THE ROLE OF THE ENVELOPE OF *MONILIFORMIS MONILIFORMIS* IN IMMUNE EVASION

A thesis presented for the degree of

DOCTOR OF PHILOSOPHY

by

VINCENT O'BRIEN, B.Sc.

Department of Biochemistry,

University of Glasgow,

January, 1988.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Acknowledgements

I would like to express my thanks to the following:

Prof. Smellie (Biochemistry) and Prof. Phillips (Zoology) for the provision of laboratory space and facilities during my stay at the University.

The Science and Engineering Research Council for financial support.

My supervisors, Drs. John Kusel and Ann Lackie, for their guidance, enthusiasm, and most of all for their friendship and understanding.

Drs. Andy McGregor, Les Fixter, John Beeley and Max Huxham for useful discussions and sound advice.

Tom Downie for cutting, sectioning and photography of EM samples.

Karen and Alan of the Medical Illustration Unit for the production of some of the figures in this thesis.

Trevor Graham and the staff of the University Photographic Unit for the production of most of the Figures in this thesis.

Liz Denton, Liz Currie and Mary Robertson for help, instruction and advice in the production of some of the figures in this thesis.

A special thanks to Mrs. Anne Mosson for her speed, skill and patience when typing this manuscript.

Janet Jones and Martina Scallan for reading parts of the manuscript.

All the members of Lab F62; Liz, Rohan, Max, Saskia, Margaret and Gwenda for their good cheer.

Finally, I would like to thank all the folk in Lab C15, especially Mary, Joyce, Ke-ying, Janet, the two Johns, Anne and Lorna for their friendship and good humour which has made my stay in the Biochemistry Department most enjoyable.

ABBREVIATIONS

The abbreviations used in this thesis are those recommended in the Instructions To Authors of the Biochemical Society (1986), with the following additions :

A_{492}	Absorbance readings at 492nm.
BM	Basement membrane.
BPB	Bromophenol blue.
BSA	Bovine serum albumin.
C_{18}AF	5-N-(octadecanoyl)-aminofluorescein.
C_{16}diI	1,1-di(hexadecyl)-3,3,3',3'-tetramethylindocarbocyanine perchlorate.
C_{18}diI	1,1-di(octadecyl)-3,3,3',3'-tetramethylindocarbocyanine perchlorate.
Con A	Concanavalin A.
CS	Chondroitin sulphate.
DBA	Dolichos biflorus agglutinin.
DMB	1,9-dimethylmethylene blue.
EDTA	Ethylenediamine tetra-acetic acid (di-sodium salt).
ELISA	Enzyme linked immunosorbent assay.
a-Env	Rabbit anti-whole cystacanth envelope antiserum.
FCS	Foetal calf serum.
FITC	Fluorescein isothiocyanate.
FRAP	Fluorescence recovery after photobleaching.
GAG	Glycosaminoglycan.
sGAG	Sulphated glycosaminoglycan.
HA	Hyaluronic acid.
HBS	HEPES-buffered insect saline.
a-HC	Rabbit anti-haemocytically encapsulated Sepharose beads antiserum.

HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid.
HP	Heparin.
HRP	Horse radish peroxidase.
HS	Heparan sulphate.
IFAT	Indirect fluorescent antibody test.
LPS	Lipopolysaccharide.
a-LS	Rabbit anti-Schistocerca gregaria serum antiserum.
Mc540	Merocyanin 540.
2-ME	2-mercaptoethanol.
M_r	Relative molecular weight.
NP40	Nonidet P40.
NRS	Normal rabbit serum.
PAGE	Polyacrylamide gel electrophoresis.
PBS	Phosphate buffered saline.
PG	Proteoglycan.
PMSF	Phenyl methyl sulphonyl fluoride.
PNA	Peanut agglutinin.
PO	Phenoloxidase.
proPO	Prophenoloxidase.
a-PS	Rabbit anti-Periplaneta americana serum antiserum.
R_f	Relative mobility.
SDS	Sodium dodecyl sulphate.
TCA	Trichloroacetic acid.
TEM	Transmission electron microscopy.
TEMED	N,N,N',N',-tetramethylenediamine.
TLC	Thin-layer chromatography.
TLCK	N-tosyl-L-Lysine chloromethyl ketone.

TPCK	L-1-tosylamide-2-ethylchloromethyl ketone.
WGA	Wheat germ agglutinin.

Contents

List of Figures

List of Tables

Summary

The biochemical composition of the envelope surrounding cystacanth larvae of Moniliformis moniliformis (Acanthocephala) was investigated in an attempt to understand how this structure protects developing larvae in the hostile environment of the haemocoele of the intermediate cockroach host Periplaneta americana.

The results have shown that the envelope has a complex composition and contains six lipid species, at least 25 polypeptides and a glycosaminoglycan/proteoglycan-like molecule. The GAG-like molecule may be present at the outer surface of the envelope.

The lipid composition is substantial and represents about 25% of the dry weight of the cystacanth envelope. At least six lipid species have been detected and three have been tentatively identified as phosphatidyl choline, phosphatidyl ethanolamine and cholesterol.

Envelope proteins have been extracted and analysed by SDS-PAGE, and have been shown by lectin overlay to be glycoproteins. The molecular weights of those glycoproteins range from about 30 to > 300Kd. One of the envelope proteins has been identified as a collagen, and a similar molecule is also present in extracts of larval bodies. Thus the envelope collagen is apparently parasite derived.

There is extensive cross-reactivity between epitopes present on envelope glycoproteins and glycoproteins from cell and plasma fractions of the blood (haemolymph) of the insect host. The cross-reactivity can, however, be attributed to carbohydrate epitopes present on the parasite and host molecules. Whether these shared epitopes are significant in the protective mechanism of the envelope or have some completely different

function is not yet known. It has, however, been demonstrated that the host lipoprotein, lipophorin, is intimately associated with the cystacanth envelope.

Two models have been proposed to explain the protective function of the envelope. One common theme of both models is that adsorption of lipophorin to the envelope surface in a native conformation is critical in the prevention of haemocyte recognition of the developing enveloped larvae.

Chapter 1

General Introduction

1. General Introduction

1.1. Introduction

All multicellular organisms have the ability to distinguish between "self" and "non-self" tissues; this is important during the reproductive process and for responding to parasitic infections.

Vertebrates have, characteristically, adaptive or acquired immune responses which demonstrate specific recognition of "non-self" with a memory component such that the organism can react more rapidly and efficiently to a secondary infection. In addition to specific responses, vertebrates also have non-specific or innate mechanisms which limit infection, for example, the dermis, and the mucous secretions that line the alimentary and genital tracts, provide a limited barrier to microbial entry into epithelial cells, while blood clotting mechanisms serve to limit the spread of infection at a wound site. After infection, other mechanisms, including complement activation and phagocytosis, limit the spread of microorganisms through the tissues. (Taussig, 1984; Roitt, 1984).

Invertebrates do not appear to have adaptive or acquired immune mechanisms similar to those present in vertebrates; immunoglobulins and lymphocytes are absent from the blood of these animals. However, they do have efficient mechanisms for protection against microorganism infection. For example, insects are passively protected against invasion by foreign organisms; the chitinous exoskeleton completely covers the outside of the body as well as the foregut, hindgut and tracheal tubes (Wigglesworth, 1984). No epithelia are directly exposed to invaders, even the intestinal epithelium of the midgut in most insects is guarded by the peritrophic membrane that encloses the gut contents (Wigglesworth, 1984).

However, insects are prone to infection by a number of organisms including bacteria, protozoa, fungi and various helminth species which live in the animal's body cavity (haemocoele) and tissues (Salt, 1963, 1970). To deal with these invading organisms insects have adopted a different strategy from the higher vertebrates based on non-specific stimulation of a number of different cell types by a variety of chemical and/or physicochemical factors that are present on the surface of, or released by intruders (Salt, 1970; Lackie, 1980). The blood cells (haemocytes), freely circulating in the insect's blood space, mediate the immune response by reactions such as phagocytosis (see section 1.3.2.) nodule formation (see section 1.3.3.) and encapsulation (see section 1.3.4.), while fat-body cells produce various humoral factors that are important in the overall response (section 1.2.). Haemocytes also take part in haemolymph (blood) coagulation (Bohn, 1986) which serves to limit the spread of infection at a wound site, and in the removal of toxic substances from the circulation (Salt, 1970). Generally the immune reactions of insects are very efficient and afford the animal ample protection against invading organisms. However, some parasites live in the haemocoele yet evade the cellular defence mechanisms of their hosts; their surfaces are not recognised as foreign and they either fail to elicit haemocyte adhesion or else produce substances which inhibit host responses. Larvae of the helminth Moniliformis moniliformis (Acanthocephala) evade cellular encapsulation in the American cockroach Periplaneta americana. The life-cycle of this parasite is easily maintained in rats and cockroaches both of which are robust experimental animals, thus making this a suitable host-parasite system with which to investigate the defence reactions of the host. One approach, which is the subject of this thesis, has been to investigate the biochemical composition of the non-cellular envelope

surrounding the developing larvae of Moniliformis; this structure represents the host-parasite interface and appears to be essential for the parasite's evasion of encapsulation. A knowledge of its constituents may lead to an understanding of why this parasite develops unmolested and so give a general indication of the properties of surfaces which do not elicit a haemocytic response.

In this general introduction, both humoral and cellular components of the insect immune system will be considered as well as some details of the possible mechanisms used by parasites to avoid recognition. Some aspects of the biology of Moniliformis are discussed including details of the structure and composition of the envelope and its possible role in protecting the developing larva from haemocytic attack in Periplaneta.

1.2. Humoral components

In this dissertation, humoral components will be considered to include any soluble factor present in cell-free haemolymph which has anti-microbial activity or may mediate haemocyte adhesion to foreign surfaces. The distinction between cellular and humoral responses may be artificial since it is possible that these soluble components may be actively synthesised and secreted by the blood cells or result from haemocyte lysis which occurs in response to wounding or contact with foreign objects (Ratcliffe, 1986). However, the distinction provides a convenient means for subdividing the immune response of insects.

1.2.1. Antibacterial proteins

When infected by bacteria some insects respond by synthesising de novo a set of proteins that accumulate in the haemolymph and produce a potent

antibacterial activity which is highly effective, if somewhat non-specific, against a number of bacterial species. Furthermore, continued synthesis of these proteins ensures that there is non-specific protection against a secondary infection with non-pathogenic or pathogenic bacteria up to two weeks after the primary infection, depending on the insect species (Boman et al., 1981; Boman and Hultmark, 1987). The induction of antibacterial activity is best characterised in various moths (Lepidoptera). In the silk moth, Hyalophora cecropia, three major classes of inducible, antibacterial proteins have been found (Boman, 1986) although it has recently been demonstrated that they can also be synthesised by haemocytes of Hyalophora cecropia in vitro (Trenczek , 1986). Recent evidence has shown that soluble peptidoglycan fragments from the cell walls of Micrococcus luteus will stimulate antibacterial protein synthesis by fat body from Manduca sexta larvae in vitro (Dunn et al., 1985). Whether peptidoglycan fragments also stimulate antibacterial production in Manduca sexta and other insects in vivo is not yet known. The first class of antibacterial proteins, the cecropins, are a family of small basic bacteriolytic peptides with molecular weights around 4000 which appear to effect bacterial membranes and cause lysis of a wide range of gram-positive and -negative bacteria but have no effect on eukaryotic cells. They have been purified and sequenced (Steiner et al., 1981) and are the products of at least three separate genes. Amino acid sequence data has revealed a lysine-rich N-terminal region in these molecules which is highly conserved among the five different cecropins. This region has the potential to form an amphipathic alpha-helix which may suggest an ionophore activity for these molecules similar to that of some transport antibiotics such as Gramicidin A (Krasne et al., 1971). As yet however, the mode of action for these molecules is not known (Boman and Hultmark, 1987).

The second set of proteins, the attacins, comprise six forms with molecular weights around 20,000 (Hultmark et al., 1982). They are the products of two genes and all six forms can be accounted for by secondary modifications of two precursor proteins which are the products of separate genes (Kockum et al., 1984). They appear to interact with the outer membrane of a variety of bacterial species and act in concert with the cecropins and lysozyme (Hultmark et al., 1983). Lysozyme (N-acetyl-muramidase; E.C.3.2.1.17) is also synthesised in these insects. This enzyme hydrolyses β1 - 4 glycosidic linkages between the monomers of the peptidoglycan that makes up part of the bacterial cell wall. Its main function may be to degrade the cell walls of bacteria killed by the other two sets of inducible proteins.

In addition to the antibacterial proteins, other proteins of unknown function are also induced by infection. Protein P4, the major component of the induced proteins in H. cecropia pupae, has been purified and sequenced. It has no antibacterial activity, but when protein P4 is injected into naive cecropia pupae it is capable of transiently switching on the synthesis of the other immune proteins (Anderson and Steiner, 1987). Also, in natural infections or after injection of purified P4, this protein accumulates in the haemolymph of the insect, suggesting that it is continually synthesised during infection and is not readily degraded by haemolymph proteinases. Immunofluorescence studies, using a monospecific antiserum directed against purified P4, have also demonstrated its accumulation in the fat body basal membrane, in certain haemocytes and in haemocytic nodules (section 1.3.3.) of infected larvae (Anderson and Steiner, 1987). This protein may play a role in regulating antibacterial protein synthesis or activity.

Inducible antibacterial proteins that cross-react with anti-cecropin antibodies have been found in the fruit fly Drosophila. Recently, the proteins responsible for the antibacterial activity have been isolated (Flyg et al., 1987). They appear to share biochemical properties with the inducible molecules found in the Lepidoptera. The powerful genetic and recombinant DNA techniques available for Drosophila should facilitate an investigation into the molecular mechanisms of the inducible antibacterial response in these insects.

A novel antibacterial peptide termed Diptericin A, has been found in the haemolymph of infected or injured larvae of the Dipteran Phormia terranova (Keppi et al., 1986). In response to injections of live bacteria or to injury, the fat body synthesises a family of antibacterial peptides, which are relatively small, heat-stable, basic proteins. They have a broad antibacterial spectrum against both gram-negative and gram-positive organisms. The amino acid compositions of the major diptericins have been determined; the results suggest that they are different from the cecropins and attacins of Lepidoptera (Keppi et al., 1986; Dimarcq et al., 1986) although they may function in a similar manner to these molecules.

Antibacterial proteins can be induced, in response to injury, in the haemolymph of flesh fly larvae Sarcophaga peregrina (Natori, 1977). One of the components of this inducible system has been purified, characterised and sequenced (Okada and Natori, 1983, 1985) and appears to be cecropin-like (Boman and Hultmark, 1987). Recent reports (Okada and Natori, 1984, 1985) suggest that this antibacterial protein acts as an ionophore when tested against E. coli. The protein is, however, ineffective at $0^{o}C$ (Okada and Natori, 1984) which excludes the possibility of it forming

membrane channels, since pore-forming (e.g. Gramicidin A) but not carrier ionophores (e.g. valinomycin), are effective at this temperature (Krasne et al., 1971). It is not yet known whether the cecropins have a similar ionophore activity.

The general occurrence of cecropin- and attacin-like molecules in insects is not yet established. These molecules appear only to be found in "higher" insects (i.e. the Holometabola which show complete metamorphosis through larva then pupa to adult) that include Lepidoptera (moths),Diptera (flies) and Coleoptera (beetles). They appear absent from the "lower" insects (i.e. the Hemimetabola in which metamorphosis is incomplete; the young, referred to as nymphs, resemble the adults in appearance and there is no striking morphological change during development as in the Holometabola) including the Dictyoptera (Cockroaches) and Orthoptera (Locusts). However, some sort of antibacterial activity is produced in Locusta migratoria (Lambert and Hoffmann, 1985) and in the bug Rhodnius prolixus (de Azambuja et al., 1986). Lysozyme appears to be a common component of the haemolymph of all insects studied and apparently increases in concentration after injection of microorganisms. However, in some cases it has been demonstrated that the enzymic activity is in fact a chitinase (E.C. 3.2.1.14.) which possesses some gratuitous lysozyme activity as a result of a similarity in the enzyme substrates (Boman and Hultmark, 1987). As such, then, this enzyme may be principally involved in tissue repair, digesting chitin fragments in the haemolymph after wounding of the cuticle, and may also play a role in normal insect morphogenesis.

1.2.2. The prophenoloxidase (proPO) system

The prophenoloxidase system comprises a phenoloxidase (E.C.1.10.3.1) and other enzymes and factors that are responsible for the initiation of melanin synthesis in insects. Melanin deposition is frequently observed to accompany the host cellular responses to wounds and parasitic or microbial invasion (Salt, 1970). Pye (1974) demonstrated that yeast, (*Saccharomyces cerevisiae*) or components of their cell walls, β1 - 3 glucans, initiated melanization reactions in insect haemolymph by activating prophenoloxidase, the key enzyme in the pathway. This lent support to Taylor's suggestion (Taylor, 1969) that activation of the prophenoloxidase pathway in insects and other arthropods, was a prerequisite for, rather than a consequence of, haemocyte adhesion to foreign objects. The quinones produced during enzymic action could also serve as the toxic agents required to bring about the intruder's death. The significance of the activating cascade to cellular responsiveness was enigmatic until Smith and Soderhall (1983) showed that elevated rates of phagocytosis by crayfish or crab (Crustacea) haemocytes *in vitro*, could be obtained by inclusion of β1 - 3 glucans in the haemocyte/bacteria mixtures. Furthermore, it is the proteins of the proPO cascade (but not PO itself) which appear to possess opsonic function when they attach to the foreign object. An opsonic role for proPO components in the insects has also been suggested recently by Ratcliffe, Leonard and Rowley (1984). These workers used haemocyte monolayers prepared from the haemolymph of the wax moth *Galleria mellonella* which were overlayed with *Baccilus cereus* and a solution of either laminarin (a β1 - 3 glucan) or *E. coli* endotoxin (lipopolysaccharide, LPS). There was a significant increase in the uptake of bacteria for both laminarin- and endotoxin-containing cultures. However, only the laminarin appeared to stimulate phenoloxidase activity.

The authors suggested, on the basis of this circumstantial evidence that proPO components had an opsonic role in insects similar to that suggested for crustaceans.

The generality of an opsonic role for proPO components in insects remains in question in view of the results obtained by Dularay and Lackie (1985). These workers have shown that the prophenoloxidase pathway in the locust Schistocerca gregaria can be partially activated by Ca^{2+} ions and strongly activated by a zymosan preparation that contains β1 - 3 glucans. They investigated whether components of a haemocyte lysate could adhere to negatively charged Sepharose beads, which were known, from previous work, to remain unencapsulated when injected into locusts (Lackie, 1983a). It was apparent from the results that, while the locust phenoloxidase and other components in the lysate did attach to the beads, they did not appear to have any opsonic function under the conditions used.

The authors suggested three possible explanations for their observations (i) a key factor for opsonic activity may not adhere to the beads under these conditions, (ii) the components may not adhere to the outer surface of the beads, thus making them inaccessible to the haemocytes or (iii) the activated proPO system of the locust possesses no opsonic activity. It should be noted, however, that the animal used in these studies has a demonstrated low acuity of non-self recognition (Lackie, 1981a) and may not be a representative model. Also, the crude zymosan preparation may contain other components which adhere and mask the opsonic factors or degrade or alter them so preventing enhanced phagocytosis.

Takle and Lackie (1986) have shown that components of the activated proPO pathway stimulate chemokinetic behaviour of haemocytes from cockroaches in vitro. The proPO components may be involved in cell-cell

interactions causing a general stimulation of the locomotary behaviour of cells such that there is an increased chance of a "random collision" between cell and invader. This might enhance the clearance of foreign objects by phagocytosis and nodule formation/encapsulation, although such a system could only operate where haemocytes were adherent to a substratum, such as the connective tissue layer covering the organs in the haemocoele, and may be important in recruitment of cells to wound sites.

It appears, then, that elements of the proPO system may play a role in phagocytosis and as mediators of haemocyte adherence, and in nodule/capsule formation. The current experimental evidence suggests that the reactions are part of a response to recognition and do not describe the initial events in non-self recognition as suggested by Leonard et al., (1985). Some evidence has emerged recently to suggest that receptors for β1 - 3 glucans are associated with plasma membranes from Bombyx mori haemocytes (Ashida et al. 1986). The initial events may involve direct interaction of receptor-bearing haemocytes with fungal surface molecules - this may result in stimulation of these cells and release of factors which subsequently stimulate proPO activation. Peptidoglycans from bacterial cell walls have also been shown to stimulate insect haemocytes to release a mixture of macromolecules including components of the proPO pathway. It could be envisaged that subclasses of haemocytes with receptors for these molecules might be involved in the initial recognition of microorganisms, with the secondary feature of proPO activation being a common response in all stimulated haemocytes. For insects however, it has not yet been demonstrated that lipopolysaccharide (LPS) will stimulate proPO activation in vitro, while this class of molecule does stimulate phagocytosis in vitro (Leonard et al., 1985), nodule formation in vivo (Gunnarsson and Lackie, 1985) and haemocyte locomotion (Takle, 1986). This may be a consequence

of the assay system - perhaps some necessary plasma component(s) or labile haemocytes are lost during the cell culture preparation. It is, however, very difficult to work with insect plasma preparations (see section 1.2.4.), so it may be difficult to establish a role for soluble haemolymph components in non-self recognition under defined, in vitro, conditions. An alternative explanation is that LPS stimulates haemocytes via a mechanism which does not also involve proPO activation. Recent evidence has emerged to suggest that Galleria mellonella haemolymph contains C3-convertase activity similar to the cobra venom factor responsible for alternative complement activation (Phipps et al., 1987). Either this is a fortuitous similarity in the substrate specificities between the insect haemolymph proteinase and the cobra venom factor or it suggests that insects may contain an analogous non-self recognition system similar to that of the mammalian complement cascade (Whaley, 1986). Whether β1 - 3 glucan and/or LPS receptors and C3 convertase activity are present in the Holometabola, including Cockroaches and Locusts, must remain, at present, a matter for speculation. The above findings should prompt an intense investigation into the role of soluble plasma factors in non-self recognition and provide detailed evidence for their occurrence throughout the insects.

1.2.3. Haemolymph lectins

Lectins are carbohydrate binding proteins that agglutinate cells and/or precipitate glycoconjugates through specific interactions with sugar residues in these molecules. In a number of organisms, from slime moulds to mammals, lectins have been shown to mediate intercellular adhesion and differentiation (Barondes, 1981, 1983) and Sharon (1984) has described a role for cell-surface lectins in mediating vertebrate cell phagocytosis.

The presence of red blood cell-agglutinating substances in invertebrate haemolymph has been known for many years (Bernheimer, 1952) but only recently has information on the binding specificity of these agglutinins been described and has been shown in most cases, to be carbohydrate-directed (Olafsen, 1986). Many insects contain lectins, both soluble and cell-associated, the biochemical characteristics of which are now becoming known (Komano et al., 1980; Stebbins and Hapner, 1985; Lackie and Vasta, 1986; Kubo and Natori, 1987; reviewed by Hapner and Stebbins, 1986). In view of the evidence that exists to suggest an opsonic role for lectins in mammalian leucocyte-bacteria interactions (Sharon, 1984), the lectins of insects are also hoped to be important components of the immune response involved in detection and neutralisation of non-self materials by phagocytosis and/or encapsulation. The in vivo function of insect lectins is, however, unknown and experimental data supportive of their involvement in immune mechanisms is inconsistent (Ey and Jenkin, 1982).

Some evidence has been provided that suggests a possible role for lectins in insect cellular defence reactions. Lackie (1981a), comparing the carbohydrate specificity of the agglutinating activity of serum from the cockroach Periplaneta and the locust, Schistocerca gregaria, found differences in the specificity and titre between the serum of these insects that coincided with differences in their acuity of immunorecognition. (Lackie, 1979). Oncospheres of the tapeworm, Hymenolepis diminuta were agglutinated strongly by cockroach serum while locust serum had no effect (Lackie, 1981b). This coincided with the observation that Hymenolepis oncospheres remained unencapsulated by haemocytes of Schistocerca, but were encapsulated in Periplaneta (Lackie, 1976).

Lackie and Vasta (1986, 1988) have also shown that the extent of the haemocytic response (encapsulation and nodule formation) in vivo is

influenced by the carbohydrate composition of a foreign surface. When injected into the haemocoele of cockroaches, Sepharose beads, that had been covalently conjugated with desialated glycoproteins, provoked a significantly thicker haemocytic capsule than did Sepharose beads conjugated to the corresponding sialated glycoprotein or bovine serum albumin. These authors have also established that the major serum lectin of Periplaneta is D-galactose specific and appears to be associated with the haemocyte surface (Lackie and Vasta, 1988). They suggest that the lectin may influence encapsulation of the glycoprotein-conjugated Sepharose beads via specific receptor-ligand interaction between the exposed D-galactose residues of the desialated glycoprotein and the serum lectin. Also the response is not charge-dependent since the hierarchy of the response is opposite to that expected for encapsulation of charged Sepharose beads in these insects (see section 1.6 and Lackie, 1986a).

Opsonic activity of insect lectins, unlike molluscan lectins, has not been demonstrated in vitro, although it has recently been reported that haemolymph factors can stimulate phagocytosis of bacteria in vitro depending on the composition of the outer surface coat (lipopolysaccharide) of the bacterial strain used (Mohrig et al., 1979a; Ratcliffe and Rowley, 1983). The phagocytosis by Periplaneta haemocyte monolayers of E. coli strain B1 is stimulated after the addition of cockroach serum to the medium (Ratcliffe and Rowley, 1983). However, if a mutant E. coli (strain K12) was used then no enhancement of phagocytosis was obtained. Similarly, sheep erythrocytes could not be opsonised. Ratcliffe and Rowley (1983), suggested that the natural isolate, B1, contained molecules in the outer cell wall which had been lost by K12 and that these molecules could have activated the proPO system (section 1.2.2.) thus promoting phagocytosis.

An alternative explanation could be that the differences in the two strains may be reflected in a difference in their cell surface carbohydrate composition (e.g. via changes in lipopolysaccharide structure) such that the serum lectin could not bind to the mutant bacteria.

The question of an opsonic role for insect serum lectins may be answered using purified insect lectins under defined in vitro conditions but as yet it remains unresolved (Renwrantz, 1986). Whether lectins represent components of the insect immune system has yet to be convincingly demonstrated, but it is probable that they will have other biological functions such as aiding ingestion of self-tissues during pupation and wound repair. The production of a lectin in larvae of the flesh fly, Sarcophaga peregrina, can be induced by injury to the body wall and by injecting larvae with sheep red blood cells, while it is constitutive during pupation. This might suggest a dual role for the lectin in wound repair/resistance to infection and insect morphogenesis (Komano et al., 1980; Takahaski et al., 1986).

A possible role for some lectins could be to agglutinate invading organisms that carry surface glycoconjugates containing the corresponding sugar residues, which might then result in their clearance by phagocytosis or nodule/capsule formation. In one case it appears that haemolymph lectins from Rhodnius prolixus agglutinate trypanosomes that are natural parasites of this insect (Pereira et al., 1981). However, in view of the fact that the vast majority of the trypanosomatids reside in the gut of their insect host and rarely invade the haemocoele (Molyneux et al., 1986) it is unlikely that the sole function of these insect lectins is for parasite recognition, and it is more likely that they are involved in other physiological processes within the insect.

Another function, linked to coagulation of haemolymph (see section 1.2.4.) has been described for M13, a glucose-specific lectin induced in the haemolymph of larvae of Manduca sexta by bacterial infection or oral administration of Bacillus thuringiensis toxin (Minnick et al., 1986). The lectin appears to stimulate release of the haemocyte coagulogen and coagulation could be inhibited in vitro by addition of glucose to the medium which also abolished the sheep red blood and agglutinating activity of the M13 lectin. Coagulation serves to plug tissue wounds so preventing blood loss and microbial infection; it may also play an essential role in encapsulation and nodule formation (Bohn, 1986; section 1.2.4.). If this is so then the results of Minnick et al., (1986) may represent the first example of a humoral lectin being implicated in cell-mediated immunity in insects.

From the data available it appears that different insects possess lectins of unique structural characteristics. Also, patterns of expression of lectin during development appear to differ in phylogenetically distant species. For example the lectin of Sarcophaga, a holometabolous insect, is composed of six subunits with a $\alpha_2\beta_4$ composition and a native molecular weight of 190,000. It is inducible in larvae in response to injury and during embryonic and pupal development but appears to be absent in the adult. The lectin of Periplaneta, a hemimetabolous insect, however, is a huge molecule of about 1,500,000 molecular weight apparently comprised of a single M_r 30,000 subunit which associated to form a filamentous structure visible by electron microscopy (Kubo and Natori, 1987). Furthermore, this lectin is present in adult insect haemolymph. In view of the fact that these lectins show differences in their structure and developmental synthesis, it may be wise to exercise caution when trying to ascribe a common function to haemolymph lectins in the immune responses of insects.

1.2.4. Haemolymph coagulation

The localised coagulation of haemolymph, upon exposure to foreign objects or upon wound healing, occurs very rapidly and may play an essential role in encapsulation and nodule formation as well as in wound healing. One practical implication of the rapidity of clotting is that it is very difficult to prepare insect plasma and thus identify soluble factors involved in immune recognition (see section 1.2.2.).

Haemolymph coagulation involves the cooperative involvement of plasma proteins and a particular class of haemocytes termed coagulocytes (Bohn, 1986). The plasma coagulogen has been identified, in all species studied, as being identical with the plasma lipoprotein (lipophorin) of insects (Brehelin, 1979; Chino, 1985; Bohn, 1986) which is present in a high concentration in haemolymph and constitutes about 50% (w/w) of the total plasma protein in some species e.g. Periplaneta (Chino, 1985). The mechanism of clotting has recently been reviewed (Bohn, 1986) and will not be considered here.

Clotting is important in wound healing; the rapid localised clotting of the haemolymph produces a "plug" at the wound site, thus minimising the risk of infection. The process may also be important in the various cellular defence reactions of insects (section 1.3.). Here, the haemocytes degranulate and release clotting substances which entrap or coat foreign particles and may promote phagocytosis or nodule formation/encapsulation. Since the plasma lipophorin is present at such a high concentration in the haemolymph it might be absorbed onto foreign surfaces entering the haemocoele, as well as onto damaged regions of host tissue during wound repair. The adsorbed lipoprotein may adopt an altered configuration or may be denatured, resulting in recognition by circulating

haemocytes, which then release their coagulation thus entrapping the intruder in a localised clot. Also, denatured lipophorin has been shown to activate the prophenoloxidase system in the silk worm Bombyx mori (Ashida et al., 1983), some of the components of which may mediate haemocyte binding to foreign objects (Ratcliffe et al., 1984). It is interesting to speculate whether the adsorption and denaturation of lipophorin onto a foreign surface, and resultant activation of the proPO pathway, could constitute a simple non-specific recognition system.

In summary it is apparent that there are a number of candidates for mediators of recognition including proPO components, serum lectins and plasma lipoprotein. As yet, no clear results have emerged implicating one class of molecule in recognition of non-self. This appears to be due to technical difficulties in purifying to homogeneity the soluble components under investigation as well as in separating the different classes of haemocyte involved in immune reactions (see section 1.3.1.) in order to allow detailed in vitro studies to be carried out. It may also be due to one or more soluble components working in concert to bring about recognition and elimination of intruders. The recent developments in haemocyte separation techniques and purification of soluble lectins and lipoproteins, may allow more precise conclusions to be drawn about the individual roles of insect plasma factors in mediating non-self recognition.

1.3. Cellular defence reactions

1.3.1. Insect blood cells

Reviews of the classification of insect haemocytes have appeared recently (Gupta, 1979; Rowley and Ratcliffe, 1981; and Brehelin and Zachary, 1986). Although there is still a great deal of controversy surrounding the subject, the basic classification scheme outlined by Rowley and Ratcliffe (1981) is now widely used by insect physiologists and immunologists.

1.3.1.1. Circulating haemocytes

The main classes of circulating blood cells involved in immune reactions are the Plasmatocytes, Granular cells and Coagulocytes (Rowley and Ratcliffe, 1981). Plasmatocytes generally contain few granules and are usually the major class of circulating haemocytes. They contain lysosomal enzymes and possibly phenoloxidase, which may be in keeping with their involvement in phagocytosis of invading microorganisms. They are also the main cell type involved in encapsulation and nodule formation and play a role in wound-healing. They give rise to the Lamellocytes of Drosophila (Rizki and Rizki, 1980a).

Granular cells are usually round or oval in vitro and contain, as their name suggests, many granular inclusions, the number of which in each cell appear to vary between different insect orders and also within species. They are involved in phagocytosis and possess phenoloxidase activity. Their main function may be in non-self recognition; they degranulate on contact with a foreign object releasing material which appears to be important in cellular (plasmatocyte) recruitment to intruders

thus ensuring rapid sequestration into cellular capsules or nodules. The third class of cell, the Coagulocyte (also referred to as cystocytes) are also classified as Granular cells in the "higher" insect orders. Coagulocytes are highly unstable in vitro, and lyse to produce a gel or coagulum. As the name suggests, these cells appear to be involved in haemolymph coagulation. Other circulating haemocytes include spherule cells of unknown function and Oenocytoids, which have been implicated in mediating transfer of lipids from the plasma lipoprotein to the epithelium below the cuticle (Katase and Chino, 1984). Other cell types are peculiar to particular species, although it is not clear if all insects contain functionally equivalent cells to some degree.

Another class of haemocytes, the prohaemocytes, are relatively small cells with a low cytoplasm:nucleus ratio. They are more common in the haemolymph of larvae than of adult and may represent the progenitor for the other haemocyte types.

1.3.1.2. Sessile haemocytes

Not all haemocytes in the haemolymph are in constant or free circulation within the haemocoele as evidenced by the rapid increase in the number of circulating haemocytes in response to certain stimuli (Hoffmann et al., 1974). They may be found in the lobes of the fat body or transiently adherent to haemocoelic surfaces (Gunnarsson, 1987) and as such do not represent true sessile cells. Other cell types, such as pericardial cells and those of the haemopoietic organs, which appear to be involved in the sequestration of injected particulate material, represent true sessile cells (Rowley and Ratcliffe, 1981).

The above classification of haemocytes is based on differences in

morphology and ultrastructure only. Recent advances have allowed the subdivision of haemocyte classes based on either physical criteria, such as density (Huxham and Lackie, 1987) and adhesive or locomotary properties (Bohn, 1977; Takle and Lackie, 1986; Huxham and Lackie, 1987) or biochemical criteria such as enzyme markers (Huxham and Lackie, 1986). Not all these techniques allow for the physical separation of haemocyte classes, but they do demonstrate functional differences within classes of cells with very similar morphologies. The problem of interpreting results from *in vitro* experiments using total haemocyte populations may be circumvented by applying physical separation techniques to haemocyte preparations and using defined cell populations to study immune responses *in vitro*.

1.3.2. Phagocytosis

Most of the early work on phagocytosis by haemocytes was a result of observations made *in vivo*; following the clearance of injected dye particles or microorganisms by blood cells (e.g. Hollande, 1930). Recent *in vitro* techniques, such as preparations of haemocyte monolayers, have provided some details of the fundamental mechanisms involved in the phagocytic process. A number of researchers have successfully demonstrated phagocytosis by insect haemocytes *in vitro*, using both abiotic (e.g. Latex beads) and biotic (fungal spores, bacteria) test particles (reviewed by Ratcliffe and Rowley, 1979). Sessile cells (see section 1.3.1.2.) also appear to be involved in phagocytosis *in vivo* (Crossley, 1975).

The predominant class of haemocyte involved in phagocytosis, both *in vitro* and *in vivo*, is the plasmatocyte (see section 1.3.1.1.) although a small contribution to the overall response is made by granulocytes and/or coagulocytes (Rowley and Ratcliffe, 1981; Huxham and Lackie, 1987). The

mechanisms of phagocyte recognition in insects are not yet known. It is possible that non-specific surface properties, such as charge or hydrophobicity, determine whether a target particle can be recognised by phagocytosing leucocytes (Edebo et al., 1980). However, it seems likely that more specific mechanisms are involved in the recognition of physiologically relevant particles such as bacteria or fungal spores. There is evidence to suggest that the carbohydrate moieties of surface glycoconjugates on target particles are recognition determinants for cell-associated lectins in non-immunological phagocytosis in mammals (review by Sharon, 1984). The widespread occurrence of soluble and cell-associated lectins in insects (see Hapner and Stebbins, 1986 for a review) has prompted suggestions that lectins may also serve an opsonic function in these animals.

Some workers have suggested that components of the prophenoloxidase pathway may play an opsonic role in insect phagocytosis (Ratcliffe et al., 1984). In all cases, although it is clear that other workers have shown that β1 - 3 glucans increase the proportion of phagocytic haemocytes in vivo (Gunnarsson, 1987) and in vitro (Ratcliffe et al., 1984; Huxham and Lackie, 1987) and stimulate phenoloxidase activation, the evidence for opsonisation and cell-cell cooperation in insect phagocytosis is only circumstantial.

The proportion of the haemocyte population that is phagocytic can also be increased by injection of Latex (Mohrig et al, 1979a) or Sepharose beads (Dularay and Lackie, 1985) or even saline (Gunnarsson, 1987) and as such represent non-specific stimulation of phagocytosis. Apparent enhancement of phagocytosis in vitro, by insect serum components has been reported by Ratcliffe and Rowley (1983), who showed that addition of insect serum to culture medium increased the ingestion of a Bacillus cereus isolate by

Periplaneta haemocytes. Also Mohrig et al., (1979a,b) have shown that the haemocytes of Galleria mellonella larvae can be stimulated to ingest a normally non-phagocytosed bacterial strain, provided that the larvae receive a prior injection of latex beads. Transfer of diluted cell-free haemolymph to naive larvae resulted in an enhanced phagocytic ability in these animals against the same bacterial strain. This suggested that a soluble factor was responsible for stimulating phagocytosis, although this interpretation must be viewed with caution, firstly because of the impossibility of preventing cell lysis in their system, which may result in stimulation, and secondly because of the observed stimulation of phagocytosis observed for locust haemocytes by Gunnarsson (1987) upon injection of saline alone. The next step in the phagocytic process, following recognition and attachment, involves ingestion of the target particle. This process, as in mammalian phagocytosis, is energy dependent (Anderson et al., 1973) and uses the glycolytic pathway as an energy source. A potent antimicrobial system that involves, hydrogen peroxide, myeloperoxidase and a halide has been found in monocytes and polymorphonuclear leucocytes (Klebanoff, 1982). Such a system has not yet been demonstrated in insect phagocytes, although acid phosphatase, β-glucuronidase and β-glucosaminidase activity have been found in some insects (M. Carr, pers. commun.; Walters and Ratcliffe, 1981). Lysozyme is also present in insect haemocytes (M. Carr, pers. commun.; Zachary and Hoffmann, 1984) and together these enzymes represent a powerful potential for killing and digesting sequestered microorganisms.

1.3.3. Nodule formation

Nodule formation (essentially haemocyte aggregation) in insects occurs in response to injected foreign particles including bacteria (Ratcliffe and

Walters, 1983) and soluble molecules of microbial origin such as β1 - glucans (Gunnarsson and Lackie, 1985). Quantitative work by Ratcliffe and Walters (1983) using various pathogenic and non-pathogenic bacteria in Galleria larvae, showed that nodule formation occurred only above a threshold dose of bacteria (more than 10^3 organisms/ul). Below this threshold, phagocytosis was the main bacterial clearance mechanism. The formation of haemocyte nodules appears to depend on the stimulation of coagulocytes with their resultant degranulation and formation of a plasma clot, in which the invading bacteria are trapped. Plasmatocytes adhere to the coagulum and flatten to form a capsule-like structure (Ratcliffe and Gagen, 1977). The pro-phenoloxidase system is rapidly activated in the developing nodule and the resulting melanization of the nodule core which occurs in some but not all insects studied so far, may be important for microbial killing (i.e. via toxic quinone production) in conjunction with the lysosomal enzymes which have been detected in these structures (Walters and Ratcliffe, 1981).

Nodules have been reported to occur in response to injection of a variety of microorganisms (reviewed by Ratcliffe and Rowley, 1979), soluble extracts of molecules of microbial origin (Gunnarsson and Lackie, 1985) certain glycoproteins (Lackie and Vasta, 1986) and in response to transfer of haemolymph between insect species (Lackie, 1986b). They also occur in response to injection of various abiotic materials (Ratcliffe and Rowley, 1979) and saline solutions (Gunnarsson, 1987) although injection of dextran or bovine serum albumin solutions are without effect (Gunnarsson and Lackie, 1985; Lackie and Vasta, 1986). Nodule formation, then, represents an extremely effective mechanism for clearing large numbers of microorganisms from the haemolymph within a very short time (Gagen and Ratcliffe, 1976). However, the details of the initial interaction between the effector cells and the foreign intruder remain unresolved.

1.3.4. Encapsulation

Foreign objects that enter the haemocoele and are too large to be phagocytosed or trapped in nodules are encapsulated by haemocytes (Salt, 1970). Capsules are formed in insects against a wide range of biological and non-biological objects including fungi, large protozoans, various helminths, biological implants, nylon fibres, glass beads and air bubbles (Ratcliffe and Rowley, 1979). However, not all foreign material introduced into the haemocoele is encapsulated; some habitual parasites apparently cannot be distinguished from "self" and avoid encapsulation by an undetermined mechanism (see section 1.6.).

1.3.4.1. Capsule ultrastructure

Light and electron microscope investigations of capsules show them to consist of three layers of cells, each of distinct morphology, comprising a completed capsule which varies in overall thickness, depending on the object being encapsulated (Grimstone et al., 1967; Lackie et al., 1985; Ennesser and Nappi, 1984). For example, capsules between 30-80μm thick are formed around Araldite implants in Ephestia larvae (Grimstone et al., 1967). The innermost layer appears to be made up of often necrotic and melanized haemocytes that have a rounded or slightly flattened morphology. The next cellular layer is composed of flattened, tightly adherent, plasmatocytes which appear to establish cell-cell contact via desmosomes. The outer layer of cells consists of mostly rounded or only slightly flattened cells (Grimstone et al., 1967; Lackie et al., 1985; Ennesser and Nappi, 1984).

Some ultrastructural studies on the time-course of encapsulation have shown that one of the first events in the process involves adhesion of

cells (granulocytes/coagulocytes) to the foreign surface with concomitant degranulation, within minutes of implantation of the foreign object (Schmit and Ratcliffe, 1977). Plasmatocytes are then recruited to the capsule which is usually complete after about 24hr. The exact details of the process may vary between insects. For example, melanization of the inner core of the capsule does not always occur (Salt, 1970) and Lackie et al., (1985) found no degranulation of coagulocytes in Periplaneta during encapsulation of Sepharose beads. Confusion may arise due to the haemocyte classification adopted by some investigators to describe the cells involved in capsule formation (e.g. Ennesser and Nappi, 1984), but on the whole the general process of cellular encapsulation is fairly similar in the species studied so far.

The thickness of capsules varies between species and within species depending on the object being encapsulated. The variation between species seems to correlate well with the total haemocyte counts in those animals (Gotz, 1986) for example, Lackie et al., (1985) have shown that the mean value for the number of cell layers around positively charged Sepharose beads is 17 in Periplaneta but only 3 in the locust, Schistocerca gregaria, and that locusts have fewer plasmatocytes than cockroaches. The variation in capsule thickness within a species has been shown to depend on the surface properties of the object being encapsulated, including surface charge and hydrophobicity (Vinson, 1974; Lackie, 1983b) and carbohydrate composition (Lackie and Vasta, 1986). The variation in thickness is due to the number of haemocytes recruited to the capsule and not due to differences in the degree of cell spreading within the capsule (Lackie et al., 1985).

Completed capsules are coated in a extracellular material which binds the cationic dye Alcian Blue 8GX, suggesting that it contains

glycosaminoglycans (Lackie et al., 1985; Scott and Dorling, 1965). This material may prevent further haemocyte adhesion to the capsule, although this may be unlikely since similar substances can also be detected between the extensively flattened plasmatocytes in completed capsules (Grimstone et al., 1967). Similarities of this material with the connective tissue of the animal in which it is formed is suggested by transplantation studies; if "coated" capsules from Periplaneta are washed and transferred to the haemocoele of an allogeneic recipient or of Schistocerca, then no further encapsulation occurs in the recipient. This lack of response is similar to that shown by Schistocerca against transplanted tissues from Periplaneta (Lackie, 1979) i.e. "coated" capsules are apparently regarded as "self" (Lackie, 1986a; Salt, 1970). How components of this material can prevent haemocyte adhesion is unclear, but it may shed light on the mechanism of evasion used by some habitual parasites of insects that develop in the haemocoele (see section 1.6.).

1.3.4.2. Recognition of "non-self"

Although there is an extensive literature describing encapsulation in insects, experimental evidence to explain the initial recognition of the encapsulated object has not been forthcoming. Recent developments, however, may help redress the situation. Ratner and Vinson (1983) have developed a system for studying encapsulation in vitro. The system has been used to investigate the interaction between Heliothis virescens haemocytes with eggs of the parasitoid wasp Cardiochiles nigriceps and has provided insight into possible mechanisms adopted by these parasites to allow them to develop, unmolested, within the haemocoele (Davies and Vinson, 1986). The in vitro system has also demonstrated the lack of encapsulation of allogeneic tissues and therefore displays

recognition/discrimination in the absence of other haemolymph components (Ratner and Vinson, 1983). Further use of in vitro systems may allow for the elucidation of the molecular events involved in host recognition and provide a tool to dissect and analyse the major steps in encapsulation, thus providing a more detailed understanding of the process.

1.4. Non-cellular (humoral) encapsulation

This phenomenon consists of the deposition of a substance on the surface of foreign objects which appears to be melanin. Humoral encapsulation can be observed in vitro using living organisms or organic material such as Sephadex beads. This response is effective against nematodes, fungi and bacteria (see Götz, 1986, for a review). From in vitro studies it is obvious that the material is deposited very quickly on the surface of the intruder with no apparent participation from host haemocytes. The material is resistant to various solvents and enzymes (Vey and Götz, 1975), and hardening of the capsule is suppressed if inhibitors of phenoloxidase (e.g. phenylthiourea) are included in the in vitro systems, suggesting that the completed capsule is a sclerotised complex of protein and polyphenols.

Humoral encapsulation is known only to occur in larvae of certain flies (Diptera); its occurrence correlates with low haemocyte counts in these animals and may therefore be a peculiar reaction of that insect order (Götz, 1986).

1.5. Killing mechanisms of haemocytes

Mammalian leucocytes produce a variety of enzymes and other proteins that are responsible for killing ingested viruses and bacteria that include proteolytic and hydrolytic enzymes and systems for generation of oxygen radicals. They also produce extracellular factors, such as the eosinophil cationic protein, that is produced in response to the surface of helminth parasites (McLaren et al., 1981).

Interestingly, oxygen-dependent killing mechanisms have also been demonstrated in molluscan haemocytes (Dikkeboom et al., 1987). They do not appear to be present in insect haemocytes although, as mentioned earlier, some workers have shown that a variety of proteolytic and hydrolytic enzymes are present in haemocyte lysosomes and represent a potent potential for killing and digestion of microorganisms within these phagocytes.

The prophenoloxidase system has been cited as an extracellular killing mechanism of haemocytes, via melanization, which may retard the physical development of the parasite, and the toxic effects of quinone radicals produced during this process. However, the role of melanization in the killing of parasites is controversial and no clear evidence exists that this system alone is important for killing bacterial, protozoan or metazoan parasites. In fact, some parasites have been shown to survive in melanized nodules and capsules (reviewed by Ratcliffe and Rowley, 1979).

Haemocytes produce and secrete lysozymes (Schneider, 1985) which are bacteriolytic, but the function of the enzyme is probably to digest bacterial cell walls from organisms killed by other mechanisms. In some cases, it has been demonstrated that the secreted enzyme activity is in fact a chitinase which possesses some lysozyme activity due to a similarity in the enzyme substrates (Boman and Hultmark, 1987).

Trenczek and Bennich (1987) have demonstrated that Hyalophora cecropia haemocytes can synthesise the proteins (cecropins, attacins and lysozyme) involved in the extracellular killing of bacteria (see section 1.2.1.). Whether haemocytes produce factors analogous to Major Basic Protein or eosinophil cationic protein for extracellular killing mechanisms of helminths is not yet known (see Butterworth, 1984, for a review of mechanisms of vertebrate cell-mediated damage to helminths).

Some parasites can survive within haemocyte nodules and capsules; whether this reflects the generally poor nature of insect killing mechanisms or highlights the parasites which have adapted to survive the cellular responses of their host remains to be determined.

1.6. Evasion of insect immune responses by parasites

The ability of parasites to avoid immune recognition due to a property of their surface, or to actively inhibit the immune response of their host, is essential if they are to survive in the hostile environment of the host animal, where the internal defence systems are designed to maintain and protect the integrity of "self" tissues.

Apparent active interference with host immune responses has been demonstrated for a number of insect parasites, including bacteria, parasitoid wasps, fungi and some nematodes in symbiosis with bacteria. Here, the parasites are involved in synthesising and secreting substances which inhibit the normal immune responses or destroy humoral factors or cells involved in mediating the immune response. Passive evasion of recognition may also occur, where "antigen sharing" between parasite and host occurs or where an intrinsic property of the parasite's surface allow it to develop unmolested in the host, or the site of development is inaccessible to the immune effector cells (Salt, 1968; Lackie 1986a)

1.6.1. Suppression of host immune responses

The bacterium, Xenorhabolus nematophilus lives in symbiosis with a nematode, Steinernema feltiae, in the haemocoele of Galleria mellonella larvae, where it helps to kill the insect host. In turn, the nematode secretes immune inhibitors which rapidly destroy the attacins and cecropins thus protecting the bacterial symbiont (Götz et al., 1981). How the nematode avoids encapsulation is unclear; however, it appears that an intact, lipoidal epicuticle is essential for non-recognition, since lipase treatment of dauer larvae rendered them susceptible to haemocytic encapsulation in vivo

The insect pathogen, Bacillus thuringiensis, secretes a proteinase termed InA, that can degrade attacins and cecropins (Boman and Hultmark, 1987). However this proteinase is only produced late in infection of Hyallophora cecropia, when the insect is already dead or dying. In any case this bacterium is resistant to cecropins and attacins, so the value of InA production for bacterial survival is not obvious (Boman and Hultmark, 1987).

Some fungi produce toxins which may be the cause of host death. They may also inhibit the cellular defence reactions of the host. One family of fungal toxins that have been purified are the "destruxins" (Vey et al., 1985; Vey and Götz, 1986; Huxham et al., 1987); cyclodepsipeptides produced by Metarhizium anisopliae, which have strong cytotoxic and insecticidal properties. Using one member of this family, destruxin E, Vey et al. (1985) have demonstrated an immunosuppressive effect on encapsulation of Aspergillus spores in Galleria larvae injected with this toxin. The mode of action of destruxin E is not yet known, although it appeared to alter haemocyte morphology resulting in impaired movement of

the blood cells *in vitro* (Huxham *et al*., 1987) and a disturbance in the mode of haemocyte association in the capsules developing around the *Aspergillus* spores (Vey *et al*., 1985).

Other examples of immunosuppression have been reported for trypanosomes and filarial nematodes. Bitokowska *et al*. (1982) have demonstrated reduced encapsulation of xenografts in the bug, *Triatoma infestans*, which had previously been infected with *Trypanosoma cruzi*. The trypanosome however, like most others, completes its development in the insect gut without entering the haemocoele, so it is difficult to envisage how it would benefit from the suppression of cellular responses. The mechanism whereby trypanosome gut infection inhibits xenograft response is not clear, although Bitokowska *et al*. (1982) have demonstrated that culture fluid rich in shed *T. cruzi* antigens, when injected intrahaemocoelically had a similar effect on the xenograft encapsulation response. However inhibition was also observed in bugs given control culture fluid i.e. the suppressor effect of experimental fluid is a result of injection of foreign macromolecules and not from the action of particular parasite antigens. The doses of administered protein ranged from 0.1 to 0.5mg in 50µl distilled water and it is possible that introducing such relatively large amounts of protein into the haemolymph could disrupt the osmolarity of the blood, which may account for the observed effects.

Evidence for evasion by acquisition of host materials is available for the larvae of the filarial nematode *Brugia pahangi* during natural infection of mosquitoes (Sutherland *et al*., 1984). Midgut penetration appears essential if the worms are to avoid melanization during their migration through the insect haemocoele to the thorax. It has been proposed that microfilariae (mff) acquire host materials on their surface during penetration (Sutherland *et al*., 1984) or receive a physiological or

developmental stimulus that enables them to adapt to avoid melanization (La Fond et al., 1985). Christensen et al. (1987) however, could demonstrate no cross-reactivity between gut-penetrated mff and midgut tissues, but did demonstrate a decrease in the negativity of the surface of mff after gut penetration, as measured by cationised colloidal iron binding, which may also influence haemocyte recognition and/or adhesion (see section 1.6.2.).

