considerations and a brief summary of the human immune system will be provided as framework for discussion of invertebrate immunity.

(I) RECOGNITION. Before turning our attention to cellular recognition, it should be noted, that recognition of a foreign epitope may, at least theoretically, occur without activation of defense cells; activation of the vertebrate complement system (Whaley 1987,

Fig. 7.1: Evolution of defense mechanisms

(A)

	RECOGNITION		ENHANCEMENT	DESTRUCTION
II[a]	Amoebocytes	+	proliferation	phagocytosis
	(other types)	+	activation	(PO secretion, etc.)
	lectins	+	synthesis	-
	(PO)	+	&	killing (melanin)
	(cecropins)	+	release	lysis
I	Amoebocytes	+	prolif./activ.	phagocytosis
	lectins	+	synth./release	-
II[b]	Dendritic cells	-		-
	Macrophages	+	proliferation	phagocytosis
	PMN	+	&	phagocytosis
	NK-cells	+	activation	cytolysis
	T-lymphocytes	***	activation &	cytolysis (T_c)
	B-lymphocytes	***	clonal expansion	-
	CRP	+++	synthesis	-
	complement	+++	& release	lysis (MAC)
	(antibody)	+++	synth./release	-



(A) A schematic presentation of a hypothetical development of the vertebrate (II^b) and the arthropod defense (II^a) from primitive mechanisms (I). + denotes enhancement; * enhancement with a memory component; ▒ specificity; humoral components in parenthesis do not occur extracellularly before activation. Analogies may exist, e.g. between macrophages and amoebocytes, and between lectins and CRP (see text). Some features of the vertebrate system may be more primitive than others, e.g. complement, NK-cells (see text). (B) A pictorial overview of the vertebrate immune system compared with more primitive defense measures.

Rother & Till 1987) may thus lead to destruction of the invader without the need for cellular involvement. It seems implausible that the refined discrimination between self and nonself needed for successful elimination of potential pathogens with minimal tissue damage can be attained by a single molecular interaction between the activation component and the foreign epitope (e.g. the reaction of C3 with -OH or $-NH_2$ groups as the first step of the alternative pathway). Indeed, discrimination occurs at several stages, providing ample means for averting untimely activation. Both activation pathways of complement are currently thought to be constitutively engaged (Nydegger 1985), but native cells are protected by (a) a molecular organization favouring interaction with regulatory components of the complement cascade (e.g. high sialic acid content), (b) the incorporation of specific complement regulatory proteins into the plasma membrane (e.g. DAF, MCP, CR1, HRP) which interact with bound complement components and avert the assembly of lytic components, and (c) the ability to internalize and destroy complement lesions (Frank 1987; Mollnes & Lachmann 1988). The dependence upon each function varies from cell to cell; RBC thus rely on the first two, but cells with more complex metabolic machinery may be protected also by the last function.

Cellular recognition: (a) direct interaction. Recognition of course also occurs at the cellular level, and this may occur directly or through soluble ligands (opsonins). In both cases, the recognition may be specific or non-specific (table 7.1). Recently, the traditional view of recognition as a specific process in the vertebrates has been challenged by the discovery that the initial recognition of antigens occurs at the level of the antigen presenting cell (APC), with T- and B- lymphocytes only becoming involved after this stage. Dendritic cells (DC) of the skin and the T-dependent areas of lymphoid tissues are derived from the macrophage precursor series (Unkeless & Springer 1986), and in vertebrates as well as invertebrates, recognition thus involves phagocytic cells of leucocytic lineage. As specific recognition factors can not conceivably be expressed on the surface of phagocytic cells (it would require the expression of an infinite number of different recognition molecules), the initial step of recognition must be essentially non-specific in vertebrates as well as invertebrates. At the cell surface, non-specific recognition may be brought about by differences in physico-chemical characteristics; the degree of **hydrophobicity** may thus determine the interaction of phagocytes and particles in the absence of humoral factors (van Oss 1978). Physico-chemical characteristics are likely to govern e.g. the reaction of phagocytes towards glass and other inert particles.

Another possibility of direct, relatively non-specific interaction between phagocytes and particles is provided by membrane associated molecules with distinct affinities for certain atomic configurations. Lectins may thus occur as integral membrane molecules as well as in a soluble state, and appear to be involved in recognition by human macrophages (Weir & Ögmundsdóttir 1977; Nakamura & Kolb 1980; Glass *et al* 1981; Sharon 1984; Hogg 1988) as well as invertebrate phagocytes (Section 7.3).

(b) Soluble recognition factors. The two main types of non-specific ligands known to possess opsonic properties in vivo are **lectins** and **complement**. It should be mentioned that ligand binding to the phagocyte membrane may be specific although binding to the foreign surface is not, as exemplified by the binding of opsonic complement (membrane bound C3b/iC3b) to receptors (CR1, CR3, CR4) on phagocytes. Constant occupation of the binding site is prevented by receptor specificity for certain activation products of the ligand (C3). The interaction of lectins with foreign particles has not been shown to involve a conformational change allowing for recognition of an activated product, and their subsequent (or indeed previous) association with phagocyte membranes may thus conceivably result from their own affinities for surface carbohydrate groups. This is well demonstrated by some recent studies of invertebrates, indicating affinity of serum lectins to phagocyte membranes even in absence of foreign particles (Section 7.3). One must also keep in mind that non-specific adsorbtion of serum proteins onto foreign surfaces may indirectly help the phagocyte by decreasing the surface hydrophobicity of the particle (van Oss 1978). Involvement of hydrophobic interactions between lectins and their determinants has been noted (Balding 1980). On the whole, until the presence of specific receptors with affinities for soluble lectins is demonstrated, it seems advisable to prefer terms such as 'acceptors' or 'determinants' to 'receptors' in opsonic studies involving lectins.

(II) AMPLIFICATION. At the cellular level, enhancement of responses may be achieved by mitogenesis, release of tissue reserves and activation of defensive cells. In vertebrates, these responses generally involve the participation of humoral messenger molecules, e.g. colony-stimulating factors, interferons, interleukins and complement components. These factors accelerate the proliferation of leucocytes in bone marrow and their departure in blood vessels, induce margination and migration into tissues of inflammation, and bring about tissue degradation and swelling which further facilitates

the entry and migration of leucocytes (Fearon & Wong 1983; Björck *et al* 1985; Nicola 1987, O'Garra 1988). The production of specific serum antibodies constitutes an even more dramatic humoral enhancement response; after initial recognition and presentation of the foreign antigen by APC, lymphocyte clones which recognize the antigen become selectively expanded, resulting in immunoglobulin production. Antibody production is regulated by a complex interaction of cells and humoral factors, one of which in fact is complement; it is strictly needed for the secondary response (Pepys 1974; Böttger & Bitter-Suermann 1987) and may provide regulatory effects by interacting with APC and lymphocytes (Feldbush et al 1984; Ross & Medof 1985; Ambrus *et al* 1987; Peters *et al* 1987). The amplifying effects of complement are further enhanced by a feed-back loop, and infection results in a substantial increase in serum levels of these and other acute phase proteins. Apart from complement, this enhanced production has not been fully explained in terms of defensive purposes, but the binding of CRP to polysaccharides containing phosphorylcholine may lead to opsonization of bacteria, fungi and metazoan parasites in the presence of complement (Pepys & Baltz 1983). This is quite interesting in the light of studies indicating antigenic similarities (Stein & Basch 1979) and 40% amino acid sequence homology (Liu, Robey & Wang 1982) between CRP and some invertebrate lectins.

