1987

Hemolymph Proteins: Origin And Developmental Changes In The Last Larval Instar Of Calpodes Ethlius (lepidoptera: Hesperiidae)

Subba Reddy Palli

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HEMOLYMPH PROTEINS: ORIGIN AND DEVELOPMENTAL CHANGES IN THE LAST LARVAL INSTAR OF *CALPODES ETHLIUS* (LEPIDOPTERA:HESPERIIDAE)

by

Subba Reddy Palli

Department of Zoology

Submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
April 1987

ISBN 0-315-36572-2

ABSTRACT

Sheets of dorsal abdominal integument, ligated tubes of midgut, sheets of fat body and suspended hemocytes from mid fifth instar larvae of Calpodes ethlius (Lepidoptera: Hesperiidae) were incubated in artificial hemolymph in the presence of [^{35}S] methionine to investigate protein synthesis and secretion. Epidermis secretes at least 13 polypeptides basally and 15 apically. Four basally secreted polypeptides were identified in the hemolymph labelled in vivo. Midgut secretes at least eight polypeptides basally and seven apically. Two basally secreted polypeptides were similar to those in the hemolymph. Fat body releases more than 14 polypeptides into the incubation medium and 12 of which are found in the hemolymph. Hemocytes secrete 11 polypeptides and seven of which could be identified in the hemolymph.

Antibodies made against hemolymph proteins recognized five epidermal, eight midgut, ten fat body and six hemocyte secretory polypeptides indicating that these four tissues contribute at least 29 polypeptides to the hemolymph protein pool. Arylphorin (Ar) is the major hemolymph protein secreted by all four tissues. Arylphorin synthesized and secreted in vitro by all four tissues has been identified through its precipitation with antibodies made against hemolymph Ar.

Fifth stage Calpodes larvae have three major hemolymph proteins SP1 (720 K), SP2 (580 K) and Ar (470 K). The 470 K protein is identified as Ar because it is rich in aromatic amino acids. Electron microscopy of negatively stained preparations show that each polymer has a

different geometrical arrangement of subunits. SP1 is a cube made from eight subunits. SP2 is a hexamer in the form of a pentahedral prism. Ar is probably an octahedron made from six subunits.

Forty six hemolymph polypeptides are stage specific in addition to ten polypeptides that are present throughout the fourth, fifth stage larval and pupal hemolymph. Each growth phase is characterized by the presence of many minor polypeptides that are either absent at all other times or present during only one other phase. Some of the most abundant polypeptides also change with development. Ar is present from early in the stadium and continues into the pupa. SP1 is synthesized from 66 hr and SP2 appears two days later. Both storage proteins start to disappear at the beginning of pupation and are absent in the pupa.

ACKNOWLEDGEMENTS

It gives me immense pleasure to acknowledge the help of my supervisor Dr M. Locke for providing me with the best training any graduate student could have. His excellent performance as teacher, guide, editor and mentor prepared me and provided a source of knowledge and inspiration throughout this study and preparation of thesis. I thank Dr Locke and Dr J. V. Collins for everything they did to make me feel at home in a place several thousand miles away from parents and family.

I am grateful to Dr B. G. Atkinson for teaching me how to run a gel and how to read a scientific paper; both skills played an important role in this study. I would like to thank my advisory committee members Dr B. G. Atkinson and Dr J. E. Steele for their interest and encouragement during the study. Dr Steele read the manuscript and offered some valuable suggestions.

Thanks to Dr G. M. Kidder, Dr C. Ketola, H. Leung, L. Wilkie, H. Kirk, H. Fife, P. Delhanty, S. Henderson, A. Jackson, I. Craig, R. Harris, Y. H. Chang and members of faculty, staff and graduate students of Zoology department for their help and encouragement.

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LIST OF ABBREVIATIONS

A	apical surface
APS	ammonium persulfate
Ar	arylphorin
B	basal surface
C	cuticle
$^{\circ}C$	degrees Celsius
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
E	epidermis
EDTA	ethylenediamine tetraacetic acid
H	hemolymph
HC	hemocytes
IEF	isoelectric focusing
IgG	immunoglobulin
K	kilodalton
M	medium
ug	microgram
ul	microliter
mCi	millicurie
mg	milligram
ml	milliliter
M_r	relative molecular mass
NEPHGE-SDS-PAGE	non-equilibrium pH gradient gel electrophoresis in the first dimension followed by sodium dodecyl sufate polyacrylamide gel electrophoresis in the second dimension
NP-40	nonidet p-40
N-PAGE	non-denaturing polyacrylamide gel electrophoresis
PAS	periodic acid-Schiff method
PBS	phosphate buffered saline
P_i	isoelectric point
PMSF	phenylmethylsulfonylfluoride
PTA	phosphotungstic acid
PTU	phenylthiourea
SB	sudan black B
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SP1	storage protein 1
SP2	storage protein 2
T	tissue
TCA	trichloroacetic acid
TEMED	tetramethylethylenediamine

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Insect hemolymph circulates freely around all tissues in the hemocoel and is therefore involved in the chemical exchanges which occur between them. Hemolymph transports nutrients for assimilation and storage and carries mobilized reserves to tissues for their growth and differentiation. These processes require the presence of many macromolecules including proteins, lipids and carbohydrates. The protein components of hemolymph comprise a functionally and structurally heterogeneous array of macromolecules such as storage proteins, arylphorins, lipophorins, enzymes (JH esterases, trehalase, phenol oxidases etc.), hormone binding proteins, polypeptide hormones, clotting proteins, immuno proteins, tanning proteins, lysozymes etc. For example the proteins in fifth larval Calpodes ethlius (Lepidoptera: Hesperiidae) hemolymph can be resolved into approximately 25 bands by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). and 60 spots by two-dimensional gel electrophoresis (non-equilibrium pH gradient gel electrophoresis in the first dimension and sodium dodecyl sulfate polyacrylamide gel electrophoresis in the second dimension (NEPHGE-SDS-PAGE, Fig. 1). The insect hemolymph thus contains a variety of proteins comparable to those in mammalian blood.

As shown in Table 1, most of the studies on the origin of hemolymph proteins were limited to a few major proteins. Although

Fig. 1. Calpodes ethlius larval hemolymph proteins are resolved into approximately 25 bands by one-dimensional and 60 spots by two-dimensional gel electrophoresis. Approximately 100 ug of hemolymph proteins were separated by two-dimensional gel electrophoresis (NEPHGE in the first dimension and 3-15% SDS-PAGE gradient in the second dimension; NEPHGE-SDS-PAGE) and stained with silver nitrate as described in Materials and Methods. The relative mobility of polypeptides with known molecular masses (M_r) is shown on the left side of the figure and the hemolymph separated only on SDS-PAGE is shown on the right side of the figure.

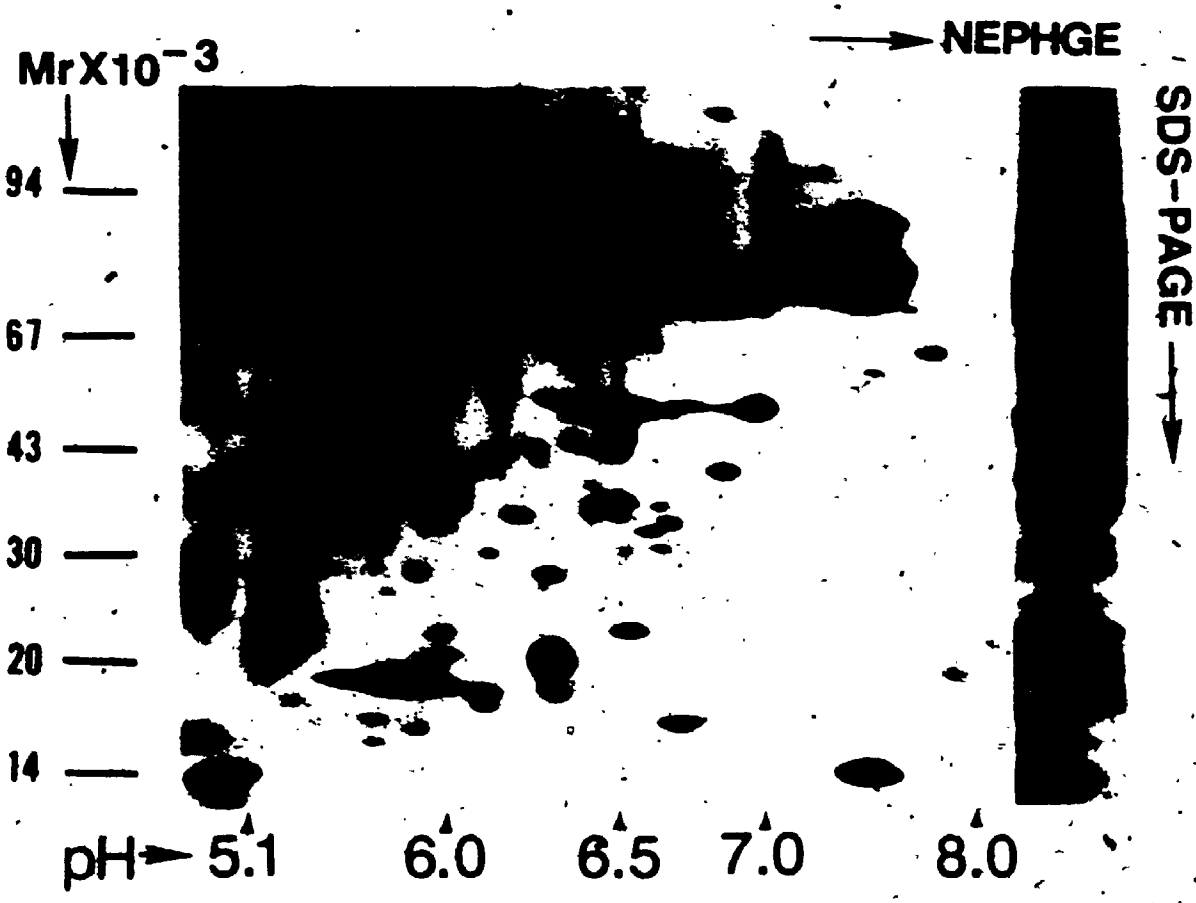

NEPHGE
MrX10⁻³
94
67
43
30
20
14
SDS-PAGE
pH
5.1
6.0
6.5
7.0
8.0

Table 1 Studies on the Origin of Hemolymph Proteins

Name of Insect	Order	Observation	Reference
Bombyx mori	Lepidoptera	Fat body secretory proteins co-migrate with hemolymph proteins on SDS gels	Shigematsu, 1958
Hyalophora cecropia	Lepidoptera	Hemocytes synthesize injury protein	Berry et al., 1964
Rhodnius prolixus	Hemiptera	Hemocytes synthesize injury protein	Coles, 1965
Calliphora erythrocephala	Diptera	Isolated fat body releases proteins into the medium	Price, 1966
Calliphora erythrocephala	Diptera	Fat body secretory proteins co-migrate with hemolymph proteins on gels	Price and Bosman, 1966
Calliphora stygia	Diptera	Fat body secretes major hemolymph protein	Martin et al., 1969
Calliphora erythrocephala	Diptera	Fat body synthesizes and secretes calliphorin	Munn et al., 1969
Diatraea grandiosella	Lepidoptera	Fat body and midgut but not hemocytes could contribute hemolymph proteins	Chippendale, 1970
Pieris brassicae	Lepidoptera	Fat body and midgut but not hemocytes could contribute hemolymph proteins.	Chippendale and Kilby, 1970
Galleria mellonella	Lepidoptera	Hemocytes synthesize cooling protein	Marek, 1970
Calliphora stygia	Diptera	Fat body secretory proteins are electrophoretically similar to hemolymph proteins	Kinnear et al.,1971
Leptinotarsa decemlineata	Coleoptera	Three diapause specific proteins are synthesized in fat body.	De Loof, 1972
Blaberus craniifer	Dictyoptera	Hemocytes synthesize hemagglutinin	Anderson et al.,1972

Table 1 Cont...

Name of Insect	Order	Observation	Reference
Hyalophora cecropia	Lepidoptera	Isolated abdomens (without midgut) secrete major hemolymph proteins	Ruh et al., 1972
Hyalophora cecropia	Lepidoptera	Fat body synthesizes lipoprotein	Thomas, 1972
Leucophaea maderae	Dictyoptera	Fat body releases labelled proteins into the medium	Wyss-Huber and Luscher, 1972
Calpodes ethlius	Lepidoptera	Fat body secretes three major hemolymph proteins	Collins, 1975
Manduca sexta	Lepidoptera	Fat body synthesizes high affinity JH binding protein	Nowock et al., 1975
Locusta migratoria	Orthoptera	Proteins secreted by fat body and heart but not midgut co-migrate with hemolymph proteins	Turner and Loughton, 1975
Sarcophaga barbata	Diptera	Hemocytes contribute protyrosinase	Hughes and Price, 1976
Lucilia cuprina	Diptera	Fat body is the source of lucilin (Storage protein)	Thomson et al., 1976
Plodia interpunctella	Lepidoptera	Fat body secretes JH binding protein	Ferkovich et al., 1977
Periplaneta americana	Dictyoptera	Hemocyte proteins are transported to the cuticle	Geiger et al., 1977
Calliphora vicina	Diptera	mRNA isolated from fat body code for calliphorin	Sekeris et al., 1977
Drosophila melanogaster	Diptera	mRNA isolated from fat body code for calliphorin like protein	Sekeris et al., 1977
Calliphora vicina	Diptera	mRNA isolated from fat body code for calliphorin.	Kemp et al., 1978

Table 1 Cont...

Name of Insect	Order	Observation	Reference
Locusta migratoria	Orthoptera	Fat body secretes lipoprotein	Harry et al.,1979
Bombyx mori	Lepidoptera	mRNA isolated from fat body code for two storage proteins	Izumi et al., 1980
Diatraea grandiosella	Lepidoptera	Fat body secretes diapause associated protein	Turunen and Chippendale, 1980
Locusta migratoria	Orthoptera	Fat body secretes lipophorin	Gellissen and Wyatt, 1981
Trichoplusia ni	Lepidoptera	JH esterase present in fat body and hemolymph have similar activity	Wing et al., 1981
Calpodes ethlius	Lepidoptera	Fat body is the source of two storage proteins and lipoprotein	Locke et al., 1982
Galleria melonella	Lepidoptera	Fat body synthesizes four major hemolymph proteins	Miller and Silhacck, 1982a
Sarcophaga peregrina	Diptera	mRNA isolated from fat body code for one storage protein	Tahara et al., 1982
Calpodes ethlius	Lepidoptera	Fat body secretes two storage proteins and lipoprotein	Webster, 1982
Ceratitis capitata	Diptera	mRNA isolated from fat body code for four major hemolymph proteins	Mintzas et al., 1983
Drosophila melanogaster	Lepidoptera	Fat body synthesizes larval storage proteins 1 and 2	Sato and Roberts, 1983
Rhynchosciara americana	Diptera	Fat body synthesizes one storage protein	De Bianchi and Marinotti, 1984

Table 1 Cont...

Name of Insect	Order	Obsevation	Reference
Sarcophaga peregrina	Diptera	Fat body secretes two injury proteins	Takahashi et al., 1984
Diatraea grandiosella	Lepidoptera	Fat body secretes JH binding protein	Dillwith et al., 1985
Manduca sexta	Lepidoptera	mRNA isolated from fat body code for three major hemolymph proteins	Riddiford and Hice, 1985
Phormia terranovae	Diptera	Fat body is the source of antibacterial hemolymph protein	Keppi et al., 1986
Diatraea grandiosella	Lepidoptera	Fat body contributes lipophorin and one diapause associated protein	Venkatesh and Chippendale, 1986
Calpodes ethlius	Lepidoptera	Pericardial cells secrete arylphorin and two other hemolymph proteins	Fife et al., 1987

there are a variety of proteins in the hemolymph, the origin and function of only a few of these are known. Most of the work on hemolymph proteins was reviewed in Wyatt and Pan (1978), Riddiford and Law (1983) and Levenbook (1985).

1.2 Epidermis as a Source of Hemolymph Proteins

Epidermal cells incorporate labelled amino acids into the cuticle in vitro showing that they have the capacity to synthesize cuticular proteins (Marks and Sowa, 1976). Manduca sexta epidermis maintained in vitro deposits larval cuticle at near normal rates without the addition of hormones or exogenous proteins and without the presence of nutritive sources such as fat body (Mitsui et al., 1980). Hyalophora cecropia larval epidermis maintained in vitro synthesizes most if not all cuticular proteins in the absence of all exogenous factors or organs (Willis et al., 1981). The above studies show that the epidermis synthesizes the cuticular proteins. In addition it has been proposed that the hemolymph may provide some of the protein matrix for the cuticle. At least two Periplaneta americana hemolymph proteins are antigenically similar to cuticular proteins (Fox et al., 1972). In Manduca sexta Koeppe and Gilbert (1973) demonstrated that proteins extracted from cuticle are immunologically similar to hemolymph proteins. The above authors postulated that haemolymph proteins are incorporated into the cuticle unchanged. Several proteins extracted from Astacus leptodoctylus cuticle exhibit total or partial immunochemical identities with the hemolymph proteins (Durliat et al., 1980). However, epidermal cells may secrete proteins at both

their basal and apical faces (Locke and Krishnan, 1971), and proteins common to hemolymph and cuticle could have been synthesized only in the epidermis. Support for this idea came from Riddiford and Hice (1985) who found mRNA coding for arylphorin in Manduca sexta epidermis. They also reported the presence of a polypeptide that cross reacted with hemolymph arylphorin antibodies in both cuticle and epidermis.

1.3 Midgut as a Source of Hemolymph Proteins

Although there are a few reports of hemolymph protein synthesis by the midgut, none of them have solid evidence to prove the point. On the basis of autoradiographic and ultrastructural observations of the fat body, ovary and midgut of blood-fed mosquitoes, it was suggested that the midgut might be the site of yolk protein synthesis (Roth and Porter, 1964). In Diatraea grandiosella, Chippendale and Kilby (1970) studied the relative protein synthetic capacity ([^{14}C] leucine incorporation into proteins) of the larval midgut, fat body and hemocytes. They concluded that the midgut could be contributing proteins to the hemolymph pool. On the other hand Turner and Loughton (1975), investigating protein synthesis by the heart, fat body and midgut of Locusta migratoria, did not find any hemolymph proteins released by the midgut. With the help of disc electrophoresis seven esterase and two lipase-like enzymes were detected in the midgut cells of Diatraea grandiosella larvae (Turunen and Chippendale, 1977). These observations (except Turner and Loughton, 1975) support the idea that the midgut can synthesize proteins but whether it synthesizes hemolymph proteins is unknown.

