THE CUTICLE PROTEINS AND CUTICLE PROTEIN GENES
OF THE SHEEP BLOWFLY, LUCILIA CUPRINA

A thesis submitted to
The Australian National University
for the degree of Doctor of Philosophy
by Patrick J. Skelly.
December 1985.
DECLARATION

The research described in this thesis was carried out by myself, under the supervision of Dr. A.J. Howells.

Patrick Skelly

Patrick Skelly
ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Tony Howells, for his advice, guidance and friendliness throughout the course of this research. Thanks also to Dr. Mike Howell for supplying assistance and chemical reagents freely, to Dr. Keith Wardhaugh for aid in obtaining serum samples, to Dr. Ron Hackman for helpful discussions and to Tom Van Gerwen and Roger Belves for supplying flies on request.

I would also like to express my gratitude to many members of the Biochemistry Department, ANU, particularly those in Dr. K. C. Reed's lab and those with whom I shared a lab - Donna Boyle, Harvey Perkins, Mandy Walker, Mary Anne Rye and Relli Kline. My special thanks to two fellow Phd students - Rick Tearle, for unceasingly scanning his computer for 'utic' references, and Abigail Elizur, for introducing me both to the techniques of molecular biology and falafas.

Thanks to my family for their support (and the odd letter), to Niall Mc Inerney for keeping out of this one, to Barbara Piper for typing this thesis without mistke and to Carmel Teahan for explaining that a mole is not necessarily a small, furry mammal.

A scholarship awarded by the Australian Wool Corporation is very gratefully acknowledged.
ABSTRACT

The cuticle of the sheep blowfly, *Lucilia cuprina*, is a multilamellate secretion of the epidermis which protects and supports the insect. The epidermis secretes five separate cuticles (three larval, a pupal and an adult) during the fly's developmental history. Each cuticle differs somewhat in form but all are composed largely of the polysaccharide chitin and protein. This thesis describes studies on the soluble proteins of the various cuticles, on the interaction between the cuticle proteins and the immune system of sheep infested with *L. cuprina* larvae and on the isolation and characterization of the genes which code for larval cuticle proteins.

The proteins which are extracted from isolated cuticles of 3rd instar larvae resolve maximally into nine prominent bands when analysed by native, gradient gel electrophoresis. Water or 7M urea or 2% SDS can be used to extract protein and while urea or SDS release significantly more protein than water the same major proteins, in the same relative proportions, are extracted by all three solutions. Around 80% of the total 3rd instar larval cuticle protein is extracted with 7M urea.

The cuticle proteins of 1st, 2nd and 3rd instar larvae display quantitatively and qualitatively unique electrophoretic profiles. However a number of major proteins are shared by all larval instars. The profiles of proteins extracted from the cuticles of 1st or 2nd instar animals at different developmental times differ quantitatively. During the 3rd instar one striking change in the protein composition of the cuticle occurs. A collection of new proteins are deposited into the cuticle at the transition from the feeding to the wandering phase. At pupariation the larval cuticle is converted into the puparium. This involves a concerted cross-linking of the major larval cuticle proteins
such that most extractable protein is rendered insoluble within 24 h of white puparium formation.

Experiments examining the effects of a number of insecticides on the larval cuticle proteins are described. Certain insecticides (e.g., diflubenzuron and cyromazine) disrupt protein deposition into the larval cuticle. However, neither polyoxin D nor 5-fluorouracil affect the larval cuticle.

The electrophoretic profiles of pupal and adult cuticle proteins differ both from each other and from the profiles of larval proteins. Maximally seven major pupal and nine major adult cuticle proteins are resolved. The profiles of proteins extracted from the unsclerotized pupal cuticle is relatively invariant throughout pupal life. In contrast the profiles of proteins extracted from the adult cuticle (which becomes sclerotized) change markedly during development. Regional variation in pupal and adult cuticle protein composition is also demonstrated in these studies.

*L. cuprina* is a myiasis-causing insect whose larvae evoke an immune response in sheep. In this thesis immunological experiments are described using sera obtained from sheep infested with larvae (struck sheep). It is shown that the larval cuticle proteins do not appear to stimulate the sheep's humoral immune response since the sera contain no antibodies directed against them. Extracts of many other larval tissues (1st, 2nd and 3rd instar larval excretions/secretions or visceral homogenates or extracts of 3rd instar salivary glands, mid guts, haemolymph) do evoke a response. The strongest antibody reaction is directed against salivary gland components. Western immunoblot analysis reveals that the pattern of *L. cuprina* larval antigens against which struck sheep respond is complex and changes between instars.
Major antigens in the larval extracts do not equate with major Coomassie-staining proteins.

Since the changing pattern of epidermal secretions of *L. cuprina* represents a useful system for studying developmental gene regulation clones containing genes which code for some of the larval cuticle proteins have been isolated. An *L. cuprina* genomic DNA library was screened with a cDNA probe synthesised using larval epidermal RNA as template to select clones which carry cuticle protein coding sequences. 13 epidermis specific clones have been isolated by this procedure. Restriction endonuclease mapping reveals that four of these (\(\lambda LoLCP 1-4\)) contain overlapping DNA inserts; a further three (\(\lambda LoLCP 5a-c\)) contain exactly the same insert. The remaining clones (\(\lambda LoES 6-11\)) contain unique inserts. Three lines of evidence suggest that \(\lambda LoLCP 1-4\) carry larval cuticle protein genes. 1) \(\lambda LoLCP 1\) DNA has been used to select larval epidermal RNA in a hybrid release translation assay. The electrophoretic mobilities of some of the polypeptides translated *in vitro* from the selected RNA are similar to those of a number of cuticle proteins. 2) A specific anti-cuticle protein antiserum immunoprecipitates these polypeptides. 3) \(\lambda LoLCP 1-4\) (and \(\lambda LoLCP 5 a-c\)) show homology to cloned *D. melanogaster* larval cuticle protein genes. Another epidermis specific clone (\(\lambda LoES 6\)) shows homology to the cloned dopa decarboxylase gene of *D. melanogaster*.

A preliminary analysis of the organization of DNA sequences in the clones that carry cuticle protein genes has been undertaken. They appear to contain more than one cuticle protein coding region, suggesting that the cuticle protein genes are present in a multi-gene cluster. The clones also contain repeated sequence DNA; the structural and functional significance of this type of DNA interspersed with unique
sequence DNA is unknown. This work on the cuticle protein genes provides a basis for future studies on their structure and regulation.
**ABBREVIATIONS.**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACP</td>
<td>adult cuticle protein</td>
</tr>
<tr>
<td>AMV</td>
<td>avian myeloblastosis virus</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>Hepes</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobase pairs</td>
</tr>
<tr>
<td>Kd</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>LCP</td>
<td>larval cuticle protein</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCP</td>
<td>pupal cuticle protein</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming unit</td>
</tr>
<tr>
<td>pipes</td>
<td>piperazine-N,N'-bis(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>Poly A⁺</td>
<td>polyadenylated</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>1 x SSC</td>
<td>0.15M NaCl/0.015M Na-citrate (pH7)</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
</tbody>
</table>

Other abbreviations used in this thesis are defined in *J. Biol. Chem.* (1966), **241**, 527-533.
**TABLE OF CONTENTS**

**CHAPTER ONE: GENERAL INTRODUCTION**

- The life history of *Lucilia cuprina* .................................................. 1
- The arthropod cuticle ............................................................................. 1
- The epicuticle ....................................................................................... 3
- The procuticle ...................................................................................... 5
- The moulting cycle .............................................................................. 6
- The biochemistry of the insect procuticle .............................................. 7
- Chitin ................................................................................................... 9
- Protein .................................................................................................. 9
- Molecular organization of the procuticle ................................................ 11
- Studies on the cuticle: scope of this thesis ........................................... 12
- .............................................................. 13

**CHAPTER TWO: THE LARVAL CUTICLE AND CUTICLE PROTEINS OF *L. CUPRINA***

- Introduction ....................................................................................... 15
  - Larval growth and the larval cuticle .................................................. 15
  - The cuticle proteins .......................................................................... 16
  - Larvicides which affect the cuticle .................................................... 17
- Materials and Methods ...................................................................... 20
  - Chemicals ........................................................................................... 20
  - Insect cultures .................................................................................. 20
  - Preparation of larval cuticles and extraction of proteins ................... 21
  - Gel electrophoresis and sample preparation ...................................... 22
  - Protein staining and quantitation ...................................................... 23
  - Larval and cuticle weight .................................................................. 23
Preparation of antisera.

1. Rabbit
2. Mouse

Western blotting
Immunological assays
Electron microscopy
Insect culture in the presence of larvicides
Extraction of proteins from the cuticles of larvicide-treated larvae

Results

1. The cuticle proteins of mid resting phase 3rd instar larvae
   a. Extraction and quantitation
   b. Properties of extracted proteins
   c. The proteins of different morphological regions of the cuticle
   d. The cuticle proteins of five lines of L. Cuprina
   e. d. Cuticle water content

2. Developmental changes in the larval cuticle
   a. Cuticle weight and protein content
   b. Electrophoretic profiles of the cuticle proteins of different larval instars
   c. Electrophoretic profiles of the cuticle proteins within the larval instars

3. Immunological relatedness of the cuticle proteins
   a. Within the 3rd instar
   b. Between the larval instars

4. Comparison of the major cuticle proteins of 3rd instar larvae of L. cuprina and D. melanogaster
5. Immunological relatedness of cuticle, haemolymph and other non-cuticular proteins

6. Effects of larvicides on the larval cuticle and cuticle proteins
   a. Larval and cuticle dry weight
   b. Cuticle protein electrophoresis

Discussion

The cuticle proteins

Regional distribution of cuticle proteins
   (i) Epicuticle and procuticle
   (ii) Anatomically-distinct regions

The multiplicity of cuticle proteins

Changes in the larval cuticle during development

Comparison of the major larval cuticle proteins of L. cuprina and D. melanogaster

Immunological relatedness of cuticle, haemolymph and other non-cuticular proteins

Effects of larvicides on cuticle structure and cuticle proteins

1. Diflubenzuron and Polyoxin D: Inhibitors of chitin synthesis
2. Cyromazine and 5-fluorouracil: disrupters of folic acid metabolism?
CHAPTER THREE: THE PROTEINS OF PUPARIA AND OF PUPAL AND ADULT CUTICLES OF L. CUPRINA

Introduction 58

The development of the pupal and adult cuticle 58

The form of the pupal and adult cuticle 59

Sclerotization 60

Materials and Methods 63

Insect culture 63

Cuticle preparation and protein extraction 63

Electrophoresis and immunological assays 64

Results 65

1. Quantitation and electrophoretic separation of cuticular protein 65
   a. Puparia 65
   b. Pupal cuticle 65
   c. Adult cuticle 66

2. Regional distribution of the major pupal and adult cuticle proteins 66

3. Immunological relatedness of the pupal and adult cuticle proteins to 3rd instar larval cuticle proteins 67

Discussion 68

Electrophoretic profiles of the cuticle proteins 68
   a. Puparia 68
   b. Pupae 70
   c. Adults 72

Relatedness of larval, pupal and adult cuticle proteins 73
CHAPTER FOUR: THE HUMORAL IMMUNE RESPONSE OF SHEEP TO BLOWFLY LARVAL ANTIGENS

Introduction

Materials and Methods

Preparation of larval extracts
Electrophoresis
Sheep sera
Immuno-dot blot assay
Western immuno blotting
Incubation of larvae in anti-cuticle protein antiserum

Results

Larval excretions/secretions
Immuno-dot blot assay
Electrophoretic profiles of larval extracts
Western immuno blot analysis
Incubation of larvae in anti-cuticle protein antiserum

Discussion

CHAPTER FIVE: CLONING AND CHARACTERIZATION OF THE LARVAL CUTICLE PROTEIN GENES OF L. CUPRINA

Introduction

Materials and Methods

Construction and screening of the L. cuprina genomic DNA library
Isolating larval integuments
Nucleic acid preparation
  a. Isolation, electrophoresis and blotting of RNA
  b. Isolation, electrophoresis and blotting of DNA
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction of DNA fragments from agarose gels</td>
<td>104</td>
</tr>
<tr>
<td>Reprobing nitrocellulose filters</td>
<td>104</td>
</tr>
<tr>
<td>DNA labelling</td>
<td>105</td>
</tr>
<tr>
<td>a. cDNA synthesis</td>
<td>105</td>
</tr>
<tr>
<td>b. Nick translation</td>
<td>106</td>
</tr>
<tr>
<td>Hybridization conditions</td>
<td>106</td>
</tr>
<tr>
<td>Hybrid release translation and immunoprecipitation</td>
<td>107</td>
</tr>
<tr>
<td>Results</td>
<td>110</td>
</tr>
<tr>
<td>Selecting cuticle protein clones</td>
<td>110</td>
</tr>
<tr>
<td>Characterization of the selected clones</td>
<td>110</td>
</tr>
<tr>
<td>a. Mapping</td>
<td>110</td>
</tr>
<tr>
<td>b. Defining coding regions within the clones</td>
<td>111</td>
</tr>
<tr>
<td>c. Repeated sequences within the clones</td>
<td>112</td>
</tr>
<tr>
<td>d. Homology to cloned <em>D. melanogaster</em> larval cuticle protein genes</td>
<td>111</td>
</tr>
<tr>
<td>Characterization of $\lambda$LoLCP 1</td>
<td>112</td>
</tr>
<tr>
<td>a. Hybrid release translation, immunoprecipitation</td>
<td>112</td>
</tr>
<tr>
<td>b. Detailed restriction mapping</td>
<td>114</td>
</tr>
<tr>
<td>c. Location of coding regions and repeated sequences in the subclones</td>
<td>114</td>
</tr>
<tr>
<td>d. Relatedness of coding regions in the epidermis specific clones</td>
<td>115</td>
</tr>
<tr>
<td>RNA transcripts encoded by subclones pLCP 5.4 S/E and pLCP 3.2 S/H</td>
<td>116</td>
</tr>
<tr>
<td>Homology between the cloned dopa decarboxylase gene of <em>D. melanogaster</em> and epidermis specific clones of <em>L. cuprina</em></td>
<td>116</td>
</tr>
<tr>
<td>Discussion</td>
<td>117</td>
</tr>
</tbody>
</table>
Characterization of $\lambda LoLCP$ 1 119
The transition from feeding to wandering phase in the 3rd larval instar 122
Repeated DNA sequences in the epidermis specific clones 124
Dopa decarboxylase 125
Future direction 126

CHAPTER SIX: GENERAL DISCUSSION AND FUTURE STUDY

The protein secretions of the epidermis 127
Regulation of the secretory programme of the epidermis 131
The eradication of the sheep blowfly 136
The moult cycle and growth in arthropods 138

REFERENCES 144
CHAPTER ONE

GENERAL INTRODUCTION
The experimental work described in this thesis is aimed at providing fundamental information about the proteins which constitute the cuticle (outer covering) of the sheep blowfly, *Lucilia cuprina*, Weidemann. The cuticle proteins of all developmental stages are examined but particular emphasis is placed on the larval cuticle and its proteins. Recombinant DNA techniques are used to clone some of the larval cuticle protein genes.

In this introduction the life cycle of the fly is detailed. In addition the ultrastructural and biochemical nature of the cuticle is reviewed.

The life history of *Lucilia cuprina*

The sheep blowfly, *Lucilia cuprina*, (Diptera, cyclorrhapha), is an economically important parasite of sheep. It is a myiasis-causing species i.e., the larvae infest living vertebrates. In fact this species is responsible for initiating the majority of cases of cutaneous myiasis in sheep (blowfly strike) (Colless and McAlpine, 1970) which, at present, costs the Australian agricultural industry around $100 million annually (R. Hart, pers. comm.)

*L. cuprina* is thought to have been introduced into Australia from South Africa or India in the 19th century (Waterhouse and Paramonov 1950). Originally the fly was a carrion feeder. However competition from native blowfly species for breeding space on carrion and the introduction and farming of a large sheep population in Australia probably combined to promote the myiasis-causing behaviour of *L. cuprina* (Foster and Whitten, 1974).

*L. cuprina* is a holometabulous insect, that is one which undergoes a complete metamorphosis; the juvenile form (the larva) transforms into the adult form during a pupal stage.
The life cycle of *L. euprina* is outlined in Fig. 1.1. Adult female flies oviposit on areas of the body of sheep (or other animals) where the wool is wet from rain, sweat or urine (Holdaway and Mulhearn, 1934). Such areas prevent the newly laid eggs from desiccating. *L. euprina* eggs hatch over a wide temperature range (10-40°C) as long as they do not dry out (Vogt and Woodburn, 1979). Whether the mouthparts of newly emerged 1st instar larvae are sufficiently developed to allow them to penetrate and survive on the skin of sheep or whether superficial damage of the skin by bacterial infection is essential for a successful fly strike is not clear. Larval activity and/or bacterial infection result in the production of a serous exudate in the fleece which provides a food source for the young larvae (Merritt and Watts, 1978).

Larvae moult twice *in situ* and during the 2nd and the early (feeding) phase of the 3rd instar larvae continue to feed and grow on the epidermis and dermis of the sheep. Skin lesions produced as a result of the activity of these older larvae not only destroy or decrease the quality of meat and wool but can also give rise to a toxaemia which may result in sheep death (Gibson *et al.*, 1984; Broadmeadow *et al.*, 1984). Larval activity also provides a suitable environment both for the promotion of further fly development and the attraction of other flies (both *L. euprina* and other species) to the area to lay their eggs.

Following the feeding phase larvae undertake a wandering phase during which they migrate from the feeding site. Wandering larvae may remain (without feeding) on their hosts for varying time periods before dropping off, a process which usually takes place between dusk and dawn. These larvae may further migrate on the ground but eventually
Fig. 1.1: The life cycle of the sheep blowfly, *Lucilia cuprina*.

- Egg
- 1st instar larva
- 2nd instar larva
- 3rd instar larva
- Adult (feeding phase)
- Pupa (within puparium)
- Puparium
- 3rd instar (wandering phase)
- 3rd instar (resting phase)
burrow into the soil where they assume a resting phase. Subsequently
the larval cuticle contracts, stiffens and darkens to form the
protective puparium. This process is known as pupariation. Within the
puparium the pupa forms (pupation) and metamorphosis ensues with the
eventual emergence of the adult. Adults emerge during the early
morning, breaking open the puparium and burrowing up through the soil.
They may remain at the surface for several hours, allowing the cuticle
to harden and the wings to expand. Adults need carbohydrate and water
to survive. In addition adult females require protein to become
sexually receptive and to successfully mature their eggs. Depending on
the ambient temperature females in the field can mature a batch of eggs
(potentially as many as 300) within 4-8 days. Field experiments suggest
that the longevity of adults is about two weeks (Vogt and Woodburn,
1979).

The Arthropod cuticle

Arthropods represent the largest and most diversified division of
the animal kingdom. The group includes insects, crustaceans and
chilicerates and its members are characterized by the possession of
metamerically segmented bodies, jointed appendages and a characteristic
outer covering - the cuticle. The arthropod cuticle is a multilamellate
secretion of the epidermal cell layer. The epidermis and cuticle
together constitute the integument. For reviews of integument form and
composition see Richards, (1951); Wigglesworth, (1972); Locke (1974);
Hackman, (1974b); Neville, (1975); Hepburn, (1976); Bereiter-Hahn et
al., (1984). Excepting specialized secretory and sensory regions the
cuticle covers all of the arthropod’s body including fore and hind gut
and the entire tracheal (respiratory) system (Neville, 1970).
As an exoskeleton the cuticle provides support for the arthropod. In addition to providing mechanical protection the cuticle also helps to protect arthropods from parasites and pathogens as well as other harmful external contaminants e.g., insecticides (Gilby, 1984). Cuticular architecture and/or the presence of pigment within the cuticle provide protection from predators by providing camouflaging colours or patterns for mimicry (Gnatzy and Romer, 1984). Cuticular colours can also provide protection against harmful ultraviolet irradiation as well as providing thermal benefits for some species by reflecting or absorbing sunlight (Hadley, 1984). In addition elements associated with the cuticle can act as signals between the individuals of a species (Emmens, 1981). The cuticle may have a number of specialized functions. For instance the mandibles (biting mouthparts) of some crustaceans are tipped with opal to increase hardness. The bombardier beetle has a special cuticle-lined chamber in which defensive, violently explosive reaction mixtures reaching 100°C are produced (Neville, 1978). The cuticle may also serve as a food reserve in times of starvation. The ability of the cuticle to restrict water loss has been of enormous evolutionary significance, enabling arthropods to invade and survive in dry terrestrial environments (Waterhouse and Norris, 1980).

In many respects arthropods represent the most successful animal group on earth. Over 75% of animal species are arthropods. The worldwide total has been conservatively estimated at 2-3 million species and more recent estimates put the total at as many as 30 million (Erwin, 1982). In view of this huge number of species and the diversity of their habitats it must be recognized that the notion of a generalized arthropod cuticle is as mythical as that of a generalized cell. Nevertheless on the basis of microscopic examination the cuticles of the
vast majority of species examined can be divided into two main regions, the *epicuticle* and the *procuticle* (Fig. 1.2). The outer region, the *epicuticle*, ranges from 0.03-4 µm in thickness and is characterized by the apparent absence of the polysaccharide chitin. The inner region, the *procuticle*, varies from 10-200 µm in thickness and is composed largely of water, chitin and protein.

The *epicuticle*

While it is difficult to reconcile the somewhat different images of the cuticle obtained by light compared with electron microscopy, the *epicuticle* is generally accepted as being composed of at least four layers (Andersen, 1979). These are, from the outside: the cement, wax, cuticulin and dense layer (inner *epicuticle*).

The *cement* is secreted by dermal glands which are derived from epidermal cells. As the outermost layer the cement may determine the surface properties of the cuticle and is considered, in some arthropods, to form a layer which protects the underlying epicuticular structures. In some cases cement may consist of cross-linked (sclerotized) protein, in others a shellac-like substance (shellac being a mixture of laccose and lipid) (Locke, 1974).

The *wax* layer consists of long chain hydrocarbons and the esters of fatty acids and alcohols. It provides a waterproofing layer for the arthropod and has been associated with the resistance of certain insects to fungal attack (Locke, 1974).

*L. cuprina* larvae lack dermal glands and both cement and wax layers are not detected. In their place a further two morphologically distinct layers - the *superficial layer* and the *outer epicuticle* - are found (Fig. 1.2). The composition of these layers is unknown, but like the cement and wax layers of other arthropods they are secreted above the
Fig. 1.2: The structure of the larval integument of *L. cuprina*. (Left: Electron micrograph; Right: Diagrammatic representation). Inset: detail of epicuticle.
cuticulin layer prior to the shedding of the old cuticle during the moulting cycle (see later). They may serve similar functions as the cement and wax of other organisms (Filshie, 1970).

The cuticulin layer often has a trilaminar appearance (Locke, 1966; Neville, 1975) and may be composed of lipoprotein (Romoser, 1973). As it is the first layer to be secreted in the cycle of cuticle formation this layer determines the surface patterning of the cuticle. It also functions as an important permeability barrier (Locke, 1974).

Below the cuticulin lies a thicker and more homogeneous layer, known as the dense layer (inner epicuticle). This layer may be lamellate and contains highly stabilized protein, lipid and phenolic substances (Weis-Fogh, 1970).

The procuticle

A series of subdivisions of the procuticle can often be recognised by histochemical means (Fig. 1.3a). An outer, chemically stabilized region, adjacent to the epicuticle is known as the exocuticle (Wigglesworth, 1972). The stabilization process, known as sclerotization or tanning, involves the cross-linking of the protein molecules of the region through metabolic derivatives of the amino acid tyrosine and water loss. By contrast the proteins of the inner procuticular region (the endocuticle) are not sclerotized. In certain arthropods (e.g., crustaceans) the procuticle may be strengthened by deposits of calcium carbonate.

Interposed between the exocuticle and endocuticle in some arthropods lies a histochemically distinct zone known as the mesocuticle (Locke, 1974). Whether this zone is chemically distinct from other procuticular regions or is a physically modified form of one of the regions is unknown.

The morphologically distinct cuticular zone immediately adjacent to
Fig. 1.3: Diagrammatic representation of a) the structure of the arthropod cuticle (showing subdivisions of the procuticle) and b) the helacoidal arrangement of chitin microfibril sheets to form a lamella.
the epidermis is called the subcuticle or Schmidt's layer. This is thought to be the region into which the endocuticular components are secreted and which are yet to be ordered.

In many arthropods there exists a system of fine channels known as pore canals which extend through the entire cuticle. At the junction of the epicuticle the cansals frequently branch and become finer. Here they may be known as wax canals. The pore and wax canals are assumed to be important in the transport of material from the epidermis through the cuticle. In L. cuprina larvae a series of 'filaments' which extend from the epidermis to the outer epicuticle probably carry out the same function (Filshie, 1970) (Fig. 1.2). Both in the case of Sarcophaga falculata and L. cuprina larvae such 'filaments' are not formed in the inner lamellae of the endocuticle (Dennell, 1946). Pore canals vary in size from one arthropod to another and may be apparently empty or may contain cellular extensions or rods of cuticular material.

The moulting cycle

The life of an arthropod is punctuated by the periodic secretion of a new and the shedding of an old cuticle. The sequence of events leading to the shedding of the old cuticle is known as moulting, while the actual process of shedding is called ecdysis (Zacharuk, 1976). In L. cuprina a new cuticle is secreted under hormonal influence prior to each of four post embryonic molts. Thus five separate cuticles, one for each of three larval instars, plus a pupal and an adult cuticle are formed during the life cycle of this insect.

The exact form of the cuticle differs between different arthropod species, at different developmental times in the life of an animal and in different regions of an individual (Noble Nesbitt, 1963a, b; Kayser-Wegmann, 1976; Bordereau, 1982). For instance, unlike adult flies, the
procuticle of *L. cuprina* larvae is completely unsclerotized (Filshie, 1970). Therefore the three subdivisions (exocuticle, mesocuticle and endocuticle) of the procuticle are not recognized (compare Figs. 1.2 and 1.3a).

Cholesterol derivatives (ecdysteroid hormones) and a series of sesquiterpenoids (the juvenile hormones) appear to play a central controlling role in the moulting process and, classically, the nature of the moult is seen as being determined by the balance between the levels of these hormones (Richards, 1981). Relatively high ecdysteroid and juvenile hormone concentrations in larvae ensure the secretion of further larval cuticles. When the juvenile hormone titre drops metamorphosis ensues with the formation of pupal and adult cuticles (Gnatzy and Romer, 1984; Riddiford and Truman, 1978).

The onset of moulting is usually first indicated by an increase in the volume of epidermal cells or in the number of epidermal mitoses (Richards, 1951; Wolfe, 1954). Subsequently a separation of the cuticle from the epidermis (apolysis) occurs and a space—the exuvial space—develops between epidermis and cuticle. It is presumed that both apolysis and the formation of the exuvial space are the result of the secretion of epidermal moulting fluids. Such secretions contain proteolytic enzymes and chitinases which, when activated, digest the procuticle (Noble-Nesbitt, 1963b). The extent of degradation of the procuticular layers varies in different species but it is generally believed that only the endocuticle is digested with the remaining layers of the cuticle (constituting the exuvium) being shed (Gnatzy and Romer, 1984).

Typically the secretion of the new epicuticle begins soon after apolysis. The cuticulin and the inner epicuticle are formed first as
patches over the tips of epidermal projections (microvilli). Material is added to the edges of these patches so that they merge and form a continuous sheet (Gnatzy and Romer, 1984). Through these layers the products of digestion of the old endocuticle are resorbed. Based on ultrastructural observations Filshie (1970) suggests that little resorption of endocuticular components occurs during the final larval moult of *L. cuprina*. During the moulting cycle of some arthropods (but not *L. cuprina* larvae) a thin, chemically stabilized membrane, apparently derived from the inner layers of the old procuticle, is noted. The structure is known as the ecdysial membrane and is shed at ecdysis (Zacharuk, 1976; Filshie, 1970). Following the formation of the cuticulin and inner epicuticle the first layers of the new procuticle are laid down and shortly thereafter the wax and cement (or outer epicuticle and superficial layers) are secreted onto the surface of the cuticulin (Zacharuk, 1976). The animal then sheds the exuvium (i.e., ecdysis occurs) exposing the new cuticle. Material continues to be deposited into the new cuticle for at least part of the intermoult period. Oenocytes (cells derived from, and often situated just below, the epidermis) may play some ill-defined role in the moulting cycle, e.g., in epicuticle secretion (Wolfe, 1954; Wigglesworth, 1972).

The biochemistry of the insect procuticle

Chitin

The polysaccharide chitin is the most abundant, organic skeletal component of invertebrates and usually makes up 20-50% of the dry insect cuticle (Andersen, 1979). Chitin is a colourless, amorphous solid, insoluble in water, all organic solvents, alcohol or dilute acids and alkalis. It is soluble in concentrated mineral acids but undergoes extensive, rapid degradation. X-ray diffraction studies suggest that
three distinct crystallographic types of chitin exist. Of the three (α, β and γ) only α-chitin (the most stable form) occurs in insect cuticles (Richards, 1978).

Chitin consists of long, linear polymers of N-acetyl-D-glucosamine residues bonded through β1-4 glycosidic linkages (Fig. 1.4). Every sixth residue along the chitin chain may not be acetylated. Adjacent chitin chains associate together to form highly stable assemblies. This occurs in the absence of interchain covalent bonds, with a large number of inter- and intrachain hydrogen (H) bonds holding the chains together (Neville, 1984). For example, since the β1-4 glycosidic link is bent, the formation of an H-bond between the ring oxygen of one residue and the oxygen of the 3-hydroxy group on the next is allowed (Fig. 1.4b). Inter chain H-bonds exist between the C=O of one chain and the NH of a neighbour. Chitin chains linked together in this way form microfibrils varying from 2.8 - 5.8 nm in diameter depending on the species examined (Filshie, 1982). The length of chitin chains and the distance separating microfibrils in various species also appear to be variable.

Observations using electron microscopy suggest that chitin microfibrils are bound to and surrounded by protein (Rudall, 1965; Filshie, 1982). Presumably only chitin chains on the periphery of a microfibril bind to protein, the remaining proteins may complex with each other or with other cuticular components to varying degrees. The site along a chitin chain at which there is a linkage with protein has yet to be clarified. In some instances it appears that microfibrils may contain some protein and may not be composed solely of chitin (Filshie, 1982).
Fig. 1.4: The structure of chitin - a linear polymer of N acetyl glucosamine residues (a),(b) indicates moieties involved in intra, (O3-O5), and inter, chain hydrogen bonding. Carbon; Oxygen; Nitrogen.
Protein

Protein can comprise up to 50% of the dry weight of the insect cuticle, and, as such, represents the second major, organic component of cuticle (Richards, 1978). There is some debate as to whether the proteins contained within the cuticle are synthesised only in the epidermis or whether they originate elsewhere and are taken up by the epidermis from the haemolymph to be deposited into the cuticle (Weis-Fogh, 1970; Fox et al., 1972; Koepppe and Gilbert, 1973).

When examined by electrophoresis or, on the basis of solubility, proteins extracted from isolated cuticles are found to represent a heterogeneous collection (Andersen, 1979; Hackman, 1974b). As many as 50 cuticle polypeptides are separable electrophoretically, of which usually less than 10 make up the major proportion. It is not known to what extent the multiplicity of cuticle proteins represents protein polymorphism, secondary modifications of a limited number of 'core' polypeptides or artifacts and how much is real.

The major soluble cuticle proteins from a variety of insects share certain properties (Hackman, 1974b; Neville, 1975); they are generally of relatively low molecular weight (< 30 Kd) and low isoelectric point (pI 3-6). Amino acid analysis of cuticle protein extracts suggest that cuticle proteins contain many amino acids with bulky side chains (aspartic acid, glutamic acid and proline) in contrast to those of fibrous proteins. Cuticle proteins also appear to be low in sulphur containing amino acids.

Most cuticle proteins are reportedly structural. However numerous enzyme activities are also exhibited within cuticles, e.g., the activity of moulting fluid enzymes or enzymes involved in the cross-linking of structural cuticle proteins (see Chapter 3). Ordinarily enzymes are
thought to constitute a small proportion of the total cuticular protein (Hackman, 1974b; Richards, 1978). The conformation of proteins within insect cuticles is not known.