There is also evidence to suggest that Brugia mff are capable of suppressing the immune response of their mosquito host, since, insects harbouring developing larvae show a diminished melanization response against a secondary inoculation with two species of blood-isolated microfilariae (Christensen and La Fond, 1986). The mechanism(s) employed by filarial worms in circumventing the immune system of their hosts is still however, unclear (see Christensen, 1986, for a review).

Parasitoid wasps can actively inhibit host responses. Rizki and Rizki (1984) have shown that eggs of the parasitoid Leptopilinia heterotoma remain unencapsulated in Drosophila larvae. These workers have demonstrated that plasmatocyte-like immune competent cells of the host principally involved in encapsulation, the Lamellocytes, undergo morphological changes and lose their adhesiveness after infection. The factor responsible for this effect termed Lamellolysin, is produced by the female wasp and injected with the eggs during infection. It is specific for the host lamellocytes with no apparent effect on the granular cells of the larvae (Rizki and Rizki, 1984). Lamellolysin has not yet been characterised but it apparently produces multiple effects in the target cells, including alterations in their surface carbohydrate composition and re-distribution of their microfilaments, which may result in cell death (Rizki and Rizki, 1986).

Other parasitoids infect hosts with viruses, which are in the calyx fluid surrounding the injected eggs or larvae (Stoltz and Vinson, 1979).

The viral particles, when injected alone, are capable of inhibiting encapsulation (Vinson et al., 1979). Furthermore, Stoltz and Vinson (1979) have shown that the nucleocapsids within the calyx particles penetrate host cells, including haemocytes, and that viral DNA is translated in these infected cells. The viral products appear to inhibit physiological processes of the host, including protein synthesis and endocrine balance (Beckage et al., 1987) and so may have a suppressive effect on host haemocyte functions (Stoltz, 1986).

In view of the considerable interference on host physiological processes as a result of parasitism which result in the arrest of host development, it is perhaps not surprising that host immune responses would be impaired (see Beckage and Templeton, 1986 for review). However, since haemocytic recognition of non-self is almost immediate, as assessed by the rapid initiation of encapsulation (Grimstone et al., 1967; Nappi and Ennessener, 1984) there must be some additional mechanism preventing encapsulation of the eggs during the first 1-2hr after oviposition before de novo synthesised viral products or other factors can alter host physiology. A possible explanation may be forthcoming from the results of Davies and Vinson (1986), who have demonstrated that the fibrous layer around the eggs of one parasitoid species may afford protection against haemocytic encapsulation in vitro and that this structure may play a similar role in vivo, providing temporary protection from encapsulation before induction of the viral or parasite "immunosuppressive" agents (Davies and Vinson, 1986). The fibrous layer in wasp eggs appears to contain neutral or acidic mucoprotein/glycoprotein components. Histochemical examination of the envelope surrounding developing larvae of Moniliformis also suggests that mucoproteins (more specifically glycosaminoglycan-containing proteoglycans) are present in the outermost

regions at the host parasite-interface (Lackie, 1986a). This envelope appears to protect the developing larva from encapsulation in the cockroach haemocoele (Robinson and Strickland, 1969).

1.6.2. Parameters important in recognition

In order to understand how surface structures in parasites can protect them from encapsulation it is necessary to ask what is recognised as "self" or "non-self" by the insect immune system.

Haemocyte encapsulation of foreign objects introduced into the haemocoele provides a measure of whether a cellular response, and thus immunorecognition has occurred. In general, two categories of object are used in these assays, biotic materials (e.g. nerve cord, ovariole and abiotic materials (e.g. Sepharose beads) used with the common aim of defining the discriminatory ability of the recognition system of the recipient insect (see Lackie, 1986c, for a review).

1.6.2.1. Biotic implants

The results from transplantation experiments have shown that allogeneic (intraspecific) recognition is absent in a wide range of insects (Salt, 1961; Lackie, 1986c), while xenogeneic recognition, for the most part, occurs except in certain restricted species combinations (i.e. between closely-related species) or where the recipient species has a low acuity of immunorecognition (Lackie, 1986c). Interestingly, all surfaces and organs of the insect body cavity are coated in a thin layer of connective-tissue (Ashhurst, 1985). Furthermore, the results from allogeneic transplantation studies suggest that the haemocytes do not adhere and encapsulate the intact basement membrane covering these tissues,

thus, it is possible that the molecular characteristics of this layer could define "self".

Early studies by Salt (1961) demonstrated that if the connective-tissue layer of allogeneic transplants was modified by physical or chemical treatment, then such transplants were encapsulated by the recipient animal's haemocytes. Also, haemocytes adhere only to the cut ends of the allogeneic transplants where tissue, and presumably, connective-tissue damage has occurred (Lackie, 1986b). Rizki and Rizki (1980b) have shown, using a temperature-sensitive mutant of Drosophila, that the basement membrane overlying the atypical cells of the larval caudal fat body becomes abnormal at the temperature at which melanotic tumours develop, resulting in haemocyte adhesion and encapsulation of this tissue. These authors have suggested that the haemocytes recognised the altered architecture of the connective tissue layer covering this organ and that normal basement membrane will not elicit an encapsulation response (Rizki and Rizki, 1986). One argument against this hypothesis is that haemocytes also invade apparently normal connective-tissue layers during metamorphosis of the insect (Crossley, 1968). However, subtle differences in the properties of "normal" connective-tissue layers, such as might occur during morphogenesis to allow autolysis of tissues, will not be detected during an ultrastructural study, and so it is difficult to argue against the hypothesis that normal connective tissue defines self by citing the above example.

1.6.2.2. Assays using abiotic objects

Assuming that characteristics of the connective-tissue layer define "self" it is necessary to consider what properties of this layer allow it to be described as "self". One approach has been to use abiotic material

of defined surface physicochemical properties (charge and hydrophobicity) and carbohydrate composition and to analyse the ability of haemocytes to recognise and respond to differences in these parameters.

1.6.2.2.1. Physicochemical properties

Vinson (1974) and Lackie (1983b, 1986c) using Heliothis larvae and Periplaneta and Schistocerca adults respectively, have found that different-charged ion-exchange beads are encapsulated to different extents when introduced into the haemocoele of these insects. Negatively charge beads are not encapsulated by Heliothis haemocytes in vivo (Vinson, 1974) or in vitro (Davies and Vinson, 1986). Lackie (1983b) has shown, using adult Periplaneta and Schistocerca, that the magnitude of the in vivo response, i.e. thickness of haemocytic capsules around abiotic objects, corresponds well to the adhesive behaviour of the haemocytes in vitro on various polystyrene substrata which differ in their net negative charge and hydrophobicity. A greater proportion of Periplaneta haemocytes adhered as the net negative charge of the substratum increased and hydrophobicity decreased. This corresponded with the in vivo encapsulation response where thicker capsules were formed around negatively charged beads than neutral beads. For Schistocerca, a low proportion of haemocytes adhered to all the polystyrene surfaces in vitro, irrespective of their charge, while in vivo, negatively charged sepharose beads were not encapsulated. Takle and Lackie (1985) have demonstrated that the haemocytes of these two insects do in fact have different net surface charges, as measured by cell electrophoresis and the thickness of the layer of bound cationised ferritin in electron microscope-visualised sections of haemocytes. Locust cells have a greater net negative charge and these authors suggested that this might explain the differences in their adhesive behaviour towards charged

substrata. Whether the charge of an object has a direct effect on cell adhesion *in vivo* is not clear but net charge may affect the adsorption of haemolymph components and their conformation once adsorbed onto the surface (i.e. whether they are denatured or not) which, in turn, may affect the secondary event of haemocyte adhesion. The relative hydrophobicity of the surface of a foreign object also affects cell adhesion (Edebo *et al.*, 1980), or cell-cell interactions between bacteria and mammalian leukocytes. The hydrophobicity of a foreign surface is also an important factor in the process of haemocyte adhesion *in vitro* (Lackie, 1983b; Takle, 1986) and *in vivo* (Salt, 1970). Pieces of paraffin wax (Salt, 1970) or glass beads coated with polyvinyl acetate provoke thick capsules (Lackie, 1986c) presumably due to their hydrophobic nature.

Surface carbohydrates of bacteria generally consist of neutral or acidic polysaccharide chains which show a great capacity to bind water. Thus, in general they possess a hydrophilic surface which appears to provide some protection against phagocytosis by animal cells (Dudman, 1977). The acidic polysaccharide of connective tissue (glycosaminoglycans) demonstrate not only a net negative charge at physiological pH, but also a capacity to bind relatively large amounts of water (Lindahl and Höök, 1978). Thus insect connective tissues represent a relatively hydrophilic surface, the physicochemical properties of which might affect haemocyte behaviour. Foreign surfaces, including parasite surfaces, which have similar physicochemical properties to those of the recipient insect's connective-tissue, may not be recognised as non-self and remain unencapsulated. However, the net surface charge of haemocytes and parasites cannot account for the effective phagocytosis by *Schistocerca* haemocytes of the protozoan *Trypanosoma rangeli* since haemocytes of *Rhodnius* the natural host of this parasite, have the same net surface

charge as Schistocerca haemocytes (Takle and Lackie, 1985) but are unable to effectively interact with this parasite as assessed by this animal's failure to clear intrahaemocoelic infections of T. rangeli (Molyneux et al., 1986). It should be noted, however, that this is a vague comparison to make since the differences in susceptibility of infection in the two insects could be due to other factors including differences in plasma components or total haemocyte counts (Takle and Lackie, 1987).

1.6.2.2.2. Carbohydrate as a recognition signal

Lackie and Vasta (1986) have shown that the extent of the haemocytic encapsulation response of in vivo by Periplaneta haemocytes, is influenced by the carbohydrate composition of a foreign surface. Sepharose beads conjugated to asialoglycoproteins provoke significantly thicker capsules than do beads conjugated to the corresponding sialoglycoproteins or bovine serum albumin. Interestingly, the difference in encapsulation is not due merely to differences in net charge of the object, since sialoglycoprotein-conjugated beads which had a higher net negative charge than the corresponding asialoglycoprotein beads, provoked thinner capsules in vivo. As mentioned earlier, (section 1.2.3.) this led Lackie and Vasta to propose that the major haemolymph lectin of Periplaneta, which is apparently galactose specific, and is found in association with the blood cell surface, could influence immunorecognition in this insect and represent a specific recognition component supplementary to the non-specific physicochemical parameters of foreign surfaces which seem to influence the responsiveness of the insect blood cells.

In summary then, it is clear that while insects can recognise broad differences in the surface properties of foreign objects, such as charge, they can also respond to subtle variations in the carbohydrate composition

of foreign objects. For the connective-tissue layer to fulfill the role of defining "self"-tissue it might be expected to remain constant in both its carbohydrate composition and physicochemical properties throughout all the surfaces and organs of the haemocoele. However, it is becoming clear that the carbohydrate composition of haemocoelic connective tissues are not constant as shown by differences in lectin-binding affinities by mosquito salivary glands (Perrone et al., 1981) and Periplaneta malpighian tubules, ovarioles and midgut (Martin and Lackie, unpublished). Variation in the surface carbohydrate composition may result in variations in the physicochemical properties of the connective-tissue surrounding the different organs of the haemocoele.

We have then, a working hypothesis to explain how helminth larvae may avoid encapsulation in their insect host; to present a surface which has both physicochemical and molecular properties similar to the host connective tissue. As mentioned earlier, histochemical evidence suggests that the envelope surrounding Moniliformis larvae contains connective-tissue-like macromolecules (Lackie, 1986a) and so this parasite may adopt the above strategy to avoid encapsulation. An investigation of the biochemical composition of the envelope and a study of the organisation of the macromolecules within the envelope might allow some insight into the observed evasion mechanism adopted by Moniliformis. It is now pertinent to consider some aspects of the biology of the phylum Acanthocephala of which Moniliformis is a member, before considering the structure and development of the protective envelope of Moniliformis larvae.

1.6.3. The Acanthocephala

The Acanthocephala represent an entirely parasitic taxon of pseudocoelomate worms. They are all dioecious, gutless endoparasitic

helminths, characteristically having an invaginable hook-bearing proboscis (Crompton and Nickol, 1985). The closest existing phylogenetic relations of the Acanthocephala are probably the Aschelminths, such as the rotifers (Morris and Crompton, 1982).

Larval development occurs in invertebrates after these hosts ingest acanthor larvae contained within eggs. Development proceeds through six acanthella stages to a cystacanth, which is the stage infective to the definitive vertebrate host and essentially represents an immature adult. Upon ingestion of the invertebrate host by the definitive host, the cystacanths are activated by bile salts in the small intestine (Graff and Kitzman, 1965; Lackie, 1974). The adult worms reside in the alimentary canal, where they are attached to the luminal surface of the gut by the proboscis.

At all stages of development, the body wall of acanthocephalans consists of a living, multinucleate, syncytial integument about 25-50μm thick (Nicholas and Mercer, 1965; Crompton and Lee, 1965; Butterworth, 1969; Wright and Lumsden, 1970). The absence of a gut means that the tegument represents the only interface across which nutrient uptake and disposal of waste material can occur. Indeed, the surface area of the parasite is greatly increased by pore canals, which are present at all stages (Wright and Lumsden, 1968) and may represent uptake sites for specific classes of molecule. Kinetic and inhibition studies of nutrient uptake have suggested that specific carrier mechanisms exist for amino acids and monosaccharides (Rothman and Fisher, 1964; Crompton and Lockwood, 1968; Crompton and Ward, 1984) while the kinetics of uptake for other classes of biomolecules such as nucleotides have not yet been investigated. At least two classes of surface hydrolase enzymes are associated with the

apical plasma membrane of the tegument. They are an aminopeptidase and a phosphatase activity (Crompton, 1963; Uglem et al., 1973, reviewed by Starling, 1985).

Unlike other helminths, there is a paucity of biochemical data on the structure and composition of the tegument which has, to date, only been investigated by ultrastructural and histochemical techniques in mainly adult worms of a limited number of species (Nicholas and Mercer, 1965; Wright and Lumsden, 1970).

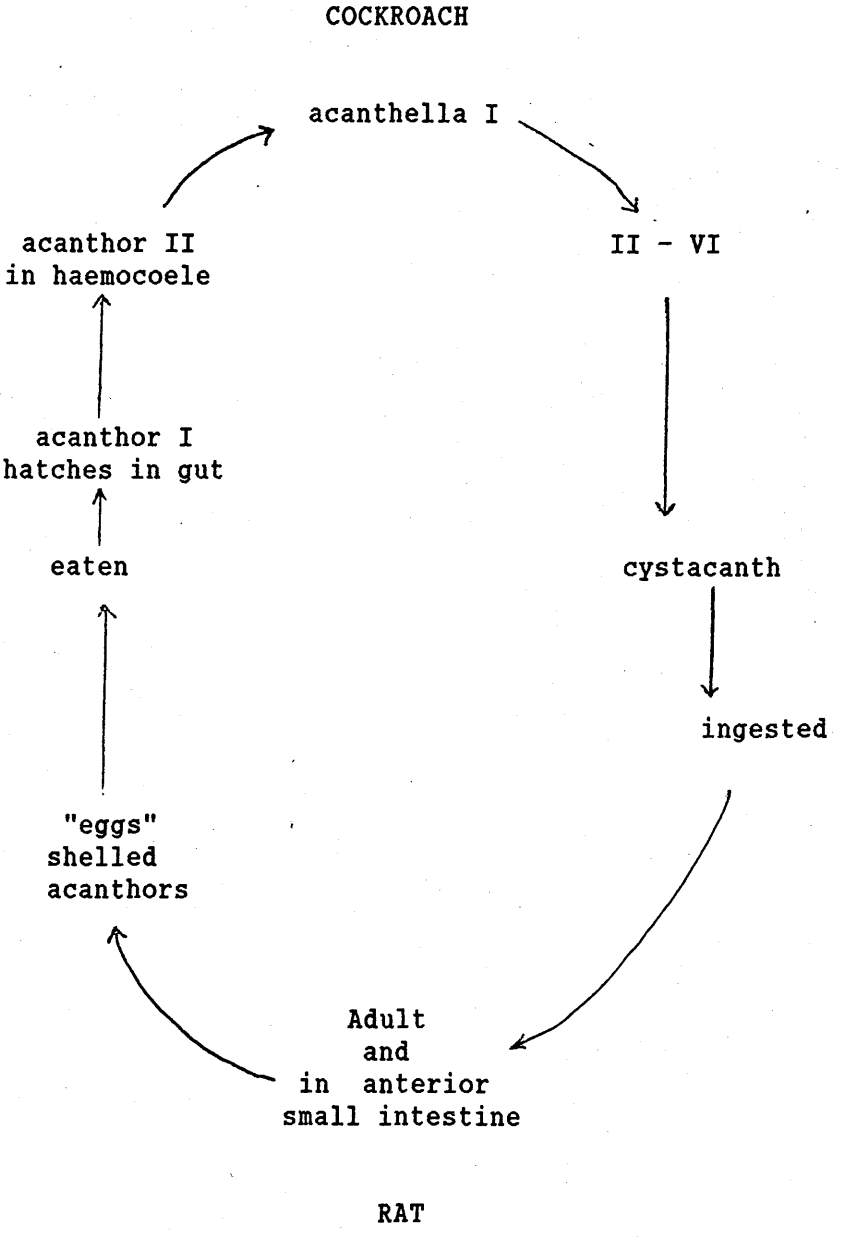
1.6.3.1. The life cycle of Moniliformis (Figure 1.1.)

This acanthocephalan is generally described as a parasite of rats and cockroaches, but it is known that other vertebrate species may be infected, including man (Sahba et al., 1970). The cockroach intermediate host, Periplaneta americana, is known to be infected under natural conditions upon ingestion of rat faeces containing embryonated eggs. Within a short period of time in the cockroach gut the acanthors hatch and migrate through the gut contents, pierce the peritrophic membrane and midgut wall to emerge in the haemocoele (Crompton, 1970). The larvae then quickly acquire a membranous coat or envelope (J. Lackie, 1973) within which they grow through a second acanthor stage and six acanthellae stages to the cystacanth. Infected cockroaches are eaten by rats and the cystacanths become established within the small intestine, where they develop to adulthood.

1.6.3.2. Haemocoelic development and envelope production

Soon after entry into the haemocoele the acanthor is surrounded by haemocytes which react initially in a manner characteristic of the response

Figure 1.1 Schematic diagram of the life cycle of Moniliformis moniliformis (after King and Robinson, 1967)



In the cockroach, acanthella I (used in some of the in vitro cultures), is present after approximately 12-14 days at 28°C; cystacanths are present from approximately 6 weeks.

to a relatively large foreign body encapsulation. However, the capsule formed is only a few cells thick (Rotheram and Crompton, 1972) and as the larva develops, the number of adherent haemocytes decreases and within about seven to ten days post-infection the surface of the larva is completely free of cells. This decline in the thickness of the capsule coincides with the production of a membranous coat, apparently produced as å microvillar proliferation of the tegumental membrane (Rotheram and Crompton, 1972). By the mid-acanthella stage the membranous coat has become elevated from the tegument, separated from it by a clear viscous material and compacted to form a transparent envelope (Lackie and Rotheram, 1972).

1.6.3.3. Origin of the envelope

The question of the origin of the envelope has created much controversy; early work on envelope structure, using light and electron microscopy, suggested that it was composed of host's haemocytes (Mercer and Nicholas, 1967), Crompton (1964) suggested that, at least in some species, the envelope originates as a result of a wound-healing response by the host to its serosa, which is stretched and damaged during the penetration of the acanthor from the lumen of the midgut. However, Robinson and Strickland (1969) injected hatched acanthors of Moniliformis moniliformis directly into the haemocoele of cockroaches and demonstrated that envelope formation does not require parasite penetration of the gut serosa. Earlier workers attributed the envelope origin to the parasite or at least suggested that it was not formed as a result of haemocyte encapsulation (e.g.Moore, 1946). J. Lackie and Rotheram (1972) and Rotheram and Crompton (1972) concluded, after electron microscopical analysis of the developing envelope, that it comprises a membranous coat formed from microvillar projections of the early acanthellar tegument which later separated from the parasite's

Figure 1.2. Morphological characteristics of the cystacanth envelope

(a) Light micrograph of an enveloped cystacanth

Bar, 10µm.

(b) Transmission electron micrograph showing a section through an enveloped cystacanth.

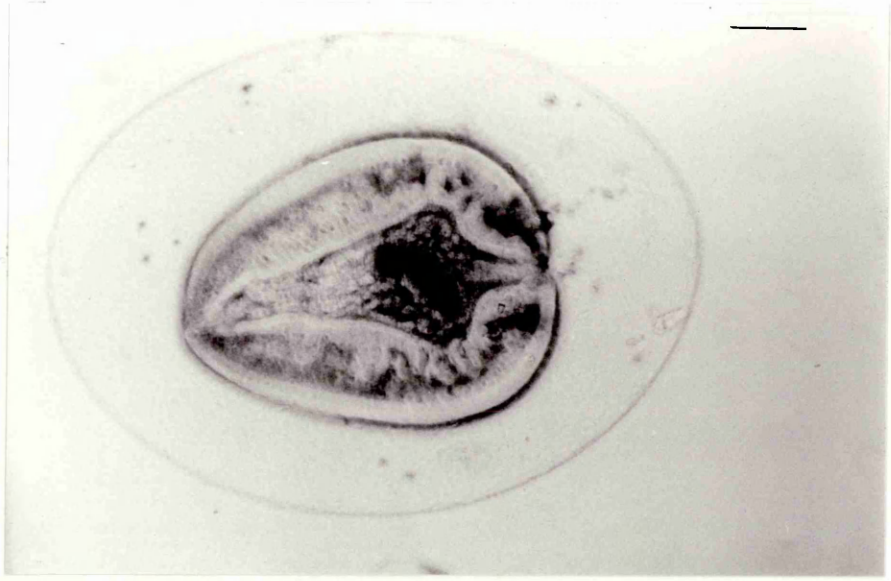
Key : E - Envelope.

L - Larva.

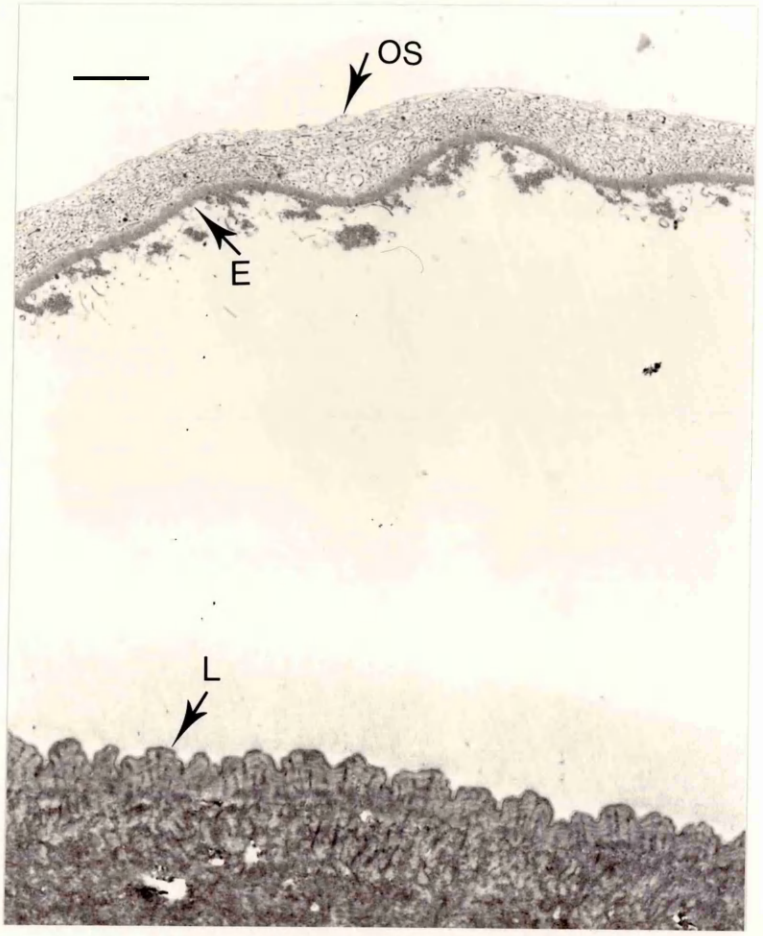
OS - outer surface

Bar, 1µm.

(a)



(b)



surface and compressed to form a trilaminate structure as visualised by electron microscopy (J. Lackie, 1973). Strong evidence from in vitro culture of hatched acanthors, that the envelope was acellular and of mainly parasite origin was provided by J. Lackie (1973). Microvillar projections were seen on the developing acanthellae, which Lackie concluded were involved in envelope formation. However, some reports still suggest the envelope is a cellular structure derived from host haemocytes (Ravindranath and Anantaraman, 1977), although, in view of the results of J. Lackie (1973) they would seem to be erroneous. The possibility, however, that the host contributes to the structure of the envelope has not yet been ruled out.

1.6.3.4. Ultrastructure and composition of the envelope (Figure 1.2.)

The outermost thin, amorphous layer of the cystacanth envelope has been shown to stain positively with the cationic dye, Alcian Blue 8GX (A. Lackie, 1986a) and may therefore, contain glycosaminoglycans which are polyanionic linear carbohydrate polymers and components of all known connective tissues (Ashhurst, 1985). The middle layer of the envelope contains numerous, loosely packed, vesicular bodies and may represent a highly folded membranous structure which appears vesicular in cross-section. The "vesicles" are bounded by double contoured membranes, resembling plasma membranes, and are devoid of electron-opaque contents. The innermost layer consists of a distinct electron dense amorphous region about 0.2μm thick. The envelope of the cystacanth is separated from the larva which is within a viscous, electron-transparent, material which has a fine granular appearance as seen by electron microscopy. (J. Lackie and Rotheram, 1972; J. Lackie, 1973).

The composition of the envelope, in terms of protein, carbohydrate and lipid each as a percentage of the dry weight, was determined by J. Lackie (1973). It was found to contain 65% protein, 25% lipid and 10% carbohydrate, the absolute proportions of which did not change during larval development. No nucleic acid could be detected in the envelope which supports the view that it is acellular (J. Lackie, 1973).

1.6.3.5. Protective role of the envelope

The envelope seems to be associated with protection from encapsulation, since if de-enveloped larvae of Moniliformis are injected into uninfected cockroaches, then the parasites are encapsulated (Robinson and Strickland, 1969). Also, Holt and Lackie (1986) demonstrated that if cockroaches, containing Moniliformis cystacanths, were also injected with hatched oncosphere larvae of Hymenolepis diminuta (Cestoda), then developing tapeworm larvae were occasionally found within the envelope of Moniliformis cystacanths, apparently having been protected from encapsulation, which is the normal fate of oncospheres in Periplaneta.

Mechanism(s) of protection

The question arises of how the Moniliformis envelope affords protection against encapsulation. It could be argued that it is not the envelope per se that is protective, but that developing larvae synthesise and release some factor(s) which inhibits the normal encapsulation response. In oral infections of cockroaches, only about 15% of the administered mature eggs of Moniliformis develop to cystacanths (J. Lackie, 1973), and encapsulated and melanized acanthors are found attached to the haemocoelic surface of the cockroach midgut (Rotheram and Crompton,

1972). Other acanthors no doubt fail to hatch or burrow through the gut wall, but the percentage which develop to cystacanths is fairly constant. When subsequent doses of eggs are given to a previously infected host then the same proportion of the oral dose develops to cystacanths in this host, thus there is neither diminished nor enhanced susceptibility to challenge infection (J. Lackie, 1972). This suggests that the immune system of the cockroach host is not suppressed, since if this were the case, a larger proportion of the subsequent doses might be expected to survive. It also suggests that the cockroach cannot be immunised against Moniliformis infection. It would appear then, that the envelope allows the parasite to achieve non-recognition in the cockroach. The envelope may adsorb host molecules which mask parasite determinants and present a "coat" of "self" molecules to the haemocytes or the host may be involved in the production of part of the envelope such that the finished structure resembles "self" tissue and is not recognised by haemocytes. Alternatively, the envelope may be inherently similar to host tissues. Antigenic similarity, in this context refers to the discrimination of differences in histocompatibility by haemocytes, which may involve differences in non-specific parameters, including surface charge (Lackie, 1980; see section 1.6.2.2.1.).

Lackie and Lackie (1979) have provided some evidence that the envelope might be totally of parasite origin. Hatched acanthors were injected directly into the haemocoele of the locust Schistocerca, where they grew and developed to cystacanths. If the envelope had incorporated host molecules, either passively or by active involvement of host tissues in envelope synthesis, then, like host's tissues, enveloped cystacanths should have been encapsulated when transplanted into the haemocoele of Periplaneta. However, locust-derived Moniliformis cystacanths remained

unencapsulated after transfer, which might suggest that it is an intrinsic property of the parasite-derived envelope that prevents haemocytes adhering to it. Alternatively, it could be argued that, although locust macromolecules are components of the envelope, they are not recognised as "non-self" in the cockroach.

Since both the host connective tissue and outermost surface of the envelope appear to contain glycosaminoglycans, and in view of the apparent importance of the physicochemical properties and carbohydrate composition of a foreign surface in eliciting a haemocytic response in Periplaneta, it is possible that the overall surface charge, hydrophobicity and certain specific molecular configurations of the cockroach connective-tissue layer and parasite envelope surface are very similar. This may be enough to prevent haemocytes recognising the envelope as "non-self" and thus explain the mechanism of protection afforded by the envelope.

1.7. Aims of the project

The aims of this project were :

(1) To determine if the envelope of Moniliformis moniliformis larva contains glycosaminoglycans and to identify the type(s) of molecule present in this structure and in the connective-tissues and haemocytes of the intermediate host, Periplaneta americana.

(2) To study the biochemical composition of the other envelope macromolecules and determine some aspects of their relative organisation within the envelope.

(3) To study the interaction of the envelope with host plasma proteins and assess the extent of serological cross-reactivity between epitopes from envelope macromolecules and host tissues.

Chapter 2

Materials and Methods

2.1. Materials

2.1.1. Animals

Stocks of Periplaneta americana (Glasgow University strain) are maintained in the Zoology Department, Glasgow University. Adult rats were obtained from the animal house of the Zoology Department, Glasgow University. The life cycle of Moniliformis moniliformis (syn. dubius) (a strain obtained originally from the laboratory of Dr. S.J. Edmonds, Australia) is maintained in cockroaches and rats in the Zoology Department, Glasgow University. Cystacanth larvae (the stage capable of infecting the definitive host) were used in this study except where specifically stated. New Zealand white rabbits were bred and maintained in the animal house, Biochemistry Department, Glasgow University.

2.1.2. Chemicals

Absolute ethanol was obtained from James Burroughs Ltd., Essex, England. Silica gel plates (plastic backed; 20cm x 20cm) were obtained from Camlab, Cambridge, England. Phenol was obtained from Formachem, Strathaven, Scotland. Bromophenol blue, Coomassie Brilliant Blue R250, Napthalene black : George T. Burr Ltd., London, England. Ethylenediamine tetra-acetic acid (EDTA) and 2-mercaptoethanol : Koch-Light Laboratories, Colnbrook, England. Ecoscint scintillation fluid : National Diagnostics, New Jersey, U.S.A. n-Butanol, chloroform, methanol : May and Baker Ltd., Dagenham, England. 1,1'-di(octadecyl)-3,3,3',3'-tetramethylindocarbocyanine perchlorate (C_{18}diI), 1,1'-di(hexadecyl) -3, 3, 3',3'-tetramethylindocarbocyanine perchlorate (C_{16}diI), and 5-N-(octadecanoyl)-aminofluorescein (C_{18}AF) : Molecular Probes, Oregon, U.S.A.

Molecular weight standards for SDS-PAGE, PD10 Sephadex G25(M) columns (1 x 12cm) and Sephadex G75(M) : Pharmacia Ltd., Milton Keynes, England. Iodogen ™ (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril) : Pierce Chemical Co. (U.K.) Ltd., Cambridge, England. 1,9-Dimethylmethylene Blue and N-(2-Hydroxyethyl)-piperazin-2-ethanesulphonic acid (HEPES) : Serva Biochemicals, Heidelberg, F.R.G. Agarose (medium E.E.O.), acid molybdate spray reagent, ceramides (types III and IV), cerebrosides, cholesterol, cholesterol oleate, chondroitin sulphate (B and C isomers), D-glucuronic acid, D-glucuronolactone, heparan sulphate, hyaluronic acid, isatin, manganese chloride, α-methyl-D-glucoside, merocyanin 540(Mc540), Nonidet P40(NP40), Orcinol-ferric chloride spray reagent, palmitic acid methylester, O-phenylene diamine (OPD), phenylmethylsulphonyl fluoride (PMSF), L-α-phosphatidyl choline (PC), L-α-phosphatidyl ethanolamine (PE), L-α-phosphatidyl serine (PS), sphingomyelin, sodium azide, sodium borohydride, sodium deoxycholate, sodium salicylate, stearic acid methyl ester, N,N,N',N'-tetramethylethylene diamine (TEMED), N-tosyl-L-lysine chloromethyl ketone (TLCK), L-1-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK), Triton X100 and Tween 20 were obtained from Sigma Chemical Co., Poole, England. All other chemicals were obtained from the British Drug House (BDH) Ltd., Poole, England.

2.1.2.1. Proteins and enzymes

Deoxyribonuclease I (E.C.3.1.4.5; 2000 Units/mg) and ribonuclease I (E.C.3.1.4.2.2; 50 Units/mg) were obtained from BDH Ltd., Poole, England. Bovine serum albumin (BSA), collagenase (E.C. 3.4.24.3; 800-1600 Units/mg), Concanavalin A, FITC-Concanavalin A, haemoglobin, pronase, pepsin (E.C.3.4.23.1; 2500-3500 Units/mg), pepstatin A, peanut agglutinin were obtained from the Sigma Chemical Co. Ltd., Poole, England. Fluoresceinated (FITC-) peanut agglutinin, FITC-wheat germ agglutinin and FITC-

Dolichos biflorus agglutinin were a gift from Dr. A.N. MacGregor, Biochemistry Department, Glasgow University.

2.1.2.2. Radiochemicals

L-[^{35}S]-methionine (1100 Ci/mmol), L-[2,3,4,5-^{3}H]-proline (108 Ci/mmol), D-[6-^{3}H]-glucosamine hydrochloride (40 Ci/mmol), $Na_2{}^{35}SO_4$ (25-40 Ci/mmol) and carrier-free Na^{125}I (100 mCi/ml) were purchased from Amersham International plc., Amersham, England.

2.1.2.3. Immunochemicals and reagents

Freund's adjuvants were obtained from Difco Laboratories Ltd., Colnbrook, England. Nitrocellulose paper (0.45µm pore size) and foetal calf serum were purchased from Gibco-BRL, Paisley, Scotland. Anti-rabbit gamma globulin, FITC- or horse radish peroxidase-conjugated and Staphylococcus aureus (Cowan I strain) cell suspension were supplied by Sigma Chemical Co., Poole, England. Normal rabbit serum was obtained from the Scottish Antibody Production Unit, Carluke, Scotland.

2.1.2.4. Photographic materials

Kodak X-Omat S film was obtained from Kodak (U.K.) Ltd., London, England. Intensifying screens (18 x 24cm) were purchased from DuPont Cronex through MAS Ltd., Stirling, Scotland. Plastic intensifying-screen holders were purchased from Anthony Monk (England) Ltd., Sutton-In-Ashfield, England.

2.1.3. Solutions

2.1.3.1. Citrate-EDTA (Calcium-, Magnesium- free) anticoagulant solutions

	Cockroaches		Locusts	
	g/L	mM	g/L	mM
Tri-sodium citrate	8.82	32.0	8.82	32.0
Citric acid	5.46	26.0	5.46	26.0
NaCl	5.30	90.6	3.74	64.0
D-glucose	18.0	100.0	18.0	100.0
EDTA	3.72	10.0	3.72	10.0
pH	4.6		4.6	
Osmolarity (mOsmol)	350		305	

2.1.3.2. HEPES-buffered saline (HBS)

	g/L	mM
NaCl	9.00	154.0
KCl	0.74	10.0
$MgCl_2.6H_2O$	0.41	2.0
$CaCl_2.6H_2O$	0.44	2.0
HEPES	2.38	10.0

pH adjusted to 7.2 using 5M NaOH solution.

2.1.3.3. Proteinase inhibitor cocktail stock solution (X100)

Phenylmethylsulphonyl fluoride (PMSF)	1.0 M
N-tosyl-L-Lysine chloromethyl ketone (TLCK)	2.0 mg/ml
L-1-tosylamide-2-phenyl-ethyl chloromethylketone (TPCK)	0.5 mg/ml
Pepstatin A	0.1 mg/ml

The inhibitors were made up, at the above concentrations, in absolute ethanol, to give a 100X concentrated stock solution which was stored at

2.1.3.4. Medium D73 (modification of Quiot's recipe; see Lackie and Huxham's medium, in Crompton and Lassiere, 1987).

Amino acids (obtained from Sigma Chemical Co., Poole, England)

	mg/200 ml
L-arginine hydrochloride	60
L-aspartic acid	50
L-cysteine hydrochloride	52
L-glutamic acid	190
glycine	130
L-histidine hydrochloride	52
L-isoleucine	48
L-leucine	50
L-methionine	100
L-phenylalanine	40
L-proline	134
L-serine	16
L-threonine	40
L-tryptophan	40
L-tyrosine	20
L-valine	30

Salts	mg/200ml	Others	mg/200ml
NaCl	1200	Cyanocobalamin (Vitamin B_{12})	$1x10^{-3}$
KCl	160	Gentamicin sulphate	40
$MgSO_4$	36	D-glucose	200
Na_2HPO_4	10	Penicillin (sodium salt)	0.3
KH_2PO_4	12	Streptomycin sulphate	20
$CaCl_2.2H_2O$	28	HEPES	952
		Flow BME Vitamin solution (X100)	1.0 ml
		Flow medium 199 (X10)	8.0 ml

The pH was adjusted to 7.2 with concentrated NaOH (5M), made up to 200ml with distilled water and sterilised by filtration.

2.2 Methods

2.2.1. Biological Methods

2.2.1.1. Collection of parasites

Carbon dioxide-anaesthetised adult cockroaches were decapitated and placed in a small glass petri dish containing HBS. Parasites were collected after opening and flushing out the abdominal cavity with insect saline (HBS) using a Pasteur pipette. The larvae were then transferred to a conical centrifuge tube and washed at least five times in HBS to remove adherent haemolymph components and any contamination by gut contents.

2.2.1.2. Collection of envelopes

Washed, enveloped larvae were transferred to a 3cm. diameter glass petri dish containing insect saline with proteinase inhibitors added. Envelopes were removed by passing the larvae through a 21 gauge syringe needle attached to a 5ml syringe. Envelopes were collected using a Pasteur pipette and washed, at least five times, by centrifugation using HBS/proteinase inhibitor solution. The larval bodies were retained and washed to remove any remaining envelope fragments, then extracted as described below or homogenised in 10mM Tris HCl pH 7.2, 0.02% (w/v) sodium azide containing proteinase inhibitors.

2.2.1.3. Infection of locusts with hatched acanthors

Adult, male Schistocerca gregaria were infected by intra-abdominal injection of acanthors hatched in vitro in 0.4M $NaHCO_3$ solution (Edmonds, 1966); thirty acanthors were injected per locust and allowed to develop for 6 weeks at 28 ± 1°C. Cystacanths were collected by dissection under HBS of CO_2-anaesthetised animals by a similar procedure to that described in section 2.2.1.1.

2.2.1.4. In vivo metabolic labelling of cockroach haemocytes

To stimulate haemopoesis, adult male, cockroaches were bled 48 and 24 hr. prior to injection, by cutting the tip off one antenna and squeezing out a drop of blood (Lackie, unpub.). Pre-bled cockroaches were then injected on consecutive days with 50μCi of $Na_2{}^{35}SO_4$ in 50μl of sterile HBS and maintained at 28 ± 1°C. Twenty-four hours after the second administration of radioisotope, haemolymph was collected from the animals, as described in section 2.2.1.5. and the washed cell pellet obtained by centrifugation of the haemolymph/anticoagulant mixture was extracted as described in section 2.2.1.8.

2.2.1.5. Haemolymph collection

Anaesthetised animals were cooled to 4°C then injected, intrahaemocoelically with approximately 200µl of cold citrate EDTA anticoagulant solution, pH 4.6. The left hind limb was then cut off and haemolymph/anticoagulant mixture collected using a Pasteur pipette. Collection was assisted by gentle, alternate, squeezing of the animal's abdomen and thorax. Diluted haemolymph was then pooled, from several cockroaches, into 5 volumes of anticoagulant and centrifuged for 5 min. at 250xg, 4°C, to pellet the cells. The supernatant constituted the diluted plasma component and macromolecules were recovered by ethanol precipitation (4.5 vol. ethanol containing 1.4% (w/v) potassium acetate, 48hr. at -20°C). The cell pellet was retained for extraction, haemocyte culture or used to prepare a cell lysate.

2.2.1.6. Preparation of a haemocyte lysate

The initial cell pellet, obtained as described in section 2.2.1.5., was washed three times in anticoagulant solution by centrifugation, then washed a further two times in 10mM Tris HCl pH 7.2, containing proteinase inhibitors. A cell lysate was obtained by resuspending the final cell pellet in the above buffer containing 1% (w/v) SDS. After incubation for 2 hr. at 37°C, with occasional gentle shaking, the sample was centrifuged (11000xg; 2 min. at room temperature). The supernatant obtained constituted the cell lysate. This was aliquoted and stored at -20°C or analysed immediately for protein content (section 2.2.3.1.), and subsequently by SDS-PAGE as described in section 2.2.3.9.

2.2.1.7. Cockroach haemocyte culture

The cell pellet, formed after centrifugation of the anticoagulant and haemolymph mixture, was resuspended in sterile anticoagulant solution and washed a total of three times by centrifugation. The cell pellet was then resuspended in D73 medium and washed a further three times in this medium by centrifugation. The cells were resuspended in the same medium to give a density of 1 x 10^6 cells/ml medium and then plated out into 3cm. diameter Nunc plastic petri dishes (1ml/dish) under sterile conditions. After 60 min. at room temperature, the medium was replaced with 1ml/dish of sterile radiolabelling medium (5% (w/v) FCS in low sulphate D73 medium, containing 30μCi/ml $^{35}SO_4^{2-}$). The cells were maintained at 28 ± 1°C in this medium before harvesting.

2.2.1.8. Extraction of haemocytes

The culture medium was decanted and the petri dishes were washed briefly with 10mM Tris HCl, pH 7.2, containing 0.02% (w/v) sodium azide, at 4°C. The cell monolayer was removed by scraping the dish with a silicone rubber policeman into a solution containing 5mg/ml pronase, 0.1% (w/v) sodium deoxycholate, 0.02% (w/v) sodium azide in 10mM Tris HCl pH 7.2. The combined cell extract from each dish was then sonicated briefly, 4 x 15 sec., at low frequency on a MSE sonicator before being incubated for 48hr. at 37°C. Fresh enzyme was added after the first 24hr. After the end of the proteinase treatment, the cell extract was centrifuged (11000xg; 2 min, 22°C) to remove insoluble material, then 100μg each of chondroitin sulphates B and C, heparan sulphate and 50μg hyaluronic acid were added to the supernatant as carriers for metabolically labelled glycosaminoglycans (GAG). Macromolecules were then precipitated by the addition of 4.5 volumes of ethanol containing 1.4% (w/v) potassium acetate with incubation

for 48hr. at -20°C. The precipitated GAG were collected by centrifugation at 10000xg for 30 min. at -5°C, in a Sorvall SS34 rotor. The pellet was solubilised in 1.0ml of 2M NaCl solution and ethanol precipitation repeated. The final pellet was air-dried, then solubilised immediately in a 2% ethanol solution and stored, aliquoted, at -20°C. Small aliquots were counted for ^{35}S incorporation in 5ml Ecoscint on a Beckmann L56800 liquid scintillation spectrometer. The dialysed culture medium contained negligible amounts of ^{35}S radioactivity. The cell pellet obtained from *in vivo* metabolic labelling of cockroaches haemocytes (section 2.2.1.4.) was also extracted as described above.

For unlabelled cells, a nuclease digestion step was included. After proteinase treatment, the sample was heated at 100°C for 2 min, to inactivate the pronase, then centrifuged (10000xg for 10 min. at 22°C). A small aliquot of mixed nuclease stock solution (10mg/ml DNase I, 10mg/ml RNase A in 0.5M citrate buffer, pH 5.0) was added to the supernatant to give a final concentration of 1mg/ml for both nucleases. After incubation for 24hr at 37°C the sample was heat-treated and centrifuged as described above. The supernatant was then ethanol precipitated and the final pellet analysed by agarose gel electrophoresis.

2.2.1.9. Preparation and extraction of cockroach midgut

Midguts were dissected from CO_2-anaesthetised adult cockroaches and, after withdrawing the peritrophic membrane from their lumens with watchmakers forceps, were washed five times in HBS, cut into small pieces using scissors then incubated for 1-2 hr with occasional agitation, in 0.35M citric acid (pH 2.5) to remove epithelial cells (Ryerse and Reisner, 1985), washed by centrifugation in first instance, 1% (v/v) Triton X100 in 10mls Tris HCl, pH 7.2 and then in 10mM Tris HCl, pH 7.2, then incubated for 48hr at 37°C with 5mg/ml pronase in 10mM Tris HCl pH 7.2, 0.02% sodium azide. Glycosaminoglycan extraction was continued as described above and

the final ethanol precipitate was retained for analysis by agarose gel electrophoresis.

2.2.1.10. Preparation and extraction of cockroach Malpighian tubules

Malpighian tubules were dissected from CO_2-anaesthetised adult cockroaches then washed five times in HBS. The tubules were then extracted as described above and analysed by agarose gel electrophoresis.

2.2.1.11. In vitro cultivation of parasite larvae

Larvae were collected as described above (see section 2.2.1.1.). They were washed twice in sterile HBS, transferred to sterile plastic conical centrifuge tubes, washed three times more in sterile medium containing 5% (v/v) FCS, then transferred to clean tubes before washing a further three times in FCS/medium. Larvae were then transferred to the wells of a sterile Linbro tray and maintained at 28°C in 1-2ml of D73 medium containing 5% (v/v) FCS at a density of 25-50 larvae/well depending on developmental stage. The medium was changed every 24hr. for up to 10 days, after which the medium was replaced with 1-2ml sterile radioisotopic labelling medium (5% (v/v) FCS, low proline D73 medium containing 10μCi/ml L-[2,3,4,5,-^{3}H]-proline). After 24hr the medium was decanted, extensively dialysed against 10mM Tris HCl, pH 7.2, 0.02% (w/v) sodium azide, then against four changes of distilled water, and finally lyophilised. Parasites were collected, their envelopes removed as described above (section 2.2.1.2.) and both envelopes and larvae extracted with 1% (w/v) SDS, 10mM Tris HCl, pH 7.2, containing proteinase inhibitors. The residue after extraction was collected, for both samples, as a pellet by centrifugation (bench top microfuge, 2 min) and re-extracted with 1% (w/v) SDS, 5% (v/v) 2-ME in Tris buffer. Aliquots of each sample were analysed for ^{3}H-proline and the protein content determined as described in section 2.2.3.1. Subsequent analysis was by SDS-PAGE/fluorography.

2.2.2. Microscopical Methods

2.2.2.1. Fluorescent lipid probes

The parasites were labelled with fluorescent lipid analogues by incubating them for 15 min. at 37°C in 1.0ml HBS to which 5µl of ethanol containing 10µg of the probe (Mc540, C_{18}diI, C_{16}diI or C_{18}AF) had been added. After washing five times in HBS, the parasites were then mounted within squares of silicone grease on glass slides for microscopic examination.

2.2.2.2. Labelling of cystacanths with fluorescent lectins

Enveloped cystacanths were incubated for 30 min. at 37°C in HBS containing 10% FCS and 20µg/ml FITC-lectin. In control experiments, the mixture also contained 0.2M inhibitor sugar appropriate for each particular lectin. After incubation, the parasites were washed five times in 10% (v/v) FCS in HBS, then mounted within squares of silicone grease on glass slides for microscopic examination.

2.2.2.3. Fluorescence Microscopy

Fluorescence photomicrographs were taken with a Leitz Ortholux II fluorescent microscope, a standard camera attachment and Kodacolor II 200 ASA daylight film. Fluorescence filter blocks I2 (for fluorescein) and N2 (for diI's and Merocyanin 540) were used within a PLOEMOPAK^R 2.2. fluorescence illuminator microscope attachment. Fluorescence was quantified using a digital-readout photomultiplier attachment.

2.2.2.4. Transmission electron microscopy

Enveloped cystacanths of Moniliformis were examined by transmission electron microscopy (TEM) after :

(i) De-lipidation, by extraction with chloroform methanol (1:2, v/v) for 2hr. at room temperature.

(ii) Extraction with 1% (w/v) SDS in 10mM Tris HCl pH 7.2 containing proteinase inhibitors and 0.02% (w/v) sodium azide, overnight at room temperature.

(iii) Extraction overnight at room temperature with 10mM Tris HCl pH 7.2 containing proteinase inhibitors and 0.02% (w/v) sodium azide.

The extracted larvae and freshly collected, untreated, cystacanths, were washed five times in 0.5M sodium cacodylate buffer, pH 7.4, containing 0.15M sucrose, then fixed overnight at 4°C in 2.5% (v/v) glutaraldehyde made up in the same buffer. Subsequent fixation steps and sectioning were performed by Iain More and Tom Downie, Pathology Department, Western Infirmary, Glasgow.

2.2.3. Chemical Methods

2.2.3.1. Protein determination

Protein content was determined by the method of Lowry et al. (1951) incorporating the modification of Markwell et al. (1978) for the determination of SDS-solubilised polypeptides. All samples were made up in a total of 100µl in which the SDS concentration did not exceed 0.2% by weight. One millilitre of reagent was added to each sample with gentle vortexing. After 15 min., 90µl of Folin reagent:H_2O (1:1, v/v) mixture was added to each sample with subsequent incubation for 45 min. at room temperature. The absorbance at 750nm was read, for all samples, on a LKB

ultraviolet spectrophotometer. Calibration curves were constructed from a stock solution of bovine serum albumin (Sigma, Grade IV) made up at 10mg/ml in distilled water and stored, aliquoted, at -20°C.

2.2.3.2. Determination of total neutral sugar content

Lyophilised cystacanth envelopes and larval homogenates were weighed in tinfoil weigh-boats on an electrobalance. The total neutral sugar content of triplicate samples was then determined using the phenol/sulphuric acid method of Dubois et al (1956). Briefly, samples were suspended in 500µl of water in an acid-washed glass tube to which was added 20µl of 80% (w/v) phenol followed by the rapid addition of 2ml concentrated sulphuric acid (specific gravity 1.84) with gentle vortexing. After 30 min. at room temperature, the absorbance at 485nm was measured for each sample, the coloration being due to the formation of a complex between phenol and the furfural derivatives of the sugar molecules. Calibration curves were constructed using a 1mg/ml stock solution of D-glucose in water. The result of each determination was expressed as a percentage of the total weight of each sample.

2.2.3.3. Gravimetric estimation of lipid in envelopes and worm bodies

Lyophilised cystacanth envelopes and larval homogenates were used. Lipids were extracted by the method of Bligh and Dyer (1959) (section 2.2.3.4.) and total lipid extracts for each sample were dried under nitrogen in pre-weighed, chloroform-washed, Eppendorf tubes, which were then re-weighed on an electrobalance. Samples were determined in triplicate and values expressed as a percentage of the total weight of each sample.

2.2.3.4. Lipid extraction from envelopes and larval homogenates

Lipids were extracted by the method of Bligh and Dyer (1959). Briefly, envelopes or larval body homogenate samples in 100-200µl HBS were extracted for 2hr at room temperature with 375-750µl of methanol:chloroform (2:1, v/v) in a chloroform-washed Eppendorf tube. After centrifugation, the pellet was re-extracted with methanol:chloroform:water (2:1:0.8, v/v/v) for 2hr. at room temperature. The extracts were combined and 250-500µl each of chloroform and distilled water added to the crude extract. After centrifugation (500xg, 5 min.) the lower chloroform layer was removed, evaporated under nitrogen and re-solubilised in a small volume of chloroform:methanol (2:1, v/v). The lipid extracts were analysed immediately by TLC or stored under nitrogen at -20°C.

2.2.3.5. Thin-layer Chromatography

2.2.3.5.1. Lipids

The lipid extracts from envelopes and larval bodies were subjected to one-dimensional thin-layer chromatography (TLC) on silica G plates (10 x 10cm) in a solvent system composed of chloroform:methanol:water:acetic acid (65:25:4:1, v/v/v/v). After chromatography the plate was air-dried then developed in a tank containing iodine crystals. Lipids were identified as brown spots. The following reference standards were included : cholesterol, cholesterol oleate, bovine brain cerebrosides and ceramides (types III and IV), L-α-phosphatidyl choline, L-α-phosphatidyl ethanolamine, L-α-phosphatidyl serine, a mixture of phosphatidyl inositides, palmitic acid methylester, stearic acid methylester and sphingomyelin. All standards were made up at 10mg/ml in

chloroform:methanol (2:1, v/v) and stored at -20°C under nitrogen. Five micrograms of each standard was loaded for each plate run. Group-specific identification of the unknown lipids was attempted by comparison of their R_f values with the standards and by staining the plates with the following spray reagents:

(i) Acid molybdate spray reagent (Sigma Chemical Co.) for lipids containing ester-linked phosphate. Phospholipids were evident as blue spots.