1.4 Fat body as a Source of Hemolymph Proteins

By using labelled amino acids, Bombyx mori fat body was observed to synthesize and secrete proteins into the in vitro incubation medium. With the help of paper electrophoresis a similarity between the mobilities of the released and the native hemolymph proteins was noticed, suggesting that the latter were being synthesized by the fat body (Shigematsu, 1958). Munn et al (1969), using immunoelectrophoresis, immunodiffusion and starch gel electrophoresis, identified the proteins secreted by Calliphora erythrocephala fat body as hemolymph proteins. In Calpodes ethlius, the fat body of fifth instar larvae synthesize and secrete three major hemolymph proteins (Collins, 1975; Locke et al., 1982). Isolated fat body of Galleria mellonella released four major proteins into the incubation medium (Miller and Silhacek, 1982a). In Bombyx mori (Izumi et al., 1980) and Manduca sexta (Riddiford and Hice, 1985), mRNA isolated from the fat body was translated into three or four major polypeptides.

Hemolymph proteins for which the fat body was identified as a source include storage proteins (Locke et al., 1982), arylphorins (Riddiford and Law, 1983), lipophorins (Gellissen and Wyatt, 1981), JH binding proteins (Nowock et al., 1975), JH esterases (Wing et al., 1981), diapause associated proteins (Venkatesh and Chippendale, 1986) and defence proteins (Keppi et al., 1986). Most of these studies have shown that the fat body secretes particular major hemolymph proteins. Due to the nature of the questions asked and also due to the limited resolution of the techniques used, almost all the above studies were

limited to one or two proteins. We still do not know how many proteins the fat body may contribute to the hemolymph for even one insect species.

1.5 Hemocytes as a Source of Hemolymph Proteins

Earlier studies in Bombyx mori suggested that hemocytes may synthesize hemolymph proteins (Sissakjan and Kuvajeva, 1957 cited by Crossley, 1979). Hyalophora cecropia pupal hemocytes collected from previouly injured pupae and implanted into actinomycin D treated hosts, released one injury protein presumably utilizing the mRNA they had made before they were exposed to actinomycin D in the hemolymph of host (Berry et al., 1964). The injury proteins in Rhodnius prolixus did not co-migrate with fat body proteins in electrophoretic gels. So they were thought to be synthesized by the hemocytes (Coles, 1965). However, Chippendale and Kilby (1970) who measured the relative rate of incorporation of [^{14}C] leucine into hemocytes, fat body and midgut proteins of Diatraea grandiosella under identical conditions showed that the rate of protein synthesis ([^{14}C] leucine incorporation into proteins; counts/min/mg) by hemocytes was only one-tenth that of fat body or midgut. They concluded that hemocytes are of minor importance in the synthesis of hemolymph proteins. Since then several studies have shown that hemocytes have the capacity to synthesize and secrete proteins (reviewed in Crossley, 1979; Gupta, 1985). In the cockroach Nauphoeta cinerea, hemocytes take up labelled amino acids and synthesize vitellogenins and other proteins (Buhlmann, 1974). Sarcophaga barbata larval hemocytes synthesize protyrosinase

(Hughes and Price, 1976). It is not known whether hemocytes synthesize and secrete any of the major hemolymph proteins such as storage proteins, arylphorins, lipophorins etc. There is also no information on the role of Calpodes ethlius hemocytes in hemolymph protein synthesis.

1.6 The Storage Proteins

During the last larval stadium holometabolous insects synthesize large amounts of a few abundant polypeptides. Munn and Greville (1969) first identified such a class of polypeptides in Calliphora erythrocephala. Later, several workers identified two to four such proteins in many insects (see Wyatt and Pan, 1978; Riddiford and Law 1983; Levenbook, 1985 for reviews). Roberts and Brock (1981) suggested that the hemolymph proteins which are (1) few in number and occur only in larval stages, (2) predominantly synthesized by the fat body and (3) increase in concentration enormously during the last larval stadium, be called storage proteins. Levenbook (1985) added three additional biochemical criteria. They should (1) have molecular weight of about 500,000, (2) be composed of six subunits and (3) should contain high proportions of phenylalanine and tyrosine. Lepidoptera synthesize at least three storage proteins during their last larval stadium. In Calpodes ethlius there are three major hemolymph proteins. Locke et al (1982) called two of them storage proteins and the other lipoprotein. There are also three major hemolymph proteins in Hyalophora cecropia two of which are storage proteins (Riddiford and Law, 1983). In Calpodes ethlius and Hylophora

Cecropia all storage proteins are present in both sexes. On the other hand Bombyx mori and Manduca sexta have three storage proteins, of which one is found only in females (Tojo et al., 1980; Riddiford and Law, 1983). In Galleria mellonella, Miller and Silhacek (1982b) reported four major hemolymph proteins and called all of them storage proteins.

Telfer et al (1983) suggested that the insect storage proteins resembling calliphorin in structure and aromatic amino acid composition be called arylphorins (Ar), to signify that they bear the aryl (aromatic) groups and also to distinguish them from other storage proteins. One of the major hemolymph proteins of Bombyx mori, Calliphora vicina, Hyalophora cecropia and Manduca sexta fall into this category (Telfer et al., 1983). Whether any one of the three Calpodes ethlius major hemolymph proteins belong to this class is not known. The single major hemolymph protein in Heliothis zea was identified as Ar (Haunerland and Bowers, 1986). Papilio polyxenes hemolymph has three major proteins, one Ar and two storage proteins (Ryan et al., 1986).

1.7 Developmental Changes of Hemolymph Proteins

The developmental changes of storage proteins are similar in most holometabolous insects. These proteins are usually detected during larval growth and the titre increases markedly during the last larval stadium. They are taken out of circulation during pupation or continue to be present in the pupa and adult depending on the protein and the insect species involved. Munn and Greville (1969) studied the

developmental profiles of the storage protein calliphorin in Calliphora erythrocephala by immunodiffusion, immunoelectrophoresis, ultracentrifugation and electron microscopy and showed that calliphorin was present from two day old larvae to the adult. In Calliphora stygia Kinnear and Thomson (1975) measured the developmental changes in four major hemolymph proteins A, B, C, D. Protein A was synthesized in both larval and adult tissues. Proteins B and C were synthesized during the larval period only and protein D was found only in adults. Changes in the titre of three major hemolymph proteins during the development of Calpodes ethlius were recorded by Locke et al (1982). Lipoprotein (470 K) was present in the hemolymph at all times but not synthesized either very early in the fifth stadium or after the second critical period (156 hr after ecdysis). The two storage proteins were synthesized between the first (66 hr after ecdysis) and the second critical periods of the last larval stadium. Similar developmental profiles were reported for other Lepidoptera such as Bombyx mori (Tojo et al., 1978; 1980), Manduca sexta (Kramer et al., 1980) and Hyalophora cecropia (Telfer et al., 1983). Manduca sexta fat body mRNA translated in a rabbit reticulocyte lysate system coded for three major hemolymph proteins, each having a different developmental profile. All of them appear some time during the phase of larval syntheses and disappear during pupation (Riddiford and Hice, 1985). All the above studies considered the developmental profiles of only a few major hemolymph proteins and ignored all others.

1.8 Thesis objectives

1. To develope an *in vitro* system for maintanance of the midgut, epidermis, fat body and hemocytes.

2. To study protein synthesis and secretion by the midgut, epidermis, fat body and hemocytes under *in vitro* and *in vivo* conditions.

3. To determine whether there is a bi-directional vectorial secretion in the midgut and epidermis.

4. To find the origin of as many hemolymph proteins as possible.

5. To purify and characterize the three major hemolymph proteins.

6. To study developmental changes of hemolymph proteins during the larval-pupal transformation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Test Animals

Larvae of Calpodes ethlius (Lepidoptera: Hesperiidae) were reared on a diet of Canna leaves in our greenhouse until ecdysis to the fifth stadium when they were transferred to an incubator set at 22^0C with a 12 hr light/dark cycle. Mid fifth instar larvae (approximately 90 hr after ecdysis) were used in this study because at this post-commitment stage many tissues are involved in massive larval syntheses (Locke, 1970). To eliminate the variation due to sex only male larvae were used for all experiments.

2.2 Chemicals

All chemicals used in this study were purchased from Bio-Rad (Mississauga, Ontario) or Fisher Scientific (Toronto) or J.T. Baker Chemical Co. Phillipsburg, N.J.) or Sigma Chemical Company (St.Louis, M.O) unless otherwise indicated. IgSorb was purchased from The Enzyme Center Inc., Malden, M.A. [^{35}S] methionine, Aquasol-2 cocktail, EN^3 Hance and Triton X-100 were purchased from New England Nuclear, Boston, M.A. Grace's insect medium was from GIBCO, Grand Island, N.Y. Ampholines were from LKB Instruments Inc., Rockville, N.J. Nonidet p-40 (NP-40) was from BDH Chemicals, Poole, England. Molecular weight standards were from Pharmacia Fine Chemicals Inc., Piscataway, N.J. Peroxidase conjugated goat anti rabbit IgG was from Jackson Immuno Research Laboratories Inc., West Grove, P.A.

2.3 Determination of an Optimal Incubation Medium

To identify the hemolymph proteins contributed by the epidermis, midgut, fat body and hemocytes separately, it was necessary to develope an *in vitro* medium. The performance of these tissues was compared in three different media. (1) Grace's insect medium. (2) *Calpodes* Ringer Solution made by copying the ion composition of *Calpodes* hemolymph determined by Irvine (1969). It contains 17 mM NaCl, 40 mM KCl, 6 mM $CaCl_2$ and 33 mM $MgCl_2$ with an osmolority of 232 mM. (3) Artificial hemolymph made by copying the composition of *Calpodes* hemolymph with respect to ion and amino acid concentrations. The artificial hemolymph has the following composition in grams/liter based on the analysis of hemolymph by Irvine (1969), KCl (1.87), Na_2HPO_4 (0.71), $MgCl_2.6H_2O$ (2.03), $CaCl_2$ (0.44), trehalose (10.26), glucose (0.898), alanine (0.50), glutamic acid (0.37), glycine (2.26), histidine (2.03), lysine (1.04), serine (1.90), threonine (0.26). To this antibiotics and pH indicator, streptomycin sulfate (0.30), penicillin (0.03) and phenol red (0.06) were added. The pH was adjusted to 7.1 and passed through a Nalgene sterile filter unit. Three criteria including (1) contraction and expansion of midgut muscles, (2) dye (Trypan blue) exclusion and (3) visual appearance (general health) were used to determine the viability of tissues. All the tissues performed better in artificial hemolymph. The cells live for at least 24 hr in this medium. Artificial hemolymph was therefore chosen as the incubation medium for the *in vitro* experiments which usually lasted for only 2 hr.

2.4 Antiserum Preparation

One milliliter of early fifth instar larval hemolymph containing approximately 5 mg of protein was mixed with 1 ml of Freund's adjuvant and injected into a rabbit. Second and third injections were given four and six weeks after the first with 2 mg of protein and 1 ml of incomplete Fruend's adjuvent. The rabbit was bled seven days after the third injection and the serum tested for antibodies by the Ouchterlony double diffusion method. The same immunization and bleeding procedures were followed to make antibodies against arylphorin (Ar) eluted from SDS gels following the method of Hager and Burgess (1980). About 0.5 ml of mid fifth instar larval (90 hr after ecdysis) hemolymph containing 5-10 mg protein was loaded on 3-15% N-PAGE into a long stacking well made by inverting the regular comb. After electrophoresis the gel was stained for 10 min (0.1% Coomassie blue R250) and destained for 10 min each in primary (50% methanol in 10% acetic acid) and secondary (10% methanol in 10% acetic acid) destain. The Ar band at 470 K was identified and excised from the gel viewed over a light box. The band was equilibrated in SBA for 15 min, boiled for 3 min and loaded into a long well of 3-15% SDS-PAGE gel. After electrophoresis the sides of the gel were cut, stained, destained and used as markers for the rest of the gel. Appropriate areas of the gel containing Ar (470 K dissociates into 82 K subunits in presence of SDS) were excised and homogenized in 2 ml of elution buffer [0.1% SDS, 0.05 M Tris-HCl (pH 7.9), 0.1 mM EDTA (ethylenediaminetetraacetic acid), 5 mM DTT (dithiothreitol) and 0.2

M NaCl]. The homogenate was mixed on a rotary shaker for 10-15 hr at 4°C and centrifuged in an Eppendorf microfuge (15,000g) for 5 min. The supernatant was then concentrated to 200 ul (Minicon concentrator, type-B 15). Protein concentrations were estimated and the purity checked by SDS-PAGE (Fig. 14a; b). About 1 mg of protein was mixed with 1 ml of complete Freund's adjuvent and injected into a rabbit. Second and third injections were given four and six weeks after the first with 0.5 mg of protein and 1 ml of incomplete Fruend's adjuvent. Crude IgG was prepared by repeated precipitation with 33% $(NH_4)_2SO_4$ and dialyzed against phosphate buffered saline. The antibodies made against hemolymph proteins as well as arylphorin subunits recognize native arylphorin (Appendix 1)

2.5 Tissue Preparation

2.5.1 Integument Preparation

The larvae were opened by ventral dissection in artificial hemolymph. The adhering fat body, heart, pericardial cells, Verson's glands and muscles were removed. The dorsal integument of four abdominal segments was cut with a razor blade from the rest of the animal. This integument sheet was then washed in artificial hemolymph and transferred to a 1.5 ml Eppendorf microfuge tube containing 0.5 ml of artificial hemolymph and a small piece of glass wool. As determined by visual appearance and dye exclusion the cells live for at least 24 hr when the integument sheet is floating in the artificial hemolymph supported by glass wool. In other experiments contamination from the cut edges of the integument was avoided by mounting the sheet of integument as a diaphragm, with the epidermis facing into the medium inside a cut down microfuge tube.

2.5.2 Preparation of Midgut Tubes

To prepare the midgut tubes, larvae were inflated with artificial hemolymph and cut at the junction between the midgut and the hindgut. The gut was detached from the rest of the animal by making a cut at the junction between the foregut and the midgut with a razor blade. The gut and contents then came out intact. It was washed free from contents with medium, cleaned, tied at both ends with dental floss and filled with artificial hemolymph by injection through the ligature. Midgut tubes with an inverted orientation were prepared by injecting 20 ml of artificial hemolymph through the head capsule. This resulted in the gut breaking at the front end and inverting through the rectum like the eversion of a finger of a rubber glove being blown up. These tubes were then ligatured and inflated as above. To match the apical and basal environments glucose (8.98 grams/liter) alone, and trehalose (10.26 grams/liter) with glucose (0.898 grams/liter) were used in apical and basal media respectively. Even though the pH of the midgut is about 8.0, midgut tubes survived well in 7.1 pH medium. After 2 hr incubation the medium in the lumen usually becomes dark pink due to an increase in pH, indicating that the midgut regulates pH, at least on the luminal face.

2.5.3 Fat body

The larvae were opened by ventral dissection in artificial hemolymph. The sheets of fat body were separated from the rest of the animal, washed in artificial hemolymph and transferred to a 1.5 ml Eppendorf microfuge tube containing 0.5 ml of artificial hemolymph and

a small piece of glass wool. As determined by visual appearance and dye exclusion the cells live for at least 24 hr when the fat body sheets float in the artificial hemolymph supported by glass wool.

2.5.4 Hemocytes

Hemocytes were prepared by collecting the hemolymph from a pricked proleg of mid fifth instar larvae into a sterile conical centrifuge tube containing 1.0 ml of artificial hemolymph. The degree of melanization varied with the dilution. Hemolymph from one larva (0.1 ml) diluted with 1.0 ml of artificial hemolymph containing 1 mM PTU kept the melanization at minimum level and gave enough hemocytes for in vitro labelling studies.

2.5.5 Hemolymph

Hemolymph from a pricked proleg was collected on a sheet of parafilm sprinkled with PTU (phenylthiourea) crytals. The hemolymph was pipetted into conical centrifuge tube and centrifuged in a Sorvall GLC-1 at 2,000g for 5 min to remove hemocytes. An equal volume of non-denaturing solubilizing solution [the solubilizing solution of Laemmli (1970) but with 2% SDS and 5% mercaptoethanol replaced by 0.1% Triton X-100 and 10 mM PMSF (phenyl methyl sulfonyl fluoride)] was added to the supernatant. The mixture was either used immediately or stored at -70^{0}C.

2.6 Labelling and Sample Preparation

2.6.1 Integument

Sheets of integument were incubated for 2 hr in an incubator maintained at 22^{0}C in the culture medium containing 0.05 mCi of

[^{35}S] methionine (specific activity 1086 mCi/m mole; New England Nuclear, Boston, M.A.) for in vitro protein secretion studies. At the end of the labelling period the integument sheet was washed in fresh artificial hemolymph and kept on ice for 5 min. The epidermis was separated from the cuticle under a microscope. The proteins present in the epidermis, cuticle and medium were solubilized in non-denaturing solubilizing solution. Proteins present in the cuticle are presumed to be from apical secretions while proteins in the medium are from basal secretions.

2.6.2 Midgut

Ligated midgut tubes were incubated for 2 hr in the culture medium containing 0.05 mCi of [^{35}S] methionine for in vitro protein secretion studies. In a typical incubation, the midgut, weighing 150 mg, was injected with 0.5 ml medium to make the ligated tubes. The ligated tube was incubated in 1 ml of medium in sterile 15 ml tissue culture tubes. Under these conditions the ligated tube is completely covered by and floats in the medium. The incubation tube was capped with parafilm leaving an air inlet. The preparation was kept at 22^{0}C for the incubation period. The proteins present in the media and the tissues were solubilized in non-denaturing solubilizing solution. Proteins present inside normally oriented tubes are presumed to be from apical secretions while proteins in the medium are from basal secretions. In tubes with an inverted orientation, proteins in the medium are from apical secretions while proteins on the inside are from basal secretions.

2.6.3 Fat body

For in vitro protein secretion studies sheets of fat body were incubated for 2 hr at 22°C in culture medium containing 0.05 mCi of [^{35}S] methionine. At the end of the labelling period the fat body sheet was washed in fresh artificial hemolymph. The proteins present in the fat body and medium were solubilized in non-denaturing solubilizing solution.

2.6.4 Hemocytes

Hemocytes suspended in dilute hemolymph were labelled with [^{35}S] methionine for 2 hr in an incubator maintained at 22°C. For solubilizing hemocyte proteins, the incubation medium (dilute hemolymph) was spun at 2,000g in a Sorvall GLC-1 for 5 min. The hemocyte pellet was washed by resuspending and recentrifuging it in a large volume of artificial hemolymph. One hundred microliters of non-denaturing solubilizing solution was added to the pellet and sonicated using a microultrasonic cell disruptor (Kontes Glass Company, Vineland, N.J.) with five ten second pulses at power and tune settings of eight and four respectively.