**Molecular organization of the procuticle**

The orientation of the chitin-protein microfibrillar complexes found within the procuticle takes place outside the epidermis, presumably in the subcuticle. Whether the epidermis directly controls this orientation or whether the components of the cuticle spontaneously self-assemble is not known (Neville, 1975; Filshie, 1980).

Cuticular microfibrils can be organized in various patterns. For instance sheets of microfibrils may be laid down in which each successive sheet is at a slight angle to its neighbour, with the angle changing progressively in one direction to form a helicoidal arrangement (Neville, 1970; 1984) (Fig. 1.3b). This arrangement is resolved by light microscopy as a series of lamellae where each lamella represents a 180° twist of the helicoid. The cuticles of *L. eupr>ina* larvae exhibit this architecture. Neville and Luke (1969) found that in *Hydrocya>rus colombia* (Hemiptera) each lamella is composed of 22-25 layers of microfibrils representing an average rotation of 7-8° per layer. Whether each layer represents a separate microfibril sheet or whether each layer is continuous with its predecessor is unresolved.

In locust endocuticle lamellae are only produced during periods of darkness. Under the influence of light all the microfibrils assume a common, preferred direction such that the cuticle is non-lamellate (Neville, 1963, 1970). Temperature appears to influence microfibril orientation in *L. eupr>ina* adults (Tyndale-Biscoe and Kitching, 1974).

The insect procuticle behaves as a two phase composite material since it consists of a substance of high tensile strength (chitin
microfibrils) in a matrix of lower tensile strength but higher elasticity (protein). The cuticle often contains appreciable amounts of water and this water appears to be necessary to reduce cuticular brittleness. These features make the cuticle a light yet very strong structure and well adapted to function (Neville, 1970; Hillerton, 1984).

Other, minor components of some cuticles include various pigments, some sugars (e.g., glucose, mannose, arabinose) and a low level of inorganic material (e.g., iron, zinc) (Richards, 1978; Hackman, 1974a, b; 1984).

Studies on the cuticle: scope of this thesis

The arthropod cuticle is the subject of much study at present, not only because of the proposed fundamental importance of this structure in the evolutionary success of this group of animals but also because of the central role the cuticle plays in the biology of each arthropod. This central role exists because a great deal of the physiology and behaviour of arthropods is geared toward the cycle of cuticle renewal (e.g., Cameron, 1985).

At a more immediately practical level, knowledge of cuticle organization and composition may be of benefit in the control of arthropod parasites. An understanding of the structure of the cuticle is basic to the interpretation of the penetration of the cuticle by chemical or biological controlling agents, e.g., insecticides or pathogenic fungi. Indeed aspects of cuticle organization have been associated with resistance to certain insecticides (Ebling, 1974; Gilby, 1984). In addition, the cuticle, having no equivalent in vertebrates, represents an invertebrate-specific target for certain insecticides.

Since protein is one major component of the cuticle about which detailed information is available for only a few species, the first
section of this thesis (chapters 2 and 3) aims at providing fundamental information about the major proteins contained within the various cuticles of *L. cuprina*. The number, nature and interrelatedness of the various proteins are examined. In addition the effects of several insecticides (some possessing the invertebrate-specificity mentioned above) on the larval cuticle and its proteins are investigated.

An additional aspect of this study involves an investigation of the immunological interaction between fly larvae and sheep. An immunological approach to fly strike prevention has been suggested in recent years. The larval cuticle of *L. cuprina* is in intimate contact with the host's immune system and is shed *in situ*. Since an immune response is mounted by sheep against the larvae (O'Donnell *et al*., 1980; Sandeman *et al*., 1985) this section (Chapter 4) examines whether the cuticle proteins act as stimulators of this response. In addition several other larval extracts (salivary gland, mid gut, haemolymph) are examined as possible sources of antigenic stimulation. In this way components of larval extracts against which a strong antibody reaction is mounted by struck sheep are identified.

The changing pattern of cuticle proteins secreted by epidermal cells during the development of *L. cuprina* suggest that here exists a useful model system for studying developmental gene regulation and, in particular, the effects of hormonal interaction on gene expression. In the final section of this thesis (Chapter 5) a number of genes coding for some of the major larval cuticle proteins are cloned and partially characterized using recombinant DNA techniques. Furthermore, since it appears that recombinant DNA techniques may be used in future parasite control strategies (Cockburn *et al*., 1984), the study of cuticle protein (or other) genes of the blowfly will provide fundamental information on the nature of the genome of this important parasite.
CHAPTER TWO

THE LARVAL CUTICLE AND CUTICLE PROTEINS OF \textit{L. CUPRINA}
INTRODUCTION

Myiasis-causing larvae of *L. cuprina* are directly responsible for the distress and damage caused to sheep and most emphasis in this study is directed toward the larval stages of the life cycle of the parasite.

**Larval growth and the larval cuticle**

The larvae are of the acephalic 'maggot' type which increase in size from 1-2mm in length during the 1st instar to approximately 5mm during the 2nd instar, to around 10mm during the 3rd instar. Larval growth, measured as an increase in live weight, is continuous and is not interrupted by the larval moults (Turnbull and Howells, 1980). Most growth occurs during the 2nd and the feeding phase of the 3rd instar, when larval weight increases more than 10 fold. Cuticles of increasing size are produced to accommodate this weight increase. Once the larvae cease feeding, live weight, understandably, decreases somewhat.

The cuticles of the three larval instars are similar in gross appearance. Externally the body of each animal can be divided into two morphologically distinct regions; regions possessing spines and regions of smooth (spineless) cuticle (Fig. 2.1). The spines are directed posteriorly and are clustered in irregular rows sequentially along the larvae. On the ventral surface they are elaborated into 'creeping welts' which function in locomotion (Hinton, 1955). Spines may be important in physically irritating and abrading sheep skin to create favourable conditions for feeding. In the anterior region the spines encircle the body. Toward the posterior progressively fewer spines are apparent on the dorsal surface. The anterior dorsal spines are presumably important for burrowing both in skin lesions and into the soil (during the wandering phase of 3rd instar life).

The cuticles of the various instars differ in epicuticular
Fig. 2.1: Scanning electron micrographs of a 3\textsuperscript{rd} instar *L. cuprina* larva (X 19)(a), showing the division of the body into spine-containing and smooth cuticular regions (X 26)(b), and detail of the spines (X 53)(c). (Note that the cuticle surrounding each spine is smooth).
ultrastructure; the superficial and outer epicuticular layers (Fig. 1.2)
are increased greatly in thickness in older larvae (Filshie, 1970). The
larval procuticle is lamellate as a result of the helicoidal
arrangement of chitin microfibrils. As in other arthropods the
procuticle is composed largely of water, chitin and protein. Chitin
constitutes 53% and protein 38% of the dry 3rd instar larval cuticle
(resting phase) (Hackman and Goldberg, 1971).

The cuticle proteins

This study is concerned with the cuticle proteins of *L. cuprina*. Only in the case of two other insect species have the cuticle proteins of a series of larval instars been examined.

In *Drosophila melanogaster* (Diptera), nine major proteins are extracted from the cuticles of each of the three larval instars. Electrophoretic studies of the extracted proteins demonstrate that the cuticles of 1st and 2nd instar animals contain the same nine major proteins and these differ from the cuticle proteins of the 3rd larval instar. The major proteins of all instars are less than 25,000 molecular weight (Chihara et al., 1982). In the case of *Tenebrio molitor* (Coleoptera) about ten major proteins are extracted from isolated cuticles although, in total, 23-38 major and minor polypeptides are detected. The electrophoretic profiles of the proteins from the three larval instars examined are the same. Again the molecular weights of the major proteins are less than 25,000 (Roberts and Willis, 1980).

Proteins extracted from isolated cuticles are assumed to be derived, largely, from the procuticle. Epicuticular proteins appear to be tightly bound and are lost, with the exuvium, at ecdysis. Certainly in *L. cuprina* larvae the cross-linking of certain epicuticular proteins (rendering them inextractable) appears to be essential for normal
epicuticle function (Turnbull et al., 1980). Moreover proteins extracted from the relatively thin epicuticle are likely to be present in relatively low amounts.

The cuticle proteins of the three larval instars of *L. euprina* are examined in this chapter. The number, nature and organization of the proteins as well as their interrelationships (both within and between instars) is investigated. Attempts are made to correlate changes in the protein composition of the cuticle during larval development with other changes occurring both within and outside the cuticle. The proteins of the spine containing and the smooth cuticular regions are compared electrophoretically and the site of synthesis of the larval cuticle proteins is looked at immunologically.

**Larvicides which affect the cuticle**

The use of insecticides is an important aspect of fly control and is likely to remain so for the foreseeable future. For some years an attractive potential target for insecticidal action has been chitin since it is not found in vertebrates and higher plants but is present in insects, other invertebrates and fungi. Therefore agents which affect chitin, or its metabolites, may be selectively toxic to these organisms. Among insecticides currently in use is the benzoylphenyl urea Diflubenzuron (Dimilin) (Fig. 2.2). This compound fulfils the above requirement since one of its major effects is to disrupt chitin biosynthesis (Post and Vincent, 1973; Verloop and Ferrell, 1977; Hajjar and Casida, 1978, 1979). Diflubenzuron may interfere with the enzyme chitin synthetase (EC24.1.16) which is involved in the final stages of chitin synthesis and is responsible for the linking of glucose derivatives (in the form of UDP-N-acetylglucosamine) to the growing chitin chain. A diagrammatic representation of a proposed pathway for
chitin biosynthesis in insects is given in Fig. 2.2. Whether diflubenzuron interferes in the proteolytic activation of the chitin synthetase zymogen, blocks chitin polymerization, disrupts the transport of UDP-N-acetylglucosamine or acts by some other means is unclear (Gijswijt et al., 1979; Leighton et al., 1981; Mayer et al., 1981; Mitsui et al., 1984).

The cuticle ultrastructure of diflubenzuron-treated larvae of *L. cuprina* is abnormal. The procuticle is not lamellate and contains globular bodies. A distinct subcuticle cannot be distinguished (Turnbull et al., 1980, Binnington, 1985). In addition numerous infoldings of the epicuticle are apparent (Binnington, 1985).

The fungicide Polyoxin D, a structural analogue of UDP-N-acetylglucosamine (Fig. 2.2), like diflubenzuron, disrupts chitin synthesis. This compound appears to act as a competitive inhibitor of chitin synthetase (Marks and Sowa, 1974; Gijswijt et al., 1979). Polyoxin D disrupts the form of the larval cuticle of *L. cuprina* in a manner similar to that of diflubenzuron. The cuticle of Polyoxin-D treated larvae is non-lamellate and lacks a distinct subcuticle. Additionally the epicuticle is greatly disrupted; it appears discontinuous and contains a thinner than usual dense layer (Binnington, 1985).

A 3rd compound, the triazine cyromazine (Vetrazin) (Fig. 2.3), is also currently in use as a blowfly insecticide (Hart et al., 1979). This compound has been shown to disrupt the structure of the larval cuticle. While the lamellate appearance of the cuticle and the form of the epicuticle appear normal in cyromazine-treated larvae, epidermal cell processes are seen to invade the procuticle. Dense deposits continuous with these invasive processes are detected and give rise to
Fig. 2.2: Postulated pathway for chitin biosynthesis and the structures of two chitin synthesis inhibitors, both of which disrupt (by different means) the final step of the pathway.
Fig. 2.3: An aspect of folic acid metabolism and the structures of two compounds thought to disrupt (by different means) this cycle.

Thymidylate synthetase

\[ \text{dUMP} \rightarrow \text{dTMP} \]

Methylene tetrahydrofolate

\[ \text{dUMP} \rightarrow \text{tetrahydrofolate} \]

Tetrahydrofolate

\[ \text{tetrahydrofolate} \rightarrow \text{Dihydrofolate} \]

\[ \text{Dihydrofolate} \rightarrow \text{Dihydrofolate reductase} \]

\[ \text{Dihydrofolate reductase} \rightarrow \text{NADP}^+ \text{NADPH} \]

Cyromazine

5-Fluorouracil
the appearance of macroscopic patches of brown/black cuticle (Binnington, 1985).

The mode of action of cyromazine is unknown. One suggestion is that the compound inhibits the enzyme dihydrofolate reductase (EC 1.5.1.3). This notion has arisen since this is also the likely mode of action of aminopterin and cuticles obtained from cyromazine and aminopterin-treated larvae have ultrastructural similarities (Binnington, 1985). Compounds which block dihydrofolate reductase cut off the supply of tetrahydrofolic acid which is essential for the production of DNA (Fig. 2.3). 5-Fluorouracil (Fig. 2.3), by inhibiting the enzyme thymidylate synthetase (EC 2.1.1.45), can also exert this effect (Fig. 2.3).

The effects of diflubenzuron, polyoxin-D, cyromazine and 5-fluorouracil on the larval cuticle and larval cuticle proteins of *L. cuprina* are examined in this chapter.
MATERIALS AND METHODS

Chemicals

All electrophoresis reagents were obtained from Bio Rad. Purified Sodium dodecyl sulphate (SDS) was obtained from BDH Biochemicals and urea from Schwarz Mann Inc., N.Y. Diflubenzuron (1-4-chlorophenyl)-3-(2,6-difluorobenzoyl)-urea) was obtained from Philips-Duphar, Amsterdam; Polyoxin D from Kaken Chemical Co., Tokyo; Cyromazine (2-cyclopropylamino-4, 6-diamino-5-triazine) from Ciba-Geigy, Australia and 5 fluorouracil from Sigma Chemical Co., USA.

Insect cultures

L. cuprina stocks, obtained from the Division of Entomology, CSIRO, Canberra were maintained at 27°C under the influence of a 12:12 h light:dark photoperiod. The animals were fed on sheep liver.

Timing of larval development commenced with egg laying which occurred over a two hour period. The times of larval moults were determined by examining the posterior spiracles as described by Williams (1975). At 27°C the larvae emerge from the eggs approximately 12 h post egg laying. The first moult occurs at about 28 h post egg laying and the second at approximately 46 h. The 3rd instar lasts a further 66 h and can be divided into three phases of development based on behavioural and physiological characteristics. During the feeding phase of the 3rd instar, lasting approximately 22 h, (until 68 h post egg laying) the larvae are located on the food. The larvae then enter a wandering phase during which they migrate from the food. This phase equates with the 'crop full' stage of Sarcophaga falcultata (Diptera) (Dennell, 1946). After a further 16 h (by 84 h post egg laying) the larvae are no longer active, the crop is empty and the larvae, having buried themselves, assume a resting phase. Pupariation occurs approximately 112 h after
egg laying.

A line of *L. cuprina* which has been maintained in the laboratory for more than 500 generations (30 years) was used in all experiments unless otherwise stated. Canton S stocks of *D. melanogaster* were also maintained at 25°C under the same photoperiod. Larvae were fed on a standard Drosophila food consisting essentially of agar, yeast and sugars.

**Preparation of larval cuticles and extraction of proteins**

Cuticles were isolated 2 ± 1 h after larval entry into each instar or each phase of the 3rd instar (early stage). Cuticles were also isolated in the middle of each instar or phase (mid stage) and 2 ± 1 h before entry into the next instar or phase (late stage).

The larvae were washed three times in cold distilled water and blotted dry. Each larva was then placed on a microscope slide and the anterior region (at the junction of the second and third segments) removed using a scalpel. The larva was everted on a dissecting needle and the viscera removed. The integument was then scraped under 70% ethanol with a scalpel and needle to remove body wall musculature and the underlying epidermis. Finally the scraped cuticle was rinsed twice in 70% ethanol and blotted dry. The resulting cuticle preparations were free of contaminating tissue when examined under a dissecting microscope. The procedure is based on that described by Hackman and Goldberg (1971).

To extract protein the cuticles were immersed in distilled water or 7M urea or 2% SDS at room temperature routinely for 30 min. Cuticles were also immersed in a 2% SDS solution for various time periods. 100 µl of solution was used per 50 first instar larval cuticles or per 15 second instar larval cuticles; 300 µl was used per third instar
larval cuticle. Cuticles were isolated from D. melanogaster larvae at various times during the 3rd instar and protein extracted by the same technique. To obtain an extract of non-cuticular material the internal tissues and haemolymph of a number of L. cuprina larvae were homogenized in water (100 µl/animal) at 4°C. The protein content of extracts was determined by the method of Lowry et al. (1951) using bovine serum albumin (fraction 5) as standard.

Protein extracts were either used immediately for electrophoresis or were frozen and stored at -20°C prior to use. The protein concentration of extracts was adjusted to 1 mg/ml and then routinely 20-40 µl samples were applied per lane to polyacrylamide gels. For comparative purposes the same amount of protein was applied to each lane of any given gel.

Gel electrophoresis and sample preparation

Vertical slab gel electrophoresis was carried out using the following systems:

(1) SDS-discontinuous (disc) gradient gels. SDS-disc gel electrophoresis was performed by the method of Laemmli (1970) in 10 - 20% polyacrylamide slab gels. Routinely samples in SDS/mercaptoethanol were boiled for 5 min before electrophoresis. However, to assess the importance of disulphide bonds in the structure of the major cuticle proteins, some samples were prepared without mercaptoethanol. Molecular weight marker proteins were obtained as high and low molecular weight calibration kits from Pharmacia. Samples were electrophoresed at 20 mA until the tracking dye entered the resolving gel and then at 40 mA until the dye was within 1 cm of the base of the gel.

(2) Native (non-denaturing) gradient gels. 10 - 20% gradient polyacrylamide slab gels were formed in 0.075 M Tris-citrate buffer (pH
8.6). The upper and lower buffer reservoirs were filled with 0.3 M boric acid adjusted to pH 8.6 with 5 M NaOH. Samples were electrophoresed at 20 mA until the tracking dye entered the gel and then at 40 mA until the dye was within 1 cm of the base of the gel. The method is essentially that described by Fristrom et al. (1978).

Cuticle proteins extracted with SDS were dialysed against distilled water for 16 h at 4°C before application to native gradient gels.

(3) 2-D electrophoresis. Samples were first separated on a native gradient gel. The gel was then cut into strips corresponding to the various lanes and the strips were applied to an SDS-disc gradient gel for electrophoresis in the second dimension as follows: The strips were soaked for 1.5 hours in stacking gel buffer and were then positioned on a layer of 1% agarose (in stacking gel buffer) which had been applied above the stacking gel. This ensured firm and even contact between the native gradient gel strip and the SDS-disc gradient gel.

Protein staining and quantitation

Gels were stained on a shaking platform for 1.5 h with Coomassie blue R (2.5%) in methanol:acetic acid:water (9:2:9) and destained for 16 h in 2-3 changes of a solution of ethanol:acetic acid:water (12:5:35). The relative amount of each stained protein band was estimated by scanning stained gel strips (in 7% acetic acid) using an RFT (Transdyne) 2955 densitometer.

To test for bound carbohydrate the Periodic acid-Schiffs reagent stain was carried out as described by Fairbanks et al. (1971).

Larval and cuticle weights

To determine the mean wet weight of the mid resting phase 3rd instar larval cuticle the anterior ends of a number of larvae were removed as previously described. The internal tissues were rapidly
extracted by applying pressure to the cuticle without inverting the carcass. The dissected anterior was scraped clean and both cuticular fragments were placed in a preweighed eppendorf tube. Cuticle wet weight was then determined by reweighing. The cuticles were subsequently examined under 70% ethanol using a dissecting microscope and only the weights of those seen to be free of contamination by internal tissues used in the calculation of wet weight (7 of 14).

Dry weights of cuticles from 2nd instar larvae (in groups of 15) and 3rd instar larvae (singly) were determined by freeze drying to a constant weight. The dry weights of whole larvae were determined in a similar manner. Replicates of five determinations were performed for each developmental stage.

Preparation of antisera

1. Rabbit

Proteins were extracted from mid resting phase 3rd instar larval cuticles with water. 1 mg of this extract was injected as a stable water-in-oil emulsion subcutaneously into the dorsal region of a New Zealand White rabbit. Freunds Complete Adjuvant (FCA) was used in this preparation in the ratio 1 part FCA:1 part cuticle extract. 20 days later this procedure was repeated. After a further 7 days blood was collected from the marginal vein of the ear. The antiserum raised in this way, which is directed against a total extract of 3rd instar larval cuticle proteins, is designated 'Rabbit anti 3LCP'.

2. Mouse

750 µg of a larval cuticle protein extract was injected intraperitoneally into each of 3 mice (WEHI outbred strain) in the manner described above for the rabbit. Blood was collected from the tail vein of each mouse. This antiserum, directed against a total
extract of 3rd instar larval cuticle proteins, is designated 'Mouse anti 3LCP'.

A single larval cuticle protein (denoted LCP 9) was purified electrophoretically as follows: Cuticle protein extracts were separated on a series of native gradient gels. The region of each gel containing cuticle protein 9 was cut out and fragmented. The protein was extracted from the gel fragments electrophoretically using 0.3M borate buffer (pH 8.6) in an ISCO sample concentrator (3 watts for 2.5 h; 97% recovery of a known amount of bovine serum albumin was obtained with this method). Two 200 μg samples of isolated LCP 9, were injected into a single mouse at 20 day intervals as described above. This antiserum, raised by the injection of only LCP 9 is designated 'Mouse anti LCP 9'. Control mice were uninjected.

**Western blotting**

Transfer of proteins from native gels onto nitrocellulose was undertaken in a 25 mM Tris, 192 mM glycine (pH 8.3) buffer using a Bio Rad electrophoresis blotter at 40 V for 16 h (room temperature). Blotting of proteins from SDS disc gels onto nitrocellulose was undertaken in the same manner in a 25 mM Tris, 192 mM glycine, 20% methanol buffer (pH 8.3).

**Immunological assays**

Protein samples were separated electrophoretically, Western blotted onto nitrocellulose sheets which were then incubated in a 1:200 dilution in Tris-saline (10mM Tris-HCl, pH 7.4, 0.9% NaCl) of an appropriate antiserum. Sheets incubated in the presence of mouse antisera were subsequently incubated in a 1:600 dilution (in Tris-saline, pH 7.4) of an IgG fraction of rabbit-anti mouse immunoglobulin G (Miles Yeda, Ltd.). With Rabbit anti 3LCP it was unnecessary to use a second
antiserum. The presence of bound antibody was then detected using Staphylococcus aureus protein A radiolabelled with $^{125}\text{I}$ as described by Erlich et al. (1979). This procedure is essentially that described by Burnette (1981). Nitrocellulose sheets were exposed to X-ray film at $-45^\circ\text{C}$ in Cronex X-ray cassettes fitted with lightening plus intensifying screens.

**Electron microscopy**

*L. cuprina* 3rd instar larvae were prepared for scanning electron microscopy by members of the Electron Microscopy Unit, Australian National University. Larvae were freeze dried on a cold stage freeze drier, mounted on stubs and coated in an evaporative coating unit with 20nm of gold. Specimens were examined at an accelerating voltage of 30KV, in the secondary electron mode using a Cambridge Stereoscan 180.

**Insect culture in the presence of larvicides**

Recently emerged 3rd instar larvae were placed on an artificial feeding medium made as described by Brust and Fraenkel (1955) which contained either the test chemical or the solvent used for dissolving the chemical. Larvae were obtained from Dr. K.C. Binnington, CSIRO Division of Entomology, Canberra, and were maintained at 25$^\circ\text{C}$ for 12 h prior to examination.

**Extraction of protein from cuticles of larvicide-treated larvae**

Cuticles were hand-dissected and scraped clean of contaminating tissue using a scalpel and needle under a dissecting microscope. To ensure that this standard method of cuticle preparation did not damage the cuticles of larvicide-treated larvae relative to controls, a series of cuticles were isolated as follows: On a microscope slide each larva was immobilised on ice and its anterior end removed with a scalpel. The larva was then inverted using a needle and forceps and the internal
tissues removed by gently washing the carcass in water. Protein from the resulting cuticle (contaminated with muscle and epidermal tissue) was then extracted with 7M urea as described earlier (p 21).
RESULTS

1. The cuticle proteins of mid resting phase third instar larvae

(a) Extraction and quantitation

Considerable amounts of protein are extracted from isolated cuticles by simply immersing them in water (see Fig. 2.4a). The amount extracted can be increased almost three-fold by using either 7M urea or 2% SDS to represent approximately 31% of the dry weight of the cuticle. Homogenizing cuticles in SDS results in the release of significantly more protein than immersion in either urea (p < 0.01, Student's t test for unpaired samples) or SDS (p < 0.02).

Within 30 min. of incubating isolated cuticles in a 2% SDS solution all extractable protein is removed (Table 2.1). Significantly more protein is released by this time compared with a 15 minute incubation (p < 0.02). Retaining cuticles in SDS for longer than 30 min. does not result in the extraction of significantly more protein.

(b) Properties of extracted cuticle proteins

The same major protein bands, in the same relative proportions, are extracted by all four extraction procedures (Fig. 2.4a). On native gradient polyacrylamide gels nine major protein bands are resolved (numbered 1-9 Fig. 2.4a). These proteins are anionic and similarly charged at this separating pH (8.6) since a single protein cluster is seen when the cuticle protein extract is separated on a 7.5% native gel (Fig. 2.4b). The degree of resolution varies somewhat from gel to gel. Proteins 4, 5 and 6 resolved clearly in this experiment. In others (see Fig. 2.6, R3) they did not. On SDS gradient gels two clusters of cuticle proteins, comprising about eight prominent protein bands, are resolved (i.e., Fig. 2.4c, CP). The major cuticle proteins are all of relatively low molecular weight. One cluster of five bands
Table 2.1: Amount of protein extracted by incubating cuticles in a 2% SDS solution for varying time periods.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Amount of protein extracted (µg/cuticle ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>284 ± 69</td>
</tr>
<tr>
<td>30</td>
<td>543 ± 105</td>
</tr>
<tr>
<td>60</td>
<td>534 ± 69</td>
</tr>
<tr>
<td>120</td>
<td>591 ± 76</td>
</tr>
<tr>
<td>1440</td>
<td>606 ± 60</td>
</tr>
</tbody>
</table>
Fig. 2.4: Electrophoretic profiles of proteins extracted from mid resting phase 3rd instar larval cuticles.

a) Amount of protein extracted by different procedures and profiles of the extracts on a native gradient gel.

<table>
<thead>
<tr>
<th>Extraction procedure</th>
<th>Immersion in water</th>
<th>Immersion in 7M urea</th>
<th>Immersion in 2% SDS</th>
<th>Homogenization in 2% SDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of protein extracted (µg/cuticle) ± SD</td>
<td>180 ± 60</td>
<td>510 ± 72</td>
<td>516 ± 108</td>
<td>648 ± 99</td>
</tr>
</tbody>
</table>
Fig. 2.4 Cont'

b) Profile of cuticle protein extracts (duplicates) on a 7.5% native gel.

c) Profile of a cuticle protein extract (CP) and a non-cuticular homogenate (NC) on an SDS gradient gel. (MWM: Molecular weight marker proteins, Kd)

d) Profiles of proteins extracted from smooth (S) and spine-containing (SC) cuticular regions on a native gradient gel. Minor proteins specific to the spine-containing region are indicated (*).
is between 23-28 Kd and the other three band cluster is 13-15.5 Kd. A comparison of the electrophoretic profile of a cuticle protein extract with that of homogenized non-cuticular material (Fig. 2.4c, NC) reveals that contamination of the cuticle protein extract with such material is minimal.

By separating cuticle proteins on a native gradient gel and then subjecting them to electrophoresis in the second dimension in an SDS gradient gel it is found that a number of major proteins resolved as single bands on native gels comprise more than one polypeptide (Fig. 2.5). Protein band 1, for instance, consists of a number of polypeptides which belong to the lower molecular weight cluster. Protein band 7 comprises two polypeptides, one belonging to the higher and one to the lower molecular weight cluster. Protein band 9, however, appears to be a single polypeptide and is the most prominent spot on a 2D gel. 2D gel electrophoresis therefore increases the resolution of the cuticle proteins extract and demonstrates that at least 12 polypeptides make up LCPs 1-9.

The major third instar cuticle proteins are essentially free of carbohydrate as none stain with Periodic acid - Schiffs reagent. No differences are observed in the electrophoretic profile of cuticle proteins prepared in the presence or absence either of the reducing agent, β-mercaptoethanol or of an inhibitor of serine esterases, phenylmethanesulfonyl fluoride (PMSF, 1mM).

(c) The proteins of different morphological regions of the cuticle

The larval cuticle is clearly divided along its length into regions of smooth cuticle and regions possessing spines (Fig. 2.1). The electrophoretic profiles of proteins extracted from both these types of cuticle are essentially the same (Fig. 2.4d). Some minor proteins can
Fig. 2.5: Electrophoretic profile of a cuticle protein extract separated in two dimensions. (1st dimension: Native gradient gel; 2nd dimension: SDS disc gradient gel). The profiles of cuticle proteins separated independently in each dimension are also shown for comparison as are molecular weight markers, MWM, Kd).

Protein bands are numbered as in fig. 2.4.
be seen in extracts of spine-containing cuticle which are not evident in extracts of smooth cuticle.

(d) The cuticle proteins of five lines of *L. cuprina*

A number of different laboratory lines of *L. cuprina* are maintained at the CSIRO, Division of Entomology. The larval cuticle proteins of five different lines were compared. Three of these lines have been maintained in the laboratory for more than 30 years; the fourth was isolated from the field about five years ago and the fifth is a recently established line (in its fourth generation in the laboratory). The more recently established lines develop more rapidly than their longer established counterparts. However when larvae at the same developmental stage from the different lines are compared, the same amount and type of cuticle proteins are extracted (data not shown).

(e) Cuticle water content

The mean wet weight of isolated mid resting phase third instar larval cuticles is 4.15mg ± 0.42 (SD) and the mean dry weight is 1.67 mg ± 0.19 (SD). Thus the cuticular water content is of the order of 60%.

2. Developmental changes in the larval cuticle

a) Cuticle weight and protein content

Between mid 2nd and mid feeding phase of the 3rd instar the increase in larval cuticle dry weight roughly parallels the increase in total larval dry weight (Table 2.2). Thus cuticle weight, expressed as a percentage of larval weight is similar in both instances (5.8 and 6.8%). Also the amount of protein released from isolated cuticles is relatively similar (about 20% of cuticle dry weight in both cases).

The situation changes during the 3rd instar. Between mid feeding and mid wandering phases the relative increase in cuticle weight is
Table 2.2: Developmental changes in the weight and protein content of larval cuticles

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>mid 2nd instar</th>
<th>3rd instar</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (hrs post egg laying + 1)</td>
<td>37</td>
<td>57</td>
<td>76</td>
<td>98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean larval dry wt. (mg) + SD</td>
<td>0.60 + 0.02</td>
<td>6.63 ± 1.11</td>
<td>11.89 ± 2.04</td>
<td>12.23 ± 0.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean cuticle dry wt. (mg) + SD</td>
<td>0.035 + 0.004</td>
<td>0.45 ± 0.07</td>
<td>1.18 ± 0.12</td>
<td>1.67 ± 0.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean cuticle wt. as a % of mean larval wt.</td>
<td>5.83</td>
<td>6.79</td>
<td>9.92</td>
<td>13.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean protein amount extracted per cuticle with 7M urea (mg) + 50</td>
<td>0.008 ± 0.001</td>
<td>0.09 ± 0.05</td>
<td>0.36 ± 0.06</td>
<td>0.52 ± 0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean protein amount extracted as a % of cuticle dry wt.</td>
<td>22.9</td>
<td>20.0</td>
<td>30.5</td>
<td>31.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n = 10 in each case
greater than the relative increase in larval weight such that the wandering phase larval cuticle accounts for 9.9% of the animal's dry weight. The amount of protein released from these cuticles increases to an even greater degree and makes up 30.5% of the dry weight of the cuticle of wandering larvae.