(ii) Ninhydrin, 0.25% (w/v) in acetone for lipids containing a primary amino group (proteolipids, amino sugars, phosphatidyl serine and phosphatidyl ethanolamine) which gave a yellow spot.

(iii) α-Naphthol 0.5% (w/v) in 50% methanol, followed by 95% concentrated sulphuric acid with heating (100°C, 15 min.) for glycolipids, which appear as pink spots.

(iv) Orcinol-ferric chloride spray reagent (Sigma Chemical Co.) for glycolipids which appear as red spots.

(v) Acetic acid:concentrated sulphuric acid in water with heating (120°C; 2 min.) for sterols which appear as brown spots.

2.2.3.5.2. Two-dimensional thin-layer chromatography of amino acids

The pooled fractions, representing the L-^{3}H-proline peak at the void volume (V_o) of a Sephadex G75 column, used to fractionate an aliquot of dialysed, concentrated, culture medium (section 2.2.3.16.) were collected, dialysed against distilled water, then lyophilised. The dried material was taken up in 1.0ml of 6N HCl and hydrolysed, in a sealed tube under vacuum for 24hr. at 110°C. The sample was dried in a desiccator as described in section 2.2.3.7., then finally solubilised in 50µl of a aqueous 10% (v/v) isopropanol solution. The sample was then centrifuged

(11000xg; 2 min. at room temperature) and the supernatant subjected to two-dimensional TLC on silica G plates (10 x 10cm) in a solvent system composed of n-butanol:glacial acetic acid:water (65:15:25 v/v/v) in the first dimension and 80% (w/v) phenol in the second dimension. A small aliquot (5µl each) of standard solutions of L-proline and L-4-hydroxyproline, both at 10mg/ml were chromatographed in a separate plate. The dried chromatograms were stained using a 0.2% (w/v) solution of isatin in acetone; imino acids (proline and hydroxyproline) were evident as pink spots, with amino acids staining blue. Two pink-staining spots from the L-^{3}H-proline chromatogram were scraped into separate Eppendorf tubes each containing 500µl of 10% isopropanol solution. After 10 min. at room temperature, the samples were centrifuged briefly and the supernatants counted for ^{3}H-radioactivity after transfer to separate vials each containing 4.5 ml of scintillation fluid.

2.2.3.6. Extraction of envelopes and larval bodies

Envelopes and larvae were extracted using the following solvents :

(i) 10mM Tris HCl, pH 7.2

(ii) 2M Urea in 10mM Tris HCl, pH 7.2.

(iii) 1% (w/v) SDS in 10mM Tris HCl, pH 7.2.

(iv) " " " + 5% (v/v) 2-ME in 10mM Tris HCl, pH 7.2.

(v) " " " /8M Urea " " " " " "

(vi) " " Triton X100 " " " " " "

(vii) " " " " /8M Urea " " " " " "

All solutions contained proteinase inhibitors (section 2.1.3.3.). Extraction was for 16-24hr. at room temperature on a rotator. In some cases there was sequential extraction of material e.g. 1% (w/v) SDS

extraction then re-extraction of the pellet, obtained after centrifugation, with the same solvent but with the addition of 5% (v/v) 2-mercaptoethanol (2-ME). Sometimes samples were boiled directly in the solvent then diluted by the addition of SDS-PAGE sample buffer (0.05% (w/v) Bromophenol Blue, 0.15M sucrose, 5% (v/v) 2-ME in 10mM Tris HCl, pH 6.8). Samples were analysed immediately by SDS-PAGE or stored at -20°C.

2.2.3.7. Automated amino acid analysis

Cystacanth envelopes and bodies were extracted with 1% SDS and then washed extensively in distilled water by centrifugation, then lyophilised. The dried samples were hydrolysed under nitrogen, in sealed tubes with 6N HCl at 110°C for a total of 72hr. on a heated block. The hydrolysates were sampled at 24, 48 and finally 72hr. then dried in a desiccator containing small beakers of concentrated sulphuric acid as desiccant and sodium hydroxide pellets to neutralise the hydrochloric acid. The samples were then dissolved in a small volume of 0.2N citrate buffer (pH 2.0) and aliquots (10-25µl) run on a LKB model 4400 automated amino acid analyser with a ninhydrin detection system. The identity of each amino acid was established by comparing elution times with those for standards and the amount of each amino acid was determined by comparison with the peak area of the norleucine internal standard, a known amount of which was added to each sample before hydrolysis.

2.2.3.8. Radio-iodinations (Fraker and Speck, 1978).

2.2.3.8.1. Whole larvae

De-enveloped and enveloped cystacanths or stage II acanthellae were washed in HBS then added to a glass tube containing 200µCi of carrier-free $Na^{125}I$, 200µM KI in HBS and 50µg of Iodogen dried down from dichloromethane

solution. The reaction was allowed to proceed for 15 min. then the larvae were removed to a conical glass tube and washed exhaustively in HBS to remove unbound radiolabel. The larvae were then extracted using 1% (w/v) SDS in 10mM Tris HCl pH 7.2 as described in section 2.2.3. In some cases, before iodination, larvae were extracted with 1M NaCl in 10mM Tris HCl pH 7.2, at 4°C. Macromolecules present in the salt extract (SE) were recovered by ethanol precipitation, in the presence of 10-20µg of BSA, and radio-iodinated as described below (2.2.3.8.2.).

2.2.3.8.2. Lectins, envelope extracts and cockroach plasma proteins

Proteins were labelled covalently with ^{125}I in a reaction mixture which consisted of 200µCi $Na^{125}I$, 50-500µg of protein, 200µM KI in HBS. For envelope proteins the reaction mixture also contained 0.1% (w/v) SDS. The reaction was allowed to proceed for 15 min. in a glass tube containing 50µg of iodogen dried down from dichloromethane solution. Unbound radiolabel was removed using a Sephadex G25(M) column (a commercially available Pharmacia PD10 column) previously equilibrated with insect saline for lectins or 0.1% SDS in 10M Tris HCl, pH 6.8, for envelope and plasma proteins.

2.2.3.9. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis (PAGE) was performed in slab gels under denaturing conditions in the presence or absence of 2-mercaptoethanol and urea at various concentrations of acrylamide using the discontinuous buffer system of Laemmli (1970).

2.2.3.9.1. Stock solutions

Acrylamide solution (50% w/v total; 48.75% (w/v) acrylamide, 1.25% (w/v) NN'-methylene bis acrylamide).

1M Tris HCl pH 6.8

1M Tris HCl pH 8.8

10% (w/v) SDS

Sample buffer (0.05% (w/v) Bromophenol Blue, 0.15M sucrose, 5% (v/v) 2-mercaptoethanol in 10mM Tris HCl pH 6.8).

Stock (5x concentrated) tank buffer - 9.6M glycine, 1.25M Tris, pH 8.3.

8M Urea.

2.2.3.9.2. Composition of SDS-polyacrylamide gels

Separating gel (volume 40ml)	Final polyacrylamide concentration		
Volume (ml)	7.5%	10%	15%
Stock acrylamide	6.0	8.0	12.0
1M Tris HCl pH 8.8	15.0	15.0	15.0
10% SDS	0.4	0.4	0.4
Water	17.7*	15.7*	11.7
1.5% ammonium persulphate (made up fresh just before use)	0.9	0.9	0.9
TEMED	0.025	0.025	0.025

Stacking gel (final volume 10ml)

Volume (ml)	Final polyacrylamide concentration	
	(1) § 3%	(2) ¶ 5%
Stock acrylamide	0.6	1.0
1M Tris HCl pH 6.8	1.25	1.25
Water	7.7*	7.3*
10% SDS	0.1	0.1
1.5% ammonium persulphate	0.35	0.35
TEMED	0.01	0.01

* In some cases the volume was made up to 40ml with a concentrated solution of deionised urea to give a final concentration of 4M urea in the gel.

§ (1) For 7.5% separating gels

¶ (2) For 10% " "

Gels were run at 50mA constant current for 4-6 hr, then stained in 0.1% (w/v) Coomassie Brilliant Blue R250 in 50% (v/v) methanol and 10% (v/v) acetic acid, then destained in 50% methanol, 10% glacial acetic acid. Collagen-like peptides were detected as pink bands in the gel by staining as above and destaining in 10% glacial acetic acid (McCormick et al., 1979).

2.2.3.10. Lectin overlay of gels and nitrocellulose blots

Lectin overlay was carried out on the stained and destained gels using a modification of the method of Tanner and Anstee (1976). Following glutaraldehyde fixation and borohydride reduction, gels were equilibrated with 120mM KCl, 20mM Tris HCl pH 8.0 containing 1mM $CaCl_2$, 1mM $MnCl_2$, 0.1% (w/v) haemoglobin, 1% (v/v) Tween 20, 0.05% sodium azide and incubated for 3 days at room temperature with shaking in 25-50ml of this buffer containing 2.5-5 x 10^6 c.p.m. ^{125}I-labelled lectin (section 2.2.3.). The gels were then washed extensively over 3-5 days using the same buffer, then dried and autoradiographed (section 2.2.3.). As a control for non-specific binding, non-glycosylated protein standards were always included in the gels along with glycoprotein standards. For nitrocellulose electroblots derived from the gels (section 2.2.4.) the same buffer system was used but incubation time with lectin was for 90 min. at 37°C after which the blot was washed extensively in buffer for at least 30 min. before being processed for autoradiography.

2.2.3.11. Autoradiography

Nitrocellulose lectin- or immuno-blots and dried SDS gels containing ^{125}I-labelled polypeptides were incubated, in the dark, at -70°C for various periods of time, with Kodak X-Omat S film in a film holder containing an intensifying screen. Films were then developed according to the manufacturer's instructions.

2.2.3.12. Fluorography (Chamberlain, 1979).

Agarose gels containing $^{35}SO_4^{2-}$-labelled molecules and SDS-gels containing ^{3}H-labelled polypeptides were washed for at least two hours under a continuous flow of tap water, then immersed in a 1M solution of sodium salicylate for 30 min. at room temperature to allow the salicylate scintillant to be absorbed into the gel matrix. After being dried onto Whatman No.3 filter paper the gels were exposed to X-Omat S film as described above (section 2.2.3.11.)

2.2.3.13. Peptide "Cleveland" mapping

Bands of interest were excised from dried polyacrylamide gels using a scalpel blade. Their position on the gel was determined using the autoradiograph obtained as described in section 2.2.3.12. The gel chips were then rehydrated by immersion in 200µl of 0.0125mM Tris HCl, pH 6.8, for 15 min. at room temperature. The filter paper, onto which the gel was dried, was carefully removed using watchmaker's forceps. The gel chips were then transferred to separate wells of an acrylamide gel (5% (w/v) acrylamide stacking gel; 15% (w/v) acrylamide separating gel) and a small volume of proteinase solution, containing 50ng proteinase in 10mM Tris HCl, pH 6.8, 0.005% bromophenol blue, 0.1% SDS, 0.3M sucrose, was added to each

well. The gel was run at 30mA until the dye front reached the stacking gel/separating gel interface. The power supply to the gel apparatus was then switched off for 30 min. to allow digestion to take place. Electrophoresis was then continued for a further 3-4 hr., after which the gel was dried down and developed for autoradiography as described in section 2.2.3.12.

2.2.3.14. Estimation of sulphated glycosaminoglycans (sGAG)

Sulphated glycosaminoglycans were estimated using the spectrophotometric assay of Farndale et al. (1986). Triplicate aliquots of sample, each in 100µl of distilled water, were mixed with 1.0ml of reagent to give a total volume of 1.1ml. The absorbance at 525nm was measured 15 sec. after sample-reagent mixing on a LKB ultraviolet spectrometer. Calibration curves were constructed from a stock solution of chondroitin sulphate B made up at 0.1mg/ml in distilled water.

Reagent : 3.04 g/l glycine, 2.37 g/l sodium chloride, 16 mg/l 1,9,-Dimethylmethylene Blue in 0.011M HCl.

2.2.3.15. Agarose gel electrophoresis

Glycosaminoglycan extracts and standards were electrophoresed on horizontal, 0.9% (w/v) agarose slab gels in 0.06M barbital buffer, pH 8.6, modified from the method of Dietrich et al. (1977). Electrophoresis was for 6-8 hr at 4°C or room temperature and 50mA constant current. Between 10-20µg of each GAG standard (chondroitin sulphates B and C, heparan sulphate) was loaded per gel.

2.2.3.15.1. Gel mixture (500ml)

Agarose (4.5g; specially purified for electrophoresis) was added to 500ml of 0.06M barbital buffer (pH 8.6) in a conical flask. After recording the total weight of the flask plus contents, the agarose was dissolved by heating the flask in a 100°C water bath for 15-20 min. The flask was then re-weighed and distilled water added to make up the original weight. About 50ml of this mixture was used to cast a slab gel of dimensions 12.5 x 11.5 x 0.3cm containing 16 wells, each with a maximum volume of approximately 30µl.

2.2.3.15.2. Electrophoresis buffer

Sodium diethylbarbiturate	10.3 g/l
Diethylbarbituric acid	1.84 g/l

The pH was adjusted to 8.6 using solid diethylbarbituric acid and the buffer made up to the required volume with distilled water.

2.2.3.15.3. Sample buffer

The sample buffer contained 0.01% (w/v) Bromophenol Blue, 0.15M sucrose in 0.06M barbital buffer, pH 8.6.

2.2.3.15.4. Gel stain and destain

Gels were stained for 2hr. at room temperature in 0.1% (w/v) Toluidine Blue in 25% (v/v) methanol, 10% (v/v) glacial acetic acid and destained in 5% (v/v) glacial acetic acid.

2.2.3.16. Chemical and enzymic treatments

2.2.3.16.1. Nitrous acid treatment of glycosaminoglycan extracts (Beeley, 1985).

Aliquots of GAG extracts from haemocytes, midgut and Malpighian tubules, as well as the heparan sulphate standard, were treated with nitrous acid, which was generated *in vitro* by mixing 100µl of each sample, in distilled water, with 50µl of a solution containing 0.1M $NaNO_2$, 0.5M acetic acid. After 4 hr. at room temperature, excess nitrous acid was destroyed by the addition of 50µl 0.5M NaOH. Control samples, containing no sodium nitrite, were treated in parallel with the same reagents. All samples were lyophilised and analysed by agarose gel electrophoresis.

2.2.3.16.2. Pepsin digestion of cystacanth envelopes (Miller, 1971).

One hundred cystacanth envelopes were collected as described before (section 2.2.1.2.), washed briefly in cold distilled water by centrifugation, then resuspended in 100µl of cold 0.5M acetic acid containing 1mg/ml pepsin. Samples were digested for 18 hr. at 4°C with constant stirring, frozen, then lyophilised. The dried material was solubilised in 50µl SDS-PAGE sample buffer (section 2.2.3.9.). Control samples contained heat-inactivated (60°C; 2 min.) pepsin.

2.2.3.16.3. Collagenase digestion of cystacanth envelopes (Bruckner *et al.*, 1985).

One hundred cystacanth envelopes were collected as described before, washed briefly in distilled water, by centrifugation, then resuspended in 60µl of 0.2% (v/v) Triton X100, 2mM $CaCl_2$, 0.1M Tris HCl pH 8.0 containing 150 units of bacterial collagenase enzyme. Samples were digested for 4 hr.

at 37°C with constant stirring. The reaction was stopped by adding 12µl of 5x concentrated SDS-PAGE sample buffer and heating the mixture for 2 min. at 100°C. Other digested samples incubated in parallel with the envelope samples contained an aliquot of cystacanth body homogenate which represented 50µg of larval protein. Control samples contained heat-inactivated (60°C; 2 min.) collagenase. All samples were analysed immediately by SDS-PAGE.

2.2.3.17. Gel filtration chromatography

Aliquots of dialysed, concentrated culture medium from *in vitro* metabolic labelling of cystacanths (section 2.2.1.11.), were chromatographed on a Sephadex G75(M) column (1 x 50cm) equilibrated with 10mM Tris HCl, pH 7.2 containing 0.02% sodium azide and proteinase inhibitors. The column was eluted with the same buffer at a flow rate of 0.5ml/min. and 80 x 1 ml fractions were collected. Radioactivity in each fraction was determined by liquid scintillation counting (0.5ml of each fraction added to vials containing 4.5ml scintillation fluid). Other aliquots of the culture medium preparation were incubated with 50 units of bacterial collagenase in a total volume of 200µl 0.1M Tris HCl, pH 8.0, 0.02% sodium azide for 24hr at 37°C, then chromatographed as described above.

2.2.4. Immunochemical Methods

2.2.4.1. Production and collection of antiserum against whole cystacanth envelopes

A 1ml suspension of envelopes from 100 cystacanths in HBS was mixed with an equal volume of Freund's complete adjuvant and sonicated. This

mixture was injected subcutaneously at ten sites (200µl/site) on the back of a New Zealand white rabbit. A booster inoculum, consisting of 100 envelopes in 2ml of HBS/Freund's incomplete adjuvant emulsion prepared as above, was given six weeks later.

The antiserum was collected by bleeding the rabbit from the ear vein 10 days after boosting. Approximately 20ml of blood was collected at each bleeding. Blood was allowed to clot at room temperature for 1hr and then at 4°C overnight. The serum was removed using a Pasteur pipette and centrifuged (500xg; 20 min) to remove any red blood cells. Serum samples were aliquoted and stored at -20°C.

The activity of the antiserum was determined by ELISA (section 2.2.4.3.), using a soluble extract of envelope macromolecules, and against enveloped parasites as measured by the indirect fluorescent antibody test (IFAT; section 2.2.4.2.).

Antisera, raised in rabbits against Periplaneta serum (a-PS), Schistocerca serum (a-LS) and against haemocytically-encapsulated Sepharose beads recovered from cockroaches (a-HC), were provided by Dr. A.M. Lackie, Zoology Department, Glasgow University.

2.2.4.2. Indirect fluorescent antibody test (IFAT)

Larvae were incubated in a 1:100 dilution of antiserum in HBS containing 10% FCS to reduce non-specific binding, for 30 min. at 37°C with occasional gentle agitation. The larvae were then washed 5 times in 10% FCS/HBS, and antiserum binding was visualised by incubation in medium containing a FITC-labelled sheep IgG fraction (1:500 dilution), from an antiserum produced against rabbit IgG. After 30 min. at 37°C unbound FITC-labelled IgG was washed off using 10% FCS/HBS solution, and the larvae were

examined on a Leitz Ortholux Fluorescent microscope (section 2.2.2.2.). Controls contained normal rabbit serum (pre-immune serum) or no first antiserum.

2.2.4.3. Enzyme linked immunosorbent assay (ELISA)

Antigen, made up to 100µl with phosphate buffered saline (PBS), was bound to the bottom of the wells of a Falcon microtitre plate by incubation at 37°C for 3hr. or 4°C overnight. Excess antigen was removed by rinsing the wells with 0.05% (w/v) Tween 20 in PBS (washing buffer). Residual binding sites were blocked by adding 200µl of washing buffer, containing 1mg/ml bovine serum albumin (BSA), to each well and incubating the plate for 2hr. at room temperature. The wells were rinsed with washing buffer, then serial dilutions of antiserum (1:50 to 1:6400), in 100µl washing buffer, added to the wells. After incubating for 1hr. at room temperature, the plate was washed 2x with buffer before adding 100µl of goat-antirabbit IgG gamma globulin conjugated to horse radish peroxidase (1:1000 dilution in PBS) to each well and incubating for a further 1hr. at room temperature. Excess conjugate was removed by washing the plate with buffer. The amount of peroxidase activity bound to the wells of the plate was determined spectrophotometrically using 100µl per well of a solution containing 0.04% (w/v) O-phenylene diamine (OPD), 0.04% (V/V) H_2O_2, 36mM citric acid and 128mM $Na_2(PO_4)_3$. After 30 min. in the dark at room temperature, the reaction was stopped by the addition of 50µl of 4N H_2SO_4 to each well. The absorbance at 492nm was determined for each well using a Titertek Multisvan spectrophotometer.

2.2.4.4. Immunoprecipitation

Immunoprecipitation was performed on various envelope and larval extracts and cockroach plasma proteins. After iodination of the extract and removal of free label (section 2.2.3.8.), a small aliquot of each

sample, containing 100-200,000 c.p.m., was diluted to 1ml in 0.5% (v/v) Nonidet P40, 0.5M NaCl, 10mM Tris HCl pH 7.2 and pre-absorbed with 50µl of a 10% (v/v) suspension in NP40 buffer, of heat-killed S. aureus (Cowan I-strain) for 30 min. at 4°C on a rotator. After centrifugation (benchtop microfuge; 11000xg, 2 min. at 4°C) the supernatant was removed and divided into 2 x 500µl aliquots, and 5µl of the appropriate antiserum added to one aliquot with 5µl pre-immune serum or normal rabbit serum (NRS) added to the other. After 30 min. at room temperature on a rotator, immune complexes were precipitated by the addition of 50µl of 10% S. aureus suspension and incubation for 1hr. at 4°C on a rotator. Precipitates were collected by centrifugation and washed, at room temperature, by centrifugation in NP40 buffer, SDS buffer (0.5% (w/v) SDS in NP40 buffer) and finally in 10mM Tris HCl pH 7.2. Precipitates were stored at -20°C or analysed immediately by SDS-PAGE.

2.2.4.5. Immunoblotting

Proteins were electrotransferred from unstained SDS-gels to nitrocellulose paper in a Bio-Rad Trans-Blot™ cell containing 3 litres of 0.02% SDS, 20% (v/v) methanol, 25mM Tris, 192mM glycine, pH 8.3. The electroblotting was performed at 40mA overnight or for 3hr. at 400mA. After electrophoresis, the nitrocellulose paper was removed and a test strip, containing Pharmacia molecular weight standards, stained for 2 min. in 0.1% (w/v) amido black, 45% methanol, 10% glacial acetic acid, to check the efficiency of transfer. The strip was destained in 45% methanol, 10% glacial acetic acid. The remainder of the nitrocellulose paper was placed in 0.15M NaCl, 0.5% (v/v) Tween 20, 20mM Tris HCl pH 7.2, 0.02% (w/v) sodium azide (incubation buffer) overnight at 4°C or for 3hr at 37°C. The nitrocellulose paper was then incubated for 90 min. at 37°C in a 1:100

dilution of the appropriate antiserum in incubation buffer containing 5% (v/v) heat-inactivated FCS or NRS. After washing, with shaking, in five changes of buffer, over a period of at least 30 min. the nitrocellulose paper was incubated, with shaking, for 1 hr at 37°C in 5 x 10^6 c.p.m. of ^{125}I-protein A in buffer. The nitrocellulose strip was finally washed with at least five changes of incubation buffer and dried between two pieces of Whatman 3mm filter paper overnight at room temperature. The dried nitrocellulose paper was then autoradiographed (section 2.2.3.11.). Controls contained a 1:100 dilution of pre-immune or normal rabbit serum in place of antiserum.

In some cases, after initial treatment with incubation buffer, nitrocellulose paper strips were treated with 0.01M sodium meta-periodate in 0.02M sodium acetate buffer, pH 5.4, containing 0.05M NaCl and 0.005M NaN_3, for 1hr at room temperature, then washed 3x with distilled water, incubated for 20 min in 0.2M glycerol in 0.15M NaCl, 20mM Tris HCl pH 7.2, then rinsed 3x in 0.15M NaCl, 20mM Tris HCl pH 7.2. Control strips of nitrocellulose were treated with acetate buffer lacking sodium meta-periodate. Both control and periodate-treated nitrocellulose strips were then processed, as described above, to assess anti-cystacanth envelope antiserum (a-Env) binding.

Chapter 3

Glycosaminoglycans of Parasite and Host

3.1. Introduction

The chitinous cuticle of insects provides the main skeletal tissue of these animals. However, the internal organs are all supported by collagenous connective tissues (Ashhurst, 1985), and all epithelia, including those which manufacture the cuticle, lie on basement membranes; these are specialised forms of connective-tissue that occur at the interface of epithelia with the interstitial fluid or matrix, and function, in part, as an endoskeletal matrix. Thus, all surfaces of the haemocoele are covered with a thin layer of connective-tissue and, as argued in section 1.6.2., the molecular characteristics of this tissue may be important in defining "self".

Despite the variety of connective-tissues present in vertebrates - for example, basement membranes and the ground substance of bone and cartilage - they all apparently contain a limited number of components, which include collagens, proteoglycans and various glycoproteins. For a long time, very little has been known about the components of insect connective-tissues. Much of the early work relied on histochemical and ultrastructural methods designed primarily to determine if acid mucopolysaccharides (glycosaminoglycans) and collagen were present (Ashhurst, 1968). Detailed biochemical evidence is now accumulating to suggest that the main components of connective-tissues (i.e. proteoglycans and collagen) from insects and all other metazoans are quite similar (Hunt, 1970: Höglund, 1976a,b; Cassaro and Dietrich, 1977; Ashhurst and Bailey, 1980; Francois et al., 1980). This has led to the suggestion that connective-tissue macromolecules serve similar functions in all organisms that exhibit some tissue organisation (Cassaro and Dietrich, 1977).

Before considering some of the possible roles of these macromolecules it is necessary to discuss some of the basic biochemical properties of the main connective-tissue components.

3.1.1. The glycosaminoglycans/proteoglycans

Glycosaminoglycans (GAG) are negatively charged, long-chain, linear polysaccharides composed of repeating disaccharide units. They comprise - with one exception - of an amino hexose, N-acetylglucosamine or N-acetylgalactosamine and a uronic acid moiety, which may be either D-glucuronic or its epimer, L-iduronic acid. The polymers include hyaluronic acid (HA), chondroitin sulphate (CS), dermatan sulphate (or chondroitin sulphate B; CSB), heparan sulphate (HS), heparin (HP) and keratan sulphate (KS), in which the uronic acid is replaced by D-glucose (Rodén, 1980). Sulphate groups are found on all GAG apart from hyaluronic acid, and all the sulphated GAG (sGAG) are covalently linked to protein to form proteoglycans (PG) (Rodén, 1980; Gallagher et al., 1986; Poole, 1986). The structures and functions of vertebrate proteoglycans have recently been reviewed (Poole, 1986; Gallagher et al, 1986). The molecules show a diversity in size from about 70,000 molecular weight to 1-2 x 10^6 molecular weight. Variations in the size of proteoglycans appear to reflect differences in both the structure of the protein cores and in the number and type(s) of GAG chain associated with particular molecules (Poole, 1986). Each type of mammalian connective-tissue appears to contain characteristic proteoglycan species. For example, basement membranes contain heparin sulphate proteoglycans (HS-PG), although there is still considerable variation in the biochemical properties of HS-PGs between comparable tissues of different vertebrate species and within the different tissues of a single species. These result from the various combinations

of sugar units and sulphate residues which produce a large number of different polymer sequences.

3.1.1.1. Insect glycosaminoglycans/proteoglycans

One of the first reviews on the occurrence of GAG and general protein-polysaccharide complexes in insects and other invertebrates was produced by Hunt (1970). Much of the evidence for the presence of GAG in insect tissues was from histochemical staining of tissue sections and/or chemical detection of uronic acid and sulphate in total hydrolysates of tissue preparations. However, there are now several publications citing the occurrence and biochemical characterisation of GAG in insects (Höglund, 1976(a); Cassaro and Dietrich, 1977; Theocharis et al., 1985; Garcia et al., 1986). The results have indicated that all species investigated contain variable amounts of one or more types of GAG and that each species has a characteristic composition, differing from each other regarding the relative amount and type of GAG present in their tissues. It has also been demonstrated that, in the insects so far investigated, there is variation in the types of GAG present in different developmental stages (Höglund, 1976(a),(b); Theocharis et al., 1985). In all these investigations extracts of whole animals were used and it was therefore not possible to determine the distribution of the individual GAG within the various tissues of each insect.

Recent results suggest that the pathways of proteoglycan biosynthesis in insects and vertebrate are quite similar (Rodén, 1980; Garcia et al., 1986) although evidence is now accumulating to suggest that some insects may produce sulphated polysaccharide protein complexes which are structurally distinct from vertebrate proteoglycans. In particular, high

molecular weight glycoproteins that contain O-glycosidically-linked sulphated chitin-like (β1 - 4 linked N-acetylglucosamine residues that are O-sulphated) carbohydrate components are synthesised in an embryonic Drosophila cell line (Kramerov et al., 1986) and in isolated imaginal discs of Drosophila (Mukha et al., 1987). It has been suggested that the biological role of these glycoproteins is in connective-tissue formation and they may represent insect-specific functional analogues of GAG-containing proteoglycans (Mukha et al., 1987).

Interestingly, Fessler et al. (1984) have demonstrated that another Drosophila cell line (KC) synthesises a number of high molecular weight glycoproteins which are released into the culture medium. One of these glycoproteins has been identified as a proteoglycan, as assessed by its staining on SDS-agarose gels with periodic acid Schiff's reagent, and also by specific metabolic incoporation of $^{35}SO_4^{2-}$ into this molecule. In its native form, this molecule exists in dimers and high oligomers which are disulphide-linked to give a molecule in the order of one million daltons in size (Fessler et al., 1984). Furthermore, antiserum raised in rabbits, against the purified "proteoglycan" was shown by immunofluorescence to bind specifically to the basement membranes of epithelial and muscle cells in Drosophila embryos, suggesting that similar molecules are present in these structures. The possibility exists then that the sulphated "proteoglycan" and the sulphated chitin-protein complexes represent the same class of molecule. No reports have yet been published of similar molecules in the connective-tissue of other insects.

3.1.1.2. Glycosaminoglycans of helminths

Histochemical demonstration of GAG associated with the tegument of

various helminth species was reported by Monné (1959). Recent biochemical studies have demonstrated that glycosaminoglycans are present in the cestodes, Eubothrium sp., and Hymenolepis diminuta, as well as in the digenean trematodes, Haematoloechus medioplexus and Schistosoma mansoni (Rahemtulla and Løvtrup, 1974; Robertson et al., 1984; Robertson and Cain, 1984; 1985). Between different phyla there may be a different anatomical distribution of GAG. For example, only 2% of the uronic acid-containing material of 10 day-old tapeworms is associated with the tegument (Robertson and Cain, 1984) while for adult schistosomes about 73% of the total GAG content is located in the tegumental membrane (Robertson and Cain, 1985). It has been suggested that these variations in GAG distribution between the two species may reflect the different functions of these molecules (see section 3.1.2.).

3.1.2. Function of glycosaminoglycans

A multitude of biological functions has been ascribed to proteoglycans. It would appear that much of the activity associated with different PGs is imparted by their GAG chains through their capacity as polyelectrolytes (Lindahl and Höök, 1978), although other phenomena, such as the anticoagulant activity of heparin, are critically dependent on the fine structure of the polysaccharide chains (Gallagher et al., 1986). Glycosaminoglycans/proteoglycans occur as both intracellular and extracellular components. As intracellular components there is some evidence to suggest that GAG are associated with cell nuclei and may regulate DNA synthesis as well as transcription and translation via direct interactions with chromatin and/or enzymes involved in DNA replication/transcription (Lindahl and Höök, 1978; Gallagher et al, 1986). A nuclear proteoglycan, probably HS-PG, is an essential determinant of

genetic regulation in sea-urchin embryos; if PG biosynthesis is specifically inhibited then embryonic development does not proceed beyond the blastula stage (Kinoshita and Yoshii, 1979).

Glycosaminoglycans have also been identified as components of lysosomes or of storage and secretory granules, where they are proposed to regulate the activity of lysosomal enzymes or facilitate storage of biologically active molecules. For example, histamine and heparin are found within the same secretory granules of mast cells, while a chondroitin sulphate proteoglycan has been identified as one of the products released by Natural Killer (NK) cells on contact with a target cell. Here it has been proposed that the CS-PG plays a dual role; to allow for safe storage of the cytotoxic material released by the NK cells and to protect the NK cells from the effects of their released cytotoxic substance (MacDermott et al., 1985).

Perhaps more interestingly, in the context of this thesis, are the functions that have been attributed to extracellular proteoglycans and glycosaminoglycans. In basement membranes (BM), heparin sulphate proteoglycans interact with other components, including type IV collagen and fibronectin, to help organise and stabilise the BM structure (Yamada, 1983). They may also regulate solute permeability through the BM to the epithelial cells, and together with another basement membrane protein, Laminin, form the BM receptor for attachment of epithelial cells (Gallagher et al, 1986). Within basement membranes and as components of the cell surface, either as integral membrane proteins or in peripheral association with the plasma membrane of a variety of mammalian cells, they may act as receptors for circulating macromolecules or macromolecules on the surface of other cells (Poole,1986; Gallagher et al, 1986). For

example the transferrin receptor in mammalian fibroblasts is thought to be a dimeric HS-PG, the monomers of which are joined by disulphide bonds (Fransson et al., 1984). Receptors for sperm cells on embryos of both vertebrate and invertebrate species appear to be sulphated polysaccharides (Ahuja, 1982; Rossignol et al., 1984) while cell surface receptors for GAG have been found on a variety of mammalian cells from macrophages to neurones (Yamada, 1983; Höök, 1984; Gallagher et al, 1986). It is not yet clear if the interaction of these cells with GAG is through primarily electrostatic interactions or through receptor-ligand interactions such as through cell-surface lectins binding GAG-sequences in PGs. As extracellular or cell-surface components GAG may be involved in regulating cell-cell interactions, cell growth, cell-substratum adhesion and cell-locomotion.

Some species-specific functions for GAG have been suggested for parasitic helminths. For example, in adult S. mansoni, 73% of the total GAG present is associated with the tegument (Robertson and Cain, 1984). This GAG fraction possesses some anticoagulant activity and these authors have suggested that it may function at the tegumental surface to prevent entrapment of the worms by the host's blood coagulation system. It has also been suggested that GAG in the tegument may protect intestinal helminths, for example tapeworms, from host digestive enzymes (Monne, 1959). However, although it is true that some GAG can inhibit some proteinases in vitro (Katchalsky, 1964) it has also been shown that tapeworms and a Planaria sp. (a free living platyhelminth) contain the same GAG in their tegument (Rahemtull and Løvtrup, 1974); these results do not contribute to the plausibility of Monné's suggestion.

3.1.3. Collagens in insects and helminths

The presence of collagen in insect tissues was suggested by transmission electron microscopy; banded fibrils characteristic of collagen were present in all connective-tissues studied and chemical analysis of tissue hydrolysates revealed the presence of amino acids characteristic of collagen, for example, L-hydroxylysine (Harper et al., 1967; reviewed by Ashhurst, 1982,1985). The collagen fibrils in insect connective-tissues are relatively thin compared with those of vertebrate cartilagenous tissues, and are randomly arranged in most tissue matrices (Ashhurst, 1985). It would appear that the random arrangement is appropriate, since most of the connective-tissue layers provide support around organs or under the epidermis where deformation can occur in many directions. Recent reports have emerged describing collagens isolated from insect connective-tissues (Ashhurst and Bailey, 1980; Francois et al., 1980). Fessler et al. (1984) have isolated a procollagen molecule released by epithelial-like Drosophila KC cells in culture which shows many similarities with type IV basement membrane collagen from vertebrates. This collagen has been shown to occur in basement membranes in Drosophila larvae (Ashhurst, 1982; Fessler et al., 1984).

The presence of collagen in helminth species is well established (Murray et al.,1982), with perhaps the best characterised helminth collagens being those of the nematode cuticle (McBride and Harrington, 1967; Cox et al., 1981). A collagen from adults of the giant acanthocephalan Macracanthorhynchus hirudinaceus has also been purified and characterised (Cain, 1970), while results in this thesis suggest that the envelope of Moniliformis larvae also contains a collagen (see Chapter 4, section 4.2.1.4.).

3.1.4. Other components

The other components of vertebrate connective-tissues, in addition to collagens and proteoglycans, include elastin and various glycoproteins. Elastin has not been detected in any invertebrate connective-tissue and may be unique to the vertebrates. Elastic fibres are present in insect tissues, however their biochemical nature remains to be determined (Ashhurst, 1985).

There is, at present, not much information on the types of glycoproteins in insect basement membranes. Drosophila KC cells appear to produce laminin, which is a glycoprotein involved in mediating basement membrane attachment to epithelial cells (Fessler et al., 1984). Other glycoproteins secreted by this cell line include an entactin-like molecule; entactin is a sulphated glycoprotein found in vertebrate basement membranes. As yet, no fibronectin-like proteins have been detected in Drosophila (Fessler et al., 1984) or associated with the cell lines established for four insect species (Goldstein and McIntosh, 1980).

For helminths, an investigation of the intestinal basement membrane of the parasitic nematode Ascaris suum has shown that it contains, in addition to collagen, at least 17 glycoproteins. Whether any of these glycoproteins are similar to the glycoproteins common to all vertebrate basement membranes (e.g. laminin, entactin) remains to be determined, but it is apparent that their organisation within the basement membrane is quite similar to that found in vertebrates (Hung et al., 1980).

3.1.5. Aims

The demonstration by Lackie (1986a) that the outer surface of the envelope of Moniliformis stains with Alcian Blue 8GX suggests that GAG are present in this structure. However, histochemical demonstrations are too imprecise to be of importance for comparative studies, in this case between the envelope putative GAG and the GAG in P. americana connective-tissues. Thus, the experiments described in this Chapter were undertaken (1) to confirm the presence of GAG in the Moniliformis envelope and host tissues by chemical and biochemical tests; (2) to further characterise the type(s) of GAG present in both the parasite envelope and host connective-tissues and (3) to investigate the ability of host tissues and Moniliformis larvae to synthesise GAG, in order to determine if the GAG of the envelope was of parasite or host origin.

3.2. Results

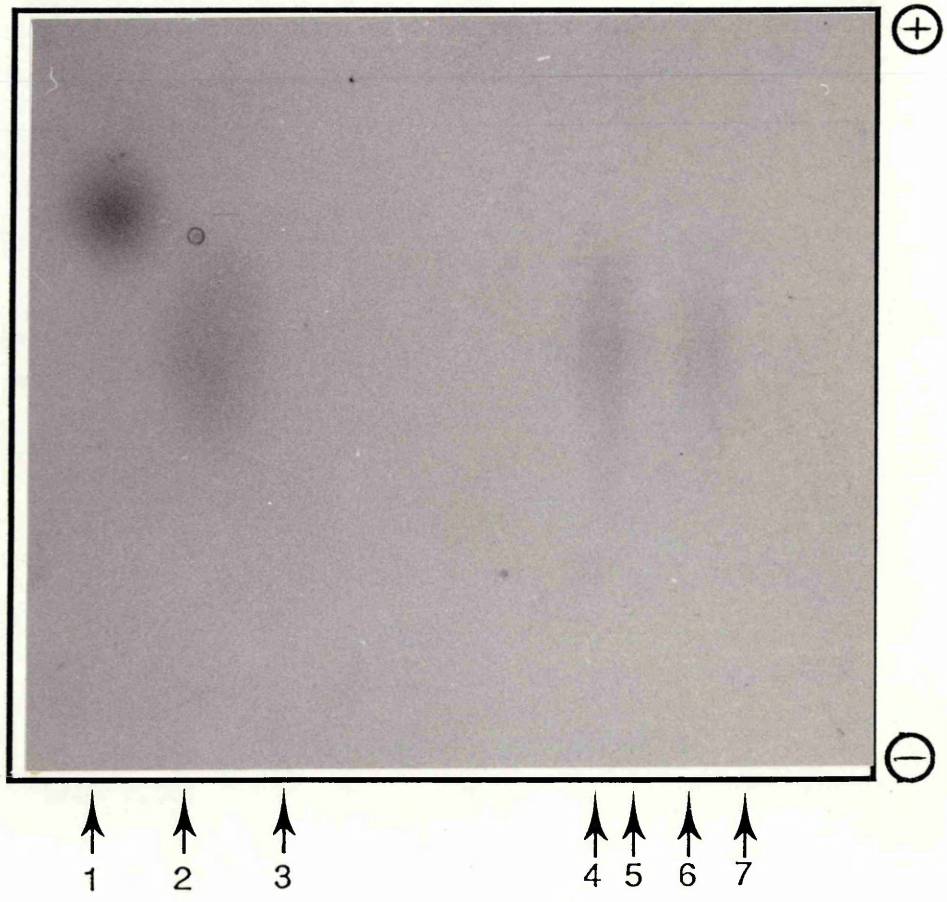
3.2.1. Midgut and Malpighian tubule GAG

Midgut and Malpighian tubules were collected from 30 adult cockroaches and extracted as described in sections 2.2.1.9. and 2.2.1.10. The final ethanol precipitates from each tissue extract were each resuspended in 100µl of 2% ethanol and divided into two aliquots of 50µl. Aliquots of both extracts were treated with nitrous acid (section 2.2.3.16.1.) which causes depolymerisation of heparin/heparan sulphated glycosaminoglycans only. Controls were treated with 0.5M acetic acid but lacked sodium nitrite. Control samples of both extracts, as well as nitrous acid-treated aliquots of both samples, were then analysed by agarose gel electrophoresis. The results are shown in Fig. 3.1. A single Toluidine

Figure 3.1. Agarose gel electrophoresis of GAG extracts from cockroach midguts and Malpighian tubules.

Midguts and Malpighian tubules from 30 adult cockroaches were extracted for GAG as described in sections 2.2.1.9. and 2.2.1.10. The final pellets were each solubilised in 200µl of distilled water and divided in 2 x 100µl aliquots, one of which was HNO_2-treated (while the other received mock treatment (section 2.2.3.16.1.).

Key:
- Track 1 Chondroitin sulphate B (CSB) standard (5µg)
- Track 2 Heparan sulphate (HS) standard (5µg)
- Track 3 HS standard (5µg) HNO_2-treated prior to elecrophoresis
- Track 4 Midgut extract
- Track 5 Midgut extract HNO_2-treated prior to electrophoresis
- Track 6 Malpighian tubule extract
- Track 7 Malpighian tubule extract HNO_2-treated prior to electrophoreis


⊕
⊖
1
2
3
4
5
6
7

Blue-staining band is present in both the midgut (track 4) and Malpighian tubule (track 6) control samples. In both cases the electrophoretic mobility of the band is very similar to the heparan sulphate standard (track 2). However, for the nitrous acid-treated midgut (track 5) and Malpighian tubule (track 7) samples there are no bands apparent. Likewise, for the HNO_2-treated HS standard (track 3) no band is visible. This suggests that the molecules present in both the midgut and Malpighian tubule extracts are degraded upon incubation in nitrous acid and therefore represent heparin or heparan sulphate glycosaminoglycan chains.

3.2.2. Haemocyte glycosaminoglycan molecule

Having established that both midguts and Malpighian tubules contain at least one species of sulphated GAG, a similar analysis of cockroach haemocytes was undertaken.

Haemocytes were collected from adult cockroaches and extracted as described in section 2.2.1.8. The extract was then analysed for the presence of GAG in untreated and nitrous acid-treated aliquots by agarose gel electrophoresis. The results are shown in Figure 3.2. They suggest that haemocytes also contain a nitrous-acid sensitive GAG with a very similar electrophoretic mobility to the midgut and Malpighian tubule molecules.

3.2.3. Metabolic labelling of cockroach haemocytes and plasma macromolecules in vivo using $^{35}SO_4^{2-}$.

In order to investigate if haemocytes could synthesise sulphated glycosaminoglycans, including the HP/HS molecule associated with the haemocytes, and to analyse any sGAG synthesised then released into the

Figure 3.2. Agarose gel electrophoresis of GAG extract from cockroach haemocytes

Cockroach haemocytes (2 x 10^7 cells) were extracted for GAG as described in section 2.2.1.8. and the final pellet resuspended in 200µl distilled water then divided into 2 x 100µl aliquots. One aliquot (100µl) was treated with HNO_2 while the other received mock treatment. Both aliquots were then analysed by agarose gel electrophoresis.

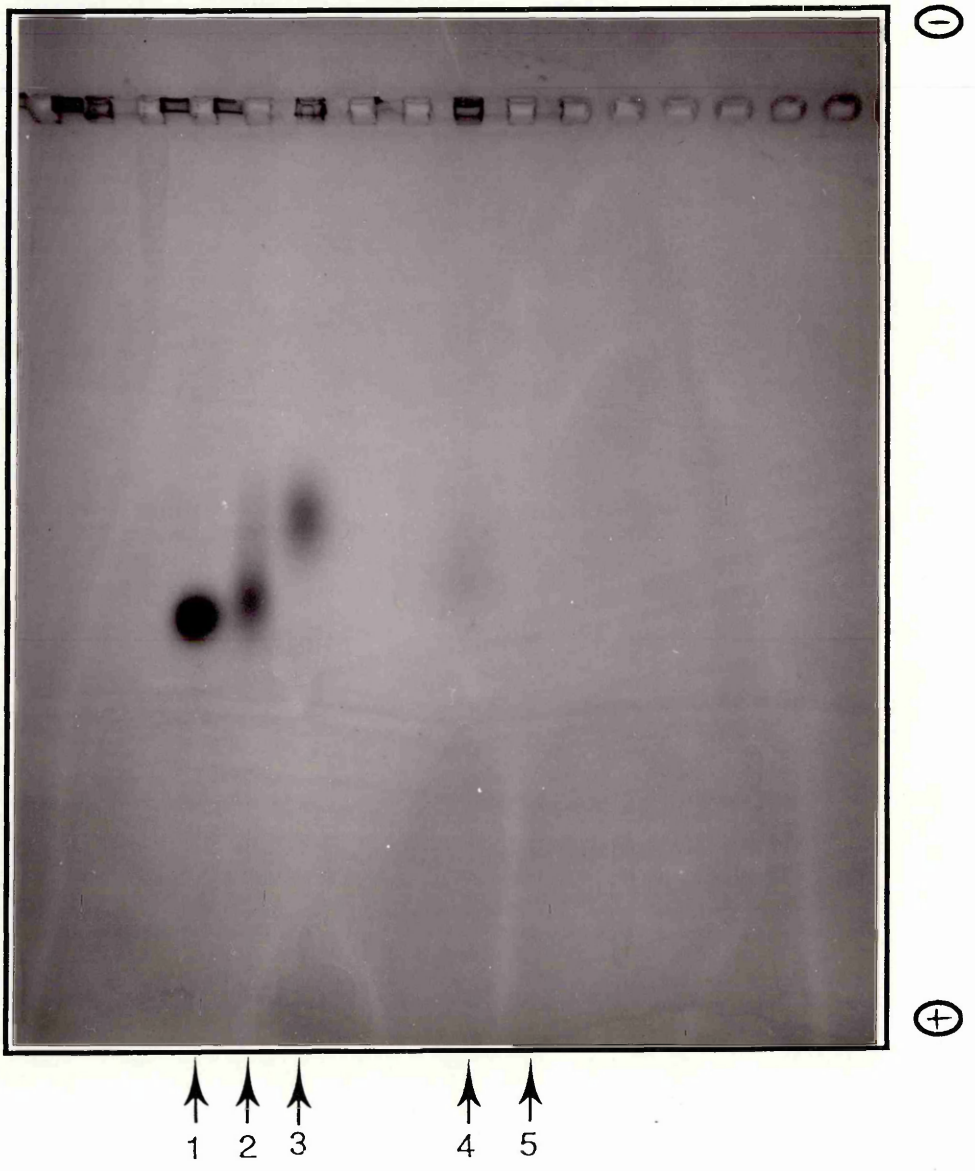
Key : Track 1 CSB (5µg)

Track 2 CSC (5µg)

Track 3 HS (5µg)

Track 4 Haemocyte extract

Track 5 Haemocyte extract HNO_2-treated prior to electrophoresis.


⊖
⊕
1
2
3
4
5

plasma fraction of the haemolymph, cockroaches were injected intra-abdominally with a radiolabelled GAG precursor, $^{35}SO_4^{2-}$, in medium D73, as described in section 2.2.14.

Haemocytes were recovered and extracted for GAG. The extract was then analysed by agarose gel electrophoresis and fluorography to detect any sGAG synthesised de novo. An aliquot of cockroach plasma macromolecules, recovered by ethanol precipitation of anticoagulant-diluted plasma (section 2.2.1.5.) was also analysed by agarose gel electrophoresis and fluorography to detect soluble sulphated proteoglycans/glycoproteins which may be synthesised and secreted by haemocytes and/or cells of some other tissue(s). The fluorograph obtained after electrophoresis is shown in Figure 3.3. No labelled sGAG could be detected in the haemocyte extract (track 1) although there was material at the point of sample application. This material may represent either very large aggregates of labelled macromolecules which are unable to enter the gel matrix, or material which is uncharged under the electrophoresis conditions. The labelled material at the point of sample application is absent in the aliquot of haemocyte extract treated with nitrous acid (track 2), suggesting that it may indeed, represent aggregated HP/HS GAG molecules.

At least three discrete radiolabelled bands are present in the plasma sample (track 3). That they represent sulphated heparan sulphate proteoglycans is suggested by their absence from the track 4, which contains the plasma sample incubated with nitrous acid prior to electrophoresis. No further information was obtained on the labelled haemocyte and plasma macromolecules.

Figure 3.3. Fluorograph from agarose gel electrophoresis of haemocyte and plasma extracts from in vivo labelling experiment using $^{35}SO_4^{2-}$.

Mock- and HNO_2-treated aliquots of haemocyte GAG extract and plasma fraction (10000 cpm each) were analysed by agarose gel electrophoresis and fluorography.

Track 1 Haemocyte GAG extract

Track 2 Haemocyte GAG extract HNO_2-treated prior to electrophoresis

Track 3 Plasma fraction

Track 4 Plasma fraction HNO_2-treated prior to electrophoresis.


⊕
⊖
1
2
3
4

3.2.4. Glycosaminoglycans in the envelope of Moniliformis larvae

Having established the presence of a single type of HP/HS associated with midgut, Malpighian tubules and haemocytes of the cockroach host, extracts from cystacanth envelopes were obtained and analysed by gel electrophoresis to determine if a similar GAG molecule was associated with the envelope.

One thousand envelopes were collected, washed, and extracted as described for midguts in section 2.2.1.9. The total extract was then analysed by agarose gel electrophoresis. The results are shown in Figure 3.4. A Toluidine Blue-staining spot is present in the envelope extract (track 1); however this molecule has an electrophoretic mobility distinct from the chondroitin sulphate B (track 1) or chondroitin sulphate C (track 2) standards. The sensitivity of this molecule to nitrous acid was not tested. Densitometric scanning of the gel containing the envelope molecule suggested that it represented about 5µg as equivalents of chondroitin sulphate B standard (results not shown). Thus, since this represents the total GAG extract from 10^3 envelopes, which is equivalent to about 1mg dry weight of material, this molecule represents only about 0.5% of the total weight of the envelope.

Attempts to metabolically-label envelope macromolecules with $^{35}SO_4^{2-}$ both in vivo and in vitro were unsuccessful. For in vivo labelling, cystacanths were recovered 24h after intra-abdominal injection of 50µCi $^{35}SO_4^{2-}$ into infected, adult, cockroaches. There were very few counts associated with washed envelopes and there was no specific incorporation of $^{35}SO_4^{2-}$ into any molecules in the GAG extract of recovered envelopes. For in vitro labelling, stage I acanthellae, stage III acanthellae and cystacanths were cultured in low sulphate-medium D73 ($MgSO_4$ replaced by

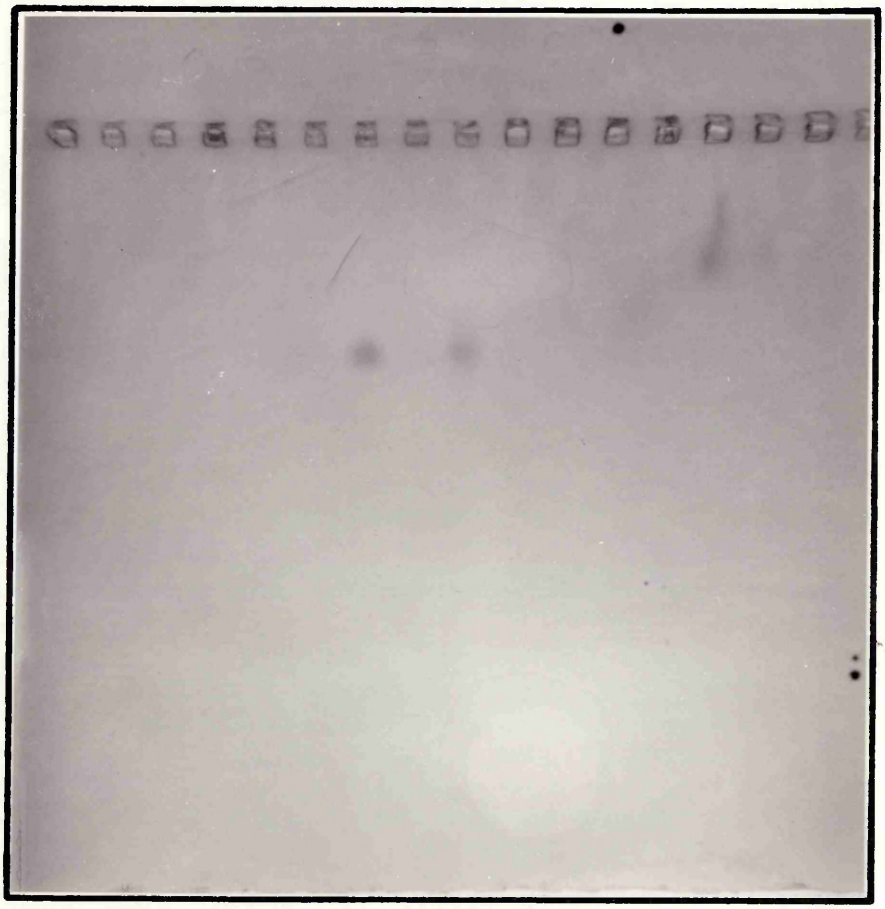
Figure 3.4. Agarose gel electrophoresis GAG extract from cystacanth envelopes

Envelopes (10^3) were extracted as described for cockroach midguts in section 2.2.1.9. The extract was then solubilised in sample buffer and analysed by gel electrophoresis.