2.6.5 In vivo Labelling

For in vivo studies 0.1 mCi of [^{35}S] methionine was injected into the feeding larvae. After 2 hr of incorporation the proteins from the hemolymph, hemocytes, cuticle, epidermis, gut lumen, midgut and fat body were solubilized in non-denaturing solubilizing solution.

2.7 Protein and Radioactivity Determination

Protein concentrations were determined by the method of Bradford

(1976) with bovine serum albumin as a standard. Radioactivity of [^{35}S] methionine labelled proteins was measured in the trichloroacetic acid (TCA) precipitable macromolecules. Five microliters of sample, 10 ul of bovine serum albumin (10 mg/ml) and 10 ul of 10 mM methionine were added to 1.5 ml distilled water. To this 0.5 ml of ice cold 50% (W/V) TCA was added. The sample was mixed thoroughly on a vortex mixer and kept on ice for 2 hr. The mixture was filtered through Whatman filter discs (GF/C) using a suction filter. The filter discs were washed with 5% TCA and air dried. The filters were then placed in a vial containing 10 ml of Aquasol-2 scintillant (New England Nuclear, Boston, M.A.). The radioactivity of [^{35}S] was measured in a Beckman LS-225 scintillation counter.

2.8 Polyacrylamide Gel Electrophoresis

One-dimensional gel electrophoresis in the presence of SDS was performed according to the method of Laemmli (1970) except that the separating gel consisted of a 3-15% or 5-15% polyacrylamide gradient overlaid with a 3% polyacrylamide stacking gel (Atkinson, 1981). The samples in the non-denaturing buffer were made up to 2% SDS and boiled for 3 min just before loading. The molecular weights of electrophoretically separated polypeptides were determined by co-electrophoresing marker proteins from a Pharmacia low molecular weight calibration kit (Phosphorylase-b, 94,000; albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100 and lactalbumin, 14,400). Gels were stained with 0.1% Coomassie brilliant blue R250 in 50% methanol and 10% acetic acid and

destained routinely in 50% methanol containing 10% acetic acid and finally in 5% methanol containing 10% acetic acid. The destained gels were stored in 7% acetic acid. N-PAGE was performed following the same procedure except that the SDS was left out from gel and buffer solutions (Locke et al., 1982). The molecular weights of electrophoretically separated proteins were determined by co-electrophoresing marker proteins from a Pharmacia high molecular weight calibration kit (Thyroglobulin, 669,000; ferritin, 440,000; catalase, 232,000; lactate dehydrogenase, 140,000 and bovine serum albumin, 67,000).

Gels were also stained with silver nitrate (Morrissey, 1981). Immediately after electrophoresis the gels were prefixed in 50% methanol, 10% acetic acid for 30 min, followed by 5% methanol, 10% acetic acid for 30 min. Then the gels were fixed in 10% glutaraldehyde for 30 min and washed with several changes of distilled water for 10 hr. The gels were soaked in 5 ug/ml DTT for 30 min prior to staining in 0.1% silver nitrate solution for 30 min. After rinsing rapidly in distilled water the gels were placed in developer solution (50 ul of 37% formaldehyde in 100 ml 3% sodium carbonate) until the desired level of staining was obtained. Staining was stopped by adding 5 ml of 2.3 M citric acid directly to the developer and leaving for a further 10 min. Finally the gels were washed several times in distilled water for 30 min and stored in distilled water.

Glycoproteins were stained with the periodic acid-Schiff method

(Clarke, 1964). Immediately after electrophoresis the gels were oxidised in 1% periodic acid in 3% acetic acid for 1 hr, washed in distilled water for another hour prior to staining with PAS for 1 hr. The gels were stored in 1% sodium meta bisulphite. Lipoproteins were stained with 0.5% Sudan black B (SB, Chippendale and Beck, 1966) in 7.5% acetic acid and 70% ethanol and destained in 70% ethanol, 7.5% acetic acid.

2.9 Two-Dimensional Gel Electrophoresis

O'Farrell's (1975) two-dimensional gel electrophoresis method was used with the modifications suggested by Jones (1980). First dimension gels containing 3.5% acrylamide, 1.8% NP-40, 9M urea, 2% ampholines (pH 3.5-10 ampholines, LKB instruments, inc., Rockville M.D), 11 ul of TEMED (tetramethylethylenediamine) and 13 ul of 10% APS (ammonium persulfate) were prepared in glass tubes as tube gels approximately 10.5 X 0.4 cm in diameter. After allowing the gels to polymerize for 6 hr, the bottom ends of the tubes were covered with a two cm^2 piece of dialysis membrane and mounted in a Bio-Rad model 150A electrophoresis tank. The upper and lower tanks were filled with 0.01 M phosphoric acid and 0.02 M NaOH respectively. The samples were prepared to a final volume of 90 ul with 5% NP-40, 5% mercaptoethanol, 5% ampholines and 9 M urea and loaded on the tops of the gels. Proteins were electrophoresed by applying 500 volts for 6 hr. The gels were extruded from glass tubes with a water filled syringe. The first dimension tube gels were stained in 0.25% Coomassie blue R250 in 40% ethanol and destained in 35% methanol containing 10% acetic acid.

The tube gels were washed with three changes of 35% methanol prior to transfer to the second dimension (Gower and Tytell, 1985). The second dimension gel slabs consisted of a 3-15% or 5-15% polyacrylamide gradient overlaid with 3% polyacrylamide stacking gel. The first dimension gels were fixed to the stacking gel with a thin layer of melted agar (1% agar in 1% SDS). To compare the relative mobility of the electrophoretically separated proteins, those proteins with known isoelectric point (P_i) and molecular mass (M_r) were co-electrophoresed (Bio-Rad IEF standards, phycocyanin, 4.65 (p_i), 232,000 (M_r); lactoglobulin, 5.10, 18,400; bovine carbonic anhydrase, 6.00, 29,000; human carbonic anhydrase, 6.50, 29,000; equine myoglobulin, 7.00, 17,500; whale myoglobulin, 8.05, 17,500; chymotrypsin, 8.80, 25,000 and cytochrome c, 9.60, 12,200 and Pharmacia low molecular weight standards). In addition, the pH gradients established in the first dimension gels were measured by slicing the companion gels and determining the pH of the water extract (Saleem and Atkinson, 1976).

2.10 Fluorography

Destained gels were washed in 1% DMSO (dimethyl sulfoxide) in 10% acetic acid, impregnated with EN^3 Hance (New England Nuclear, Boston, M.A.), washed in excess cold water and dried on Whatman 3MM filter paper with a Bio-Rad model 224 slab gel drier. Fluorograms were prepared by apposing dried gels at -70^{0}C to pre-flashed Kodak X-omat AR film (Laskey and Mills, 1975).

2.11 Immunoprecipitation

The immunoprecipitation method of Ivarie and Jones (1979) was

followed with the modifications suggested by Riddiford (1982). In a typical procedure 100 ul of labelled protein solution was incubated with 20 ul of 10% heat inactivated Staphylococcus aureus Cowan type-1 (SAS; IgSorb, The Enzyme Center, inc., Malden M.A.) for 10 min in an Eppendorf microfuge tube on ice. The SAS was removed by centrifugation for 5 min in an Eppendorf microfuge (15,000g). The supernatant was incubated with 50 ul of antiserum (approximately 2.00 units at 280 nm) on ice for 30 min, 20 ul of SAS was added for an additional 15 min. The mixture was centrifuged in an Eppendorf microfuge for 3 min and the resultant pellet washed thrice with PBSE [0.025 M potassium phosphate (pH 7.6), 0.1 M NaCl containing 0.001 M EDTA and 0.25% NP-40]. The washed pellet was dissolved in 100 ul of Laemmli (1970) solubilizing solution and the radioactivity was measured as described in section 2.7. In control experiments the antiserum was replaced with the equal quantity of preimmune serum. Under the above described conditions the radioactivity in the control samples was equal to or less than that in the blank (Aquasol-2 scintillant, New England Nuclear, Boston, M.A.) for all the data presented in this thesis. Prior to electrophoresis the sample was boiled for 3 min and centrifuged in the Eppendorf microfuge for 2 minutes to remove the SAS.

2.12 Immunoblotting

Immediately after electrophoresis the proteins were transferred from gel to nitrocellulose by the method described by Towbin et al (1979) with the following modifications. The proteins were transferred electrophoretically for 2 hr at 60 volts and approximately

0.5 Amps current with cool water circulating through the coil and with constant stirring of transfer buffer. After the transfer the blot was incubated for 6 hr at 4^0C in a blocking buffer [140 mM NaCl, 10 mM KPO_4 (pH 7.5) containing 10 mg BSA per ml (fraction V)]. The blot was then incubated in 50 ml of primary antibody buffer [PAB;140 mM NaCl, 10 mM KPO_4 (pH 7.5), 10 mg BSA per ml, 0.1% Triton X-100 and 0.02% SDS] containing 0.05 ml of either hemolymph or Ar crude IgG for 12 hr at 4^0C and washed for 3 hr with three changes of PAB. After washing the blot was soaked in 50 ml of secondary antibody buffer [SAB;560 mM NaCl, 10 mM KPO_4 (pH 7.5), 0.1% Triton X-100 and 0.02% SDS] containing 0.005 ml of peroxidase conjugated goat anti rabbit IgG (Jackson Immuno Research Laboratories Inc., West Grove P.A.) for 12 hr at 4^0C and washed for 6 hr with six changes of SAB. The peroxidase was visualized by staining with 0.06% 4-chloro-naphthol, 0.02% hydrogen peroxide and 10 mM Tris-HCl (pH 7.2) prepared in 20% methanol (Hawkes et al., 1982).

2.13 Amino acid Analysis

One hundred micrograms of Ar and two storage proteins (SP1 and SP2) were hydrolysed in one milliliter of 6 N HCl containing 5% mercaptoethanol for 18 hr at 115^0C (Houston, 1971). The hydrolysates were analysed on a Beckman model 119 CL single column analyzer. Cysteine was determined as cysteic acid after performic acid oxidation (Moore, 1963) and tryptophan was determined by hydrolysis of proteins with p-toluene sulfonic acid as described in Liu and Chang (1971).

2.14 Electron Microscopy

Ar, SP1 and SP2 samples were obtained from unfixed unstained non-denaturing gels. Five hundred microliters of hemolymph containing 5-10 mg of protein was separated in a 3-15% non-denaturing gel with a long well made by inverting the regular comb. The sides of the gel were cut, stained briefly, destained and used as markers for unstained areas of the gels. Appropriate areas of the unstained gel containing Ar, SP1 and SP2 were excised and the bands suspended in 2 ml distilled water agitated in a rotary shaker for 10 hr in a cold room (4°C). The liquid was centrifuged in an Eppendorf microfuge (15,000g) for 5 min and the supernatant containing protein was concentrated to 100 ul in a concentrator (Minicon, Type B 15). A drop of protein solution was placed on a grid with a carbon coated formvar film and blotted off by touching the edge of the grid with filter paper. Proteins were nagatively stained with phosphotungstic acid (PTA) by placing a drop of PTA (1.5 %, pH 7.4) on the grid and blotting with filter paper. The grids were dried and viewed at 80 KV with a Philips 300 electron microscope. Although I learned the technique, took many pictures and prepared all the material used in figures 30-32, these actual photographs were taken by Dr. Locke for publication in Archives of Insect Biochemistry and Physiology.

CHAPTER 3

BI-DIRECTIONAL SECRETION IN THE EPIDERMIS

3.1 Introduction

Although our knowledge of insect hemolymph proteins has advanced greatly during the last decade we still know the origin and function of only a few of the major proteins (see Wyatt and Pan, 1978; Riddiford and Law 1983; Levenbook, 1985 for reviews). For example, the proteins of fifth larval Calpodes hemolymph can be resolved into approximately 25 bands by SDS-PAGE and 60 spots by NEPHGE-SDS-PAGE (Fig. 1), but we only know the origin of three major ones (Locke et al., 1982).

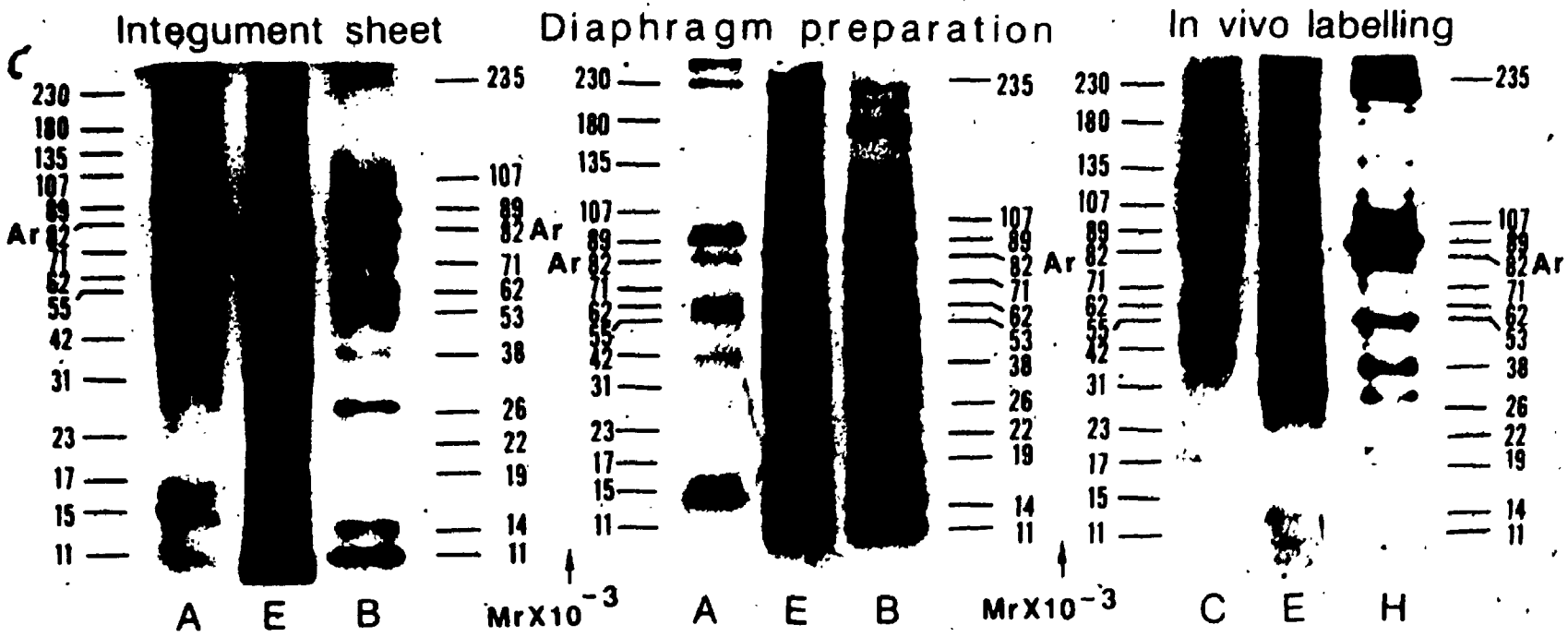
The incorporation of labelled amino acids into cuticle by the integument in vitro showed that epidermal cells have the capacity to synthesize cuticular proteins (Marks and Sowa, 1976). By using tissue culture and electrophoresis the epidermis was confirmed as the site of cuticular protein synthesis (Willis et al., 1981). It has also been proposed that cuticular matrix proteins may come from the hemolymph, since immunological studies indicated that certain cuticular and hemolymph proteins were similar. It was therefore postulated that haemolymph proteins are incorporated into the cuticle unchanged (Fox et al., 1972; Koeppe and Gilbert, 1973; Durliat et al., 1980). However, epidermal cells may secrete proteins at both their basal and apical faces (Locke and Krishanan, 1971), and an alternative explanation supposed that the epidermis synthesizes proteins common to both hemolymph and cuticle. I have investigated this possibility under in vitro and in vivo conditions.

3.2 Results

3.2.1 Protein Synthesis In vitro

Sheets of integument kept *in vitro* survived for at least 24 hr. Cells kept for short periods *in vitro* should therefore behave in a similar way to those *in vivo*. Proteins released on the apical surface will be deposited into cuticle whereas those released on the basal surface will accumulate in the medium, making it possible to determine whether the epidermis secretes proteins on both faces. When sheets of integument were incubated in artificial hemolymph in the presence of [^{35}S] methionine for 2 hr, approximately 30 polypeptides in the molecular weight range of 10-200 K were labelled in the epidermis (Fig. 2, integument sheet, E). Thirteen polypeptides (235, 107, 89, 82, 71, 62, 53, 38, 26, 22, 19, 14 and 11 K) were released from the basal surface (Fig. 2, integument Sheet, B) and fifteen (230, 180, 135, 107, 89, 82, 71, 62, 55, 42, 31, 23, 17, 15 and 11 K) from the apical surface (Fig. 2, integument Sheet, A). When sheets of integument were mounted as diaphragms inside cut down microfuge tubes there were no cut ends of integument exposed to the medium which only faced the epidermis. Labelling then occurred in all 13 basally secreted polypeptides and 15 apically secreted polpeptides as well as almost all 30 polypeptides in the tissue (Fig. 2, Diaphragm preparation). Although there are some quantitative differences, qualitatively the polypeptides are similar under both conditions indicating that the cut ends of integument do not contribute polypeptides to the medium. Since the polypeptides present in the

Fig. 2. Bi-directional secretion by the epidermis. Sheets of dorsal abdominal integument were incubated in artificial hemolymph in the presence of 0.05 mCi of [^{35}S] methionine for 2 hr. The proteins present in the epidermis, cuticle and medium were separated on a 5-15% SDS-PAGE gradient. Approximately 10,000 counts/min were loaded in each well and the fluorograms were developed for 15 days at -70^{0}C. Thirteen [^{35}S] methionine labelled polypeptides were secreted in vitro basally and 15 apically. The same polypeptide pattern occurred when integument was mounted as diaphragm with the epidermis facing the medium in a cut off microfuge tube. Since the 13 basal polypeptides occur in the absence of cut edges and there is no evidence for cell breakdown, these polypeptides are probably normal basal secretions. Similarly the 15 apical secretions are probably normal cuticular proteins. Whole larvae were also injected with [^{35}S] methionine and after 2 hr of incorporation in vivo the proteins labelled in cuticle, epidermis and hemolymph were electrophoresed. Note the correspondence between polypeptides labelled in vitro and in vivo in their respective compartments. The left side of each panel shows the M_r of apical secretions and the right side of each panel shows the M_r of the basal secretions.