(III) DESTRUCTION/INACTIVATION. In addition to two main phagocyte types, neutrophils and macrophages (Mϕ), vertebrates possess special cells capable of contact lysis of pathogens and malignant cells (NK cells, K cells, cytotoxic T-lymphocytes). The lytic action of these cells is mediated by the release of **perforins**, 60-65 kd molecules which become inserted into target cell membranes in a polymerized form as circular lesions (Young & Cohn 1986). Killing may also be effected by serum molecules, i.e. **complement** (Whaley 1987, Rother & Till 1987). The lytic action of complement is due to the insertion into target cell membranes of C5-C9, the membrane attack complex MAC), the last and most effective of which is a 62-65 kd molecule (C9) with striking antigenic and structural resemblance to the perforins (Young & Cohn 1986; Rother & Till 1986, p. 248). The complement system can be activated by various foreign surfaces and molecules, after which ligands are formed which may be bound by special complement receptors on defensive cells (see above). The central component of this system, C3, can be directly activated by many pathogens through the alternative pathway, forming reactive

components which by binding to specific receptors on defence cells, or by interacting with other complement components are able to mediate a number of functions, notably inflammation (including leucocytosis, margination and chemotaxis), phagocytosis, and cytolysis. After the evolution of immunoglobulins, a link connecting these to C3 (the classical pathway) has become superimposed upon the original system. The system was apparently also integrated into immunoregulatory circuits (see above), and took on a very important role by preventing the precipitation (in vessels and tissues) of immune complexes (Schifferli *et al* 1986; Harkin *et al* 1988). The complement system has clearly been expanded through evolution. The nurse shark, representing the most primitive vertebrate for which a complete complement system has been described, has thus only 6 functionally distinct classical pathway components, reacting in the order C1n, C2n, C3n, C4n, C8n, and C9n (Smith & Jensen 1986). The last component is clearly distinct from mammalian C9, as revealed by size (185 kd), charge and reactivity (Jensen *et al* 1973), and in lamprey, C3 is found to participate in opsonization but not haemolytic activity (Nonaka *et al* 1984). The opsonic activity of cyclostomes is associated with components from the alternative pathway, the classical pathway being absent (Day *et al* 1970); it would thus seem likely that complement, if present at all in invertebrate phyla, is restricted to a few components, such as C3, B, factors with regulatory activity (e.g. D, H, I, P) and opsonic receptors (CR1, CR3).

7.3 Humoral factors in invertebrates.

In invertebrates, the absence of serum components corresponding to the globulin fraction of vertebrates (Woods *et al* 1958; Cheng 1969; Scott 1972; Pauley 1974[a]) and of reactions characterized by specificity and memory (e.g. Taylor *et al* 1964; Teague & Friou 1964; Chadwick 1967; Bang 1967; Evans, Weinheimer *et al* 1969[b]; Bayne 1973[a]; Acton & Weinheimer 1974; Coffaro 1978) together with the finding of molecules in tunicates with some resemblance to vertebrate heavy chains (Warr & Marchalonis 1982) implies that the evolution of immunoglobulins occurred after, or close to the separation of the chordate and the non-chordate stock. In invertebrates, the enhancement step thus consists mainly of cellular processes such as multiplication, mobilization and activation. Humoral factors however may be involved in recognition and destruction, and in some cases, a moderate

increase in lectins and/or lysins may be observed after challenge. Before turning our attention to cellular defence, it therefore seems useful to briefly review our current knowledge of invertebrate lectins and lytic factors.

(I) LECTINS. Lectins are probably ubiquitous in invertebrates (Yeaton 1981[a,b]), and an increasing body of evidence speaks for their involvement in defense as soluble recognition factors, or opsonins (Section 6.4). Many lectins are shown to have affinity for bacterial surface components, e.g. KDO (2-keto-3-deoxyoctonate; Rostam-Abadi & Pistole 1982; Stein & Cooper 1983) or phosphorylcholine (Roche & Monsigny 1974; Stein & Basch 1979); the latter in fact provides an interesting comparison to vertebrate CRP (Pepys & Baltz 1983). The specificity of other lectins appears to be directed towards more commonly represented carbohydrate residues (Yeaton 1981[a,b]), but the ability to adsorb onto various particles appears to characterize all invertebrate lectins, and their ability to act as bridges connecting these to phagocytic cells forms the basis of their opsonic activity. As previously stated, the nature of the lectin-phagocyte interaction has not been determined at present, and the need for conclusive evidence of specific receptor-ligand interactions is emphasized by the cytophilic nature of native lectins (see below) as well as the ability of non-native lectins to mediate attachment (Renwrantz & Cheng 1977[a,b]; Cheng, Huang et al 1980; Renwrantz, Daniels & Hansen 1985) and even ingestion (Sminia, Winsemius & van der Knaap 1981) in invertebrate test systems *in vitro*.

The *in vivo* importance of lectins is indicated by the correlation of lectin titers and resistance to ciliate infection in individual spider crabs (Bang 1962, 1967), inverse correlation between lectin activity and bacterial pathogenicity in lobsters (Cornick & Stewart 1968[a]), crabs (Cornick & Stewart 1968[b], 1975; Schapiro 1978), and sea hares (Pauley et al 1971[a]), and resistance to *S. mansoni* infection in *B. glabrata* strains which are able to agglutinate the parasite (Bayne, Boswell *et al* 1985). The original role of lectins however may be associated with molecular transport or cellular integration (Yeaton 1982); in algae, lectins thus function in gamete recognition and subsequent reproductive cell fusion (Ingram 1985). Like pentaxins (Pepys & Baltz 1983), lectins are composed of subunits (Pauley 1974[a]), but form a double-stacked hexamer (Marchalonis & Edelman 1968[a]; Liu *et al* 1982) rather than the typical pentamer structure of CRP. Both depend on Ca^{++} for their function, and EDTA causes disassociation of lectin subunits (Pauley 1974[a], Jenkin & Hardy 1975). Reaggregation of subunits in the presence of Ca^{++} can restore

activity of adsorbed crayfish serum, and this has been used to explain binding activity heterogeneity (see Section 6.4) by random aggregation of non-identical subunits (Jenkin & Hardy 1975). This notion has received further elaboration by Parish (1977), who suggests that the subunits may be related to 5-glycosyl-transferases.