Key : 1 CSB (5µg)

2 CSC (5µg)

3 Envelope extract


⊖
⊕
1
2
3

$MgCl_2$, no gentamicin or streptomycin sulphates; see section 2.1.3.4.) supplemented with 5% FCS. There was no specific incorporation of $^{35}SO_4^{2-}$ into molecules from the envelopes of stage III acanthellae and cystacanths or from homogenates of the bodies of all three larval stages.

In all cases, there was incorporation of $^{35}SO_4^{2-}$ into the dialysed culture fluid, as measured by ethanol precipitation of counts. However these counts were also associated with control medium (i.e. containing no larvae) and appeared to be associated with the calf serum albumin (see section 3.4.).

3.3. Discussion

The results presented, although preliminary, have demonstrated that cockroach midgut, Malpighian tubules and haemolymph components contain glycosaminoglycans. Midgut, tubules and haemocytes contain a polyanion that shares, with authentic heparan sulphate, the same electrophoretic mobility on agarose gels and susceptibility to nitrous acid, suggesting that it represents heparin/heparan sulphate. The gel electrophoresis system used in this investigation separates molecules on the basis of their net charge (Dietrich et al., 1977) thus allowing separation of different GAG classes and isomers independent of the relative size of the GAG chain. Thus, although midgut, Malpighian tubules and haemocytes all appear to contain the same class of GAG molecule, namely heparin/heparan sulphate, it is possible that the size of GAG chains associated with the different tissues varies. The results presented here are in good agreement with previous reports of GAG in Periplaneta americana by Cassaro and Dietrich (1977). However, these workers used extracts from whole adult animals and so could not locate the origin of the GAG detected to one particular

tissue. They reported the presence of a single band on agarose gels with an electrophoretic mobility similar to heparan sulphate. This material was degraded to disaccharides and oligosaccharides using bacterial enzymes which are specific for GAGs. From characterisation of the disaccharides produced upon degradation, these workers concluded that the material contained a mixture of both heparan sulphate and chondroitin sulphate (Cassaro and Deitrich, 1977). In the results reported here, only HS/HP could be detected in midgut, Malpighian tubule and haemocyte extracts. The tissues may contain no CS or it may be present only in very small amounts and so would not be detected by Toluidine Blue staining of the nitrous acid-treated extract.

The results of the in vivo metabolic labelling experiments imply that cockroach haemocytes might be involved in the synthesis of some sulphated macromolecules, since labelled material was detected by fluorography in the GAG extract from haemocytes. However, labelled material was detected only at the point of sample application to the gel while none was located at the position corresponding to the HS/HP detected in unlabelled haemocyte extracts by Toluidine Blue staining of the agarose gels. This could be because : (1) the cells were not synthesising the HS/HP molecule during the time course of labelling or (2) the labelled material represents a sulphated oligosaccharide fraction from cell glycoproteins or another unidentified sulphated polysaccharide and that these molecules are uncharged under the conditions of electrophoresis. However, the fact that the haemocyte material at the origin is lost by treatment of the extract with nitrous acid prior to electrophoresis suggests that the radioactive material does represent heparin or heparan sulphate polysaccharides. The labelled molecule may represent an undersulphated (sulphation is one of the

last modifications of GAG chains during their biosynthesis) precursor of the Toluidine Blue-staining band and therefore have a smaller negative charge, and hence electrophoretic mobility. For any one aliquot of the $^{35}SO_4^{2-}$-GAG extract of haemocytes, the ratio of labelled mature molecule to labelled precursor molecule may be low, and so it would be necessary to expose the fluorograph for a longer period to detect a labelled molecule corresponding to the Toluidine Blue-staining band from the unlabelled cell extract.

Other workers have reported that a $^{35}SO_4^{2-}$-labelled polyanion is associated with the spherulocytes of the insect Malacosoma disstria (Lepidoptera) which had been injected with $^{35}SO_4^{2-}$ in buffer (Cook et al., 1985). Spherule cells are haemocytes of, as yet, unknown function, although they do not appear to be involved in the immune response of the insect (see Chapter 1). The identity of the sulphated molecule associated with these cells has not yet been confirmed, although it was apparent that its electrophoretic mobility on agarose gels was not coincident with any of the GAG standards used and had a decreased mobility relative to heparan sulphate (Cook et al., 1985). In theory, however, this could be a result of minor differences (i.e. decreased sulphation) between the spherulocyte macromolecule and the authentic heparan sulphate standard. Likewise, the envelope polyanion may possibly represent a GAG which, although it has an electrophoretic mobility distinctly less than the molecule in the cockroach tissues, may be derived from a very similar molecule by relatively minor modifications of the polymer structure.

Turning to the labelled plasma extract, it appears that some plasma macromolecules are sulphated proteoglycans, the GAG chains of which are sensitive to nitrous acid and are therefore HS/HP-like. The possibility

cannot be ruled out at this stage that the envelope GAG is a host molecule, perhaps synthesised as a proteoglycan by haemocytes or cells of other tissues, which is taken up by the envelope and modified (see Chapter 6). Alternatively, the envelope molecule may be synthesised by the parasite, as a proteoglycan, and integrated into the envelope at its outermost surface. Either way, the envelope polyanion is the only macromolecule likely to be responsible for the observed Alcian Blue staining of the outer envelope surface (Lackie, 1986a).

To confirm the identity of the cockroach GAG molecules suitable amounts of material must be collected for analysis of the relative proportions of the main constituents and disaccharides produced by enzymic and chemical cleavage, each of which are characteristic of a particular GAG species. Indeed, HS and HP can only be distinguished by quantitative differences in their sulphate and uronic acid epimer content (Beeley, 1985). For the cockroach tissues, obtaining large enough amounts of GAG material should be no problem (Cassaro and Dietrich, 1977). Furthermore, the preliminary work in this Chapter indicates that relatively small amounts of material could be used if they are first metabolically labelled by injection of cockroaches with suitable radioactive precursors. However, the small amounts of material available from the envelope (10^3 envelopes contain only about 5µg of GAG-like molecule) and the failure to metabolically label the components of this molecule, will hinder progress towards the unequivocal demonstration of the nature of this envelope polyanion. Recently, however, more sensitive methods have become available to detect very small amounts of GAG/PG (Heimer and Sampson, 1987; Heimer et al., 1987). These methods should facilitate future attempts to isolate and characterise the envelope proteoglycan/glycosaminoglycan for comparison with the molecules present in host tissues.

In spite of the considerable technical difficulties which have arisen in attempts to characterise the envelope GAG-like molecule it is still promising that this molecule is present at, or near, the outer surface of the envelope and may, therefore, have a possible physiological role in the mechanism of protection afforded by the Moniliformis envelope against haemocytic attack.

3.4. Appendix : Binding of $^{35}SO_4^{2-}$ to serum albumin from culture medium.

For all *in vitro* metabolic labelling experiments with haemocytes, midguts, Malpighian tubules and *Moniliformis* larvae, using $^{35}SO_4^{2-}$ as the precursor, there was no incorporation of label into any haemocyte, cockroach tissue or parasite macromolecules although, in all cases, the dialysed culture fluid did contain ethanol-precipitable counts, suggesting that labelled macromolecules were present. However, the counts were also detected in the control well containing culture medium and isotope only. Analysis of both control and experimental culture fluid by SDS-PAGE and fluorography revealed a major labelled band at 67,000 coincident with serum albumin (results not shown but see section 4.2.3. and Figure 4.1.1). This labelled band probably represents calf serum albumin binding the radioactive sulphate non-specifically but with high affinity.

Chapter 4

Biochemical Composition of the Envelope

4.1. Introduction

Ultrastructural examination of the envelope surrounding the cystacanth stage shows that it is a trilaminate structure, about 1um thick (J. Lackie and Rotheram, 1972). The outer surface of the envelope binds Alcian Blue 8GX (A. Lackie, 1986a) under conditions specific for labelling glycosaminoglycans (GAG). However, since electron-dense deposits of dye are found only at the outermost surface of the envelope, even when applied to sectioned material (A. Lackie and L. Tetley, unpublished), it appears that the inner regions of the envelope do not contain this class of molecule.

In section, it is seen that most of the envelope comprises vesicular structures; it is possible that they are cross-sections through highly-folded membranous structures, such as the long microvillar protrusions of the larval tegument seen in developing acanthors (Rotheram and Crompton, 1972). In contrast, the inner layer of the envelope appears as an amorphous matrix, with no discernible ultrastructure. The ultrastructural cytochemistry and the results from Chapter 3 suggest that GAGs might comprise only a very small part of the envelope and in order to understand how the envelope protects the parasite from the haemocytic response, it is important to investigate its molecular composition and how this relates to the ultrastructure. The aims of the work described in this chapter were to investigate the total composition of the envelope, to extract and characterise envelope lipids and proteins, and to use fluorescence microscopy and transmission electron microscopy to gain some insight into how these molecules are organised within the envelope. It was hoped that this information might allow a clearer understanding of the synthesis and origin of the component macromolecules and how they are organised, which in turn might allow some conclusions to be drawn with

regard to the protective mechanism afforded by the envelope to the developing larvae.

4.2. Results

4.2.1. Composition of the cystacanth envelope

The values for protein, lipid and carbohydrate as a percentage of the total dry weight of the cystacanth envelope are shown in Table 4.1.

4.2.1.1. Envelope lipids

Results showing a relatively high lipid content prompted an investigation into the classes of lipid (e.g. phospholipid, sterol, glycolipid) present in the envelope. Envelopes were extracted using a scaled-down version of the method described by Bligh and Dyer (1959). Lipid extracts were analysed by one-dimensional thin-layer chromatography (TLC) using a solvent system developed for phospholipid separation, but which was shown to resolve all classes of lipid standard used in this investigation. A representation of a typical chromatogram is shown in Figure 4.1. The envelope contains at least six iodine-staining lipids. The classification of each lipid species was attempted using various staining reagents in conjunction with appropriate standards (as described in section 2.2.3.5.1.). The results, summarised in Table 4.2, suggest that the envelope contains three phospholipids (E1-E3). Phospholipid E3 has a similar relative mobility (R_f value) to L-α-phosphatidylcholine and shows a similar staining pattern with the various reagents. Likewise, E2 was tentatively identified as L-α-phosphatidylethanolamine. Phospholipid E1 matched none of the standards used in this investigation. Lipid E4 had

Table 4.1. Composition of the envelope

For protein, the values represent the mean ± the standard deviation for six separate experiments in which each sample was determined in triplicate.

For carbohydrate and lipid the values represent the average of triplicate determinations from two separate experiments.

Table 4.1 Composition of the cystacanth envelope

		Percentage of total dry weight (%)	
Protein	(total)	65 ± 2.3	(n = 6)
	(1% SDS-solubilised)	58 ± 3.2	(n = 6)
Lipid		22.5 26.3	(n = 2)
Carbohydrate		11.8 9.8	(n = 2)

Figure 4.1. TLC chromatogram showing envelope and larval body lipids

Envelopes and larval body lipids were extracted as described in section 2.2.3.4. and analysed by TLC. For envelopes and larvae 5-10µg of lipid were loaded per chromatogram with 1µg of each standard included in each run.

Solvent : chloroform:methanol:water:acetic acid

65:25:4:1 (v/v/v/v).

The arrow indicates the direction of chromatography.

Key: E - Envelope extract

B - Cystacanth body extract

PC - L-α-phosphatidyl choline

PE - L-α-phosphatidyl ethanolamine

PS - L-α-phosphatidyl serine

CH - Cholesterol

CO - Cholesterol oleate

PI - L-α-phosphatidyl inositol.

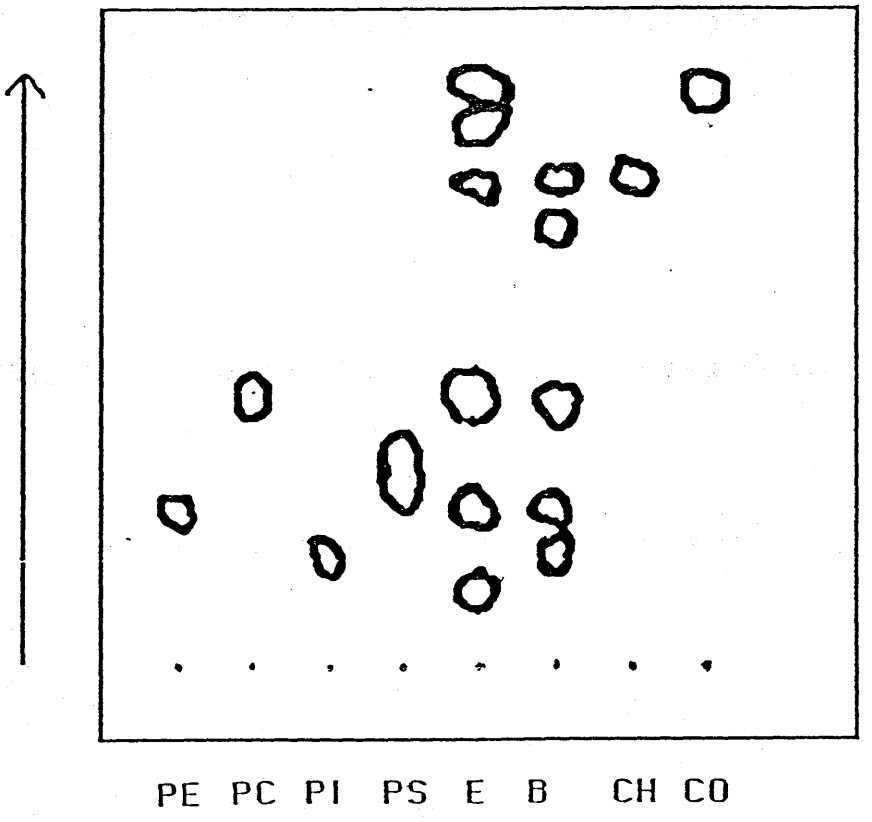

PE PC PI PS E B CH CO

Table 4.2. Summary table of lipids from cystacanth envelopes and bodies

The specificity of each spray reagent is given in section 2.2.3.5.1.

✓ indicates a positive reaction.

X indicates a negative reaction.

Key :

PC	L-α-phosphatidyl choline
PE	L-α-phosphatidyl ethanolamine
PI	L-α-phosphatidyl inositol
PS	L-α-phosphatidyl serine
CH	Cholesterol
CO	Cholesterol oleate

Table 4.2. Envelope and larval-body lipids

(a) Envelope	R_f	Ester Phosphate	Sterol	Amino Group	Carbohydrate	Identity
E1	0.16	√	X	X	X	?
E2	0.32	√	X	√	X	PE
E3	0.47	√	X	√	X	PC
E4	0.78	X	√	X	X	CH
E5	0.86	X	X	X	X	?
E6	0.93	X	X	X	X	?

(b) Larvae	R_f	Ester Phosphate	Sterol	Amino Group	Carbohydrate	Identity
L1	0.24	√	X	X	X	PI
L2	0.30	√	X	√	X	PE
L3	0.45	√	X	√	X	PC
L4	0.67	X	X	X	X	?
L5	0.74	X	X	X	X	?

(c) Standards	R_f	Ester Phosphate	Sterol	Amino Group	Carbohydrate
PC	0.45	√	X	√	X
PE	0.31	√	X	√	X
PI	0.21	√	X	X	X
PS	0.34	√	X	√	X
CH	0.76	X	√	X	X
CO	0.84	X	√	X	X

similar properties to cholesterol, while the remaining two non-polar lipids (as assessed by their high R_f values in the solvent used), E5 and E6, which may be sterol or fatty acid methyl esters, could not be identified with this solvent system and standards. That none of the envelope lipid is glycolipid is suggested by a negative reaction for both the orcinol:ferric chloride and α-napthol spray test which give a red and pink colour, respectively, if carbohydrate is present.

4.2.1.1.1. Integration of fluorescent lipid analogues into the cystacanth envelope

The results of this experiment are shown in Figure 4.2. Both C_{18}AF and Mc540 are taken up by the envelope suggesting that it contains lipophilic regions or domains. In some cases there was differential uptake of dyes by the envelope which was dependent on the length of the dye alkyl chains, since C_{16}diI was taken up by the cystacanth envelope while C_{18}diI was not. Binding of dyes appeared confined to the envelope only and, in experiments carried out in parallel with de-enveloped cystacanths, only a faint fluorescence was found associated with the larvae themselves.

4.2.1.2. Proteins in the envelope

The total protein content of the envelope represents 65% of the dry weight (Table 4.1.). In an attempt to characterise the envelope protein samples were extracted using a variety of solvents as outlined in section 2.2.3.6. For each solvent 100 envelopes were used representing 50-60µg of protein. Extracts were then analysed by sodium dodecylsuphate-polyacrylamide gel electrophoresis and stained for protein using Coomassie Blue R250. The results are shown in Figure 4.3. Only the combined solvents SDS/Urea or Triton/Urea and SDS alone extracted appreciable

Figure 4.2. Integration of fluorescent lipid analogues into the cystacanth envelope

Cystacanths were labelled with C_{18}AF (a) or Mc540 (b) as described in section 2.2.2.1.

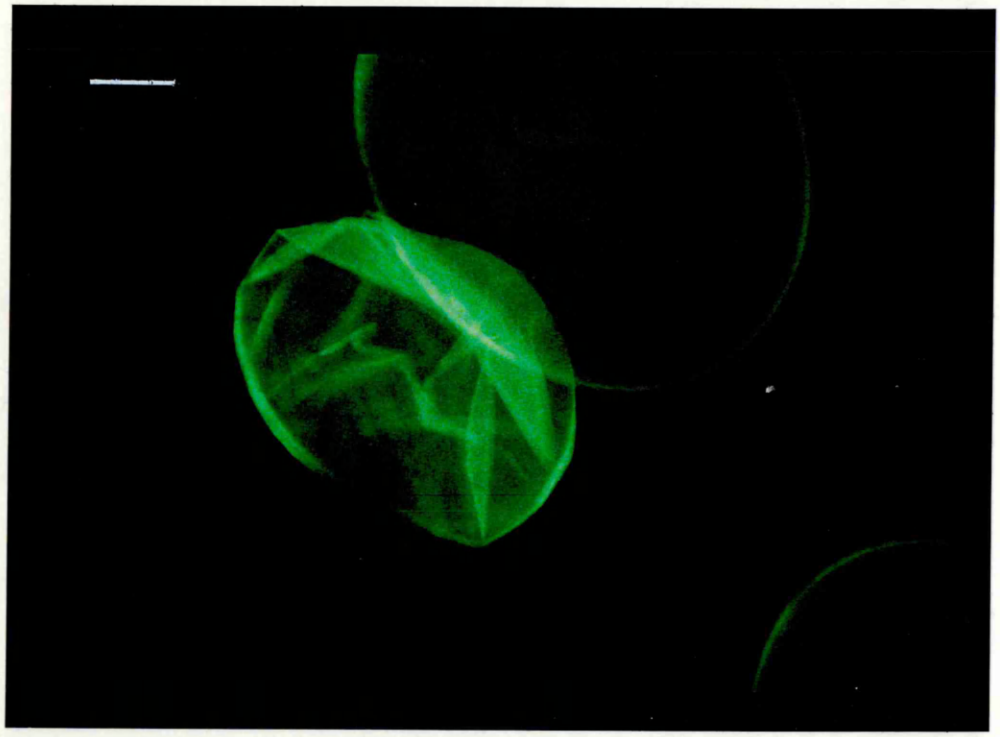
(a) Cystacanths labelled with C_{18}AF.

Bar, 0.2mm

(b) Cystacanth labelled with Mc540

Bar, 0.1mm

(a)



(b)

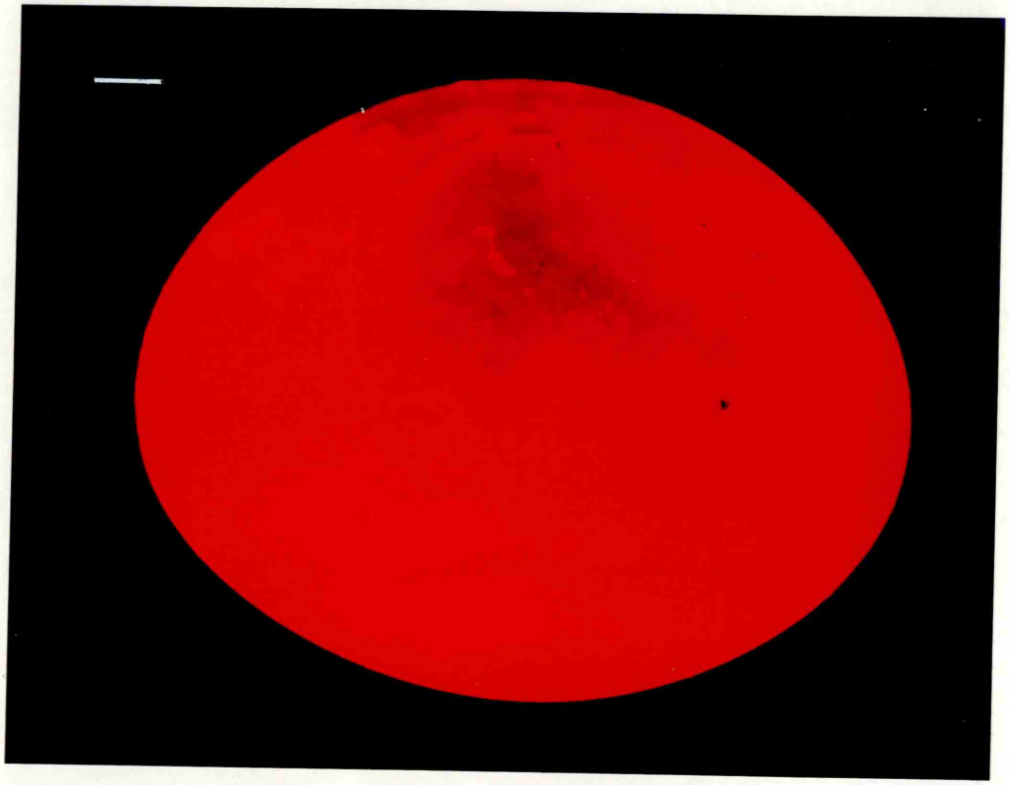


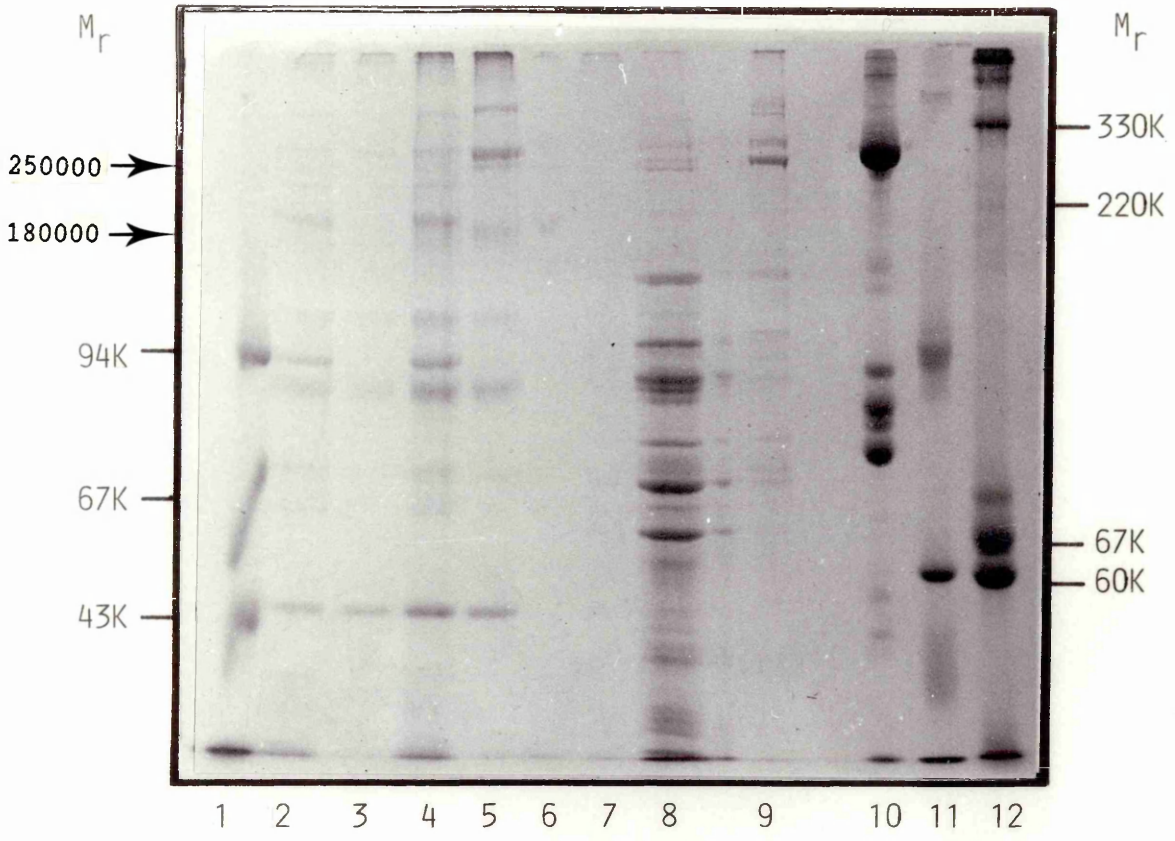
Figure 4.3. SDS-PAGE analysis of solvent extracts from cystacanth envelopes

Envelopes (100) were extracted with the solvents as described in section 2.2.3.6. Extracts were then analysed by electrophoresis on a 10% (w/v) acrylamide slab gel (anode represents the bottom of the gel). All samples contained 40-50µg protein, except the molecular weight markers which both contained 25µg protein.

Key : Track 1 Pharmacia low molecular weight markers
Track 2 1% SDS/8M urea extract of cystacanth envelopes
Track 3 1% Triton/8M urea extract of cystacanth envelopes
Track 4 1% SDS extract of cystacanth envelopes
Track 5 1% SDS/5% 2-ME extract of cystacanth envelopes residue
Track 6 1% Triton extract of cystacanth envelopes
Track 7 2M urea extract of cystacanth envelopes
Track 8 1% SDS extract of cystacanth bodies
Track 9 1% SDS/5% 2-ME extract of cystacanth bodies
Track 10 Cockroach plasma
Track 11 Pharmacia low molecular weight markers
Track 12 Pharmacia high molecular weight markers

The molecular weights of the Pharmacia markers are indiated in thousands (K) of daltons.

The gel was destained in 10% acetic acid to detect collagen-like polypeptides (see page 122).


Mr
250000
180000
94K
67K
43K
Mr
330K
220K
67K
60K
1 2 3 4 5 6 7 8 9 10 11 12

amounts of protein. For SDS extraction this represented 90% of the total envelope protein as determined by the Lowry et al. (1951) method of protein determination. Solutions of 1% (v/v) Triton X100 or 2M Urea extracted negligible amounts of protein, (Figure 4.3. tracks 6 and 7. The amount of protein extracted by 1% SDS and 1% SDS/8M Urea appears similar with perhaps SDS alone representing the better solvent, however, there were no differences in the pattern of polypeptides obtained using these solvents. A 1% SDS solution in 10mM Tris HCl, pH 7.2 and in some cases supplemented with 5% (v/v) 2-mercaptoethanol (2-ME) (Figure 4.3., track 5) was used for subsequent extractions.

The pattern of polypeptides extracted with 1% SDS in 10mM Tris HCl pH 7.2 and stained with Coomassie Blue R250 is shown in Figure 4.4, track 2. The envelope contains at least 25 polypeptides with a wide molecular weight range (M_r 30K->300K). Some of the envelope polypeptides apparently exist as disulphide-linked oligomers, notably at apparent M_r 160000 in track 3 (non-reducing conditions). This polypeptide is absent (or substantially diminished) in Figure 4.4 track 2 (reducing conditions); however, there is the concomitant appearance of a M_r 80000 polypeptide not observed in track 3 that may be the result of dissociation of two disulphide-linked M_r 80000 monomers which possibly represent the M_r 160000 polypeptide observed in track 3.

Figure 4.4. SDS-PAGE gel electrophoresis of 1% SDS extracts of cystacanth envelopes and bodies

Extracts from envelopes and larval bodies were electrophoresed in the presence (tracks 2 and 4) or absence (tracks 3 and 5) of 5% 2-ME on a 10% (w/v) acrylamide slab gel.

Key:
- Track 1 Low molecular weight markers
- Track 2 1% SDS extract of cystacanth envelopes (reducing conditions)
- Track 3 1% SDS extract of cystacanth envelopes (non-reducing conditions)
- Track 4 1% SDS extract cystacanth bodies (reducing conditions)
- Track 5 1% SDS extract cystacanth bodies (non-reducing conditions)
- Track 6 Cockroach plasma
- Track 7 High molecular weight markers

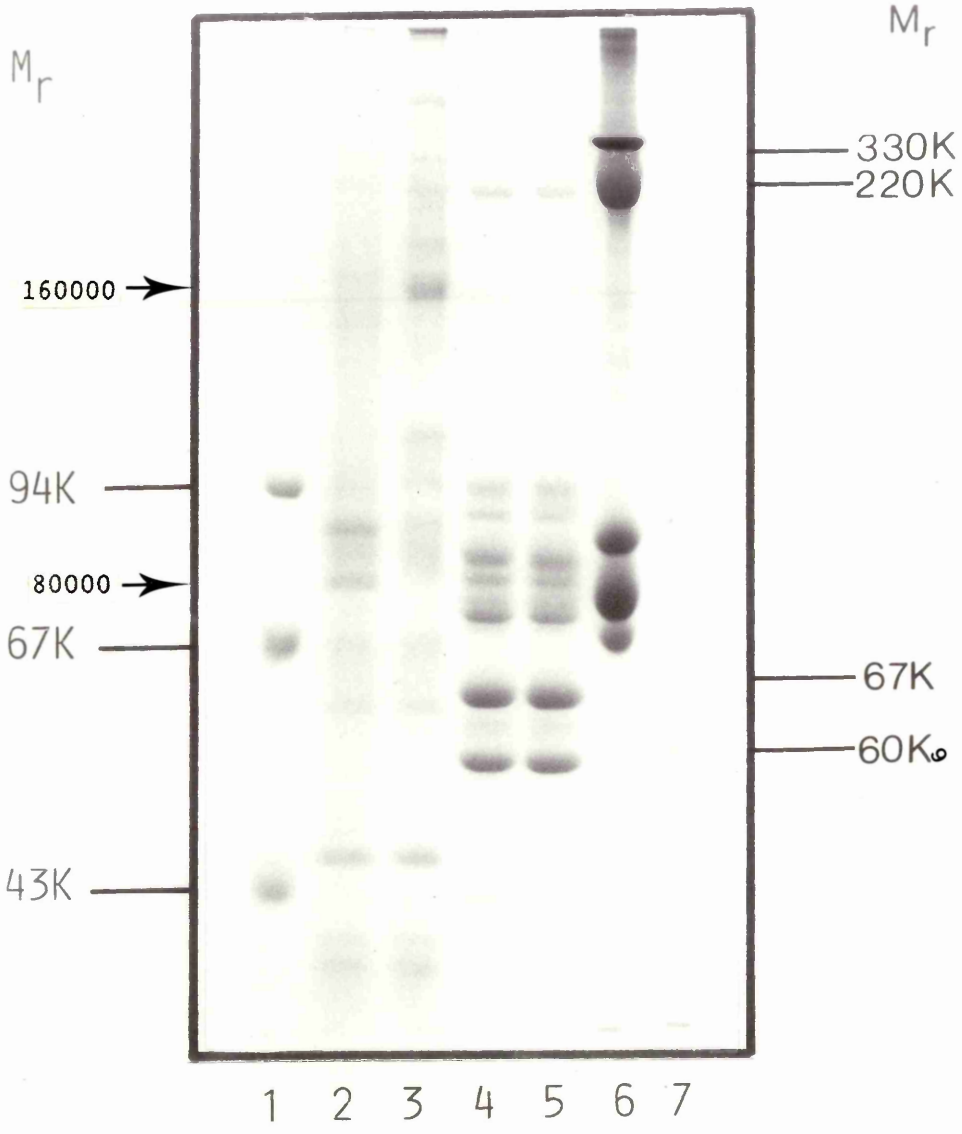

Mr
Mr
330K
220K
160000
94K
80000
67K
67K
60K
43K
1 2 3 4 5 6 7

Figure 4.5. shows the autoradiograph obtained after ^{125}I-Concanavalin A (^{125}I-Con A; a lectin which binds terminal and internal D-glucose and D-mannose residues in the oligosaccharides of glycoproteins) overlay of the gel shown in Figure 4.4. Many of the envelope polypeptides (tracks 5 and 6) can be visualised by lectin staining suggesting that they are glycoproteins. Interestingly, there appears to be little binding of the lectin to the proteins of the cystacanth bodies (Figure 4.5, tracks 3 and 4), suggesting that the major proteins present are not glycoproteins. Cockroach plasma proteins are readily stained with the lectin (track 2), including minor bands not visualised by Coomassie Blue staining, suggesting that they are all glycoproteins.

Having established that envelope polypeptides were glycosylated, a number of fluoresceinated lectins were used as probes to study the carbohydrate ligands present in intact envelopes surrounding living cystacanths, as described in section 2.2.2.2. The results are summarised in Table 4.3. Of the four lectins used, only peanut agglutinin (PNA) bound specifically to the enveloped cystacanth, as inclusion of 0.2M D-galactose in the buffer abolished lectin binding. The other lectins bound non-specifically, since inclusion of their respective competitor sugars in the buffer, at concentrations up to 0.5M, did not abolish binding (Table 4.3). In all cases the fluorescence associated with the envelope was uniform over the entire surface and there was little or no binding of any of the lectins used in this investigation, to the de-enveloped larvae.

The results of ^{125}I-PNA overlay of an SDS gel containing SDS-solubilised envelope and larval proteins as well as cockroach plasma proteins are shown in Figure 4.6. With lectin staining, only a few

Figure 4.5. Autoradiograph of ^{125}I-ConA overlay of electrophoretically separated polypeptides from cystacanth envelopes, larvae and cockroach plasma

The SDS-gel shown in Figure 4.4. was overlayed with ^{125}I-ConA as described in section 2.2.3.10., dried and then autoradiographed.

Key : Track 1 High molecular weight markers

Track 2 Cockroach plasma

Track 3 1% SDS extract, cystacanth bodies (non-reducing conditions)

Track 4 1% SDS extract, cystacanth bodies (reducing conditions)

Track 5 1% SDS extract, cystacanth envelopes (non-reducing conditions

Track 6 1% SDS extract, cystacanth enveloped (reducing conditions)

Track 7 Low molecular weight markers

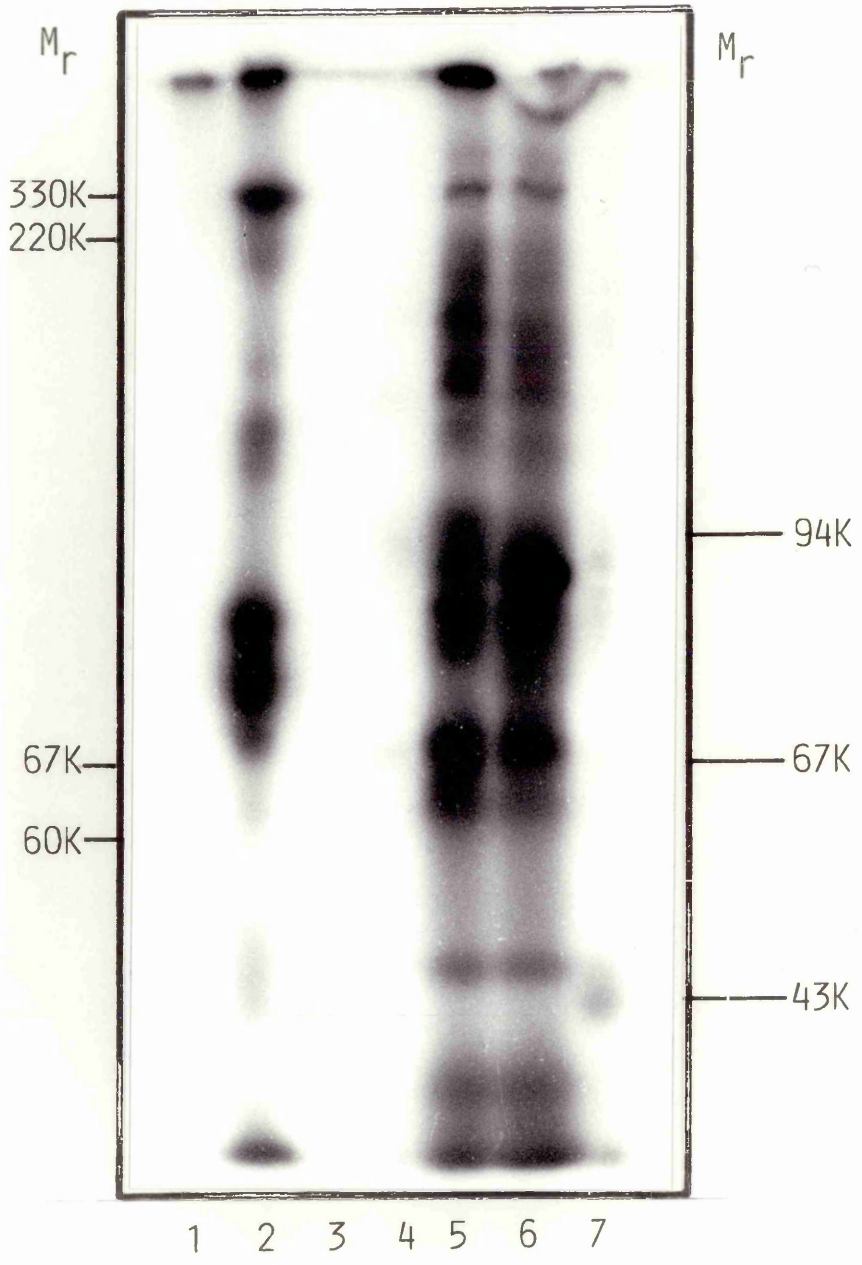

Mr
330K
220K
67K
60K
Mr
94K
67K
43K
1 2 3 4 5 6 7

Table 4.3. Fluoresceinated lectin binding to cystacanths

Enveloped cystacanths were incubated for 30 min. at 37°C in HBS containing 10% (v/v) FCS and 20µg/ml FITC-lectin. In control experiments, the mixture also contained 0.2M inhibitor sugar appropriate for each particular lectin.

Binding of lectin was scored on an arbitrary basis as indicated in the key.

Table 4.3. Fluoresceinated-lectin binding to cystacanths

Lectin	Sugar specificity	Enveloped cystacanths	De-enveloped cystacanths
Concanavalin A (Con A)	D-mannose, D-glucose	+++*	/
Con A + 0.2M ∝-methylglucoside		++	/
Dolichos biflorus agglutinin (DBA)	N-acetylgalactosamine	+	/
DBA + 0.2M (Gal NAc)	(Gal NAc)	+	/
Peanut agglutinin (PNA)	D-galactose, (D-gal)	+++	/
PNA + 0.2M D-gal	*β(1 - 3)N-acetyl-D-glucosamine, (GlcNAc)	/	/
Wheat germ agglutinin (WGA)	N-acetyl-D-glucosamine (GlcNAc),	++	/
WGA + 0.2M NANA	N-acetylneuraminic acid (NANA)	++	/

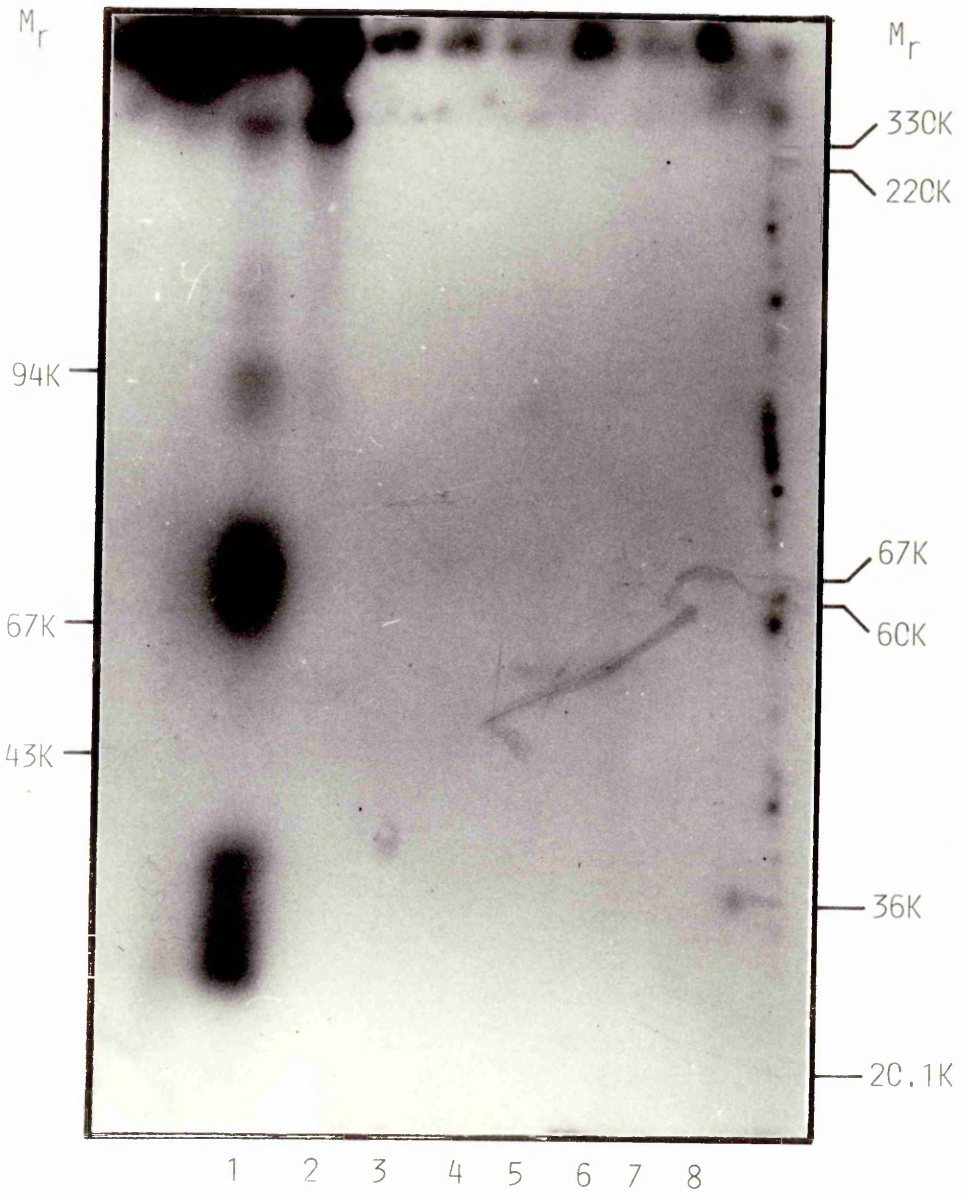
* Key: / : No binding
+ : Faint "
++ : Distinct "
+++ : Strong "

*Forsythe et al., (1984)

Figure 4.6. Autoradiograph of ^{125}I-PNA overlay of electrophoretically-separated polypeptides from cystacanth envelopes, larvae and cockroach plasma

Envelopes and larvae were extracted sequentially with first 1% SDS then re-extracted with 1% SDS/5% 2-ME as described in section 2.2.3.6. All extracts were then electrophoresed on a 10% (w/v) acrylamide slab gel which subsequently overlayed with ^{125}I-PNA.

Key :
- Track 1 1% SDS extract, cystacanth envelopes
- Track 2 1% SDS/5% 2-ME extract, cystacanth envelope residue
- Track 3 1% SDS extract, cystacanth bodies
- Track 4 1% SDS/5% 2-ME extract, cystacanth body residue
- Track 5 Cockroach plasma (reduced)
- Track 6 Cockroach plasma (non-reduced)
- Track 7 Low molecular weight markers
- Track 8 High molecular weight markers


Mr
94K
67K
43K
Mr
330K
220K
67K
60K
36K
20.1K
1 2 3 4 5 6 7 8

envelope polypeptides are visualised (Figure 4.6., tracks 1 and 2). Furthermore, there appears to be little lectin binding to the proteins of the cystacanth bodies (Figure 4.6., tracks 3 and 4) or cockroach plasma (Figure 4.6., track 5), therefore PNA might be most suitable as a tool for the study of a particular subset of envelope glycoproteins.

Lectin overlay of SDS-gels was also used to compare cockroach haemocyte and larval envelope glycoproteins. Figure 4.7 shows the Coomassie Blue staining pattern obtained after electrophoretic separation of proteins in a cockroach haemocyte lysate (track 2) and parasite envelope (track 3), both of which had been solubilised in a 1% SDS solution. The overall pattern of polypeptides in the two samples appears quite different. Figure 4.8 shows the autoradiograph obtained after ^{125}I-ConA overlay of a nitrocellulose strip containing electrophoretically separated lysate (track 1) and envelope (track 2) polypeptides. The patterns obtained for lectin staining are quite different, except for a polypeptide with the same electrophoretic mobility as cockroach plasma protein at M_r 85000. There is also faint staining of the gel lane containing proteins from the larval bodies (track 5), but they do not appear coincident with any envelope polypeptides.

4.2.1.3. Amino acid analysis of envelope residue after solvent extraction

It was observed that intact cystacanths which had been extracted in a 1% SDS solution were surrounded by a collapsed, but apparently complete, envelope structure which was indistinguishable from the normal envelope when visualised by light microscopy. This suggested that the residue might represent a structural framework or scaffold for the envelope. Furthermore, this residual material was apparently completely solubilised

Figure 4.7. SDS-PAGE gel electrophoresis of cystacanth envelope, and cockroach haemocyte lysate-polypeptides.

A haemocyte lysate was prepared as described in section 2.2.1.6. and an aliquot containing 50µg of protein was analysed by electrophoresis, along with the other samples mentioned below, on a 10% (w/v) acrylamide gel.

Key : Track 1 High molecular weight markers

Track 2 Haemocyte lysate

Track 3 1% SDS extract of cystacanth envelopes

Track 4 Cockroach lipophorin

Track 5 Cockroach plasma

Track 6 1% SDS extract of cystacanth bodies

Track 7 Type VI collagen

Track 8 High molecular weight markers

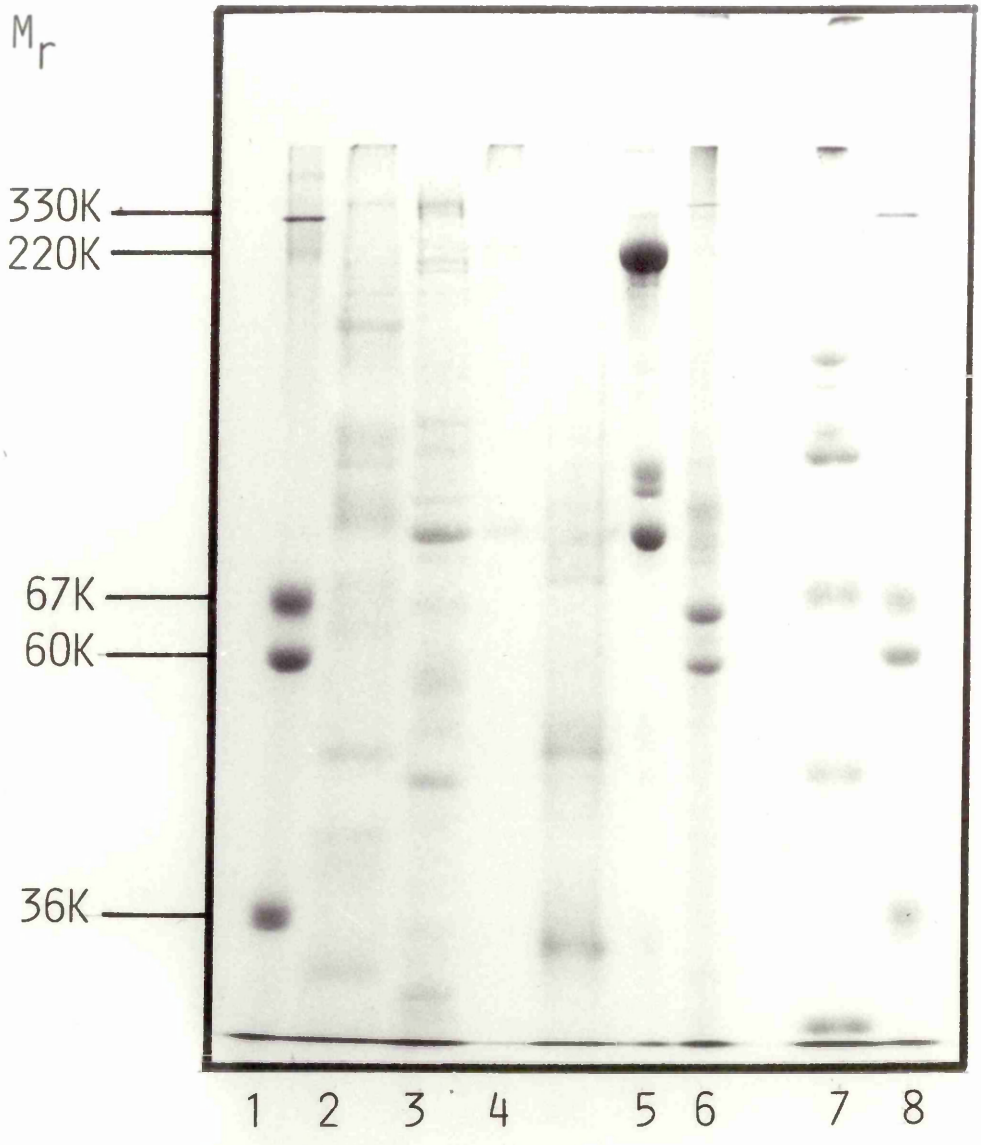
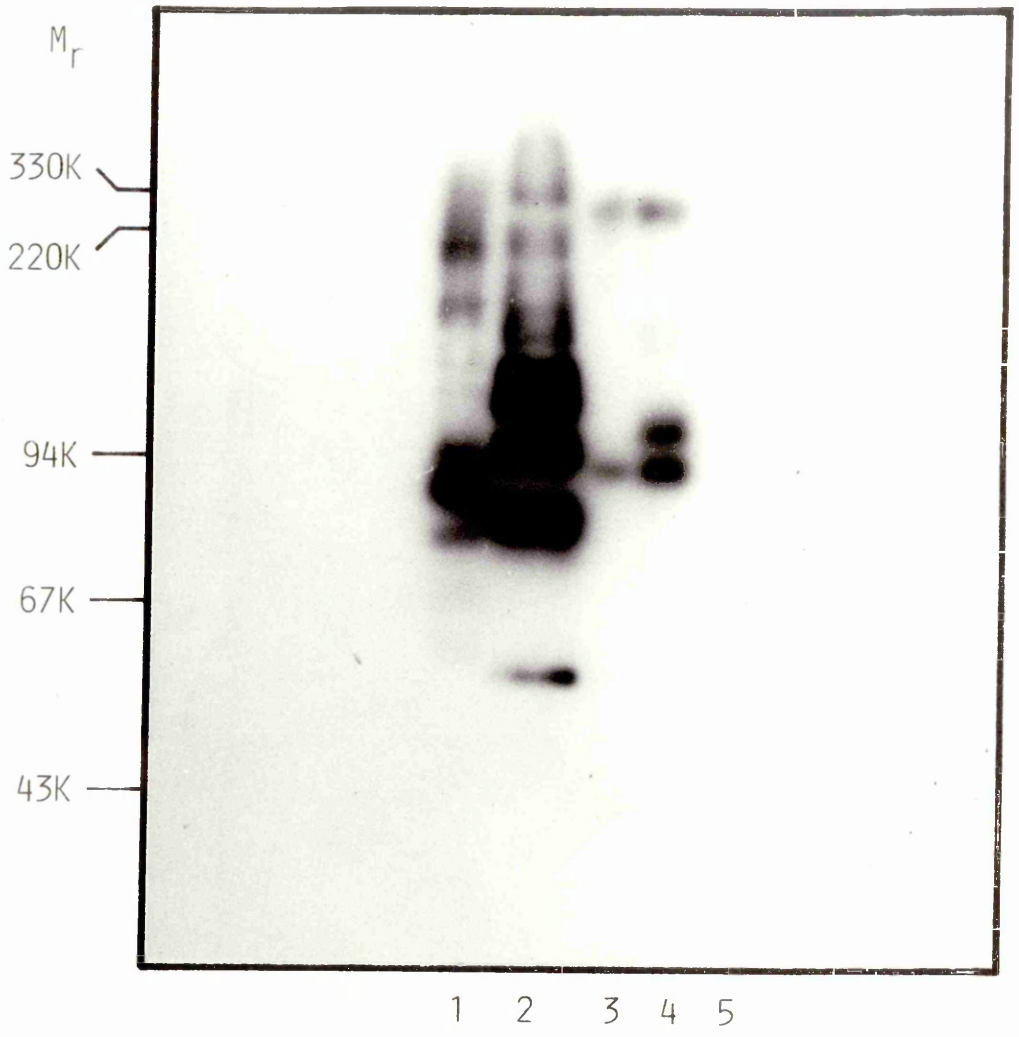

Mr
330K
220K
67K
60K
36K
1 2 3 4 5 6 7 8

Figure 4.8. Autoradiograph of ^{125}I-ConA overlay of electrophoretically-separated polypeptides from cystacanth envelopes and larvae and cockroach haemocyte lysate, lipophorin and plasma preparations.