Since the larvae have ceased feeding, it is not surprising that between the wandering and resting phases of the 3rd instar, larval dry weight does not increase significantly. Cuticle dry weight however does increase significantly (p < 0.05, Student's t test); the resting phase larval cuticle now comprises 13.7% of total larval dry weight. The protein amount released from such cuticles is relatively the same as that released from the cuticles of wandering phase larvae (31%).

(b) Electrophoretic profiles of the cuticle proteins of different larval instars

Cuticle protein extracts of each larval instar display quantitatively and qualitatively unique electrophoretic profiles (Fig. 2.6). Two major protein bands (designated 1 and 9) are resolved in the electrophoretic profile of 1st instar larval cuticle extracts. These proteins are common to all larval instar cuticle extracts; protein band 1 being present in approximately the same relative amount in all extracts (Table 2.3). Protein 9 more than doubles in mean relative amount between mid 1st and mid 2nd instar and again between mid 2nd and the middle of the feeding phase of the 3rd instar. At all stages it is the most prominent protein band.

In addition to these, protein 8 is resolved in the electrophoretic profile of 2nd instar larval cuticle extracts and remains evident, in low levels, in electrophoretic profiles of all 3rd instar extracts. Extracts of both 1st and 2nd instar larval cuticles contain a high
Fig. 2.6: Developmental changes in the electrophoretic profile of the larval cuticle proteins. (Native gradient gel).

M1: Mid 1st instar (20 h); M2: Mid 2nd instar (37 h); F3: Mid feeding phase (3rd instar, 57 h); W3: Mid wandering phase (3rd instar, 76 h); R3: Mid resting phase (3rd instar, 98 h).
All times are ±1h after egg laying.
### Table 2.3: Relative proportions of the major cuticle protein bands at different developmental stages

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Band 1</th>
<th>Band 2</th>
<th>Band 3</th>
<th>Bands 4/5</th>
<th>Bands 6/7</th>
<th>Band 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mid 1st instar</td>
<td>2.8</td>
<td>1.76</td>
<td>1.34</td>
<td>1.34</td>
<td>3.08</td>
<td>4.34</td>
</tr>
<tr>
<td></td>
<td>± 0.88</td>
<td>± 0.59</td>
<td>± 0.34</td>
<td>± 0.37</td>
<td>± 1.11</td>
<td>± 1.42</td>
</tr>
<tr>
<td>Mid 2nd instar</td>
<td>4.4</td>
<td>2.24</td>
<td>2.24</td>
<td>2.24</td>
<td>4.66</td>
<td>9.70</td>
</tr>
<tr>
<td></td>
<td>± 1.61</td>
<td>± 1.00</td>
<td>± 1.00</td>
<td>± 1.00</td>
<td>± 1.39</td>
<td>± 4.01</td>
</tr>
<tr>
<td>Mid feeding phase (3rd instar)</td>
<td>4.5</td>
<td>2.84</td>
<td>2.30</td>
<td>2.36</td>
<td>10.42</td>
<td>21.62</td>
</tr>
<tr>
<td></td>
<td>± 0.66</td>
<td>± 1.51</td>
<td>± 0.73</td>
<td>± 0.87</td>
<td>± 4.08</td>
<td>± 8.92</td>
</tr>
<tr>
<td>Mid wandering (3rd instar)</td>
<td>4.8</td>
<td>5.78</td>
<td>5.74</td>
<td>5.12</td>
<td>15.16</td>
<td>26.54</td>
</tr>
<tr>
<td></td>
<td>± 0.54</td>
<td>± 1.97</td>
<td>± 1.48</td>
<td>± 1.08</td>
<td>± 3.12</td>
<td>± 1.50</td>
</tr>
<tr>
<td>Mid resting (3rd instar)</td>
<td>4.4</td>
<td>4.08</td>
<td>6.68</td>
<td>7.5</td>
<td>13.35</td>
<td>20.53</td>
</tr>
<tr>
<td></td>
<td>± 1.03</td>
<td>± 1.18</td>
<td>± 1.96</td>
<td>± 2.81</td>
<td>± 2.24</td>
<td>± 3.41</td>
</tr>
</tbody>
</table>

Polyacrylamide gels (such as that shown in Fig. 2.4a) were scanned using an RFT transdyne Densitometer. From the tracings obtained the area under each scanned peak (corresponding to different cuticle protein bands) was calculated and expressed as a percentage (± SD) of the total scanned area. Replicates of 5 were performed.
proportion of heterogeneous proteins of low electrophoretic mobilities which resolve poorly on native gels.

The electrophoretic profiles of 3rd instar larval cuticle extracts display additional bands (2, 3, 4, 5, 6 and 7) and less heterogeneous material. The bands become more prominent as the instar progresses.

c) Electrophoretic profiles of the cuticle proteins within the larval instars.

As shown in Figure 2.6 the electrophoretic profiles of proteins extracted from larval cuticles change markedly during the 3rd instar. Protein bands 2, 3, 4 and 5 increase more than two-fold in relative amount between feeding and wandering phase larval cuticle extracts (Table 2.3). Figure 2.7 shows that the appearance of these protein bands in the electrophoretic profile is very closely correlated with the entry of the larvae into the wandering phase of the 3rd instar. In the four hour interval between the late feeding and early wandering phase these proteins change in amount from being detectable at a low level to being prominent. Thereafter few qualitative changes occur; the electrophoretic profiles of the cuticle proteins of wandering and resting phase larvae are similar.

The electrophoretic profiles of proteins extracted from larval cuticles at various times within the 1st or 2nd instar differ quantitatively. In addition, protein band 2 appears in the electrophoretic profile of late 2nd instar larval cuticle extracts, indicating that it is not a 3rd instar specific protein (data not shown).
Fig. 2.7: Developmental changes in the electrophoretic profile of the larval cuticle proteins during the 3rd instar. (Native gradient gel).

EF: Early feeding phase (48 h); MF: Mid feeding phase (57 h); LF: Late feeding phase (66 h); EW: Early wandering phase (70 h); MW: Mid wandering phase (76 h); LW: Late wandering phase (82 h); ER: Early resting phase (86 h); MR: Mid resting phase (98 h); LR: Late resting phase (110 h). All times are ± 1 h after egg laying.
3. **Immunological relatedness of the cuticle protein**

(a) Within the 3rd instar

The 3rd instar larval cuticle proteins were separated electrophoretically, transferred to nitrocellulose and probed with Mouse anti 3LCP in a Western immuno blot assay. As expected this antiserum reacts with all 3rd instar larval cuticle proteins (Fig. 2.8b,i). The antiserum raised against the single larval cuticle protein (Mouse anti LCP9) reacts only with this protein (LCP9) (Fig. 2.8b,ii). Control serum is unreactive (Fig. 2.8b,iii).

(b) Between the larval instars

Rabbit anti LCP3 reacts with all 3rd instar cuticle proteins (in common with Mouse anti 3LCP above) (Fig. 2.9b, 3). In addition some of the minor proteins of lower electrophoretic mobility (which stain poorly with Coomassie blue) also react. The intensity of reaction to various proteins differs between the mouse and rabbit antisera.

The major 1st and 2nd instar larval cuticle proteins (LCP1 and 9) are recognised by Rabbit anti 3LCP as is LCP2, isolated from 2nd instar larval cuticles (Fig. 2.9b, 2 and 1). Longer exposures of this Western immuno blot reveal the presence of LCP 2 in 1st instar extracts as well as reactivity to 1st and 2nd instar material of low electrophoretic mobility.

4. **Comparison of the major cuticle proteins of 3rd instar larvae of**

*L. cuprina* and *D. melanogaster*

On SDS gradient polyacrylamide gels the 7M urea soluble cuticle proteins of *D. melanogaster* larvae resolve, like those of *L. cuprina*, into two relatively low molecular weight clusters (Fig. 2.10a). In contrast to *L. cuprina* however the most prominent *D. melanogaster* cuticle protein cluster has a molecular weight below 18 Kd. The more
Fig. 2.8: a) Electrophoretic profile of 3rd instar larval cuticle proteins (resting phase),

b) Autoradiograph showing Western immuno blot analysis of (a) probed with the antiserum indicated.
Fig. 2.9: a) Electrophoretic profiles of 1<sup>st</sup> (1), 2<sup>nd</sup> (2) and 3<sup>rd</sup> (3) instar larval cuticle proteins.

b) Autoradiograph showing Western immunoblot analysis of gel (a) probed with Rabbit anti 3LCP.
minor cluster has a molecular weight within the range 25-30 Kd. When the larval cuticle proteins of *D. melanogaster* are resolved on a native gradient gel nine bands are seen (Fig. 2.10b). By transferring these proteins to nitrocellulose and probing with Rabbit anti 3LCP in a Western immuno blot assay it was found that many of these proteins react with the antiserum (Fig. 2.10c).

5. Immunological relatedness of cuticle, haemolymph and other non-cuticular proteins

Extracts of cuticle, haemolymph and non-cuticular material were separated on an SDS gradient gel and stained with Coomassie blue (Fig. 2.11a). The three extracts contain proteins with shared electrophoretic mobilities (Fig. 2.11a). When the electrophoretically separated extracts are Western blotted onto nitrocellulose and probed with Rabbit anti 3LCP it is revealed that they contain few common antigens (Fig. 2.11b). The major reactive species in the cuticle extract (the two low molecular weight cuticle protein clusters) are not present in either of the other extracts. The only antigens detected in all three extracts are of relatively high molecular weight (greater than 40 Kd) (Fig. 2.11b*). These results show not only that the major cuticle proteins are not located in the haemolymph but also that the cuticle protein extract is not contaminated, to any extent, with proteins either from the haemolymph or other non-cuticular tissues.

6. Effect of larvicides on the larval cuticle and cuticle proteins

(a) Larval and cuticle dry weight

Both diflubenzuron and cyromazine-treated larvae are significantly lighter (*p < 0.001*, Student's t test) than controls (Table 2.4). These larvae also possess significantly lighter cuticles (*p < 0.001*) and yield significantly less protein from isolated cuticles (*p < 0.001*) than controls (Table 2.4). In contrast both polyoxin D and 5-fluorouracil-
Fig. 2.10: A comparison of the 3rd instar larval cuticle proteins of *D. melanogaster* (Dm) and *L. cuprina* (Lc).

a) Electrophoretic profiles of the cuticle proteins of both species on an SDS disc gradient gel. (MWM: Molecular weight marker proteins, Kd).

b) Electrophoretic profiles of the cuticle proteins of both species on a native gradient gel.

c) Autoradiograph showing Western immuno blot analysis of gel (b) probed with Rabbit anti 3LCP.
Fig. 2.11: a) Electrophoretic profiles of larval non-cuticular (NC), haemolymph (H) and cuticle (C) extracts.

b) Autoradiograph showing Western immunoblot analysis of gel (a) probed with Rabbit anti 3LCP.

The arrow indicates the major haemolymph storage protein, lucilin.
Table 2.4: Effects of larvicides on larval dry weight, cuticle dry weight and the amount of extractable cuticular protein

<table>
<thead>
<tr>
<th>Larvicidal treatment</th>
<th>Mean larval dry weight (mg) ± SD</th>
<th>Mean cuticle dry weight (mg) ± SD</th>
<th>Mean amount of extractable protein per cuticle (mg) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no treatment)</td>
<td>7.09 ± 0.78</td>
<td>1.31 ± 0.22</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td>Diflubenzuron (100)*</td>
<td>4.85 ± 0.90</td>
<td>0.37 ± 0.06</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Polyoxin D (50)*</td>
<td>7.29 ± 0.76</td>
<td>1.24 ± 0.15</td>
<td>0.30 ± 0.06</td>
</tr>
<tr>
<td>Cyromazine (10)*</td>
<td>4.58 ± 0.75</td>
<td>0.48 ± 0.04</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>5-fluorouracil (100)*</td>
<td>-</td>
<td>1.33 ± 0.06</td>
<td>0.31 ± 0.05</td>
</tr>
</tbody>
</table>

*Concentrations of chemicals in mg/kg of feeding medium.
treated animals do not differ from controls in any of the measured parameters.

When comparing diflubenzuron-treated larvae with cyromazine-treated larvae it is clear that there is no significant difference between the larval dry weights of the two groups. However the dry weights of the cuticles of the two larval groups differ significantly from each other ($p < 0.001$). There is no difference between the amount of protein extracted per cuticle from diflubenzuron or cyromazine-treated larvae.

(b) Cuticle protein electrophoresis

The electrophoretic profiles of proteins extracted from the isolated cuticles of larvicide-treated and control animals are shown in Fig. 2.12. All larvae contain the same major proteins, in the same relative proportions. A similar result is obtained when the proteins isolated from cuticles contaminated with muscle and epidermal tissue are examined by electrophoresis. Again the major cuticle proteins are discernable in all cases yet the amounts of these proteins are greatly reduced in the cuticle extracts of diflubenzuron and cyromazine-treated larvae (data not shown). This suggests that the cuticles of diflubenzuron and cyromazine-treated animals are affected by the larvicides and the reduced level of protein extracted is not due to damage caused to the cuticle during isolation.
Fig. 2.12: Electrophoretic profiles of the cuticle proteins extracted from larvae treated with the larvicides indicated and control larvae.

5f: 5-fluorouracil; Di: Diflubenzuron; P: Polyoxin D; V: Vetrazin; C: Control.
DISCUSSION

The cuticle proteins

The major components of the dry insect cuticle are chitin and protein. The chitin is arranged into a series of microfibrils which are bound to and surrounded by hydrated protein (Rudall, 1965; Filshie, 1982). Any differences in solubility of the proteins within the unsclerotized larval cuticle of *L. cuprina* must depend on the bonding between these proteins and other cuticular components.

Proteins which are extracted from the cuticles of resting phase 3rd instar larvae by water resolve into nine major bands on native gradient polyacrylamide gels. Water is considered to remove soluble, unbound proteins and also those held via weak electrostatic or hydrogen bonded interactions. Water-extracted proteins are presumably not intimately associated with chitin but surround chitin-protein complexes. The amount of protein extracted by water equals 10.8% of the dry weight of the cuticle. In view of the high water content of the *L. cuprina* larval cuticle, it is unclear how these water-extractable proteins are organized within the cuticle.

Since extraction of protein with water or neutral salt solutions does not significantly change the cuticular x-ray diffraction pattern but extraction with solutions of urea does (Rudall and Kenchington, 1973), it has been suggested that the additional proteins extracted with urea are those bonded to chitin and constitute part of the chitin-protein complex. Urea, like SDS, is capable of disrupting hydrogen bonds. Extraction of *L. cuprina* larval cuticles with 7M urea or 2% SDS yields about three times the amount of protein obtained with water. Electrophoresis shows that this protein is simply more of the same types as are extracted with water. It therefore appears that the proteins
which are strongly associated with chitin are the same as those unbound, or weakly bound, within the protein matrix of *L. cuprina* larval cuticles. Nevertheless, the physical location of each of the major larval cuticle proteins (e.g., with regard to intimacy of association with chitin microfibrils) is unknown.

The observation that essentially the same cuticle proteins are extracted using water or compounds capable of disrupting hydrogen bonds (e.g., urea, SDS, formamide) is also recorded by Willis *et al.* (1981) for *Sarcophaga bullata* (Diptera) larval cuticles and by Roberts and Willis (1980) for *Tenebrio molitor* cuticles. However the electrophoretic profiles of proteins obtained by successive extractions of *Agrioname spinicollis* (Coleoptera) larval cuticles with water, KCl and 7M urea differ (Hackman, 1972). This is also the case for other arthropod species such as *Scylla serrata* (Decapoda), *Boophilus microplus* (Acarina), *Astacus leptodactylus* (Decapoda) (Hackman, 1974a; Vranckx and Durliat, 1980). In these studies it was found that although some of the proteins in the successive cuticular extracts appear to be electrophoretically identical others are not. Similar successive cuticle protein extracts of *Galleria mellonella* (Lepidoptera), *Calliphora erythrocephala* (Diptera) or *Philosamia ricina* (Lepidoptera) differ in amino acid composition (Karlson *et al.*, 1969a; Srivastava, 1971; Pant and Sharma, 1974). These studies suggest that in these species certain proteins make up the unbound protein matrix and that the chitin-protein complex contains some other, different proteins. It is clear, therefore, that in these species different proteins have distinct spatial distributions within the cuticle.

The total protein content of a resting phase 3rd instar *L. cuprina* larval cuticle has been estimated by amino acid analysis to equal 37.9%
of the weight of a water free cuticle (Hackman and Goldberg, 1971). It
has been shown in the present study that protein extracted from isolated
cuticles using 7M urea amounts to 31% of the cuticle dry weight. Thus,
of the total protein within such a cuticle, most (82%) is removed by
using 7M urea. Some of the remaining protein may be hydrogen bonded to
chitin but inaccessible to the extracting solution since homogenization
of the cuticle in SDS releases more of the same proteins. Other
unextracted proteins may be covalently bound to chitin or within the
epicuticle. Whether the protein which is not extracted with 7M urea (<
20% of the total) is made up of the same nine major soluble proteins is
unknown. Extracted and unextracted proteins may be of the same type in
those species (e.g., Schistocerca gregaria (Orthoptera), B. microplus,
Rhodnius prolixus (Hemiptera)) in which the amino acid composition of
extracted proteins is similar to that of the whole cuticle or to the
cuticle residue remaining after protein removal (Andersen, 1971;
Hackman, 1974a, 1975; Vranckx and Durliat, 1980). However (even in
different cuticular regions of a single animal) this is not always the
case (Andersen, 1971). In Sarcophaga bullata larvae the amino termini
of the soluble cuticle proteins are heterogeneous while the insoluble
protein has only aspartic acid in this position (Lipke et al., 1981).
In S. bullata, then, the extracted and unextracted proteins are not
identical.

A proportion of the unextracted L. cuprina cuticle protein
presumably constitutes part of the cystine-rich region, located
immediately below the epicuticle, which is about one-tenth the total
width of the cuticle (Hackman, 1971). Cysteine is proposed to play a
role in cuticular protein stabilization through the formation of
disulphide linkages. Since no differences are observed in the
electrophoretic profile of cuticle proteins extracted in the presence or absence of β-mercaptoethanol, it is clear that disulphide cross-linked proteins were not extracted to a detectable extent in this study.

The proteins of the resting phase larval cuticle of *L. cuprina* are remarkably soluble. Of the total protein present in the cuticle 28% is extracted with water and this represents one of the higher values for such an extraction reported in the literature. Almost no protein is extracted from adult *B. micropus* cuticles with water (Hackman, 1974a) and less than 5% is extracted from *Locusta migratoria* (Orthoptera) larval or adult cuticles (this value increases, however, at ecdysis) (Phillips and Loughton, 1976, 1981). Water removes 2% of the cuticle protein of 2nd instar, around 13% of 3rd, 4th and 5th instar *Philosamia ricini* and 14% of *Agrianome spinicollis* larvae (Pant and Sharma, 1974; Hackman and Golberg, 1958).

Only approximately one fifth of the total protein of the *L. cuprina* cuticle appears to be covalently bound (i.e., is not removed by any of the extraction solutions used). While *P. ricini* 3rd, 4th and 5th instar larval cuticles appear to have a similar percentage of protein bound covalently, in 2nd instar larvae and in *S. bullata* larvae about one third (and in *A. spinicollis* larvae more than one half) of the protein is said to be covalently bound (Pant and Sharma, 1974; Lipke *et al.*, 1981; Hackman and Goldberg, 1958). Undoubtedly these percentages change at various developmental stages.

The major extractable cuticle proteins of *L. cuprina* larvae are of similar low molecular weight and low isoelectric point (pI 4.5 - 5.5) (Hackman, 1980). In these properties they resemble the extractable protein fraction of many other arthropod cuticles (Larsen, 1975; Welinder, 1975; Hackman, 1976; Fristrom *et al.*, 1978; Hackman and
Goldberg, 1979; Roberts and Willis, 1980; Vranck and Durliat, 1980; Lipke et al., 1981).

**Regional distribution of cuticle proteins**

(i) Epicuticle and procuticle

The relationship between the proteins contained within the two broad subdivisions of the cuticle (epicuticle and procuticle) are largely unknown. Proteins appear to be shared between both regions in the case of *Calliphora vomitoria* (Diptera) and in the carapace of *Limulus polyphemus* (Merostomata) in which the amino acid composition of the procuticle is the same as that of the isolated epicuticle (Dennell, 1958; Karlson et al., 1969b). In other cases (and in different cuticular regions of *L. polyphemus*) different proteins appear to constitute the two regions since the amino acid composition and/or the electrophoretic profiles of proteins extracted from isolated outer regions of the cuticle differ from inner cuticular regions (Welinder, 1975; Bordereau and Andersen, 1978; Karlson et al., 1969b). Whether there are shared proteins in the epicuticle and procuticle of *L. cuprina* larvae is unknown.

(ii) Anatomically-distinct regions

The cuticles of all arthropods display regional specialisations. Since protein is a fundamental constituent of the cuticle it is not surprising that such different cuticular regions contain different proteins. For instance proteins extracted from anatomically distinct regions of larvae, pupae or adult *Hyalophora cecropia* (Lepidoptera) often differ in electrophoretic profile (Cox and Willis, 1985). The profiles and amino acid composition of the proteins of various regions of *Macrotermes bellicosus* (Isoptera) queen cuticle also differ somewhat (Bordereau and Andersen, 1978). The proteins of the abdominal sclerites
(i.e., the cuticle covering the dorsal region of segments) and intersegmental membranes (i.e., the cuticle connecting segments) of *S. gregaria* differ markedly in amino acid composition as do those from the abdominal tergites (i.e., cuticle covering the ventral region of segments) and intersegmental membranes of *Anabrus simplex* (Orthoptera) (Andersen, 1971; De Hass et al., 1957). Presumably the different proteins are important in determining the nature of particular cuticular regions. In contrast the spine-containing and smooth regions of *L. cuprina* 3rd instar larval cuticles contain largely the same proteins. Thus it appears that the morphological differences between these regions is not determined by their proteins. Filshie (1970), in electron microscopy studies of *L. cuprina* larval cuticle, has shown that one difference between spine-containing and smooth cuticle is the presence of an enlarged dense layer in the epicuticle of the spines. The additional minor proteins extracted from spine-containing cuticle may therefore be derived from this epicuticular dense layer. Electrophoretically similar proteins have also been extracted from the sclerites and intersegmental membranes of both *H. cecropia* (Lepidoptera) larval cuticles and *Antheraea polyphemus* (Lepidoptera) pupal and adult cuticles (Cox and Willis, 1985; Willis et al., 1981; Sridhara, 1983). In these cases it appears that there must be a different organisation of the same macromolecules within different regions of the cuticle. It may be that the amount of protein or, probably more importantly, the chitin:protein ratio is the prime determinant of the nature of these different cuticular regions (especially since it has been suggested that the cuticle may be a self-assembling entity (Neville, 1975)). However it must be emphasised that these studies looked only at the soluble cuticle proteins of the various regions. The unextracted proteins of
each region may, of course, differ. This is certainly so in the case of adult *T. molitor* where the morphologically distinct ventral and dorsal cuticles contain electrophoretically identical extractable proteins but the total amino acid composition of the two cuticular types differs (Andersen *et al.*, 1973; Roberts and Willis, 1980).

The multiplicity of cuticle proteins

It is now widely regarded that cuticles contain a heterogeneous assemblage of similar proteins (Hackman, 1976; Andersen, 1979). It has been suggested that the presence of many similar proteins in cuticles is the result of mutation leading to protein polymorphisms and that such a multiplicity of cuticular proteins reflects the fact that amino acid sequence specificity (required for the active sites of enzymes) is not necessary for the structural proteins of the cuticle (Hackman and Golberg, 1976). Our observations on the different laboratory lines of *L. cuprina*, some of which have been in isolation for more than 30 years, show that the major extractable larval cuticle proteins have been conserved. This suggests that individual proteins have precise roles to play at specific developmental stages. Similarly the major cuticle proteins of other insect species examined do not exhibit a high degree of polymorphism (Fristrom *et al.*, 1978; Roberts and Willis, 1980; Willis *et al.*, 1981).

It has also been suggested that many cuticle proteins represent variants of a few 'core' polypeptides or that these 'core' polypeptides complex chemically with each other to give rise to a series of related multimers (Mills *et al.*, 1967). Certainly some cuticle proteins have been shown to associate *in vitro* (Hackman, 1972, 1974a; Welinder, 1975; Hackman and Golberg, 1979). The notion of a limited number of 'core' polypeptide units interacting to make up an apparently complex cuticle
protein mixture is attractive since the cross-linking of cuticle proteins by tyrosine derivatives is responsible, in part, for the stiffening of insect cuticles. The larval cuticle proteins of *L. cuprina* may, therefore, comprise only a few interacting polypeptide units. Thus the heterogeneous material of low electrophoretic mobility which reacts with cuticle protein antiserum (Rabbit anti 3LCP) in cuticle extracts of all larval instars may represent further complexed forms of these polypeptides. However the antiserum raised against a single larval cuticle protein (Mouse anti LCP 9) binds only to LCP 9 showing that this prominent protein at least does not exist in any aggregated form with other proteins. It is also clear that no other protein band represents a variant of LCP 9.

An alternative explanation for the heterogeneous nature of cuticle proteins is that many are unique and encoded by unique genes. This notion is supported by biochemical data. Several of the major cuticle proteins of a variety of arthropods have been isolated and by limited proteolysis ('fingerprinting') studies and/or amino acid analysis it has been shown that most are unique (Larsen, 1975; Willis *et al*., 1981; Lipke *et al*., 1981; Sridhara, 1983). Statistical analysis of the amino acid composition of the cuticle proteins of *H. cecropia* has led Willis *et al.* (1981) to suggest that cuticle proteins are encoded by unique but related genes which belong to multigene families. Molecular biological studies carried out on *D. melanogaster* support this contention (Snyder *et al*., 1981). Thus, four of the major larval cuticle proteins of *D. melanogaster* are encoded by a cluster of closely related genes and all are immunoprecipitated by an antiserum raised against one of the four proteins. However a fifth protein appears to differ from these; it is not immunoprecipitated by the antiserum mentioned above and the gene
encoding it maps to a different chromosome (Fristrom et al., 1978; Snyder et al., 1981). In *L. cuprina* LCP 9 is immunologically unrelated to other larval cuticle proteins. Therefore it is likely that the gene encoding it does not belong to a multigene family. The relatedness of the other larval cuticle proteins and whether they are encoded by a cluster of related genes will be considered in detail in Chapter 5.

It is noteworthy that most studies of cuticle protein composition describe only about 10 major proteins. It is these proteins which have been examined by 'fingerprinting', amino acid analysis or with respect to protein polymorphism and it is these proteins, in many cases, which appear to be unique. The relationships of these major proteins to the numerous minor protein species also extracted from cuticles, with respect to the question of cuticle protein multiplicity, is not clear. Whether the minor proteins are unique, exist in variant forms, are polymorphic or are artifacts derived from epidermal contamination of cuticle preparations, has not been investigated in most cases.

Changes in the larval cuticle during development

Both the larva and larval cuticle increase in weight more than 10-fold between mid 2nd and mid feeding phase of the 3rd instar. The dry weight of the cuticle therefore, expressed as a percentage of the dry weight of the whole larva, remains similar. However the relative size of the cuticle increases with subsequent development during the 3rd larval instar. Between the feeding and wandering phases of the 3rd instar not only does the relative weight of the cuticle increase but the proportion of protein extracted from the cuticle increases from 20 to 31% of cuticle dry weight. The dry cuticle is composed to a very large extent of chitin and protein and chitin deposition continues throughout the third instar (Retnakaran and Hackman, 1985). Nevertheless, the
increased proportion of protein extracted from wandering larvae (31%) indicates a greater input of cuticular protein relative to chitin at this time.

Over the final stages of the 3rd instar, between wandering and resting phases, larval weight does not change appreciably yet cuticle weight continues to increase. The deposition of cuticular protein (as well as chitin) continues over this period so that the cuticle protein:chitin ratio (of 1:1.4, calculated from present and previously published data (Retnakaran and Hackman, 1985; Hackman and Goldberg, 1971)) is maintained during the later stages of the 3rd instar. The amount of protein extracted from cuticles expressed as a percentage of cuticle dry weight also remains constant (at approximately 31%).

Of the various major proteins which are found in the larval cuticles of *L. cuprina* a number (protein bands 1 and 9) are common to all instars. The proteins comprising band 1, for example, are found in relatively constant amounts for all instar cuticles indicating their essential nature in cuticle structure. Since this band has been shown by 2D electrophoresis to consist of more than one protein the relative importance of each constituent protein for each instar is now known. Band 9 (a single protein) is also found in the cuticles of all instars but its proportion increases as larval development proceeds.

The antiserum directed against 3rd instar larval cuticle proteins (Rabbit anti 3LCP) reacts with bands 1 and 9 from all larval instars, lending support to the notion that these proteins are common to all instars and are not simply different proteins of shared electrophoretic mobility. Similarly, the reaction of Rabbit anti 3LCP with protein band 2 in extracts of 1st and 2nd instar cuticles suggests that this protein also (while not detected by Coomassie staining of gels) is present in
low amounts and is common to the cuticles of all instars.

Some proteins are specific for a particular instar or a particular period within an instar and it appears likely that these are involved with the specific and differing functions of the various cuticles. 1st instar larvae live, in nature, in the fleece or on the skin of sheep (or other animals) where they feed on serous exudate produced as a result of bacterial activity (Merritt and Watts, 1978). The cuticles of these animals contain two easily extractable major protein bands (1 and 9). During the 2nd instar larvae actively invade the epidermis and dermis of the sheep and the cuticles of these animals contain an additional protein band (8). Its presence, like that of protein band 2 (which is extracted in greater amounts from late 2nd instar larval cuticles) may be necessary in some as yet undefined way during this invasive process.

The most striking change in the electrophoretic profile of larval cuticle proteins occurs within the 3rd instar. During this instar (at the beginning of wandering phase) larvae leave the moist conditions in which they feed and enter a more desiccating environment (on and into the soil). Here the larvae enter a resting phase and the cuticle subsequently becomes sclerotized to form the protective puparium. Coincident with the onset of the wandering phase is a dramatic increase in the levels of a series of proteins (2, 3, 4 and 5) within the cuticle. These presumably have a directly protective role for the animals in their new environment and are necessary for puparium formation. Accompanying the increase in the dry weight of the cuticle during the later stages of the 3rd instar is an increase in the levels of extractable protein. It follows that proteins 2, 3, 4 and 5 are extracted in greater total amounts over this time (their relative...
amounts either remain unchanged or increase, as determined by densitometry). It is therefore apparent that these proteins continue to be deposited into the cuticle at least until the middle of the resting phase.

It is possible that in insects whose adult form generally resembles the larval form (ametabolous and hemimetabolous insects) the nature and composition of the cuticle will remain relatively unchanged throughout development. For example, in the hemimetabolous *S. gregaria* the same proteins are extracted from larval and adult femur cuticles (Andersen, 1973). Similarly the electrophoretic profiles of the cuticle proteins of early adult and fifth instar *L. migratoria* are alike (Phillips and Loughton, 1976). Also, in those holometabolous insects which inhabit the same environment during different developmental stages, whose cuticles are subjected therefore to the same environmental 'pressures' and perform the same functions, it is likely that the composition of the different cuticles will remain relatively unchanged. *T. molitor* larvae, for instance, ordinarily live and pupate in meal or stored grain. The electrophoretic profiles of the cuticle proteins of the final three larval instars of *T. molitor* are identical (Roberts and Willis, 1980). Similarly, in *D. melanogaster* the first two larval instars are spent in the same environment and proteins extracted from cuticles of these larvae appear to be the same (Chihara et al., 1982). However in the 3rd instar the animals undertake a migration following the completion of a feeding phase and, as with *L. cuprina*, marked differences are observed in the electrophoretic profile of cuticle proteins extracted from late 3rd instar animals relative to earlier instars.