Results from the crayfish, *Cherax destructor* (Tyson & Jenkin 1974[a]) and the snail, *Lymnaea stagnalis* (van der Knaap, Sminia et al 1983; van der Knaap, Boots & Sminia 1983) demonstrate that phagocytic activity of haemocytes may be lost upon trypsinization and regained by serum incubation, and, in the latter case, the activity varies in paralell with the opsonic activity of serum. Lectins thus appear to function as reversibly bound cytophilic receptors (Renwrantz & Stahmer 1983; Vasta, Cheng & Marchalonis 1984). These results may help to explain the different degrees of serum dependence found in different test particle-haemocyte models (Tyson & Jenkin 1974[a]). The ability of lobster (Paterson *et al* 1976) and crayfish haemocytes (Tyson *et al* 1974) to ingest bacteria but not RBC thus suggests different requirements for cytophilic opsonin density. Claims of absolute requirements for opsonins in the pulmonate *Helix aspersa* (Prowse & Tait 1969) *vs.* absence of serum opsonins in insects (Scott 1971; Anderson *et al* 1973[a]; Rowley & Ratcliffe 1980; Wago & Ichikawa 1979[b]) may similarly be taken to suggest differences in opsonin cytophilicity or lectin determinant-density on the test particle used (Tyson & Jenkin 1974[a]) although it is not inconceivable that the potent inducible antimicrobial factors of insect haemolymph (see below) have reduced the need for lectin involvement in phagocytosis in this invertebrate group. The importance of lectins as recognition factors in phagocytosis may apply also to cell mediated cytolysis (CMC); this function is shown to depend upon target adhesion by a 81.5 kd membrane factor in the crayfish, which shares with lectins the six subunit composition (Jenkin & Hardy 1975). Lysis of injected RBC also depends upon lectin activity in the flesh-fly, *Sarcophaga peregrina* (Komano & Natori 1985), although the nature of the lytic activity (cellular or humoral) was not established.

Lectins apparently originate in haemocytes in most or all invertebrates with a circulatory system (Tyson & Jenkin 1973; Cornick & Stewart 1973, 1978; Amirante & Mazzalai 1978; van der Knaap, Barendsen *et al* 1981; Wright & Cooper 1982; see however Leclerc *et al* 1980 for a possible exception). Mucus-associated lectins may be secreted by epidermal glandular cells (Marthy 1974), and in sponges, which do not contain a circulatory system, the spherulous cells have been implicated (Bretting & Königsmann 1979). A

moderate increase in lectin titers upon challenge has been recorded for insects (Luther *et al* 1975; Komano *et al* 1980; Pendland & Boucias 1985), annelids (Stein *et al* 1982; Wojdani *et al* 1982), bivalves (Hardy, *et al* 1977[a]) and gastropods (the current thesis; also see Jeong, Sussman *et al* 1981). These results are particularly interesting in connection with the opsonic properties of invertebrate lectins and their possible relationship to vertebrate CRP (see above). Incidentally, factors with functional resemblance to invertebrate lectins have also been reported in studies of lamprey (Marchalonis & Edelman 1968[b]) and other lower vertebrates (Gold & Balding 1975; Sigel 1973; Balding & Gold 1976) but their relationship to invertebrate lectins (or indeed to CRP) is not known.

Lectins thus function as soluble and cytophilic haemocyte-derived recognition factors for phagocytes and (probably) cytolytic cells. A degree of heterogeneity has been detected (Section 6.4), featuring perhaps also molecules with opsonizing but not agglutinating activity (Goldenberg & Greenberg 1983), and their action may exhibit class specificity; at least 10 relatively class-specific lectins have been reported from lobster serum (Tyler & Scheer 1945), and class specificity is also observed in crayfish (McKay & Jenkin 1970[a]) and starfish (Tyler 1946). This provides an interesting parallel to vertebrate antibodies, and offers some support to the notion (Boyden 1966; Burnet 1968; Acton & Weinheimer 1974) that lectins may be viewed as antibody forerunners. The accumulated evidence gives new appeal to the term 'antisomes', originally suggested by Bang (1973[a]).

It should be noted, however, that although lectins are by far the likeliest candidate for opsonic activity in invertebrate sera, other molecules may be implicated, e.g. components of the prophenoloxidase system (Söderhäll 1982; Johansson & Söderhäll 1986), complement-like molecules (Bertheussen 1981[a,b]; Laulan *et al* 1988) and possibly even haemocyanin (Stuart 1968; Gilbride & Pistole 1981; also see van der Knaap, Barendsen et al 1981, and Yoshino 1983). Lectins may thus conceivably be non-opsonic in insects, and supplemented by other opsonic molecules in other invertebrates. Future studies should aim to clarify these matters as well as establishing the nature of the lectin-phagocyte membrane interaction.

(II) LYTIC FACTORS. Factors with antibacterial activity appear to be common in arthropods, annelids and sipunculids, but studies on echinoderms (Johnson & Chapman 1971; Wardlaw & Unkles 1978) and mollusks (Weinheimer *et al* 1969[a]; Bayne 1973[a]) have

proved negative, apart from one isolated report on abalones (Cushing *et al* 1971). Among the antibacterial factors, the best characterized are the inducible bactericidins of insects, which were intensively studied in the 1920's (see Huff 1940) and rediscovered several decades later (Briggs 1958; Wagner 1961; Stephens & Marshall 1962; Gingrich 1964). They are released from the fat body upon stimulation, and recent studies have revealed three main categories (Hultmark 1986), i.e. dialyzable cecropins (35-37 aa), attacins (ca. 20 kd. proteins which affect the permeability barrier at the outer membrane of Gram(-) bacteria) and lysozyme. Recent studies indicate that their release may be induced by a haemocyte-derived factor (DeVerno *et al* 1984), or, artificially, by phospholipase A2 from bee venom (Rheins & Karp 1984). Cecropins and attacins appear to be unique to insects, but lysozyme is also found in echinoderms (Jollés & Jollés 1975), annelids (Schubert & Messner 1971) and molluscs (McHenery, Birkbeck & Allen 1979). The ca. 14 kd insect molecule is closely related to its chicken counterpart, and the same may apply for the mussel lysozyme (Hardy *et al* 1976), whereas in oyster, it appears acidic and much larger (Feng 1974). In insects, it is released from pericardial cells (Crossley 1972) as well as haemocytes (Anderson & Cook 1979), but in the oyster it is released from haemocytes during phagocytosis or encapsulation (Cheng *et al* 1975, 1977; Kassim & Richards 1978; see Section 7.4) and is present in mucus as well as blood (McDade & Tripp 1967[b,c]). The widespread occurrence of lysozyme in invertebrates demands comparison of this entity to other thermostable bactericidins reported (e.g. Li 1969).

Insects and other arthropods are also able to kill pathogens by melanization (see Unestam & Nyhlén 1974), and the response is shown to be heavier *in vitro* and *in vivo* in blood from resistant crayfish (Unestam & Weiss 1970). Recent studies have led to the characterization of some of the components acting within this system (Söderhäll *et al* 1986; Johansson & Söderhäll 1986; Ashida *et al* 1986). Briefly, stimulation by bacterial cell wall products leads to the release of prophenoloxidase and its activating enzyme (a serine protease) from granular cells, leading to activation of the phenoloxidase and its deposition on the foreign object. The serine protease may also mediate blood coagulation by converting cystocyte-derived coagulinogen to coagulin (Rowley 1977; Söderhäll 1982). A close inspection of earlier reports on inducible bactericidins in *Limulus* (Johannsen *et al* 1973; Furman & Pistole 1976; Pistole & Furman 1976), spiny lobsters (Evans *et al* 1967; Evans, Cushing *et al* 1969) and lobsters (Acton *et al* 1969[b]) indicates similarity with the prophenoloxidase system. The lobster bactericidin is thus adsorbed by various bacteria

although only active against certain strains (Mori *et al* 1976) and appears to be formed by the interaction of a cellular and a humoral component (Stewart & Zwicker 1972, 1974[a,b]); injected particles are seen to darken with time (Cornick & Stewart 1968[a]). A plasma-haemocyte interaction is also seen in the horseshoe crab (Furman & Pistole 1976).