A=Apical secretion
B=Basal secretion

E=Epidermis
C=Cuticle

H=Hemolymph
Ar=Arylphorin

cuticle and basal medium are generally specific for the respective surfaces and do not include the main polypeptides labelled in the cells themselves but do include a known secretory protein (Ar), it is most likely that there is little or no contamination from tissue breakdown or cell death. Since 13 polypeptides are secreted from the basal surface into the medium without access to the cut edges of the integument or apical surface (diaphragm preparations) these 13 are probably normally secreted by the epidermis into the hemocoel. Similarly, the secretion of 15 polypeptides occurs in the cuticle without access to any other tissues, so these are probably secreted by the epidermis into the cuticle. These experiments show that Calpodes epidermis has bi-directional secretion in vitro. I therefore wished to know if there might be similar bi-directional secretion in vivo.

3.2.2 Protein Synthesis In vivo

Protein synthesis and secretion by the epidermis were studied in vivo to determine whether the in vitro results represent normal behaviour. [^{35}S] methionine was injected into mid fifth instar Calpodes larvae and allowed to incorporate for 2 hr before sampling the epidermis, cuticle and the hemolymph.. Approximately 30 polypeptides were labelled in the epidermis (Fig. 2, In vivo E) compared to 15 in the apical cuticular compartment (Fig. 2, In vivo C) and more than 25 in the basal compartment=hemolymph (Fig. 2, In vivo H). The polypeptides labelled in the epidermis under in vivo and in vitro conditions show similar patterns on these SDS gels. The similar labelling of cellular proteins under in vitro and in vivo

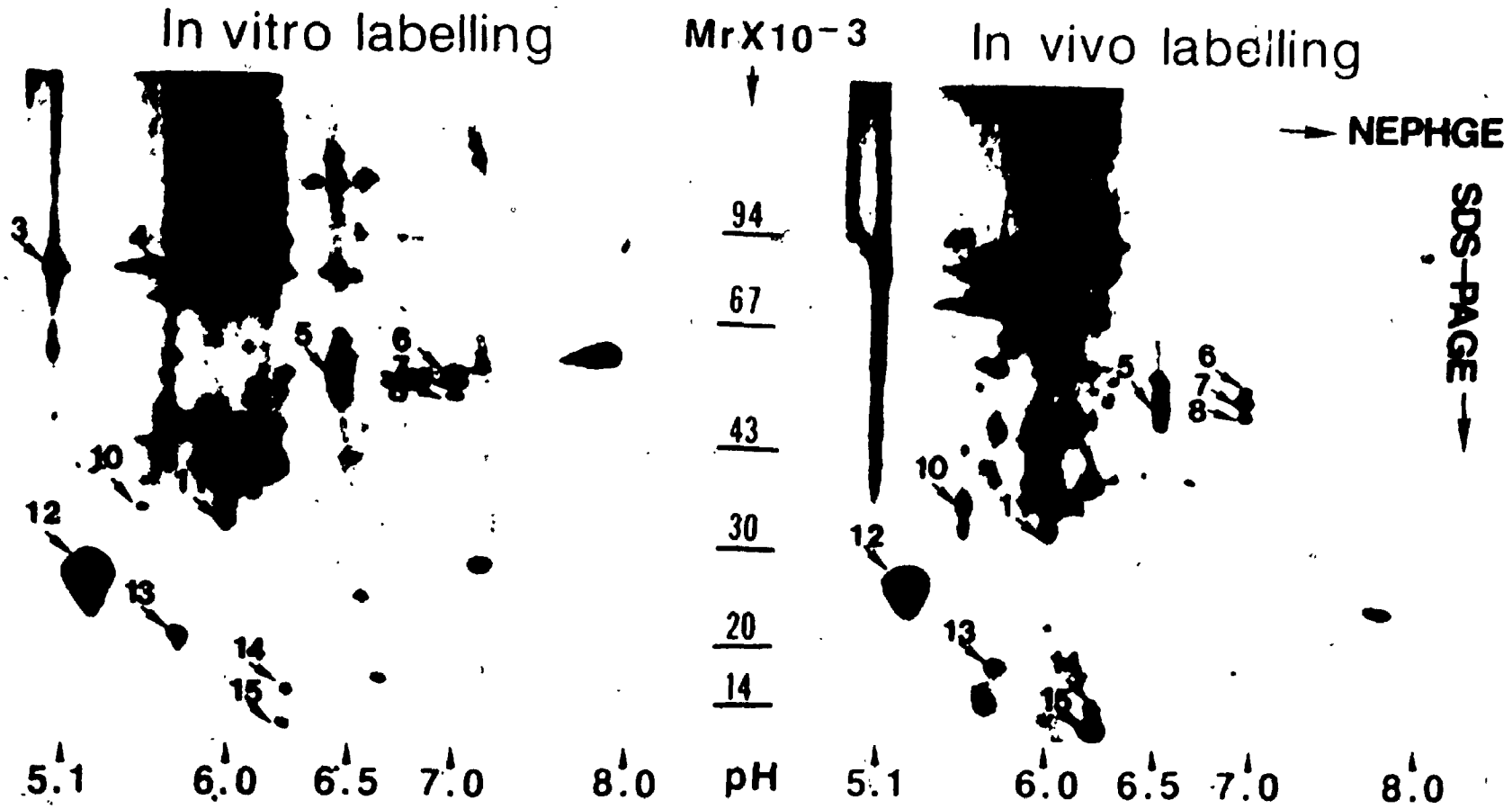
conditions suggests that the *in vitro* system closely mimics the natural conditions, at least as far as protein synthesis is concerned. Since the cellular proteins synthesized *in vitro* and *in vivo* are similar, it was of interest to determine whether proteins secreted *in vitro* are also present in their respective compartments after labelling *in vivo*.

3.2.3 The Similarity between Apical Secretions and Naturally Occurring Cuticular Proteins

If the pattern of protein secretion observed *in vitro* is a natural process, the [^{35}S] methionine labelled proteins in the apical secretions should match the cuticular proteins labelled *in vivo*. Cuticular proteins labelled *in vitro* and *in vivo* were separated on SDS-PAGE and NEPHGE-SDS-PAGE. One-dimensional SDS-PAGE analysis showed that all of the 15 polypeptides synthesized and secreted apically *in vitro* are present among the cuticular polypeptides labelled *in vivo* (Fig. 2). Two-dimensional separation of labelled proteins from the *in vitro* and *in vivo* apical compartments showed that most of the apical polypeptides synthesized and secreted into the cuticle *in vitro* are also present in the cuticle labelled *in vivo* although not necessarily in identical proportions (Fig. 3). These experiments showed that all apical protein secretions labelled *in vitro* are similar to those labelled *in vivo*, confirming the suggestion that the *in vitro* system closely mimics the natural conditions for protein synthesis.

3.2.4 The Similarity between Basal Secretions and Naturally Occurring Hemolymph Proteins

Fig. 3. The similarity between epidermal apical secretions labelled *in vitro* and *in vivo*. Proteins from the cuticle (apical compartment) after [^{35}S] methionine labelling *in vitro* and from cuticle labelled *in vivo* were separated by NEPHGE-SDS-PAGE employing 5-15% SDS polyacrylamide gradient gels in the second dimension. Approximately 50,000 counts/min were loaded onto each gel and the fluorograms were developed for 28 days. pH and M_r were determined as described in Materials and Methods. The arrows point to 15 of the most pronounced spots that are present in both fluorograms.

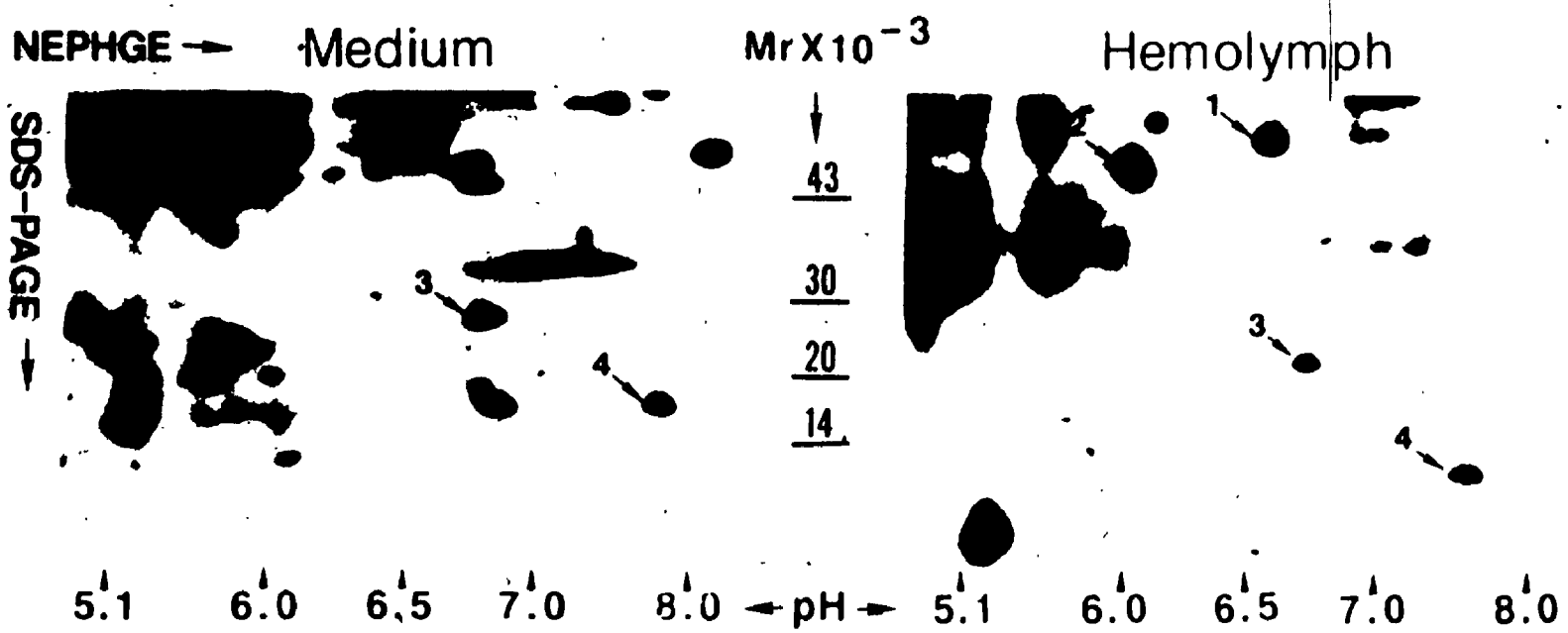

In vitro labelling
MrX10-3
In vivo labelling
NEPHGE
SDS-PAGE
94
67
43
30
20
14
3
5
6
10
12
13
14
15
1
7
8
5.1
6.0
6.5
7.0
8.0
pH

The degree of correspondence between basal secretions labelled *in vitro* and hemolymph labelled *in vivo* was also determined by SDS-PAGE and NEPHGE-SDS-PAGE. Most bands in the basal secretion co-migrated with similar bands from hemolymph (Fig. 2,B; H). Four of these polypeptides (Fig. 4, 1-4) could be identified in the hemolymph NEPHGE-SDS-PAGE gel. There is also correspondence between some other spots but because of the overcrowding many spots could not be separately identified. Since there are so many polypeptides in the hemolymph it becomes very difficult to point unequivocally to those that are secreted by the epidermis. I therefore used an immunological approach to demonstrate the presence of epidermal basal secretions in the hemolymph.

3.2.5 The Immunological Similarity between Epidermal Basal Secretions and Naturally Occurring Hmolymph Proteins

Antibodies were made against whole hemolymph from early fifth instar larvae. The rabbit made antibodies to almost all hemolymph proteins (details are presented in Chapter 4, Fig. 12a; b). Antibodies were therefore probably made against epidermal basal secretions present in the hemolymph. These antibodies should recognize those *in vitro* labelled basal secretions that are normal constituents of hemolymph. To verify this, *in vitro* labelled proteins from the cuticle, epidermis and medium were precipitated using antibodies to the hemolymph. The resultant precipitates were separated on SDS-PAGE and the gels processed for fluorography. Antibodies made against hemolymph proteins recognized at least five

Fig. 4. The similarity between proteins secreted by the epidermis and those occurring naturally in the hemolymph. Proteins from the basal compartment labelled with [^{35}S] methionine *in vitro* and hemolymph labelled *in vivo* were resolved on NEPHGE-SDS-PAGE employing 5-15% SDS polyacrylamide gradient gels in the second dimension. Approximately 50,000 counts/min were loaded onto each gel and the fluorograms were developed for 28 days. pH and M_r were determined as described in Materials and Methods. The arrows point to the spots that are present in both fluorograms.

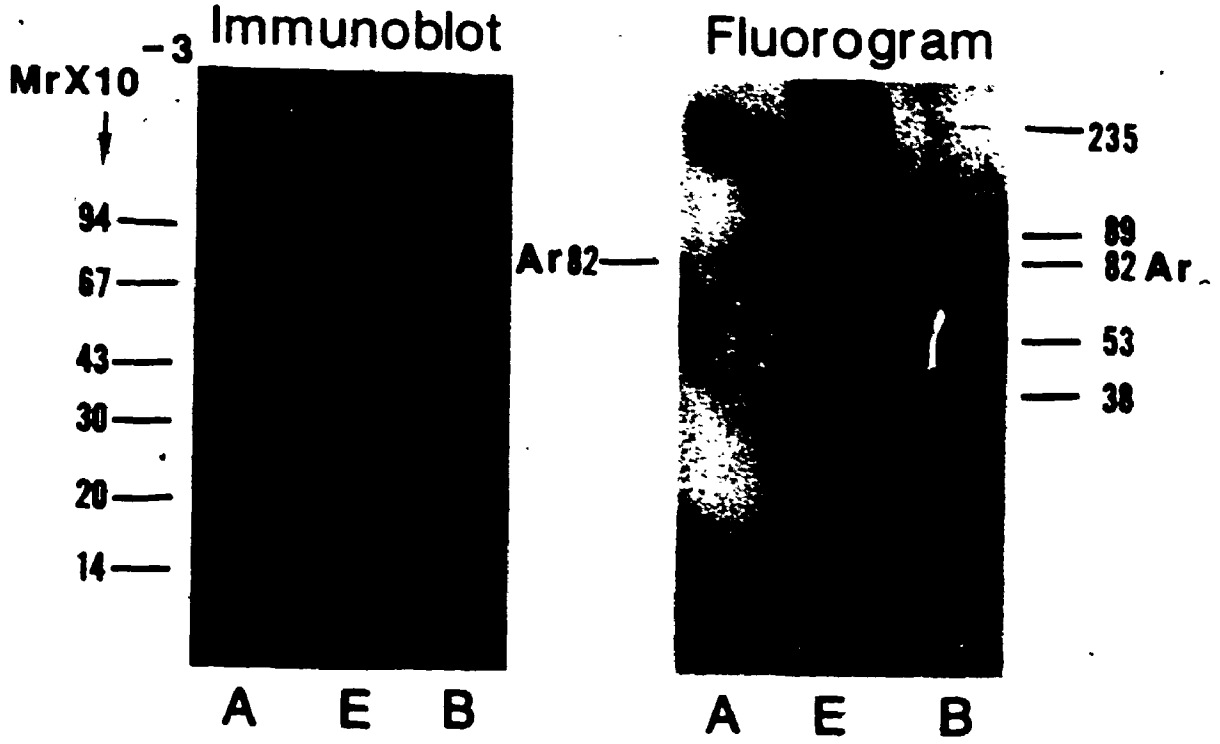

NEPHGE →
Medium
Mr X 10⁻³
Hemolymph
SDS-PAGE →
43
30
20
14
1
3
4
1
3
4
5.1
6.0
6.5
7.0
8.0
← pH →
5.1
6.0
6.5
7.0
8.0

polypeptides (235, 89, 82, 53 and 38 K) present in the epidermis and the same five polypeptides present in the basal medium. The single apically secreted polypeptide recognized co-migrated with polypeptides from both the epidermis and basal medium (Fig. 5). The protein sample was pretreated with SAS (IgSorb) and a non-ionic detergent (NP-40) was included in the assay to reduce the background. These precautions make it likely that the antibodies to the hemolymph proteins were recognizing basally secreted polypeptides because of immunological similarity. The results obtained by immunoprecipitation were confirmed with immunoblotting. Hemolymph antibodies also recognized most of the same polypeptides present in epidermis, medium and cuticle on immunoblots (Fig. 5). Although the epidermis secretes 13 polypeptides basally only five major polypeptides were recognized by antibodies prepared against whole hemolymph. The remaining polypeptides which appear as minor bands may be at too low a concentration in the hemolymph, so that antibodies might not have been made to them. The absence of antibodies to all the basally secreted polypeptides could also be due to the stage of development. The hemolymph used to prepare antibodies was deliberately taken from larvae just prior to commitment, when the larval storage proteins secreted by the fat body are not the main component (Chapter 8). Minor hemolymph components from the epidermis might also not have been secreted at that time. Whatever may be the case, these results established that at least five of the polypeptides present in the hemolymph can be synthesized and secreted by epidermal cells.

Fig. 5. The immunological similarity between epidermal basal secretions and naturally occurring hemolymph proteins.

Immunoblot: One hundred micrograms of protein from cuticle, epidermis and basal medium were separated on a 5-15% SDS-PAGE gradient and transferred to nitrocellulose. The blot was stained with antibodies prepared against the hemolymph proteins. Five basally secreted polypeptides and one apically secreted polypeptide were recognized by antibodies prepared against the hemolymph proteins. The relative mobility of co-electrophoresed Pharmacia low molecular weight standards is shown on the left side of the figure.

Fluorogram: The proteins from cuticle (100,000 counts/min), epidermis (500,000 counts/min) and basal medium (500,000 counts/min) labelled with [^{35}S] methionine in vitro, were precipitated with antibodies to hemolymph (see Materials and Methods for details). Immunoprecipitates of cuticle, epidermis and basal medium were separated on a 5-15% SDS-PAGE gradient. The fluorogram was developed for two weeks at -70^{o}C. Five basally secreted polypeptides and one apically secreted polypeptide were recognized by antibodies prepared against the hemolymph proteins.