Samples were electrophoresed on a 7.5% (w/v) acrylamide gel then electrophoretically transferred to nitrocellulose paper which was overlayed with ^{125}I-ConA as described in section 2.2.3.10.

Key : Track 1 Haemocyte lysate
Track 2 1% SDS/5% 2-ME extract of cystacanth envelopes
Track 3 Cockroach lipophorin
Track 4 Cockroach plasma
Track 5 1% SDS/5% 2-ME extract of cystacanth bodies

The positions of the molecular weight markers are given on the left-hand side of the Figure.


M_r
330K
220K
94K
67K
43K
1
2
3
4
5

in a 1% SDS solution supplemented with the reducing agent 2-mercaptoethanol (2-ME; 5% (v/v)), suggesting that it comprised protein(s) covalently cross-linked by disulphide bonds.

The residue obtained after 1% SDS extraction of isolated envelopes was collected and exhaustively washed, by centrifugation, first in 1% SDS solution and finally in distilled water. The residue was lyophilised then taken up in 1.0ml of 6N HCl. After hydrolysis, the dried, neutralised sample was dissolved in 125µl of 0.2N citrate buffer, pH 2.5 and 25µl aliquots were run on an LKB automated amino acid analyser. The results are shown in Table 4.4. Interestingly, L-proline, L-hydroxyproline and L-hydroxylysine are present which strongly suggests that a collagen molecule is a component of the residue. This is supported by the high glycine content (~15% of the total residues) which, however, does not represent one-third of the total residues (as expected for a collagen molecule) suggesting that proteins other than the collagen are also present in the envelope residue.

Envelopes were then sequentially extracted with first 1% SDS solution then with 1% SDS supplemented with 5% (v/v) 2-ME to disrupt disulphide-linked polypeptides. The extracts were analysed by SDS-PAGE, and the gels were processed in such a way as to detect collagen-like polypeptides as pink coloured bands, while non-collagenous polypeptides stain as blue bands. This is achieved by staining gels in a 0.1% (w/v) solution of Coomassie Blue R250 in 25% methanol, 10% acetic acid, with destaining in 10% acetic acid only. Under these conditions protein-Coomassie Blue complexes have absorbance maxima at 550-560nm and appear blue, while collagen-dye complexes have maxima at 520-535nm and appear as pink bands. The absorbance shift may be due to the high L-proline content, since human salivary glycoproteins, which are non-collagenous but do have a high L-

Table 4.4. Amino acid composition of cystacanth envelope residue after extraction with 1% SDS

The residue from 1000 cystacanth envelopes after extraction, with 1% SDS, was hydrolysed in 6N HCl at 110°C for 72 hr. Aliquots of the residue were removed and analysed on a LKB automated amino acid analyser as described in section 2.2.3.7.

Values represents the average for three separate analyses.

Table 4.4. Amino acid composition of the residue remaining after extraction of envelopes with 1% SDS

Amino acid	Residues per 1000 total residues
Cys SH	19.6
Hyp	4.4
Asp	100.0
Thr	51.2
Ser	53.3
Glu	109.0
Pro	65.8
Gly	149.0
Ala	42.3
Cys	12.0
Val	44.3
Met	6.5
Ile	51.2
Leu	90.0
Tyr	22.9
Phe	37.9
Hyl	39.1
His	18.9
Lys	32.0
Arg	49.8

proline content, also appear as pink bands using the above protocol (Beeley, J.A., pers. comm.). The results of applying this staining procedure to a 1% SDS extract of envelope and a 1% SDS + 5% 2-ME extract of envelope residue polypeptides are shown in Figure 4.3. The SDS/2-ME residue, track 5, contains a high molecular weight ($M_r \sim$ 200K) pink-staining band which is not observed in the 1% SDS extract (track 4) i.e. this band appears only when 2-mercaptoethanol is present in the extraction buffer suggesting that the molecules are cross-linked, perhaps by inter- as well as intra-molecular disulphide bonds. There are other polypeptides present in track 5 which are either absent or present only in relatively small amounts in the 1% SDS extract, notably at M_r 180000.

4.2.1.4. Envelope collagen molecule

Having established the presence of a collagen in the envelope an attempt was made to characterise certain aspects of the molecule in more detail, such as the number of chains and their relative molecular weights, for comparison with the cockroach molecule which has been characterised (Francois et al., 1980).

Bearing in mind the aberrant behaviour of collagenous polypeptides on SDS gels (i.e. they migrate slower than "globular" proteins of the same molecular weight (McCormick et al., 1979)), the value for the molecular weight of the envelope collagen was calculated to be 250000, by comparison with the migration of globular protein standards, or 150000 compared with the migration of the three major pepsin-soluble chains of the type VI collagen standard which have molecular weights of 73000, 60000 and 40000 (Trüeb and Bornstein, 1984).

4.2.1.5. Pepsin and Collagenase treatment of cystacanth envelopes

The triple-helical region of collagen molecules is insensitive to the proteinase pepsin - a fact which has been exploited during the isolation of collagen from a variety of tissues (e.g. Miller, 1971). Duplicate samples, each containing 100 envelopes, were pepsin digested and digests were analysed on an SDS gel for comparison with the pepsin-treated type VI collagen standard (Figure 4.9, tracks 8 and 9); it was hoped that the pattern of polypeptides thus produced would reveal whether the envelope molecule contains any regions of helical conformation and would also reveal some information on the organisation of the monomer polypeptides within the collagen trimer. Duplicate samples of washed envelope residues resulting from extraction with either 1% SDS/2-ME in 10mM Tris HCL, pH 7.2 or 1% SDS/8M Urea also in Tris buffer, were also digested with pepsin in parallel experiments. The results (Figure 4.9, tracks 1-7) show that a faint pink-staining band is visible in the whole envelope extract (tracks 2 and 3) at apparent M_r 120000. This band is not visible in the tracks representing the pepsin-digested SDS/2-ME- or SDS/urea/2-ME- extracted envelope residues (tracks 4-6). Pink staining bands of similar relative molecular weight to the envelope polypeptide are present in the collagen-standard tracks (8 and 9), while the untreated SDS/2-ME extract of whole envelopes contains a pink-staining band at M_r 250000 (track 1).

No pink-staining bands are present in the envelope samples treated with collagenase, which suggests that the envelope molecule is susceptible to an activity present in the commercial collagenase preparation. However, there is extensive degradation of a number of cystacanth body polypeptides after incubation of an aliquot of larval homogenate with bacterial collagenase (Figure 4.9, track 13), which suggests that the

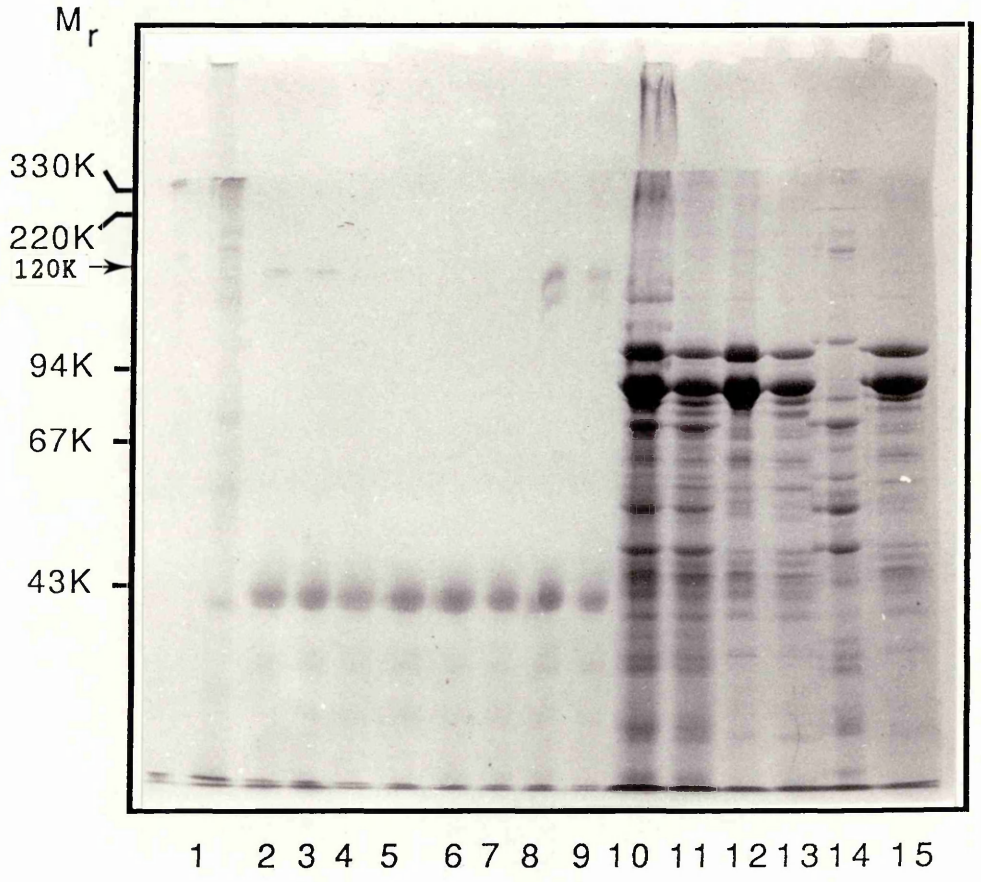
Figure 4.9. Pepsin and collagenase digestion of cystacanth envelopes

Whole cystacanth envelopes* or the envelope residues obtained after extraction with 1% SDS/5% 2-ME or 1% SDS/8M urea were digested with pepsin as described in section 2.2.3.16.2. (track 1-9).

Cystacanth envelopes, larvae and type VI collagen (50µg) were treated with collagenase as described in section 2.2.3.16.3. Digests were then analysed by SDS-PAGE on a 10% (w/v) acrylamide gel (tracks 10-15).

Key :
1 1% SDS/5% 2-ME extract of cystacanth envelopes
2 Duplicate samples of cystacanth envelopes
3 digested with pepsin.
4 Duplicate samples of 1% SDS/5% 2-ME
5 envelope residue digested with pepsin
6 Duplicate samples of 1% SDS/8M urea
7 envelope residue digested with pepsin
8 Duplicate samples of type VI collagen
9 digested with pepsin
10 Collagenase-digested typed VI collagen
11 Duplicate samples of cystacanth
12 envelope digested with collagenase
13 Cystacanth body homogenate collagenase digested
14 Cystacanth body homogenate
15 Collagenase

*The original gel showed two pink-staining bands in tracks 2 and 3 i.e. at M_r 120000 and a faint band at M_r 140000.


Mr
330K
220K
120K
94K
67K
43K
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

collagenase preparation contains other proteinase activities, present as contaminants, which may also account for the observed degradation of the envelope collagen molecule (Figure 4.9, track 11).

In conclusion, the partial resistance of the envelope collagen molecule to pepsin digestion suggests that it contains region(s) of helical conformation, but with at least one non-helical domain which contains a pepsin cleavage site.

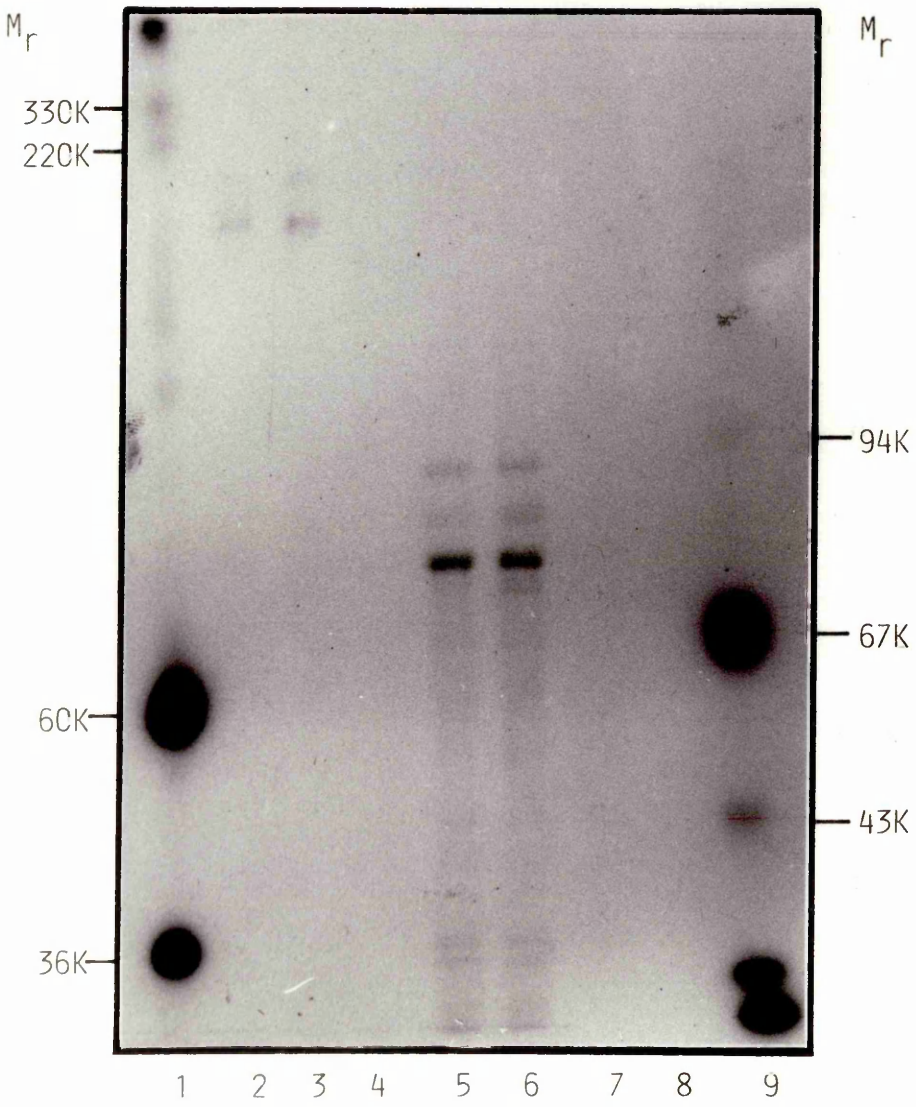
4.2.3. Metabolic radioisotopic labelling of Moniliformis larvae macromolecules

Stage V-VI larvae, either enveloped or de-enveloped by dissection, were cultured *in vitro* using 5% (v/v) foetal calf serum (FCS)- supplemented medium D73 (section 2.1.3.4.). L-[^{3}H-2,3,4,5.] - proline was then added to fresh medium containing no FCS or proline, and cultures were maintained for a further 24hr. Larvae were then collected and envelopes removed as described before. Envelopes and larvae were sequentially extracted with first 1% SDS in 10mM Tris HCl pH 7.2 and then 1% SDS/5% 2-ME in this buffer. Extracts were analysed by SDS-PAGE and metabolically radiolabelled polypeptides were detected by fluorography (section 2.2.3.12.). The results shown in Figure 4.10. suggest that there is negligible incorporation of the label into the envelope and very low incorporation into the larval bodies. The pattern of labelled polypeptides detected in the larvae for 1% SDS extracts and 1% SDS/2-ME extracts are different, with the appearance of two high molecular weight polypeptides in the SDS/2-ME extract from enveloped or de-enveloped larvae, which are not solubilised by SDS extraction alone; this suggests that they

Figure 4.10. Fluorograph of electrophoretically-separated stage V-VI acanthellae polypeptides metabolically labelled with L-[^{3}H]- proline in vitro.

Enveloped or de-enveloped larvae were culture in vitro for 24 hr. in the presence of L-[^{3}H]-proline in medium D73 supplemented with 5% (v/v) FCS (section 2.2.1.11.). Parasites were then collected and dissected envelopes and enveloped and de-enveloped larvae were then extracted sequentially with first, 1% SDS then 1% SDS containing 5% 2-ME. All extracts were then analysed by SDS-PAGE on a 7.5% (w/v) polyacrylamide gel which was subsequently processed for fluorography as described in section 2.2.3.12.

Key : 1 ^{125}I-labelled high molecular weight markers
2 1% SDS/5% 2-ME extract of larval body residue after 1% SDS extraction.
3 1% SDS/5% 2-ME extract of enveloped larval residue after 1% SDS extraction.
4 1% SDS/5% 2-ME extract of envelope residue after 1% SDS extraction.
5 1% SDS extract of de-enveloped larvae
6 1% SDS extract of enveloped larvae
7 1% SDS extract of envelopes
8 larval culture medium
9 ^{125}I-labelled low molecular weight markers


Mr
330K
220K
60K
36K
Mr
94K
67K
43K
1
2
3
4
5
6
7
8
9

are disulphide linked, and are only solubilised in the presence of 2-ME. Interestingly, these polypeptides have similar molecular weights (~140K) to the pink-staining envelope molecules observed after pepsin extraction of cystacanth envelopes (Figure 4.10., track 2 and 3). No trichloroacetic acid (TCA) - precipitable tritium counts were detected in the dialysed, concentrated culture fluid, suggesting that no secretory-excretory products were metabolically labelled.

In vitro labelling experiments with L-^{35}S-methionine and D-^{3}H-glucosamine produced similar results even if earlier larval stages were used; there was negligible incorporation into envelopes, and, since such low levels were incorporated into larval bodies, impractically long exposure times (3-6 months) were required to detect radiolabelled macromolecules by fluorography. For $Na_2{}^{35}SO_4$ labelling in vitro TCA- and ethanol- precipitable counts were measured in the dialysed, concentrated culture fluid for experiments using stages II, V-VI and cystacanth larvae labelled in the presence of FCS. Analysis by SDS-PAGE/fluorography revealed the presence of a sulphate-labelled polypeptide of M_r 67000. This band was, however, absent if serum-free medium was used, which suggested that it represented serum albumin (from FCS) which was tightly binding the labelled sulphate. There have been many reports of serum albumin binding a variety of small biomolecules and drugs (Allen, Hill and Stokes, 1977). Evidence that the molecule was not parasite-derived was obtained by including control wells, during culturing, which contained only 5% (v/v) FCS in medium plus the isotope. The M_r 67000 molecule was also present in the pooled sample, suggesting that it represented labelled serum albumin (see section 3.4.).

In one radiolabelling experiment using cystacanths, with $Na_2{}^{35}SO_4$ and L-[^{3}H-2,3,4,5]-proline present in the culture medium, both ^{3}H-proline and $^{35}SO_4{}^{2-}$ counts could be detected in the ethanol-precipitable fraction of the dialysed culture medium. Analysis of an aliquot of this fraction by SDS-PAGE, followed by fluorography, revealed the presence of two radiolabelled bands at M_r 250000 and 67000 (Figure 4.11.). Those regions of the dried-down gel containing the two bands were cut out, heated (to elute the labelled polypeptide from the gel matrix) at 60°C for 1hr. in sealed vials containing 5ml of scintillation fluid, then counted on a scintillation counter. The results revealed that, while the M_r 67000 band contained predominantly $^{35}SO_4{}^{2-}$-counts as observed before, the M_r 250000 band contained predominantly ^{3}H-proline counts. Also, gel filtration on Sephadex G75(M) of labelled medium which had been incubated with bacterial collagenase demonstrated a shift of elution for the tritium counts from the void volume (V_o) for undigested material, to the total volume (V_t) for the collagenase-treated material (Figure 4.12(a) and (b)). This suggested that the ^{3}H-proline was incorporated into a molecule which was susceptible to a proteinase activity present in the commercial preparation of collagenase (i.e. collagenase or another proteinase activity).

An aliquot of the dialysed culture medium was heated in 6N HCl at 110°C for 24hr. to hydrolyse the proteins present to their constituent amino acids. After vacuum drying, the sample was taken up in a small volume of isopropanol:water (9:1, v/v) and analysed by two-dimensional thin-layer chromatography (TLC) on silica plates (section 2.2.3.5.2.). The results are shown in Figure 4.13. For the labelled medium, two pink spots were

Figure 4.11. Fluorograph of radiolabelled culture medium polypeptides, separated by SDS-PAGE, from in vitro maintenance of cystacanths in the presence of L-[^{3}H]-proline and $^{35}SO_4^{2-}$.

Twenty-five cystacanths in 1.0ml of D73 medium, supplemented with 5% (v/v) FCS, were cultured in vitro in the presence of 10μCi/ml L-[^{3}H]-proline and 50μCi/ml $^{35}SO_4^{2-}$. The culture medium was extensively dialysed and a small aliquot of the dialysed culture medium containing about 20000 cpm was ethanol precipitated. The ethanol precipitate was solubilised in SDS-PAGE sample buffer containing 5% 2-ME and analysed by electrophoresis and fluorography.

The relative molecular weights of the radiolabelled polypeptides are indicated at the right-hand side of the Figure.

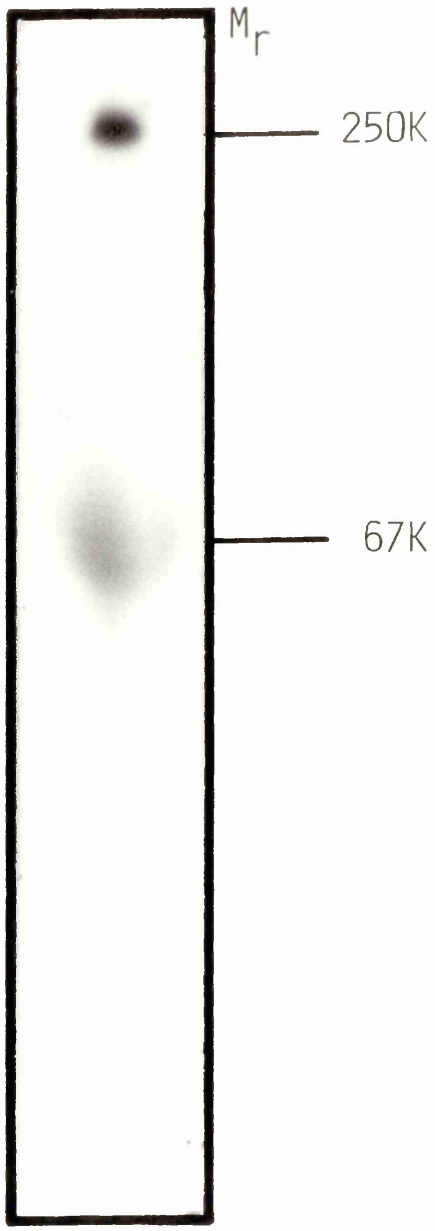
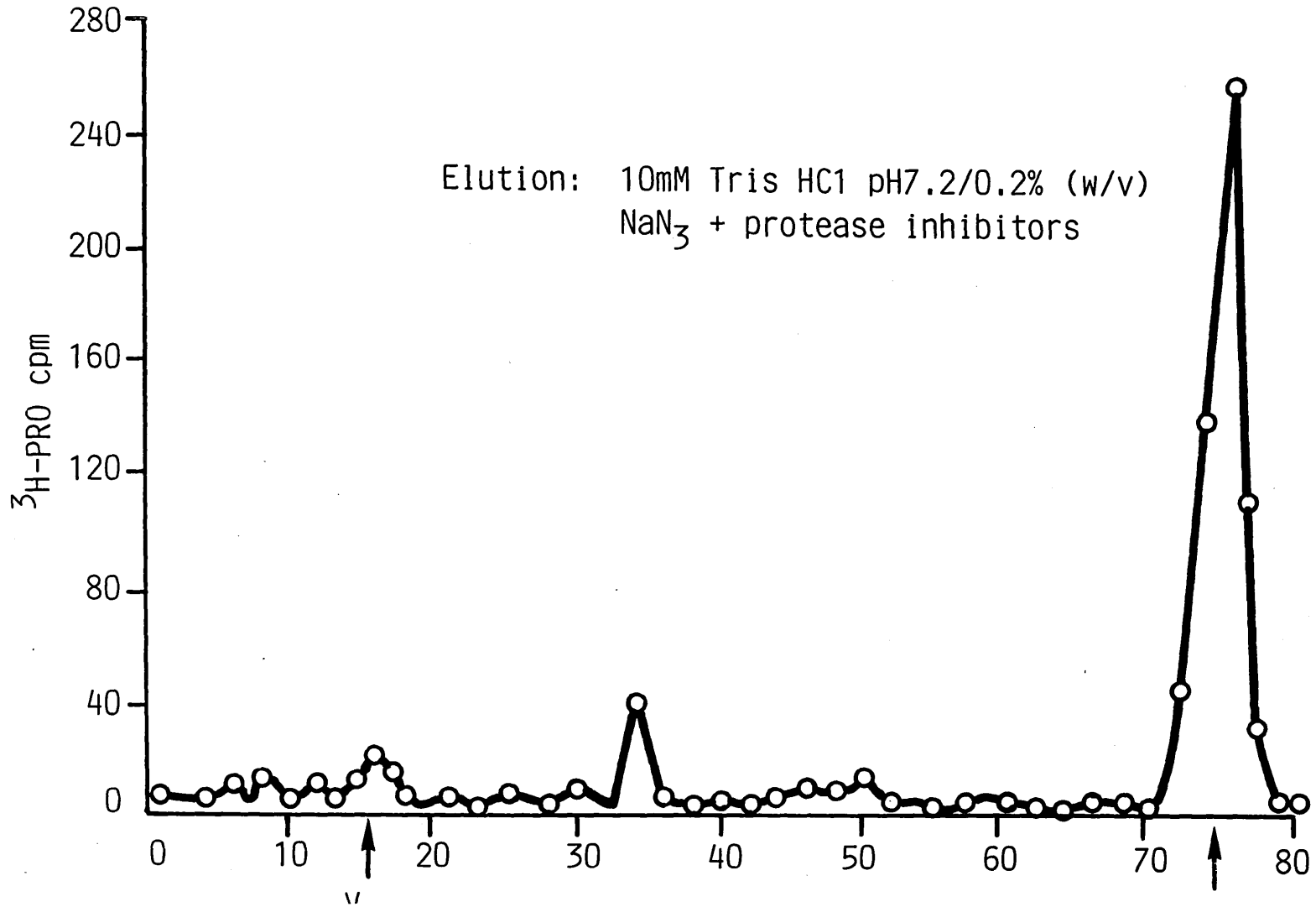

M_r
250K
67K

Figure 4.12. Gel filtration of collagenase-digested L-[^{3}H]-proline/$^{35}SO_4^{2-}$-culture medium from in vitro maintenance of cystacanths.

Aliquots of the dialysed culture medium (each containing 1000 cpm of tritium) were incubated for 24 hr. at 37°C in 200µl of 0.1M Tris HCl pH 8.0, 0.02% sodium azide, containing 50 Units of bacterial collagenase (section 2.2.3.17.) or heat-inactivated (60°C; 2 min.) enzyme. Digested and mock-digested samples were then chromatographed on a 1 x 50 cm. Sephadex G75(M) column which had been pre-equilibrated with 10mM Tris HCl pH 7.2. The column was eluted with the same buffer and 80 x 1.0ml fractions were collected for each sample. Radioactivity in each sample was determined by liquid scintillation counting.

(a) Elution profile of collagenase-digested culture medium.

(b) Elution profile of mock-digested culture medium.


280
240
200
160
120
80
40
0
^{3}H-PRO cpm
Elution: 10mM Tris HC1 pH7.2/0.2% (w/v)
NaN_3 + protease inhibitors
0
10
20
30
40
50
60
70
80
V

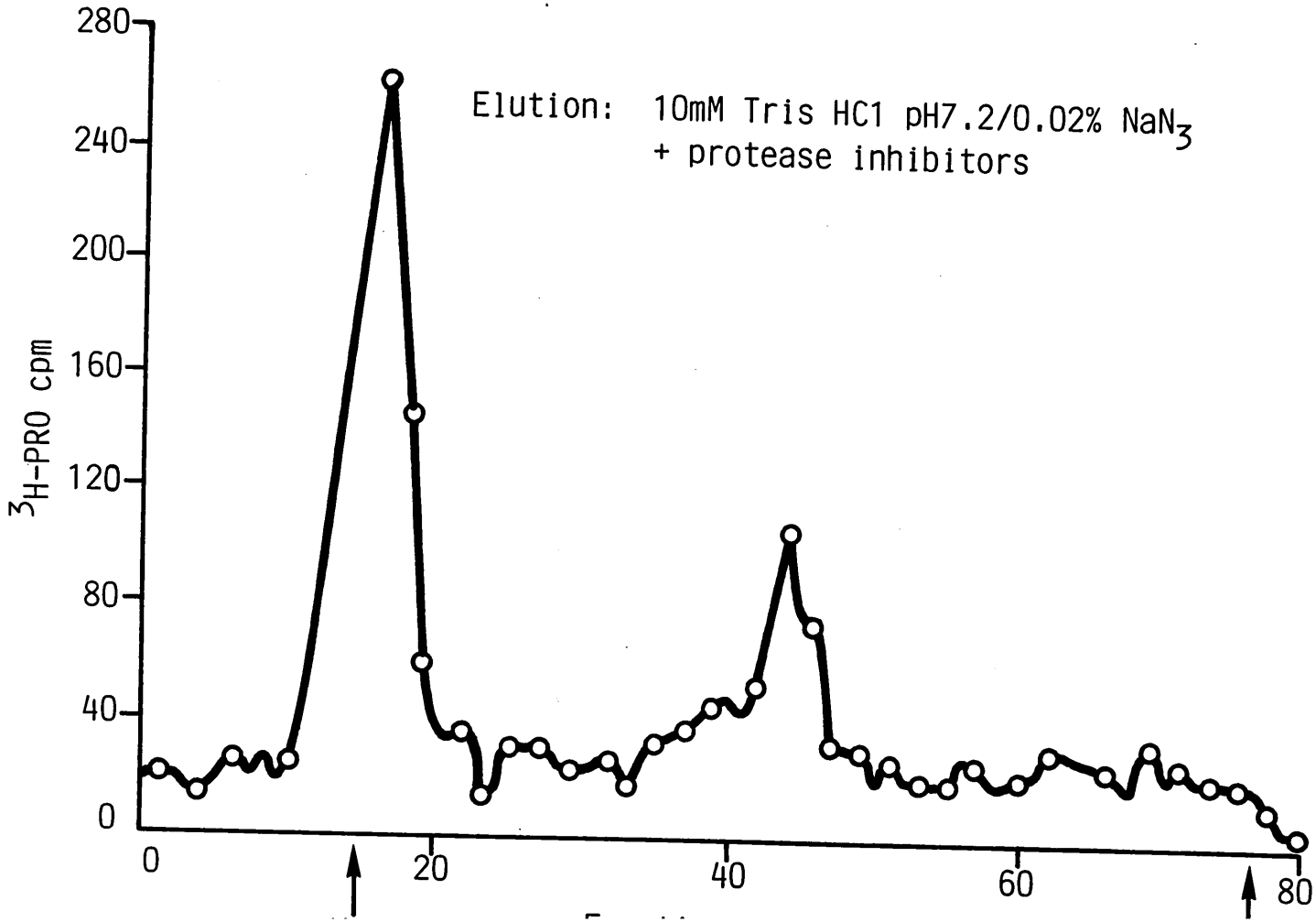

Elution: 10mM Tris HC1 pH7.2/0.02% NaN3
+ protease inhibitors
3H-PRO cpm
280
240
200
160
120
80
40
0
0
20
40
60
80

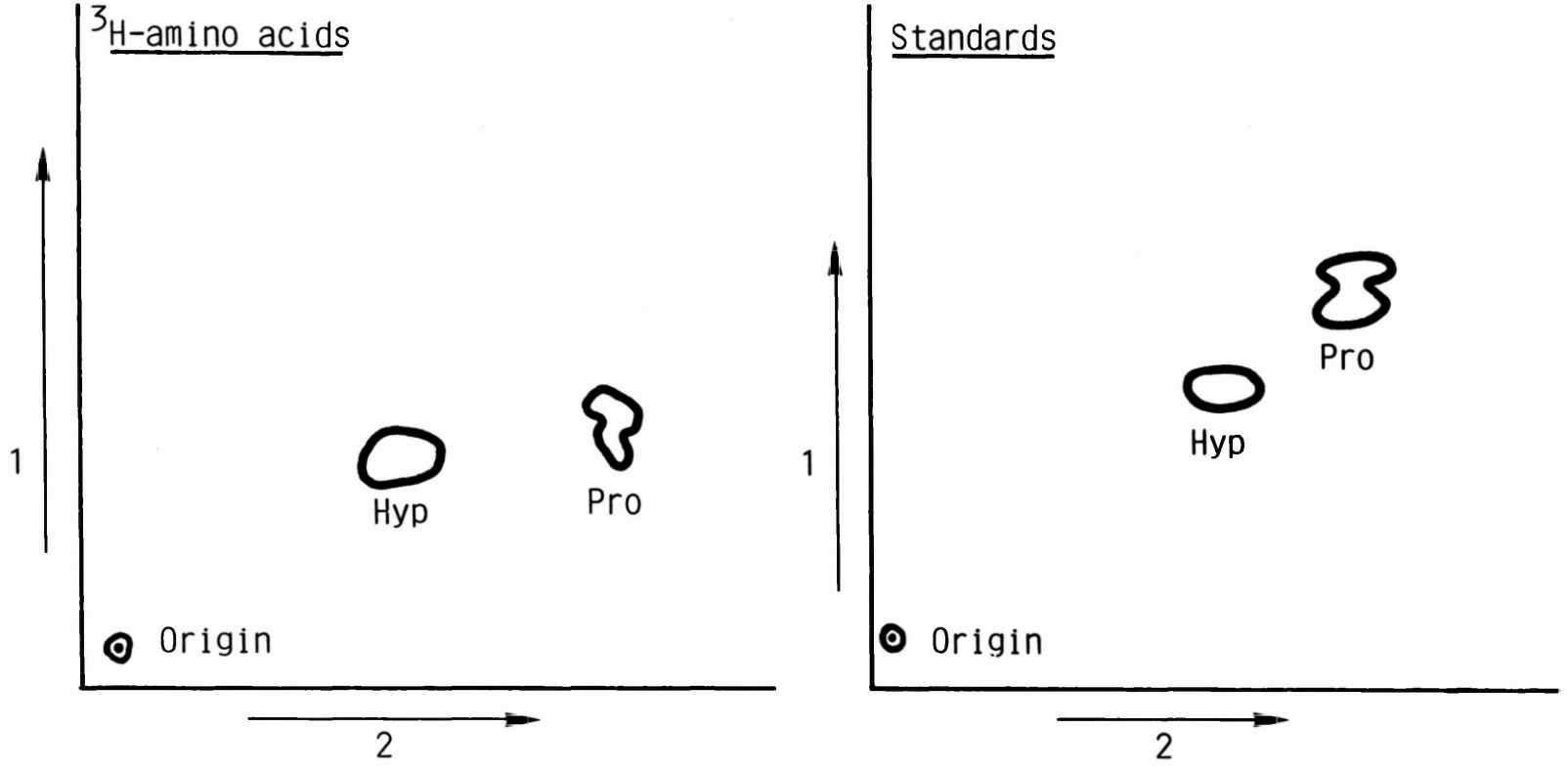
Figure 4.13. Two-dimensional (2D) thin-layer chromatograph (TLC) of ^{3}H-amino acids in an acid-hydrolysed aliquot of L-[^{3}H]-proline/$^{35}SO_4^{2-}$-culture medium

An aliquot of the dialysed culture medium was hydrolysed at 110°C for 24 hr. in 6N HCl under vacuum as described in section 2.2.3.7. After neutralisation of the acid and vacuum drying, the sample was taken up in a small volume of isopropanol:water (9:1 v/v) and analysed by 2D-TLC as described in section 2.2.3.5.2.

Key : Hyp L-4-hydroxyproline

Pro L-proline

Arrows indicate the direction of chromatography in the first dimension (1) and the second dimension (2).



Solvent 1: n-butanol: acetic acid: H_2O (65:15:25 v/v)
Solvent 2: 80% phenol in H_2O
Stain: 0.2% (w/v) isatin in acetone

apparent after isatin staining, characteristic of the imino acids proline and hydroxyproline. Also, the positions of these spots on the plate corresponded fairly well to those of the standard imino acids; the presence of amino acids and other material probably prevented an exact correlation between these standards and the pink-staining spots from the dialysed culture medium. The isatin-stained spots from the culture medium were scraped off the plate into separate vials containing 5ml of scintillation fluid. Both spots contained tritium counts suggesting that the labelled molecule contained both proline and, more significantly, hydroxyproline, indicating metabolic incorporation into a molecule which may represent a parasite collagen. Unfortunately, this result could not be repeated with cystacanths or earlier larval stages of the parasite.

4.2.4. Radioiodination of cystacanths and stage II acanthellae

As mentioned in Chapter 1 (section 1.6.3.4.) the absolute values of envelope protein, lipid and carbohydrate do not change during larval development. However, whether or not there is variation in the actual component macromolecules of the envelope during development is not known. In order to investigate whether the pattern of proteins present in the envelope varies during larval development, enveloped cystacanths or stage II acanthellae (whose envelope is just visible, elevated from the larval surface) were used for radioiodination studies. After iodogen-catalysed radioiodination of intact larvae, of saline washed stage II parasite larvae and of envelopes collected from labelled cystacanths, all tissues were sequentially extracted using 1% SDS followed by 1% SDS/2-ME as described elsewhere (section 2.2.3.6.). For acanthellae it was impossible to remove the envelope so whole larvae were extracted. The extracts were analysed by SDS-PAGE and radiolabelled macromolecules detected by direct

autoradiography of dried gels (Figure 4.14.). The results suggest that the pattern of polypeptides labelled for the two developmental stages is very similar, both for 1% SDS and 1% SDS/2-ME extracts. Furthermore, for the cystacanths, only a limited number of envelope polypeptides are labelled, suggesting some restriction in the accessibility of the label for the proteins in the intact envelope. In a 1% SDS extract of cystacanth envelopes all the Coomassie Blue-staining bands can be radioiodinated using iodogen-coated tubes (see Figures 4.4 and 5.9). All the labelled polypeptides appear to be components of the envelope. Also, the cystacanth envelope SDS/2-ME extract contains a polypeptide of similar M_r to the envelope collagen molecule (i.e. at 250000) which is also only solubilised in SDS solutions supplemented with reducing agent (Figure 4.14, track 4). A labelled molecule of similar M_r is also present in the stage II extract (track 9). This suggests that the collagen is present at, or near, the outer surface of the envelope in both cystacanths and acanthellae. Other major labelled proteins in both cystacanth and acanthellae extracts have similar M_r values to host plasma proteins.

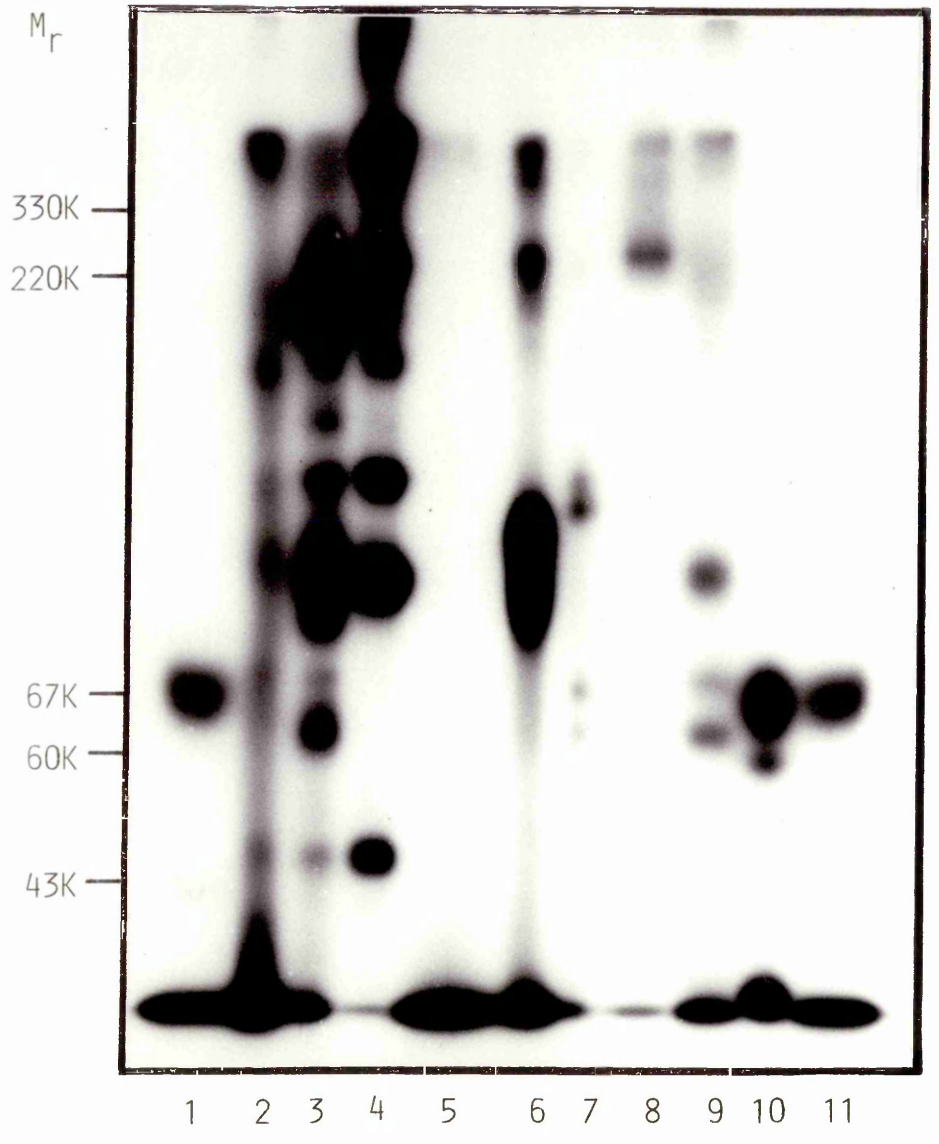
4.2.5. Radioiodination of Locust-grown cystacanths

In order to compare the component macromolecules of cystacanths grown in their normal hosts, cockroaches, and experimental hosts, locusts, intact cystacanths from each species were radioiodinated then their envelopes extracted with 1% SDS. *In vitro*-hatched acanthors were introduced by injection into the haemocoele of *Schistocerca gregaria* (see section 2.2.1.3.). Parasites were collected six-weeks post-injection of acanthors and enveloped larvae were radiolabelled, then their envelopes were removed and extracted in a 1% SDS solution. The extracts and radiolabelled locust plasma proteins were analysed by SDS-PAGE (Figure 4.15.). With the

Figure 4.14. Autoradiograph of SDS-gel showing ^{125}I-labelled proteins extracted from enveloped and de-enveloped cystacanths and stage II acanthellae

Intact cystacanths, de-enveloped cystacanths and stage II acanthellae were radioiodinated as described in section 2.2.3.8.1. Envelopes were recovered from the cystacanths as described in section 2.2.1.2. and both larvae and isolated envelopes were then extracted sequentially with first 1% SDS, then with 1% SDS/5% 2-ME. Proteins in 1% SDS extracts of cystacanth envelopes and larval bodies were also radioiodinated as described in section 2.2.3.8.2. and aliquots of all the samples were analysed by SDS-PAGE and autoradiography.

Key : Track 1 ^{125}I-labelled high molecular weight markers
Track 2 ^{125}I-labelled envelope proteins, extracted with 1% SDS then iodinated
Track 3 ^{125}I-labelled proteins extracted with 1% SDS from iodinated envelopes
Track 4 ^{125}I-labelled proteins extracted with 1% SDS/5% 2-ME from the iodinated envelope residue
Track 5 ^{125}I-labelled cockroach lipophorin
Track 6 ^{125}I-cockroach plasma proteins
Track 7 ^{125}I-labelled larval body proteins, extracted with 1% SDS then iodinated
Track 8 ^{125}I-labelled proteins extracted with 1% SDS/5% 2-ME from de-enveloped larvae
Track 9 ^{125}I-labelled proteins extracted with 1% SDS/5% 2-ME from stage II acanthellae
Track 10 ^{125}I-labelled low molecular weight markers
Track 11 ^{125}I-labelled high molecular weight markers


Mr
330K
220K
67K
60K
43K
1
2
3
4
5
6
7
8
9
10
11

exception of the two (cockroach) lipoprotein subunits at M_r 250000 and 85000, there is a similar pattern of ^{125}I-labelled polypeptides in envelopes from cystacanths from locusts and from cockroaches. Interestingly, none of the envelope-associated polypeptides, from locust-grown cystacanths, have electrophoretic mobilities very similar to locust plasma proteins. This suggests that either the locust plasma proteins are not associated with the envelope or that they are associated with the envelope but cannot be radiolabelled under these conditions.

4.2.6. Transmission electron microscopy of normal and solvent-extracted cystacanth envelopes

Having established that the envelope represented a complex, organised structure containing a number of lipids and proteins, transmission electron microscopy was utilised to examine normal and solvent extracted envelope sections, in order to gain some insight into how these macromolecules are organised in the envelope. Two solvents were used: Chloroform:methanol (1:2 v/v), which had been shown to extract envelope lipids, and 1% SDS in 10mM Tris HCl pH 7.2, which had been shown to extract about 90% of the envelope protein, but, significantly, did not extract the envelope collagen and some other proteins. The results are shown in Figure 4.16(a),(b) and (c). After chloroform:methanol extraction the "vesicles" of the cystacanth envelope were no longer visible; the envelope width was compressed to about 0.5μm and electron-dense granular material was present between the outer layer and the inner amorphous region (Figure 16(b)). The "vesicle" structures may therefore represent sections through membranous profiles which are disrupted by lipophilic solvent extraction.

Figure 4.15. Autoradiograph of SDS-gel showing ^{125}I-labelled proteins extracted from radioiodinated cystacanths recovered from experimental locust hosts.

Cystacanths were recovered from experimental locust hosts 6 weeks after intra-abdominal injection of hatched acanthor larvae (section 2.2.1.3.). Enveloped larvae were radioiodinated, then their envelopes were removed and extracted with 1% SDS. The extracts were analysed by SDS-PAGE on a 10% (w/v) acrylamide slab gel.

Key:
- Track 1 ^{125}I-labelled low molecular weight markers
- Track 2 ^{125}I-labelled locust plasma proteins
- Track 3 ^{125}I-labelled proteins extracted from envelopes with 1% SDS then iodinated
- Track 4 ^{125}I-labelled proteins extracted from cystacanth envelopes using 1% SDS
- Track 5 ^{125}I-labelled proteins extracted from enveloped cystacanths using 1% SDS
- Track 6 ^{125}I-labelled proteins extracted from cystacanth bodies with 1% SDS then iodinated
- Track 7 ^{125}I-labelled cockroach plasma proteins
- Track 8 ^{125}I-labelled high molecular weight standards

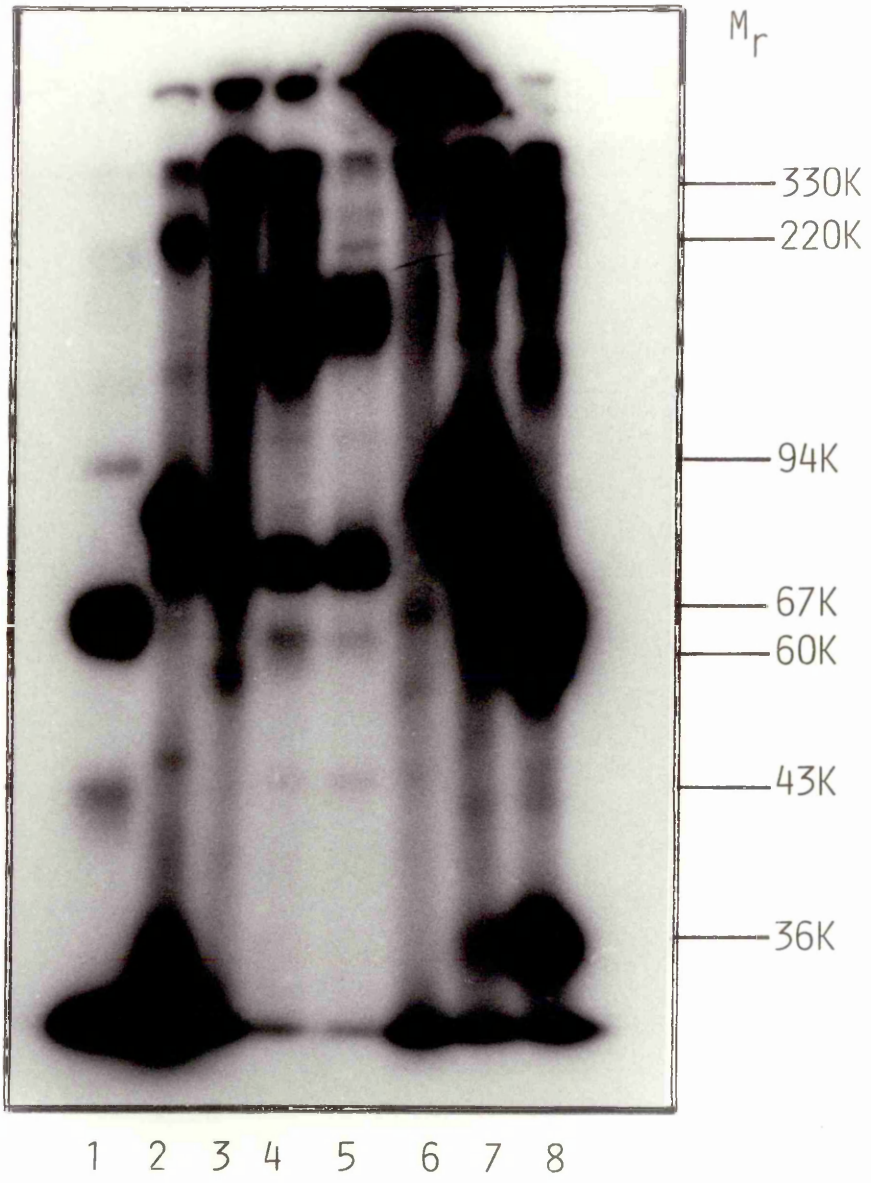

Mr
330K
220K
94K
67K
60K
43K
36K
1 2 3 4 5 6 7 8

Figure 4.16. Transmission electron microscopy of normal and solvent-extracted cystacanth envelopes.

Enveloped cystacanths were extracted with chloroform:methanol (1:2, v/v) or 1% SDS in 10mM Tris HCl, pH 7.2, as described in section 2.2.2.4. Normal and solvent-extracted cystacanths were then processed for transmission electron microscopy (section 2.2.2.4.).