The bacteriostatic factors of annelid coelomic fluid are also being characterized; the activity appears to be associated with heterogenous molecules of 20, 40 and 45 kd (Vailler *et al* 1985). Much less is known about factors in sipunculids with lytic or arresting properties against ciliates (Bang 1962, 1966; Bang & Shin 1981), ciliates and flagellates (Cushing *et al* 1969) and bacteria (Evans, Weinheimer *et al* 1969[a]; Evans *et al* 1973). Comparison of the reported characteristics of these factors suggests that they may be identical. Sipunculids appear unique among invertebrates in possessing a natural bactericidin (Bang & Krassner 1958; Krassner & Flory 1970). This factor is dialyzable, and thus clearly distinct from other factors reported from sipunculids. It appears to be released from circulating cells (Rabin & Bang 1964). Mollusks are generally found to lack factors with antibacterial activity, and the identity of factors which bring about the immobilization of parasites (Michelson 1964; Cheng 1968; Ratanarat-Brockelman 1977; Lie & Heyneman 1979; Sullivan *et al* 1982) is uncertain; it may not be proven that the factors originate within the host and do not represent parasite competition for 'food reserves'.

The presence of lytic activity in invertebrate body fluids has from an early stage prompted comparison to vertebrate complement. Bacteriolytic factors are generally found to be relatively heat-resistant (e.g. inactivated at 65 °C in the spiny lobster; Evans *et al* 1968), and more attention has thus been given to the thermolabile haemolysins of arthropods (Weinheimer *et al* 1969[b]), sipunculids (Weinheimer, Acton *et al* 1970), annelids (Cooper *et al* 1974; Garte & Russell 1976; Roch 1979; Anderson 1980; Dales 1982), molluscs (Hardy *et al* 1976; Michelson & Dubois 1977; Anderson 1981[a]) and echinoderms (Ryoyama 1973, Parrinello *et al* 1979). The defensive value of haemolysins is uncertain; annelid haemolysins apparently contribute to the bacteriostatic effect of the coelomic fluid (Vailler *et al* 1985; Kauschke & Mohrig 1987) but holothuroid haemolysins are active only against membranes containing sphingomyelin, with no effect on bacteria (Canicatti *et al* 1987). Haemolysins apparently differ from complement in many important aspects. The haemolysin of the clam, *Mercenaria mercenaria* (Anderson 1981) appears to be released from haemocytes upon activation (Leippe & Renwrantz 1988). Sipunculid haemolysin

does not require divalent cations (Weinheimer, Acton *et al* 1970), whereas *Limulus* haemolysin requires Ca^{++} and partitions chiefly with the pseudoglobulins (Gewurz *et al* 1970). In earthworms, the activity is associated with two lipoproteins of 40 and 45 kd (Roch 1979) and can be inhibited by simple sugars (Tucková *et al* 1986). The latter also applies to the polychaete *Neoamphitrite figulus* (Dales 1982), whereas *Glycera dibranchiata* may differ from other annelids in this respect (Anderson 1980; Chain & Anderson 1983). The involvement of complement in invertebrate haemolytic systems has thus not been substantiated, but recent studies on echinoderms (Bertheussen 1983) suggest that further studies may be warranted, at least in deuterostomes. There is also some support for the presence of individual molecules with functional resemblance to complement components. Starfish haemolymph is thus shown to support haemolysis in the presence of frog lytic complement and CVF (Day *et al* 1970), with similar (although less convincing) results reported from other invertebrates (Day *et al* 1970; Anderson *et al* 1972). This may be explained by the presence of a serine esterase, capable of cleaving vertebrate C3 into C3b- and C3a-like fragments after activation by CVF (Day *et al* 1972[b]; Phipps *et al* 1987); however, the small size of this factor in starfish (ca 2 kd; Day *et al* 1972[a]) does not indicate relationship to vertebrate complement, and the presence, in insect haemolymph, of a serine protease functioning in the prophenol-oxidase system (Andersson *et al* 1986) demands a comparison between the two activities. Whatever the identity of this factor, its activation by CVF may provide an explanation for the depressive effect of CVF on the induced resistance of *Galleria mellonella* (Aston & Chadwick 1981). The finding of structural as well as functional similarities between the main haemolymph protein of the insect, *Spodoptera frugiperda* and mammalian C4 binding protein (D'Cruz & Day 1985) is rather curious, since the classical pathway of complement, in which C4-bp functions, is considered absent in invertebrates (Day *et al* 1970). However, a clue may be provided by the ability of *Spodoptera* serum to regulate alternative pathway activity (D'Cruz & Day 1984) and the relationship of C4-bp to a number of C3 binding proteins, including B, H and CR1 (Reid *et al* 1986). The presence of receptors for opsonic complement (i.e C3 fragments) on circulating cells of echinoids (Bertheussen 1981[a,b], 1982; Bertheussen & Seljelid 1982) and earthworms (Laulan *et al* 1988) suggests that corresponding ligands may be present in the body fluids of these invertebrates. The ability of pulmonate serum factors (Koch & Nielsen 1984) including lectins (Stein & Basch 1979; Gold & Thompson 1969) to activate vertebrate complement on the other hand may not indicate complement

relationship, as the cascade can be activated by complement-unrelated compounds. Taken together, these results suggest that factors with functional resemblance to B and/or C3 of vertebrate complement may be present in invertebrates, but further studies are needed for final conclusions. It is by no means inconceivable that complement evolved for opsonic purposes, lytic components being added later. The latter may in fact even be present from an early stage although not coupled to B/C3 predecessors; the possible relationship of the ca. 80 kd. coelomocyte-derived haemolysin of *Holothuria polii* (Canicatti *et al* 1987) to perforins (Canicatti & Ciulla 1987, 1988) and the close relationship of perforins to C9 (Young & Cohn 1986) suggests that a relationship between C9 and invertebrate haemolysins should not be overlooked. The evolution of complement will no doubt become clearer in future, as direct studies on the structure of invertebrate haemolysins and B/C3-like factors are now feasible with modern recombination techniques.

7.4 Cellular responses.

(I) EFFECTOR CELLS. The consensus of work on defence reactions in invertebrates emphasizes phagocytosis as the main mechanism of defense (e.g. Sindermann 1971; Coffaro 1978; Mori & Stewart 1978; Sminia & van der Knaap 1986), and enhanced secondary responses, where they occur, are generally traced to increased numbers and/or activity of phagocytes (Taylor *et al* 1964; Acton *et al* 1969[a]; McKay & Jenkin 1969, 1970[b,c]; Lie *et al* 1975[a]; Paterson *et al* 1976; Bayne 1980; van der Knaap, Tensen *et al* 1982; van der Knaap, Boots *et al* 1983) except in insects (Chadwick 1967) where potent bacteristatic and bactericidal principles may be induced (Walters & Ratcliffe 1983; see Section 7.3). In accordance with this, responses against foreign objects are generally considered to lack the high degree of specificity and memory shown by advanced vertebrates. Claims to the contrary are based entirely upon studies of allograft rejection (see Hildemann, Bigger & Johnston 1979) in which the complexity of responses provoked puts extremely rigid demands on objectivity in interpretation as well as technical skills. As an example, the original claims of specificity and memory in second set xenograft rejections by earthworms (Valembois 1974; Lemmi *et al* 1974; Hostetter & Cooper 1972, 1974; Cooper 1975) have not been confirmed by later studies (Parry 1978; Dales 1978[a,b]) and the supporting arguments are open to speculation; later studies suggest that the

responding coelomic cells form a developmental series to phagocytes (Stein *et al* 1977; Linthicum *et al* 1977; Lemmi 1982), contradicting previous assumptions of lymphocyte analogy (Toupin & Lamoureaux 1976), and critical analysis of the original data indicates that the enhanced response may be due to increased proliferation of non-specific phagocytes at the time when the second graft was received. In fact, the response may be against dying tissue rather than foreignness (Parry 1978), and graft survival may thus arguably depend partly upon the suitability of the host tissue for the donor tissue, which may be expected to vary with the degree of relatedness (cf. Lackie 1979) as well as the tissue type.