It is noteworthy that the 3rd instar larvae of *L. cuprina* undergo a behavioural change (from a feeding to a wandering phase) which coincides
with the appearance of specific and prominent new proteins in the cuticle. One may speculate that the mechanisms controlling both events are related, for example through the release/activation of hormones. A peak in the level of ecdysteroid in larval Manduca sexta (Lepidoptera) is known to provoke a change in behaviour of the caterpillar from feeding to seeking a pupation site followed by the construction of a pupation chamber (Truman and Riddiford, 1974; Bollenbacker et al., 1975, 1981). This ecdysteroid peak is coincident with changes in the epidermal mRNA populations of the larvae (Riddiford, 1981). A later and larger ecdysteroid peak initiates epidermal apolysis, the degradation of larval cuticle and the synthesis of pupal cuticle. Similar changes in ecdysteroid titre have been reported to occur prior to the larval-pupal transformation of several other insects, including four blowfly species, Sarcoophaga bullata, Phormia terrae-novae, Calliphora vicina and Lucilia caesar (Briers et al., 1983; Berreur et al., 1979; Dean et al., 1980; Sehnal et al., 1981; Scheller, 1982). It therefore seems likely that changes in ecdysteroid concentrations occur in L. cuprina larvae during the transition from feeding to wandering and may play a role in controlling the changes in cuticle composition which occur.

Comparison of the major larval cuticle proteins of L. cuprina and D. melanogaster

The major larval cuticle proteins of L. cuprina and D. melanogaster share a number of properties. In both cases the proteins resolve electrophoretically into two relatively low molecular weight clusters and are essentially free of carbohydrate (Snyder et al., 1981). They have few or none of the sulphur-containing amino acids, cysteine and methionine, and are relatively rich in the dicarboxylic amino acids, aspartic and glutamic acid (Hackman and Goldberg, 1971; Fristrom et al.,
A number of differences exist between the organization of the proteins in the cuticles of the two species. In the case of *L. cuprina* the major larval cuticle proteins can be extracted with water. In contrast, *D. melanogaster* larval cuticle proteins are insoluble in neutral buffers and require compounds capable of disrupting hydrogen bonds to extract them (Silvert *et al.*, 1984). Furthermore, all *L. cuprina* larval cuticles share a number of major proteins. This is not the case for *D. melanogaster* (Chiara *et al.*, 1982). Nevertheless the cross reactivity of Rabbit anti 3LCP with many of the major larval cuticle proteins of *D. melanogaster* illustrates a high degree of relatedness between some of the proteins of both species suggesting that they have been strongly conserved during dipteran evolution (see Chapter 5). Support for this idea comes from the finding that three proteins from the larval cuticle of the dipteran *Sarcophaga bullata* have been isolated and partially sequenced and all three show a high degree of homology with related proteins of *D. melanogaster* (Henzel *et al.*, 1985). On the other hand, the immunological cross reactivity between the cuticle proteins of *L. cuprina* and *D. melanogaster* reported here may reflect regions of conformational similarity between the proteins of both species which exist because the proteins play similar roles within the cuticle. Such considerations may explain the general similarities of the cuticle proteins of many arthropods.

**Immunological relatedness of cuticle, haemolymph and other non-cuticular proteins**

Some proteins present in extracts of insect cuticles have similar electrophoretic mobilities to haemolymph proteins. On this basis and, more convincingly, since some antisera raised against cuticle protein extracts have been found to react with haemolymph extracts, it has been
postulated that cuticle and haemolymph contain some common proteins (Fox et al., 1972; Koepppe and Gilbert, 1973; Phillips and Loughton, 1976). It has been suggested that the presence of shared proteins arises because some tissue other than the epidermis, for example the fat body, synthesises proteins which are transported via the haemolymph and are incorporated unaltered into the cuticle. Using immunocytochemical techniques, the presence of major haemolymph storage proteins of some insects have been detected in the cuticles of these insects (Scheller et al., 1980; Kaliafas et al., 1984). However, Willis et al. (1981) have shown that the majority of the larval cuticle proteins of *H. cecropia* can be synthesised by the epidermis alone *in vitro* and are incorporated into the cuticle. Other workers have also demonstrated the ability of the epidermis to synthesise cuticle proteins *in vitro* (Weis-Fogh, 1970; Silvert, 1981). Results reported here support the view that the epidermis is the primary site of synthesis of cuticle proteins. Clearly the major Coomassie-staining cuticle proteins of *L. cuprina* are found exclusively in the cuticle. Haemolymph or other non cuticular proteins having the same electrophoretic mobilities as these do not react with Rabbit anti 3LCP indicating that they are different proteins.

It is noteworthy that in *Calliphora vicina* (Diptera) a major haemolymph storage protein (called calliphorin) is detected in pupal and adult cuticles (Scheller et al., 1980). Similarly the equivalent haemolymph protein in *Ceratitis capitata* (Diptera) (ceratitin) is detected in the 'white pupal' and pharate adult cuticle but not in the larval cuticle (Kaliafas et al., 1984). The equivalent storage protein in *L. cuprina* (lucilin) is visible in haemolymph extracts separated electrophoretically (Fig. 2.11 arrow). Clearly however it is not recognized by Rabbit anti 3LCP following Western immuno blot analysis.
It may, of course, (like calliphorin and ceratitin) be incorporated into later (pupal or adult) cuticles.

Effects of larvicides on cuticle structure and cuticle proteins

1) Diflubenzuron and Polyoxin D: Inhibitors of chitin synthesis.

Diflubenzuron exerts a number of effects when applied to insects (Grosscurt, 1978a,b; Soltani et al., 1984). Perhaps its primary, and certainly its best documented, effect is its inhibition of chitin synthesis (Deul et al., 1978; Gijswijt et al., 1979; Van Eck, 1979). A reduction in chitin levels in the peritrophic membrane (a complex of polysaccharides, including chitin and protein in the larval mid gut) of *L. cuprina* larvae brought about by diflubenzuron may upset food intake or digestive metabolism and may thereby result in decreased larval weight as reported in this study. Such effects have been reported previously (Clarke et al., 1977; Becker, 1978; Mitsui et al., 1984; Soltani, 1984).

The ability of diflubenzuron to block chitin synthesis in the larval cuticle of *L. cuprina* has also been demonstrated (Turnbull and Howells, 1982). Reduced chitin levels within the cuticles of diflubenzuron-treated larvae relative to controls can account for the reduced weight of the cuticles of these larvae.

Diflubenzuron-treated larvae also possess less 7M urea-extractable cuticular protein than controls. An effect by benzylphenyl ureas (like diflubenzuron) on cuticle protein as well as cuticular chitin has been noted in previous studies. Post et al. (1974), for instance, report that the pattern of incorporation of injected radiolabelled amino acids into the cuticles of *Pieris brassica* (Lepidoptera) larvae differ markedly in larvicide-treated and control animals. Other workers report that the endocuticle does not grow (increase in thickness) in
diflubenzuron-treated insects (Mulder and Gijswijt, 1973; Grosscurt, 1978a; Soltani et al., 1984). This suggests that not only is chitin synthesis inhibited in these insects but that protein synthesis or protein incorporation into the cuticle is similarly affected. It appears therefore that this larvicide either exerts an additional direct effect on the levels of cuticle protein synthesis or that the effect on protein levels is indirectly brought about by a lowering of cuticular chitin levels. Since protein and chitin constitute the major proportion of dry insect cuticles it may be that the levels of these two major constituents are in some way balanced within the cuticle such that a decrease in the level of one constituent results in a concomitant decrease in the level of synthesis of the other. In support of this Clarke et al. (1977) report that coinciding with a decrease in the chitin levels in the peritrophic membranes of diflubenzuron-treated L. migratoria larvae is an equivalent decrease in protein levels. The chitin:protein ratio of the peritrophic membranes therefore remained constant in insecticide-treated and control animals. Silvert (1981), too, reports that coinciding with chitin synthesis inhibition in diflubenzuron-treated imaginal discs of D. melanogaster protein is not secreted in vitro. The lower protein recovery from the cuticles of diflubenzuron-treated L. cuprina larvae reported in this study support the proposal that the biosynthetic processes of cuticular chitin and protein are coupled.

In contrast to this, however, Grosscurt (1978b) reports that while chitin synthesis is arrested in the elytra (cuticular wing covering) of diflubenzuron-treated Leptinotarsa decemlineata (Coleoptera) larvae the cuticle nevertheless increases in thickness. He proposes therefore that cuticle protein synthesis and deposition are ongoing and are unaffected
by diflubenzuron in these larvae. Also Mulder and Gijswijt (1973) and Grosscurt (1978a), while not recording endocuticular growth in diflubenzuron-treated insects, do observe the presence of scattered globules in the endocuticle of these animals. It has been suggested that the globules may be proteinaceous (Ker, 1978) and may represent that protein which has continued to be deposited after diflubenzuron treatment. As discussed earlier most cuticular proteins are associated to varying degrees with cuticular chitin. However, in diflubenzuron-treated larvae in which chitin synthesis is arrested, the secreted proteins may associate with each other and perhaps cuticular lipids to form the observed globular structures. Since similar globules have been noted in the cuticles of diflubenzuron-treated L. cuprina larvae (Binnington, 1985) it is possible that the lower recovery of cuticular protein from diflubenzuron-treated L. cuprina larvae is a result of the inability of 7M urea to dissociate these globules. The proteins extracted may therefore be derived only from that normal cuticle which was deposited prior to diflubenzuron treatment, hence giving an electrophoretic pattern identical to that obtained with normal cuticle. Whether only the organization of the proteins within the cuticle of L. cuprina is affected by diflubenzuron or whether the amount of protein synthesised/deposited is also affected is unknown.

In direct contrast to the results presented here Hunter and Vincent (1974) report no difference in the amount of protein extracted with 7M urea from diflubenzuron-treated or untreated adult locusts. As expected chitin production is inhibited in diflubenzuron-treated locusts. Similarly, administration of diflubenzuron to last instar nymphs of Chrotogonus trachypterus (Orthoptera) or to larvae of Musca domestica (Diptera) results in a reduction in cuticular chitin content but no
similar decrease in cuticle protein content (Ishaaya and Casida, 1974; Saxena and Kumar, 1981). In these cases it appears that protein deposition continues in diflubenzuron-treated insects and that the deposited proteins are stabilized even in the absence of chitin. Clearly no chitin:protein balance operates in these instances. How the proteins are organized in chitin-depleted cuticles is unknown.

Polyoxin D, like diflubenzuron, is a well documented and potent inhibitor of chitin synthesis (Marks and Sowa, 1974; Gijswijt et al., 1979; Van Eck, 1979) which can disrupt the organization of L. cuprina 1st and 2nd instar larval cuticles (Binnington, 1985). Incubation of isolated integuments of 3rd instar L. cuprina larvae in the presence of either polyoxin D or diflubenzuron results in a similar degree of chitin synthesis inhibition in both cases (Turnbull and Howells, 1982). Nevertheless larvae treated with polyoxin D in this study do not differ significantly in dry weight, cuticle dry weight or cuticle protein content relative to controls. Also electron micrographs of these polyoxin D-treated larvae reveal only minimal disruption of the lamellate endocuticle (K. Binnington, pers. comm.). This can be explained since 3rd instar larvae were used in this study and since polyoxin D is more highly disruptive when injected into some insects (including L. cuprina larvae) than when fed to the insects (Gijswijt et al., 1979; Turnbull and Howells, 1982). This suggests that polyoxin D, at the concentration used in this study, is either rapidly detoxified in feeding 3rd instar larvae or else that it is not readily absorbed by the gut. This compound, therefore, appears to be an efficient inhibitor of chitin synthesis but is a poor insecticide.

2) Cyromazine and 5-fluorouracil: Disrupters of folic acid metabolism?

The effects of cyromazine on the ultrastructure of the larval
cuticle of *L. cuprina* differs from those of diflubenzuron; cyromazine causes epidermal cells to invade the cuticle and produce necrotic lesions whereas diflubenzuron markedly inhibits the formation of new cuticle (Binnington, 1985). Nevertheless in this study both compounds cause a number of similar effects. For instance treatment of *L. cuprina* larvae with cyromazine or diflubenzuron results in a large reduction in the dry weight of the larvae and their cuticles in both cases. The amount of protein extracted from isolated cuticles of the two larval groups is also the same. The mechanisms by which the larvicides bring about these similar effects differs, however. Unlike diflubenzuron, cyromazine does not inhibit chitin synthesis in *L. cuprina* larvae (Turnbull and Howells, 1982). Since the cuticle is composed to a large extent of protein and chitin and chitin deposition appears unaffected by cyromazine the reduced cuticular weight is probably due to an inhibition of cuticle protein synthesis and/or deposition. The protein extracted from the cuticles of cyromazine-treated larvae with 7M urea may represent, as postulated for diflubenzuron, that protein which was deposited prior to larvicide treatment and has a normal electrophoretic pattern.

In addition to causing a reduction in cuticle dry weight cyromazine also causes a reduction in total larval dry weight. While cuticular ultrastructure is clearly adversely affected by cyromazine (Binnington, 1985) this decrease in larval weight suggests that the inhibitory effects of the larvicide may be more widespread and may extend beyond an effect on the larval cuticle. Studies with other insects suggest that cyromazine does not specifically disrupt cuticle formation. Histological examination of cyromazine-treated *Musca domestica* or *Culex quinquefasciatus* (Diptera) larvae demonstrate no cuticular abnormalities
and no interference by the larvicide with normal cuticle deposition (Awad and Mulla, 1984a, b). (In fact a disruption of the muscle tissue of *M. domestica* was reported (Awad and Mulla, 1984a)).

Recently cyromazine has been shown to inhibit mammalian dihydrofolate reductase (K. Binnington, *pers. comm.*). Other inhibitors of this enzyme, such as aminopterin or methotrexate, are known to disrupt the development of, and kill, dipteran larvae (La Brecque *et al.*, 1960; Perry and Miller, 1965; Akov, 1967). An inhibition by cyromazine of *L. cuprina* larval dehydrofolate reductase could disrupt nucleic acid metabolism and inhibit larval growth with concomitant effects on the larval cuticle. This notion is supported by the finding that thymidine administration slightly alleviates cyromazine toxicity in *M. domestica* (Pochon and Casida, 1983). However this proposal for the mode of action of cyromazine does not explain all the effects of the compound. For instance an insecticide which acts primarily through the inhibition of dihydrofolate reductase would be expected to severely disrupt normal insect embryonic development and presumably prevent larval hatching. However eggs laid by cyromazine-treated adult *L. cuprina* hatch normally (larvae die early in development) (Levot and Shipp, 1983, 1984; Friedel and McDonell, 1985). Moreover cyromazine reportedly does not kill external parasites of sheep other than blowfly larvae (Hart *et al.*, 1979). Therefore while the exact mode of action of cyromazine remains unknown some effect by the compound on nucleic acid metabolism is suggested.

In the present study 5-fluorouracil exerted no observed effect on *L. cuprina* larvae. This compound, like polyoxin D, may be metabolised rapidly or may not be absorbed by the gut. The application of a higher concentration of 5-fluorouracil might exert detectable effects.
Finally, it is noteworthy that many insecticides exert more than one effect, making the elucidation of their modes of action at the molecular level difficult. Diflubenzuron, in addition to disrupting chitin biosynthesis also affects epidermal mitoses as well as DNA synthesis and other processes (Verloop and Ferrell, 1977; Soltani et al., 1983, 1984). It has been suggested that the hydrophobicity and the structural simplicity of most insecticides means that they can interact with a number of lipoprotein matrices. The resulting disruption of diverse lipoprotein complexes may explain the multiplicity of effects of many insecticides (Marks et al., 1982).
CHAPTER THREE

THE PROTEINS OF PUPARIA AND OF PUPAL
AND ADULT CUTICLES OF L. CUPRINA
INTRODUCTION

The development of the pupal and adult cuticle.

As discussed in the general introduction *L. cuprina* forms five different cuticles during its life cycle. While the cuticles of the three larval instars are similar in external appearance, the form of the last (3rd) instar cuticle changes markedly at the end of larval life. The cuticle is not shed at this time but contracts to form a structure known as the white puparium which subsequently darkens and is chemically stabilized in the processes of sclerotization. The chemistry of stiffening can be distinguished from that of darkening (Hackman and Goldberg, 1971).

A pupal cuticle forms beneath and separates from the puparium within 12h (at 25°C) of white puparium formation. The separation process, termed apolysis, begins at the anterior end of the developing animal. The pupal cuticle formed in this region is derived from imaginal cells. In contrast, the abdominal pupal cuticle, the last to separate from the puparium, is derived very largely from larval epidermal cells. Within the pupal cuticle metamorphosis occurs. Metamorphosis involves the histolysis of larval tissues and the concomitant histogenesis of adult ones. Most adult organs are derived from small nests of cells called imaginal discs. These are committed in early development to form specific (non-larval) organs and they persist in an undifferentiated state in larvae. Once the adult form is assumed within the pupal cuticle (and puparium), and prior to emergence, the insect is known as a pharate adult.

By 24h after white puparium formation the imaginal head has everted. Barritt and Birt (1971) suggest that the separation of the
pupal cuticle from the newly-forming adult cuticle takes place at around this time. However, based on developmental studies on other species, this notion has been contradicted (Fraenkel and Bhaskaran, 1973). It is likely that pupal/adult apolysis does not occur until some time after head eversion. Certainly it is not possible to isolate the intact pharate adult cuticle from beneath the pupal cuticle (and puparium) until 120h post white puparium formation. Unlike larval/pupal apolysis the separation of the pupal cuticle starts at the posterior of the animal and progresses forward. Following pupal/adult apolysis the adult is confined within three cuticles; outermost the puparium within which lies the pupal cuticle and innermost the adult cuticle. At 25°C *L. cuprina* adults break out from the pupal cuticle and puparium and emerge approximately 204h after white puparium formation. After emergence the adults are relatively immobile for a time during which various regions of the cuticle stiffen and assume their characteristic metallic green appearance.

The form of the pupal and adult cuticle.

The ultrastructures of pupal and adult *L. cuprina* cuticles have not been examined in detail, but they may differ in molecular organization from the larval cuticle. In *T. molitor* larvae (as in *L. cuprina* larvae) the procuticle is lamellate throughout its thickness but in adults a pseudo-orthogonal architecture is found (Neville, 1970). This architecture arises by virtue of the fact that chitin microfibrils are laid down in a series of sheets with successive sheets being aligned at regularly changing angles. In this case, the sheets do not complete one full helicoid (i.e. 180°) before a series is oriented in one 'preferred'
direction. Such architecture may be common in Lepidopteran and Dipteran (e.g. L. cuprina) adults (Neville, 1970). The advantages of the pseudo-orthogonal, as opposed to the straight helicoidal, arrangement of cuticle microfibrils are not clear.

Throughout development the entire pupal cuticle is a thin, flexible and transparent entity. In contrast the adult cuticle changes markedly during development and numerous morphologically distinct regions (undoubtedly differing in ultrastructure) form. For instance the cuticle covering the insect eye becomes stiff yet transparent and is free of pore canals (Neville, 1970) or cuticular perturbations (Noble-Nesbitt, 1963a) common to other regions of the cuticle. The adult cuticle also remains flexible in some regions of the body but becomes dark and stiff in others. This stiffening arises by virtue of the processes of sclerotization.

**Sclerotization**

Sclerotization involves the reorientation of the components of the cuticle, the addition of cross-links between the components, a decrease in their solubility and the elimination of cuticular water (Lipke et al., 1983). Tyrosine derivatives are central to the cross-linking process. N-acetyl dopamine, generated enzymatically from tyrosine as shown in Fig. 3.1, is transferred into the cuticle where it is oxidised to an ortho-quinone by phenoloxidase (Karlson and Sekeris, 1976). The ortho-quinone then reacts spontaneously with available amino or sulphydryl groups to form a protein-linked catechol derivative. This may further react with the amino or sulphydryl group of a second protein molecule to form a protein-protein complex; a quinone polymer may be
Fig. 3.1: Reaction pathways in sclerotization.

Tyrosine → Phenyloxidase → Dopa → Dopa decarboxylase → Dopamine → Dopamine N-acetyl transferase → N-acetyl dopamine → N-acetyl dopamine quinone

Protein → Protein → Protein → Protein → Protein → Protein → Protein → Protein → Protein

β SCLEROTIZATION

QUINONE TANNING
formed before the crosslinking of the second protein occurs. This form of sclerotization is known as quinone tanning (Hackman, 1984).

A second form of sclerotization, called β sclerotization, also occurs and again involves N-acetyl dopamine as the protein linking group. In this case however, the reactive groups of the proteins bond through the β carbon atom of the linking compound (Fig. 3.1) (Andersen, 1975; Karlson and Sekeris, 1976). β sclerotization is postulated to give rise to the stiffened yet colourless cuticle covering insect photoreceptors (Andersen, 1979).

More recently it has been suggested that dehydration of the cuticle (brought about by tyrosine derivatives) will induce sufficient non-covalent associations between cuticular components to account for the observed stiffening and insolubility of sclerotized cuticle (Vincent and Hillerton, 1979; Hillerton and Vincent, 1979). The involvement of chitin in these proposed sclerotization processes has not been documented.

Many studies have shown that the solubility of cuticle protein decreases rapidly following the onset of sclerotization (Andersen, 1971; Srivastava, 1971; Hackman, 1974b; Willis et al., 1981) and the electrophoretic profiles of extracted cuticle proteins usually change during the stabilization processes (Srivastava, 1971; Roberts and Willis, 1981; Phillips and Loughton, 1981; Chihara et al., 1982; Roter et al., 1985). In this chapter the effects of sclerotization on L. cuprina cuticle protein solubility and composition are studied. The proteins of the puparium and the adult cuticle are examined at different times during the sclerotization process. The proteins of the apparently unsclerotized pupal cuticle are also examined. In addition, to
determine whether the cuticle proteins of the various metamorphic states differ (as appears to be the case in many other species, (Chihara et al., 1982; Kiely and Riddiford, 1985; Riddiford, 1992; Silvert et al., 1984; Sridhara, 1983) of the soluble proteins of L. euprina puparia, as well as pupal and adult cuticles are compared electrophoretically and immunologically. Finally, regional variation in protein composition is demonstrated in pupal and adult cuticles.
MATERIALS AND METHODS

Insect culture

*L. euprina* stocks, obtained from the Division of Entomology, CSIRO, Canberra, were maintained as previously described (p 20). From late in the 3rd instar until emergence the animals were kept at 25°C in a bed of vermiculite, under the influence of a 12:12 h light:dark photoperiod.

Cuticle preparation and protein extraction

Cuticles were isolated from *L. euprina* larvae midway through the 1st instar (20 h ± 1 after egg laying), midway through the 2nd instar (37 h ± 1 after egg laying) and midway through the resting phase of the 3rd instar (96 h ± 1 after egg laying). Protein was extracted from these cuticles as previously described (p 21).

Puparia of *L. euprina* were separated from the underlying pupal and pharate adult structures at various times by dissection. Pupal and pharate adult cuticles were obtained at 12-hour intervals; timing of pupal and adult development began with the formation of the white puparium. It is possible to isolate the pupal cuticle from 12 h after white puparium formation. The pharate adult cuticle cannot be isolated until 120 h after this time. Cuticles were also isolated from newly emerged adults (0-2 h after emergence).

Pupal cuticles were isolated by dissection and pharate (and newly emerged) adult cuticles were recovered by opening the animals longitudinally, removing the internal organs, and then scraping the cuticle with a needle and scalpel. Cuticles appeared to be free of contaminating tissue when examined under a dissecting microscope. Isolated cuticles were rinsed 3 times in water and blotted dry. Groups of 3 were then immersed in 100 µl 7M urea for 30 minutes at room
temperature to extract protein.

To obtain an extract of non-cuticular material the internal tissues of resting phase 3rd instar larvae were homogenized in water (100 µl/animal) at 4°C. Samples of haemolymph were obtained by piercing larvae using a 5 µl Hamilton syringe and collecting the exudate. The protein content of extracts was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Electrophoresis and Immunological assays

Vertical slab gel electrophoresis was carried out using either the SDS discontinuous gel system of Laemmli (1970) or a native (non-denaturing) gel system as previously described (p. 22). 10-20% gradient polyacrylamide gels were used in all cases. Western blotting and immunological assays were carried out as described previously (p 25).
RESULTS

1. Quantitation and electrophoretic separation of cuticular protein

(a) Puparia

Protein was extracted from isolated puparia at various developmental times after white puparium formation. The greatest amount of protein was obtained from puparia isolated at 12 h (Fig. 3.2, 12 h). While this is $3 \frac{1}{2}$ times less protein than can be extracted from 3rd instar larval cuticles (Fig. 3.2, - 15 h) it is significantly more (approximately $5 \frac{1}{2}$ times) than that extracted from puparia at 24 h. Thereafter there is no significant change in protein extractability.

All extracts contain the same series of nine major cuticle protein bands found in the 3rd instar larval cuticle (Fig. 3.2). This group of proteins becomes less distinct with puparial maturation. In addition puparial extracts contain a collection of heterogeneous polypeptides which give rise to a background stain on polyacrylamide gels.

(b) Pupal cuticle

The electrophoretic profile of the 7M urea soluble pupal cuticle proteins is relatively constant throughout development (Fig. 3.3). Maximally seven prominent protein bands are resolved (designated pupal cuticle proteins, PCPs, 1-7). Of these, four (PCP 2, 5, 6 and 7) are present in about the same relative amount throughout pupal and pharate adult life. However a change in the protein profile does occur at 108 h (after white puparium formation). A heterogeneous collection of polypeptides of low electrophoretic mobility (PCP 0) is no longer apparent at this
Fig. 3.2: Amount of protein extracted from puparia at various developmental stages and electrophoretic profiles of the extracts.

<table>
<thead>
<tr>
<th>Time (H±1, from white puparium formation)</th>
<th>Protein extracted per puparium, (µg)± SD.</th>
</tr>
</thead>
<tbody>
<tr>
<td>-15*</td>
<td>15 12 24 84 120 192</td>
</tr>
<tr>
<td>516*</td>
<td>145 26 17 25 ±12 ±8 ±12</td>
</tr>
<tr>
<td>±108</td>
<td>±14 ±8 ±12</td>
</tr>
</tbody>
</table>

*, resting phase 3rd instar larvae.
Fig. 3.3: Electrophoretic profiles of pupal cuticle proteins.

Each lane represents the protein (30µg) extracted from a single cuticle.
time, and another major protein (PCP 3) is resolved. After 108 h PCP 1 gradually diminishes in relative amount and is not seen in the extract of 168 h pupal cuticle. In addition the amount of PCP 4 extracted is somewhat variable. The profiles of the cuticle proteins of older pupae do not differ from that seen at 168 h (data not shown).

(c) Adult cuticle

The electrophoretic profile of the 7M urea soluble adult cuticle proteins (ACPs) is given in Fig. 3.4. The protein composition of the adult cuticle appears more dynamic than that of the pupal cuticle. Several major changes in the protein profile are seen. Firstly, a series of proteins, relatively abundant in early pharate adult cuticle extracts (ACP 1, 2, 5, 6, 7), are found in diminished amounts in later cuticle extracts. Secondly, some proteins (ACP 4, 9) become relatively more prominent during the middle stages of pharate adult life (156-180 h) but then diminish. ACP 8 exhibits a similar change in profile except that it is also relatively prominent in the cuticle of newly emerged animals. Finally, on emergence, ACP 3 (a previously prominent protein) and heterogeneous material of low electrophoretic mobility (designated ACP 0) are no longer apparent.

2. Regional distribution of the major pupal and adult cuticle proteins

The electrophoretic profiles of extracts of pupal and adult cuticle obtained from different body regions are shown in Fig. 3.5. The distribution and relative abundance of the major cuticle proteins differs in the different regions. For instance PCPs 2 and 3 are present in the pupal head and thorax but are absent from the pupal abdomen. In
Fig. 3.4: Electrophoretic profiles of adult cuticle proteins.

120 144 168 196 NE
132 156 180 204

Time (H±1, post white puparium formation).

0
-- 1
-- 2
-- 3
-- 4
-- 5
-- 6
-- 7
-- 8
-- 9

NE: Newly emerged adults.
Fig. 3.5: Electrophoretic comparison of cuticle proteins extracted from different regions of the insect body.

PH: Pupal head; PT: Pupal thorax; PA: Pupal abdomen; AH: Adult head; AT: Adult thorax; AA: Adult abdomen; AW: Whole adult.

Proteins were extracted from pupal cuticles 120-156 h, and from adult cuticles 180 h post white puparium formation. (Numbering of proteins as in figs. 3.3 and 3.4).
contrast PCP4 appears to be specific to the abdomen and PCP 5 is relatively more abundant in this region compared with the head or thorax. In the case of adult proteins, ACP 4 and 9 can be isolated from the adult head but not from the thorax or abdomen and ACP 3 is more prominent in the thorax and abdomen compared with the head. Most protein material of low electrophoretic mobility (ACP 0) appears to be derived from the thorax.

3. Immunological relatedness of the pupal and adult cuticle proteins to 3rd instar larval cuticle proteins.

Pupal and adult cuticle proteins (from several different developmental stages) were separated electrophoretically and Western blotted onto a nitrocellulose filter. The filter was probed with the antiserum directed against 3rd instar larval cuticle proteins (Rabbit Anti 3LCP) to examine the immunological relatedness of the proteins of the different metamorphic states.

Of the major pupal cuticle proteins only PCP 5 shows clear reactivity with the Rabbit Anti 3LCP (Fig. 3.6b, PI). The electrophoretic mobility of this protein is similar to that of larval cuticle protein 2. PCP 6 also reacts with the antiserum but to a lesser degree.

Of the major adult cuticle proteins, isolated at three different times during adult development, only ACP 9 reacts with Rabbit Anti 3LCP (Fig. 3.6b, A1, A2, A3). The electrophoretic mobility of ACP 9 is similar to that of larval cuticle protein 7. Reactive material of low electrophoretic mobility (although clearly not ACP 0 or 1) is seen, particularly in extracts of newly emerged adult cuticles.
Fig. 3.6: a) Electrophoretic profiles of pupal and adult cuticle proteins,

b) Autoradiograph of Western blotted gel (a) probed with Rabbit anti 3LCP.

Profile of larval cuticle proteins.

Pl: Pupal cuticle extract (132 h); A1: Adult cuticle extract (120 h); A2: Adult cuticle extract (168 h); A3: Newly emerged adult cuticle extract. All times (±1 h) post white puparium formation.
DISCUSSION

Electrophoretic profiles of the cuticle proteins

(a) Puparia

Sclerotization may involve both covalent and non-covalent binding of cuticle proteins in the stabilization and stiffening of the insect cuticle. Amino acid analysis of the larval cuticle and puparium of *L. ouprina* suggests that the number of amino acids which could participate in covalent cross-links within the cuticle (cysteine, lysine, N-terminal amino acids) is low (Hackman and Goldberg, 1977). This fact, coupled with the large amount of tanning material introduced into the developing puparium, has led Hackman and Goldberg (1977) to suggest that a significant fraction of the cuticular material within the puparium of *L. ouprina* is non-covalently bound. By whatever means, then, the proteins of the 3rd instar larval cuticle of *L. ouprina* interact to form the stiff protective puparium. This occurs within 24 h of white puparium formation and is reflected in the lower amount of protein which is extracted with 7M urea from older puparia. Nevertheless the same major proteins are extracted from *L. ouprina* puparia at all time intervals. This indicates that the interactions of the proteins occur in concert and that no single protein or group of proteins is preferentially bound up at any time. Similarly the same major proteins are extracted from the larval cuticles and puparia of *Sarcophaga bullata* (Willis *et al.*, 1981; Lipke *et al.*, 1981).

One change in the electrophoretic profile of puparial extracts seen in the present study and in that of Willis *et al.* (1981) is the increase in extraction of heterogeneous protein material which accompanies sclerotization and appears as a background stain on polyacrylamide
gels. Since cuticle protein modification may be a prerequisite for cross-linking this heterogeneous material may represent a series of modified intermediates of the sclerotization process. The stabilization of the insect cuticle is not always accompanied by an increase in the background stain of electrophoretic profiles of cuticle extracts. During the sclerotization of the larval and pupal cuticles of *T. molitor*, and the adult cuticles of *L. migratoria*, *D. melanogaster* and *L. cuprina* (see later) no appreciable increase in background staining is observed (Roberts and Willis, 1980; Phillips and Loughton, 1981; Chihara et al., 1982). In addition, while the same major proteins are extracted from puparia during sclerotization, the electrophoretic profiles of the other cuticles mentioned above change during sclerotization. For example, certain early, major proteins decrease in prominence or disappear during the stabilization process, suggesting that these are selectively rendered insoluble. These differences probably reflect the fact that the larval cuticle is fully sclerotized to form the puparium whereas other cuticles are sclerotized only in selected areas. A variation within the cuticle of particular proteins and/or of cross-linking reagents is implicated.