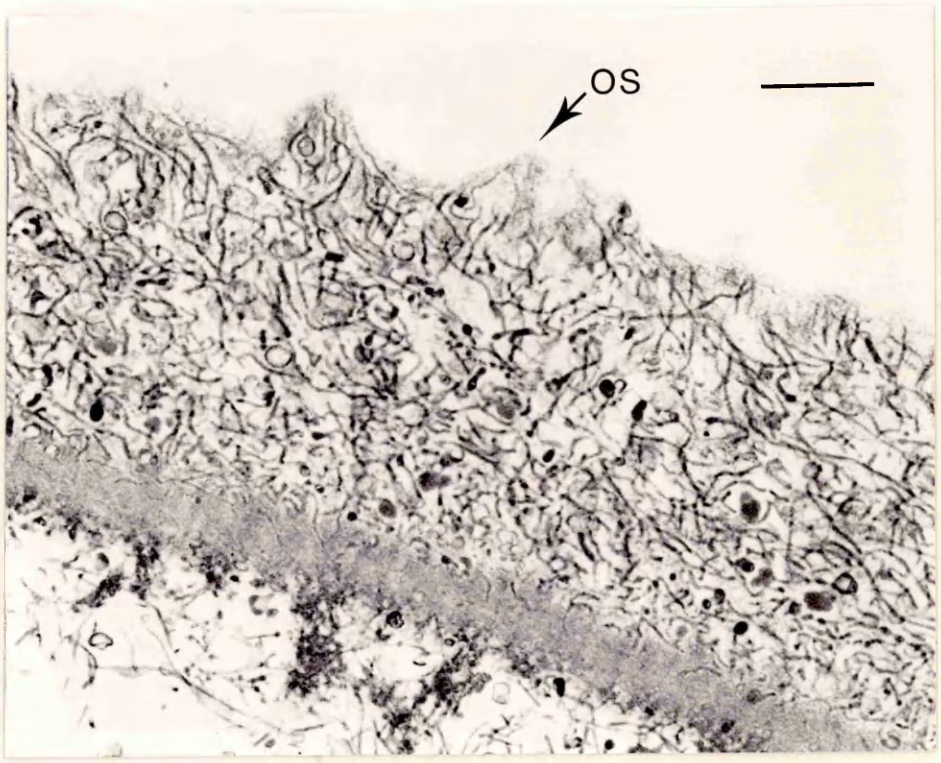
(a) Electronmicrograph of a normal cystacanth envelope.
Bar, 0.25µm.

(b) Electronmicrograph of a chloroform:methanol-extracted cystacanth envelope.
Bar, 0.1µm.

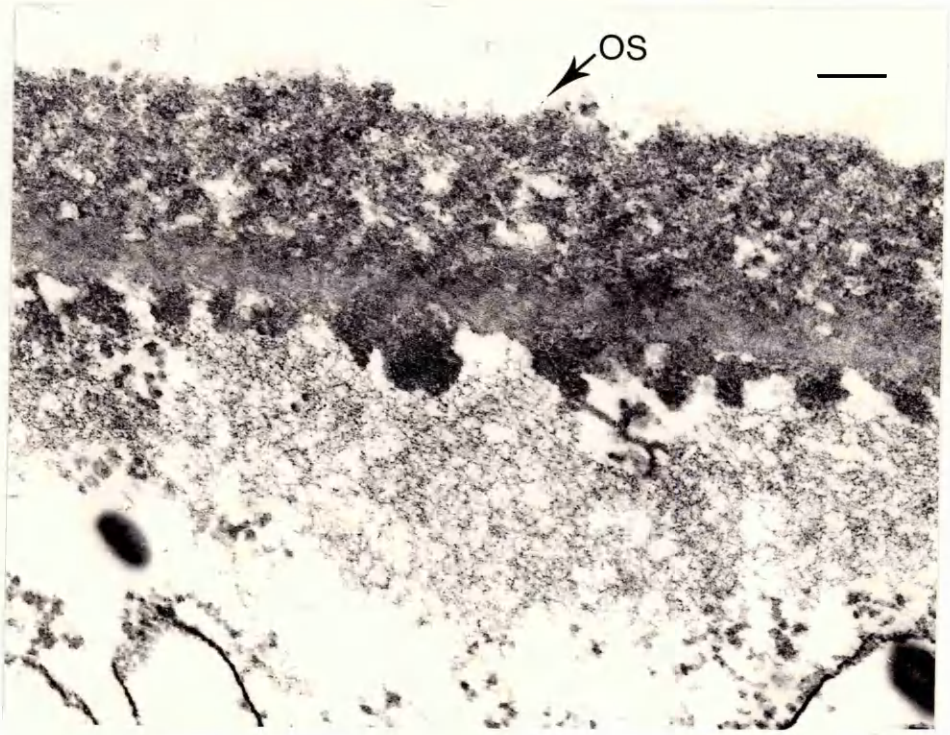
(c) Electronmicrograph showing a section through a cystacanth envelope fixed after extraction with 1% SDS
Bar, 0.25µm.

OS Outer surface

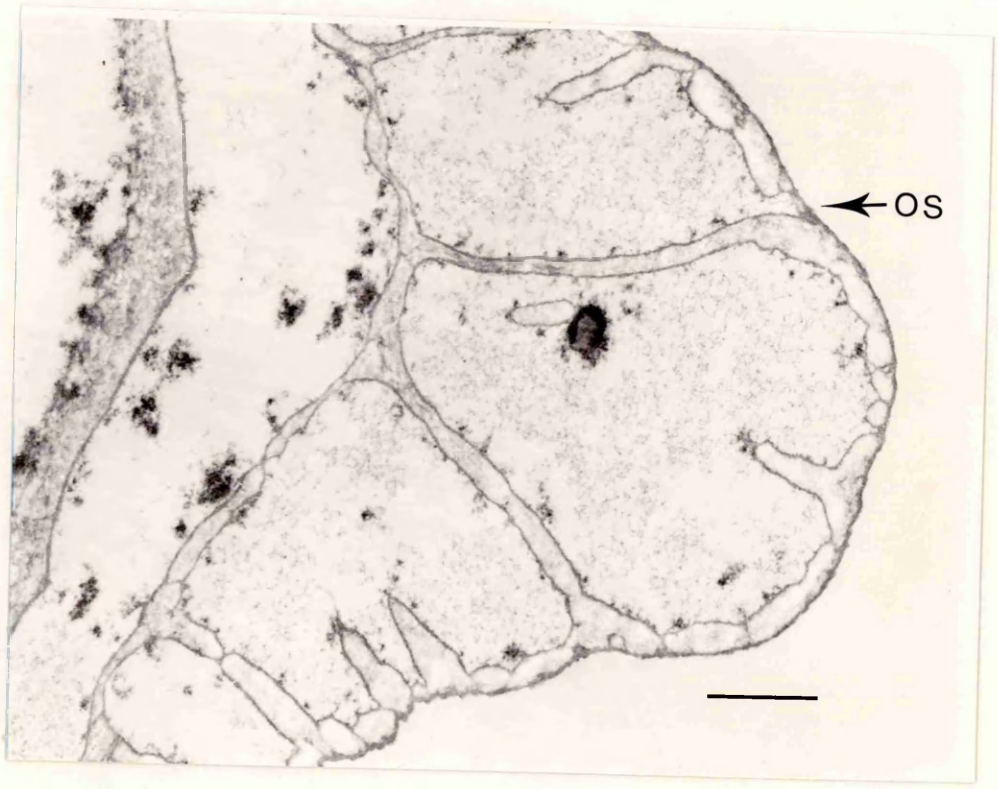
(a)



(b)



(c)



After extraction with 1% SDS the organisation of the envelope into three distinct layers was drastically affected. No "vesicle" structures were apparent while the innermost amorphous layer was highly disrupted. However, after extraction the envelope does appear as an intact structure surrounding the larvae, as observed by light microscopy, but is somewhat collapsed and deformed compared to the normal envelope.

4.3. Discussion

The results from the compositional analysis of the envelope for protein, carbohydrate and lipid are in close agreement with those obtained by J. Lackie (1973). Furthermore, the results from transmission electron microscopy also confirm Lackie's finding that the envelope is a complex trilaminate structure about 1μm thick.

4.3.1. Lipids

The results from lipid analysis show that three phospholipids, a sterol and two unidentified non-polar lipids are components of the envelope. Transmission electron microscopy in conjunction with solvent extraction has provided some evidence that the "vesicular" region of the envelope represents organised lipid assemblies, such as compressed or deformed lipid-rich vesicles or cross-sections through foldings in a compacted, continuous membrane bilayer which may derive from the microvillar protrusions of the acanthellar tegument observed by J. Lackie and Rotheram (1972). The uptake of fluorescent lipid analogues by the envelope confirms that there are lipid domains within the envelope. It has been demonstrated that the alkyl chain length of lipids in bacterial membranes influences membrane fluidity, with short-chain fatty acids promoting a more fluid bilayer organisation. Perhaps the observed

differential uptake of the fluorescent lipid analogues, that is dependent on the alkyl chain length of their non-polar "tail regions", may reflect the physical properties of the envelope lipid, such as fluidity. Perhaps more interesting are the observed differences in the uptake of lipid analogues by envelopes and by larval bodies, suggesting that fundamental differences exist between the physical properties of the lipid in the envelopes and in the larval surfaces. These differences may be crucial in helping to explain why de-enveloped intact larvae are readily encapsulated after injection into the abdominal haemocoele of a naive host; the differences in lipid fluidity may reflect differences in the hydrophobicity of the envelope and larval surfaces - which, as mentioned in Chapter 1, does appear to influence the adhesion of haemocytes to foreign surfaces.

Further characterisation and identification of the envelope lipids should be possible using two dimensional thin-layer chromatography in conjunction with differential staining techniques. The physical environment of the envelope phospholipids could also be investigated using ^{31}P-nuclear magnetic resonance (NMR) spectroscopy. This technique would allow for non-destructive analysis of component macromolecules using isolated envelopes or enveloped larvae. A study of fluorescence recovery after photobleaching (FRAP), would yield information on the molecular associations (i.e. lipid-lipid and lipid-protein interactions) of envelope lipids and would measure the fluidity of envelope bilayer organisations.

4.3.2. Envelope proteins

The inability to extract appreciable amounts of protein in solvents lacking detergents or chaotropic agents such as urea, suggests that many of the envelope proteins are in close association with lipid, perhaps as

integral or peripheral membrane proteins, or are interacting through strong non-covalent associations such as hydrophobic domain interactions or hydrogen bonds. A 1% (w/v) SDS solution proved to be a suitable solvent for about 90% of the total envelope protein as determined by the Lowry method. This extract, when analysed by SDS-PAGE, was resolved into at least 25 polypeptides which could be visualised by Coomassie Blue R250 staining of gels. ^{125}I-labelled lectin staining suggests that all these proteins were glycoproteins, as expected for extracellular proteins. Although some polypeptides had similar electrophoretic mobilities to host plasma proteins (see Chapter 5) the majority appeared to be unique to the envelope as suggested by the absence of similar glycoproteins in the larval body extracts or haemocyte lysate preparations. Re-extraction of the 1% SDS envelope residue with 1% SDS supplemented with 5% (v/v) 2-mercaptoethanol (2-ME) resulted in the appearance on SDS-gels of envelope polypeptides not present in the 1% SDS extract, or enrichment of polypeptides present in this extract. One of the disulphide-linked molecules is a collagen as determined by its staining properties on SDS gels, its partial resistance to pepsin digestion and the characteristic amino acid content of the 1% SDS residue containing this molecule.

The results from collagenase digestion cannot be unequivocally interpreted as indicating the presence of a collagen in the envelope, owing to the contamination of the commercial collagenase preparation with other proteinase activities. However, although taken separately these results do not conclusively demonstrate the presence of a collagen in the envelope, taken together they strongly suggest the presence of such a molecule.

Examination of the envelope after solvent extraction, by transmission electron microscopy, suggests that most of the envelope protein is in the inner amorphous region, with perhaps the collagen molecule and some others

(i.e. those radiolabelled with ^{125}I in intact enveloped cystacanths) in the outermost Alcian Blue-staining layer, where they may be in close association with the acidic macromolecules (GAGs) responsible for dye binding. Other proteins may, as mentioned above, be in association with the envelope lipid and may represent the electron-dense layer seen between the outer and inner layers after chloroform-methanol extraction. The innermost amorphous layer, although highly disrupted in parasites extracted with a 1% SDS solution, does appear to remain as an intact structure surrounding the larva and it is possible that covalently (disulphide) cross-linked proteins constitute this matrix, perhaps including a collagen and/or some other proteins.

The absence of incorporation of radioisotopic precursors including amino acids, sugars, sulphate and phosphate (for phospholipid labelling), suggests a lack of metabolic turnover in the envelope. Taken together with J. Lackie's (1973) demonstration that the total nitrogen content and volume of the envelope does not change between early acanthellae and cystacanths, this suggests that the envelope may be an inert but flexible structure, the composition of which does not change during larval development. Studies on radioiodinated extracted cystacanths and stage II acanthellae show that the pattern of labelled polypeptides appears to be the same for both developmental stages, providing some evidence for the statement above. Also, not all of the polypeptides which can be extracted from envelopes by 1% SDS are labelled, suggesting that some envelope proteins are inaccessible to the reagent due to their physical environment and may, for example, be partially wholly buried within a lipid bilayer. Of those which are labelled, some have R_f values similar to host plasma proteins and, if radioiodination is restricted to the polypeptides at or near the envelope outer surface, then this raises the intriguing possibility that absorption of host plasma protein may be important in

parasite disguise (see Chapter 5) and/or nutrition. Many of the results obtained in this chapter, including the radioiodination of cystacanths grown in locusts as experimental hosts, suggest that many of the envelope macromolecules are parasite-derived.

It should be possible to demonstrate unequivocally that these proteins are of parasite origin using available techniques. For example, two-dimensional electrophoretic separation of envelope proteins extracted in 1% Triton/8M Urea represents a powerful tool to confirm the number of proteins in the envelope and distinguish whether polypeptides of the same electrophoretic mobility in two separate samples on a one-dimensional SDS-gel do in fact represent the same polypeptide, and not two distinct polypeptides with the same molecular weight. Unequivocal demonstration that envelope proteins are parasite-derived might come from <u>in vitro</u> translation experiments using total mRNA isolated from larvae. The polypeptides synthesised <u>de novo</u> in this system, from a parasite mRNA template, could be compared with those extracted from cystacanth envelopes by isoelectric focusing (IEF) in one dimension, and SDS-PAGE of the IEF gel in the second dimension on a polyacrylamide gradient gel. Further comparisons could be made between the two samples by peptide ("Cleveland") mapping of individual polypeptide bands resolved on the 2D-gels.

The topography of envelope molecules can also be further investigated; protein cross-linking reagents in conjunction with solvent extraction, would be useful in determining the organisation of envelope molecules, while biophysical techniques such as ^{31}P-NMR and fluorescence recovery after photobleaching (FRAP) experiments would yield useful data on the organisation of lipid molecules within the envelope.

No information has been obtained on the possible function(s) of the majority of envelope proteins (with the exception of collagen and other disulphide-linked proteins which may form a matrix or scaffold for the

envelope). Preliminary results suggest that fibronectin (a conserved component in both vertebrate and invertebrate extracellular matrices) is not present in the envelope; no labelled polypeptides could be detected by immunoprecipitation of ^{125}I-labelled envelope polypeptides extracted using 1% SDS, with rabbit anti-human fibronectin antiserum (obtained from the Scottish Antibody Production Unit, Carluke).

Substrate gels were used to assess proteinase activity in the envelope. In this technique, a proteinase substrate - in this case gelatin (0.1% w/v) - is included in the matrix of an SDS gel. Samples are then electrophoresed as normal and the gel fixed briefly in 5% acetic acid solution, incubated overnight (15-18hr.) in physiological buffer at 37°C, and then stained with Coomassie Blue. On destaining, only regions in which the gelatin has been digested by proteinase activity are clear. Using this technique no proteinase activity could be detected in any of the envelope polypeptides. However, only monomeric proteinases which can rapidly renature will be detected by this technique. The envelope may contain oligomeric proteinases which have an activity highly dependent on protein quaternary structure and so their activity would not be detected under these conditions.

It is possible, to speculate that some envelope proteins may function as transporter systems for biomolecules, while others may be enzymes such as glycohydrolases or proteinases involved in initial breakdown of nutrients in the host haemolymph. Envelope proteins however will be difficult to analyse in their native state due to their insolubility in non-denaturing solvents.

The details of envelope synthesis and organisation have not been elucidated due to the difficulty in metabolically labelling the constituent macromolecules. This may prove to be a hindrance in understanding the mechanism of protection afforded by the envelope.

Chapter 5

Acquisition of host molecules by the envelope

5.1. Introduction

The host-parasite interface represents the site of interaction between host immune effector mechanisms and the parasite. It is usually at this interface that the host may recognise the parasite as "non-self". It is also the possible site at which mechanisms of parasite evasion of immunity are operational. The envelope of the Moniliformis larva represents the host-parasite interface, and it would appear that this structure allows the parasite to achieve non-recognition in the cockroach (see section 1.6.3.5.). The mechanism of the protection afforded by the envelope is, however, unclear.

The mechanisms by which parasites evade the vertebrate immune response are likely to be complex. However, they can be divided into, (a) features decreasing the immunogenicity or recognition of the parasite, (b) suppression of host immune responses through the generation of T-suppressor cells, or (c) antigenic competition or diversion of the immune response into inappropriate effector mechanisms (for example, induction of the wrong class of antibody with no opsonic function or generation of irrelevant antibody with no capacity to bind parasite antigens).

The processes which decrease the recognition of the parasite may be the most relevant to explain the observed protective function of the Moniliformis envelope in its invertebrate host. They can be further subdivided into : (a) host antigen acquisition, in which host molecules insert into the parasite surface and obscure parasite epitopes or (b) "molecular mimicry", where parasite antigens are synthesised to carry epitopes identical to those found in the host.

Examples of host antigen acquisition have been found for Schistosomes which take up host glycolipids bearing blood-group antigens (Goldring et al., 1976). It has been claimed that acquired host antigens play a protective role during parasite maturation. However, the evidence that resistance to immune killing is due to host antigen acquisition is only circumstantial, and other workers have argued that the turnover of tegumental membrane components and/or changes in the physical properties of the tegumental membrane during schistosome development are more important in the development of intrinsic resistance to immune killing (McLaren and Terry, 1982; Samuelson et al., 1982). Host antigen acquisition has also been demonstrated for the filarial nematode Onchocerca gibsoni; microfilariae bind host serum albumin to their cuticle via covalent linkage of sulphydryl groups of the albumin and a molecule on the cuticle surface (Forsythe et al., 1984). However, the role for adsorbed albumin in immune evasion has not been demonstrated, and it may play another, as yet undetermined, function in larval development.

The idea that parasites might synthesise antigens to carry epitopes identical to those found on host antigens (Damian, 1964) is not well established at the experimental level. However, examples of molecular mimicry have recently emerged with the demonstration that schistosomes synthesise an oligosaccharide structure on glycolipids identical to that found on host glycoproteins (Nayame et al., 1987). Again, however, the evidence that mimicry of host epitopes is an important strategy in immune evasion is only circumstantial (Damian, 1987).

For invertebrate host-parasite systems, the molecular mechanisms underlying either host immune recognition or parasite immune evasion strategies have not yet been elucidated. Although the ideas proposed for

vertebrate-parasite immune evasion have been adopted by investigators to explain evasion of the insect host response by habitual parasites, they can really only form part of a working model due to the paucity of information available concerning the specificity and molecular interactions involved during the insect immune response. However, an important step in understanding parasitic evasion of the host's resultant response is to identify parasite surface components that may potentially interact with and influence host defence mechanisms, and to determine whether these components are synthesised by the host or by the parasite. For example, they may represent host serum molecules acquired by the parasite or parasite molecules which mimic host structures, or actively or passively inhibit haemocyte interactions with the parasite surface.

Serological techniques have had important applications in studies directed towards elucidating the mechanisms whereby parasites evade vertebrate immune responses. In this study these methods were adopted to address two aims. The first was to determine if epitopes are shared between the Moniliformis envelope and host haemolymph components and tissues, and if so, to determine on which molecules the shared epitopes reside i.e. on host macromolecules acquired by the parasite or those of parasite origin. The second aim was to determine if any host components are at, or near, the surface of the envelope, which may point to them as candidates for molecules directly involved in the lack of recognition of the parasite by the host.

5.2. Results

5.2.1. Indirect fluorescent antibody test (IFAT)

As a preliminary test to investigate the possibility of shared epitopes between macromolecules of the cystacanth envelope and host haemolymph, the indirect fluorescent antibody test was used.

The binding of antibodies raised in rabbits against (a) host serum components (i.e. the supernatant fraction after centrifugation of clotted haemolymph) and (b) haemocytically-encapsulated Sepharose beads recovered from adult cockroaches, to enveloped and de-enveloped cystacanth larvae, was assessed by this method. Also, cross-reactivity between anti-cystacanth envelope antiserum (section 2.2.4.) and paraformaldehyde-fixed cockroach haemocytes, and the specificity of binding of anti-cystacanth envelope antiserum to enveloped and de-enveloped larvae, was determined.

The results, quantified as described in section 2.2.2.3., with statistical analysis by the Student's t-test, are summarised as histograms in Figure 5.1. Analysis of the data in Figure 5.1 suggests that antisera raised against host serum or haemocytic capsules cross-react with epitopes present in the envelope. The binding is specific, since there is significantly less fluorescence associated with larvae incubated initially in a 5% (v/v) solution of normal rabbit serum (NRS). Furthermore, no fluoresence was observed associated with control samples in which the first antiserum was omitted and only FITC-sheep anti-rabbit IgG antiserum was present. None of the three antisera bound to de-enveloped larvae in experiments run in parallel, while anti-envelope antiserum was shown to bind specifically to earlier enveloped stages of the parasite i.e. Stage I

Figure 5.1. Indirect immunofluorescence data for a-Env, a-HC and a-PS binding to enveloped cystacanths

Enveloped cystacanths were reacted with rabbit a-PS, a-HC or a-Env followed by fluorescein-labelled anti-rabbit IgG as described in section 2.2.4.2. Fluorescence, associated with the larvae, was quantified using a digital-readout photomultiplier attachment. The binding of all antisera to the larvae was significantly greater than the binding of NRS i.e. $p < 0.05$ in all cases.

a-Env Rabbit anti-cystacanth envelope antiserum.

a-HC Rabbit anti-haemocytically encapsulated Sepharose bead antiserum.

a-PS Rabbit anti-cockroach serum antiserum.

NRS Normal rabbit serum.

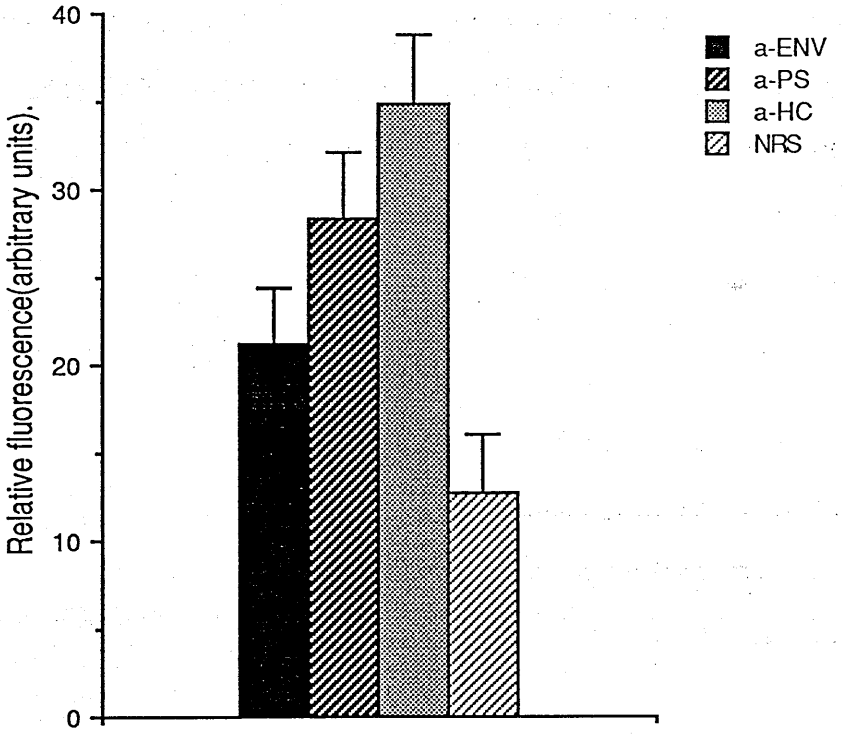

40
30
20
10
0
Relative fluorescence(arbitrary units).
a-ENV
a-PS
a-HC
NRS

Figure 5.2. Immunofluorescence microscopy showing the binding of a-Env to stage I and III acanthellae

Stage I and stage III acanthellae were reacted with a-Env followed by FITC-labelled anti-rabbit IgG to detect first antiserum binding, as described in section 2.2.4.2.

(a) Photomicrograph of acanthella I under bright-field illumination

Bar, 0.12mm

(b) Corresponding fluorescence photomicrograph showing the binding of a-Env.

Bar, 0.12mm

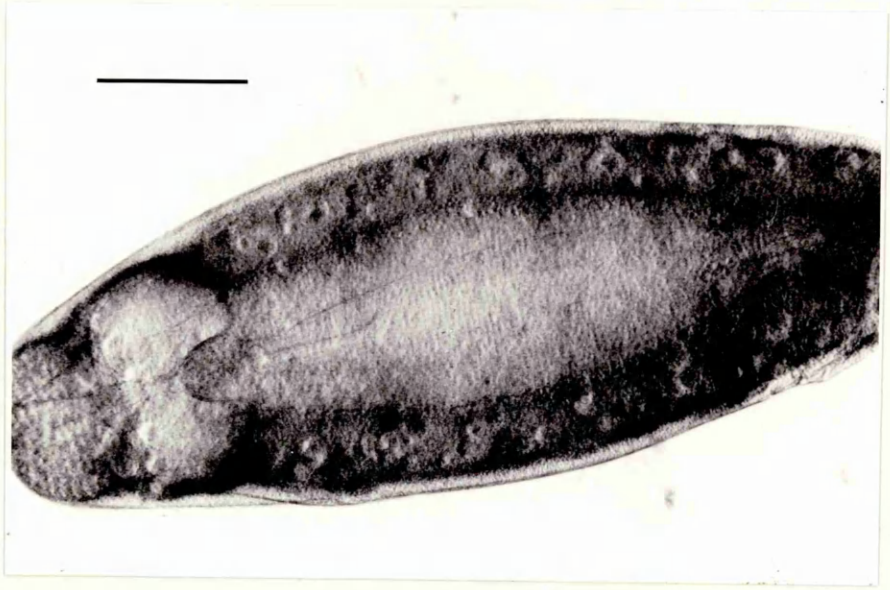
(c) Photomicrograph of acanthella III under bright-field illumination

Bar, 0.1mm

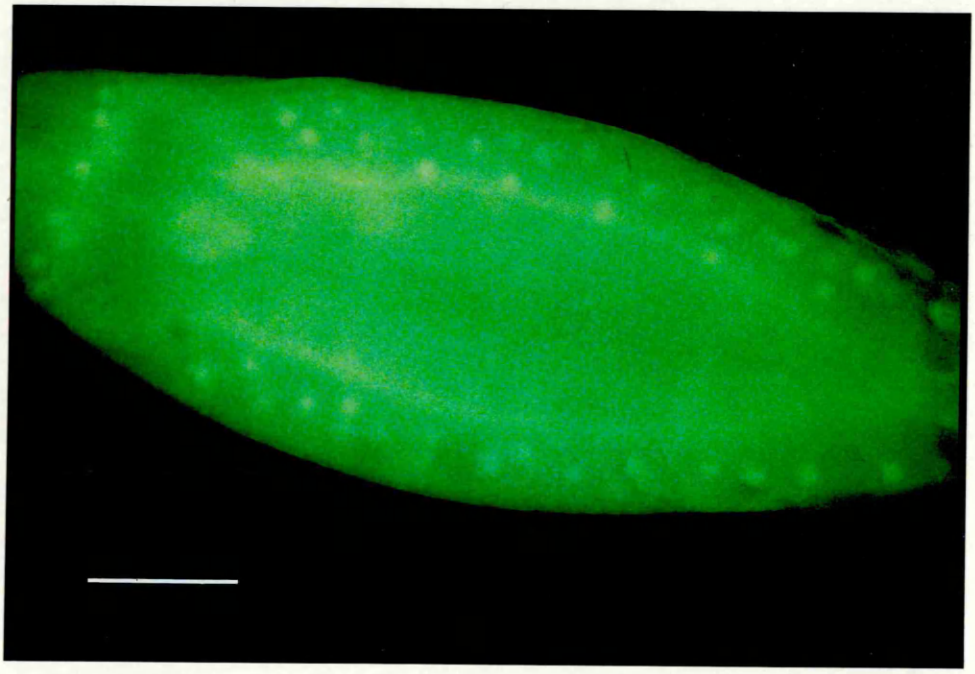
(d) Corresponding fluorescence photomicrograph showing the binding of a-Env.

Bar, 0.1mm

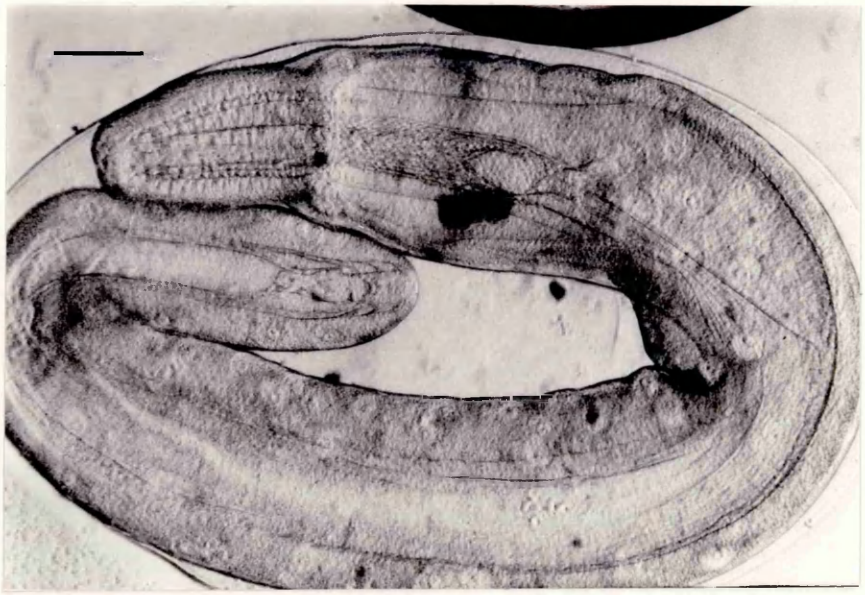
(a)



(b)



(c)



(d)

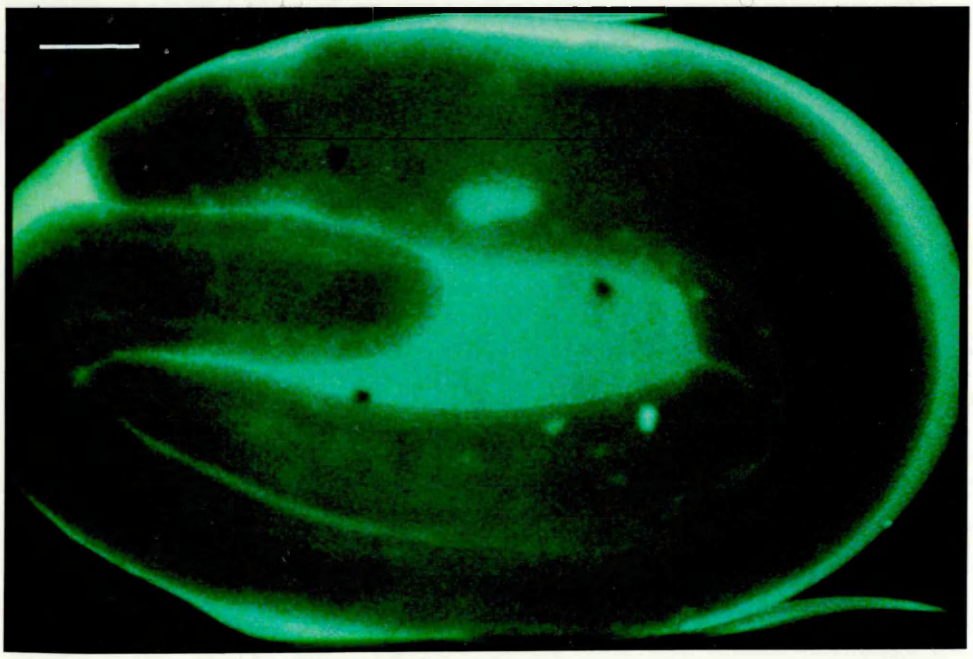


Figure 5.3. Immunofluorescence data for a-PS, a-Env binding to cockroach haemocytes

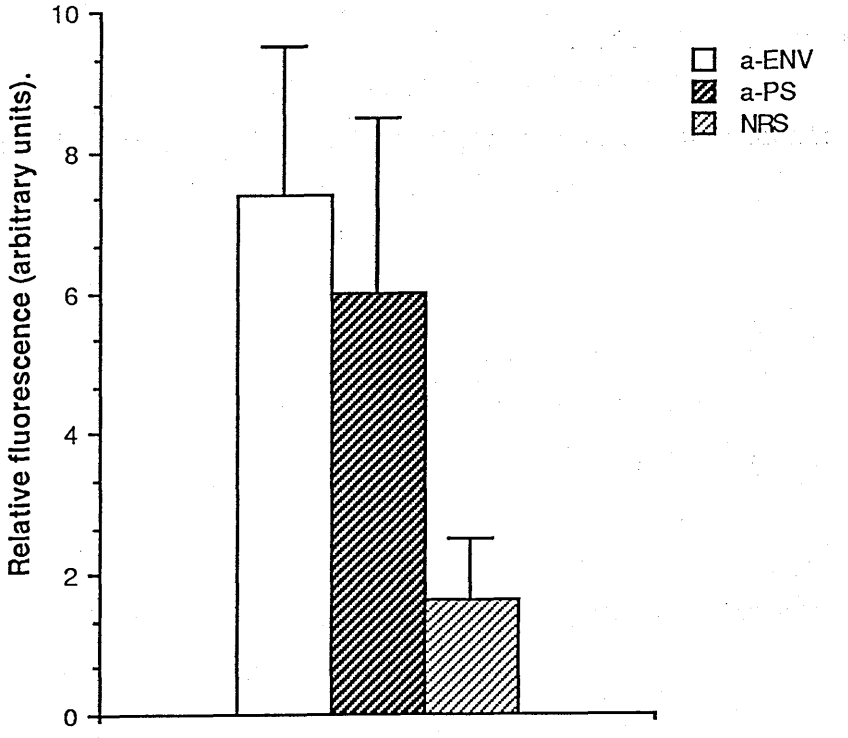
Paraformaldehyde-fixed cockroach haemocytes were reacted with a-Env or a-PS followed by FITC-labelled anti-rabbit IgG to detect first antiserum binding (section 2.2.4.2.).

The binding of a-Env and a-PS to the cells was significantly greater than the binding of NRS.

a-Env Rabbit anti-cystacanth envelope antiserum

a-PS Rabbit anti-cockroach serum antiserum

NRS Normal rabbit serum


Relative fluorescence (arbitrary units).
10
8
6
4
2
0
a-ENV
a-PS
NRS

and Stage III acanthellae (Figure 5.2.).

Analysis of the data presented in Figure 5.3. suggests that anti-envelope antiserum cross-reacts with epitopes present in insect haemocytes. Again, controls, run in parallel, with no first antiserum or with NRS, did not show fluorescence associated with the haemocytes.

5.2.2. Enzyme-linked immunosorbent assay (ELISA) studies

The cross-reactivity between epitopes of the parasite envelope and those present in host haemolymph was confirmed using ELISA (section 2.4.2.) with (1) a diluted 1% SDS extract of cystacanth envelope macromolecules (final SDS concentration 0.002%), (2) a total haemocyte lysate (section 2.2.4.) and (3) a host plasma preparation (section 2.2.4.). The results are summarised in Figure 5.4. None of the antisera bound specifically to wells coated with diluted 1% SDS extract of cystacanth bodies, which suggests that cross-reactivity between host tissue and parasite macromolecules is confined to those present in the envelope.

5.2.3. Immunoprecipitation and immunoblotting studies

In an attempt to identify which, if any, protein components in the envelope or host tissues were responsible for the observed cross-reactivities, immunoprecipitation (section 2.4.3.) followed by SDS-PAGE (section 2.2.3.9.) and immunoblotting (section 2.4.4.) were carried out. Figure 5.5. shows the autoradiograph obtained from the SDS-gel of ^{125}I-surface-labelled envelope proteins while Figure 5.6. shows the results of immunoprecipitation studies using the ^{125}I-labelled parasite proteins and anti-cockroach serum antiserum (a-PS). Two major polypeptides in the immunoprecipitates at M_r 250000 and 85000 (Figure 5.6. tracks 4-6) have

Figure 5.4. Composite ELISA plots for envelope (1% SDS extract) and cockroach plasma and haemocyte lysate fractions

Plates were coated with 2µg of the appropriate antigen and ELISA was performed as described in section 2.2.4.3.

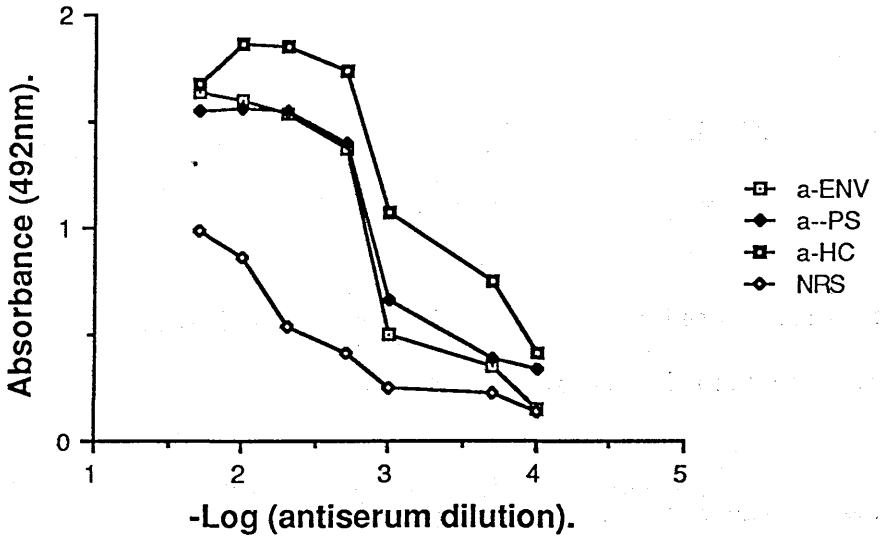
a-Env Rabbit anti-envelope antiserum

a-HC Rabbit anti-haemocytically-encapsulated Sepharose bead antiserum

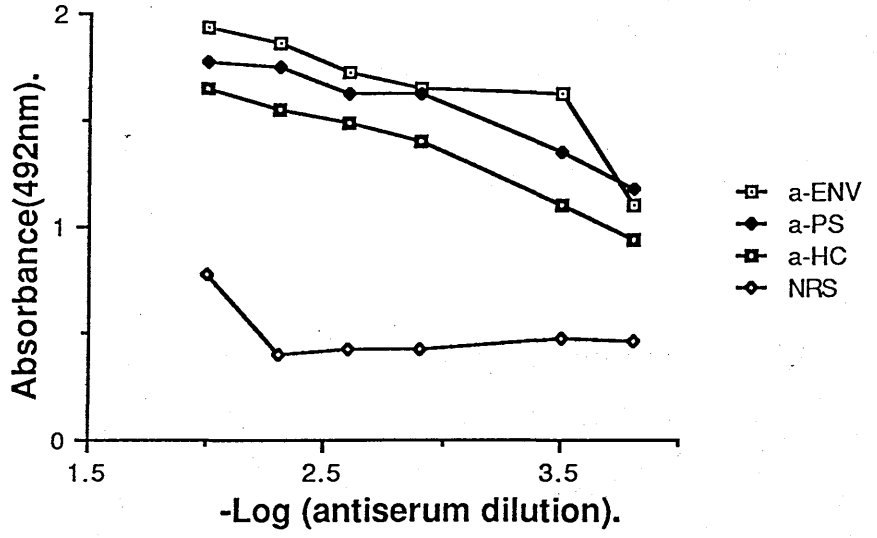
a-PS Rabbit anti-cockroach serum antiserum

NRS Normal rabbit serum

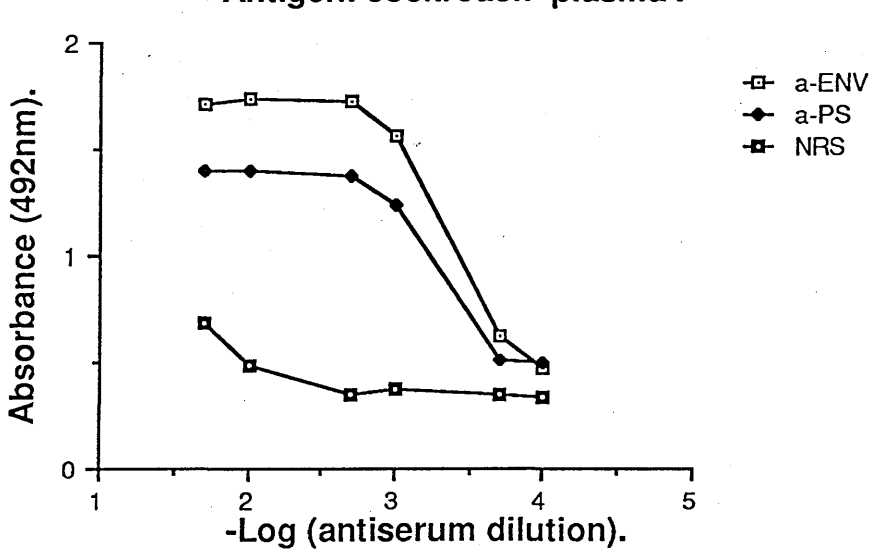
(a).


Antigen: 1%SDS-Env
Absorbance (492nm).
2
1
0
1
2
3
4
5
-Log (antiserum dilution).
a-ENV
a--PS
a-HC
NRS

(b).


Antigen:haemocyte lysate.
Absorbance(492nm).
2
1
0
1.5
2.5
3.5
-Log (antiserum dilution).
a-ENV
a-PS
a-HC
NRS

(c).


Antigen: cockroach plasma .
Absorbance (492nm).
2
1
0
1
2
3
4
5
-Log (antiserum dilution).
a-ENV
a-PS
NRS

very similar M_r values to the two major polypeptides of host plasma (Figure 5.5., track 1) which have been shown by other workers to represent the polypeptide subunits of the host lipoprotein or lipophorin (Chino, 1981). The identity of these two envelope-associated polypeptides was confirmed by one-dimensional peptide ("Cleveland") mapping (section 2.2.3.13.) after excision of the relevant bands from the dried gels that were used to obtain the autoradiographs in Figures 5.5. and 5.6. The pattern of ^{125}I-labelled-peptide fragments obtained (using 50ng/track of pronase from Streptomyces griseus) was compared with those obtained for peptide mapping of the corresponding plasma polypeptides. The results are shown in Figure 5.7. For the envelope-associated polypeptide of M_r 250k (Figure 5.7., tracks 2 and 3) the pattern of polypeptides obtained is very similar to that obtained for digestion of the plasma protein of corresponding molecular weight (Figure 5.7., track 1). Also, the patterns for envelope-associated polypeptide of M_r 85k (Figure 5.7., tracks 5-7) and the plasma 85k (Figure 5.7., track 4) bands are very similar. This suggests that the ^{125}I-labelled envelope-associated polypeptides of M_r 250k and 85k are host plasma proteins, namely the subunits of the lipoprotein.

A similar immunoprecipitation experiment was also performed using ^{125}I-labelled envelope extracts from cystacanths grown in experimental locust hosts. Here, antisera raised in rabbits against locust serum (i.e. the supernatant from centrifugation of clotted haemolymph; a-LS) and Periplaneta serum (a-PS), were used to immunoprecipitate macromolecules from ^{125}I-labelled envelope extracts collected from parasites grown in locust hosts and from ^{125}I-labelled cockroach and locust plasma proteins. The results are shown in Figure 5.8. There is specific precipitation of labelled envelope polypeptides using a-PS (Figure 5.8., section A, tracks 1 and 2). These labelled polypeptides do not represent cockroach plasma

Figure 5.5. Autoradiograph of SDS-gel showing ^{125}I-labelled proteins extracted from radioiodinated cystacanths

Untreated and salt-extracted cystacanths and isolated envelopes were radioiodinated as described in section 2.2.3.8. Envelopes and larvae were then extracted with 1% SDS and extract analysed by SDS-PAGE on a 10% (w/v) acrylamide slab gel.

Key : 1 ^{125}I-labelled cockroach plasma

2 ^{125}I-labelled proteins from untreated cystacanths

3 ^{125}I-labelled proteins from salt-extracted cystacanths

4 ^{125}I-labelled proteins from salt-extracted envelopes

5 ^{125}I-labelled proteins from untreated envelopes

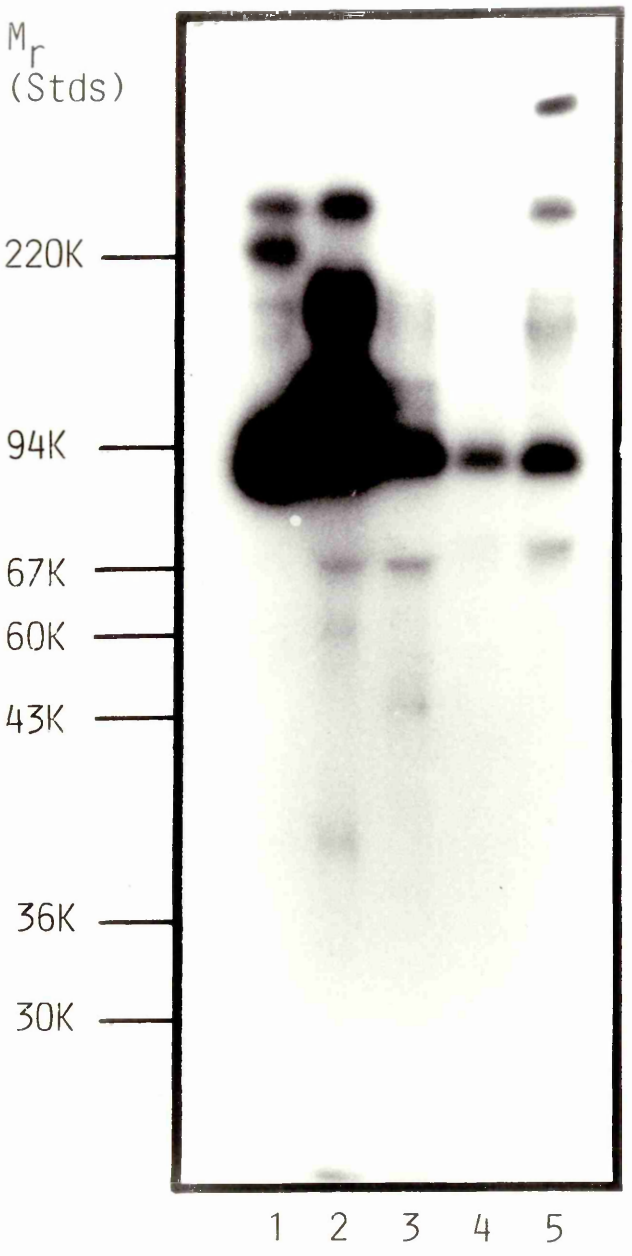

Mr
(Stds)
220K
94K
67K
60K
43K
36K
30K
1
2
3
4
5

Figure 5.6. Immunoprecipitation of ^{125}I-labelled envelope polypeptides using a-PS

Immunoprecipitation of the ^{125}I-labelled parasite proteins was performed as described in section 2.2.4.4.

Key : 1 ^{125}I-labelled polypeptides from the salt extract of envelopes

2 ^{125}I-labelled polypeptides from salt-extracted cystacanths

3 ^{125}I-labelled polypeptides extracted from cystacanths.

4 - 6 Corresponding immunoprecipitates of 1 - 3

5 - 9 Normal rabbit serum (NRS) controls for samples 1 - 3

The relative molecular weights of the major labelled proteins in the immunoprecipitates are indicated at the left-hand side of the Figure.

The labelled band at M_r 67000 in track 1 is BSA which served as a carrier for ethanol precipitation of parasite macromolecules in the 1M NaCl extract of cystacanths (section 2.2.3.8.1.), and as a internal control for non-specific precipitation.

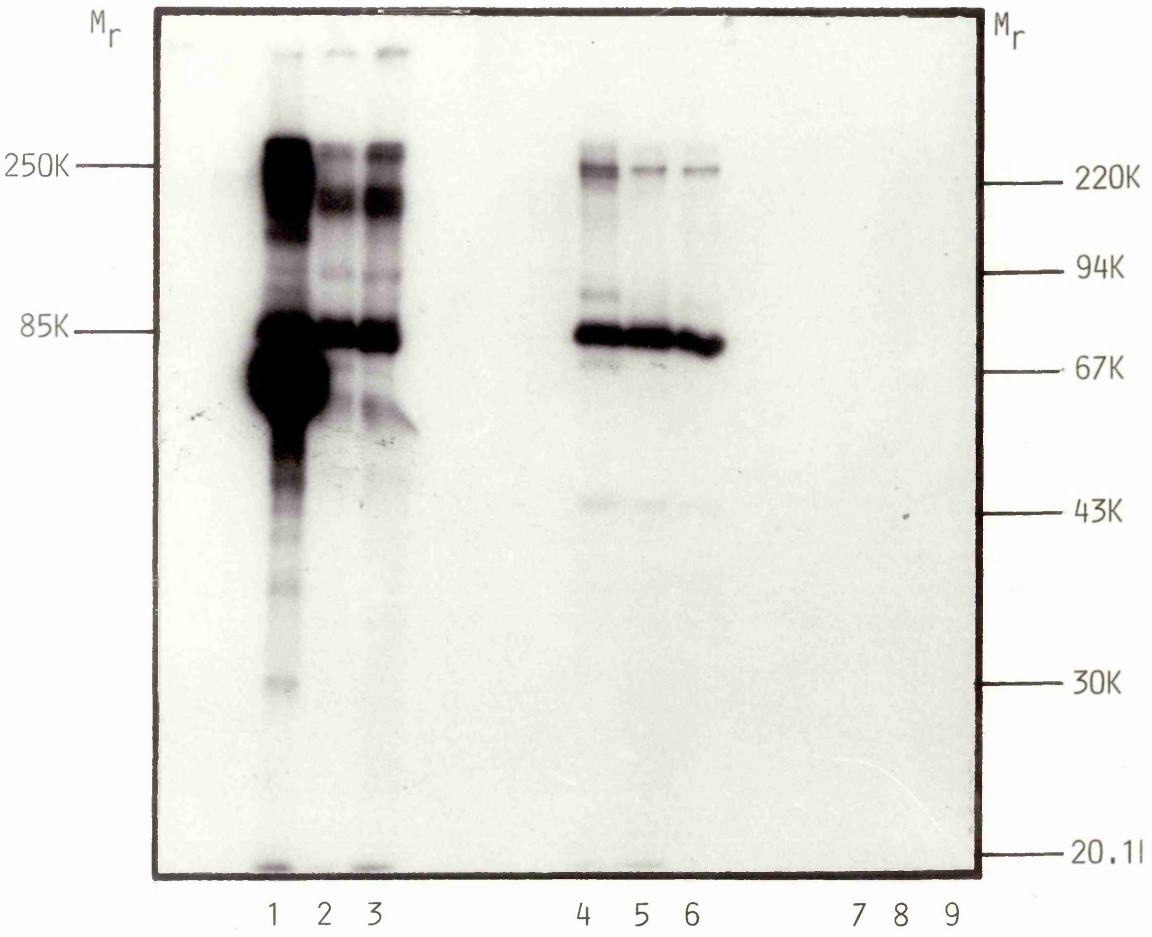

Mr
250K
85K
Mr
220K
94K
67K
43K
30K
20.1
1 2 3 4 5 6 7 8 9

Figure 5.7. Peptide "Cleveland" maps of ^{125}I-labelled parasite-associated and cockroach plasma 250K and 85K polypeptides

The ^{125}I-labelled polypeptides, with Mr's 250K and 85K, together with the ^{125}I-labelled cockroach plasma polypeptides of similar Mr, were excised from dried acrylamide gels, rehydrated and digested with 50ng *S. griseus* proteinase as described in section 2.2.3.13. Peptide fragments produced by proteolysis of each sample were detected by autoradiography of the dried gel.