A=Apical secretion E=Epidermis

B=Basal secretion Ar=Arylphorin

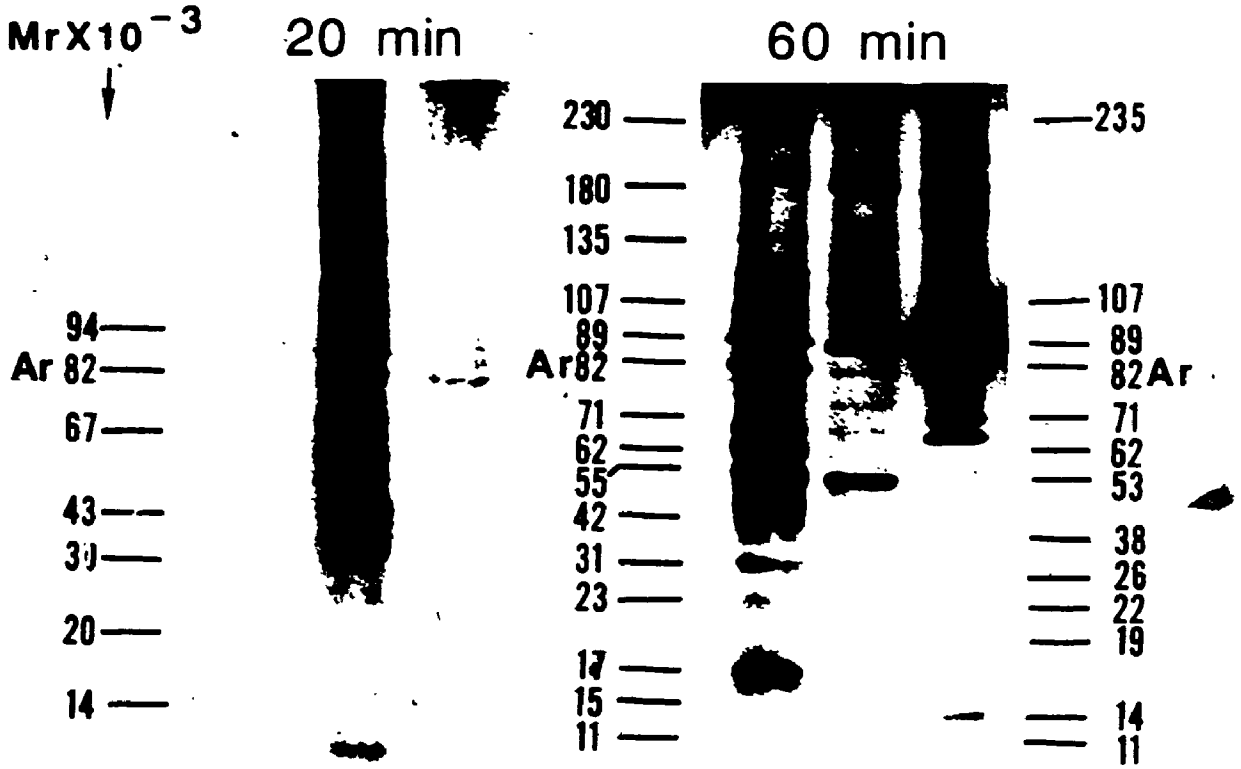
3.2.6 The Sequential Appearance of Basal Secretions in the Epidermis Prior to their Appearance in the Hemolymph

The disadvantage with *in vivo* labelling is that the epidermis might have taken up proteins newly synthesized by other tissues during the 2 hr of labelling. The comparison between *in vitro* and *in vivo* protein synthesis and secretion could be in error unless it can be shown that the proteins in question are synthesized first by the epidermis. Tissues were exposed to [^{35}S] methionine *in vivo* for 20 and 60 min to show the relative times of appearance of the basal secretion in the epidermis and hemolymph. Labelling the mid fifth instar *Calpodes* larvae with [^{35}S] methionine for 20 and 60 min showed that labelled polypeptides appeared in the epidermis after 20 min at a time when neither hemolymph nor cuticle was labelled (Fig. 6). By 60 min labelled polypeptides also occurred in the hemolymph (which has Ar and other basal secretions) and cuticle (which has Ar and other apical secretions). This agrees with earlier autoradiographic studies, where a variety of amino acids were incorporated either in layers or diffusely into the cuticle after 2 hr (Condoulis and Locke, 1966). Since most of the bands corresponding to secretory polypeptides appeared in the epidermis 20 min after the beginning of labelling, when nothing was labelled in the hemolymph, the epidermis must be the source of such polypeptides.

3.2.7 The Secretion of Arylphorin by the Epidermis

Previous studies on *Calpodes* (Locke et al., 1982) showed that one of the three major hemolymph proteins is a lipoglycoprotein with a

Fig. 6. The sequential appearance of epidermal secretory proteins, first in the tissue and later in the cuticle (apical compartment) and hemolymph (basal compartment). Epidermis was labelled <u>In vivo</u> by injecting [^{35}S] methionine into mid fifth instar larvae. After incorporation for 20 and 60 min proteins were solubilized from cuticle, epidermis and hemolymph. Approximately 20,000 counts/min were loaded onto each well (except in the case of C and H at 20 min where the incorporation was so low that, 100 ul of samples were loaded, which are at least 5X more than that loaded for 60 min) and the fluorograms were developed for a week at -70°C. Both apical (cuticle) and basal (hemolymph) secretions appear first in the epidermis before either cuticle or hemolymph are labelled (20 min). By 60 min labelled polypeptides also appear in the cuticle and hemolymph. The relative mobility of Pharmacia low molecular weight standards is shown on the left side of the figure and the M_r of the basal secretions are shown on the right. The M_r of apical secretions are shown in the middle.



C=Cuticle

E=Epidermis

H=Hemolymph

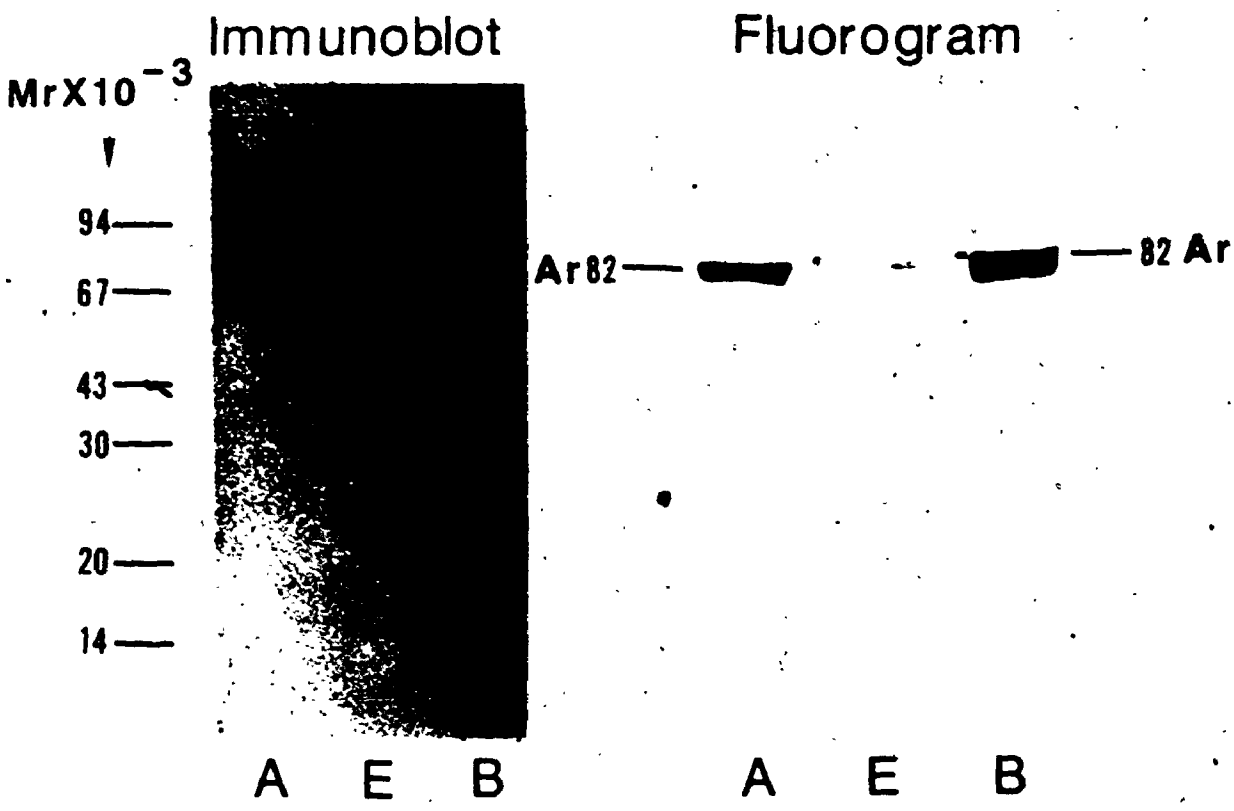
Ar=Arylphorin

molecular weight of 470 K and dissociating into six similar subunits each with a molecular weight of 82 K in the presence of SDS. This protein is now called as arylphorin (Ar, details are presented in Chapter 7). Arylphorin was present in epidermis (Fig. 2, E), cuticle (Fig. 2, A) and medium (Fig. 2, B) labelled *in vitro*, and in epidermis (Fig. 2, E), cuticle (Fig. 2, C) and hemolymph (Fig. 2, H) labelled *in vivo*. The experiments in which fifth instar larvae were exposed to [^{35}S] methionine for 20 and 60 min showed that Ar occurred in the epidermis (by 20 min), prior to its appearance in the hemolymph and cuticle (Fig. 6). Arylphorin was also labelled in epidermis, cuticle and basal medium *in vitro* (Fig. 2) in the absence of other tissues. To check the possibility that Ar from either hemolymph or medium might be sticking to cuticle, larvae were skinned to obtain a cuticle sample without exposing it to hemolymph. Arylphorin was also present in this skinned cuticle. The cuticle separated from epidermis was stained with feulgen (Thompson, 1966) and bismuth (Locke and Huie, 1977) to check for contamination. Neither nuclei nor nucleoli are present in this cuticle. Isolated cuticle was incubated with [^{35}S] methionine for 2 hr, proteins were solubilized, separated on gels and the gels processed for fluorography. None of the cuticular proteins were labelled, indicating that the cuticle is neither contaminated with a few epidermal cells nor is [^{35}S] methionine sticking to cuticular proteins. Similar results were observed when the isolated cuticle was incubated in the presence of [^{35}S] methionine labelled hemolymph proteins. These results

Fig.7. The immunological evidence that arylphorin is secreted by the epidermis.

Immunoblot: One hundred micrograms of protein from cuticle, epidermis and basal medium were separated on a 5-15% SDS-PAGE gradient and transferred to nitrocellulose. The blot was stained with antibodies to hemolymph Ar. The Ar present in cuticle, epidermis and basal medium was recognized by antibodies prepared against hemolymph Ar. The relative mobility of co-electrophoresed low molecular weight stadards is shown on the left side of the figure.

Fluorogram: [^{35}S] methionine labelled proteins from cuticle (100,000 counts/min), epidermis (500,000 counts/min) and basal medium (500,000 counts/min) were precipitated with antibodies to hemolymph Ar. Immunoprecipitates of cuticle, epidermis and basal medium containing approximately 10,000 counts/min were separated on a 5-15% SDS-PAGE gradient. Fluorograms were developed for two weeks at -70^{o}C. The Ar present in the cuticle, epidermis and basal medium has been precipitated by antibodies prepared against hemolymph Ar.



A=Apical secretion E=Epidermis

B=Basal secretion Ar=Arylphorin

suggested that Ar might arise in the epidermis as well as in the fat body, where it had previously been shown to be made. The synthesis of Ar by the epidermis has been confirmed by immunological studies. Antibodies to the hemolymph proteins recognized Ar present in epidermis, cuticle and medium (Fig. 5). Antibodies to Ar itself (purified by electrophoretic separation from hemolymph, Chapter 2, Fig. 14a; b) recognized the Ar from epidermis, cuticle and basal medium (Fig. 7). All these findings suggest that the epidermis synthesizes and secretes Ar on both apical and basal surfaces.

3.3. Discussion

3.3.1 Hemolymph Protein Secretion by the Epidermis

The main finding is that Calpodes epidermis secretes certain hemolymph proteins from the basal surface as well as others in the expected apical cuticle direction. In preparations where the integument was mounted as a diaphragm, the basally secreted proteins accumulate inside the tube where there is no contamination from cut edges. Since the epidermis normally releases these proteins basally it is reasonable to assume that they contribute directly to the hemolymph. Sources of error such as cell breakdown, membrane blebbing or transepithelial leakage are unlikely, since: (a) all *in vitro* apical secretions occur in the *in vivo* cuticular proteins, (b) at least four basally secreted epidermal polypeptides occur naturally in the hemolymph, (c) there are few bands in the medium compared to many in the tissue, and (d) five polypeptides secreted basally and one secreted apically are immunologically similar to naturally occurring

hemolymph proteins. It may also be significant that electron microscopy shows epidermal Golgi complexes often orient their secretory faces towards the hemolymph surface (the orientation is easily recognizable by the position of the GC beads, Locke, 1984). These observations strongly suggest that the protein secretion observed in the in vitro system is natural. The data suggest that this insect epidermis secretes hemolymph proteins from the basal surface as well as cuticular proteins from the apical surface. The epidermis now joins a lengthening list of tissues (fat body, hemocytes and pericardial cells) that contribute to the hemolymph pool of proteins.

3.3.2 Bi-directional Protein Secretion in the Epidermis

The second finding is that Calpodes epidermis secretes proteins from both apical and basal surfaces. Proteins seen in the basal medium are not due to leakage from the cuticle since two-dimensional analysis shows that most of the cuticular polypeptides are different from those in the medium. Although six polypeptides from apical and basal secretions co-migrated on SDS gels (107, 89, 82, 71, 62 and 11 K, Fig. 2) only one of these (82 K) has matching spots on two-dimensional gels (parts of medium and hemolymph two-dimensional gels were eliminated for clear comparison). The remaining polypeptides could be different but migrating at the same position on SDS gels. Almost all apical secretions match the cuticular proteins labelled in vivo (Fig. 3) and five basally secreted polypeptides and only one cuticular polypeptide showed immunological similarity with hemolymph

polypeptides (Fig. 5). The bi-directional secretion could come about by regional or temporal localization, by cell specific directional secretion, by bi-directional secretion in individual cells. Ultrastructural observations on the orientation of epidermal Golgi complexes (Locke, 1984) suggest the later interpretation. In other polarized epithelial cells that have distinct apical and basolateral surfaces, it has been shown that the viral protein hemagglutinin is directed to the apical surface, whereas the G protein of vesicular stomatitis virus is directed preferentially towards the basolateral surface (Rindler et al., 1985). Madin-Darby canine kidney (MDCK) cells have also been shown to secrete different proteins on their apical and basolateral surfaces (Caplan et al., 1985). The presence of apical and basolateral membrane domains in epithelial cells, itself implies vectorial membrane secretion (Locke, 1984).

The observation of bi-directional secretion in Calpodes epidermis gives clues to another longstanding problem the purported transport of hemolymph proteins into cuticle. Immunological evidence suggests an antigenic similarity between hemolymph and cuticular proteins (Fox et al., 1972; Koeppe and Gilbert, 1973; Durliat et al., 1980) and it was postulated that those hemolymph proteins are incorporated into the cuticle unchanged. These results show that the epidermis can secrete the same protein (Ar) on both surfaces. Proteins common to the hemolymph and cuticle might therefore be synthesized in the epidermis itself, as suggested by Locke and Krishnan (1971).

3.3.3 Synthesis and Secretion of Arylphorin by the Epidermis

The third finding is that the epidermis secretes Ar on both faces. Arylphorin (470 k protein with 82 K sub units on SDS gels) is present in cuticle, epidermis and basal medium (Fig. 2). and is precipitated by antibodies to hemolymph Ar (Fig. 7). Arylphorin is synthesized by the fat body in many insects (Riddiford and Law, 1983; Riddiford and Hice, 1985). Since proteins can be labelled in epidermis isolated *in vitro* the possibility, that the Ar has been taken up from the fat body is excluded. It is also unlikely that the epidermal Ar is an error due to contamination by a few fat body cells. The epidermis was carefully cleaned from all fat body. The two-dimensional gels of medium from the fat body and epidermis are very different (Figs. 4; 17), so it is most unlikely that the presence of Ar is due to fat body contamination. The idea that tissues other than the fat body may make Ar is not entirely new. The mRNA for Ar in *Manduca sexta* are found in the epidermis (Riddiford and Hice, 1985). The synthesis and secretion of Ar was demonstrated in *Calpodes* pericardial cells (Fife et al., 1987). From these observations it appears that hemolymph Ar may be synthesized by many tissues. The surprising result is the presence of Ar in the cuticle. Riddiford and Hice (1985) also reported the presence of a polypeptide in the cuticle that reacts with Ar antibodies. The functional significance of this observation is not clear, but based on its high aromatic amino acid content, one may speculate that Ar could be concerned with the stabilization of cuticle and perhaps cuticular

repairs, including the stabilization of hemolymph clots. It seems unlikely that Ar is merely concerned with the supply of phenolics, since massive amounts of tyrosine are accumulated in fat body vacuoles prior to new cuticle formation (McDermid and Locke, 1983).

CHAPTER 4

BI-DIRECTIONAL SECRETION IN THE MIDGUT

4.1 Introduction

The hemolymph of holometabolous insects comprise a functionally and structurally heterogeneous array of macromolecules such as proteins, carbohydrates and lipids (see Wyatt and Pan, 1978; Riddiford and Law, 1983; Levenbook, 1985 for reviews). For example, the protein components of fifth larval Calpodes hemolymph can be separated into approximately 25 bands by one dimensional and 60 spots by two dimensional gel electrophoresis (Fig. 1). Although, there many proteins in the hemolymph, the origin and function of only a few of these are known (Wyatt and Pan, 1978).