The amino acid composition of the puparium of *L. cuprina* differs markedly from that of the larval cuticle (Hackman and Goldberg, 1971). This suggests that puparium formation involves more than the 'simple' linking together of previously deposited cuticle proteins. The same applies in the case of the sclerotized and unsclerotized cuticles of other holometabolous insects (Hackman and Goldberg, 1977). In contrast the amino acid composition of the sclerotized and unsclerotized cuticles of the hemimetabolous insects examined are similar (Andersen, 1973;
Hackman and Goldberg, 1977). In addition it may be recalled that the proteins extracted from larval and adult hemimetabolous insects are similar in electrophoretic profile (Andersen, 1973; Phillips and Loughton, 1976, 1981). These facts suggest that sclerotization may differ between hemimetabolous and holometabolous insects and may be a biochemically less complex operation in hemimetabolous forms.

(b) Pupae

The relative invariance of the profiles of the pupal cuticle proteins of *L. cuprina* (and *D. melanogaster* (Chihara et al., 1982)) may be related to the unusual mode of development of dipterans in which the pupa is enclosed at all times within the puparium. In more typical situations the last larval cuticle is lost at ecdysis and the pupal cuticle itself becomes sclerotized. The pupal cuticle proteins therefore decrease in extractability with age (Srivastava, 1971). *T. molitor* and *Galleria mellonella* (Lepidoptera), for instance, develop in this more typical way and the profiles of the pupal cuticle proteins of these species are more dynamic than those of *L. cuprina* and *D. melanogaster* (Roberts and Willis, 1980; Srivastava, 1970). Presumably this is because different proteins secreted into the cuticle at various times confer different properties on the cuticle. The pupal cuticles of *T. molitor* and *G. mellonella* must provide not only the strength and flexibility to allow for imaginal disc evertion but also must provide environmental protection for the insect during metamorphosis.

The separation of the pupal and adult cuticles of *L. cuprina* is known to occur before 120 h (post white puparium formation). The change in electrophoretic profile of the pupal cuticle proteins at 108 h may, therefore, be related to the processes involved at, or after,
apolysis. For instance the heterogeneous material designated PCP 0 and PCP 1, which begins to disappear at this time, may be selectively resorbed following apolysis. However, even late in pharate adult life, it is still possible to isolate many major proteins from the pupal cuticle. It appears, therefore, that these major proteins are not broken down and resorbed to any great extent after apolysis (as expected from classical descriptions of the moulting process) but are lost at adult emergence. A similar situation exists in the case of D. melanogaster (Chihara et al., 1982).

The pupal cuticle is derived from a number of groups of cells with different developmental histories. The abdominal cuticle is secreted by epidermal cells which previously secreted larval cuticle. The cuticles of the head and thorax are produced by the cells of the head and thoracic imaginal discs. In L. cuprina these different groups of cells secrete a similar collection of major proteins to form the pupal cuticle. There are however a number of notable differences in the types of proteins secreted by the groups of cells; PCPs 2 and 3, for instance are found in the head and thoracic cuticles but not in the abdominal cuticle. Whether these proteins fulfil a particular role in the cuticles of the head and thorax which is not required in the abdomen is unknown. PCP 4, on the other hand, is an abdomen-specific protein. This clear distinction between the cuticle proteins secreted by imaginal cells and those produced by 'larval' epidermal cells may be a characteristic of dipterans since a major protein extracted from the cuticles of the head and thorax of D. melanogaster pupae is absent from the abdominal cuticle (Chihara et al., 1982). It is not, however, a general phenomenon. Proteins extracted from the pupal sclerites of
Hyalophora cecropia (derived from the 'larval' epidermis) are very largely identical in electrophoretic profile to those extracted from pupal forewing cuticle (derived from wing imaginal discs) (Willis et al., 1981; Cox and Willis, 1985). Similarly, in the case of Antheraea polyphemus (Lepidoptera) the cuticle proteins of pupal abdomens are identical in profile to those of the pupal wing (Sridhara, 1983).

(c) Adults

The incorporation of different proteins into the pharate adult cuticle of L. cuprina at various times during development may account for the observed changes in electrophoretic profile of the cuticle extracts. The proteins may fulfil specific functions in specialized areas of the developing adult cuticle. It is clear (as seen in the case of the pupal cuticle) that different proteins are located in different regions of the adult cuticle. ACPs 4 and 9, for instance, are head specific and the extracted levels of these proteins are greatest mid way through pharate adult life (156-180 h post white puparium formation). Thereafter the levels of these proteins diminish. This indicates that mid way through pharate adult development ACPs 4 and 9 are secreted into the cuticle of the head. Once incorporated, the proteins may stabilize (through interaction with other proteins or other cuticular components) and thus decrease in solubility.

Proteins prominent in the early pharate adult cuticle (ACPs 1, 2, 5, 6 and 7), which diminish in amount in later extracts, may similarly be incorporated within specialized cuticular regions. However it is also possible that these proteins are laid down at an early stage, in the outer layers of the cuticle. Subsequently, other proteins (for instance ACPs 0, 2, 8) may be secreted into the more inner regions.
On emergence a number of previously prominent cuticle proteins are no longer seen. The simplest explanation for this observation is that these proteins are also stabilized through sclerotization soon after emergence and therefore cannot be extracted. The possession of a stiffened cuticle is of obvious importance for the newly emerged adult, providing protection and support as well as facilitating flight. The inability to extract certain major pharate adult cuticle proteins soon after the final ecdysis, again presumably due to the binding up of these proteins, has also been recorded in the case of *L. migratoria*, *H. cecropia* and *D. melanogaster* (Phillips and Loughton, 1981; Andersen, 1973; Cox and Willis, 1985; Chihara *et al*., 1982; Roter *et al*., 1985).

As stated previously, in some species larval haemolymph proteins are incorporated into pupal or adult cuticles. It is possible therefore that some pupal or adult cuticle proteins of *L. cuprina* are derived from the major haemolymph storage protein of this species (lucilin).

**Relatedness of larval, pupal and adult cuticle proteins**

Since the electrophoretic profiles of *L. cuprina* larval, pupal and adult cuticle proteins differ it is likely that the cuticles of each stage contains a largely unique set of 7M urea soluble proteins. This view is confirmed by the finding that Rabbit anti 3LCP reacts with 3rd instar larval cuticle proteins but with few major pupal and adult cuticle proteins. This suggests that the pupal and adult cuticles are mainly composed of proteins which differ from those of the larval cuticle.

In most other species also the cuticles of different developmental stages have been shown (electrophoretically, immunologically or by the techniques of molecular biology) to contain many different proteins
(Roberts and Willis, 1980; Willis et al., 1981; Chihara et al., 1982; Riddiford, 1982; Silvert et al., 1984; Sridhara, 1985; Kiely and Riddiford, 1985). However it is important to note that proteins supposedly specific to one metamorphic stage may be located in limited regions of another stage, where they may be overlooked (Cox and Willis, 1985).

Some major L. cuprina pupal and adult proteins do react with Rabbit anti-3LCP (PCP 5, ACP 9) and these have similar electrophoretic mobilities to prominent larval cuticle proteins (LCP 2, LCP 7). It appears, therefore, that certain proteins may be common to the cuticles of two different developmental stages. If this is the case then the gene coding for LCP 2 would also be expressed through pupal life to form PCP 5. The gene encoding LCP 7, not expressed through pupal life, would be re-expressed in adults to form ACP 9. The expression of a set of genes in larvae and adults but not in pupae has been documented previously in D. melanogaster (Snyder and Davidson, 1983).

Alternatively, the pupal and adult proteins may differ from the corresponding larval proteins yet may share antigenic determinants with them. Evidence in support of this interpretation comes from the fact that some pupal and adult cuticle proteins of D. melanogaster are related immunologically to larval cuticle proteins, yet 'fingerprinting' and molecular biological analysis suggest that the larval proteins differ from those of later developmental stages (Chihara et al., 1982; Silvert et al., 1984). Also in A. polyphemus an antiserum raised against pupal cuticle proteins cross reacts with many adult cuticle proteins in Western immuno-blot experiments and vice versa. Again 'fingerprinting', amino acid and molecular biological analysis show that
the pupal and adult proteins differ and are encoded by different genes (Sridhara, 1983, 1985). The immunological cross reactivity observed in some of the above studies and in the present one may be due to the fact that cuticle proteins share certain structural features associated, for instance, with their capacity to complex with cuticular chitin.

The fact that some adult cuticle proteins of *L. cuprina* have similar electrophoretic mobilities to larval proteins but show no immunological reactivity (e.g. ACP 7, 8) highlights the danger of assuming that cuticle proteins having similar electrophoretic mobilities are identical (cf. Cox and Willis, 1985).
CHAPTER FOUR

THE HUMORAL IMMUNE RESPONSE OF SHEEP

TO BLOWFLY LARVAL ANTIGENS
INTRODUCTION

As was pointed out earlier insecticides currently provide protection for sheep from myiasis-causing insects such as \textit{L. cuprina}. However the development among fly populations of resistance to insecticides (Shanahan and Roxburgh, 1974) and the adverse environmental side-effects of insecticide application have resulted in the investigation of other potential fly control measures.

The idea of using blowfly parasites, pathogens or predators in a biological control regime has been suggested. The European wasp, \textit{Alyssia manducator}, which is a parasite of blowfly larvae, was released in one such regime. However the release was unsuccessful and the wasp did not establish itself in Australia (Kitching, 1974). In fact most predators or parasitoids of \textit{L. cuprina} are associated with carrion so that when the fly feeds on sheep it appears to be relatively free from their depradation (Kitching, 1981).

Attempts are being made to lessen fly strike by increasing the resistance of sheep by selective breeding and genetic manipulation of \textit{L. cuprina} is being undertaken to develop fly strains that will spread infertility or blindness to field populations (Foster and Whitten, 1974). In addition immunological approaches to providing protection for sheep from fly infection have recently been discussed (O'Donnell \textit{et al.}, 1980; Sandeman \textit{et al.}, 1985). This chapter examines one aspect of the immunological interaction of sheep and fly.

Traditionally, the study of arthropods in medicine has emphasised their role as vectors in the transmission of disease between vertebrates. In addition, however, arthropods can directly affect vertebrates by injecting toxins or irritants or by actively invading tissues (Gaafar, 1972; James, 1982). Some myiasis-causing organisms
undertake extensive migrations (and often for extended periods, e.g., 8-10 months) within host tissues e.g., Hypoderma spp (warble flies) which cause subdermal myiasis in cattle and deer; others undertake more limited wanderings e.g., Cuterebra spp. (bot flies) which cause dermal myiasis of rodents and lagomorphs and Gastrophilus spp (stomach bot flies) which cause enteric myiasis in horses and their relatives. Others do not migrate from their original site of penetration e.g., Cordylolia spp., Dermatobia spp. (Askew, 1973; Philipp and Rumjaneck, 1984). Although L. cuprina larvae can cause extensive lesions in sheep skin the exact extent of their penetration into the host's body has not been documented.

The close association of these and other arthropod parasites with their hosts means that an immune response can be mounted by the host against them. However, whether such a response is protective to the host is unclear. Many parasites have been shown to have the ability to circumvent strong host immune reactions. For instance certain parasites may inhabit immunologically privileged sites i.e., regions of the body which for morphological and physiological reasons are relatively isolated from immune effector mechanisms. They include the inner eye, cerebrum and testicles. Other parasites, by living within cells, appear to be relatively free from immunological attack (Bloom, 1979). However, myiasis-causing organisms like L. cuprina larvae, while largely external, do invade host skin and therefore clearly do not inhabit anatomically-secluded sites. Parasites which do not inhabit secluded sites may have other means of evading host immune mechanisms. Some alter their repertoire of antigens between developmental stages (Cohen, 1982; Parkhouse and Ortega-Pierres, 1984; Phillip and Rumjaneck, 1984); others vary the serological specificity of their surface
antigens within a single stage. For example, infection of vertebrates by African trypanosomes (protozoan blood parasites), results in antibody production against the parasites' surface glycoprotein coat. By virtue of molecular rearrangements of the parasites' genome the secretion of a new surface glycoprotein can be directed. Numerous variant antigenic glycoproteins exist, only one of which is expressed at a given time (Myler et al., 1984; Donelson and Turner, 1985). Still other parasites reportedly use 'molecular mimicry' to overcome host immune reactions. Schistosomes (platyhelminth parasites of vertebrate blood systems) are said to actively acquire host biomolecules which 'camouflage' them by obscuring their own antigens (Clegg, 1972; Bloom, 1979; Phillipp and Rumjaneck, 1984).

A large number of parasites appear to have the ability to modify or suppress host immune reactions (Cohen, 1982). The interaction of parasite-derived suppressive factors with T cell or macrophage functions are often implicated in immunosuppression (Askonas, 1984). Parasite excretory/secretory products may inactivate effector functions of specific lymphocyte populations (Goose, 1978). In addition certain parasites can cleave antibody by the action of parasite proteases and others have the ability to inactivate complement. Several protozoans induce an apparently misdirected humoral response in their hosts which can be attributed to the action of polyclonal B cell mitogens of parasite origin (Cohen, 1982).

The question of whether arthropod parasites utilize any or all of these mechanisms to evade the hosts' immunological response has been largely unstudied, although there is some evidence that arthropods depress the immunocompetence of their hosts (Wikel, 1982, 1984). Nevertheless, as early as the 1920s it was shown that the immune
response to myiasis-causing larvae could be protective to the host. Blacklock and colleagues demonstrated that resistance to infestation by *Cordylobia antropophaga* (Diptera, Calliphoridae) larvae in older guinea pigs was the result of an immunity acquired through previous larval attack and could not be attributed to a failure of larvae to penetrate older skin (Blacklock and Thompson, 1923; Blacklock and Gordon, 1927; Blacklock *et al.*, 1930). Resistance manifested itself in a lowering of the survival rate of larvae feeding in such immune hosts. Thus, 49% of larvae survive an initial experimental infection of guinea pigs with *C. antropophaga*; however, only 7% of larvae develop when the infection is repeated (Blacklock and Gordon, 1927). Similarly, 80% of *Cuterebra fontinella* (Diptera, Cuterebridae) larvae survive on previously uninfected hosts, whereas only 50% survive following a second experimental infection (Gingrich and Barrett, 1976). And while 53% of *Hypoderma lineatum* (Diptera, Oestridae) survive a primary infection of cattle only 31% survive when applied to cattle previously exposed to the parasite (Gingrich, 1980). As a final example, guinea pigs reinfected with tissue-invading *Cochliomyia americana* (Diptera, Calliphoridae) larvae after a 20 day interval, survive the injurious effects of a dose of larvae which prove lethal to control (previously uninfected) guinea pigs (Borgstrom, 1938). Moreover it has been possible to vaccinate certain hosts against arthropod parasites. Injection of cattle with a crude preparation of the salivary gland enzyme, collagenase, derived from the larvae of *H. lineatum*, provided these animals with a measure of protection from further fly attack (Magat and Boulard, 1970). It has also been possible to immunize against blood sucking, ectoparasitic ticks by injecting hosts with salivary gland or other tick extracts (Allen and Humphreys, 1979; Davidson, 1985).
Thus the notion of protecting sheep from \textit{L. cuprina} strike by vaccinating them against blowfly larvae is at least a valid theoretical concept. It has been shown that sheep infected with \textit{L. cuprina} respond immunologically to the presence of the parasitic larvae. Anti-\textit{L. cuprina} antibodies have been detected in the sera of struck sheep (O'Donnell \textit{et al.}, 1980; Sandeman \textit{et al.}, 1985). In this chapter the \textit{L. cuprina} larval antigens which stimulate the immune response of sheep when struck are examined. Of particular interest in the context of this thesis is the involvement of the larval cuticle in the sheep's humoral immune response.

\textbf{MATERIALS AND METHODS}

\textbf{Preparation of larval extracts}

1st instar extracts were prepared at $20 \pm 1$ h after egg laying (mid 1st instar); 2nd instar extracts at $37 \pm 1$ h (mid 2nd instar) and 3rd instar extracts at $76 \pm 1$ h (mid wandering phase of the 3rd instar). Larvae were washed 3 times in cold, distilled water before extracts were prepared.

Excretions/secretions: A preparation of larval excretions/secretions was obtained by placing groups of larvae (30 1st instar larvae, 15 2nd instar larvae or 6 3rd instar larvae) in 100 µl distilled water for 30 min at room temperature. After this time larvae were removed and examined under a binocular microscope to ensure that all were still viable. To ensure that material released into the water did not consist of surface bacterial or other contaminating debris 10 groups of 6 washed 3rd instar larvae were frozen at $-20^\circ$C. After 5 hours the larvae were thawed and placed in 100 µl of water as just described.
Viscera: An extract of larval viscera was obtained by removing the integuments of six 1st, 2nd or 3rd instar larvae under a microscope and by then homogenising the internal tissues of each group in 100 µl distilled water at 4°C.

Salivary gland and Mid gut: 5 pairs of 3rd instar salivary glands and 5 mid guts were isolated by dissection. Tissues were maintained in cold saline (0.96% NaCl) throughout the procedure. Extracts were prepared by homogenizing the tissues in 100 µl distilled water at 4°C. Each mid gut included the alimentary tract from above the gastric caecae to immediately above the malpighian tubules.

Cuticle: 3rd instar larval cuticles were isolated by dissection. A cuticle extract was obtained by incubating cuticles in groups of 3 in 100 µl water for 1 h at room temperature.

Haemolymph: A 3rd instar larval haemolymph extract was obtained by piercing larvae with a 5 µl Hamilton syringe and collecting the exudate.

The protein content of all extracts was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. Extracts were used fresh or were frozen and stored at -20°C prior to use.

Electrophoresis

Extracts (30 µg protein) were separated on 10-20% gradient SDS discontinuous polyacrylamide gels by the method of Laemmli (1971, p. 22).

Sheep sera

Samples of sheep serum were generously donated by Margaret Broadmeadow, Department of Primary Industry, Queensland, Australia. Ten 3 year old wethers and two 3 year old ewes were taken from the field and maintained at the Department of Primary Industry laboratories. After a 6 month settling in period the animals were divided into two groups. One group of 6 wethers was subjected to implants of 4,000 wild type 1st
instar *L. cuprina* larvae over an 8 day period. The remaining 6 sheep were not struck and served as controls. Blood was collected approximately 8-10 days after the strike. Blood was also obtained from a single 3 year old previously unstruck wether maintained at the Division of Entomology, CSIRO, Canberra. This animal was then struck experimentally with 1,000 1st instar larvae and blood was collected 10 days after the strike. Blood was also obtained from 2 sheep struck naturally in the field.

**Immuno-dot blot assay**

Larval extracts were serially diluted with standard phosphate buffered saline (PBS, 150 mM NaCl, 40 mM phosphate, pH 7.4). 2 µl samples of each dilution (covering the range 250 - 0.5 ng protein) were dotted in rows on sheets of nitrocellulose. These sheets were then processed largely as described by Burnette (1981) for Western immuno blots. To determine whether the serum from struck sheep (or controls) contained antibodies against components of the larval extracts, each nitrocellulose sheet was incubated in a 1:200 dilution of struck or control sheep serum in Tris-saline (10 mM Tris-HCl, pH 7.4, 0.9% NaCl). After washing in Tris-saline each sheet was then incubated in a 1:600 dilution (in Tris saline) of rabbit anti-sheep immunoglobulin antiserum (Pel Freez, Arkansas). The presence of bound antibody was then detected using *Staphylococcus aureus* protein A radiolabelled with $^{125}$I as described by Erlich et al. (1979). Nitrocellulose sheets were exposed to x-ray film at $-45^\circ$C in Cronex x-ray cassettes fitted with lightening plus intensifying screens.

**Western immuno-blotting**

Western blotting of proteins from polyacrylamide slab gels onto nitrocellulose was undertaken in a 25 mM Tris, 192 mM glycine, 20%
methanol buffer (pH 8.3) using a Bio-Rad electrophoresis blotter at 40 V for 16 h (room temperature). Nitrocellulose sheets were subsequently processed as described above for immuno-dot blots.

**Incubation of larvae in anti-cuticle protein antiserum**

Ten groups of five 1st instar larvae (15 h ± 1 after egg laying) were incubated in glass vials containing 500 µl antiserum raised against 3rd instar larval cuticle proteins (Rabbit anti 3LCP) or control serum absorbed in cotton wool. The sera were fortified with 2% yeast extract plus 0.5% KH₂PO₄ as recommended by Green and Connole (1981) for larval survival. The number of larvae per vial which survived after 48 h at 25°C was assessed.
RESULTS

Larval excretions/secretions

A minimal amount of protein is detected in water in which frozen/thawed 3rd instar larvae are incubated (0.35 µg ± 0.03 per larva, n = 10). Approximately 100-fold more is found in the water in which live animals are incubated (37.0 µg ± 16.7 per larva, n = 9). Therefore the material introduced into the water by living larvae represents their excretions/secretions and contains little (surface or bacterial) contamination.

Immuno-dot blot assay

Sera from struck sheep clearly recognize all but one of the larval extracts. All sera react to the same relative degree with the various extracts (Figs. 4.1a, b). In each case the sera react most intensely with 3rd instar salivary gland extracts (1-2 ng salivary gland protein is clearly detected). Other 3rd instar extracts (excretions/secretions, mid gut or visceral homogenates) also react strongly; 4-8 ng of these extracts being detected with struck sheep serum. Another 3rd instar extract (larval haemolymph) as well as all 1st and 2nd instar larval extracts are lower in reactivity. At least 16 ng of protein is required for clear recognition. An extract of the 3rd instar larval cuticle is recognized by sheep sera only to a very low degree.

Serum from unstruck (control) sheep also react with larval extracts but the level of reactivity is generally 2-4 fold lower than that seen with struck sheep serum (Fig. 4.1c). Similarly the reaction of serum obtained from an animal before being experimentally struck is 4 fold lower than that of serum taken 10 days after fly strike (data not shown).
Fig. 4.1: Immuno dot blot analysis: Autoradiographs showing the reactivity of serum from a) sheep struck in the field, b) sheep struck in the laboratory and c) unstruck (control) sheep to *L. cuprina* larval antigens.

- **1E/S**: 1st instar excretions/secretions;
- **1V**: 1st instar visceral homogenate;
- **2E/S**: 2nd instar excretions/secretions;
- **2V**: 2nd instar visceral homogenate;
- **3E/S**: 3rd instar excretions/secretions;
- **3V**: 3rd instar visceral homogenate;
- **C**: 3rd instar cuticle extract;
- **S**: 3rd instar salivary gland extract;
- **H**: 3rd instar haemolymph extract;
- **MG**: 3rd instar mid-gut extract.
Electrophoretic profiles of larval extracts

Larval extracts were separated electrophoretically and stained with Coomassie blue (Fig. 4.2). The electrophoretic profile of 1st instar larval excretory/secretory material is very similar to that of 2nd instar larval excretory/secretory matter (Fig. 4.2, 1 E/S, 2 E/S). Similarly the profile of the 1st instar visceral homogenate (1V) is very similar to that of the 2nd instar (2V). The profile of a 3rd instar visceral extract (3V), however, (3V) differs from these. The proteins of 3rd instar internal tissue extracts (salivary gland, mid gut and haemolymph) are, of course, contained within the profile of the 3rd instar visceral homogenate but in lower amounts. 3rd instar excretory/secretory material (3 E/S) fails to resolve electrophoretically to give a discrete profile. However this material is highly reactive in immuno-dot and Western blot assays (see later) which suggests that the material may have a very low affinity for Coomassie blue. The profiles of all extracts (including that of 3rd instar excretory/secretory material) are highly reproducible.

Western immuno-blot analysis

Sera from struck sheep react with a wide array of components from all Western blotted larval extracts (Fig. 4.3a, b). The reaction to 1st instar excretory/secretory material and a homogenate of larval viscera is similar (Fig. 4.3, 1 E/S, 1V). Antibody reaction is most intense in three regions of these extracts. (If these antigens are pure protein they correspond to approximately 91, 72 and 45 kd components).

Reaction with 2nd instar extracts (excretory/secretory material, visceral homogenate) is similar in many respects to that with extracts of the 1st instar larvae. However the prominent 45 kd 1st instar antigens are evident only to a low extent in 2nd instar extracts. Many
Fig. 4.2: Electrophoretic profiles of larval extracts on an SDS, disc gradient gel.

1E/S: 1st instar excretions/secretions; 1V: 1st instar visceral homogenate; 2E/S: 2nd instar excretions/secretions; 2V: 2nd instar visceral homogenate; 3E/S: 3rd instar excretions/secretions; 3V: 3rd instar visceral homogenate; S: 3rd instar salivary gland extract; MG: 3rd instar mid-gut extract; H: 3rd instar haemolymph extract.
Fig. 4.3: Western immuno blot analysis: Autoradiographs showing the reactivity of serum from a) sheep struck in the field, b) sheep struck in the laboratory and, c) unstruck (control) sheep to larval extracts.

(MWM: Molecular weight markers, kDa)
1E/S: 1st instar excretions/secretions; 1V: 1st instar visceral homogenate; 2E/S: 2nd instar excretions/secretions; 2V: 2nd instar visceral homogenate; 3E/S: 3rd instar excretions/secretions; 3V: 3rd instar visceral homogenate; S: 3rd instar salivary gland extract; H: 3rd instar haemolymph extract; MG: 3rd instar mid gut extract.
of the prominent 1st or 2nd instar excretory/secretory proteins which stain with Coomassie blue do not appear to react with sheep sera. Thus the electrophoretic profiles of the extracts and the patterns of antibody reactivity are very different. In fact, this is a general phenomenon for all extracts.

Of the 3rd instar extracts the most intense antibody reaction is to the salivary gland preparation (as expected from the immuno-dot blot assay) (Fig. 4.3, S). While the antisera react with salivary gland components covering a wide molecular weight range, a cluster of about three components of approximately 43 kd is consistently and strongly recognised (Fig. 4.3, S*). A relatively intense reaction is also noted to an array of 3rd instar excretory/secretory components. A number of these, most notably the lower molecular weight group (20-26 kd) have counterparts in the mid gut extract (Fig. 4.3, MG). Antisera react to a low degree with a 3rd instar haemolymph extract. As expected the 3rd instar visceral homogenate contains all 3rd instar antigens but in lower amounts.

The reaction of different sheep sera to the various *L. cuprina* antigens often differs in relative intensity. In addition certain antigens (sometimes quite prominent ones) are recognized by some but not other sera (compare Fig. 4.3a and b, arrow).

Some Western blotted antigenic material is contained within the stacking gel although larval excretions/secrections appear to be relatively free of such material. However all extracts contain antigens which remain in the electrophoresis loading wells and do not enter the polyacrylamide gel.
Control sera react weakly with some larval antigens (Fig. 4.3c). Two groups of antigens however are prominently and invariably recognized. One group, in the 1st instar excretory/secretory material is of the order of 72 kd; the second, in the 3rd instar salivary gland extract, is approximately 159 kd.

**Incubation of larvae in anti-cuticle protein antiserum**

There is no significant difference between the number of larvae which survive incubation in anti-cuticle protein antiserum (2.4 larvae per vial ± 1.4) compared with the number surviving in control serum (2.9 larvae per vial ± 0.9).
DISCUSSION

When incubated in water, *L. cuprina* larvae excrete/secrete a range of components. Immuno-dot and Western immuno blot analysis demonstrates that some of the excretions/secretions are exposed, in nature, to effector elements of the sheep's immune response, resulting in the production of antibodies against them. Strong antibody reaction is seen in three regions of Western blotted 1st instar larval excretions/secretions, yet these regions do not equate with major Coomassie-staining groups. The major 1st instar antigens therefore, appear to be relatively minor components of the excretory/secretory mixture. As expected a 1st instar visceral homogenate also contains these antigens. No additional antigens are revealed in this extract.

While the electrophoretic profiles of 1st instar extracts are similar to their 2nd instar counterparts, the pattern of antibody reactivity differs somewhat between the instars. Most notably the prominent 45 kd group of 1st instar antigens are no longer present (or are present to a considerably lesser extent) in 2nd instar extracts. Presumably these antigens play an important role during the life of the 1st instar larvae but are no longer necessary for 2nd instar larvae. The antigens may represent proteolytic enzymes (keratinases or collagenases, perhaps) which may be important in ensuring a continuing food supply for the parasites or in facilitating the invasion of the host's skin by the larvae.

Sera from struck sheep recognize a complex array of 3rd instar larval components. A salivary gland extract reacts most intensly with all antisera. This lends support to the view that oral secretions (derived from the salivary glands) act as prominent antigens not only in the case of haematophagous (blood feeding) arthropods but also in the
case of invasive arthropods such as *L. cuprina* (Barriga, 1981).

Sera obtained from struck sheep also react strongly with material excreted/secreted by 3rd instar larvae. Some of the antigenic excreted/secreted material is obviously derived from the larval alimentary tract since many antigens contained within the excretory/secretory mix have counterparts in an extract of the 3rd instar mid gut. Other major antigens, found only in the excretory/secretory mix, may be derived from portions of the alimentary tract other than the mid gut. No major salivary gland antigen is detected in the excreted/secreted material although some minor antigens may be present. It is noteworthy that in the blowfly *L. sericata* (Diptera, Calliphoridae) many proteolytic enzymes (tryptase, peptidase and collagenase) and lipolytic enzymes are detected in extracts of larval alimentary tracts or larval 'excreta' but not larval salivary glands (Hobson, 1931a, b). Similarly in *Cordylobia antropophaga* proteolytic enzymes are present in gut extracts but not in extracts of salivary glands (Blacklock *et al.*, 1930). In these insects, larval excretions may be of greater importance than salivary secretions in providing a suitable environment for larval survival and development.

In the study of Blacklock *et al.* (1930), using the ring precipitin test, no reaction was detected between serum obtained from animals infected with *C. antropophaga* and a salivary gland preparation, but a reaction was clearly seen with larval 'excreta'. Similarly, serum taken from rabbits infected with *Cuterebra buccata* reacted strongly with a larval alimentary tract preparation (Weisbroth *et al.*, 1973). In these and in the present study, it is clear that the gut of invading parasitic larvae is invariably a rich source of antigens.
Sera obtained from struck sheep react to a relatively low degree with 3rd instar larval haemolymph. In contrast strong reactions are seen when the sera of animals infected with *C. antropophaga* or *C. buccata* are tested with 3rd instar larval haemolymph (Blacklock *et al.*, 1930; Weisbroth *et al.*, 1973). It is possible that immune, or other, mechanisms result in the death *in situ* of some parasites resulting in the subsequent release of larval somatic material (e.g., haemolymph). An antibody response could then be mounted against the exposed somatic components. This has been proposed to occur in the case of infection of rabbits with the myiasis-causing larvae of *Hypoderma bovis* and *H. lineatum* (Bouard and Weintraub, 1973). The lower antibody response in the case of *L. cuprina* may be due to the exposure of the host to relatively low levels of haemolymph or to a lower immunogenicity of the *L. cuprina* haemolymph components.

In addition to the salivary glands and the alimentary tract, the cuticle might also be suspected of being a potential source of antigens. The cuticle of *L. cuprina* is shed twice *in situ* during development and proteins can easily be extracted with water from isolated cuticles. In the case of parasitic nematodes whose cuticles (like those of all insects) are derived from secretions of the epidermal cell layer, it is believed that the cuticle is a major source of antigens (Ogilvie and Mackenzie, 1981; Parkhouse and Ortega-Pierres, 1984). However the reaction of serum from struck sheep with a 3rd instar larval cuticle extract is barely detectable. This may mean that shed cuticles (exuviae) contain little extractable protein (much having been reabsorbed during the moulting cycle) and that (unlike nematodes) there is no shedding or turnover between ecdyses of the components of the outer region of the insect's cuticle (epicuticle). In the case of
C. antropophaga and also C. buccata, no reaction between host serum and a cuticle extract is seen (Blacklock et al., 1930; Weisbroth et al., 1973).