Digests :

1 Mr 250K polypeptide from cockroach serum (ser).

2 Mr 250K polypeptide from salt extract (SE) of cystacanths

3 Mr 250K polypeptide from untreated parasites (UP)

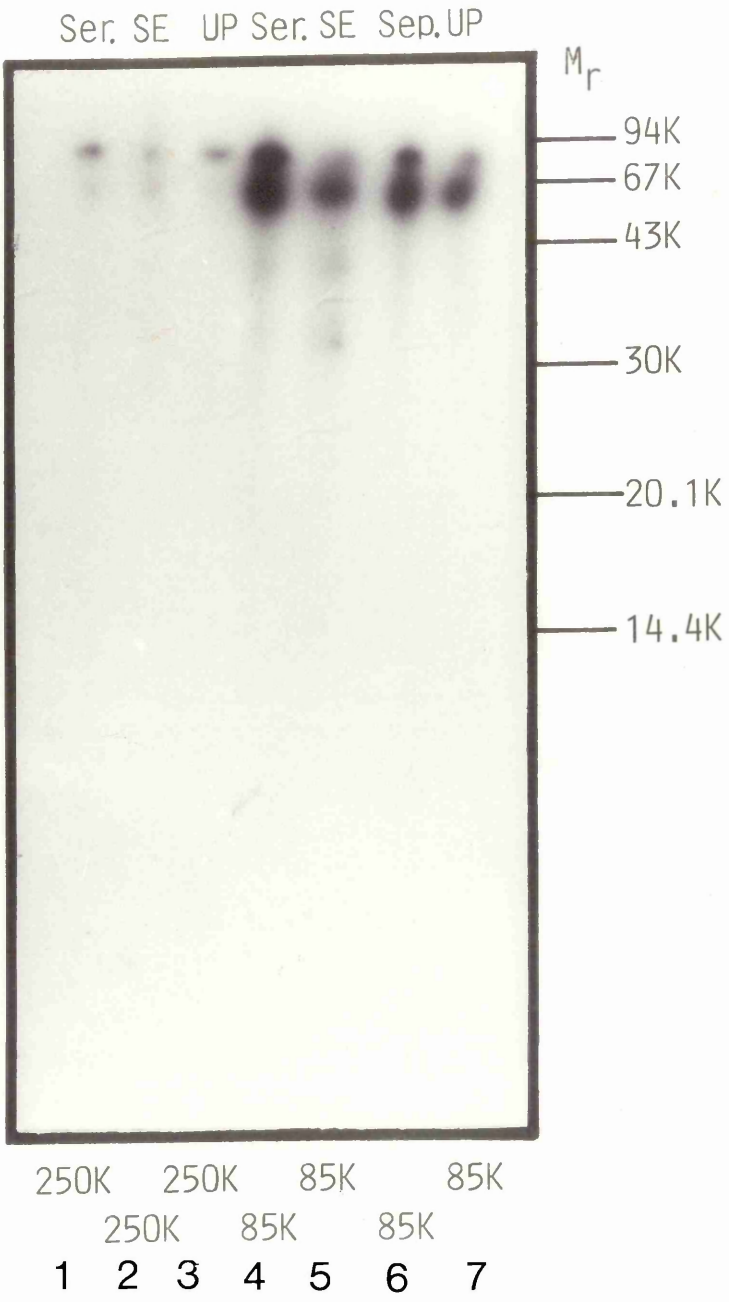
4 Mr 85K polypeptides from cockroach serum (ser)

5 Mr 85K polypeptide from salt extract (SE) of cystacanths

6 Mr 85K polypeptide from salt extract parasites (sep)

7 Mr 85K polypeptide from untreated parasites (UP)

The positions of molecular weight markers are given at the right-hand side of the Figure.


Ser. SE UP Ser. SE Sep. UP
Mr
94K
67K
43K
30K
20.1K
14.4K
250K 250K 85K 85K
250K 85K 85K
1 2 3 4 5 6 7

proteins but they must contain epitopes in common with cockroach serum. Surprisingly, there is also specific precipitation of all major locust plasma proteins (section A, track 4). Likewise, three of the four major cockroach plasma proteins (section B, track 3) and the labelled envelope polypeptides (section B, tracks 1 and 2) can be immunoprecipitated with a-LS. Two of the labelled envelope polypeptides have very similar molecular weights at about 80000. There is also a locust serum protein at M_r 80000; it is possible that one of the envelope-associated polypeptides represents this polypeptide and this explains its specific immunoprecipitation from the envelope extract using a-LS. However, there are no locust plasma proteins with relative mobilities co-incident with the other two major labelled envelope polypeptides at M_r about 160-180000, although they may represent partially digested locust plasma polypeptides, for example, the plasma protein at M_r 250000 (track 4). Alternatively, all the labelled envelope proteins may be synthesised by the parasite, but contain epitopes also found on locust serum components. The cross-reactivity between locust and cockroach serum components suggests that macromolecules in both sera have some common epitopes and it is possible that envelope proteins also contain these epitopes.

If immunoprecipitation experiments are performed using envelope proteins (from larvae derived from cockroach hosts), iodinated after extraction with a 1% solution of SDS in 10mM Tris buffer (see section 2.2.3.), then most of the labelled envelope polypeptides are specifically precipitated (Figure 5.9., track 3) using a-PS. A similar result was also obtained if a-HC (Figure 5.9., track 4) or a-Env (Figure 5.9., track 5) antisera were used, although there were quantitative differences in the relative amounts of some of the polypeptides precipitated by the different antisera, as assessed by differing intensities of bands on the

Figure 5.8. Immunoprecipitation of ^{125}I-labelled envelope proteins extracted from cystacanths recovered from experimental locust hosts

Cystacanths were recovered from experimental locust hosts 6 weeks after intra-abdominal injection of hatched acanthor larvae (section 2.2.1.3.). Enveloped larvae were radioiodinated and whole larvae or their envelopes were extracted with 1% SDS. Immunoprecipitates of the extracts were analysed by SDS-PAGE on a 10% (w/v) acrylamide gel.

1 ^{125}I-labelled polypeptides extracted from cystacanth envelopes

2 ^{125}I-labelled polypeptides extracted from enveloped cystacanths

3 ^{125}I-labelled cockroach plasma polypeptides

4 ^{125}I-labelled locust plasma polypeptides

Panel A Immunoprecipitated using anti-cockroach serum antiserum (a-PS)

Panel B Immunoprecipitated using anti-locust serum antiserum (a-LS)

Panel C NRS controls for non-specific precipitation

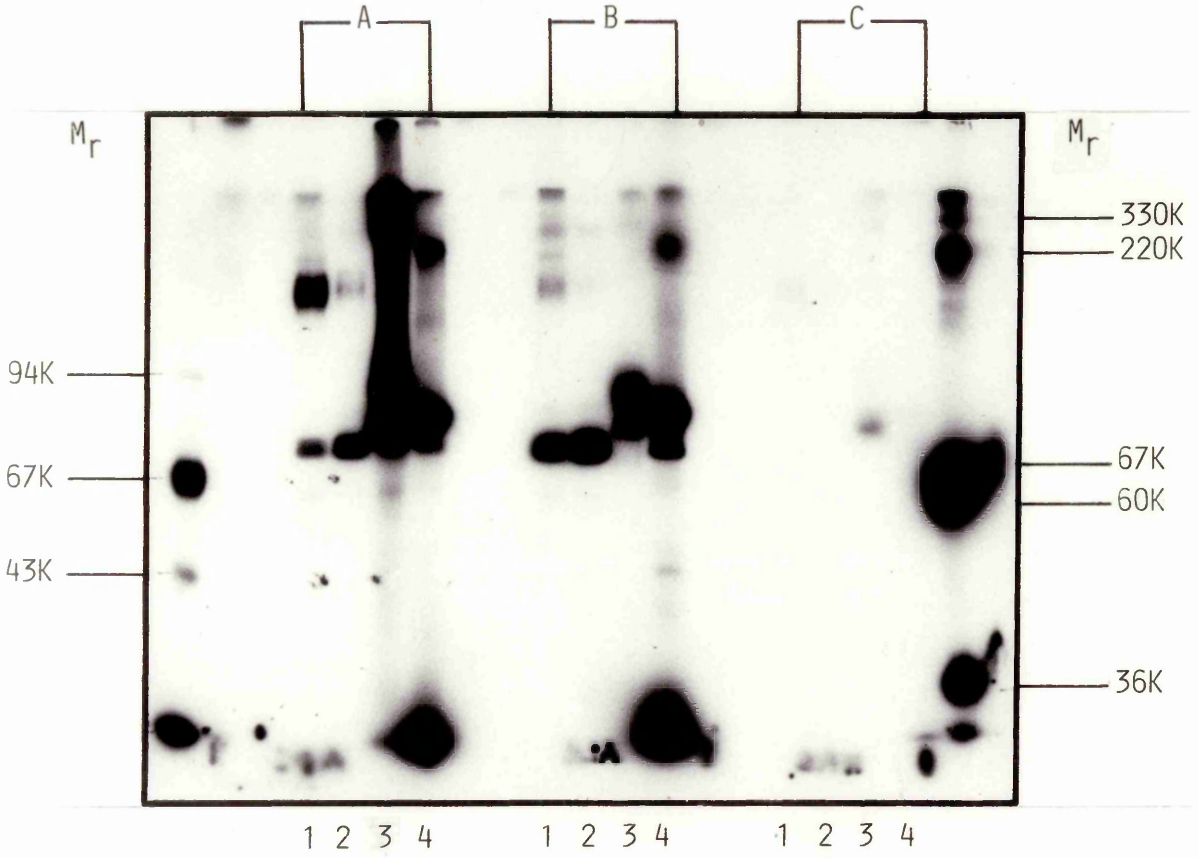

A
B
C
Mr
Mr
330K
220K
94K
67K
60K
43K
36K
1 2 3 4
1 2 3 4
1 2 3 4

Figure 5.9. Immunoprecipitation of ^{125}I-labelled envelope polypeptides with a-Env, a-HC and a-PS

Envelope polypeptides, ^{125}I-labelled after extraction with 1% SDS, were immunoprecipitated using a-Env, a-HC and a-PS as described in section 2.2.4.4.

1 ^{125}I-labelled high molecular weight markers

2 ^{125}I-envelope polypeptides

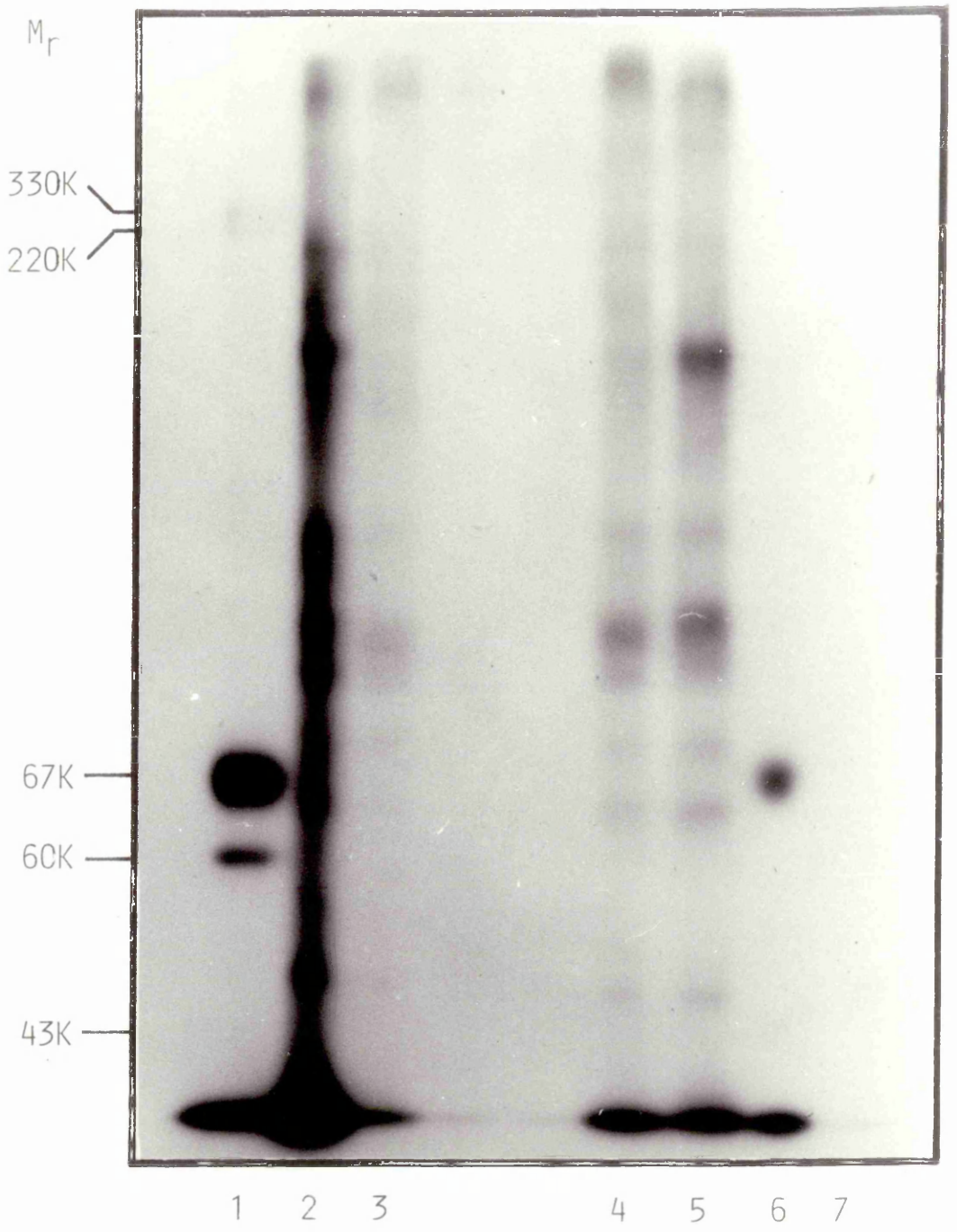
3 ^{125}I-envelope polypeptides immunoprecipitated using a-Env

4 ^{125}I-envelope polypeptides immunoprecipitated using a-HC

5 ^{125}I-envelope polypeptides immunoprecipitated using a-PS

6 ^{125}I-labelled low molecular weight markers

7 NRS control for non-specific precipitation of envelope proteins


Mr
330K
220K
67K
60K
43K
1
2
3
4
5
6
7

autoradiograph.

To ensure that precipitation of all the labelled envelope polypeptides was not due to co-precipitation of cross-reactive polypeptides and other recognised polypeptides, present in mixed detergent micelles (which was an unlikely possibility due to the stringent washing conditions of the immunoprecipitates) immunoblotting experiments were performed. Figure 5.10 shows the autoradiograph obtained from an immunoblotting experiment in which a-Env and a-PS were used to probe SDS-gel blots of total haemocyte lysate, envelope (1% SDS extract), cystacanth bodies (1% SDS extract) and Periplaneta plasma proteins. As before, a-PS bound specifically to most of the envelope polypeptides (section B, track 1). In addition, both antisera cross-reacted with epitopes present on polypeptides in the haemocyte lysate (Figure 5.10., section A, track 2; section B, track 2). The pattern of haemocyte polypeptides recognised by the two antisera differ. However, they do show some similarity in molecular weight with polypeptides present in the envelope extract that are also recognised by the two antisera. Surprisingly, a-Env only binds to one cockroach plasma protein at M_r 250000, which is apparently the large subunit of lipophorin (section A, track 3). The results from Figure 5.7. demonstrate that both lipophorin subunits are associated with the envelope, but a-Env apparently contains no antibodies directed against the 85000 lipophorin subunits. This apparent contradiction may be a result of one or more of the possibilities below : (1) the loss of the small lipophorin subunit during washing of the envelope preparation used for immunisation, (2) the insensitivity of the blotting technique to detect the lipophorin subunits - these probably represent only a small proportion of the total envelope protein, so that the antibody response against them will, correspondingly, be a small proportion of the total response against all the envelope

Figure 5.10. Immunoblotting of parasite and haemolymph proteins with a-Env and a-PS

Polypeptides, separated by SDS-PAGE, were electrophoretically transferred to nitrocellulose. Nitrocellulose strips were then probed with a-Env, a-PS or NRS and immune complexes were visualised by overlaying the nitrocellulose with ^{125}I-labelled protein A (section 2.2.4.5.).

1 1% SDS/5% 2-ME extract of cystacanth envelopes

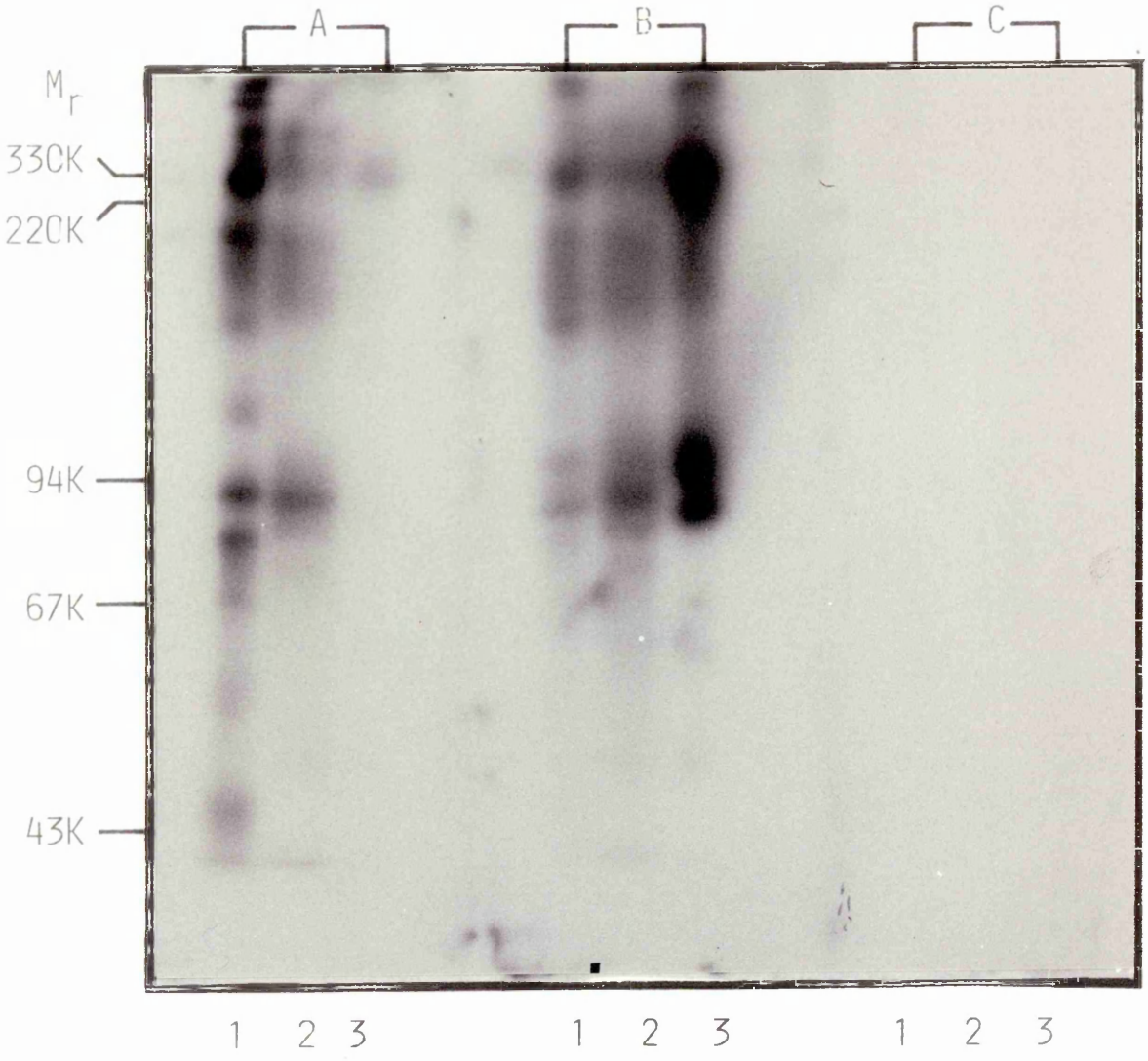
2 Cockroach haemocyte lysate

3 Cockroach plasma fraction

Panel A Probed with a-Env then ^{125}I-Protein A (PrA)

Panel B Probed with a-PS then ^{125}I-PrA

Panel C Probed with NRS then ^{125}I-PrA


A
B
C
M_r
330K
220K
94K
67K
43K
1 2 3
1 2 3
1 2 3

macromolecules, (3) the antibodies directed against the lipophorin subunit do not bind the ^{125}I-protein A used to detect first antibody binding e.g. they may be predominantly of the IgM subclass of immunoglobulins.

In summary then, it would appear that at least one cockroach plasma protein (i.e. lipophorin) is associated with the envelope. However, there is cross-reactivity between a-PS and other envelope polypeptides which are apparently not host plasma proteins, as assessed by comparing their molecular weights with plasma proteins and by the limited cross-reactivity of anti-envelope antiserum with cockroach plasma proteins. Nevertheless, a-Env does cross-react with a number of polypeptides present in a cockroach haemocyte lysate, and, in turn, a-HC immunoprecipitates all ^{125}I-labelled envelope polypeptides (Figure 5.9., track 4). One common feature of a-PS and a-HC is that, by the nature of the antigen preparation used for immunisation, they contain a significant proportion of antibodies directed against haemocyte antigens. This is because a plasma preparation will be contaminated with haemocyte macromolecules as a result of lysis of unstable haemocytes and/or exocytosis of granules from granular haemocytes during haemolymph collection. One interpretation of the results is that cross-reactivity between a-HC, a-PS and envelope polypeptides may be due to the derivation of these polypeptides from haemocytes in the course of envelope synthesis. However, another explanation is that envelope, cockroach haemocyte, and locust and cockroach plasma macromolecules contain common epitopes that do not lose their antigenicity under the highly denaturing conditions of SDS-PAGE, thus accounting for the observed cross-reactivities. The carbohydrate moieties of glycoproteins may represent such epitopes.

5.2.4. Sodium meta-periodate treatment of nitrocellulose strips

In an attempt to establish the nature of the cross-reactive determinants on envelope and haemolymph macromolecules, periodate treatment, which destroys predominantly carbohydrate epitopes (Mattes and Steiner, 1978) was used. The effects of a-Env binding to periodate-treated nitrocellulose strips containing SDS-gel blots of envelope, cockroach haemocyte lysate and plasma proteins is shown in Figure 5.11. It is apparent that prior periodate treatment of the nitrocellulose blot significantly decreases the extent of the cross-reactivity of a-Env antiserum with haemocyte lysate polypeptides (section B, track 2). There is also a significant decrease in the binding of this antiserum to the track containing periodate-treated envelope polypeptides, indicating that a significant proportion of the antibody response against envelope macromolecules is against the carbohydrate moieties of glycoproteins. Periodate treatment did not alter, either qualitatively or quantitatively, the pattern of adsorbed polypeptides on the nitrocellulose strips used to obtain the autoradiographs in Figure 5.11. or on test strips containing molecular weight markers, as assessed by Naphthalene Black staining (results not shown). Thus periodate treatment does not promote dissociation of transferred polypeptides. The results suggest that the cross-reactivity between envelope and haemolymph macromolecules is due to shared carbohydrate epitopes on glycoproteins from haemocytes and envelopes. The high molecular weight material (>330k) in the haemocyte lysate and plasma tracks (section B, track 2 and 3 respectively) may represent glycoprotein(s) in which the oligosaccharides are insensitive or inaccessible to periodate reagent but can bind antibody. Shared carbohydrate epitopes on envelope, cockroach plasma and locust plasma proteins may also explain the observed cross-reactivity of a-LS with

Figure 5.11. Immunoblotting with a-Env antiserum of untreated and periodate-treated nitrocellulose strips containing electrophoretically-separated envelope, haemocyte and plasma polypeptides

Polypeptides separated by SDS-PAGE were electrophoretically transferred to nitrocellulose. One strip of nitrocellulose was treated with sodium meta-periodate (Panel B) while the other two strips (Panels A and C) received mock periodate treatment before continuing with the immunoblotting procedure (section 2.2.4.5.).

1 1% SDS/5% 2-ME extract of cystacanth envelopes

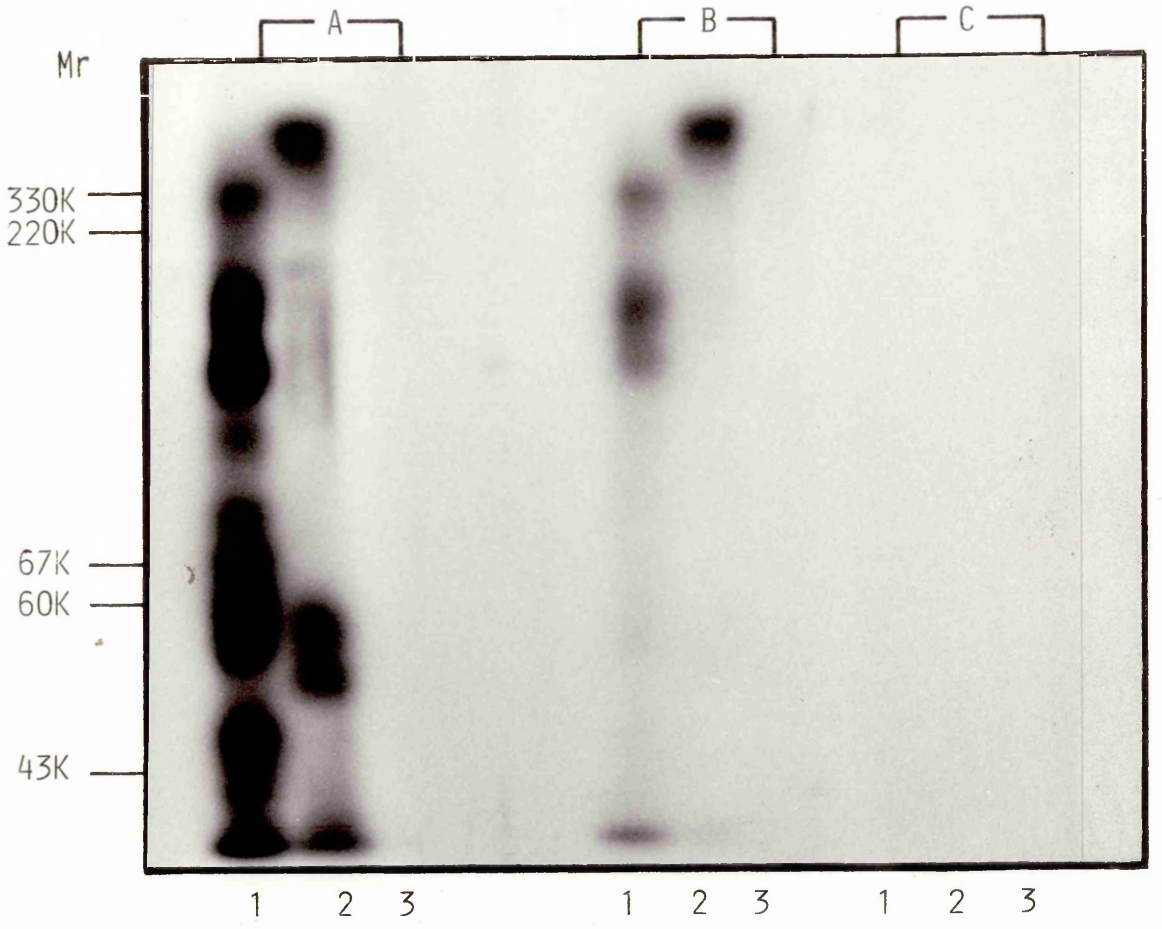
2 Cockroach haemocyte lysate

3 Cockroach plasma fraction

Panel A Probed with a-Env then ^{125}I-PrA

Panel B Probed with a-Env then ^{125}I-PrA

Panel C Probed with NRS then ^{125}I-PrA


A
B
C
Mr
330K
220K
67K
60K
43K
1 2 3
1 2 3
1 2 3

cockroach plasma proteins and ^{125}I-labelled envelope polypeptides shown in Figure 5.8.

In summary, it would appear that although the host lipoprotein (lipophorin) is associated with the cystacanth envelope, the cross-reactivity between envelope and haemolymph components may be predominantly due to shared carbohydrates on component macromolecules. The origin of the envelope glycoproteins containing the shared epitopes has not been resolved and it is possible that a contribution to envelope production is made by host haemocytes.

5.3. Discussion

The results from immunofluorescence studies on enveloped and de-enveloped cystacanths, using antisera raised against host serum components or haemocytically encapsulated Sepharose beads, suggest that envelope macromolecules share epitopes with host haemolymph components. Furthermore, cross-reactivity of anti-envelope antiserum with earlier enveloped larval stages of Moniliformis suggests that the composition of the envelope may not change much during development, a state which may be necessary for protection from host haemocyte responses. With none of the antisera was fluorescence observed associated with de-enveloped larvae, although results in Chapter 4 have shown that only one polypeptide can be labelled when intact de-enveloped larvae are radioiodinated. None of the fluorescinated lectins bound to de-enveloped larvae. Thus the larval surface may have a relatively simple antigenic composition with the exposed polypeptides lacking oligosaccharide moieties.

Cross-reactivity was also observed between solubilised envelope

macromolecules and host haemolymph components using ELISA (Figure 5.4.) Preliminary results, using this assay, have shown that an antiserum raised against soluble extracts from soft tissues of the fresh water snail, Biomphalaria glabrata, cross-reacts with envelope macromolecules (results not shown). Immunoprecipitation studies of radiolabelled envelope polypeptides suggested that a major host plasma protein, lipophorin, is associated with the envelope. Since the polypeptides corresponding to the lipophorin subunits (i.e. at M_r 250000 and 850000) are major components of the proteins in intact enveloped cystacanths that can be radioiodinated, using the iodogen method, and since they represent only a small proportion of the envelope polypeptides that can be solubilised and visualised on SDS-gels, this suggests that the lipophorin is at or near the outer surface of the envelope. It is not known from these experiments in what form the host lipophorin is associated with the envelope, that is, whether it is in a native conformation or denatured, although it is clear that both subunits can be extracted from envelopes using a 1M NaCl solution (Figure 5.5., track 2).

The possibility that all the host plasma proteins may be associated with the envelope is suggested by immunoprecipitation of envelope polypeptides, which have been radiolabelled with ^{125}I after extraction from the envelope. Polypeptides with very similar electrophoretic mobilities to host plasma proteins are found associated with the envelope and can be specifically immunoprecipitated using a-PS or a-HC. However, it is clear that cross-reactive antibodies in a-PS and a-HC are directed against all the labelled envelope polypeptides, the majority of which do not have molecular weights similar to plasma protein components. A similar result was obtained for immunoblotting experiments in which a-PS was used to probe a strip of nitrocellulose paper containing 1% SDS-solubilised envelope

polypeptides (Figure 5.10.). In fact, although a number of envelope polypeptides are recognised by this antiserum, only one of these polypeptides has a molecular weight similar to that of a host plasma polypeptide (i.e. at M_r 250000). Also, a-PS cross-reacts with all locust plasma polypeptides and with ^{125}I-labelled polypeptides from envelopes removed from cystacanths grown in experimental locust hosts. The cross-reactive epitopes on envelope polypeptides, haemocyte polypeptides and plasma proteins were suspected to be carbohydrate. This was because the antigens used in both immunoprecipitation and immunoblotting represent denatured polypeptide extracts (i.e. originally in 1% SDS or 1M NaCl, tris-buffered solutions) or were reduced and denatured during SDS-PAGE sample preparation. It seemed unlikely then, that the cross-reactivity was due to conformation-dependent determinants, although the possibility that they were directed against peptide determinants could not be ruled out, since such determinants might remain antigenic even under the harsh denaturing conditions (i.e. 1% SDS, 100°C temperature) of extraction and preparation for SDS-PAGE. The sensitivity of the cross-reactive determinant(s) on haemocyte polypeptides to periodate treatment (Figure 5.11., track 2), which causes oxidation and subsequent preferential modification of carbohydrate residues on glycoproteins, suggests that the cross-reactivity between envelope polypeptides and host haemolymph components may be due to shared oligosaccharide moieties on unrelated glycoproteins. Lectin overlay studies (Figure 4.5. and 4.8.) suggest that all the major envelope and plasma proteins are glycoproteins. Moreover, the pattern of haemocyte polypeptides (Figure 4.8., track 1) stained with ^{125}I-conA and cross-reactive with a-Env are very similar, that is, all cross-reactive polypeptides are glycosylated.

Recent results for a trematode-mollusc host-parasite system have demonstrated that the epitopes shared between the tegumental proteins of the sporocyst stage of Schistosoma mansoni and the plasma proteins of its host, Biomphalaria glabrata, are carbohydrate (Bayne et al., 1987) as assessed by their sensitivity to periodate treatment (Boswell et al., 1987). These authors have cautioned that the conclusions drawn from serological assays using antisera raised in mammals against very distantly related antigens may be misleading in implying phylogenetic relatedness of different molecules, especially in host-parasite interactions where both host and parasite are invertebrates. As mentioned in Chapter 3, section 1, there is good evidence to suggest that some of the oligosaccharide moieties of glycoproteins from various invertebrates may be quite different from those found in "higher" animals such as mammals. Such oligosaccharides are thus potentially very good immunogens. Furthermore, the adjuvants and regimes commonly used to prepare antisera may favour production of predominantly anti-carbohydrate antibodies (Feizi and Childs, 1987). It may not be surprising then to generate a large antibody response against carbohydrate epitopes when immunising with invertebrate tissue extracts.

The present study indicates that the observed cross-reactivity between host haemolymph components and a spectrum of cystacanth envelope glycoproteins of widely different molecular weights may be due to shared carbohydrate epitopes. Whether this antigen sharing is relevant to the envelope protective function is not known since it has yet to be clearly demonstrated that carbohydrates are recognised as descriptors of "self" from "non-self" in insects. It could, however, be speculated that carbohydrate residues are important for the envelope's protective role; de-enveloped cystacanths contain no ligands on their surfaces for the four

lectins used in this investigation (Chapter 4, Table 4.3.) and are readily encapsulated when introduced into naive cockroach hosts. Enveloped cystacanths, containing an envelope rich in a number of glycoproteins, are not encapsulated. How could carbohydrates act as recognition signals ? It could be postulated that similarities or differences in the oligosaccharide sequence or length associated with parasite glycoconjugates could provide a system for diversification of surface macromolecules which may influence molecular interactions between the parasite and host. For example, parasites may mimic a particular molecular configuration on their surface glycoconjugates and therefore be recognised as "self", or the oligosaccharides may sterically block the binding of host effector molecules and thereby cause a localised inhibition of the host response. Alternatively, the serological data on the cross-reactivity between carbohydrate epitopes of invertebrate host and parasite glycoconjugates may introduce a misleading complexity into the interpretation of host-parasite interactions. This is because cross-reacting epitopes may form part of an oligosaccharide structure that is common throughout the invertebrate phyla but absent in "higher" vertebrates. Antisera against a variety of invertebrate tissues may cross-react due to shared carbohydrate moieties on glycoproteins which, otherwise, have no structural homology. This may explain the preliminary ELISA data (not shown) that demonstrates cross-reactivity between snail tissue and envelope macromolecules. It seems appropriate that more attention should be given to determining what is recognised at the molecular level in the histocompatibility system of insects and other invertebrates before more positive conclusions can be drawn about the relevance of shared oligosaccharides between host and parasite. However, at present, the problem of interpreting serological data, in studies in which both parasite and host are invertebrates, should

be highlighted, in view of the growing number of investigators who are adopting immunochemical methods for use in studying insect and, in general, invertebrate immune responses to parasites.

Chapter 6

General discussion

6. General discussion

6.1. Introduction

The overall aim of this project was to understand the mechanism whereby the envelope surrounding the Moniliformis larva affords protection in the haemocoele of P. americana. The main emphasis of this discussion is to reconsider the hypotheses described in Chapter 1 and to discuss which hypothesis, if any, is the most appropriate to explain the envelope's protective function.

6.2. Non-recognition of enveloped larvae

There is evidence from the results presented in this thesis that the envelope contains molecules which are inherently similar to host connective-tissue macromolecules (i.e. the GAG-like molecule described in Chapter 3 and the collagen molecule described in Chapter 4) and other molecules which may originate from the host plasma (i.e. Lipophorin, see Chapter 5). The possible mechanisms whereby these molecules might protect the parasite from immunorecognition will be considered below.

6.2.1. Inherent similarity between envelope and host tissue macromolecules (molecular mimicry).

The results in Chapter 3 suggest that the envelope contains a GAG-like macromolecule and that this molecule may be responsible for the observed Alcian Blue staining of the outer surface of the envelope (Lackie, 1986a). Furthermore, the results in Chapter 4 suggest that a collagen molecule may also be present in the envelope at, or near, its outer surface. It is possible to speculate that the collagen molecule and the envelope

proteoglycan may interact and provide an envelope surface with properties similar to the host connective-tissue present on all the haemocoelic surfaces. For the purpose of this discussion it will be assumed that both the collagen and the GAG-like molecule in the envelope are synthesised by the parasite.

6.2.1.1. The envelope surface as a physical barrier

Glycosaminoglycans are hydrated at physiological pH and can occupy a solution volume that is many times their dry weight (Poole, 1986). Thus the envelope GAG-like molecule may form part of a hydrophilic barrier which prevents adhesion of the haemocytes. The situation may be analagous to the interaction of enterobacteria with mammalian mucosal membranes and leucocytes. The capsules surrounding these bacteria consist largely of hydrated neutral or acidic polysaccharides which form a hydrophilic cell envelope; this appears to influence the virulence of the organism, since mutant strains lacking a complete capsule show a reduced virulence or are non-infective. From investigations of bacterial phagocytosis *in vitro*, in the absence of serum antibody or active complement components, it has been shown that the hydrophilic surface, resulting from the hydrated surface carbohydrates on both the bacterium and the phagocyte, act as a physical barrier to their adhesive interaction (Edebo *et al.*, 1980). Mutant strains of bacteria which lack the complete cell envelope are demonstrably more hydrophobic and are more readily phagocytosed *in vitro* or cleared from the circulation *in vivo*, than the wild type bacteria.

The hydrophobicity of a foreign surface is also an important factor in haemocyte adhesion *in vitro* and *in vivo* (see section 1.6.2.2.1.); for example, glass beads coated with hydrophobic polymers (e.g. polyHEYA) when injected into the haemocoele of *P. americana*, are more thickly encapsulated

than uncoated beads (Takle, 1986). Thus, in order to prevent haemocyte adhesion, enveloped larvae may avoid presenting a hydrophobic surface to the haemocoele. Experiments designed to test this hypothesis might be difficult to devise. One approach would be to use the *in vitro* encapsulation system of Davies and Vinson (1983), using cockroach haemocytes and both normal enveloped larvae and larvae treated with specific GAG-degrading enzymes. Preliminary experiments have already shown that intact, enveloped cystacanths remain unencapsulated in this system (R. Holt, pers. commun.). Larvae in which the enveloped GAG molecule has been selectively removed might then present a surface to which haemocytes could adhere. However, although this assay might allow the interaction of haemocytes with the envelope to be studied in the absence of any other haemolymph components, it would be unclear whether the putative encapsulation of larvae treated with mucopolysaccharidases was due to removal of the hydrophilic barrier or to exposure of molecules that might be specifically recognised by the haemocytes. Alternatively, increased adhesion might result from alteration of the envelope surface charge (see section 6.2.1.2.) or hydrophobicity, due to removal of the polyanionic GAG-like molecule and/or adsorption of exogenous degradative enzymes.

6.2.1.2. Surface charge of the envelope

In addition to their capacity to bind relatively large amounts of water, glycosaminoglycans also have a net negative charge at physiological pH, imparted by the carboxyl and sulphate groups of their constituents (Lindahl and Höök, 1978). Thus, the envelope may present a surface which is negatively charged *in vivo*. It has been shown by cell electrophoresis and cationised ferritin binding that *Periplaneta* haemocytes also have a net negative surface charge (Takle and Lackie, 1985). Thus, on the basis of

charge repulsion, it might be difficult to envisage adhesion of negatively-charged haemocytes to the envelope surface which also hold a net negative charge imparted by the GAG chains at the outer surface. However, although limited interaction because of electrostatic repulsion might hold true for cell-substratum interactions *in vitro*, the situation *in vivo* is probably much more complex. Thus, *Periplaneta* haemocytes interact with negatively charged abiotic objects (Sepharose beads) and the negatively charged protozoan, *Trypanosoma rangeli*, *in vivo*. On this basis, it is probably unlikely that charge interactions are directly responsible for the failure of haemocytes to encapsulate *Moniliformis* larvae. It is however, possible that the polyelectrolyte properties of the envelope might allow adsorption of host molecules which, depending on their nature or conformation may prevent recognition (see section 6.2.2.).

6.2.1.3. The influence of the carbohydrate composition of the envelope

It is clear that insect haemocytes can respond to differences in the physicochemical properties of the surface of foreign objects. In addition, they appear also to respond to more subtle molecular variations, such as the carbohydrate composition of foreign surfaces (see section 1.6.2.2.2.). For the *Moniliformis* envelope, the GAG-like molecule at the outer surface represents a molecule which may have a very similar structure to the glycosaminoglycans of cockroach connective-tissues.

Although the polymers from the cockroach connective-tissues and the envelope do not appear to be identical, in that they differ in their net negative charge (see section 3.2.), this may result from minor differences in structure. One possibility is that the sulphation of the envelope molecule is decreased by a putative sulphatase activity that could be

associated with one or more of the envelope proteins. Perhaps critical structural features, such as the uronic acid epimer content and/or the position of the sulphate groups on some of the disaccharide repeat units, are common to both the envelope polymer and the GAG chains of the host connective-tissue. The importance of the conformation of GAG chains is illustrated by the specific anticoagulant activity of heparin : a pentasaccharide sequence containing N-sulphated glucosamine, which is sulphated at the C-3 hydroxyl group, is essential for the anticoagulant function of this polymer (Gallagher et al., 1986). The 3-O-sulphated unit is absent in N-sulphated residues of most heparan sulphates which correspondingly, possess no anticoagulant activity. This example serves as an illustration that, although the GAG molecules may be similar, differences in the positioning of functional groups are critical for polymer function.

A recent report has suggested that cells from a variety of species of sponges (Porifera) may contain surface-associated receptors for sulphated proteoglycans. The lectin binding activity was different for each species and the binding affinity of each lectin was most probably determined by the orientation of the sulphate groups on the different polysaccharides (Coombe et al., 1987). These authors suggested that sulphated proteoglycans, synthesised by the sponge cells, mediated intercellular adhesion by interacting with and thus bridging the gap between cell-surface lectins on adjacent cells. Since the lectin specificity was different for each species, the hypothesis was presented that sulphated polysaccharide-binding lectins may be involved in cell-cell interactions via recognition of a species-specific proteoglycan molecule (Coombe et al., 1987).

It is possible that sulphated polysaccharides could play a similar

role in insect immunorecognition, in that specific sulphation patterns of sugar constituents on connective—tissue GAG could be recognised by haemocytes as "self" tissue, to which they do not adhere. The envelope GAG-like molecule may share particular molecular configurations (distribution of sulphate groups) in common with the host GAG so that the haemocytes would recognise it as "self" tissue. However, it is also possible that mimicry of the host GAG may allow adsorption of certain host molecules which serve to "disguise" the envelope as "self".

The results from Chapter 4 suggest that the envelope contains exposed D-galactose residues which bind the fluoresceinated lectin peanut agglutinin (PNA), and the results from lectin overlays of SDS-polyacrylamide gels suggest that envelope glycoproteins may also specifically bind this lectin. Thus, the D-galactose-specific lectin, PNA, binds to intact envelopes and to SDS-polyacrylamide gels of envelope proteins (Chapter 4), suggesting that D-galactose residues are not only present but are also exposed at the surface of the envelope. It has been found that the major serum lectin of Periplaneta americana is D-galactose specific (Lackie and Vasta, 1986; Kubo and Natori, 1987). If, as has been suggested, haemolymph lectins interact with and opsonise foreign objects for haemocyte adhesion, then it would seem to be a perilous strategy for the Moniliformis envelope to contain exposed D-galactose residues. However, we must consider the specificity of the lectins involved. Peanut agglutinin, although inhibited in its binding by D-galactose, recognises both D-galactose and N-acetylglucosamine (GlcNAc). The binding of PNA to envelope molecules could occur through interaction with exposed GalNAc residues in the GAG molecule and other envelope glycoproteins. The cockroach serum lectin is, however, specific for D-galactose and so may not bind to enveloped larvae.

In summary then, the envelope surface may contain similar carbohydrate structures to the host connective-tissue, which may prevent haemocytic recognition of the larvae. Non-recognition due to similarities between the carbohydrate moieties of the envelope GAG-like molecule and the insect connective-tissue GAG, could be either due to the epitopes on the GAGs or an indirect result of the adsorption of soluble molecules that prevent haemocyte recognition/adhesion.

6.2.1.4. Molecular mimicry to allow adsorption of host plasma components

If we are to assume that adsorption of certain host plasma molecules to a surface makes that surface appear like "self" then it is possible that the sole function of the envelope is to provide a suitable surface for the adsorption of host components. Some possible mechanisms whereby adsorbed host molecules might prevent encapsulation of enveloped larvae will be discussed below. (section 6.2.2.).

6.2.2. Acquisition of host plasma components by the envelope

The results from Chapter 5 suggest that there is extensive cross-reactivity between envelope glycoproteins and glycoproteins from cockroach haemocytes and plasma fractions. However, it now seems likely that this cross-reactivity is largely, if not totally, a result of carbohydrate epitopes common to the envelope glycoproteins and the haemolymph components. For vertebrate-host parasite systems there is at least precedence to explain the relevance of shared epitopes on host molecules and molecules at the parasite surface; they may be involved in evasion of the specific immune response of the vertebrate host by decreasing the

ability of the host to recognise the parasite as foreign. However, until more information is available on the details of immunorecognition in insects the relevance of shared carbohydrate epitopes to immune evasion must remain questionable.

The cross-reactivity between oligosaccharides on envelope and host glycoproteins has confused the interpretation of experiments designed to determine whether plasma proteins or other host molecules are associated with the envelope. Despite these difficulties, there is good evidence that the host high-density lipoprotein, lipophorin, is associated with the envelope, possibly as a result of adsorption to the outer surface.

6.2.2.1. The possible significance of the adsorbed lipophorin

Lipophorin is the major plasma protein of Periplaneta haemolymph and accounts for about 50% (w/w) of the total protein (Chino et al., 1981). As it is present in such high concentrations in the haemolymph (~10mg/ml) there is a strong possibility that it could become bound to surfaces that represent either damaged self or non-self. The lipophorin may be denatured (or have its conformation slightly altered) upon contact with surfaces which differ from intact self in their physicochemical properties; the adsorbed lipophorin may either be recognised directly by the haemocytes or may indirectly stimulate recognition by its interaction with other plasma factors.

Lipophorin is involved in haemolymph coagulation (section 1.2.4.) where it interacts with a coagulagen released locally by the haemocytes at the wound site, to form a insoluble covalently cross-linked clot (Bohn, 1986). The plasma clot not only contributes to haemostasis, but may also trap invading microorganisms (Gagen and Ratcliffe, 1976).

Denatured lipophorin activates the haemolymph prophenoloxidase system in the silk worm Bombyx mori (Ashida et al., 1983), some of the components of which may mediate haemocyte binding to foreign objects (see section 1.2.2.). Thus, it is interesting to speculate that the adsorption and denaturation of lipoprotein onto a foreign surface may be the first step in non-self recognition. The proposed process is reminiscent of the intrinsic blood clotting mechanism which is initiated by contact of Factor XII (Hageman Factor) with a foreign surface. This activated factor initiates the proteolytic cascade which results in fibrinogenesis (Taussig, 1984).

This model would explain why there is indiscriminate recognition and encapsulation of a variety of abiotic objects of widely differing physicochemical properties from hydrophobic paraffin wax to negatively charged Sepharose beads; all these surfaces might be incompatible for adsorption and denaturation of the lipophorin would stimulate cellular encapsulation of that object. Thus for parasites, one strategy might be to present a suitable surface for the adsorption and stabilisation of the host lipophorin in a native conformation, thus preventing recognition.

The interaction between low-density lipoprotein (LDL) in the plasma of mammals with the heparan sulphate proteoglycan on the surface of endothelial cells is well documented. The interaction is thought to be principally ionic and involves Ca^{2+} ions, which bridge the polar head groups of the LDL phospholipids and the GAG-chains of the proteoglycan. These PGs also bind lipoprotein lipase, and it has been suggested that they serve to stimulate lipolysis by bringing the substrate and the enzyme into close opposition (Lindahl and Höök, 1978). The interaction between lipoproteins and proteoglycans may also have some relevance to the

Moniliformis envelope. In this case the GAG-like molecule of the envelope may also serve to bind the host lipoprotein in a stable conformation thus preventing recognition of the envelope. To test this hypothesis it is necessary to demonstrate that the lipoprotein associated with the envelope is in a native conformation. For a number of insects including Periplaneta, it has been shown that lipophorin serves as a carrier for Juvenile hormone III (deKort and Koopmanschap, 1986). Thus it might be possible to label the lipophorin associated with the envelope using a specific radioactive photoaffinity label (10-[10,11 ^{3}H-]-epoxyfarnesyl diazoacetate; ^{3}H-EFDA) which is an analogue of Juvenile hormone III (Koeppe et al., 1984; deKort and Koopmanschap, 1986). This label attaches to the lipoprotein at the binding site of Juvenile hormone III and, after UV-photolysis, covalently labels the apoprotein subunits. Presumably, the binding of this analogue is highly dependent on the conformation of the lipophorin, and so could be used to probe the nature of the association of lipophorin with the Moniliformis envelope. If freshly collected envelope larvae were incubated in vitro with the label then, after UV-photolysis, the envelopes could be removed and the proteins extracted and analysed by SDS-PAGE and fluorography. If the lipophorin subunits were labelled under these conditions then it would seem likely that the molecule was associated with the envelope in a native conformation.

It might also be possible to detect lipophorin associated with the envelope using antibodies which bind to conformation-dependent epitopes, for example, antibodies that bind only to the native protein. However, our own attempts to raise antibodies against purified, denatured lipophorin (a gift from Prof. H. Chino, Japan) were unsuccessful on two separate occasions during this investigation. Also, the difficulty of interpreting

serological cross-reactivity between envelope and haemolymph molecules, as discussed in Chapter 5, suggests that an immunochemical approach to studying lipophorin-envelope interactions might be inappropriate.

6.2.3. The influence of other components on non-recognition

Until now, this discussion has concentrated on the importance of the envelope GAG-like molecule in the process of non-recognition. However, it is clear, from the results presented in Chapter 4, that the envelope also contains a number of other proteins and lipids, and it is possible that these molecules play some role in the protective function of the envelope.

One important molecule that has been identified and characterised is the envelope collagen. If the collagen is at or near the outer surface, it could interact with and modulate the physiology of haemocytes that come into contact with the envelope. Alternatively, the collagen may have only a structural role, to orientate the envelope proteoglycan so as to allow maximum binding of lipophorin or minimum interaction of the haemocytes with the envelope.

When mammalian or avian epithelial cells are grown *in vitro*, the provision of a collagen substratum has been shown to alter the cell phenotype and to change the pattern of glycosaminoglycans synthesised and incorporated into the extracellular matrix (e.g. Parry *et al.*, 1985). Since haemocytes are involved in the repair of wounds at the basement membranes or elsewhere (Smith *et al.*, 1986) it is plausible that the envelope collagen may also influence the biosynthetic processes of haemocytes which come into contact with it. Cockroach haemocytes are adherent to the acanthor II and early acanthellae of *Moniliformis* (Rotheram and Crompton, 1972) and thus the possibility arises that the cells interact

with the parasite-derived collagen and are stimulated (as in wound repair ?) to produce the GAG-like molecule associated with the envelope. After production of this molecule (completion of healing ?) the cells return to the circulation. This hypothesis presents the envelope as a substratum that resembles damaged or incomplete "self" tissue which the haemocytes "repair". Other molecules identified in the envelope comprise a variety of glycoproteins. Whether any of these envelope glycoproteins possess cell-binding capacity is a matter for speculation, although preliminary results from immunoprecipitation studies suggest that a fibronectin-like protein is not a component of the envelope (see Chapter 4, section 4.3.). Other possible functions of these protein might include transport of small biomolecules or in degradation of haemolymph components for nutritive purposes (see below).

The lipids in the envelope may be components of lipid-rich vesicles or possibly be organised in a highly folded, but continuous, membrane structure. The main function of a lipid-rich membrane is to form a permeability barrier which restricts the flow of charged molecules. However, for enveloped larvae this is likely to be disadvantageous, since the presence of a membrane with low permeability would limit the flow of nutrients and waste products from the haemolymph to the larval tegument and vice versa. This would require the duplication of transport systems for sugars, amino acids and other small biomolecules at the surfaces of both the larval tegument and the envelope. However, such an arrangement could explain the surprising complexity of the envelope structure - the collagenous component could provide a structural framework within which some of the other proteins, as integral components of the membranous structures, function as transporters for small molecules and larval waste

products. Other proteins may represent proteinases, lipases and glycohydrolases necessary for degradation of haemolymph components for nutrition.

This model, then, presents the envelope as a dynamic structure which may bind, internalise and degrade host haemolymph proteins and may also specifically adsorb amino acids and simple sugars from the rich pool of nutrients present in the haemolymph. However, all attempts to metabolically label protein, carbohydrate or lipid (phospholipids using ^{32}P; results not presented) components of the envelope *in vitro* or *in vivo* were unsuccessful even when early acanthellae, which presumably are actively producing the envelope, were used.