In addition to phagocytosis, cell-mediated cytolysis (CMC) by specific (T_c/K) or non-specific cells (NK) forms an important effector mechanism in vertebrates. Cells with superficial recemblance to lymphocytes occur in many invertebrates, but clear differences exist (Valembois & Boiledieu 1980; Anderson 1981[b]) since even in tunicates, they are found to be progenitor cells, giving rise to other circulating cells (Freeman 1970; Warr *et al* 1977). An analogy to cytotoxic T-lymphocytes is also contradicted by studies indicating lack of specificity and memory in invertebrate responses to foreign objects (see above). The common reference to cytolytic cells in the destruction of grafts or other foreign materials does not appear to be based on sound evidence, and in fact, even comparison to non-specific NK-cells (see review by Savary & Lotzová 1986) remains speculative; although true contact lysis may be observed in some cases (e.g. Tyson & Jenkin 1974[b]), the lytic action may in other cases be due to release of lytic substances without the need for target adhesion (Yoshino & Tuan 1985; Wittke & Renwrantz 1984; Leippe & Renwrantz 1988). It remains to be demonstrated that cytotoxicity is mediated by cells distinct from phagocytes, and until evidence is produced to the contrary, it may even be attributed to the release of factors during phagocytosis. It seems reasonable to expect the response towards grafts to resemble that shown towards parasites, in which phagocytosis constitutes the only well characterized effector mechanism (Bayne 1982). Further studies thus appear warranted for positive demonstration of the involvement of CMC in invertebrate responses.

The importance of phagocytosis in invertebrate defense responses has mainly been questioned on basis of studies indicating higly discriminative elimination of molecules and viruses (Teague & Friou 1964; Stewart & Foley 1969), considered to correlate with the degree of foreignness (Chrichton & Lafferty 1975; Sloan *et al* 1975; McCumber & Clem

1977; McCumber *et al* 1979). The detection of markers in the seawater surrounding the animal however suggests that filtration rather than immune recognition may be involved, and this is supported by recent studies which demonstrate that this kind of elimination depends largely upon the molecular size of the marker (Mullainadhan *et al* 1983, 1984). In fact, the presence of proteolytic enzymes in the haemolymph of many invertebrates (e.g. Tuckova *et al* 1986) also opens the possibility of enzyme degradation. The general importance of phagocytosis in cellular responses is emphasized by all studies in which the effector cells have been characterized. The most important effector cells are circulating amoebocytes, i.e. haemocytes or coelomocytes, but the number of different types and the relative contribution of each to the overall defense differs between taxonomic groups (Ratcliffe & Rowley 1981). Fixed or non-circulating phagocytes also occur, at least in arthropods (Reade 1968, Cornick & Stewart 1968[a]; Johnson *et al* 1981; Johnson 1987) and advanced molluscs (Section 5). Let us now look more closely at the main features of phagocytic cells, and the events occurring during phagocytosis.

(II) PHAGOCYTOSIS. The anti-microbial activity of phagocytes can conveniently be divided into 8 stages (modified after Bridges & Valdimarsson 1985); (a) proliferation and development, (b) recruitment and margination, (c) migration, (d) activation, (e) attachment, (f) engulfment, (g) killing and (h) digestion. Factors in the circulating fluid may assist by mediating directional migration (chemotactic factors), or act as ligands between the antigen and special receptors at the phagocyte surface (opsonins). In vertebrates, serum factors may also be required for succesful activation of the killing mechanism (Bridges & Valdimarsson 1985). Most aspects of phagocyte behaviour are much less well studied among invertebrates, but a brief synopsis of the current knowledge will be presented, paying special attention to gastropods. Encapsulation will not be discussed, as it is considered to represent a special case of phagocytosis (Bang 1973[b]; Hostetter & Cooper 1972; Wago 1982[b]).

(a) **Proliferation and development.** The haemocyte levels of marine invertebrates appear to be higher than those of terrestrial forms; in pulmonates, this figure is commonly in the range of 0.1-1 million/ml, but 10x higher in marine gastropods and bivalves (Sminia 1981; Cheng 1981), as well as in decapods (McKay *et al* 1969; Paterson & Stewart 1974). Gastropod haemocytes proliferate by mitotic division of circulating stem cells or

possibly even mature haemocytes (Brown & Brown 1965; Sminia 1974, 1981; Sminia, van der Knaap & van Asselt 1983; Ottaviani 1983) but stem cells may also reside in the connective tissue. The proliferation of haemocytes may be enhanced upon stimulation, and is especially prominent in the mantle-kidney region of planorbids (Lie *et al* 1975[b], 1976; Jeong *et al* 1983; Joky *et al* 1983; Sullivan *et al* 1984) and other pulmonates (Rondelaud *et al* 1981) after trematode infection, hence considered by many to compose a primitive amoebocyte producing organ (APO). The white body of cephalopods (Stuart 1968; Cowden 1972; Cowden & Curtis 1981) is the only known case of a fully developed haemopoietic organ within Mollusca, but in crustaceans, haemocytes are formed in nodules in the foregut wall (Ghiretti-Magaldi *et al* 1977; Johnson *et al* 1981); as in molluscs, the ability to divide is retained after release in blood. The heart and associated vessels and tissues is the main site of blood cell formation in other arthropods, but the coelomocytes of lophophorates and annelid-related phyla are generally thought to originate in the peritoneum (see apropriate sections in Ratcliffe & Rowley 1981); the same applies to echinoderms, although the axial organ (VJ Smith 1981) or corresponding structures (AC Smith 1978) are also implicated. The wall of the intestine serves as a haemopoietic organ in tunicates (Ermak 1976, Wright 1981) as well as primitive vertebrates (Rijkers 1981); the importance of the spleen and to some extent also the thymus and the gut associated lymphoid tissue in this respect is seen in ontogeny as well as phylogeny of terrestrial vertebrates, although in adult life, bone marrow becomes the main site of haemopoiesis in terrestrial species.