The fact that sera from control sheep also react with larval extracts (but to a lesser degree) suggests that some of these sheep may have been previously exposed to L. cuprina. This phenomenon has been noted in other studies involving tissue-invading insects (Elliott et al., 1980; Robertson, 1980). It is known that apparently unstruck and overtly healthy sheep can sometimes have small pockets of larval infestation which are difficult to detect. This is known as covert strike. Sera from control (and struck) animals react prominently with a collection of 72 kd 1st instar excretory/secretory antigens and 159 kd 3rd instar salivary gland antigens. In general, control sera react minimally with most other larval components indicating that the response to the antigenic groups just mentioned is prolonged and suggests that these antigens are worthy of further study.

As discussed in the introduction to this chapter, it is clear that the hosts of many myiasis-causing flies can become resistant to fly strike (Blacklock and Gordon, 1927; Borgstrom, 1938; Gingrich and Barrett, 1976; Gingrich, 1980). The exact relationship between such acquired resistance and the host's immune response has not been identified. In many cases anti-parasite antibodies have been detected in the sera of hosts (Boulard et al., 1970; Robertson, 1980; Barriga, 1981). For instance, rats which develop strong resistance to C. fontinella have high anti-C. fontinella antibody levels in their blood (Pruett and Barrett, 1983). Whether this humoral response is protective remains unclear since infected mice also have elevated antibody levels yet they remain susceptible to reinfection (Pruett and Barrett,
Moreover Gingrich (1982) finds no correlation between circulating antibody levels (detected by a passive haemagglutination assay) and resistance in cattle to *H. lineatum* and suggests that components of the cellular immune response play a more important role in the acquisition of resistance.

Sheep with myiasis caused by the larvae of *L. cuprina* also possess elevated serum antibody levels relative to uninfected controls (O'Donnell et al., 1980; Sandeman et al., 1985). The present study has demonstrated that many such antibodies are directed against a wide array of components from all larval instars. The full range of antigens has not been completely revealed since some do not enter the resolving polyacrylamide gel but remain in the sample loading well or in the stacking gel. In fact additional antigens might be observed if wild flies from the field were used as the source of antigen in immunological assays (Elliott et al., 1980).

Whether the response of sheep to some or all of the many *L. cuprina* larval antigens detected in this study results in any impairment of larval development is unknown. Certainly infection of previously struck sheep with *L. cuprina* does not result in a decrease in survival of the reinfected insects (Sandeman et al., 1985). In fact, it has been suggested that host immune inflammatory reactions may be injurious to host tissues and may create better conditions for parasite survival (O'Donnell, 1979; O'Donnell et al., 1980; Broadmeadow et al., 1984). Nevertheless, the suggestion that previously exposed animals do possess some form of protection is supported by the finding that reinfected animals have significantly smaller wounds than sheep infected for the first time (Sandeman et al., 1985). Moreover while sheep immunized against *L. cuprina* (by the injection of a homogenate of 3rd instar
larvae) remain susceptible to reinfection, serum from these sheep disrupts the development of larvae incubated in it in vitro (O'Donnell et al., 1981). Preliminary observations also suggest that reaginic antibody may have a protective role for sheep (Sandeman et al., 1985). It should also be noted that young sheep are more susceptible to fly strike than older animals, which could reflect the acquisition of immunity following a previous strike. On the other hand, this might be due simply to change in the biochemistry of the epidermis as the animals mature.

Whether the larvae of *L. cuprina* can impair or evade host immunological responses in a manner similar to other parasites is also unknown (Wikel, 1982, 1984; Barriga, 1981; Cohen, 1982). It is possible that *L. cuprina* prevents a protective antibody response from being mounted against those important proteins which, when bound by antibody, might result in the disruption of larval development, by exposing to the host a very wide variety of unimportant antigens. In the case of other parasites and many tumours (metabolic parasites) it has been suggested that an array of unimportant antigens comprising a 'smokescreen' prevent the host's immune mechanisms from attacking the parasite (Barriga, 1981). In fact selective pressures would act on parasites to reduce the immunogenicity of vital antigens or, if possible, to conceal them. In this regard, and with vaccination in mind, it should be noted that the important antigens of *L. cuprina* larvae (if such antigens exist) may not be contained within the wide array detected with struck sheep serum in this study. Schlein and Lewis (1976) have clearly shown that by exposing *Stomoxys calcitrans* (Diptera, Muscidae) to antibodies directed against thoracic muscle (a tissue which would not normally be exposed to the host's immune response) it is possible to greatly disrupt the
development and decrease the survival rates of these parasites. In contrast, exposing *L. cuprina* larvae to antibody directed against the larval cuticle proteins (which are also, apparently, not exposed to the host's immune response) has no real effect on larval survival. These proteins are derived mainly from the procuticle and presumably antibody cannot penetrate either the epicuticle from the outside or the gut wall/epidermis from the inside to bind to them (even during ecdysis). Moreover, the 'simple' binding of antibody to antigen is no guarantee of any impairment of larval survival since such binding may not necessarily disrupt cuticular structure.

Clearly the humoral immune response of sheep to blowfly larvae is complex and whether the response, as documented in this chapter, assists the sheep which mounts it or the fly which stimulates it is, as yet, unresolved. Work on cellular aspects of sheep immunity and how cellular and humoral responses are integrated in struck sheep is required to increase our understanding of the immunological interaction of sheep and fly and to indicate how this interaction can be modified to help protect livestock.
CHAPTER FIVE

CLONING AND CHARACTERIZATION OF THE LARVAL CUTICLE PROTEIN GENES OF L. CUPRINA
INTRODUCTION

In *L. euprina* the epidermis directs four moults and secretes five cuticles all differing in form and composition. Each cuticle consists, in part, of a different set of extractable cuticle proteins (although some proteins are shared by the three larval instars).

The molecular mechanisms by which the epidermal secretions of invertebrates are temporally and spatially regulated are largely unknown. Of particular interest are the mechanisms whereby regulatory hormones interact with the genome to affect the cuticle. The changing pattern of epidermal secretions may serve as a model system for studying the principles which underly developmental gene regulation.

An understanding of the mechanisms of controlled gene expression must be based on a knowledge of gene structure and organization. To this end a number of genes coding for cuticle proteins have been cloned and characterized in two invertebrate systems to date: in the nematode *Caenorhabditis elegans* and the insect *Drosophila melanogaster* (Kramer *et al.*, 1982; Snyder *et al.*, 1981, 1982).

The cuticle of nematodes (a group of unsegmented worms) consists of an outer epicuticle and a number of morphologically distinct inner zones (Bird, 1984). This cuticle is largely a proteinaceous matrix with the proteins being cross-linked by disulphide bonds; cuticular chitin is not present (Cox *et al.*, 1981a). Most nematode cuticle proteins belong to that group of structural proteins called collagens. (Collagens are characterized by their triple helical form, by being composed of about 30% glycine residues and, commonly, 20-25% imino acid residues (proline and hydroxyproline) (Cox *et al.*, 1981b; Bird, 1984)). The cuticle of nematodes, like that of arthropods, is periodically replaced during the processes of moulting.
To clone the nematode cuticle protein genes the similarity between vertebrate and invertebrate collagens was exploited. A *C. elegans* recombinant DNA library was screened with a probe synthesised from a previously cloned chicken collagen gene. This resulted in the isolation of numerous nematode collagen genes. The nucleotide sequence of two of the genes has been determined. The genes are 67% homologous in DNA protein coding sequence and differ from chicken (and other vertebrate) collagen genes by being smaller and possessing fewer (1 or 2 as opposed to around 50) intervening sequences (introns). 50 or more cuticle collagen genes may be present in the genome of *C. elegans* (Kramer *et al.*, 1982).

Arthropod cuticle proteins are not collagens so to isolate the cuticle protein genes of *D. melanogaster* a different strategy was employed. This strategy is based on the knowledge that the primary function of the epidermis is to secrete the cuticle. Larval integuments were isolated late in the 3rd instar when a high proportion of the mRNA species of the epidermis code for cuticle proteins. Isolated integumental poly A+ RNA was used as a template to synthesise a cDNA probe and this was used to screen *D. melanogaster* recombinant DNA libraries. Clones which hybridized strongly to the cDNA probe were selected and rescreened by differential plaque hybridization. Clones which hybridized to the above probe and did not hybridize to cDNA probes synthesised using embryonic or pupal poly A+ RNA as template were isolated and characterized.

This characterization revealed that four of the major 3rd instar larval cuticle protein genes are clustered within 7.9Kb of DNA which maps to locus 44D (on chromosome two) of *D. melanogaster*. The genes are abundantly expressed in the 3rd instar but not in embryos, pupae or
adults. (2nd instar larvae also express the genes but only at about one
tenth the level of 3rd instar larvae). The protein coding DNA sequence
of gene I and II are extremely homologous (91%). Similarly genes III
and IV are highly (85%) homologous. Additionally, between genes I or II
and III or IV DNA homology greater than or equal to 59% exists (Snyder
et al., 1982). Not surprisingly the organization of such homologous
genes is similar. Each gene encodes a short (15 amino acid) signal
peptide within which lies a small intron (around 60bp in length) at a
conserved site. In addition the mRNA 5' untranslated regions are highly
conserved and contain an identical start sequence (ATCAGTC) 24-25
nucleotides downstream from the TATA box. (The TATA or Goldberg/Hogness
box is a DNA sequence common to almost all eukaryotic genes which lies a
short distance 5' to the mRNA transcription initiation site and plays
some role to ensure that RNA polymerase initiates transcription at the
correct site). The DNA sequence upstream from the TATA box of the
cuticle protein genes, in common with spacer segments between many
eukaryotic genes, is AT rich. The 3' untranslated regions have similar
poly A+ addition sequences and several other short homologous
segments. Because of the similarities between the four cuticle protein
genes it is suggested that this small gene cluster arose by the
duplication of a single ancestral gene (Snyder et al., 1982). The genes
encoding a fifth major larval cuticle protein and other more minor
proteins map to the third chromosome.

To obtain some insight into the structure and organization of the
larval cuticle protein genes of *L. cuprina* and to provide a basis for
future work on the regulation of their expression some of these genes
have been cloned. The cloning strategy follows that of Snyder et al.
(1981) and isolated *L. cuprina* clones are examined for homology to the
cloned cuticle protein genes of *D. melanogaster*. This chapter describes the cloning procedure and a preliminary characterization of the clones obtained, some of which contain cuticle protein genes.
MATERIALS AND METHODS

Construction and screening of the *L. cuprina* genomic DNA library

The construction of the library used in this study was carried out by A. Elizur and A.J. Howells in this laboratory using procedures described by Maniatis *et al.* (1982). Genomic DNA from the standard wild type strain of *L. cuprina* was partially digested with Sau 3A, size fractionated on an NaCl gradient and ligated into the Bam HI sites of the *λ*-derived cloning vector EMBL3A (Frischauf *et al.*, 1983). EMBL3A is a replacement vector in which polylinkers consisting of Sal I, Bam HI and Eco RI sites have been placed at each end of the 'non-essential', replaceable central region. When the Bam HI sites are used as the cloning sites the inserted DNA can be detached from the *λ* arms using Sal I. The recombinant DNA molecules were packaged *in vitro* and the resulting recombinant phage were plated on *E. coli* strain LE 392; a yield of approximately $3 \times 10^5$ pfu/ug genomic DNA was obtained.

The library was screened for recombinant phages containing inserts which code for larval cuticle proteins in a manner similar to that of Snyder *et al.* (1981). Unamplified preparations of the library were plated on large petri dishes (about 10,000 plaques/plate) and were lifted in triplicate onto nitrocellulose filters. The filters were denatured in 0.5M NaOH, 0.5M NaCl, neutralized in 0.5M Tris HCl (pH 7.4), 2M NaCl and then baked at 80°C under reduced pressure for 2 h. One set of filters was screened with a cDNA probe made using wandering phase larval epidermal RNA as template. In this way clones containing inserts which code for mRNAs abundantly expressed in the epidermis of wandering phase larvae were selected. (All of the major larval cuticle proteins are deposited by the wandering phase larval epidermis). As a negative control a second set of filters was screened with a cDNA probe made
using embryonic RNA as template. Embryonic RNA is not expected to be rich in mRNAs coding for cuticle proteins. Previous experience in this laboratory has shown that clones containing inserts which code for ribosomal RNAs can sometimes hybridize with cDNA probes made using total (as opposed to poly A⁺) RNA as template. This can lead to the isolation of ribosomal genes. To circumvent this possibility (and exploiting the fact that ribosomal genes tend to be conserved between species) the third set of filter lifts was screened with a nick translated probe synthesised from a clone containing the total 28S and about 2Kb of the 18S ribosomal genes of *D. melanogaster*. (This clone, denoted pDM238, was obtained from Dr. D. Glover, Department of Biochemistry, Imperial College of Science and Technology, London). Only those phage plaques hybridizing to the epidermal cDNA probe and not to the latter two probes were selected. Phage from these plaques were rescreened by differential plaque hybridization. Pure clones which hybridized with a greater intensity to the epidermal cDNA probe relative to the embryonic cDNA probe were obtained after two such rounds of rescreening. These clones carry genes which are expressed abundantly in mid 3rd instar epidermal cells and are designated the 'epidermis-specific clones' in this chapter.

Isolating larval integuments

3rd instar larval integuments of *L. cuprina* were isolated largely as described by Hirsh and Davidson (1981) for *D. melanogaster*. Each larva whose anterior (segments 1-3) had been removed was placed on and covered by a sheet of 3MM filter paper. Groups of 10 animals were then eviscerated by rolling a test tube with heavy arm pressure over them in a posterior-to-anterior direction. The integuments were quickly frozen in liquid nitrogen and RNA was later isolated from the epidermal cells
which adhere to cuticles obtained by this procedure.

Larval integuments were isolated from 3rd instar larvae at 48-54 h after egg laying (early feeding phase) and at 70-76 h after egg laying (early-mid wandering phase).

Nucleic acid preparation

(a) Isolation, electrophoresis and blotting of RNA

Total cellular RNA was prepared by the method of Chirgwin et al. (1979). Isolated integuments or 2-4 h old embryos were homogenized in 4M guanidinium thiocyanate, 1M β-mercaptoethanol, 0.05 M sodium acetate, 1% sarcosyl and 50 mM EDTA. The homogenate was made 2.9 M with respect to caesium chloride and the RNA was pelleted through a 5.7 M caesium chloride cushion by centrifugation at 35,000 rpm for 16 h (25°C) in the SW 50.1 rotor of a Beckman ultracentrifuge. The RNA recovered was not degraded to any marked extent since agarose gel electrophoresis clearly resolved the ribosomal RNA species (see Fig.5.10a). Also RNA samples demonstrated a high translation efficiency in wheat germ in vitro translation mixtures (see later).

Electrophoresis and blotting of RNA was carried out by the procedure of Thomas (1983). RNA samples, denatured in a solution of 75% (v/v) dimethylsulphoxide (DMSO), 1 M glyoxal, 10 mM sodium phosphate buffer (pH 7.0), were separated electrophoretically in a 1.5% agarose gel using 10 mM sodium phosphate buffer (pH 7.0). The gels were run at 150 V for approximately 3.5 h (4°C). DNA fragments of known size, in the range 14.4 - 0.5 Kb (denatured as for the RNA samples), served as size markers. RNA gels for staining were placed in a solution of 33 µg/ml acridine orange in gel buffer with shaking for 20 min and were destained in three changes of gel buffer over 16h. RNA gels for blotting were transferred (without staining) to nitrocellulose filters
(Northern blotted) using 20 × SSC. After transfer filters were baked at 80°C under reduced pressure. Filters were prehybridized for 16 h with shaking, in 3 × SSC, 10 × denhardtts (0.2% ficoll, 0.2% BSA, 0.2% polyvinylpyrrolidone), 0.1% SDS, 250 μg/ml salmon sperm DNA, 10 μg/ml poly A and 50% (v/v) formamide at 42°C. Hybridizations were performed for 16-20 h in fresh solutions under the same conditions with ³²P-labelled nick-translated probes. Following hybridization filters were rinsed with 2 × SSC and then washed with shaking in 2 × SSC, 0.1% SDS at 65°C for a total of 1.5 h with 6-8 changes of solution. Filters were then wrapped in Glad Wrap and exposed to x-ray film at -45°C in Cronex cassettes fitted with lightening plus intensifying screens.

(b) Isolation, electrophoresis and blotting of DNA.

Genomic DNA from the standard wild type strain of L. cuprina, prepared by the method of Miklos et al. (1984), was supplied by A. Elizur.

Recombinant phages were grown in the E. coli strain LE 392 in small or large scale liquid cultures and phage DNA was extracted using standard protocols (Maniatis et al., 1982). About 5 μg was recovered from 5ml preparations and 70-100 μg from 450 ml preparations.

DNA fragments from recombinant λ clones were subcloned into the plasmid pUC 13. The DNA fragments and the plasmid, cut with appropriate enzymes, were ligated using T4 DNA ligase (Genesearch) under standard conditions (Maniatis et al., 1982). E. coli strain JM 83 was made competent by CaCl₂ treatment and was then transformed with recombinant plasmids. Transformed (white) colonies were selected on L agar plates containing 50mg/ml ampicillin and 0.7 mg/ml of the chromogenic substrate x-gal (5-bromo-4-chloro-3-indolyl- β-D galactopyranoside; Sigma Chemical Co.). To prepare plasmid DNA cultures were grown
overnight in L broth plus 50 mg/ml ampicillin and plasmid DNA was isolated from 1.5 ml batches by the alkaline lysis method described by Maniatis et al. (1982).

Isolated DNA was digested with one or more commercially obtained restriction endonucleases in HAE buffer (10 mM Tris-HCl, pH 7.4, 7 mM MgCl₂, 0.8 mM dithiothreitol (DTT, Calbiochem), 200 µg/ml nuclease free BSA (BRL) and varying NaCl or KCl concentrations according to the enzyme suppliers' instructions. Digestions were usually for 2 h at 37°C.

Large DNA fragments (greater than 500 bp) were separated electrophoretically on 0.75 - 1% agarose gels; smaller DNA fragments were sometimes separated on 5% polyacrylamide gels. Phage λ DNA, digested with Hind III, to give rise to fragments in the size range 23-0.6 Kb, was separated in adjacent lanes and served as a size marker on agarose gels. Occasionally the plasmid, pBR322, digested with Alu I, was also used as a size marker. pBR322 DNA digested with Taq I, to give rise to fragments in the size range 1.4-0.1 Kb, was used as a size marker on polyacrylamide gels.

Agarose gel electrophoresis was routinely carried out in large (15 x 20 x 0.5 cm) gels using TBE buffer (89 mM Tris borate, 89 mM boric acid, 2 mM EDTA) at 30 V for 16 h (room temperature). Agarose mini gels (6 x 9 x 0.5 cm) were also carried out in TBE at 40 V for 2 h. Ethidium bromide (2 µg/ml) was added to mini gels and gel buffer. Acrylamide gel electrophoresis was undertaken in thin (1 mm) slab (18 x 16 cm) gels using TBE at 100 V for 4 h. Large gels were stained in a solution of 1 mg/ml ethidium bromide in water for 20 min and were rinsed twice in water prior to examination and photography under UV light.
DNA separated electrophoretically on agarose gels was transferred to nitrocellulose filters (Southern blotted) following the procedure of Southern (1975).

**Extraction of DNA fragments from agarose gels**

DNA digests were separated electrophoretically on agarose gels and the gels were stained with ethidium bromide as just described. The piece of gel containing a DNA fragment of interest was identified under UV light and was recovered using a scalpel. The fragment of agarose gel was then placed in a dialysis sac containing 400 µl TBE and the sac was put into an electrophoresis apparatus containing TBE. The DNA fragment was electroeluted from the gel at 100V for 1 h. To remove any DNA which may cling to the dialysis tubing the current was reversed for 1 min at the end of the procedure. The electroeluted DNA was recovered by ethanol precipitation of the liquid in the dialysis sac (i.e., the addition of 1/10 the volume of 3 M sodium acetate (pH 5.5) followed by 2 volumes of ethanol; the mixture was left at -45°C overnight). The DNA was pelleted by centrifugation (10 min, Eppendorf centrifuge), rinsed with 70% ethanol and then dissolved in a small volume (20 - 200 µl) of a suitable solution.

**Reprobing nitrocellulose filters**

Nitrocellulose filters which had been probed in the past were occasionally deprobed so that they could be used in further experiments. To deprobe a filter it was incubated with shaking for 20 - 30 min in a freshly prepared 20 mM NaOH solution at room temperature. The filter was then neutralized by treating it for 5 - 10 min in 0.5 M Tris HCl (pH 7.4), 2 M NaCl and then in 2 x SSC for a further 5 - 10 min. The filter was then ready for prehybridization and hybridization using a new probe.
DNA labelling

(a) cDNA synthesis

50 µg total RNA was used to synthesise each oligo-(dT) (Boehringer-Mannheim) primed cDNA probe. The RNA was denatured in sterile, deionized water containing 0.02 mg/ml oligo-(dT) by heating this mixture at 80°C for 90 sec and immediately chilling in ice-water. (Increasing oligo-(dT) concentration five-fold only marginally increased the amount of radiolabel incorporated into the probe [by 2-4%]). To the denatured chilled template/primer solution the following were added (final concentration): BSA (0.02 µg/ml) (nuclease free); Tris HCl (pH 8.3) (50 mM); DTT (20 mM); magnesium acetate (7.5 mM); dATP (0.1 mM); dGTP (0.1 mM); dTTP (0.1 mM); dCTP (0.02 µM); and 32P-dCTP (25 µCi). (32P-dCTP was obtained either from Bresa - 1700 Ci/mmol - or from Amersham - 3000 Ci/mmol). This solution was then mixed and to it 300 U AMV reverse transcriptase (Life Sciences Inc.) was added. The mixture was then incubated at 42°C for 60 min. After this time the reaction was stopped by making the solution 0.2 M with respect to EDTA and 0.4% with SDS. The cDNA formed was separated from unincorporated nucleotides by spun column chromatography. This was undertaken using mini (DC7) plastic columns (Amicon) which were packed first with 0.4 ml of 70 mg/ml Bio gel P 60 (50-100 mesh) beads (Bio-Rad) equilibrated in TE (10 mM Tris HCl, pH 7.4, 1 mM EDTA), 0.1% SDS and then with 2.5 ml of 70 mg/ml Bio gel P 60 (100-200 mesh) beads (Bio-Rad) equilibrated as above. Prior to applying the incubation mixture the columns were washed 3 times; 100 µl TE plus 0.1% SDS was added to the top of the column which was placed in a plastic centrifuge tube and centrifuged at 60 g for 30 sec (the final wash was carried out at 60 g for 90 sec). The cDNA incubation mixture was then added to the top of the column and an Eppendorf tube was placed
at its base. The column was centrifuged (60 g, 30 sec) and subsequently washed 3 times, each with 100 µl TE plus 0.1% SDS, (60 g, 90 sec) to elute the cDNA probe. The amount of $^{32}$P-dCTP incorporated into cDNA (routinely 50-60% of the added radiolabel) was measured by determining the radioactivity in an aliquot of the recovered probe, using a Beckman LS 100 radioisotope counter.

(b) Nick translation

Nick translated probes were made using a modified method developed in the laboratory of K.C. Reed in the Department of Biochemistry, ANU. To 'nick' the DNA 0.4 µg DNA was incubated in a 20µl solution of 50 mM Tris HCl (pH 7.5), 5 mM MgCl$_2$, 10 mM DTT, 0.02% BSA and 100 pg DNAase I (Bromeega Biotec) at 14°C for 90 min. For labelling, the solution was made 15 µM with respect to dGTP, dATP and dTTP; then 5 units DNA polymerase I (Pharmacia) and 25 µCi $^{32}$p dCTP were added (total volume: 40 µl). The solution was incubated at 14°C for 60 min. Nick translated probes were then separated from unincorporated nucleotides by spun column chromatography as described above. Routinely greater than 70% of the $^{32}$p dCTP was incorporated, giving probes with specific radioactivities of approximately $10^8$ dpm/µg DNA.

Hybridization conditions

Hybridization of cDNA probes to filter-bound DNA was performed as follows: Filters (maximally two/bag) were prehybridized in a solution of 50 mM Hepes (pH 7.0), 3 x SSC, 0.1% SDS, 10 x Denhardts, 1 mM EDTA, 50 mg/ml sheared salmon sperm DNA with shaking for at least 4 h at 60°C. $^{32}$p labelled probes were added to the bags after this time and hybridized for 16-20h under the same conditions. Following hybridizations filters were rinsed in 2 x SSC and then washed in 2 changes of 2 x SSC, 0.1% SDS at 60°C with shaking for 1 h. Filters were
briefly blotted on 3 MM filter paper to remove excess liquid and were then wrapped in Glad Wrap and exposed to x-ray film at -45°C (as described earlier).

Hybridizations involving nick translated probes synthesized from *L. cuprina* DNA (cloned or total genomic) or from the clone containing the ribosomal RNA genes of *D. melanogaster* were carried out as described above for cDNA probes. Hybridizations using nick translated probes synthesised from other *D. melanogaster* DNA (e.g., clones containing the larval cuticle protein genes or the dopa decarboxylase gene of *D. melanogaster*) were undertaken in a similar manner except that prehybridization and hybridization took place at 55°C and washing at 50°C.

**Hybrid release translation and immunoprecipitation**

DNA from epidermis specific clones was used to select homologous RNA sequences from the preparation of total (wandering phase) epidermal RNA largely as described by Snyder *et al.* (1981). 10 µg of cloned phage DNA in 10 x SSC was filtered onto a nitrocellulose disc (0.5 cm diameter) which was then washed (by filtration) 3 times with 2 ml 10 x SSC. The disc was dried and baked at 80°C under reduced pressure for 3h. Prior to prehybridization the filter was rinsed for 60 sec in boiling distilled, sterilized water to remove any loosely bound DNA. Filters were prehybridized in a solution of 70% formamide, 0.4 M NaCl, 0.01 M Pipes, pH 6.5 at 50°C for 1 h. This solution was then discarded and 50 µg total wandering phase epidermal RNA, in fresh prehybridization solution, was added to the filter. Hybridization took place at 50°C for 4 h. After this time the filter was washed 9 times with 1 ml 1 x SSC, 0.5% SDS at 65°C and then twice with 1 ml TE (pH 7.8). Bound RNA was then eluted by incubating the filter in 250 µl boiling sterile,
deionized water for 1 min. The recovered RNA was quick frozen using liquid nitrogen and 250 µl 4 M ammonium acetate and 5 µl of a 1 mg/ml preparation of *E. coli* tRNA (Boehringer-Mannheim) were added. Two volumes (1 ml) of ethanol was then added and the RNA was pelleted by centrifugation (10 min, Eppendorf centrifuge).

Translation of selected RNAs took place in a commercial wheat germ translation lysate (Amersham) with all of the recovered RNA being used in a single incubation. Optimal translation occurred in a solution containing (final concentration) 1 mM potassium acetate, 67 mM amino acid mix (minus L-methionine) (Amersham), 50 µCi of 1120 Ci/ m mol 35S-methionine (Amersham), 50% wheat germ lysate at 25°C for 1 h. (Taking the potassium ion content of the batch of wheat germ lysate used in these experiments into account the total potassium ion content per reaction was 90 mM). As a positive control 2 µg total wandering phase epidermal RNA was also translated *in vitro*. As a negative control an *in vitro* translation reaction was carried out without adding RNA to the mixture.

Immunoprecipitation of products translated *in vitro* using an antiserum directed against mature 3rd instar larval cuticle proteins (Rabbit anti 3LCP) was undertaken largely as described by Kessler (1975). PBS was added to an aliquot of each translation mixture (containing 100,000 cpm) to give a final volume of 50 µl. An equal volume of Rabbit anti 3LCP was added, and incubated at 4°C for 16 - 24 h. After this time 500 µl of a 10% (w/v) *Staphylococcus aureus* (Cowan I strain) suspension in NET buffer (100 mM NaCl, 5 mM EDTA, 50 mM Tris HCl, pH 7.4, 0.02% sodium azide) was added and the mixture was incubated at room temperature for 30 min. The *S. aureus*-immunoprecipitated complex was recovered by centrifugation (10 sec, eppendorf centrifuge)
and was washed 3 times by resuspension and centrifugation (as above) in 500 µl NET buffer plus 0.05% NP 40. Translated products were recovered from the final precipitated complexes by boiling for 2 min in 7M urea, 2% mercaptoethanol (for native gels) or in 2% SDS, 2% mercaptoethanol (for SDS, disc gels) followed by centrifugation (1 min, eppendorf centrifuge) to pellet the bacteria.

Translated products as well as immunoprecipitated translated products were separated electrophoretically on 10-20% gradient native or SDS, discontinuous gels prepared as described earlier (Chapter 2, p 22). To visualize the 35S-labelled translation products and immunoprecipitated translation products fluorography was undertaken largely as described by Hames (1981). After staining and destaining as described (p. 23) the gel, it was immersed in 400 ml DMSO and shaken for 30 min. The gel was then soaked in fresh DMSO for a further 30 min. After this time the gel was incubated in 4 volumes of 18% (w/w), 2,5'diphenyloxazole (PPO) in DMSO for 3 h and was then rinsed in a bath of running water for 2 h. The gel was dried under vacuum and exposed to a preflashed x-ray film at -45°C in a Cronex cassette fitted with a lightening-plus intensifying screen.
RESULTS

Selecting cuticle protein clones

Selection of clones was based on the relative intensity with which phage plaques hybridized to cDNA probes made using larval epidermal RNA ('epidermal cDNA probes') compared with probes made using embryonic RNA ('embryonic cDNA probes'). Of 80,000 phages screened, 20 were selected which hybridized more intensely to the epidermal cDNA probes. None of the 20 showed homology to the *D. melanogaster* ribosomal RNA genes. The selected phages were rescreened a further 2 times in the same manner and a final 13, which gave the greatest hybridization signal with the epidermal cDNA probe, were purified. These clones are regarded as carrying epidermis specific genes.

Characterization of the selected clones

(a) Mapping

DNA prepared from each of the 13 phages was digested with the restriction endonucleases Sal I, Eco RI and Bam HI, separated on agarose gels and stained with ethidium bromide to generate the maps given in Fig. 5.1. Four of the clones (designated \( \lambda LcLCP \ 1-4 \)) contain overlapping inserts. The size of corresponding fragments in each clone is not always constant. For example the largest Sal I - Eco RI fragment varies in size from 5.4 Kb in \( \lambda LcLCP1 \) to 4.5 Kb in \( \lambda LcLCP \ 4 \). A further 3 clones contain the same insert (\( \lambda LcLCP5a, b, c \), Fig. 5.1), which is different from the insert in \( \lambda LcLCP1-4 \); the remaining clones contain unique inserts (\( \lambda LcES \ 6-11 \)). Ethidium bromide staining of the DNA (digested and separated on an agarose gel) of two sample clones is shown in Fig. 5.2a.
Fig. 5.1: Restriction endonuclease maps of insert DNA from the epidermis specific clones.

S: Sal I; E: Eco RI; B: Bam HI.

The terminal Sal I sites are those of the vector EMBL 3A. (Three clones having exactly the same restriction map, λLoLCP 5, a, b and c, were obtained)

Fragments which hybridize to an epidermal cDNA probe are underlined in red (cDNA). Fragments which hybridize to D. melanogaster larval cuticle protein genes are underlined in blue (Dm). Fragments containing highly repeated DNA sequences are indicated (•••••).