There is general agreement that fat body is a main source of hemolymph proteins (Dean et al., 1985) and others may come from hemocytes (Hughes and Price, 1976). Fluorograms of proteins synthesized by fifth larval Calpodes fat body detected only the main storage proteins and lipoprotein (Locke et al., 1982). Most of the other proteins do not appear to be synthesized by the fat body, at least at the age of the larvae used in the experiments. Other tissues such as midgut and epidermis may therefore contribute proteins to the hemolymph. Except for the attempts by Chippendale (Chippendale, 1970; Chippendale and Kilby, 1970) and Turner and Loughton (1975), there is no information on hemolymph protein synthesis by other tissues. The structure of larval midgut with

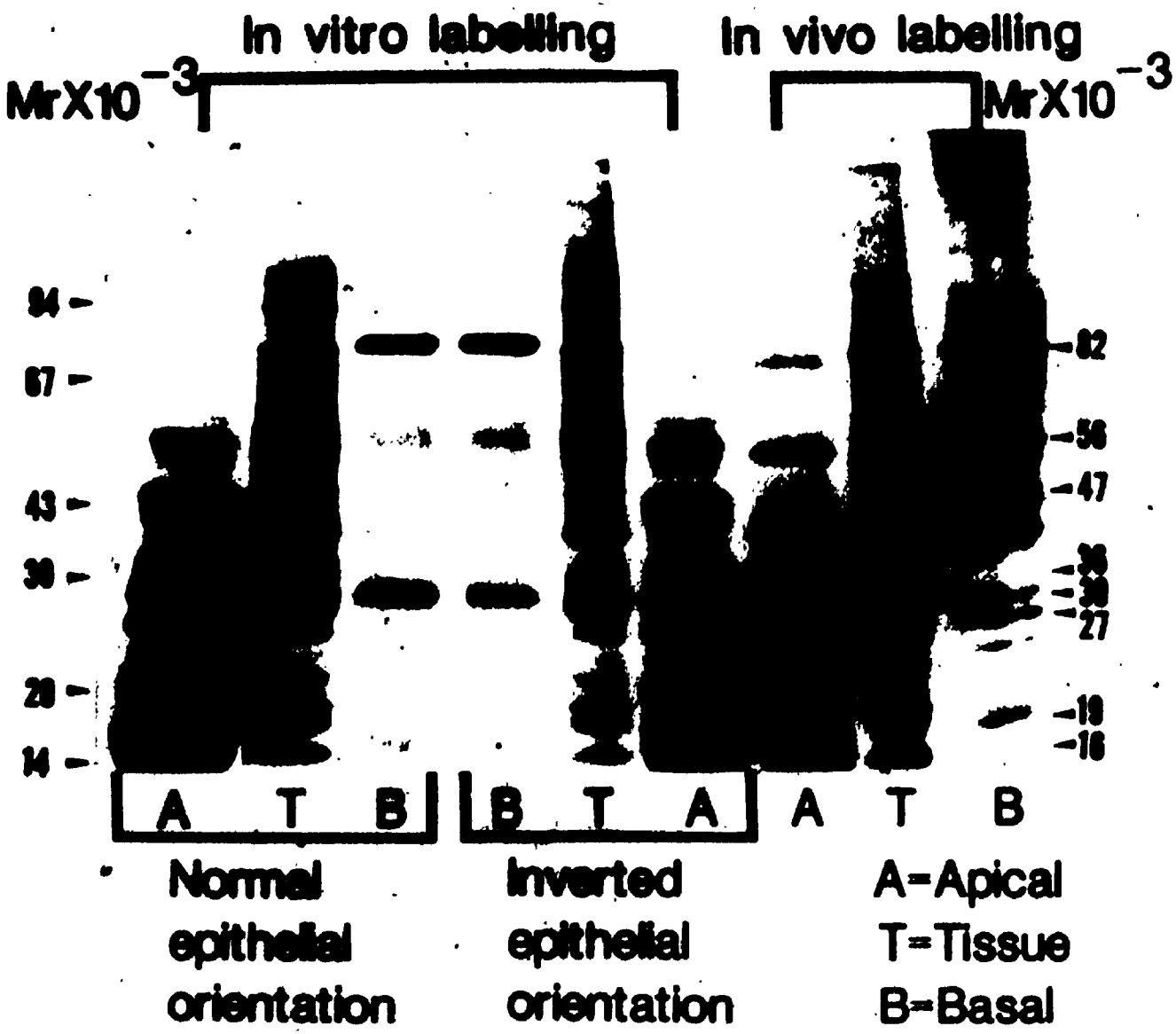
abundant RER (Gioffi, 1979; Locke and Leung, 1984), suggests that it is capable of synthesizing proteins for secretion that might be destined for the hemolymph as well as the gut lumen. I have investigated this possibility under *in vitro* and *in vivo* conditions.

4.2 Results

4.2.1 Protein Synthesis In vitro

Ligated tubes of midgut kept *in vitro* survived for at least 24 hr and therefore the cells should behave normally for the few hours of the experiments. Proteins released from either surface of the tube can be collected from the lumen without cross contamination, or from the medium with minimal contamination of the ligated ends, making it possible to determine whether the midgut secretes proteins on both faces. When ligated tubes of midgut with normal orientation (i.e. apical secretions into the lumen and basal into the medium) were incubated in artificial hemolymph in the presence of [^{35}S] methionine for 2 hr, approximately 25 polypeptides in the molecular weight range of 10-200 K were labelled in the midgut cells (Fig. 8, T). Eight polypeptides (82, 56, 47, 36, 30, 27, 19, and 16 K) were released from the basal surface (Fig. 8, B) and seven (65, 56, 47, 30, 27, 22, and 16 K) from the apical surface (Fig. 8, A). The same pattern of polypeptides occurred when inverted tubes (i.e. apical secretions into the medium and basal into the lumen) were used in place of those with normal orientation. It can be concluded that the ligated ends of the tubes, where short sections of both surfaces are

Fig. 8. Bi-directional secretion by the midgut. Ligated tubes of midgut with either normal (i.e.apical secretion into the lumen basal into the hemocoel) or inverted orientation (i.e.basal into the lumen and apical into the hemocoel) were incubated in artificial hemolymph in the presence of 0.05 mCi of [^{35}S] methionine for 2 hr. The proteins present in the tissue and in the media at the apical and basal surfaces were separated on a 5-15 % SDS-PAGE gradient. Approximately 10,000 counts/min were loaded for each well and the fluorogram was developed for 15 days at -70^{0}C. Eight [^{35}S] methionine labelled polypeptides were secreted *in vitro* basally and seven apically in tubes with normal orientation. Additional [^{35}S] methionine labelled polypeptides which do not appear at either surface were present in the tissue. The same peptide pattern occurred in midgut tubes with inverted orientation. Since the eight basal polypeptides occur in the absence of an apical surface and there is no evidence for cell breakdown, these polypeptides are probably normal basal secretions. Similarly the seven apical secretions are normal luminal proteins. Whole larvae were also injected with [^{35}S] methionine *in vivo* and the labelled proteins present in lumen, midgut and hemolymph were electrophoresed. Note the correspondence between polypeptides labelled *in vitro* and *in vivo* in their respective compartments. The relative mobility of polypeptides with known M_r is shown on the left side of the figure and the M_r of the basal secretions is shown on the right side.

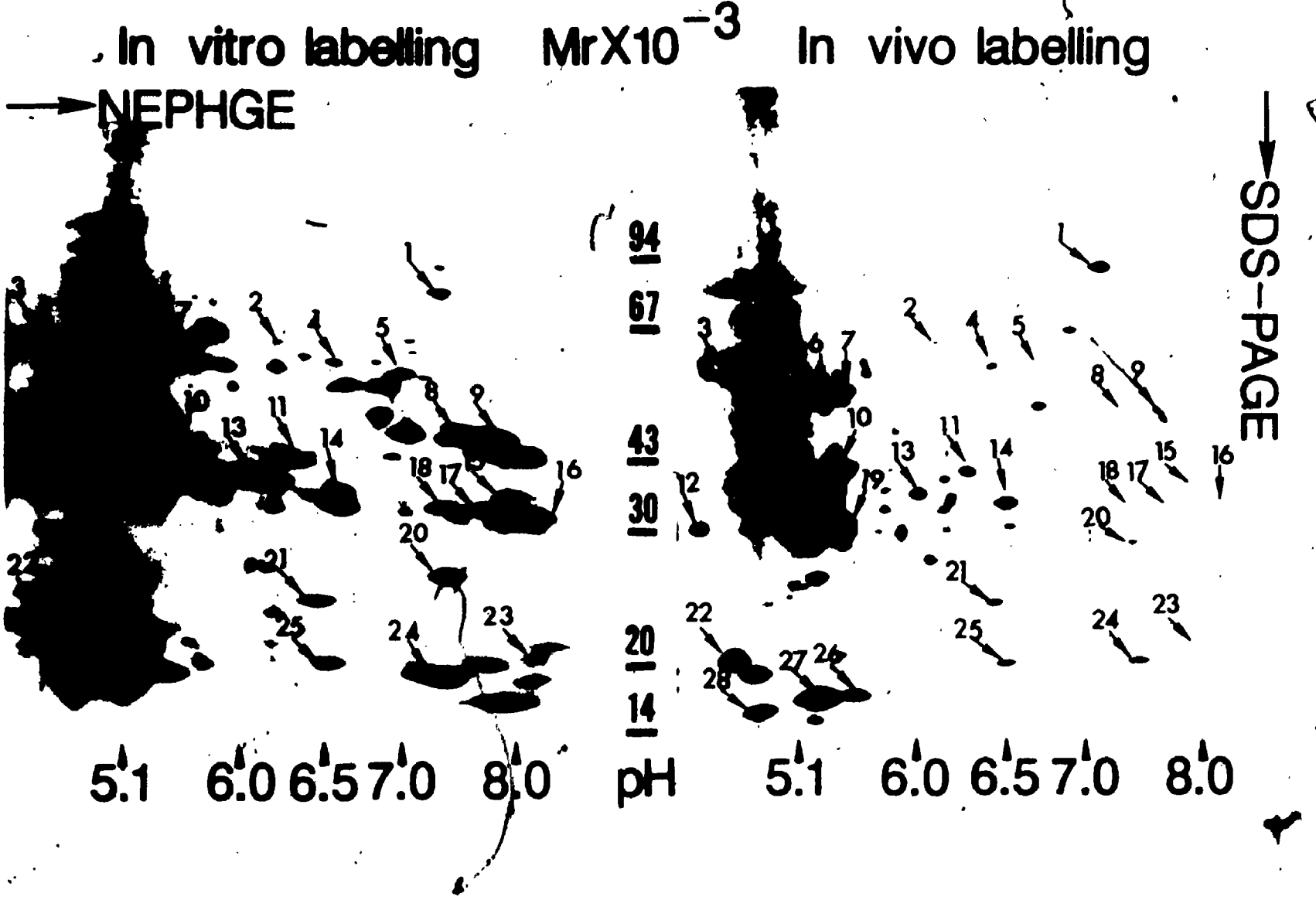

In vitro labelling
In vivo labelling
MrX10^{-3}
MrX10^{-3}
94
67
43
30
20
14
82
56
47
36
30
27
19
16
A T B
B T A
A T B
Normal epithelial orientation
Inverted epithelial orientation
A=Apical
T=Tissue
B=Basal

exposed to the medium, do not result in detectable contamination of the outer incubating medium in experiments with either epithelial orientation. The absence of almost all tissue proteins from both apical and basal secretions suggests that there is also no contamination from tissue breakdown or cell death, confirming the cytochemical tests for cell survival (contraction and expansion of muscles and dye exclusion). Since eight polypeptides are secreted from the basal surface into the lumen of tubes with an inverted epithelial orientation, these eight are probably normally secreted by the midgut into the hemocoel. Similarly the secretion of seven polypeptides across the apical surface of tubes with normal epithelial orientation, suggests that this is normal luminal secretion. These experiments show that Calpodes midgut has bi-directional secretion in vitro. I therefore wished to know if there might be similar bi-directional secretion in vivo.

4.2.2. Protein Synthesis In vivo

Protein synthesis and secretion by the midgut were studied in vivo by injecting [^{35}S] methionine into mid fifth instar Calpodes larvae and allowing to incorporate for 2 hr before sampling the midgut lumen, midgut and the hemolymph. Approximately 25 polypeptides were labelled in the midgut cells (Fig. 8, In vivo T) compared to ten in the apical luminal compartment (Fig. 8, In vivo A) and more than 25 in the basal compartment=hemolymph (Fig. 8, In vivo B). The polypeptides labelled in the midgut cells under in vivo and in vitro

Fig. 9. The similarity between midgut proteins labelled *in vitro* and *in vivo*. [^{35}S] methionine labelled midgut proteins from *in vitro* and *in vivo* experiments were separated on NEPHGE-SDS-PAGE employing 5-15% SDS-PAGE gradient in the second dimension. Approximately 100,000 counts/min were loaded onto each gel and the fluorograms were developed for two weeks. pH and M_r were determined as described in Materials and Methods. The arrows point to 28 of the most pronounced spots present in both fluorograms.

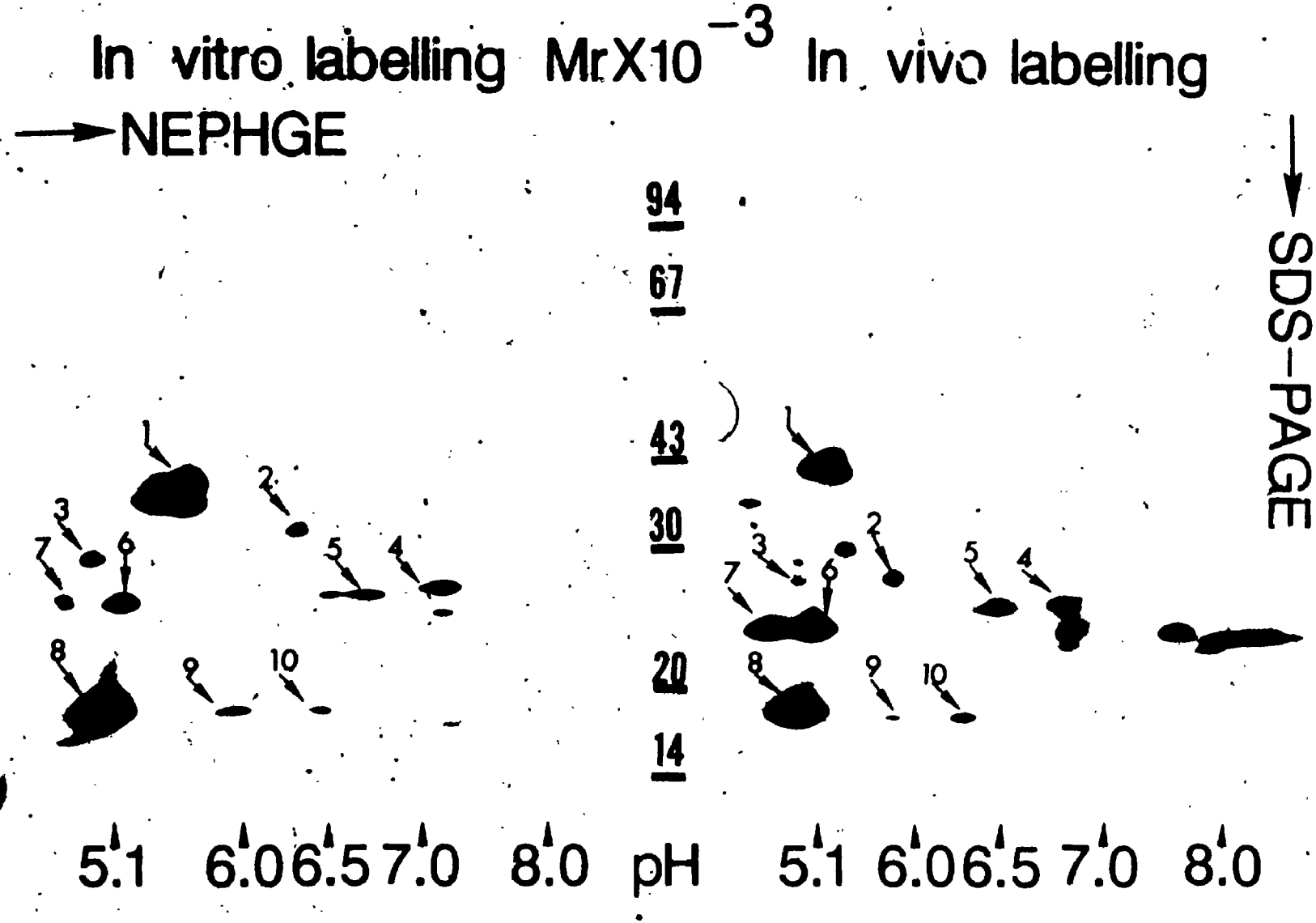

In vitro labelling
MrX10^{-3}
In vivo labelling
NEPHGE
SDS-PAGE
94
67
43
30
20
14
5.1
6.0
6.5
7.0
8.0
pH

conditions showed similar patterns on these SDS gels. Two-dimensional gels of midgut proteins labelled *in vivo* and *in vitro* confirmed the similarity and showed that 70 out of 80 of the most distinct spots were common to both gels (Fig. 9). The similar labelling of cellular proteins under *in vitro* and *in vivo* conditions suggests that the *in vitro* system functions normally.

4.2.3 The Similarity between Apical Secretions and Naturally Occurring Luminal Proteins

In vitro labelled apical secretions and luminal proteins labelled *in vivo* were separated on SDS-PAGE and NEPHGE-SDS-PAGE. All seven polypeptides secreted apically *in vitro* were present in the co-electrophoresed *in vivo* luminal secretions (Fig. 8, *In vitro* A and *In vivo* A). Three additional polypeptides in the *in vivo* luminal secretions presumably arise from tissues upstream from the midgut, such as salivary glands. Two-dimensional gels of labelled proteins from *in vitro* and *in vivo* apical compartments showed that ten out of 18 apical polypeptides discernible *in vitro* were also present in the lumen *in vivo* (Fig. 10). The additional luminal polypeptides occurring *in vivo* presumably arise from upstream regions not present in isolated midgut tubes, as mentioned above. These experiments show that all apical protein secretions labelled *in vitro* are similar to those labelled *in vivo*, confirming the suggestion that the *in vitro* system functions normally.

Fig. 10. The similarity between midgut apical secretions labelled *in vitro* and *in vivo* . Proteins from the apical compartment after [^{35}S] methionine labelling *in vitro* and from the lumen labelled *in vivo* were separated by ,NEPHGE-SDS-PAGE employing 5-15% SDS-PAGE gradient in the second dimension. Approximately 50,000 counts/min were loaded onto each gel and the fluorograms were developed for a month. pH and M_r were determined as described in Materials and Methods. The arrows point to ten of the most pronounced spots that are present in both fluorograms.