The available information on the development of molluscan haemocytes indicates that in most cases, a maturation series of cells ranging from stem cells to mature haemocytes are present in the circulating fluid (Sminia, van der Knaap & van Asselt 1983). In other cases the situation may be complicated by the presence of haemopoietic tissues (see above), and the presence of two distinct cell lines, apparently developing from a common precursor cell, is indicated in bivalves and perhaps some gastropods (Brown & Brown 1965; Sminia 1981; Cheng 1981). This situation is also observed in other invertebrates (Ratcliffe & Rowley 1981), with the number of different haemocyte types varying; as many as 6 different forms are described from insects (Wago 1982[b]). In decapods, haemopoiesis also gives rise to cyanoblasts (Fahrenbach 1970) and fixed phagocytes surrounding hepatic arterioles (Johnson *et al* 1981; Johnson 1987). The relationship between circulating and tissue resident phagocytes of pulmonates (Sminia, van

der Knaap & Kroese 1979) may be similar, or even closer (see Section 5). The description of haemocyte developmental stages is apparently limited to a few morphological studies with bivalves (Mix 1976, Moore & Lowe 1977, Cheng 1981) and crustaceans (Johnson *et al* 1981); in the latter case, it is suggested that the formation of cytoplasmic granules during development results in decreased phagocytic powers, but the reverse is demonstrated in bivalves (Cheng 1981). The morphology and biochemistry of invertebrate haemocytes appears to resemble that of vertebrate macrophages (Anderson 1981[b]; Sminia, van der Knaap & van Asselt 1983).

(b) **Mobilization and margination.** In vertebrates, humoral inflammatory factors cause the mobilization of PMNs from bone marrow and their margination in areas of inflammation (Fearon & Wong 1983). Leucocytosis is commonly observed in invertebrates after stimulation (Cheng 1966; Abdul-Salaam & Michelson 1980[a]), but the relative importance of cell division and release of pre-existing tissue pools has not been investigated. An initial reduction in haemocyte numbers after challenge with foreign materials is commonly observed in studies on molluscs (Bayne & Kime 1970; Pauley *et al* 1971[b]); it appears to be due to haemocyte aggregation (Bang 1961) and adherence to blood vessels (Renwrantz *et al* 1981; van der Knaap, Sminia *et al* 1981). A similar response is formed at decreased temperatures (Feng & Feng 1974). This point merits further attention, as accurate estimates of absolute haemocyte numbers can not be made until the relationship between circulating and tissue associated haemocytes under different conditions is better understood. A correct assessment of the contribution of circulating cells towards the clearance of injected markers (e.g. Bayne 1974) can also not be made in the absence of data on the relative proportion of phagocytic and non-phagocytic cells leaving the circulation. The accumulation of particle-laden haemocytes in capillary and lacunar networks is a well known phenomenon in chelicerates and decapods, and may result in reduced haemocyte numbers (Maynard 1960; Bang 1956; Cornick & Stewart 1968[a]; McKay *et al* 1969; Stagner & Redmond 1975; Paterson *et al* 1976; Smith & Ratcliffe 1980[a]). That this is due to increased adherence of haemocytes to the walls of vascular channels has been amply demonstrated (Cornick & Stewart 1968[a]; Smith & Ratcliffe 1980[b]; Johnson *et al* 1981). Increased haemocyte aggregation is commonly also seen (Smith & Ratcliffe 1980[a,b]; Johnson *et al* 1981; Unestam & Nyhlén 1974), implying a general increase in cell adhesiveness. It has been suggested that cellular aggregation

around injected particles, followed by tissue deposition may be the initial response of crustacean haemocytes towards injected materials, phagocytosis taking place later (Smith & Ratcliffe 1980[a]). The low (2-10%) ingestion values obtained with crustacean haemocytes *in vitro* (McKay & Jenkin 1970[a]; Paterson & Stewart 1974; Smith & Ratcliffe 1978) may thus be explained partly by the inability to mimick these conditions. Reduction in numbers of circulating cells and appearance of cellular aggregates containing marker has also been observed in molluscs (above; also see Section 4 of the current thesis), insects (Gagen & Ratcliffe 1976), polychaetes (Fitzgerald & Ratcliffe 1983) and echinoderms (Dybas & Fankboner 1986).

(c) **Migration and chemotaxis.** The migration of phagocytes towards their target is in vertebrates accomplished by means of subplasmalemmal actin and myosin (Valerius *et al* 1981; Ryder *et al* 1984), or actin alone (Wright & Silverstein 1986). A similar mechanism appears to be responsible for capping (Alberts *et al* 1984, p. 278-9) and phagocytosis (see below). Studies on phagocyte locomotion in invertebrates are few and far between, but the involvement of microfilaments has been confirmed for *Limulus* (Armstrong 1979) and *Patella* haemocytes (Partridge & Davies 1974; Jones & Partridge 1974, Jones *et al* 1976). The latter studies also emphasize the similarity between spreading on glass, haemocyte aggregation and migration. Studies on capping in earthworm (Roch & Valembois 1978), pulmonate (Yoshino 1981[a,b], 1982) and oyster haemocytes (Yoshino & Davies 1983) have not been extended to reveal the involvement of microfilaments and microtubules; in the oyster however, capping depends on cellular metabolism and is thus an active phenomenon (Yoshino *et al* 1979).

Vertebrate phagocytes move in response to chemokinetic and chemotactic factors (e.g. C5a, fMLP, PAF, LTB_4) released from inflammatory sites or cell damage (Gallin & Seligmann 1984; Hugli & Morgan 1984). Chemotaxis has been studied in the primitive prosobranch *Viviparus malleatus* and the oyster, *Crassostrea virginica*. The migration of *Viviparus* haemocytes is influenced by the interaction of a serum derived factor with heat-killed *St. aureus*, and the current evidence points to one of the two lectins detected in the serum of this species; the exact mechanism, and the identity of the corresponding surface receptors is not known, but the author suggests that chemotactic factors are released from haemocytes upon contact with a lectin-covered microbe. In fact the binding of lectin to its monosaccharide determinant appears to be sufficient for the

response to occur (Schmid 1975). The serum of the oyster apparently contains no chemotactic substances; haemocytes migrate in response to a substance released by certain bacteria (Cheng & Rudo 1976[a], Cheng & Howland 1979) or parasites (Cheng 1966, Cheng *et al* 1974). The ability of haemocytes to respond to this factor is reduced after 'vaccination' (Cheng, Bui *et al* 1981), indicating loss (or blocking) of surface receptors or serum derived helper factors, or the recruitment of immature blood cells to replace mature haemocytes laden with bacteria. The main factors implicated are bacterial cell wall or envelope proteins of 10 kd, lacking carbohydrate moieties, but soluble factors may also be involved (Howland & Cheng 1982). Earthworm coelomocytes are attracted to foreign grafts by factors which are smaller than 10 kd (Marks *et al* 1979). Insect haemocytes are often seen to attract to melanin-related substances deposited on foreign surfaces by granular cells (Ratcliffe & Gagen 1977) suggesting chemotaxis in response to factors of the prophenoloxidase system (Söderhäll 1982).

In addition to serum derived factors and metabolites from the intruders, directional migration of haemocytes is *in vivo* probably assisted also by the release of chemotactic substances from damaged host cells and moribund parasite tissues (Font 1980). The accumulation of crayfish haemocytes to the exposed epidermis of areas which have had the integument removed (Unestam & Nyhlén 1974) has been attributed to osmotic factors (Nyhlén & Unestam 1980). Haemocytes are also attracted to hyphae of the fungus *Aphanomyces astacii* as soon as the integument has been penetrated, but the relative contribution of each stimulus, i.e. tissue damage and microbial invasion has not been resolved.