---

1 Kb.

*About 5% of the phage which constitute λLoLCP 3 appear to possess a 250 bp deletion in the 1.6 Kb Sal I-Sal I fragment. This smaller fragment is visible in Fig. 5.2a and hybridizes with an epidermal cDNA probe (Fig. 5.2b).*
\(\lambda LcLCP\ 1\)

\[
\begin{array}{ccc}
S & E & S \\
\end{array}
\]

\(cDNA\)

\[
\begin{array}{ccc}
\text{Red line} & \text{Red line} & \text{Red line} \\
\end{array}
\]

\(Dm\)

\[
\begin{array}{ccc}
\text{Red line} & \text{Red line} & \text{Red line} \\
\end{array}
\]

\(\lambda LcLCP\ 2\)

\[
\begin{array}{ccc}
S & E & S \\
\end{array}
\]

\[
\begin{array}{ccc}
\text{Red line} & \text{Red line} & \text{Red line} \\
\end{array}
\]

\[
\begin{array}{ccc}
\text{Red line} & \text{Red line} & \text{Red line} \\
\end{array}
\]

\[
\begin{array}{ccc}
\text{Red line} & \text{Red line} & \text{Red line} \\
\end{array}
\]

\(\lambda LcLCP\ 3\)

\[
\begin{array}{ccc}
S & S & E \\
\end{array}
\]

\[
\begin{array}{ccc}
\text{Red line} & \text{Red line} & \text{Red line} \\
\end{array}
\]

\[
\begin{array}{ccc}
\text{Red line} & \text{Red line} & \text{Red line} \\
\end{array}
\]

\(\lambda LcLCP\ 4\)

\[
\begin{array}{ccc}
S & E & S \\
\end{array}
\]

\[
\begin{array}{ccc}
\text{Red line} & \text{Red line} & \text{Red line} \\
\end{array}
\]

\[
\begin{array}{ccc}
\text{Red line} & \text{Red line} & \text{Red line} \\
\end{array}
\]

\(\lambda LcLCP\ 5\)

\[
\begin{array}{ccc}
S & E & B & E & ES \\
\end{array}
\]

\[
\begin{array}{ccc}
\text{Red line} & \text{Red line} & \text{Red line} \\
\end{array}
\]

\[
\begin{array}{ccc}
\text{Red line} & \text{Red line} & \text{Red line} \\
\end{array}
\]
(b) Defining coding regions within the clones

The epidermis specific clones were digested with various restriction endonucleases, separated electrophoretically, blotted onto nitrocellulose and probed with a cDNA probe made using wandering phase larval epidermal RNA as template. Fig. 5.2b shows the pattern of fragments from clones \( \lambda LcLCP \) 2 and 3 which hybridize. These and fragments from the other clones which hybridize to this probe are underlined in red in Fig. 5.1 and must contain gene coding sequences. The same regions of the clones hybridize to a cDNA probe made using feeding phase larval epidermal RNA as template, although with a somewhat lower intensity.

(c) Repeated sequences within the clones

DNA from \( \lambda LcLCP \) 1, 2 and 5 were digested with the enzymes Sal I and Eco RI and were separated on an agarose gel as shown in Fig. 5.3a. These fragments were blotted onto nitrocellulose and probed first with an epidermal cDNA probe (Fig. 5.3b) and then (following deprobing) with a nick translated probe synthesised from total \( L. cuprina \) DNA (Fig. 5.3c). These and fragments from other clones which hybridize to the latter probe are indicated in Fig. 5.1 (---, above the fragment). Such fragments contain DNA sequences which are highly repeated in the fly genome. The intensity of hybridization to the various fragments is not uniform. It is likely that the greater the intensity of hybridization the more highly repeated within the genome are the DNA sequences within the fragments.

(d) Homology to cloned \( D. melanogaster \) larval cuticle protein genes

The organization of the cloned larval cuticle protein genes of \( D. melanogaster \) is illustrated in Fig. 5.4a. Clones containing these genes have been generously sent to this laboratory by Dr. N. Davidson,
Fig. 5.2: Southern blot analysis of epidermis specific clones.

\( \lambda LcLCP \) 2 and \( \lambda LcLCP \) 3 were digested with the restriction endonucleases indicated and separated on an agarose gel. (S, Sal I; E, Eco RI; B, Bam HI). (a) shows the gel stained with ethidium bromide and (b) shows the pattern of fragments which hybridize to an epidermal cDNA probe. (\( \lambda M \): phage \( \lambda \) DNA size markers, Kb)

\( \lambda M \) \( \lambda LcLCP \) 3 , \( \lambda LcLCP \) 2

\( \lambda LcLCP \) 3 , \( \lambda LcLCP \) 2
Fig. 5.3: Repeated DNA sequences within the epidermis specific clones.

$\lambda LoLCP$ 1, 2 and 5 were digested with Sal I and Eco RI and were then separated on an agarose gel and transferred to nitrocellulose. (a, d and g) show the ethidium bromide stained gel, (b, e and h) show the pattern of fragments which hybridize to an epidermal cDNA probe, (c, f and i) show the pattern of fragments which hybridize to a nick translated probe synthesised from total $L. cuprina$ genomic DNA. $\lambda M$: phage $\lambda$ DNA size markers, Kb.
Fig. 5.4: Homology between *L. cuprina* epidermis specific clones and cloned *D. melanogaster* larval cuticle protein genes.

a) Restriction endonuclease map of the region of the *D. melanogaster* genome containing larval cuticle protein genes I-IV (solid boxes) (from Snyder *et al.*, 1981). The region subcloned in this study (pDm 3E) and an artificial Eco RI site used in subcloning (E') are indicated.

DNA from all epidermis specific clones (numbered 1-11) were digested with Sal I and Eco RI and were then separated electrophoretically. (b) shows the ethidium bromide stained gel. (c) shows the pattern of fragments which hybridize to a nick translated probe synthesised from a DNA subclone containing *D. melanogaster* larval cuticle protein genes II and III (pDm 3E). (λM: phage λ DNA size markers, Kb).

A number of faint bands (e.g. those indicated; b·) represent incompletely digested DNA fragments. Some of these hybridize to the probe (c·). (The two top most fragments, which show hybridization in all cases, represent uncut phage DNA and the left arm of the cloning vector respectively. Hybridization is due to some homology between sequences in the left arm of EMBL 3A and in pUC 13).
a)

E

E

E

--- 1 Kb

E: Eco RI

b)

\[ \lambda M \ 1 \ 2 \ 3 \ 4 \ 5a \ 5b \ 5c \ 6 \ 7 \ 8 \ 9 \ 10 \ 11 \]

23.1

9.4

6.6

4.4

2.3

2.0

c)
California Institute of Technology, USA. Using these clones a 3 Kb Eco RI fragment containing cuticle protein genes II and III has been subcloned into pUC 13. This subclone is designated pDm 3E.

In a preliminary experiment in which Southern blotted L. cuprina genomic DNA, cut with Hind III, was probed with nick translated pDm 3 DNA, weak hybridization was detected to an 8.7 Kb fragment (data not shown). Despite this, when 80,000 phage plaques from the L. cuprina genomic DNA library were screened with this probe none gave a clear hybridization signal. However when the DNA from the epidermis specific clones selected by differential hybridization are digested, separated electrophoretically, blotted onto nitrocellulose and probed with this D. melanogaster cuticle protein gene probe, fragments from a number of clones hybridize (Fig. 5.4c). Of the 13 clones selected only \( \lambda LcLCP 1 \) show strong hybridization to the D. melanogaster genes and of these the largest Sal I-Eco RI (4.8 - 5.6 Kb) fragment of \( \lambda LcLCP 1 \) hybridize most intensely (Fig. 5.4c, 1-4). Regions of homology between larval cuticle protein genes II and III of D. melanogaster (pDm 3E) and the epidermis specific clones of L. cuprina are underlined in blue in Fig. 5.1.

Characterization of \( \lambda LcLCP 1 \)

\( \lambda LcLCP 1 \), as a representative of the four clones with overlapping inserts which show strong homology to the cloned D. melanogaster cuticle protein genes, was chosen for further detailed characterization. It appears likely that this clone contains genes coding for some of the major L. cuprina larval cuticle proteins characterized in Chapter 2.

(a) Hybrid release translation, immunoprecipitation

DNA from \( \lambda LcLCP 1 \) was used to select homologous RNA species from
total RNA prepared from integuments of wandering phase 3rd instar larvae. The selected RNA species were then translated *in vitro* in a wheat germ translation system.

A number of tests were used to determine whether the *in vitro* translation products of the RNAs selected by λLoLCP 1 DNA are cuticle proteins or cuticle protein precursors. Firstly the electrophoretic mobilities of the translated products were compared with the mobilities of mature larval cuticle proteins. The 5 major translation products (designated TP 1-5) migrate to the same region of a native gradient gel as do mature cuticle proteins (Fig. 5.5a, 3CP and TP2). Each band in this single dimension separation may represent more than one translation product. As expected the *in vitro* translation of wandering phase larval epidermal RNA alone results in the production of these, and other, translated products (Fig. 5.5a, TP1). Translation product 3 (and another minor translation product) are seen in translations in which no exogenous RNA is added (Fig. 5.5a, TP3). These translated products are therefore clearly derived from RNA contained within the wheat germ *in vitro* translation mix and are not cuticle proteins.

Immunoprecipitation assays also indicate that some translated products are cuticle proteins. An antiserum directed against a larval cuticle protein mixture (Rabbit anti 3LCP) immunoprecipitates TPs 1, 2 and, less clearly, 4 and 5 (Fig. 5.5a, IMM1, IMM2). λLoLCP 2, another of the four clones with overlapping inserts, gives an identical result to λLo LCP 1 in a hybrid release translation immunoprecipitation assay (data not shown). An additional minor translated product (TP 0) is immunoprecipitated from the *in vitro* translation mix obtained using total epidermal RNA (Fig. 5.5a, IMM1). The immunoprecipitated translated products obtained from this mix have also been applied to an
Fig. 5.5: Hybrid release translations using $\lambda LcLCP 1$ DNA: Identification of translated products by immunoprecipitation and electrophoresis. (All in vitro translations were carried out in a wheat germ translation system).

(a) Native gradient gel.

3CP: Electrophoretic profile of 3rd instar larval cuticle proteins; TOTAL RNA, TP1: Profile of products translated from total wandering phase larval epidermal RNA, (Major translation products are designated TP 1-5); Imm1: Translated products (from TP1) immunoprecipitated with Rabbit anti 3LCP. $\lambda LcLCP 1$, TP2: Profile of products translated from RNA selected by $\lambda LcLCP 1$; Imm2: Translated products (from TP2) immunoprecipitated with Rabbit anti 3LCP; CONTROL, TP3: Profile of products translated from endogenous RNA in the in vitro mix; Imm3: Translated products (from TP3) immunoprecipitated with Rabbit anti 3LCP. (All 'Imm' lanes have been exposed for longer periods than 'TP' lanes).
Fig. 5.5 cont'

(b) SDS, disc gradient gel.

MWM: Molecular weight markers, Kd.; 3CP: Electrophoretic profile of 3rd instar larval cuticle proteins;
TOTAL RNA, TP1: Profile of products translated from total wandering phase larval epidermal RNA;
Imml: Translated products (from TP1) immunoprecipitated with Rabbit anti 3LCP.
SDS disc gradient gel. Here these products resolve to give polypeptides of molecular weight 30, 29, 21, 20 and 18 Kd (Fig. 5.5b). Although these products fall within the molecular weight range of mature cuticle proteins they do not share electrophoretic mobilities with the mature proteins and therefore probably represent cuticle protein precursors.

(b) Detailed restriction mapping

As discussed earlier λLoLCP 1 contains regions both of unique sequence and repeat sequence DNA. In order to further define the positions of these regions with respect to one another λLoLCP 1 was subcloned and mapped more fully.

In addition to Sal I and Eco RI, λLoLCP 1 is also cut by the restriction endonucleases Bgl II and Hind III. These sites have been incorporated into the map of this clone given in Fig. 5.6a.

The 5.4 Kb Sal I-Eco RI fragment of λLoLCP 1 which shows strong hybridization to the larval cuticle protein genes of D. melanogaster (and also contains repeated sequence DNA) was subcloned into pUC 13. This subclone is designated pLCP 5.4 S/E. The insert of pLCP 5.4 S/E is cut by the restriction endonucleases ACC I, Ava II and Hha I (Fig. 5.6b). The following restriction endonucleases do not digest the 5.4 Kb insert: Ava I, Hind II, Pvu I, Pvu II, Kpn I, Pst I, Xma III, Sph I, Hpa I, Bcl I, Eco RII, Sma I and Cla I.

The 3.2 Kb Sal I-Hind III fragment of pLCP 5.4 S/E was itself subcloned into pUC 13 and was further mapped with the restriction endonucleases Alu I and Sau 3A. The map of this subclone, denoted pLCP 3.2 S/H, is given in Fig. 5.6c.

(c) Location of coding regions and repeated sequences in the subclones

Digests of the subclones of λLoLCP 1 (pLCP 5.4 S/E and pLCP 3.2 S/H) were separated electrophoretically and blotted onto nitrocellulose.
Fig. 5.6: Restriction endonuclease maps of \(\lambda Lc LCP 1\) (a), and its subclones, pLCP 5.4S/E (b) and pLCP 3.2S/H (c).

Fragments containing protein coding regions are underlined in red. Regions containing repeat sequence DNA are indicated (•••••••).

S: Sal I; E: Eco RI; Bg: Bgl II; H: Hind III; Hh: Hha I; A: Alu I; Av: Ava II.
Map co-ordinates:

a) 1 Kb

b) 0.5 Kb

c) 0.25 Kb

\(\lambda LcLCP\ 1\)

\(pLCP\ 5.4\ S/E\)

\(pLCP\ 3.2 S/H\)
These were screened with a cDNA probe to determine which regions of the subclones contain coding regions and also with a nick translated probe synthesised from total *L. cuprina* DNA to show which contain repeated sequence DNA. Fragments which hybridize with the cDNA probe (and contain coding regions) are underlined in red in Fig. 5.6, while those that hybridize with the nick translated probe (and contain repeated sequence DNA) are indicated with a black broken line. Sample blots used to generate the data presented in Fig. 5.6 are shown in Fig. 5.71 and II. Note that the nick translated probe hybridizes intensely with the large internal (1.5 Kb) Sau 3A fragment of pLCP 3.2 S/H (Fig. 5.6c) suggesting that this fragment contains very highly repeated DNA sequences. A longer exposure of this Southern blot reveals a low degree of hybridization to the terminal 0.65 Kb Sau 3A-Hind III fragment. This terminal fragment also contains protein coding sequences.

In order to assess the level of repetition of the sequences in this 0.65 Kb fragment it was used to probe a Southern blot of Hind III cut *L. cuprina* genomic DNA. To generate the probe the fragment was cut out of an agarose gel, electroeluted and nick translated. The probe hybridizes to numerous DNA fragments spanning a wide molecular weight range suggesting that the DNA sequences within it are also highly repeated, probably in a dispersed fashion, through the fly genome (Fig. 5.8b). No band corresponding to the unique portion of the probe can be recognised.

(d) Relatedness of coding regions in the epidermis specific clones

To test whether sequences present in the 0.65 Kb fragment described above are present elsewhere in the epidermis specific clones all 13 clones were Southern blotted and probed with this fragment. The probe hybridizes to a number of fragments from *λLcLCP* 1-5 and *λLcES* 6 and 8 (Fig. 5.9b). In the case of *λLcLCP* 1 it is noteworthy that the 2.6 Kb
Fig. 5.7: Southern blot analysis of the subclones of \( \lambda LcLCP \) 1.

(I) pLCP 5.4S/E

pLCP 5.4S/E DNA was digested with Sal I, Eco RI and Hind III and was then separated electrophoretically and transferred to nitrocellulose.

(a and c) show the ethidium bromide stained gel (\( \lambda M: \) phage \( \lambda \) DNA size markers, Kb), (c) shows the pattern of fragments which hybridize to an epidermal cDNA probe, (d) shows the pattern of fragments which hybridize to a nick translated probe synthesised using total \( L. ouprîna \) genomic DNA.

Additional enzymes used to digest the subclone are indicated. Av: Ava II; E\(_{II}\): Eco RII.

* indicates pUC 13 DNA.
(I) pLCP 5.4S/E

a)  

b)  

c)  

d)  

λM  Av  Av  EII  EII

23.1 -  
9.4 -  
6.6 -  
4.4 -  

2.3 -  
2.0 -  

0.6 -  

*  
*  
*  
*  

Fig. 5.7: (II) pLCP 3.2S/H

pLCP 3.2S/H DNA was digested with the enzymes indicated and was then separated electrophoretically and transferred to nitrocellulose. (a and d) show the ethidium bromide stained gel (pBRM: plasmid pBR322 (cut with the restriction enzyme Alu I) DNA size markers, Kb), (b) shows the pattern of fragments which hybridize to an epidermal cDNA probe, (c and e) show the pattern of fragments which hybridize to a nick translated probe synthesised using total L. cuprina genomic DNA.

S/H/Sa: Sal I/ Hind III/ Sau 3A;
H/Hh: Hind III/ Hha I.
p indicates pUC 13 DNA.
(II) pLCP 3.2S/H

a)

b)

c)

d)

e)
Fig. 5.8: Repeated sequence DNA within the 0.65 Kb fragment of pLCP 3.2S/H.

Hind III digested *L. cuprina* DNA was separated electrophoretically and blotted onto nitrocellulose. (a) shows the ethidium bromide stained gel. The blot was probed with a nick translated probe synthesised from the 0.65 Kb fragment of pLCP 3.2S/H. (b) shows an autoradiograph of this blot. (λM: phage λ DNA size markers. Kb)
Fig. 5.9: Homologous sequences within and between epidermis specific clones.

Epidermis specific clones were digested with Eco RI and Sal I and were then separated electrophoretically and blotted onto nitrocellulose.

(a) shows an ethidium bromide stained portion of the gel, (b) shows the pattern of fragments which hybridize to a nick translated probe synthesised from the 0.65 Kb fragment of pLCP 3.2S/H, (c) shows restriction maps of epidermis specific clones (as fig. 5.1). Fragments to which the 0.65 Kb probe hybridizes are indicated (---).

(λM: phage λ DNA size markers, Kb)
2: λLoLCP 2
8: λLoES 8
5: λLoLCP 5
1: λLoLCP 1

* The equivalent fragments of λLoLCP 3 and 4 (containing inserts which overlap those of λLoLCP 1 and 2) also hybridize to this probe.
c) 

\( \lambda LcLCP \ 1^* \)

\[ \begin{array}{cccc}
S & E & S & E & E & S \\
\end{array} \]

\( \lambda LcLCP \ 2^* \)

\[ \begin{array}{cccc}
S & E & S & E & E & ES \\
\end{array} \]

\( \lambda LcLCP \ 5 \)

\[ \begin{array}{cccc}
S & E & B & E & S \\
\end{array} \]

\( \lambda LcES \ 6 \)

\[ \begin{array}{cccc}
S & E & B & E & S \\
\end{array} \]

\( \lambda LcES \ 8 \)

\[ \begin{array}{cccc}
S & E & E & E & S \\
\end{array} \]

1 Kb
Sal I-Eco RI (Co-ord: 0 - 2.6, Fig. 5.6) and the 2.8 Kb Eco RI-Sal I (Co-ord: 2.6 - 5.4) fragments, which contain coding regions but appear to be free of repeats (Figs. 5.3 and 5.6a), nevertheless hybridize strongly to this probe. In contrast, the 3.4 Kb Eco RI - Eco RI (Co-ord: 10.8 - 14.2) fragment which contains repeated sequence DNA, hybridizes only to a very low degree. The 3 fragments which comprise λLoLCP 5 (each of which contains repeated sequence DNA) hybridize with this probe. In λLoLCP 5 both fragments which contain repeats also hybridize with the probe. These data are illustrated in Fig. 5.9c.

RNA transcripts encoded by subclones pLCP 5.4 S/E and pLCP 3.2 S/H.

Duplicate samples of total RNA isolated from the epidermis of feeding and wandering phase 3rd instar larvae were separated on an agarose gel and transferred to nitrocellulose. The nitrocellulose was cut into two strips and one strip was probed with a nick translated probe synthesised from subclone pLCP 5.4 S/E. The second strip was probed with a nick translated probe synthesised from subclone pLCP 3.2 S/H. The results of this experiment are shown in Fig. 5.10. Both probes hybridize to the same major components. In feeding phase epidermal RNA the transcripts are in the size range 500 - 680 nucleotides while in wandering phase epidermal RNA the transcripts are 500 - 600 nucleotides in size.

Homology between the cloned dopa decarboxylase gene of D. melanogaster and epidermis specific clones of L. cuprina

Since dopa decarboxylase is relatively abundant in wandering phase 3rd instar L. cuprina larvae (Turnbull and Howells, 1980) a preliminary experiment was carried out to determine whether any of the epidermis specific clones might carry the gene for this enzyme. DNA from the epidermis specific clones was Southern blotted and screened with a nick
Fig. 5.10: RNA transcripts encoded by subclones of \( \lambda \)LoLCP 1.

Wandering (W) and feeding (F) phase epidermal total RNAs were separated on an agarose gel and transferred to nitrocellulose. (a) shows the acridine orange stained gel and (b) shows the pattern of fragments which hybridize to a nick translated probe synthesised from the DNA of pLCP 5.4 S/E (a subclone of \( \lambda \)LoLCP 1). M: size markers, nucleotides.
translated probe synthesised from a 7.5 Kb Pst I fragment containing the complete dopa decarboxylase gene of *D. melanogaster* (Hirsh and Davidson, 1981). A clone containing this gene (denoted pDCC1046) was generously sent to this laboratory by Dr. J. Hirsh, Harvard Medical School, Boston, USA. Of the 13 epidermis specific clones, one, λLoES 6 hybridized strongly to this probe (Fig. 5.11b). Regions of the clone which hybridize are indicated in Fig. 5.11c. A further 2 clones, λLoES 7 and 10 show a weak degree of homology with the DNA fragment containing the dopa decarboxylase gene. The homologous regions in these clones are also shown in Fig. 5.11c).

**DISCUSSION**

Of the epidermis specific clones selected by differential hybridization in this study a number clearly contain genes which code for some of the major larval cuticle proteins characterized in chapter 2. The clone designated λLoLCP 1 selects RNA, in a hybrid-release translation assay, which translates to give rise to polypeptides of similar electrophoretic mobilities to cuticle proteins. That some of these translated products are indeed the cuticle proteins of interest is shown by the fact that an anti-cuticle protein antiserum (Rabbit anti 3LCP) immunoprecipitates them. This antiserum has been shown to be highly specific for cuticle proteins and to react only minimally with non-cuticular material (Chapter 2, p. 34). This clone, then, contains some of the larval cuticle protein genes of *L. cuprina*.

The existence of regions of homology between a number of epidermis specific clones (λLoLCP 1-5) and larval cuticle protein genes II and III of *D. melanogaster* provides further evidence that these clones contain *L. cuprina* cuticle protein genes. Larval cuticle protein genes I and II
Fig. 5.11: Homology between the dopa decarboxylase gene of *D. melanogaster* and epidermis specific clones.

Epidermis specific clones were digested with Sal I and Eco RI and were then separated electrophoretically and transferred to nitrocellulose.

(a) shows the ethidium bromide stained portion of the gel containing λLoES 6. (λM: phage λ DNA size markers, Kb). Incompletely digested fragments are indicated (·). (b) shows the pattern of fragments which hybridize to a nick translated probe synthesised from the DNA of pDDC 1046, a subclone which carries the dopa decarboxylase gene of *D. melanogaster*. (c) shows the restriction maps of selected epidermis specific clones (as fig. 5.1). Fragments which hybridize to the probe are indicated (······).
and larval cuticle protein genes II and IV of *D. melanogaster* are very similar in DNA sequence (Snyder *et al.*, 1982). Thus by using genes II and III (pDM 3E to probe the *L. cuprina* epidermis specific clones it is very likely that all regions of homology (covering the four cloned cuticle protein genes) between the two species have been detected. It has already been shown that the larval cuticle proteins of *L. cuprina* are immunologically related to many of their counterparts in *D. melanogaster* (Chapter 2, p. 33). The observed hybridization between cuticle protein genes of *D. melanogaster* and λ LoLCP 1-5 suggests that there must be extensive regions of highly conserved amino acid sequence in the proteins, since nucleotide homologies of at least 60% would be required to permit stable hybrids to form under the conditions used. It is noteworthy that a comparison of the white and scarlet eye colour genes of *L. cuprina* and *D. melanogaster* shows a high degree of nucleotide homology (60-70%) between the genes of both species and a higher degree (80-90%) of predicted amino acid homology (A. Elizur, A.J. Howells, *pers. comm*). This arises because of the degeneracy of the genetic code and third position changes in codons.

λ LoLCP 1-4 exhibit some differences in their restriction maps; for instance some overlapping fragments differ in size. This size variation may be a reflection of DNA sequence polymorphism within the fly population which has arisen through the insertion or deletion of DNA sequences within these regions. Alternatively the variation in size may represent *in vitro* artifacts. Other workers report that DNA fragments containing repeated sequences can sometimes delete during cloning (A. Elizur *pers. comm*). Regions of λ LoLCP 1-4 which contain repeat sequence DNA may, therefore, have deleted to varying degrees to give rise to fragments of different sizes.
The restriction map of $\lambda LcLCP$ 5 differs from those of the other clones showing homology to the $D. melanogaster$ larval cuticle protein genes. It seems unlikely that this clone can be regarded as an extreme polymorphic variant of $\lambda LcLCP$ 1-4. Presumably it represents a distinct (perhaps adjacent region) containing cuticle protein genes.

Characterization of $\lambda LcLCP$ 1

Since a number of products are translated from RNA selected by $\lambda LcLCP$ 1 and are immunoprecipitated by Rabbit anti $3LCP$ (TP 1, 2, 4 and 5) it is likely that the clone contains more than one cuticle protein gene. In $D. melanogaster$ the four cloned cuticle protein genes are clustered within 7.9 Kb of DNA (Snyder et al., 1981, 1982). In higher organisms generally groups of genes which are expressed simultaneously and have a common function tend to be located close to one another (e.g., histone genes, chorion genes, heat shock genes (Spradling et al., 1980; Corces et al., 1980)). However this is not always the case (e.g., actin genes, Fyrberg et al. [1980]).

Which of the larval cuticle proteins of $L. cuprina$ (LCPs 1-9) are encoded by the genes on $\lambda LcLCP$ 1 is unknown. This is because it is not possible to ascribe with certainty a particular immunoprecipitated translation product with a particular mature cuticle protein. One reason for this is that the translated products are not processed correctly in vitro. In $D. melanogaster$ the primary translation products of the cuticle protein genes, in common with all secreted proteins, possess a signal peptide. The same is certain to be the case with the cuticle proteins of $L. cuprina$. In mature cuticle proteins this signal sequence is enzymatically removed. Furthermore cuticle proteins may be modified in other ways in vivo before or after deposition into the cuticle. Thus unprocessed translated products are likely to differ
somewhat in size and charge and therefore in electrophoretic mobility from mature cuticle proteins.

The clone can, however, be divided into three broad coding regions: the central region (which extends from the Eco RI site at coordinate 2.6 to the Eco RI site at coordinate 10.8 (Fig. 5.6)) shows homology with cloned cuticle protein genes of *D. melanogaster*. (In fact fine scale restriction mapping shows that within this region both coding and non-coding DNA sequences can be found). In addition two further coding regions exist, one at each end of the clone, and these do not show homology to the *D. melanogaster* genes. The central zone undoubtedly contains some cuticle protein gene(s) of *L. cuprina* which have DNA sequences in common with cuticle protein genes of *D. melanogaster*. The terminal zones may contain cuticle protein genes specific to *L. cuprina*. Further hybrid release translation experiments using subcloned DNA from these various regions would help to clarify this issue.

RNA transcript data may aid in the localization of particular genes within these regions. Part of the region with homology to *D. melanogaster*'s cuticle protein genes was subcloned (pLCP 5.4 S/E) and was shown to hybridize strongly to epidermal RNA species 500-680 nucleotides in size. This indicates that genes on pLCP 5.4 S/E code for RNA species of this size. An RNA molecule of 680 nucleotides can maximally encode a protein of about 200 amino acids (approximately 20 Kd). Mature proteins derived from this RNA transcript may, following processing, be substantially smaller. A cluster of three larval cuticle proteins of *L. cuprina* are less than 20 Kd (a second major cuticle protein cluster is 23 - 28 Kd, Chapter 2, p. 28). This three protein cluster is comprised of LCP 1 and LCP 7 (both of these protein bands contain more than one polypeptide as revealed by 2D electrophoresis. The polypeptides of LCP 1 are of low molecular weight and one
polypeptide of LCP 7 belongs to the low molecular weight cluster, Chapter 2, p. 29). It may be, therefore, that pLCP 5.4 S/E contains the genes coding for this smaller cuticle protein group. LcLCP 1 (from which pLCP 5.4 S/E is derived) selects RNA which translates in vitro to give a series of proteins which are immunoprecipitated with Rabbit anti 3LCP. Three immunoprecipitated proteins are 18, 20 and 21 Kd and these may represent the precursors of the three proteins comprising the low molecular weight cluster. It is noteworthy that the larval cuticle protein genes of D. melanogaster which have been characterized encode small (less than 18 Kd) proteins and that the regions of homology between these genes and LcLCP 1 also appear to contain genes which code for the smaller cuticle proteins.

A further two immunoprecipitated translation products are 29 and 30 Kd. Presumably the genes coding for these proteins lie outside the sequences contained in pLCP 5.4 S/E; the larger RNAs required to code for these proteins are not detected in the RNA transcript experiments described. The terminal regions of LcLCP 1 (possessing coding regions with no homology to the cloned cuticle protein genes of D. melanogaster) may contain the genes coding for these larger proteins. The larger translation products may represent precursors of some of the proteins comprising the higher molecular weight cuticle protein cluster (i.e., LCP 2-6, 8, 9 or the high molecular weight component of LCP 7).

It is important to point out that due to a similarity in sequence LcLCP 1 may select mRNAs for proteins whose genes are not encoded by the clone.

The interrelationships of different regions of LcLCP 1 (and other clones) was investigated using the 0.65 Kb Sau 3A – Hind III fragment of pLCP 3.2 S/H (Fig. 6c). This fragment has been shown to contain both
unique and repeated DNA sequences. When the epidermis specific clones are electrophoretically separated, blotted onto nitrocellulose and probed with a nick translated probe synthesised from the 0.65 Kb fragment a number of fragments hybridize. Some such fragments (e.g., the 3.2 Kb Eco RI-Sal I fragment of \( \lambda \text{LoES} \) 6, Fig. 5.1) do not contain (epidermis specific) gene coding regions. Therefore hybridizations between these fragments and the probe is likely to indicate the presence of shared repeats on both DNA fragments. In contrast this probe also hybridizes to DNA fragments which contain gene coding regions which appear to be free of repeats, for instance the 2.6 Kb Sal I-Eco RI and 2.8 Kb Eco RI-Sal I fragments of \( \lambda \text{LoLCP} \) 1 (Figs. 5.1 and 5.9). It is likely that the hybridization in this case represents homology between gene coding regions in the fragments. Note that the 2.6 Kb Sal I-Eco RI fragment mentioned above is a terminal zone of \( \lambda \text{LoLCP} \) 1 which does not hybridize with the \( D. \text{melanogaster} \) genes. This suggests that the 0.65 Kb fragment contains DNA sequences which hybridize to the presumed \( L. \text{cuprina} \) cuticle protein gene(s) on the terminal fragment.

The available evidence suggests that a number of related gene coding segments are clustered on \( \lambda \text{LoLCP} \) 1. This clone appears to contain a number of related cuticle protein genes. The cuticle proteins of other invertebrates are also encoded by related genes. In the nematode, \( C. \text{elegans} \), two cloned cuticle protein genes, both being collagen genes, are highly homologous (Kramer et al., 1982). Similarly the cloned larval cuticle protein genes of \( D. \text{melanogaster} \) are very closely related in DNA sequence (Snyder et al., 1982).