In vitro labelling
MrX10^{-3}
In vivo labelling
NEPHGE
SDS-PAGE
94
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pH
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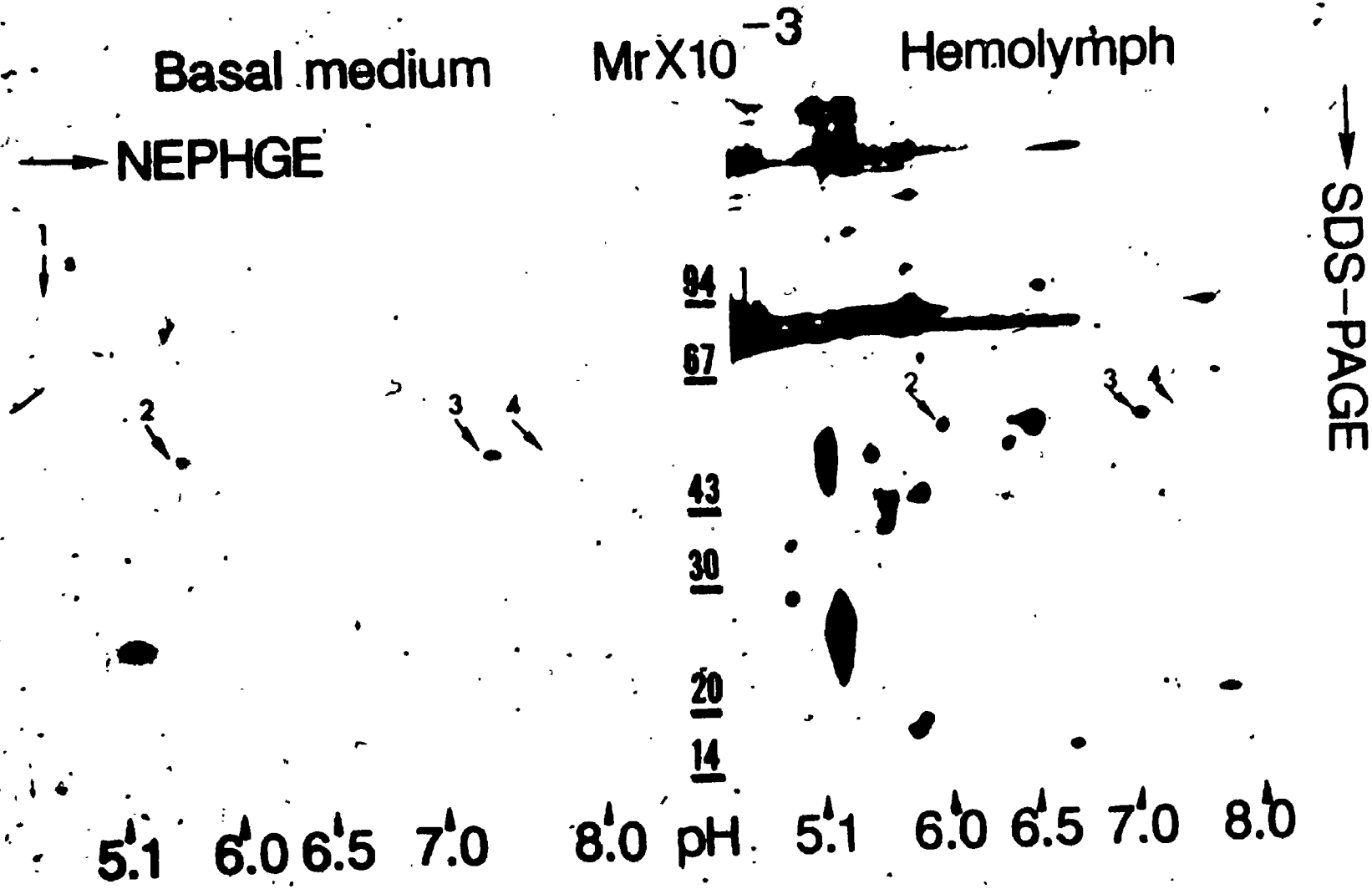
4.2.4 The Similarity between Basal Secretions and Naturally Occurring Hemolymph Proteins

Basal secretions labelled *in vitro* and hemolymph labelled *in vivo* were analysed by SDS-PAGE and NEPHGE-SDS-PAGE to determine the degree of correspondence between them. Most bands in the basal secretion co-migrated with similar bands from hemolymph (Fig. 8, B). Two of these polypeptides (82 K migrates on the acidic end, Fig. 11, 1; 56 K dissociates into three spots, Fig. 11, 2-4) could be identified in the hemolymph NEPHGE-SDS-PAGE gel. Other basal secretions may also be there but they could not be distinguished because of the overcrowding of spots. I therefore used immunoprecipitation to demonstrate the presence of midgut basal secretions in the hemolymph.

4.2.5 The Immunological Similarity between Midgut Basal Secretions and Naturally Occurring Hemolymph Proteins

The hemolymph collected from early fifth instar larvae was used to make antibodies (Fig. 12a). The rabbit made antibodies to almost all 25 polypeptides (Fig. 12b). Antibodies had therefore probably been made against all the midgut basal secretions present in the hemolymph and should recognize them. To verify this, *in vitro* labelled proteins from the lumen, tissue and basal medium were precipitated using antibodies to the hemolymph. The resultant precipitates were separated on SDS-PAGE and the gels processed for fluorography. Antibodies made against hemolymph proteins recognized almost all basal secretions present in the tissue and basal medium (Fig. 12c). No apical secretions were precipitated by antibodies to

Fig. 11. The similarity between proteins secreted by the midgut and those occurring naturally in the hemolymph. Proteins from the basal compartment labelled with [^{35}S] methionine *in vitro* and hemolymph labelled *in vivo* were resolved on NEPHGE-SDS-PAGE employing 5-15% gradient in the second dimension. Approximately 50,000 counts/min were loaded onto each gel and the fluorograms were developed for a month. pH and M_r are determined as described in Materials and Methods. The arrows point to the spots that are present in both fluorograms and indicate polypeptides 82 K (1) and 56 K (2-4). Arylphorin does not enter NEPHGE-SDS-PAGE gel very well so, it was resolved as a minor spot instead of an expected major spot.


Basal medium
MrX10 -3
Hemolymph
NEPHGE
SDS-PAGE
94
67
43
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14
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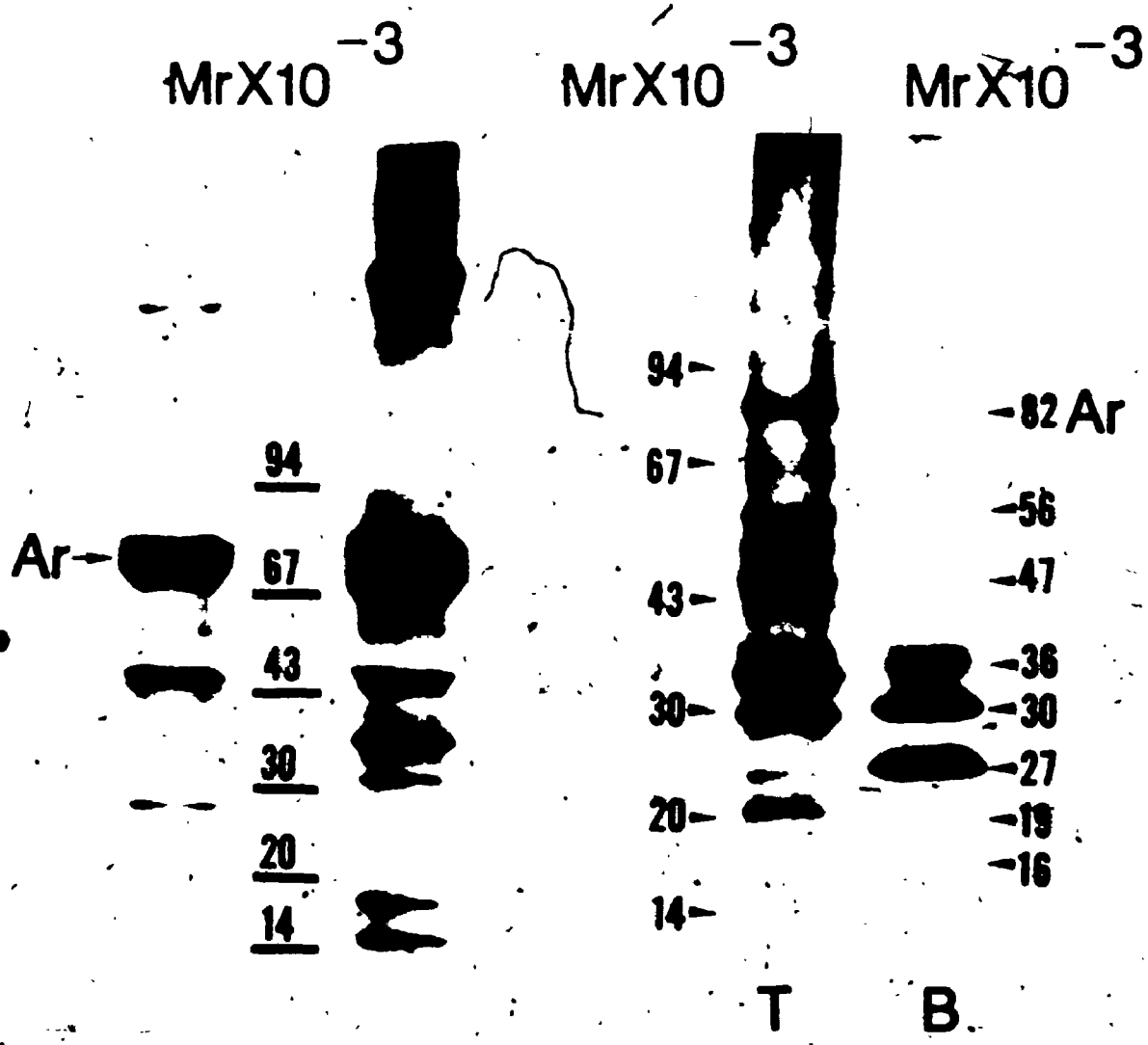
2
3
4
5.1
6.0
6.5
7.0
8.0
pH

Fig. 12. The immunological similarity between midgut basal secretions and naturally occurring hemolymph proteins.

(a) The hemolymph proteins used as antigens. Ten microliters of early fifth instar larval hemolymph (which was used for making antibodies) was separated on a 3-15% SDS-PAGE gradient and stained with Coomassie blue R250. Hemolymph was separated into approximately 25 bands.

(b) The immunoprecipitate of hemolymph. [^{35}S] methionine labelled fifth instar larval hemolymph was precipitated with hemolymph antibodies and separated on a 3-15% SDS-PAGE gradient. Approximately 20,000 counts/min were loaded and the fluorogram was developed for a week at -70°C. Antibodies were made to almost all 25 polypeptides present in the hemolymph.

(c) The immunological similarity between midgut proteins and hemolymph proteins. The proteins from midgut and the apical and basal compartments were labelled with [^{35}S] methionine in vitro. They were precipitated with antibodies to hemolymph proteins. Immunoprecipitates of midgut tissue (T) and basal medium (M) containing approximately 10,000 counts/min were separated on a 5-15% SDS-PAGE gradient. The fluorogram was developed for two weeks at -70°C. Note the presence of all eight midgut basal secretions in both the tissue (T) and basal medium (M). Although Ar is a major protein secreted into the basal medium, it did not show up as a major band in immunoprecipitation, probably due to competition between labelled and unlabelled Ar present in the medium and the proportion of Ar antibody in the whole hemolymph antibodies. The relative mobility of Pharmacia low molecular weight standards and the M_r of basal secretions are shown on the left and right sides of the figure.

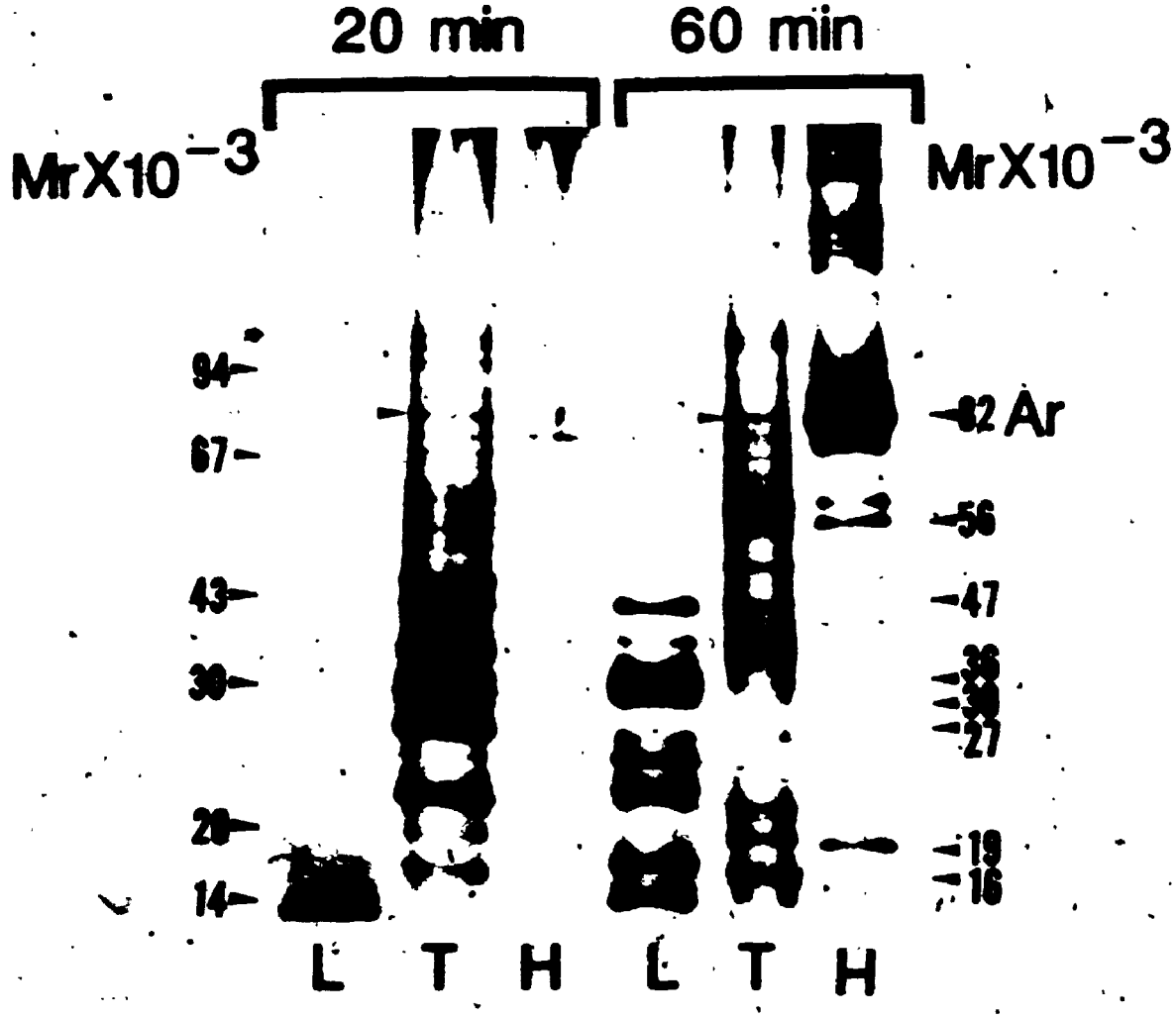

MrX10 -3
MrX10 -3
MrX10 -3
Ar
94
67
43
30
20
14
94
67
43
30
20
14
82 Ar
56
47
36
30
27
19
16
T
B
Ar=Arylphorin
T=Midgut tissue
B=Basal medium
a
b
c

the hemolymph. The protein sample was pretreated with SAS (IgSorb) and a non-ionic detergent (NP-40) was included in the assay to reduce the background. These precautions make it likely that the antibodies to the hemolymph proteins were recognizing basally secreted polypeptides because of immunological similarity.

4.2.6 The Sequential Appearance of Basal Secretions in Midgut Cells Prior to their Appearance in the Hemolymph

A source of error with in vivo labelling is the possibility that the midgut might take up proteins newly synthesized and secreted by other tissues during the 2 hr of labelling. The comparison between in vitro and in vivo protein synthesis and secretion could be in error unless it can be shown that the proteins in question are synthesized first by the midgut. Tissues were exposed to [^{35}S] methionine in vivo for 20 and 60 min to show the relative times of appearance of the basal secretion in the midgut tissue and hemolymph. Labelling the mid-fifth instar Calpodes larvae with [^{35}S] methionine for 20 and 60 min showed that labelled polypeptides appeared in the midgut cells after 20 min when neither hemolymph nor lumen were labelled (Fig. 13). By 60 min labelled polypeptides also occurred in the hemolymph and lumen. Since all bands corresponding to basally secreted polypeptides appeared in the midgut by 20 min after the beginning of labelling, when nothing was labelled in the hemolymph, the midgut is probably the source of such polypeptides.

Fig. 13. The sequential appearance of midgut secretory proteins first in the tissue and later in the apical and basal compartments. Midgut proteins were labelled in vivo by injecting [^{35}S] methionine into mid fifth instar larvae. After incorporation for 20 and 60 min proteins were extracted from the midgut tissue and its apical and basal compartments. Approximately 20,000 counts/min were loaded onto each well (except in the case of L and H at 20 min where the incorporation was minimal, 100 ul of sample were loaded) and the fluorogram was developed for a week at -70°C. Note the appearance of both apical and basal secretions in the midgut tissue when neither lumen nor hemolymph are labelled (20 min). By 60 min labelled polypeptides also occurred in the lumen and hemolymph. The relative mobility of low molecular weight standards is shown on the left side and the M_r of the basal secretions are shown on the right side.



L=Lumen T=Tissue H=Hemolymph

Ar=Arylphorin

4.2.7 The Secretion of Arylphorin by the Midgut

One of the three major larval hemolymph proteins of Calpodes is a lipoglycoprotein with a molecular weight of 470 K and dissociates into 82 K subunits in presence of SDS (Locke et al., 1982). This protein is now called as arylphorin (Ar; Chapter 7). Arylphorin was present in midgut tissue (Fig. 8, T) and basal medium (Fig. 8, B) labelled in vitro, and in midgut tissue (Fig. 8, T) and hemolymph (Fig. 8, B) labelled in vivo (Fig. 11). The experiments in which fifth instar larvae were exposed to [^{35}S] methionine for 20 and 60 min showed that Ar occurred in the midgut cells by 20 min, prior to its appearance in the hemolymph (Fig. 13). It was also labelled in both midgut cells and basal medium in vitro (Fig. 8, T; B) in the absence of other tissues. The synthesis and secretion of Ar by midgut was confirmed by immunological studies. Antibodies to the hemolymph proteins recognized Ar present in midgut cells and basal medium (Fig. 12c). Antibodies to Ar itself (purified by electrophoretic separation from hemolymph, Figs.14 a; b) recognized the Ar from midgut cells and basal medium (Fig. 14c). All these findings suggest that the midgut contributes Ar to the hemolymph pool.