(d) **Activation.** Vertebrate mononuclear phagocytes may exist in four states of activation, depending upon state of maturation, regulation by lymphokines and experience with endocytosis (Nathan & Tsunawaki 1986). PMNs can also be activated by challenge (Wandall 1982; Brummer *et al* 1984). Invertebrate phagocytes appear to be active as soon as they are mature, but an extra activation step has also been observed; LPS and prolonged glass adherence cause a marked increase in phagocytosis by lobster haemocytes, thought to be due to the recruitment of a quiescent precursor population and an increased number of opsonin binding sites (Goldenberg et al 1984). This confirms earlier indications on haemocyte activation upon challenge (e.g. McKay & Jenkin 1970[c]), and suggests that activation may be a contributing factor in other known cases of enhanced secondary

reactions in invertebrates (see above discussion), in addition to cellular proliferation. In vertebrates, prolonged macrophage activity may result in cell fusion (Werb 1987). A similar phenomenon has been noted in echinoid coelomocytes after *in vitro* incubation (Bertheussen & Seljelid 1978), and multinucleate giant cells have been reported from various invertebrates (Sparks & Pauley 1964; Cooper 1968; Cheng & Galloway 1970; Fontaine & Lightner 1975); however, the origin or significance of these cells is not known, and the results are open to speculation.

(e) **Attachment.** Adhesion of particles to membrane receptors (with or without opsonin involvement) is a physical reaction in vertebrates, unlike ingestion which is an active process, abolished by metabolic inhibitors or at low temperatures (Wright & Silverstein 1986). The difference between the attachment and the ingestive phase may however not be revealed in all test systems, as microfilaments appear to be involved in the attachment of some particles (e.g. untreated RBC, Anderson 1981[b]). Observations on echinoid (Bertheussen 1981[b]) and insect haemocytes (Anderson 1977; Wago 1982[a,c], 1983) reveal similarity to vertebrate leucocytes in their sensitivity to metabolic inhibitors, microfilament-affecting agents and decreased temperatures for attachment of untreated RBC, but as in vertebrates, the attachment stage is not strictly temperature dependent (Wago 1983). The involvement of microfilaments is explained in terms of receptor redistribution (Bertheussen 1981[b]) or filopodial elongation (Wago 1982[b]).

Invertebrates are generally considered to lack the classical vertebrate opsonins (IgG, C3b, iC3b) and their receptors ($Fc_{\gamma}R$, CR1, CR3) but phagocytosis is commonly enhanced by serum adsorbtion of the foreign particles. The current evidence (reviewed in Sections 5.5 & 7.3) supports that lectins are commonly, perhaps even universally responsible for the observed effect. It should be noted, however, that opsonic involvement of lectins may not be shown unless the glycocalyx of the particles used contains suitable carbohydrate types in a terminal position, and so, the presence of lectins in serum does not guarantee opsonization in all experimental models. Moreover, a weak affinity of a lectin towards a given test-particle may not be detected in agglutinating studies although it may conceivably provide opsonization. In insects, filopodial elongation is enhanced by the action of a humoral factor, acting directly on the haemocyte, but the adhesive properties of insect plasmatocytes and granulocytes are in general non-specific (Wago 1983) and lectins and other serum factors provide no opsonization (Rowley &

Ratcliffe 1980). The involvement of cytophilic lectins can however not be excluded (Tyson & Jenkin 1974[a]), and lectins are known to be secreted by haemocytes (Amirante & Mazzalai 1978). That receptors are involved is supported by sensitivity to trypsin (Scott 1971).

(f) **Engulfment.** In vertebrates, engulfment is a metabolic event, requiring energy from glycolysis. Studies on receptor movement in the membrane bilayer support that sub-plasmalemmal microfilaments play a part in the process in a similar fashion as suggested for locomotion and capping (see above). IgG-coated particles are engulfed by the sequential, circumferential attachment of membrane bound receptors to determinants on the particle surface, resulting in the formation of cytoplasmic extensions which fuse on the distal side of the particle (Wright & Silverstein 1986) whereas C3-coated particles sink into the cytoplasm without the requirement for full circumferential attachment (Kaplan 1977). The same apparently applies to the ingestion of C3b-coated particles by sea urchin coelomocytes (Kaplan & Bertheussen 1977). In insects, bacterial phagocytosis by plasmatocytes may occur by pseudopod formation, but uptake of larger particles (e.g. cell debris) by invagination of the cell membrane (Ratcliffe & Rowley 1974). RBC ingestion by insect granulocytes occurs by filopodial elongation and extension of veil-like membrane processes (Wago 1983). Particles have also been observed to attach to filopodia and slide along the filopodium to the cell body before being internalized (Bang 1961). Phagocytosis of lectin-treated particles appears to proceed by a rather unusual mechanism in bivalves (Renwrantz *et al* 1979) and planorbids (Schoenberg & Cheng 1980); the haemocyte extends a 'funnel'-shaped pseudopod into the direction of the particle, and the particle slides through the funnel into the cytoplasm. Attachment of particles with high lectin determinant-density (R-RBC) appears to involve similar morphological changes in periwinkle haemocytes (Plate 3.1[g]). Phagocytosis may thus proceed in various ways in invertebrate haemocytes, but nothing is known about the receptors or the signals involved. As in vertebrate models, glycolysis supplies energy for the engulfment stage in the snail, *Biomphalaria glabrata* (Abdul-Salam et al 1980[a]), the clam, *Mercenaria mercenaria* (Cheng 1976), the cockroach, *Blaberus craniifer* (Anderson et al 1973[b], Anderson 1975) and the sea urchin, *Strongylocentrotus droebachensis* (Bertheussen 1981[b]).

(g) **Killing.** Ligation of chemotactic receptors (e.g. C5aR, fMLP-R, PAF-R,

LTB4-R) and certain opsonic receptors (FcR) induces the release of toxic oxygen radicals from vertebrate phagocytes (Sklar 1988). This step is separate from the digestive process, and situated at the plasma membrane; it is marked by increased oxygen consumption and activation of the HMS pathway. Myeloperoxidase (MPO), delivered by the fusion of lysosomes to the phagosome (after ligation of opsonic receptors) may act in conjunction with hydrogen peroxide, iodide and ferric compounds to kill the microbe (Sugar *et al* 1984; McPhail & Snyderman 1984). Others however observe low oxygen requirements for killing (Vel *et al* 1984; Dr. I Kramer, pers. comm.) and according to an alternative theory, killing is mediated by lysosomal cationic proteins, at pH conditions provided by the respiratory burst (Segal et al 1981). Recent studies confirm the potency of cationic proteins in killing Gram(-) bacteria at low pH (Shafer et al 1986). The absence of MPO from insect (Anderson et al 1973b) and bivalve (Cheng 1976) haemocytes has been taken to suggest closer relationship to macrophages than PMNs (Anderson 1981b). Since this time, however, MPO has been detected in sipunculid (Dybas 1981) and pulmonate haemocytes (Carter & Bogitch 1975; Sminia & Barendsen 1980) although not universally occurring, as it is lacking from the fixed phagocytes of Lymnaea (Sminia, van der Knaap & Kroese 1979) as well as from haemocytes of the only Stylommatophoran species tested (Sminia, van der Knaap & Barendsen 1982). Recent studies on clams (Nakamura, Mori et al 1985) and pulmonates (Dikkeboom, Tijnagel et al 1987; Shozawa 1986, see Sminia & van der Knaap 1987) also demonstrate an increased oxygen consumption and the production and release of toxic oxygen intermediates, in contrast to the original studies on insects and clams (Anderson et al 1973[b]; Cheng 1976). A detailed comparison of the killing process in vertebrate and invertebrate leucocytes should include a full assessment of the contribution of cationic proteins to the process in different taxonomic groups.