The transition from feeding to wandering phase in the 3rd larval instar

The change in behaviour of 3rd instar larvae from feeding to wandering is, as pointed out earlier, accompanied by marked changes
associated with the cuticle. For instance a collection of proteins (LCPs 2-5) become prominent in the cuticles of wandering phase animals (Chapter 2, p. 32). It was suggested that a hormonal trigger might switch 'on' the genes coding for that collection of proteins at the transition. It follows therefore that in the epidermis of wandering phase animals the mRNAs for these proteins will be present in relatively high amounts but in the epidermis of early feeding phase animals these mRNAs will be absent. By virtue of the technique used in their selection the clones obtained in this study are specific to the epidermis of wandering phase animals. Among those clones, then, one or more should contain genes coding for cuticle proteins 2-5. Thus clones which hybridize with the cDNA probe made using epidermal RNA from wandering phase larvae but which do not hybridize with a cDNA probe made using epidermal RNA from early feeding phase larvae are expected to contain the genes for LCPs 2-5. However none of the selected clones exhibit this pattern of hybridization. Most clones hybridize with a somewhat lesser intensity to the feeding phase probe. This suggests that either none of the selected clones contain the genes in question or that the mRNAs for these proteins are, in fact, present in the epidermis of early feeding phase animals. It is possible to detect LCPs 2-5 in low amounts during the mid-late feeding phase (Chapter 2, p. 32), showing that the mRNAs are present and the genes 'on' at this later time. Thus the regulation of the deposition of these proteins into the larval cuticle at the feeding/wandering transition appears to involve controls other than a simple switching 'on' of a collection of cuticle protein genes.

In the Northern blot experiments in which epidermal RNA from feeding and wandering phase larvae was probed with nick translated
probes synthesised from subclones of \( \lambda LoLCP \) 1 (pLCP 5.4 S/E and pLCP 3.2 S/H) slightly different patterns of hybridization are observed in the two RNA populations. Whereas the epidermal transcripts of feeding phase larvae are 500-680 nucleotides in size those of wandering phase larvae are slightly smaller, 500-600 nucleotides. The wandering phase transcripts (and the equivalent sized feeding phase transcripts (500-600 nucleotides)) may represent more mature RNAs and the larger feeding phase transcripts (600-680 nucleotides) may represent unprocessed, immature RNA species. While it is unresolved whether \( \lambda LoLCP \) 1 contains any of the genes coding for LCPs 2-5, one form of post-transcriptional regulation of these genes at the feeding/wandering transition suggested by the transcript data above is that an increase in the rate of processing and translation of preformed mRNAs may occur.

Repeated DNA sequences in the epidermis specific clones

10 of the 13 epidermis specific genomic DNA clones selected in this study contain some highly repeated DNA sequences. The relatedness of the repeats found in the various clones is unknown. However the 0.65 Kb Sau 3A-Hind III fragment of pLCP 3.2 S/H which contains unique and repeated DNA sequence hybridizes only to \( \lambda LoLCP \) 1-5 and \( \lambda LoES \) 6 and 8. (Since \( \lambda LoES \) 6 and 8 are not cuticle protein clones the repeats of the 0.65 Kb fragment are not specific to regions carrying such genes). The repeated sequences found in the remaining clones clearly differ from the repeats of the 0.65 Kb fragment. When this fragment is used to probe a Southern blot of \( L. cuprina \) genomic DNA numerous DNA fragments hybridize. This suggests that the repeated sequences of the 0.65 Kb fragment are dispersed in many copies about the genome and do not exist in tandem arrays.
Many L. cuprina genes appear to be close to or surrounded by repeated sequence DNA. This is not just the case for cuticle proteins genes but also for other genes (e.g., eye pigment genes) studied in this laboratory (A. Elizur, A.J. Howells, *pers. comm.*). In this regard they differ from their counterparts in D. melanogaster. The relationship between the repeats and the coding regions of the larval cuticle proteins is unclear at present. In the case of the white and topaz genes (two genes which are involved in the production of eye pigments in L. cuprina) the dispersed repeats have been found to lie outside the genes. A similar situation may exist in the case of the cuticle protein genes. In addition repeated sequence DNA may lie in the introns of these genes. It is noteworthy that in the case of the white and topaz genes restriction fragment length polymorphisms are common, particularly in regions containing repeated sequence DNA (A. Elizur, A.J. Howells, *pers. comm*). This is also the case with LcLCP 1-4.

**Dopa decarboxylase**

A well documented effect of ecdysteroids on insects at the molecular level is their induction of the enzyme dopa decarboxylase (Fragoulis and Sekeris, 1975; Karlson and Sekeris, 1976). This enzyme plays an important role in sclerotization, being involved in the biochemical pathway for the production of reactive quinones from tyrosine (Chapter 3, p. 60). If ecdysteroids are involved in the changes associated with the cuticle at the feeding/wandering transition then the induction of dopa decarboxylase might be expected at this time. Certainly there is a peak in dopa decarboxylase activity in L. cuprina larvae which coincides with the cessation of larval feeding (Turnbull and Howells, 1980). Therefore among the epidermis specific clones selected by virtue of their hybridization to a wandering phase
epidermal cDNA probe the gene coding for dopa decarboxylase could be
found. One epidermis specific clone ($\lambda Lc$ ES 6) shows strong homology
to the dopa decarboxylase gene of $D. melanogaster$. It appears likely
therefore that $\lambda Lc$ ES 6 contains at least part of the dopa decarboxylase
gene of $L. cuprina$. As is the case with all of the epidermis specific
clones the intensity with which $\lambda Lc$ ES 6 hybridizes with a feeding phase
epidermal cDNA probe is similar to that with a wandering phase epidermal
cDNA probe. This is compatible with the fact that dopa decarboxylase is
found in early 3rd instar larvae (and in the earlier instars) where it
plays a role in epicuticle sclerotization (Turnbull and Howells, 1980)
and in the stabilization of the spiracles (tracheal openings) and
cuticular spines (Wolfe, 1954). The activity of dopa decarboxylase
increases dramatically at the time of pupariation (Turnbull and Howells,
1980) and so high levels of mRNA coding for this enzyme are expected in
the epidermal cells at this time. As yet the molecular mechanisms by
which the expression of dopa decarboxylase is increased at the
feeding/wandering transition or at pupariation are not known.

Future direction

In this chapter the cloning of some of the larval cuticle protein
genes of $L. cuprina$ has been described. The characterization of the
isolated clones containing these genes has been preliminary in nature
and a number of major questions about them are, as yet, unanswered. The
exact number and location of the cuticle protein genes, their
interrelatedness and their copy number in the fly genome are unknown as
is the organization of these genes relative to flanking sequences.
Experimental approaches toward answering some of these questions will be
considered in the final chapter.
CHAPTER SIX

GENERAL DISCUSSION AND FUTURE STUDY
The protein secretions of the epidermis

The epidermis of *Lucilia cuprina* exhibits a complex changing pattern of secretions during the fly’s developmental history. The epidermis directs cuticle formation, moulting and sclerotization.

The protein secretions of the epidermis represent the particular topic of much of this thesis. Some of the secreted proteins appear to be closely related to the cuticle proteins of other species. Immunological and molecular biological analysis suggests a high degree of homology between some larval cuticle proteins of *L. cuprina* and *D. melanogaster*. *D. melanogaster* and another dipteran, *Sarcophaga bullata* also contain some highly homologous proteins (Henzel *et al.*, 1985) and the cuticle proteins of many *Drosophila* species are related (Del Puerto *et al.*, 1985). Presumably the greater the evolutionary distance between any two species the less related are their cuticle proteins. Since *S. bullata* and *L. cuprina* belong to the same family within the order Diptera (Calliphoridae) whereas *D. melanogaster* belongs to a different family (Drosophilidae) it might be expected that the cuticle proteins of *L. cuprina* will be more similar to those of *S. bullata* than they are to those of *D. melanogaster*.

Many of the proteins which constitute the cuticles of different developmental stages of *L. cuprina* differ. All larval cuticles contain some shared proteins. Electrophoretic and immunological analysis suggests that the larval cuticle proteins differ from those of later developmental stages. Nevertheless a few larval proteins may be present in either pupal or adult cuticles. In other species too, the cuticle protein composition can vary between developmental stages (Roberts and Willis, 1980; Snyder *et al.*, 1981; Chihara *et al.*, 1982; Sridhara, 1983; Silvert *et al.*, 1984; Cox and Willis, 1985; Riddiford and Kiely,
1985). However proteins common to a number of developmental stages have been reported in some instances (Roberts and Willis, 1980; Cox and Willis, 1985; Riddiford and Kiely, 1985).

In *L. euprina* epidermal protein deposition into different anatomical regions of the cuticle of an individual can vary. For instance the cuticle of the head, thorax and abdomen of pupae or adults contain some different proteins. By contrast the proteins of the spine-containing and smooth larval cuticle differ only to a very minor degree. In other species cuticles from different anatomical regions often, but not always, contain different soluble proteins (Andersen et al., 1973; Chihara et al., 1982; Sridhara, 1983; Cox and Willis, 1985).

Within the cuticle of *L. euprina* it is not known whether proteins secreted by the epidermis into the epicuticle are the same as those secreted into the procuticle. In other species the proteins of the epicuticle may (Dennell, 1958; Karlson et al., 1969b) or may not (Karlson et al., 1969b; Welinder, 1975; Bordereau and Andersen, 1978) differ from those of the procuticle.

Within the larval procuticle of *L. euprina* the proteins hydrogen-bonded to chitin are of the same type as those which constitute the protein matrix of the cuticle. In other species this may (Roberts and Willis, 1980; Willis et al., 1981) or may not (Hackman, 1972, 1974a; Vranckx and Durlat, 1980) be the case.

This inability to say almost anything definitively about the general distributions of cuticle proteins may be a reflection not so much of the fact that relatively few arthropod species have been examined in this regard (and so no definitive trend has emerged) but rather that the huge numbers of arthropods in existence (from 3-30
millon species) suggest that many exceptions to every 'rule' will invariably exist. Each species may therefore have to be considered as possessing a cuticle specialized to its own living conditions.

One question of interest, although not investigated in this thesis, concerns the spatial distribution of the individual proteins within the cuticle. Within the larval cuticle of *L. cuprina*, for instance, is protein 9 (LCP 9) always associated with a specific site on the chitin microfibrils and in the protein matrix or has this protein got a random distribution throughout the procuticle? Is this protein present in the epicuticle? How is its distribution altered at the feeding/wandering transition when a further collection of proteins (LCP 2-5) are secreted into the cuticle? And are the latter group of proteins (secreted in large amounts about one third of the way through the final instar) only associated with the inner regions of the cuticle? One approach towards answering these questions is suggested by virtue of the fact that an anti LCP 9 antiserum has been raised in a mouse in this study. By sectioning larval cuticle, incubating it in mouse anti LCP 9 and then in an anti-mouse immunoglobulin antiserum (which has been labelled either with a radioactive isotope or a fluorescent dye) the site of binding of mouse anti LCP 9 (and therefore the site of protein 9 itself) can be visualized. For ultrastructural analysis the anti-antibody would be labelled with gold to facilitate studies using electron microscopy. In this way the location of the protein could be pinpointed and variations in its distribution both during development and in different cuticular zones could be examined.

To undertake the same task for other proteins it would be necessary to purify them and raise antibodies in a manner similar to that described in this thesis for LCP 9. This would be a difficult task.
given the similarities in size and charge of most of them. Alternatively monoclonal antibodies could be produced against the cuticle proteins. To produce monoclonal antibodies a cuticle protein extract would be injected into a series of mice. The spleens of those animals producing anti-larval cuticle protein antibodies (as determined by the immuno dot-blot assay described, p. 82) would be fused with a myeloma cell line and the resulting hybridomas cultured in vitro. Hybridoma lines secreting antibody would first be screened using the immuno dot-blot procedure. The individual cuticle protein against which the antibody of a given hybridoma line is directed would be determined by Western immuno blotting (using a complete cuticle protein extract as the source of antigen).

The possession of a set of specific monoclonal antibodies might also facilitate more detailed studies on the properties of the different cuticle proteins. Just as the possession of a purified protein will allow the production of an antiserum against it, so the possession of a specific antiserum will facilitate (theoretically) the purification of the protein. Affinity columns can be prepared using the monospecific antisera. A crude cuticle protein extract can then be passed through the column and the protein of interest will bind to its antibody. Other proteins, having no affinity for the antiserum used, pass through the column. Following the elution of the bound protein amino acid analysis, sequencing, conformational or other studies can be undertaken on it. In addition, the relatedness of the various cuticle proteins could be examined by using monoclonal antibodies to look for shared epitopes on the proteins.
given the similarities in size and charge of most of them. Alternatively monoclonal antibodies could be produced against the cuticle proteins. To produce monoclonal antibodies a cuticle protein extract would be injected into a series of mice. The spleens of those animals producing anti-larval cuticle protein antibodies (as determined by the immuno dot-blot assay described, p. 82) would be fused with a myeloma cell line and the resulting hybridomas cultured in vitro. Hybridoma lines secreting antibody would first be screened using the immuno dot-blot procedure. The individual cuticle protein against which the antibody of a given hybridoma line is directed would be determined by Western immuno blotting (using a complete cuticle protein extract as the source of antigen).

The possession of a set of specific monoclonal antibodies might also facilitate more detailed studies on the properties of the different cuticle proteins. Just as the possession of a purified protein will allow the production of an antiserum against it, so the possession of a specific antiserum will facilitate (theoretically) the purification of the protein. Affinity columns can be prepared using the monospecific antisera. A crude cuticle protein extract can then be passed through the column and the protein of interest will bind to its antibody. Other proteins, having no affinity for the antiserum used, pass through the column. Following the elution of the bound protein amino acid analysis, sequencing, conformational or other studies can be undertaken on it. In addition, the relatedness of the various cuticle proteins could be examined by using monoclonal antibodies to look for shared epitopes on the proteins.
Regulation of the secretory programme of the epidermis

One fundamental question to arise from the documentation in this thesis of temporal and spatial changes in epidermal secretions is how the changes are coordinated. This question has been complicated by the suggestion that proteins, synthesised in tissues other than the epidermis, are nevertheless taken up by the epidermis and deposited into the cuticle. However in the case of *L. euprina* the immunological evidence presented in this study indicates that the epidermis is the sole source of the proteins deposited into the 3rd instar larval cuticle.

Whether proteins derived from other tissues are taken up from the haemolymph and are deposited into the pupal and adult cuticles of *L. euprina* has not been determined. In some dipterans, haemolymph proteins, rich in tyrosine, are incorporated into the pupal and adult cuticles where the tyrosine is suggested to play a cross-linking role in sclerotization (Scheller, 1982).

One aspect of the epidermal secretory programme which was highlighted in this study is that change which occurs at the feeding/wandering transition during the 3rd larval instar. Changes in larval behaviour and cuticle composition coincide at this time. Changes in cuticular phenoloxidases are also seen (M. Barrett *pers. comm.*) and other changes associated with the cuticle also probably take place. In view of the fact that larvae migrate into a more desiccating environment and the superficial and outer epicuticular layers (which may act as do the water-retaining and protective wax and cement layers of other insects) increase in thickness in older larvae (Filshie, 1970) it is likely that the deposition of the components of these layers increases toward the end of larval feeding. Other changes associated with the
integument which occur in *L. cuprina* larvae at the feeding/wandering transition include an increase in the dopa decarboxylase titre (Turnbull and Howells, 1980).

The precise physiological mechanisms which trigger this series of changes has not been determined. Perhaps neurological receptors in the gut or integument register that a critical weight has been achieved and signal the brain to initiate the transition. In *Rhodnius prolitus* the distention of the abdomen (e.g., after a blood meal) stimulates stretch receptors which signal the brain to initiate the moult (Wigglesworth, 1972). It has been suggested that ecdysteroids regulate various aspects of the feeding/wandering transition of *L. cuprina*. Neurological stimulation of the brain might result in the release of prothoracicotrophic hormone which would, in turn, trigger ecdysteroid release. In some species, and perhaps in *L. cuprina*, a peak in ecdysteroid titre coincides with this transition. In a *Calliphora* species the biosynthesis of the major haemolymph protein (calliphorin) ceases at the end of larval feeding 'when the ecdysteroid titre in the haemolymph arises for a short period' (Scheller, 1982). However, in other cases it appears that a decrease in ecdysteroid titre promotes cuticle formation (Fristrom *et al.*, 1982) or dopa decarboxylase synthesis (Hiruma and Riddiford, 1985). The transition from feeding to wandering may represent a very suitable time for studying ecdysteroid action since the effects observed are not complicated by the processes of moulting.

Ecdysteroids are thought to exert their effects in much the same way as vertebrate steroids (Riddiford and Truman, 1978). The hormone binds to a cytoplasmic receptor molecule in a target cell and is transported into the nucleus where the steroid/receptor complex
interacts with the nuclear chromatin (Richards, 1981). This may result in a conformational rearrangement of the chromatin and the transcription of genes. There is much evidence that active genes have an 'exposed' chromatin structure such that they are sensitive to digestion by the enzyme DNAase I (Macintyre, 1982). At the feeding/wandering transition a part of this 'exposed' DNA may encode larval cuticle proteins 2-5 (LCPs 2-5) (since these are deposited in relatively large amounts in wandering phase cuticles). However it is known that these proteins are present in the cuticle (at a low level) prior to wandering. Molecular biological data also suggest that the genes for LCP 2-5 may be expressed prior to wandering. The effect of the hormone therefore may be to increase their rate of transcription (perhaps by exposing a stronger promoter (i.e., a DNA binding site with greater affinity for RNA polymerase) or by initiating transcription of other groups of LCP 2-5 genes present elsewhere in the genome - if multiple copies of these genes exist). It is also possible that an increase in the rate of translation of LCP 2-5 mRNAs in the epidermis may occur.

It is necessary to establish conclusively that ecdysteroids do exert a fundamental control over the processes occurring in the cuticle at the end of larval feeding. It would be interesting to test whether the injection of feeding phase 3rd instar larvae with the hormone could induce the epidermis to deposit LCPs 2-5. Alternatively (or additionally) integuments from such larvae could be incubated in vitro in the presence of ecdysteroids and the effects on the rate of expression of LCPs 2-5 determined.

To gain a better insight into the molecular nature of both the feeding/wandering transition as well as other aspects of the changing secretory programme of the epidermis genes coding for some of the larval
cuticle proteins have been cloned. Preliminary analysis suggests that some of the clones isolated in this study (λLoLCP 1-4) may contain the genes coding for the smaller *L. cuprina* larval cuticle protein cluster (i.e., LCP 1 and a component of LCP 7) as well as some other larval cuticle protein gene(s). To verify this suggestion and further characterize these clones it is necessary to localize more precisely the positions of the genes on the cloned DNA. This may be undertaken by finer scale restriction analysis coupled with the screening of Southern blots with epidermal cDNA probes. Alternatively SI nuclease mapping or electron microscopy and R looping (as was carried out to help localize the larval cuticle protein genes of *D. melanogaster* (Snyder *et al.*, 1981)) could be undertaken. Once a unique sequence DNA fragment containing a cuticle protein gene is isolated it will be possible to examine the relatedness of the various genes more fully (by Southern blot analysis) and to localize their position (locus) on the chromosomes. This can be achieved by screening polytene chromosome preparations with a radiolabelled probe synthesised using individual cuticle protein genes. Detailed photographic maps of the banding patterns of polytene chromosomes obtained from the trichogen cells of *L. cuprina* have been prepared and a variety of genes have been positioned on these chromosomes using conventional cytogenetic techniques. By using the *in situ* chromosome hybridization technique it will be possible to locate the cuticle protein genes on the chromosomes and to determine whether they exist at more than one chromosomal site (the white eye colour gene was recently localized on the *L. cuprina* polytene chromosomes using the *in situ* hybridization technique, D. Bedo pers. comm.).
Once a cuticle protein gene is identified it can be subcloned into a sequencing vector (e.g. the bacteriophage M13) and its DNA sequence can be determined. Then, to correlate a particular gene with the cuticle protein it encodes, the gene-containing DNA fragment could be used in a hybrid release translation assay (as described p.107) followed by the immunoprecipitation of the translated product(s) with a monospecific antiserum (as previously discussed p. 130). Ideally the amino acid sequence predicted from the DNA sequence of the coding region of the gene would be compared with the amino acid sequence of the purified protein (see p.130).

Such a fundamental understanding of the cloned larval cuticle protein genes is necessary before an examination of the regulation of the genes can begin. It is not known whether the dispersed, repeated DNA sequences identified in many epidermis specific clones exert a regulatory role. If they do how does this regulation compare with the that exerted on similar epidermally controlled events in D. melanogaster - an organism with a genome size about one quarter that of L. cuprina and whose genes are not bounded by such repeated sequence DNA?

Also of interest is the nature of the sequences in the remaining isolated clones which contain genes that are expressed in the epidermis of 3rd instar larvae. Some clones may contain genes which encode other major larval cuticle proteins which show no homology with the cloned D. melanogaster cuticle protein genes. One clone (\( \lambda \) LoES 6) appears to contain at least part of the dopa decarboxylase gene. Other clones may contain genes which code for membrane or transport proteins or for other enzymes used in the conversion of tyrosine to the reactive quinones which function in cuticular stabilization e.g., phenyloxidase, dopamine N-acetyl transferase (see Fig. 3.1). Some clones may carry genes
which encode enzymes involved in chitin synthesis (see Fig. 1.10) since the deposition of chitin continues throughout the 3rd larval instar (Retnakaran and Hackman, 1985).

The eradication of the sheep blowfly

The problem of fly strike in sheep is a man made one and, as such, could perhaps be reduced by a change in farming practices. An increase in the number of mixed farming operations and the adoption of the principle of more farmers stocking less sheep could lessen the incidence of fly strike. Nevertheless the eradication of the fly under present farming conditions is seen as an important objective by agriculturalists and many measures are being investigated toward this end.

One measure designed to prevent fly strike is the vaccination of sheep against larvae. With a view toward vaccination one aspect of the immunological interaction of sheep and larvae was examined in this study. The pattern of larval antigens which stimulate the sheep's immune response and which change between (and perhaps within) larval instars was documented. Overall a large number of such antigens exist, demonstrating that the humoral immune response of sheep to *L. cuprina* larvae is a complicated one. Since it is not known which, if any, of the various antigens are protective (i.e., induce a host protective immune response) the development of a vaccine will not be an easy task. However a host protective immune response does appear to be mounted naturally against other myiasis-causing larvae (and perhaps, although this has not been shown conclusively, also in the case of *L. cuprina*) (Blacklock and Gordon, 1927; Borgstrom, 1938; Gingrich and Barrett, 1976; Gingrich, 1980). In no instance does such a protective response result in the production of sterile (complete) immunity. Nevertheless, if a host protective antigen can be located in one of
these myiasis-causing flies and its equivalent isolated from *L. cuprina* then vaccination of sheep may be possible. An antigen not normally exposed to the sheep's immune system (perhaps derived from the larval gut or salivary glands) may also evoke a protective response. Recombinant DNA techniques may be useful in the longer term to obtain such a protective antigen in sufficient quantities for use in vaccine development and production.

Fundamental research such as much of the present study on more general aspects of fly biochemistry and physiology may also lead to the development of new fly control measures. Locke (1976) emphasises a number of features associated with the integument which may be important in the future development of insect control. These include not only the importance of the integument with regard to insecticide penetration but also the means by which insects control both their surface microflora (some of which may be pathogenic) and the action of their integumentary enzymes. Turnbull *et al.* (1980) have shown that certain dopa decarboxylase inhibitors can disrupt epicuticle formation in *L. cuprina* larvae thereby exerting a larvicidal effect. Thus studies on the biochemistry of the epicuticle may provide further insights into potential fly eradication mechanisms.

However before initiating any widespread eradication programme it is important to consider the ecological implications of such a programme. The effects, for example, of fly eradication on other invertebrate (e.g., spider) or vertebrate (e.g., bird) populations or the effects of increased sheep numbers on plant growth, soil quality and wildlife habitats. Overgrazing in the past has cleared plant cover, damaged the soil and rendered many areas in Australia unsuitable for sheep (Ryder, 1983). One final, and most important consideration, is
whether there are further sheep parasites (other fly species, for instance) which would assume an economic importance following the elimination of *L. cuprina*.

**The moult cycle and growth in arthropods**

As emphasised throughout this thesis the life of an arthropod is divided into a series of discrete developmental stages or instars, each involving the secretion of a new cuticle (moulting) and the shedding of an old cuticle (ecdysis). An interesting and important final question to consider is why such a growth pattern exists, particularly in soft bodied insects such as the larvae of *L. cuprina*.

Conventionally moultting/ecdysis is said to accommodate growth (Wigglesworth, 1972). In many other life forms growth is a more overtly gradual process achieved by regular cell division and enlargement. While these growth processes also occur in arthropods resulting in an increase in body weight throughout the growth phase of the life cycle, the question arises as to why a continual laying down of cuticular material does not also occur to accommodate an increase in size and thereby eliminate the need for a moult. Continual growth would surely be of survival value since an animal, especially in the later stages of moultting, is essentially helpless. It can neither feed nor move. The presence of a stiff, largely inextensible ecoskeleton in many arthropods (formed by the processes of sclerotization) is said to prohibit continual growth and to necessitate ecdysis. In these animals the increase in size occurs immediately after ecdysis and before sclerotization of the new cuticle. This is brought about by an increase in internal pressure which is achieved by muscular contraction and the uptake of air or water.
However, many arthropods, e.g., *L. euprina* larvae and the larvae of all holometabolous insects as well as argasid ticks etc., possess largely unsclerotized cuticles. Why then do these animals not grow continually, particularly as there is clear evidence that the cuticle does grow between molts? In the present study the deposition of material into the cuticle throughout the 3rd larval instar of *L. euprina* was recorded. Williams (1980) reports that the increase in weight of *M. sexta* during the 4th and 5th larval instar proceeds at the same relative rate as the deposition of cuticular chitin. If such deposited material is secreted only into the subcuticle then, in order for an arthropod to increase in size, the outer lamellae of the cuticle and the epicuticle would be required to stretch or unfold. Since a physical limit presumably exists on the amount of stretching/unfolding which is possible an animal would have to shed its stretched cuticle and secrete a new larger one to further increase in size.

However there is evidence to suggest that some arthropods can deposit cuticular material, not only at the epidermal surface, but also at the outer layers of the cuticle. In *Calpodes ethlius* larvae the distance between the outermost lamellae actually increases during the 5th instar despite concomitant cuticular stretching (Condoulis and Locke, 1966). Injected tritiated amino acids are seen by electron microscopy observations to be incorporated not only in discrete layers within the cuticles of these larvae but also in a more diffuse yet stable manner throughout the cuticle (Condoulis and Locke, 1966). It is suggested that the increased separation of the 1st formed lamellae during an instar is achieved as a result of material added in the diffuse form of incorporation. Such growth may also occur in the larval cuticle of *Spodoptera littoralis* (Lepidoptera) (Hepburn, 1978). In a
number of other instances the deposition of material into the outer endocuticular layers has been intimated to facilitate growth. The three-fold increase in thickness of the outer endocuticle of *Sarcophaga falculata* larvae despite its separation from the epidermis by the morphologically distinct inner endocuticle is reported to be an example of growth by intussception (Dennell, 1946). Amorphous material is secreted into the outermost lamellae of the post ecdysis cuticle of adult *Boophilus microplus* (Hackman and Filshie, 1982). Structural changes in the outer procuticle of *Podura aquatica* after ecdysis and changes in the appearance and relative sizes of inner and outer endocuticles of *Ixodes ricinus* females as they feed are suggestive of epidermal secretion into the outer cuticle (Noble-Nesbitt, 1963b; Lees, 1952).

In the case of *L. cuprina* larvae, while the cuticle reportedly does stretch to facilitate growth, it is clear that the epidermis exerts a direct control on, and deposits material into, the outer endocuticle. Histological techniques have revealed that a zone, rich in disulphide groups, exists immediately below the epicuticle of 3rd instar larvae (Hackman, 1971). Moreover this zone increases in thickness as the larvae age. Thus it appears that compounds containing disulphide groups pass through the procuticle to become deposited in the outer layers of the cuticle during the last larval instar (Hackman, 1971).

A number of links exist between the epidermis and the various layers of the cuticle which provide possible routes for these secretions. Pore canals, (or cuticular filaments) for instance, extend through the entire cuticle. The size, distribution and content of the canals differs between animals and in different regions of an individual (Kayser-Wegmann, 1976). Dermal glands, derived from the epidermis, also
provide channels through the cuticle. In some animals a number of morphologically distinct glands exist. While these glands reportedly provide the material of the cuticular cement layer there is evidence that their activity is not restricted to the time of current deposition. Moreover, while ixodid (hard) and argasid (soft) ticks both possess dermal glands only the argasids have a cement layer (Hackman and Filshie, 1982). These glands, therefore, may have more than one biological function. It is possible that many secretions pass through the body of the cuticle and not via any well-defined ducts. $^3$H-tyrosine, for instance, injected into 3rd instar *D. melanogaster* larvae accumulates at the inner surface of the cuticle and is subsequently 'transported' through the procuticle 'toward the epicuticle' (Mitchell *et al.*, 1971). This is seen in an animal lacking both pore canals and dermal glands. While the deposition of this radiolabel is thought to be involved, not in larval growth, but rather in the sclerotization process it does illustrate the control that the arthropod, via its epidermal cells, exerts on the outer cuticular regions. In fact the general hypothesis for sclerotization is that it occurs primarily in those cuticular regions farthest from the epidermis and progresses inward (Andersen, 1971; Hackman, 1974b). Similarly, the cross-linking of resilin (a structural cuticle protein found in some insects and which possesses elastic-like properties) molecules via di-, tri- and tetra-tyrosine groups occurs some distance from the cuticle/epidermis interface (Weis Fogh, 1970). Also, surface damaged cuticle can be repaired through the action of epidermal cells. These points illustrate that the arthropod cuticle, while it is a secreted product, is a dynamic entity and, unlike the epidermal secretions of annelids (soft bodied, segmented worms) or vertebrates, remains very largely within the
arthropod's metabolic pool.

Equipped with this control of the composition of its cuticle, a non-sclerotized insect should be capable of a continual increase in size. It has been suggested, however, that a barrier to continual growth still exists. Hackman (1975) reports that the ultimate size attainable by arthropods is determined by the epicuticle and Locke (1974) maintains that the cuticulin layer, within the epicuticle, determines the limitation of growth. Thus in blood-sucking insects and ticks which grow within an instar by expanding the integument the epicuticle is deeply pleated and simply unfolds as the animal expands. This expansion, it is said, continues to the limit of the cuticulin layer. If the cuticulin is sclerotized (Turnbull et al., 1980) then it is likely that moulting would be required to accommodate growth. Filshie (1976) however, noted that in B. micropius considerable growth of the cuticulin occurs even after the layer expands to form a continuous sheet. Based on changes in ultrastructural appearance Zacharuk (1972) inferred that a continual deposition of material into the epicuticle of elaterid larvae occurs between moulting periods. Epicuticular growth is also recorded during the expansion of termite queen cuticle (Bordereau, 1982). (While the growth of the termite queen epicuticle may be considered an extreme specializations (since the queen is a relatively large individual) the ultrastructural and histochemical characteristics of this epicuticle are identical to those of other insects). Therefore if not only the outer procuticular layers but also the epicuticle can grow then what necessitates moulting in soft-bodied arthropods?

It is possible that moulting in these animals is a consequence of having evolved from an ancestor which moulted. Onycophorans, which have
anatomical and developmental characteristics similar to those of annelids and arthropods (Boudreaux, 1979), are often regarded as a primitive link between the two groups. The cuticle of Onycophorans is similar to that of Arthropods; consisting of an epicuticle and a procuticle. These animals have tanned (sclerotized) terminal claws on their legs (Bordreaux, 1979) and may have a tanned region within the epicuticle (Weis-Fogh, 1970; Storch, 1984). Presumably the possession of tanned cuticular regions confers on Onycophorans some adaptive advantages but it also necessitates molting. Early arthropods (perhaps derived from an onycophoran-like stock) may have stabilized their cuticle to an even greater extent to provide a more energetically economical supporting structure. Thereafter a major adaptive radiation among the arthropods took place. Molting in the soft-bodied larvae of the more recently evolved holometabolous insects may be regarded therefore, as an evolutionary hangover. A measure of support for this view comes from the fact that the larvae of holometabolous insects undergo, on average, fewer molts than their more 'ancient' hemimetabolous or ametabolous counterparts (Williams, 1980). The acquisition of a soft cuticle by these animals may be viewed as an evolutionary device to minimize the number of molts which take place during the animal's life.
REFERENCES