Thus, the situation is apparently paradoxical : on the one hand, the envelope apparently interacts with the major host plasma protein and must function as a point of exchange of nutrients and waste materials between the parasite and the haemolymph, yet, on the other hand, it does not appear to turn over any of its components. The paradox may be resolved, however, if a consideration of the conditions under which metabolic labelling of the envelope components was attempted. For *in vivo* labelling it is clear that any radioactive precursor used to label envelope molecules in infected cockroaches (e.g. sugars, amino acids, $^{32}PO_4^{2-}$ or $^{35}SO_4^{2-}$) will be considerably diluted upon injection into the haemolymph, since this is very rich in small biomolecules (Mullins, 1985). There will also be competition between the parasite and the host tissues for the nutrients (including the radiolabelled precursors) present in the haemolymph. Moreover, precursor such as $^{35}SO_4^{2-}$, may be actively removed by the insect's Malpighian tubules (Maddrell and Gardiner, 1980). Thus, the possibility of labelling any envelope molecules to produce a sufficient

specific activity for detection will be remote. In vitro, even if culture medium low in L-proline or SO_4^{2-} was used to minimise isotope dilution, then there was still no, or very little, incorporation of ^3H-proline or $^{35}SO_4^{2-}$ into envelope molecules. Perhaps the larvae are relatively biosynthetically inactive in this medium, which may lack some essential factor present in the cockroach haemolymph. Alternatively, the choice of metabolic precursor may be wrong; for example dipeptides or other amino acid oligomers may be taken up by the larvae in preference to single amino acids, as has been suggested for adult Moniliformis cultured in vitro (Uglem and Lewis, 1986). In order to investigate whether envelope molecules are host or parasite derived and to determine how this fascinating structure is synthesised and assembled, it would seem essential to achieve metabolic labelling of the envelope constituents with radioactive precursors. A large part of any future work on the envelope should involve devising a suitable culture medium which could be used for this purpose.

6.3. Summary

The results in this thesis have shown that the Moniliformis envelope is a complex, structured organisation of proteins/glycoproteins and lipids.

Two models have been proposed to explain the protective function of the envelope. In the first model, the envelope collagen acts as a substratum to interact with and modulate the function of host haemocytes. The adherent cells synthesise and release a proteoglycan which is deposited at the surface of the envelope where it interacts with the parasite-derived collagen to produce a surface which is similar to host connective-tissues. Alternatively, the envelope, with adsorbed proteoglycan from the

haemocytes, may represent a substratum which can adsorb the host lipophorin, which in turn prevents haemocytic recognition.

In the second model, the collagen molecule has only a structural role, serving as a framework for the rest of the envelope macromolecules. The envelope can be thought of as a dynamic structure which readily adsorbs, internalises and degrades haemolymph components, including lipophorin, for nutritive purposes. As a consequence of the adsorption of the lipophorin, initially in a native conformation, the envelope is not recognised as foreign. Common to both models is the adsorption of lipophorin which prevents the haemocytes from recognising the envelope. Perhaps a further investigation into the role of lipophorin adsorption onto the Moniliformis envelope might reveal a central role for this molecule in the general process of immune recognition in insects.

An asterisk (*) indicates that a reference is missing at this position and is listed instead on page 221.

AHUJA, K.K. (1982). Fertilization studies in the hamster : the role of cell-surface carbohydrates. Exp. Cell Res. 140, 353-362.

AKIYAMA, S.K. AND JOHNSON, M.D. (1983). Fibronectin in evolution: presence in invertebrates and isolation from Microciona prolifera. Comp. biochem. physiol. 76B, 687-694.

ALLEN, P.C., HILL, E.A. AND STOKES, A.M. (1977). Plasma proteins : analytical and preparative techniques. Chapter 13. Blackwell Scientific Publications, Oxford.

ANDERSON, R.S., HOLMES, B. AND GOOD, R.A. (1973). Comparative biochemistry of phagocytizing insect haemocytes. Comp. biochem. physiol. 46B, 595-602.

ANDERSON, K. AND STEINER, H. (1987). Structure and properties of protein P4, the major bacteria-induced protein in pupae of Hyalophora cecropia. Insect. Biochem. 17, 133-140.

ASHHURST, D.E. (1982). The structure and development of insect connective tissues. In "Insect Ultractructure" (Eds. R.C. King and H. Akai). Vol.I, pp. 313-345. Plenum, New York.

ASHHURST, D.E. (1985). Connective tissues. In "Comprehensive Insect Biochemistry, Physiology and Pharmacology" (Eds. G.A. Kerkut and L.I. Gilbert). Vol. 3, pp. 249-288. Pergamon, Oxford.

ASHHURST, D.E. AND BAILEY, A.J. (1980). Insect collagen, morphological and biochemical characterization. Eur. J. Biochem. 103, 75-83.

ASHIDA, M., ISHIZAKI, Y. AND IWAHANA, H. (1983). Activation of prophenoloxidase by bacterial cell walls or B1,3-glucans in plasma of the silkworm, Bombyx mori. Biochem. Biophys. Res. Comm. 113, 562-568.

ASHIDA, M., OCHIAI, M., AND YOSHIDA, H. (1986). B1, 3-glucan receptor and peptidoglycan receptor within prophenoloxidase activating system in an insect. Dev. Comp. Immunol. 10, 623.

ASHIDA, M., ISHIZAKI, Y. AND ISAHANA, H. (1983). Activation of prophenoloxidase by bacterial cell walls or B-1, 3-glucans in plasma of the silkworm, Bombyx mori. Biochem. Biophys. Res. Comm. 113, 562-568.

de AZAMBUJA, P., FREITAS, C.C., AND GARCIA, F.S. (1986). Evidence and partial characterisation of an inducible antibacterial factor in the haemolymph of Rhodnius prolixus. J. Insect. Physiol. 32, 807-812.

BARONDES, S.H. (1981). Lectins : their multiple endogenous cellular functions. Ann. Rev. Biochem. 50, 107-231.

BARONDES, S.H. (1983). Developmentally regulated lectins In : Cell interactions and development : molecular mechanisms. (Ed. K.M. Yamada) pp. 185-202. John Wiley and Sons, New York.

BAYNE, C.J., BOSWELL, C.A. AND YUI, M.A. (1987). Widespread antigenic cross-reactivity between plasma proteins of a gastropod, and its trematode parasite. Dev. Comp. Immunol. 11, 321-329.

BEAMES, C.G. AND FISHER, F.M. (1964). A study on the neutral lipids and phospholipids of the Acanthocephala Macracanthorhynchus hirudinaceus and Moniliformis dubius Comp. Biochem. Physiol. 13, 401-412.

BECKAGE, N.E. AND TEMPLETON, T.J. (1986). Physiological effects of parasitism by Apanteles congregatus in terminal-stage tobacco hornworm larvae. J. Insect. Physiol. 32, 299-314.

BECKAGE, N.E., TEMPLETON, T.J., NIELSEN, B.D., COOK, D.I. AND STOLTZ, D.B. (1987). Parasitism-induced haemolymph polypeptides in Manduca sexta larvae parasitized by the braconial wasp Cotesia congregata (Say). Insect. Biochem. 17, 439-455.

BEELEY, J.G. (1985). Glycoprotein and proteoglycan techniques. Elsevier Press. Amsterdam.

BERNHEIMER, A.W. (1952). Haemagglutinins in caterpillar blood. Science 155, 150-151.

BITKOWSKA, E., DZBENKI, T.H., SZADZIEWSKA, M. AND WEGNER, Z. (1982). Inhibition of xenograft rejection reaction in the bug Triatoma infestans during infection with the protozoan Trypanosoma cruzi. J. Invert. Path. 40, 186-189.

BLIGH, E.G. AND DYER, W.J. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911-917.

BOHN, H. (1977). Differential adhesion of the haemocytes of Leucophaea maderae (Blattaria) to a glass surface. J. Insect. Physiol. 23, 185-194.

BOHN, H. (1986). Hemolymph clotting in insects. In "Immunity in Invertebrates". (Ed. M. Brehelin) pp. 188-207. Springer-Verlag. Berlin.

BOMAN, H.G. (1986). Antibacterial immune proteins in insects. In "Immune mechanisms in invertebrate vectors" (Ed. A.M. Lackie). Zoological Society of London Symposia, 56, pp. 45-58. Oxford University Press, Oxford.

BOMAN, H.G., BOMAN, A. AND PIGON, A. (1981). Immune and injury responses in Cecropia pupae - RNA isolation and comparison of protein synthesis in vivo and in vitro. Insect. Biochem. 11, 33-42.

BOMAN, H.G. AND HULTMARK, D. (1987). Cell-free immunity in insects. Ann. Rev. Microbiol. 41, 103-126.

BOSWELL, C.A., YOSHINO, T.P. AND DUNN, T.S. (1987). Analysis of tegumental surface proteins of Schistosoma mansoni primary sporocysts. J. Parasit. 73, 778-786.

BREHÉLIN, M. (1979). Hemolymph coagulation in Locusta migratoria: evidence for a functional equivalent of fibrinogen. Comp. Biochem. Physiol. 62B, 329-334.

BREHELIN, M. AND ZACHARY, D. (1986). Insect haemocytes: a new classification to rule out the controversy. In "Immunity in invertebrates" (Ed. M. Brehelin) pp. 36-48. Springer-Verlag. Berlin.

BRUCKNER, P., VAUGHAN, L. AND WINTERHALTER, K.H. (1985). Type IX collagen from sternal cartilage of chicken embryo contains covalently bound glycosaminoglycans. Proc. Natl. Acad. Sci. 82, 2608-2612.

BUTTERWORTH, A.E. (1984). Cell-mediated damage to helminths. Adv. Parasit. 23, 143-235.

BUTTERWORTH, P.E. (1969). The development of the body wall of Polymorphus minutus (Acanthocephala) in its intermediate host Gammarus pulex. Parasitology 59, 373-388.

CAIN, G.D. (1970). Collagen from the giant acanthocephalan Macracanthorhynchus hirudinaceus. Arch. Biochem. Biophys. 141, 264-270.

CASSARO, C.M.F. AND DIETRICH, C.P. (1977). Distribution of sulphated mucopolysaccharides in invertebrates. J. Biol. Chem. 252, 2254-2261.

CHAMBERLAIN, J.P. (1979). Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor sodium salicylate. Anal. Biochem. 98, 132-135.

CHINO, H. (1985). Lipid transport : biochemistry of hemolymph lipophorin. In: "Comprehensive biochemistry, physiology and pharmacology of insects" (Eds. G.A. Kerkut and L.I. Gilbert) vol. 10, pp. 115-135. Pergamon Press, Oxford.

CHINO, H., KATASE, H., DOWNER, R.G.H. AND TAKAHASHI, K. (1981). Diacylglycerol-carrying lipoprotein of haemolymph of the American cockroach : purification, characterisation, and function. J. Lipid Res. 22, 7-15.

CHRISTENSEN, B.M. (1986). Immune mechanisms and mosquito-filarial worm relationships. In: "Immune mechanisms in invertebrate vectors" (Ed. A.M. Lackie). Zoological Society of London Symposia. 56, pp. 145-160. Oxford University Press, Oxford.

CHRISTENSEN, B.M. AND LA FOND, M.M. (1986). Parasite-induced suppression of the immune response in Aedes aegypti by Brugia pahangi. J. Parasit. 72, 216-219.

CHRISTENSEN, B.M., FORTON, K.F., LA FOND, M.M. AND GRIEVE, R.B. (1987). Surface changes on Brugia pahangi microfilariae and their association with immune evasion in Aedes aegypti. J. Invert. Path.

CLEVELAND, D.W., FISCHER, S.G., KIRSCHNER, M.W. AND LAEMMLI, U.K. (1977). Peptide mapping by limited proteolysis in sodium dodecyl sulphate and analysis by gel electrophoresis. J. Biol. Chem. 252, 1102-1106.

COOK, D., STOLTZ, D.B. AND PAULEY, C. (1985). Purification and preliminary characterisation of insect spherulocytes. Insect. Biochem. 15, 419-426.

COOMBE, D.R., JACOBSEN, K.B. AND PARISH, C.R. (1987). A role for sulphated polysaccharide recognition in sponge cell aggregation. Exp. Cell Res. 170, 381-401.

COX, G.N., KUSCH, M. AND EDGAR, R.S. (1981). Cuticle of Caenorhabditis elegans : its isolation and partial characterization. J. Cell Biol. 90, 7-17.

CROMPTON, D.W.T. (1963). Morphological and histochemical observations on Polymorphus minutus (Goeze, 1782) with special reference to the body wall. Parasitology 53, 663-685.

CROMPTON, D.W.T. (1964). The envelope surrounding Polymorphus minutus (Goeze, 1782) (Acanthocephala) during its development in the intermediate host, Gammerus pulex. Parasitology 54, 721-735.

CROMPTON, D.W.T. (1970). An ecological approach to Acanthoecephalan physiology. Cambridge monographs in experimental biology No. 17. Cambridge University Press, Cambridge.

CROMPTON, D.W.T. AND LASSIERE, O. (1987). Acanthocephala In "In vitro methods of parasite cultivation". (Eds. A.E.R. Taylor and J.R. Baker). pp. 394-406. Academic Press, New York and London.

CROMPTON, D.W.T. AND LEE, D.L. (1965). The fine structure of the body wall of Polymorphus minutus (Goeze, 1782) (Acanthocephala). Parasitology 55, 357-364.

CROMPTON, D.W.T. AND LOCKWOOD, A.P.M. (1968). Studies on the absorption and metabolism of D-[U-^{14}C]-glucose by Polymorphus minutus (Acanthocephala) in vitro. J. Exp. Biol. 48, 411-425.

CROMPTON, D.W.T. AND NICKOL, B.B. (1985). Biology of the Acanthocephala. (Eds. D.W.T. Crompton and B.B. Nickol). Cambridge University Press, Cambridge.

CROMPTON, D.W.T. AND WARD, P.F.V. (1984). Selective metabolism of L-serine by Moniliformis (Acanthocephala) in vitro. Parasitology. 89, 133-144.

CROSSLEY, A.C. (1968). The fine structure and mechanism of breakdown of larval intersegmental muscles in the blowfly, Calliphora erythrocephala. J. Insect. Physiol. 11, 117-221.

CROSSLEY, A.C. (1975). The cytophysiology of insect blood cells. Adv. Insect Physiol. 11, 117-221.

DAMIAN, R.T. (1964). Molecular mimicry : antigen sharing by parasite and hosts and its consequences. Amer. Natur. 98, 129-149.

DAMIAN, R.T. (1987). Molecular mimicry revisited. Parasitology Today 3, 263-266.

DAVIES, D.H. AND VINSON, S.B. (1986). Passive evasion by eggs of Braconid parasitoid Cardiochiles nigriceps of encapsulation in vitro by haemocytes of host Heliothis virescens. Possible role for the fibrous layer in immunity. 32, 1003-1010.

DIETRICH, C.P., SAMPAIO, L.O., TOLEDO, O.M.S. AND CASSARO, C.M.F. (1977). Cell recognition and adhesiveness : a possible biological role for the sulphated mucopolysaccharides. Biochem. Biophys. Res. Commun. 75, 329-336.

DIKKEBOOM, R., TIJNAGEL, J.M.G.H., MULDER, E.C. AND VAN DER KNAPP, W.P.W. (1987). Haemocytes of the pond snail Lymnaea stagnalis generate reactive forms of oxygen. J. Invert. Path. 44, 321-331.

DIMARCQ, J.I., KEPPI, E., LAMBERT, J., ZACHARY, D. AND HOFFMAN, D. (1986). Diptericin A, a novel antibacterial peptide induced by immunisation or injury in larvae of the dipteran insect Pharmia terranovae Dev. Comp. Immunol. 10, p.626.

DISSOUS, C., GRZYCH, T.M. AND CAPRON, A. (1986). Schistosoma mansoni shares a protective oligosaccharide epitope with freshwater and marine snails. Nature 323, 443-445.

DUBOIS, M., GILES, K.A., HAMILTON, J.K., REBERS, P.A. AND SMITH, F. (1956). Colourimetric method for determination of sugars and related substances. Anal. Chem. 28, 350-356.

DUDMAN, W.F. (1977). The role of surface polysaccharides in natural environments. In : Surface carbohydrates of the prokaryotic cell (Ed. I.W. Sutherland) pp. 357-414. Academic Press, London and New York.

DULARAY, B. AND LACKIE, A.M. (1985). Haemocytic encapsulation and the prophenoloxidase activation pathway in the locust Schistocerca gregaria. Insect Biochem. 15, 827-834.

DUNN, P.E., DAI, W., KANOST, M.R., AND GENG, C. (1985). Soluble peptidoglycan fragments stimulate antibacterial protein synthesis by fat body from larvae of Manduca sexta. Dev. Comp. Immunol. 9, 559-568.

EDEBO, L., KIHLSTROM, E., MAGNUSSON, K. AND STENDAHL, O. (1980). The hydrophobic effect and charge effects in adhesion of enterobacteria to animal cell surfaces and the influence of antibodies of different immunoglobulin classes. In "Cell Adhesion and Motility" (Eds. A.S.G. Curtis and J.D. Pitts). B.S.C.B. Symposium Vol. 2, pp. 65-101. Cambridge University Press, Cambridge.

EDMONDS, S.J. (1966). Hatching of the eggs of Moniliformis dubius. Exp. Parasit. 19, 216-226.

ENNESSER, C.A. AND NAPPI, A.J. (1984). Ultrastructural study of the encapsulation response of the American cockroach, Periplaneta americana. J. Ultrastruct. Res. 87, 31-45.

EY, P.L. AND JENKIN, C.R. (1982). Molecular basis of self/non-self discrimination in the invertebrata. In: the reticuloendothelial system : a comprehensive treatise. (Eds. N. Cohen, and M.M. Sigel). pp. 321-391. Plenum Press, New York.

FARNDALE, R.W., BUTTLE, D.J. AND BARRETT, A.J. (1986). Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochem. Biophys. Acta. 883, 173-177.

FEIZI, T. AND CHILDS, R.A. (1987). Carbohydrates as antigenic determinants of glycoproteins. Biochem. J. 245, 1-11.

FESSLER, J.H., LUNSTRUM, G., DUNCAN, K.G., CAMPBELL, A.G., STERNE, R., BACHINGER, H.P. AND FESSLER, L.I. (1984). Evolutionary constancy of basement membrane components. In "The Role of the Extracellular Matrix in Development (Ed. R.L. Trelstad) pp. 207-219. Alan Liss Inc., New York.

FLYG, C., DALHAMMAR, G., RASMUSON, B. AND BOMAN, H.G. (1987). Insect Immunity: Inducible antibacterial activity in Drosophila. Insect. Biochem. 17, 153-160.

FORSYTHE, K.P., COPEMAN, D.B. AND MITCHELL, G.F. (1984). Differences in the surface radioiodinated proteins of skin and uterine microfilariae of Onchocerca gibsoni. Molec. Biochem. Parasit. 10, 217-229.

FRAKER, P.J. AND SPECK, J.C. (1978). Protein and cell membrane iodinations with a sparingly soluble chloroamide 1, 3, 4, 6-tetrachloro-3a,6a-diphenylglycoluril. Biochem. Biophys. Res. Commun. 80, 849-857.

FRANCOIS, J., HERBAGE, D. AND JUNQUA, S. (1980). Cockroach collagen : isolation, biochemical and biophysical characterisation. Eur. J. Biochem. 112, 389-396.

FRANSSON, L-A., COSTER, L. AND MALMSTROM, A. (1984). Binding of transferrin to the core protein of fibroblast protoheparan sulphate. Proc. Natl. Acad. Sci. 81, 5657-5661.

GAGEN, S.J. AND RATCLIFFE, N.A. (1976). Studies on the in vivo cellular reactions and fate of injected bacteria in Galleria mellonella and Pieris brassicae larvae. J. Invert. Path. 28, 17-24.

GALLAGHER, J.T., LYON, M. AND STEWARD, W.P. (1986). Structure and function of heparan sulphate proteoglycans. Biochem. J. 236, 313-325.

GARCIA, E.S., de AZAMBUJA, P., NADER, H.B. AND DIETRICH, C.P. (1986). Biosynthesis of sulphated glycosaminoglycans in the Hemipteran Rhodnius prolixus. Insect. Biochem. 16, 347-352.

GOLDRING, O.L., CLEGG, J.A., SMITHERS, S.R. AND TERRY, R.J. (1976). Acquisition of human blood group antigens by Schistosoma mansoni. Clin. exp. Immunol. 26, 181-187.

GOLDSTEIN, N.I. AND McINTOSH, A.H. (1980). The proteins and glycoproteins of insect cells in culture. Insect Biochem. 10, 419-427.

GÖTZ, P. (1986). Mechanisms of encapsulation in dipteran hosts. In "Immune mechanisms in invertebrate vectors" (Ed. A.M. Lackie). Zoological Society of London Symposia. 56, pp. 1-20. Oxford University Press, Oxford.

GÖTZ, P., BOMAN, A. AND BOMAN, H.G. (1981). Interactions between insect immunity and an insect pathogenic nematode with symbiotic bacteria. Proc. R. Soc. Lond. (B). 212, 333-350.

GRAFF, D.J. AND KITZMAN, W.B. (1965). Factors influencing the activation of acanthocephalan cystacanths. J. Parasit. 51, 424-429.

GRIMSTONE, A.V., ROTHERAM, S. AND SALT, G.S. (1967). An electron-microscope study of capsule formation by insect blood cells. J. Cell Sci. 2, 281-292.

GUNNARSSON, S. (1987). Cellular immune reactions in the desert locust, Schistocerca gregaria, infected by the fungus, Metarhizium anisopliae Ph.D. thesis, University of Uppsala. Acta Universitas Upsaliensis 81.

GUNNARSSON, S.G.S. AND LACKIE, A.M. (1985). Hemocytic aggregation in Schistocerca gregaria and Periplaneta americana as a response to injected substances of microbial origin. J. Invert. Path. 46, 312-319.

GUPTA, A.P. (1979). Hemocyte types : their structures, synonymies, interrelationships and taxonomic significance. In 'Insect Hemocytes' (Ed. A.P. Gupta) pp. 86-127. Cambridge University Press, Cambridge.

HAPNER, K.D. AND STEBBINS, M.R. (1986). Biochemistry of Arthropod agglutinins. In "Hemocytic and humoral immunity in Arthropods" (Ed. A.P. Gupta) pp. 227-250. John Wiley, New York.

HARPER, E., SEIFTER, S. AND SCHARRER, B. (1967). Electron microscopic and biochemical characterization of collagen in blattarian insects. J. Cell Biol. 33, 385-393.

HEIMER, R., MOLINARO, L. AND SAMPSON, P. (1987). Detection by ^{125}I-cationized cytochrome c of proteoglycans immobilized on unmodified and on positively-charged Nylon 66. Anal. Biochem. 165, 448-455.

HEIMER, R. AND SAMPSON, P.M. (1987). Detecting proteoglycans immobilised on positively charged nylon. Anal. Biochem. 162, 330-336.

HOFFMAN, D., BREHELIN, M. AND HOFFMANN, J.A. (1974). Modifications of the hemogram and of the hemocytopoietic tissue of male adults of Locusta migratoria (Orthoptera) after injection of Bacillus thuringiensis. J. Invert. Path. 24, 238-247.

HÖGLUND, L. (1976a). The comparative biochemistry of invertebrate mucopolysaccharides. V. Insecta (Calliphora erythrocephala). Comp. Biochem. Physiol. 53, 9-14.

HÖGLUND, L. (1976b). Changes in acid mucopolysaccharides during the development of the blowfly Calliphora erythrocephala. J. Insect. Physiol. 22, 917-923.

HOLLANDE, A.C. (1930). La digestion des bacilles tuberculeux par les leucocytes du sang des chenilles. Arch. Zool. Exp. Gen. 70, 231-280.

HOLT, R. AND LACKIE, A.M. (1986). Immune evasion by larvae of the tapeworm Hymenolepis diminuta in the insect host. Dev. Comp. Immunol. 10, 632.

HÖÖK, M. (1984). Cell-surface glycosaminoglycans. Ann. Rev. Biochem. 53, 847-869.

HULTMARK, D., ENGSTROM, A., BENNICH, H., KAPUR, R. AND BOMAN, H.G. (1982). Insect immunity : isolation and structure of cecropin D and four minor antibacterial components from Cecropia pupae. Eur. J. Biochem. 127, 207-217.

HULTMARK, D., ENGSTROM, A., ANDERSSON, K., STEINER, H., BENNICH, H. AND BOMAN, H.G. (1983). Insect immunity. Attacins, a family of antibacterial proteins from Hyalophora cecropia. EMBO J. 2, 571-576.

HUNG, C-H., BUTOWSKI, R.J. AND HUDSON, B.G. (1980). Intestinal basement membrane of Ascaris suum. J. Biol. Chem. 255, 4964-4971.

HUNT, S. (1970). Polysaccharide-protein complexes in invertebrates. Academic Press, New York.

HUXHAM, I.M. AND LACKIE, A.M. (1986). A simple visual method for assessing the activation and inhibition of phenoloxidase production by insect haemocytes in vitro. J. Immunol. Methods 94, 271-277.

HUXHAM, I.M. AND LACKIE, A.M. (1987). Behaviour of separated fractions of locust haemocytes in vitro. Cell Tissue Res. (in press).

HUXHAM, I.M., LACKIE, A.M. AND McCORKINDALE, N.J. (1987). Inhibitory of cyclodexipeptides, destruxins, from the fungus, Metarhizium anisopliae, on cellular immunity in insects. Eur. J. Immunol. (submitted).

KATASE, H. AND CHINO, H. (1984). Transport of hydrocarbons by haemolymph lipophorin in Locusta migratoria. Insect. Biochem. 14, 1-6.

KATCHALSKY, A. (1964). Polyelectrolytes and their biological interactions. Biophys. J. 4, 9-42.

KEPPI, E., ZACHARY, D., ROBERTSON, M., HOFFMAN, D. AND HOFFMAN, J.A. (1986). Induced antibacterial proteins in the haemolymph of Phormia terranova (Diptera). Insect. biochem. 16, 395-402.

KING, D. AND ROBINSON, E.S. (1967). Aspects of the development of Moniliformis dubius. J. Parasitol. 53, 142-149.

KINOSHITA, S. AND YOSHII, K. (1979). The role of proteoglycan synthesis in the development of sea urchins. Exp. Cell Res. 124, 361-369.

KLEBANOFF, S.J. (1982). Oxygen-dependent cytotoxic mechanisms of phagocytosis. In: Advances in host defence mechanisms (Eds. J.I. Gallin and A.S. Fauci). pp. 111-162. Raven Press, New York.

KOEPPE, J.K., KOVALICK, G.E. AND PRESTWICH, G.D. (1984). A specific photoaffinity label for haemolymph and ovarian juvenile hormone-binding proteins in Leucophaea moderae. J. Biol. Chem.. 259, 3219-3223.

de KORT, C.A.D. AND KOOPMANSCHAP, A.B. (1986). High molecular weight transport proteins for JH-III in insect haemolymph. Experientia 42, 834-836.

KNIBIEHLER, B., MIRRE, C., CECCHINI, J-P. AND LePARCO, Y. (1987). Haemocytes accumulate collagen transcripts during Drosophila melanogaster metamorphosis. Roux's Arch. Dev. Biol. 196, 243-247.

KOCKUM, K., FAYE, I., VANFFOFSTEN, P., LEE, J-Y., XANTHANOPOULOS, K.G. AND BOMAN, H.G. (1984). Insect immunity. Isolation and sequence of two cDNA clones corresponding to acidic and basic attacins from Hyalophora cecropia. EMBO J. 3, 2071-2075.

KOMANO, H., MIXUNO, D. AND NATORI, S. (1980). Purification of a lectin induced in the haemolymph of Sarcophaga peregrina larvae on injury. J. Biol. Chem. 255, 2919-2924.

KRAMEROV, A.A., MUKHA, D.V., METAKOVSKY, E.V. AND GVOSDEV, V.A. (1986). Glycoproteins containing sulfated chitin-like carbohydrate moiety are synthesised in an established Drosophila melanogaster cell line. Insect Biochem. 16, 417-432.

KRASNE, S., EISENMAN, G. AND SZABO, G. (1971). Freezing and melting of lipid bilayers and the mode of action of nonactin, valinomycin and gramicidin. Science 174, 412-415.

KUBO, T. AND NATORI, S. (1987). Purification and some properties of a lectin from the haemolymph of Periplaneta americana (American cockroach). Eur. J. Biochem. 168, 75-82.

LaFOND, M.M., CHRISTENSEN, B.M. AND LASEE, B.A. (1985). Defense mechanisms of mosquitoes to filarial worms : potential mechanism for avoidance of the response by Brugia pahangi microfilariae. J. Invert. Path. 46, 26-30.

* LACKIE, A.M. (1974). The activation of cystacanths of Polymorphus minutus (Acanthocephala) in vitro. Parasitology 68, 135-146.

LACKIE, A.M. (1976). Evasion of the haemocytic defence reaction of certain insects to larvae of Hymenolepis diminuta (Cestoda). Parasitology 73, 97-107.

LACKIE, A.M. (1979). Cellular recognition of foreign-ness in two insect species, the American cockroach and the desert locust. Immunology 36, 909-914

LACKIE, A.M. (1980). Invertebrate immunity. Parasitology 80, 393-412.

LACKIE, A.M. (1981a). The specificity of the serum agglutinins of Periplaneta americana and Schistocerca gregaria and its relationship to the insects' immune response. J. Insect Physiol. 27, 139-147.

LACKIE, A.M. (1981b). Humoral mechanisms in the immune response of insects to larvae of Hymenolepis diminuta (Cestoda) Parasite Immunol. 3, 201-208.

LACKIE, A.M. (1983a). Immunological recognition of cuticular transplants in insects. Dev. Comp. Immunol. 7, 41-50.

LACKIE, A.M. (1983b). Effect of substratum wettability and charge on adhesion in vitro and encapsulation in vivo by insect haemocytes. J. Cell Sci. 63, 181-190.

LACKIE, A.M. (1986a). Evasion of insect immunity by helminth larvae. In "Immune mechanisms in invertebrate vectors" (Ed. A.M. Lackie). Zoological Society of London Symposia, 56, pp. 161-178, Oxford University Press, Oxford.

LACKIE, A.M. (1986b). Haemolymph transfer as an assay for immunorecognition in the cockroach Periplaneta americana and the locust Schistocerca gregaria. Transplantation 41, 360-363.

LACKIE, A.M. (1986c). Transplantation : the limits of recognition. In "Hemocytic and humoral immunity in Arthropods". (Ed. A.P. Gupta) pp. 191-223. John Wiley, New York.

LACKIE, A.M. AND LACKIE, J.M. (1979). Evasion of the insect immune response by Moniliformis dubius (Acanthocephala) : further observations on the origin of the envelope. Parasitology 79, 297-301.

LACKIE, A.M., TAKLE, G.B. AND TETLEY, L. (1985). Haemocytic encapsulation in the locust Schistocerca gregaria (Orthoptera) and in the cockroach Periplaneta americana (Dictyoptera). Cell Tiss. Res. 240, 343-351.

LACKIE, A.M. AND VASTA, G.R. (1986). Biochemical characterisation and biological role of a serum lectin from the cockroach Periplaneta americana. Dev. Comp. Immunol. 10, 637.

LACKIE, A.M. AND VASTA, G. (1988). The role of galactosyl-binding lectin in the cellular immune response of the cockroach Periplaneta americana (Dictyoptera). Immunology (in press).

LACKIE, J.M. (1973). Studies on interactions between Moniliformis (Acanthocephala) and its intermediate hosts. Ph.D. thesis, Cambridge University.

LACKIE, J.M. AND ROTHERAM, S. (1972). Observations on the envelope surrounding Moniliformis dubius (Acanthocephala) in the intermediqate host, Periplaneta americana. Parasitology 65, 303-308.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685.

LAMBERT, J. AND HOFFMANN, D. (1985). Mise en evidence d'un facteur actif centre des bacteries gram negatives dans le sang de Locusta migratoria. C.R. Acad. Sci. Paris III, 300, 425-430.

LEONARD, C., RATCLIFFE, N.A. AND ROWLEY, A.F. (1985). The role of prophenoloxidase activation in non-self recognition and phagocytosis by insect blood cells. J. Insect. Physiol. 31, 789-799.

LINDAHL, U. AND HOOK, M. (1978). Glycosaminoglycans and their binding to biological macromolecules. Ann. Rev. Biochem. 47, 385-417.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, L. AND RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

MADDRELL, S.H.P. AND GARDINER, B.O.C. (1980). The retention of amino acids in the haemolymph during diuresis in Rhodnius. J. Exp. Biol. 87, 315-329.

MARKWELL, M.A.K., HAAS, S.M., BIEBER, L.L. AND TOLBERT, N.E. (1978). A modification of the Lowry procedure to simplify protein determinations in membrane and lipoprotein samples. Anal. Biochem. 87, 206-210.

MATTES, M.J. AND STEINER, L.A. (1978). Antisera to frog immunoglobulins cross-react with periodate-sensitive cell-surface determinant. Nature, 273, 761-763.

MacDERMOTT, R.P., SCHMIDT, R.E., CAULFIELD, J.P., HEIN, A., BARTLEY, G.T., RITZ, J., SCHLOSSMAN, S.F., AUSTEN, K.F. AND STEVENS, R.L. (1985). Proteoglycans in cell-mediated cytotoxicity. J. Exp. Med. 162, 1771-1787.

McBRIDE, O.W. AND HARRINGTON, W.P. (1967). Ascaris cuticle collagen : on the disulphid cross-linkages and the molecular properties of the subunits. Biochemistry, 6, 1484-1498.

McCORMICK, P.J., CHANDRASEKHAR, S. AND MILLIS, A.J.T. (1979). Direct visualisation of collagens and procollagens in polyacrylamide gels. Anal. Biochem. 97, 359-366.

McLAREN, D.J., McKEAN, J.R., OLSSON, I., VENGE, P. AND KAY, A.B. (1981). Morphological studies on the killing of schistosomula of Schistosoma mansoni by human eosinophil and neutrophil cationic proteins in vitro. Parasite Immunol. 3, 359-373.

McLAREN, D.J. AND TERRY, R.J. (1982). The protective role of acquired host antigens during schistosome maturation. Parasite Immunol. 4, 129-148.

MERCER, E.H. AND NICHOLAS, W.L. (1967). The ultrastructure of the capsule of the larval stages of Moniliformis dubius (Acanthocephala) in the cockroach Periplaneta americana. Parasitology 57, 169-174.

MILLER, E.J. (1971). Structural studies of cartilage collagen employing limited cleavage and solubilisation with pepsin. Biochemistry 11, 4903-4909.

MINNICK, M.F., RUPP, R.A. AND SPENCE, K.D. (1986). A bacterial-induced lectin which triggers hemocyte coagulation in Manduca sexta. Biochem. Biophys. Res. Comm. 37, 729-735.

MOHRIG, W., SCHITTEK, D. AND HANSCHKE, R. (1979a). Investigations on cellular defence reactions with Galleria mellonella against Bacillus thuringiensis. J. Invert. Path. 34, 207-212.

MOHRIG, W. et al. (1979b). Immunological activation of phagocytic cells in Galleria mellonella. J. invert. Path. 34, 84-87.

MOLYNEUX, D.H., TAKLE, G., IBRAHIM, E.A. AND INGRAM, G.A. (1986). Insect immunity to trypanosomatidae. In "Immune mechanisms in invertebrate vectors" (Ed. A.M. Lackie). Zoological Society of London Symposia, 56, pp. 117-144. Oxford University Press.

MOLYNEUX, D.H., TAKLE, G., IBRAHIM, E.A. AND INGRAM, G.A. (1986). Insect immunity to Trypanosomatidae In: Immune mechanisms in invertebrate vectors. (Ed. A.M. Lackie). Symposium of the Zoological Society of London, No. 56. pp. 117-138.

MONNÉ, L. (1959). On the external cuticles of various helminths and their role in the host-parasite relationship : a histochemical study. Arkiv. Zool. 12, 343-358.

MORRIS, S.C. AND CROMPTON, D.W.T. (1982). The origins and evolution of the Acanthocephala. Biol. Rev. 57, 85-115.

MUKHA, D.V., KRAMEROV, A.A. AND GVOZDEV, V.A. (1987). Nascent glycoproteins with a chitin-like carbohydrate component in Drosophila melanogaster imaginal discs. Insect Biochem. 17, 919-927.

MULLINS, D.E. (1985). Chemistry and physiology of the haemolymph. In: Comprehensive insect Physiology, Biochemistry and Pharmacology. (Ed. G.A. Gilbert and L.I. Gilbert) vol. 3, pp. 355-400. Pergamon Press, Oxford.

MURRAY, L.W., WAITE, J.H., TANZER, M.L. AND HAUSCHKA, P.V. (1982). Preparation and characterization of invertebrate collagens. In: Methods in Enzymology vol. 82A. (Ed. L.W. Cunningham and D.W. Frederikson) pp. 65-96. Academic Press, New York.

NATORI, S. (1977). Bactericidal substance induced in the haemolymph of Sarcophaga peregrina larvae. J. Insect. Physiol. 23, 1169-1173.

NAYAME, K., CUMMINGS, R.D. AND DAMIAN, R.T. (1987). Schistosoma mansoni synthesises glycoproteins containing terminal O-linked N-acetylglucosamine residues. J. Biol. Chem. 262, 7990-7995.

NICHOLAS, W.L. AND MERCER, E.H. (1965). The ultrastructure of the tegument of Moniliformis dubius (Acanthocephala). Quart. J. Microsc. Sci. 106, 137-146.

OKADA, M. AND NATORI, S. (1983). Purification and characterization of an antibacterial protein from haemolymph of Sarcophago peregrina (flesh-fly) larvae. Biochem. J. 211, 727-734.

OKADA, M. AND NATORI, S. (1984). Mode of action of a bactericidal protein induced in the haemolymph of Sarcophago peregrina (flesh-fly) larvae. Biochem. J. 222, 119-124.

OKADA, M. AND NATORI, S. (1985). Ionophore activity of sarcotoxin I, a bactericidal protein of Sarcophago peregrina. Biochem. J. 229, 453-458.

OLAFSEN, J.A. (1986). Invertebrate lectins In: Immunity in invertebrates : cells molecules and defence reactions. (Ed. M. Brehelin) pp. 94-111. Springer-Verlag, Berlin.

PARRY, G., LEE, E., FARSON, D., KOVAL, M. AND BISSELL, M.J. (1985). Collagenous substrata regulate the nature and distribution of glycosaminoglycans produced by differentiated cultures of mouse mammary epithelial cells. Exp. Cell. Res. 156, 487-499.

PEREIRA, M.E.A., ANDRADE, A.F.B. AND RIBEIRO, J.M.C. (1981). Lectins of distinct specificity in Rhodnius prolixus interact selectively with Trypanosoma cruzi Science 211, 597-600.

PERRONE, J.B., DeMAIO, J. AND SPIELMAN, A. (1986). Regions of mosquito salivary glands distinguished by surface lectin-binding characteristics. Insect Biochem. 16, 315-318.

PHIPPS, D., MENGER, M., CHADWICK, J. AND ASTON, W.P. (1987). A cobra venom factor (CVF)-induced C3 convertase activity in the haemolymph of Galleria mellonella. Dev. Comp. Immun. 11, 37-46.

POOLE, A.R. (1986). Proteoglycans in health and disease : structures and functions. Biochem. J. 236, 1-4.

PYE, A.E. (1974). Microbial activation of prophenoloxidase from immune insect larvae. Nature, Lond. 251, 610-612.

RAHEMTULLA, F. AND LOVTRUP, S. (1974). The comparative biochemistry of invertebrate mucopolysaccharides - I. Methods; platyhelminths. Comp. Biochem. Physiol. 49B, 631-637.

RATCLIFFE, N.A. (1986). Insect cellular immunity and the recognition of foreignness. In "Immune Mechanisms in Invertebrate Vectors" (Ed. A.M. Lackie), pp. 21-44. Zoological Society of London Symposia, 56, Oxford University Press, Oxford.

RATCLIFFE, N.A. AND GAGEN, S.J. (1977). Studies on the in vivo cellular reactions of insects : on ultrastructural analysis of nodule formation in Galleria mellonella. Tissue Cell 9, 73-85.

RATCLIFFE, N.A., LEONARD, C. AND ROWLEY, A.F. (1984). Prophenoloxidase activation: non-self recognition and cell cooperation in insect immunity. Science 225, 557-559.

RATCLIFFE, N.A. AND ROWLEY, A.F. (1979). Role of hemocytes in defence against biological agents. In "Insect Hemocytes" (Ed. A.P. Gupta), pp. 331-414. Cambridge University Press, Cambridge.

RATCLIFFE, N.A. AND ROWLEY, A.F. (1983). Recognition factors in insect haemolymph. Dev. Comp. Immunol. 7, 653-656.

RATCLIFFE, N.A. AND WALTERS, J.B. (1983). Studies on the in vivo cellular reactions of insects : clearance of pathogenic and non-pathogenic bacteria in Galleria mellonella larvae. J. Insect Physiol. 29, 407-415.

RATNER, S. AND VINSON, S.B. (1983). Encapsulation reactions in in vitro by haemocytes of Heliothis virescens. J. Insect Physiol. 11, 855-863.

RAVINDRANATH, M.H. AND ANANTARAMAN, S. (1977). The cystacanth of Moniliformis moniliformis (Bremser, 1811) and its relationship with the haemocytes of the intermediate host (Periplaneta americana). Zeits. Parasit. 53, 225-237.

RENWRANTZ, L. (1986). Lectins in molluscs and arthropods: their occurrence, origin and roles in immunity. In "Immune mechanisms in invertebrate vectors" (Ed. A.M. Lackie) pp. 81-94. Zoological Society of London Symposia. 56, Oxford University Press, Oxford.

RIZKI, T.M. AND RIZKI, R.M. (1980a). Properties of the larval haemocytes of Drosophila melanogaster. Experientia 36, 1223-1226.

RIZKI, T.M. AND RIZKI, R.M. (1980b). Developmental analysis of a temperature-sensitive melanotic tumour mutant in Drosophila melanogaster. Wilhelm Roux's Arch. Dev. Biol. 189, 197-206.

RIZKI, R.M. AND RIZKI, T.M. (1984). Selective destruction of a host blood cell type by a parasitoid wasp. Proc. Natl. Acad. Sci. 81, 6154-6158.

RIZKI, T.M. AND RIZKI, R.M. (1986). Surface changes in haemocytes during encapsulation in Drosophila melanogaster meign. In Haemocytic and Humoral Immunity in Arthropods (Ed. A.P. Gupta). pp. 157-190. John Wiley and Sons, New York.

ROBERTSON, N.P. AND CAIN, G.D. (1984). Glycosaminoglycans of tegumental fractions of Hymenolepis diminuta. Molec. Biochem. Parasit. 12, 173-183.

ROBERTSON, N.P. AND CAIN, G.D. (1985). Isolation and characterisation of glycosaminoglycans from Schistosoma mansoni. Comp. Biochem. Physiol. 82B, 299-306.

ROBERTSON, N.P., OAKS, J.A. AND CAIN, G.D. (1984). Biochemical characterisation of polysaccharides of the eggs and adults of Hymenolepis diminuta. Molec. Biochem. Parasit. 10, 99-109.

ROBINSON, E.S. AND STRICKLAND, B.C. (1969). Cellular responses of Periplaneta americana to Acanthocephalan larvae. Exp. Parasit. 26, 384-392.

RODEN, L. (1980). Structure and metabolism of connective-tissue proteoglycans. In: The biochemistry of glycoproteins and proteoglycans. (Ed. W.J. Lennarz). p. 267-371. Plenum Press, New York.

ROITT, I. (1984). Essential Immunology (5th edition). Blackwell Scientific Publications, Oxford.

ROSSIGNOL, D.P., EARLES, B.J., DECKER, G.L. AND LENNARZ, W.J. (1984). Characterisation of the sperm receptor on the surface of eggs of Strongylocentrotus purpuratus. Dev. Biol. 104, 308-316.

ROTHERAM, S. AND CROMPTON, D.W.T. (1972). Observations on the early relationship between Moniliformis dubius (Acanthocephala) and the haemocytes of the intermediate host Periplaneta americana. Parasitology 64, 15-21.

ROTHMAN, A.H. AND FISHER, F.M. (1964). Permeation of amino acids in Moniliformis and Macracanthorhynchus (Acanthocephala). J. Parasitol. 50, 410-414.

ROWLEY, A.F. AND RATCLIFFE, N.A. (1981). Insects. In "Invertebrate Blood Cells". (Eds. N.A. Ratcliffe and A.F. Rowley). Vol. 2, pp. 471-490. Academic Press, New York.

RYERSE, J.S. AND REISNER, M.W. (1985). A new technique for the isolation and purification of the basal lamina from insect tissues. Tissue and Cell 17, 287-292.

SALT, G. (1961). The haemocytic reaction of insects to foreign bodies. In "The Cell and the Organism" (Eds. J.A. Ramsay and V.B. Wigglesworth) pp. 175-192. Cambridge University Press, Cambridge.

SALT, G.S. (1963). The defence reactions of insects to metazoan parasites. Parasitology 53, 527-642.

SALT, G. (1968). The resistance of insect parasitoids to the defence reactions of their hosts. Biol. Rev. 43, 200-232.

SALT, G.S. (1970). The cellular defence reactions of insects. Cambridge monographs in experimental biology No. 16. Cambridge University Press, Cambridge.

SAHBA, G.H., ARFAA, F. AND RASTEGAR, M. (1970). Human infection with Moniliformis dubius (Acanthocephala) (Meyer, 1932). (syn. M. moniliformis (Bremser, 1811)) (Travassos, 1915) in Iran. Trans. R. Soc. Trop. Med. Hyg. 64, 284-286.

SAMUELSON, J.C., CAULFIELD, J.P. AND DAVID, J.R. (1982). Schistosomula of Schistosoma mansoni clear Concanavalin A from their surface by sloughing. J. Cell Biol. 94, 355-362.

SCHMIT, A.R. AND RATCLIFFE, N.A. (1977). The encapsulation of foreign tissue implants in Galleria mellonella larvae. J. Insect Physiol. 23, 175-184.

SCHNEIDER, P.M. (1985). Purification and properties of 3 lysozymes from hemolymph of the cricket Gryllus bimaculatus (De Greer). Insect Biochem. 15, 463-470.

SCOTT, J.E. AND DORLING, J. (1965). Differential staining of acid glycosaminoglycans (mucopolysaccharides) by Alcian blue in salt solutions. Histochemie 5, 221-233.

SHARON, N. (1984). Surface carbohydrates and surface lectins are recognition determinants in phagocytosis. Immunol. today 5, 143-147.

SMITH, P.J.S., HOWES, E.A., LEECH, C.A. AND TREHERNE, J.E. (1986). Haemocyte involvement in the repair of the insect central nervous system after selective glial disruption. Cell Tissue Res. 243, 367-374.

SMITH, V.J. AND SODERHALL, K. (1983). B1,3-glucan activation of crustacean haemocytes in vitro and in vivo. Biol. Bull. mar. Lab. Woods Hole. 164, 299-314.

STARLING, J.A. (1985). Feeding, nutrition and metabolism. In: Biology of the Acanthocephala. (Eds. D.W.T. Crompton and B.B. Nickol). pp. 125-212. Cambridge University Press, Cambridge.

STEBBINS, M.R. AND HAPNER, K.D. (1985). Preparation and properties of haemagglutinin from haemolymph of Acrididae (grasshoppers). Insect Biochem. 15, 451-462.

STEINER, H., HULTMARK, D., ENGSTROM, BENNICH, H. AND BOMAN, H.G. (1981). Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature 292, 246-248.

STOLTZ, D.B. (1986). Interactions between parasitoid-associated products and host insects : a brief overview. J. Insect. Physiol. 32, 347-350.

STOLTZ, D.B. AND VINSON, S.B. (1979). Viruses and parasitism in insects. Adv. Virus Res. 24, 125-171.

SUTHERLAND, D.R., CHRISTENSEN, B.M. AND FORTON, K.F. (1984). Defence reactions of mosquitoes to filarial worms : role of the microfilarial sheath in the response of mosquitoes to inoculated Brugia pahangi microfilariae. J. Invert. Path. 44, 275-281.

TAKAHASKI, H., KOMANO, H. AND NATORI, S. (1986). Expression of the lectin gene in Sarcophaga peregrina during normal development and under conditions where the defence mechanism is activated. J. Insect. Physiol. 32, 771-779.

TAKLE, G.B. (1986). Adhesion and encapsulation by insect haemocytes. Ph.D. thesis, University of Glasgow.

TAKLE, G.B. AND LACKIE, A.M. (1985). Surface charge of insect haemocytes, examined using cell electrophoresis and cationised ferritin-binding. J. Cell Sci. 75, 205-214.

TAKLE, G.B. AND LACKIE, A.M. (1986). Chemokinetic behaviour of insect haemocytes in vitro. J. Cell Sci., 85, 85-94.

TAKLE, G.B. AND LACKIE, A.M. (1987). Investigations of the possible role of haemocyte and parasite surface charge in the clearance of Trypanosoma rangelia from the insect haemocoele. J. Invert. Path. 50, 336-338.

TANNER, M.J.A. AND ANSTEE, D.J. (1976). A method for the direct demonstration of the lectin-binding components of the erythrocyte membrane. Biochem. J. 153, 265-270.

TAUSSIG, M.J. (1984). Processes in Pathology. (2nd Edition). Blackwell Scientific Publications, Oxford.

TAYLOR, R.L. (1969). A suggested role for the polyphenol-phenoloxidase system in invertebrate immunity. J. Invert Path. 14, 427-428.

THEOCHARIS, D.A., ANAGNOSTIDES, S.T. AND TSIGANOS, C.P. (1985). Distribution and changes of glycosaminoglycans in the three stages of development of the insect Ceratitis capitata. Comp. Biochem. Phys. 81B, 933-937.

TRENCZEK, T. (1986). Injury and immunity in insects. Dev. Comp. Immunol. 10, 627.

TRENCZEK, T. AND BENNICH. H. (1987). Insect immunity : antibacterial proteins in insect hemocytes. J. Immunol. (in press).

TRUEB, B. AND BORNSTEIN, P. (1984). Characterisation and precursor form of type VI collagen. J. Biol. Chem. 259, 8597-8604.

UGLEM, G.L. AND LEWIS, M.C. (1986). Specificity of the surface aminopeptidase in Moniliformis moniliformis (Acanthocephala). J. Helminthology. 60, 288-292.

UGLEM, G.L., PAPPAS, P.W. AND READ, C.P. (1973). Surface aminopeptidase in Moniliformis dubius and its relations to amino acid uptake. Parasitology. 67, 185-195.

VEY, A., QUIOT, J.M., VAGO, C. AND FARGUES, J. (1985). Effet immunodepresseur de toxines fongiques; inhibition de la reaction d'encapsulement multicellulaire par les destruxines. C.R. Acad. Sci. III, 300, 647-651.

VEY, A. AND GOTZ, P. (1975). Humoral encapsulation in Diptera (Insecta) : comparative studies in vitro. Parasitology. 70, 77-86.

VEY, A. AND GOTZ, P. (1986). Antifungal cellular defence mechanisms in insects. In: haemocytic and humoral immunity in arthropods (Ed. A.P. Gupta) pp. 89-116. John Wiley and Sons, New York.

VINSON, S.F. (1974). The role of the foreign surface and female parasitoid secretions on the immune response of an insect. Parasitology 79, 297-306.

VINSON, S.B., EDSON, K.M. AND STOLTZ, D.B. (1979). Effect of a virus associated with the reproductive system of the parasitoid wasp, Campoletis sonorensis on host weight gain. J. Invert. Path. 34, 133-137.

WALTERS, J.B. AND RATCLIFFE, N.A. (1981). A comparison of the immune response of the waxmoth, Galleria mellonella, to pathogenic and non-pathogenic bacteria. In "Aspects of Developmental and Comparative Immunology I" (Ed. J.B. Solomon) pp. 147-152. Pergamon Press, Oxford.

WHALEY, K. (1986). The complement system. In: complement in Health and Disease (Ed. K. Whaley) pp. 1-35. MTP Press Ltd., Lancaster.

WIGGLESWORTH, V.B. (1984). Insect physiology, 8th edition. Chapman and Hall, London.

WRIGHT, R.D. AND LUMSDEN, R.D. (1968). Ultrastructural and histochemical properties of the Acanthocephalan epicuticle. J. Parasit. 54, 1111-1123.

WRIGHT, R.D. AND LUMSDEN, R.D. (1970). The acanthor tegument of Moniliformis dubius. J. Parasitol. 56, 727-735.

YAMADA, K. (1983). Cell surface interactions with extracellular materials. Ann. Rev. Biochem. 52, 761-799.

YOSHINO, T.P. AND BOSWELL, C.A. (1986). Antigen-sharing between larval trematodes and their snail hosts: how real a phenomenon in immune evasion ? In: "Immune mechanisms in invertebrate vectors" (Ed. A.M. Lackie). Zoological Society of London Symposia. 56, pp. 221-238. Oxford University Press, Oxford.

ZACHARY, D. AND HOFFMANN, D. (1984). Lysozyme is stored in the granules of certain haemocyte types. J. insect. Physiol. 30, 405-411.

ASHHURST, D.E. (1968). The connective tissues of insects. Ann. Rev. Ent. 13, 45-74.

LACKIE, J.M. (1972). The course of infection and growth of Moniliformis dubius (Acanthocephala) in the intermediate host Periplaneta americana. Parasitology. 64, 95-106.

MOORE, D.V. (1946). Studies on the life history and development of Moniliformis dubius Meyer, 1933. J. Parasitol. 32, 257-271.