(h) **Digestion.** Concomitant with the phagosome formation, the granules of vertebrate PMNs fuse with the phagosomal membrane and discharge their contents into the vacuolar space (Goldstein 1984). Granule fusion occurs sequentially, and secretion of lysozyme and lactoferrin attests to the earlier and perhaps more widespread release of specific granule contents (Baggiolini & Dewald 1984). Particles too large for ingestion apparently induce the same response, resulting in lysosomal discharge due to the fusion of granules to the forming 'phagosome' (Weissman *et al* 1971). Specific granules have not been reported from invertebrate haemocytes, and digestion is probably mediated by

lysomes alone (Eble & Tripp 1969; Dybas 1981). Bacterial degradation by invertebrate haemocytes has been studied by following the incorporation of bacterial end products into cellular products in insects (Anderson et al 1973[b]) and bivalves (Cheng & Rudo 1976[b]). Glycogen, synthesized from sugar of bacterial origin becomes distributed to all tissues following its release from haemocytes. The release of lysosomal enzymes during the ingestive phase has been noted by several authors working with bivalves and pulmonates (Michelson & Dubois 1973; Douglass & Haskin 1976; Cooper-Willis 1979; see review by Cheng 1986). This is known to occur by degranulation (Foley & Cheng 1977), even by extrusion (budding) of entire lysosomes surrounded by plasma membrane (Mohandas *et al* 1985). Formation of unsealed vacuoles, as noted in the present study (Plate 5.9[b]) and in vertebrate PMNs (Cech & Lehrer 1984) may also arguably contribute to the escape of lysosomal enzymes during phagocytosis. The defensive value of lysosomal discharge is uncertain; the only known microbe-specific enzyme of bivalve lysosomes is lysozyme, which is active only against a few Gram(+) bacteria (Young & Cohn 1987). The positive effects of lysozyme have to be balanced against tissue damage due to other lysosomal enzymes. Further studies are needed to test whether chemotactic stimuli are formed in the process, as has been suggested for vertebrate phagocytes (Wilkinson & Bradley 1981). Lysosomal discharge may be an advantage against parasites and other non-phagocytosable objects (Harris 1975; Bayne 1982), and the formation of haemocytic capsules may serve to confine enzymatic activity to the interface between parasites and the surrounding cells.

(III) PHAGOCYTIC DEFECTS. The destructive action of phagocytes may of course be evaded by special adaptations of pathogens, which may operate at any step in the phagocytic process. Most however appear to operate at the killing stage, the most striking example of which is perhaps the infection of *Aerococcus viridans* var. *homari* in the lobster (Johnson *et al* 1981). This bacterium is phagocytosed by fixed phagocytes as well as circulating haemocytes, but survives, and multiplies within haemocytes, causing aggregation and the formation of emboli which may block arteries. Bacteraemia is first observed 4-6 day after infection, and death ensues in 10 more days; the lethal dose is as low as 4-5 bacteria per individual. Infectivity is apparently associated with the presence of an acid mucopolysaccharide in the capsule (Kenne *et al* 1976). Similar examples of evasive adaptations to the killing mechanism are found in other combinations of invertebrates and pathogens, e.g. *Minchinia* infection in the oyster, *Crassostrea* (Farley

1968), and bacterial infection in the cockroach, *Blaberus craniifer* (Anderson *et al* 1973[a]) and the sea-hare, *Aplysia californica* (Pauley et al 1971[b]). In vertebrates, the intracellular lysis of RBC (as well as fast ingestion) depends upon activation of CR3 (Rothlein & Springer 1985), but nothing is known of the receptors or signal pathways responsible for activation of the killing mechanism in invertebrates. Perhaps a clue may be provided by the inverse correlation of lectin activity and susceptibility in some of the above examples (Cornick & Stewart 1968[a,b], 1975; Pauley et al 1971[b]). This has also been noted in *Paranophrys* infection of the crab, *Carcinus maenas* (Bang 1962), but in this case, ingestion as well as killing may be affected by the absence of lectins, as the ciliate actively destroys haemocytes.

The relationship of parasites with their natural hosts may be further complicated by specific evasive mechanisms, e.g. the adsorbtion of host molecules or production of cross-reactive epitopes (Bayne & Stephens 1983; Bayne *et al* 1987; Yoshino & Cheng 1978, Yoshino *et al* 1977). Release of factors with inhibitory effects on phagocytes has also been suggested (Abdul-Salaam *et al* 1980[a]), and the 'attenuating' effects of gamma-radiation on the the parasite (Lie *et al* 1975[a]; Michelson & DuBois 1981) may be taken to indicate decreased ability to produce this factor. The resistance to even untreated parasites induced by 'vaccination' with irradiated parasites is difficult to explain, but it is worth noting that the inability of haemocytes from susceptible snails to phagocytose may be reversed by adding serum factors from resistant snails (Bayne *et al* 1980[a,b]; Loker *et al* 1982), and the suggestion that this is an opsonizing lectin (Bayne 1982) has received some confirmation (Bayne *et al* 1985); the phagocytosis-inhibitory factor may thus conceivably be of carbohydrate or glycoprotein nature, with the ability to bind protective factors like soluble and/or integral lectins and block their binding sites.

In summary, phagocytosis appears to be the main effector mechanism against microbial or parasite invasion in the invertebrates. Humoral factors assist the phagocytes by opsonizing the intruders, and chemotactic factors may also occur. Lectins are the main accessory molecules implicated, except in arthropods, which possess a system involving prophenoloxidase and interacting molecules. Phagocytosis may also be aided by other effector mechanisms; in arthropods, pathogens escaping from phagocyte capsules may thus be killed by humoral factors released from the fat body at later stages of infection. The bacteristatic and bacterilytic factors (e.g. cecropins, attacins and lysozyme, the last of

which may be more widely distributed in the invertebrates) may also cause rapid destruction of pathogens upon a secondary infection, assisted by an increase in cellular activity (phagocyte proliferation and activation). The latter appears to be the main factor amplifying secondary responses in non-arthropod invertebrates, although antibacterial factors also occur in sipunculids and annelids. Of course, invertebrates constitute a polyphyletic group, the diversity of which precludes all but the most cautious generalizations to be made, and the overall scheme outlined here should thus be regarded as a working hypothesis rather than a rigid statement. Studies on invertebrate defense mechanisms are becoming increasingly sophisticated with the continuing adaptation of modern techniques to experiments in the field. Centrifugal elutriation of phagocytes and studies on their membrane constituents as well as the intracellular signals induced by ligand binding have now become feasible, and with modern recombination techniques, even direct comparison on the relationship of invertebrate receptors and ligands to vertebrate counterparts may be in sight. The questions surrounding the internal defense system of invertebrates, including the prosobranch *Littorina littorea* remain countless in spite of some advances made. It is the author's hope that they will continue to inspire further work in the field.

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