4.3 Discussion

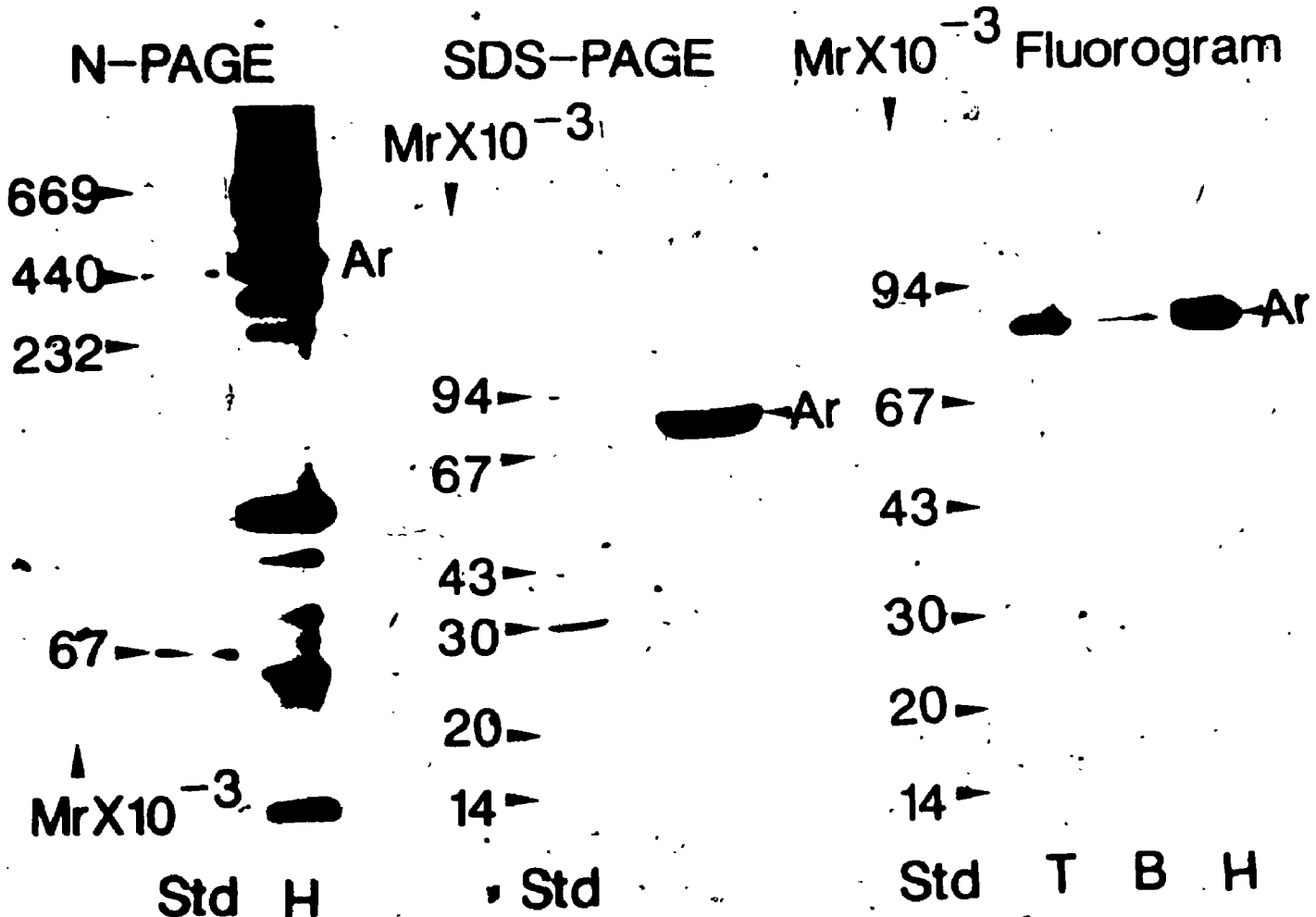
The main finding is that Calpodes midgut secretes certain hemolymph proteins from the basal surface as well as others in the expected apical luminal direction. In inverted tubes the basally secreted proteins accumulate luminally where there is no contamination

Fig. 14. The immunological evidence that arylphorin is secreted by the midgut.

(a) The purification of Ar from hemolymph. Twenty microliters of hemolymph of mid fifth instar larvae were separated on a 3-15% N-PAGE gradient and stained with Coomassie blue R250. The 470 K Ar band was excised and run on an SDS gel to obtain Ar. The relative mobility of Pharmacia high molecular weight standards is shown on the left side of the figure.

(b) The Ar used as an antigen. The 470 K bands excised from non-denaturing gels were run on SDS gels. Arylphorin dissociates into subunits with a molecular weight of 82 K. The 82 K bands from SDS gels were excised, the protein eluted and used for immunization. Fifty microliters of eluted protein was separated on a 3-15% SDS-PAGE gradient and stained with Coomassie blue R250 to show the purity of the polypeptide used as an antigen. The relative mobility of Pharmacia low molecular weight standards is shown on the left side of the figure.

(c) The immunological similarity between midgut and hemolymph Ar. [^{35}S] methionine labelled proteins (100,000 counts/min) from midgut tissue, basal medium and hemolymph were precipitated with antibodies to hemolymph Ar. Immunoprecipitates of midgut tissue (T), basal medium (M) and hemolymph (H) were separated on a 5-15% SDS-PAGE gradient. Fluorograms were developed for two weeks at -70^{o}C. The Ar present in the midgut tissue, basal medium and hemolymph was precipitated by the hemolymph Ar antibodies.


N-PAGE
SDS-PAGE
MrX10 -3
Fluorogram
669
440
232
Ar
94
67
43
30
20
14
Std H
Std
Std T B H
H=Hemolymph
Ar=Arylphorin
T=Midgut tissue
Std=Molecular weight standards
B=Basal medium
a
b
c

from apical surface. Since the midgut normally releases these proteins basally it is reasonable to assume that they contribute directly to the hemolymph. Sources of error such as cell breakdown, membrane blebbing or transepithelial leakage are unlikely, since: (a) protein synthesis is similar *in vivo* and *in vitro*, (b) all *in vitro* apical secretions occur in the *in vivo* luminal proteins but not in the hemolymph, (c) basally secreted midgut proteins occur naturally in the hemolymph, (d) there are few bands/spots in the media compared to many in the tissue, and (e) midgut basal secretions are immunologically similar to naturally occurring hemolymph proteins. These observations strongly suggest that the protein secretion observed *in vitro* is natural and the midgut secretes hemolymph proteins from the basal surface as well as proteins from the apical surface.

Chippendale (1970) and Chippendale and Kilby (1970) concluded that the midgut could be contributing proteins to the hemolymph pool mainly based on the relative incorporation of [^{14}C] leucine by hemocytes, fat body and midgut of *Pieris brassicae* and *Diatraea grandiosella*. However, Turner and Loughton (1975) could find only luminal protein synthesis in *Locusta migratoria* midgut maintained *in vitro*. The inability of those authors to demonstrate hemolymph protein synthesis by the midgut may have been due to the technical limitation of labelling with [^{3}H] leucine.

The second finding is that *Calpodes* midgut secretes a different set of proteins from each surface. Proteins found outside the ligated tubes are not due to leakage since two-dimensional analysis showed

that the apical and basal proteins are different. Almost all apical secretions match the luminal proteins labelled in vivo (Fig. 10) and almost all basal secretions showed immunological similarity with hemolymph proteins (Fig. 12c). As in the epidermis it is not known how the bi-directional secretion is achieved. The midgut has a few very fine muscles embedded with tracheae. These cells have very little RER and are unlikely sources of secretory protein. However, they have not been excluded as protein source. A more likely candidate for midgut hemolymph protein secretion is a fourth cell type (in addition to the columnar, goblet and regenerative cells) in the midgut epithelium which does have a structure appropriate for secreting proteins into the hemocoel. These cells are similar to the endocrine cells described in the midgut of Lepidopteran larvae by Endo and Nishiitsutsuji-Uwo (1981) and they could be secreting polypeptides into the hemolymph. However, preliminary results from immunoflorescence staining of midgut sections with antibodies to hemolymph show that staining is generalized rather than specific to a few cells, suggesting the presence of hemolymph proteins in most cells. The observation of bi-directional secretion in Calpodes midgut may provide another system (other systems for studies related to protein sorting are discussed in Chater 3) for studies related to protein sorting.

The third finding is that the midgut secretes Ar. Arylphorin (470 k protein with 82 K subunits on SDS gels) is the major band in the midgut basal medium (Fig. 8, B) and is precipitated by the

antibodies to hemolymph Ar (Fig. 14c). Arylphorin is synthesized by the fat body in Calpodes and many other insects (Locke et al., 1982; Riddiford and Hice, 1985). Since the proteins are labelled in midgut isolated in vitro, the possibility that the Ar has been taken up from the fat body is excluded. It is also unlikely that the midgut Ar is an error due to contamination by a few fat body cells sticking to midgut or tracheoles. The midgut was carefully cleaned from fat body and large tracheae before making the tubes. The absence of other major fat body secretory proteins (storage proteins and others) also makes it most unlikely that the presence of Ar is due to fat body contamination. These observations suggest that hemolymph Ar may be synthesized by the midgut as well as fat body and epidermis.

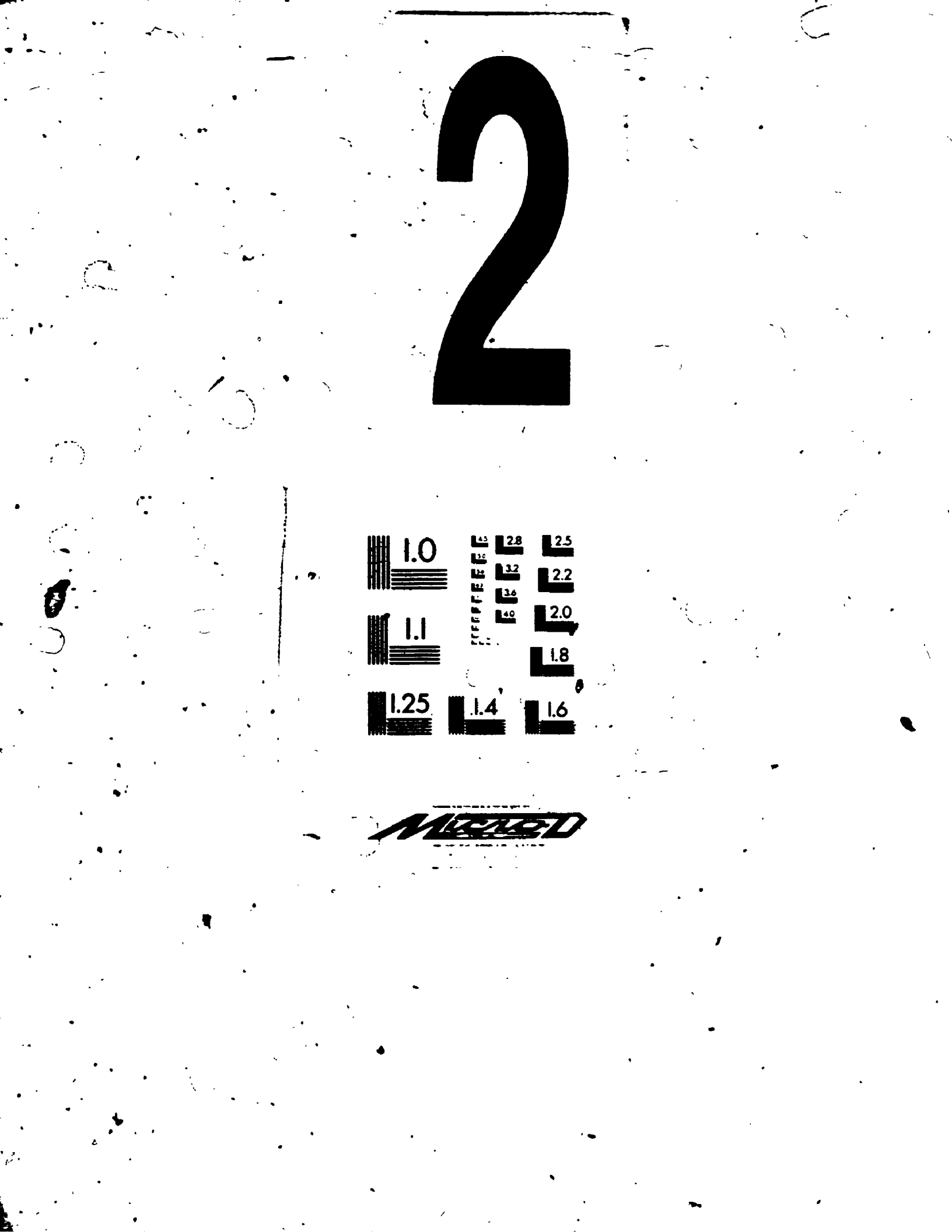
CHAPTER 5

HEMOLYMPH PROTEIN SYNTHESIS BY THE FAT BODY

5.1 Introduction

Ever since Shigematsu (1958) reported that fat body synthesizes and secretes hemolymph proteins, the fat body has been the center of attention for researchers studying hemolymph protein synthesis, resulting in many publications (see Wyatt and Pan, 1978; Roberts and Brock, 1982; Riddiford and Law 1983; Dean et al., 1985; Keeley, 1985; Levenbook, 1985 for reviews). Most studies have concentrated on a few major hemolymph proteins such as storage proteins (Munn et al., 1969), arylphorins (Kramer et al., 1980; Telfer et al., 1983), lipophorins (Gellissen and Wyatt, 1981), diapause proteins (Venkatesh and Chippendale, 1986), JH binding proteins (Nowock et al., 1975) and JH esterases (Wing et al., 1981). There has been no estimate of the relative role of the fat body contributing to the many proteins known to be in the hemolymph. Although there are many proteins in the Calpodes hemolymph only three major hemolymph proteins were shown to be secreted by the fat body (Locke et al., 1982).

To understand the role of hemolymph proteins in metamorphosis, it is necessary to construct a balance sheet for all hemolymph proteins throughout an insect's life. We do not know the source of all hemolymph proteins for even a single insect although we now know that epidermis (Chapter 3), midgut (Chapter 4) and pericardial cells (Fife et al., 1987) contribute to the hemolymph protein pool. The present


2
1.0
1.1
1.25
1.4
1.6
2.8
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4.0
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2.2
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1.8

study aimed to identify the proteins secreted by larval fat body. Comparison with hemolymph proteins synthesized by the whole animal would then show not only the role of the fat body but also the proteins that are synthesized and secreted by other tissues.

5.2 Results

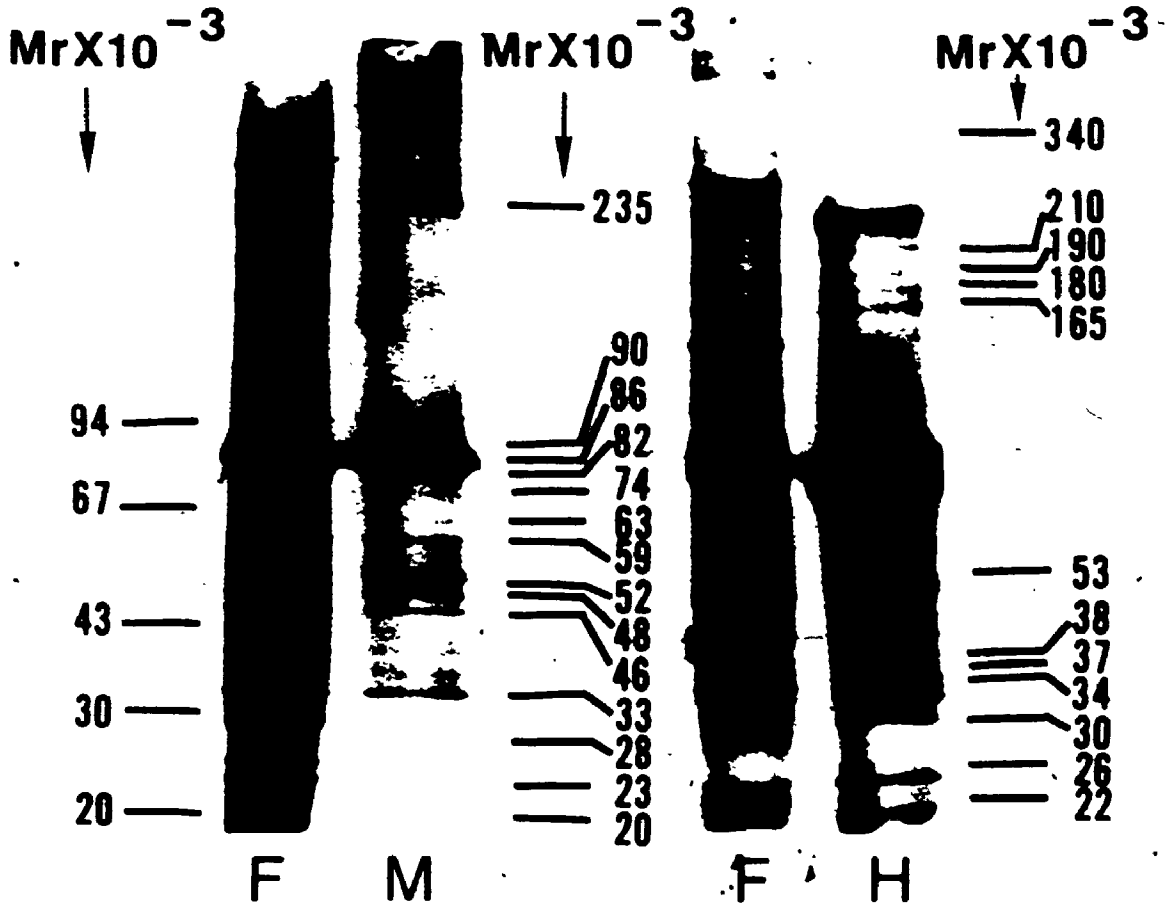
5.2.1 Protein Synthesis In vitro

Sheets of fat body were incubated in artificial hemolymph in the presence of [^{35}S] methionine for 2 hr. The proteins present in the fat body and medium were solubilized in non-denaturing solubilization solution, separated on SDS-PAGE and the gels processed for fluorography to identify newly synthesized polypeptides. Approximately 30 polypeptides in the molecular weight range of 20-270 K were labelled in the fat body (Fig. 15, in vitro F). Fourteen polypeptides (235, 90, 86, 82, 74, 63, 59, 52, 48, 46, 33, 28, 23 and 20 K) were released into the medium (Fig. 15, in vitro M). Since the polypeptides present in the medium are consistent during many repetitions and include a known secretory protein (Ar) but not the main polypeptides labelled in the cells, there is no likelihood of contamination from tissue breakdown or cell death. Since these polypeptides are secreted into the bathing medium it is reasonable to assume that they are normally secreted by the fat body into the hemocoel.

5.2.2 Protein Synthesis In vivo

[^{35}S] methionine was injected into mid fifth instar Calpodes larvae and allowed to incorporate for 2 hr. Proteins were solubilized

Fig. 15. Secretion of hemolymph proteins by the fat body. Sheets of fat body were incubated in artificial hemolymph in the presence of 0.05 mCi of [^{35}S] methionine for 2 hr. The proteins present in the fat body and medium were separated on a 5-15% SDS-PAGE gradient. Approximately 20,000 counts/min were loaded in each well and the fluorograms were developed for seven days at -70^{0}C. Fourteen [^{35}S] methionine labelled polypeptides were secreted in vitro into the medium. Since these 14 polypeptides are secreted in absence of other tissues and there is no evidence for cell breakdown, these polypeptides are probably normally secreted by fat body into the hemolymph. Whole larvae were also injected with [^{35}S] methionine and after 2 hr of incorporation in vivo the proteins labelled in fat body and hemolymph were electrophoresed. Note the correspondence between polypeptides labelled in vitro and in vivo in their respective compartments. The relative mobility of Pharmacia low molecular weight standards is shown on the left side of the figure. The M_r of fat body secretory polypeptides are shown in the middle and the M_r of non-fat body hemolymph polypeptides are shown on the right side of the figure.

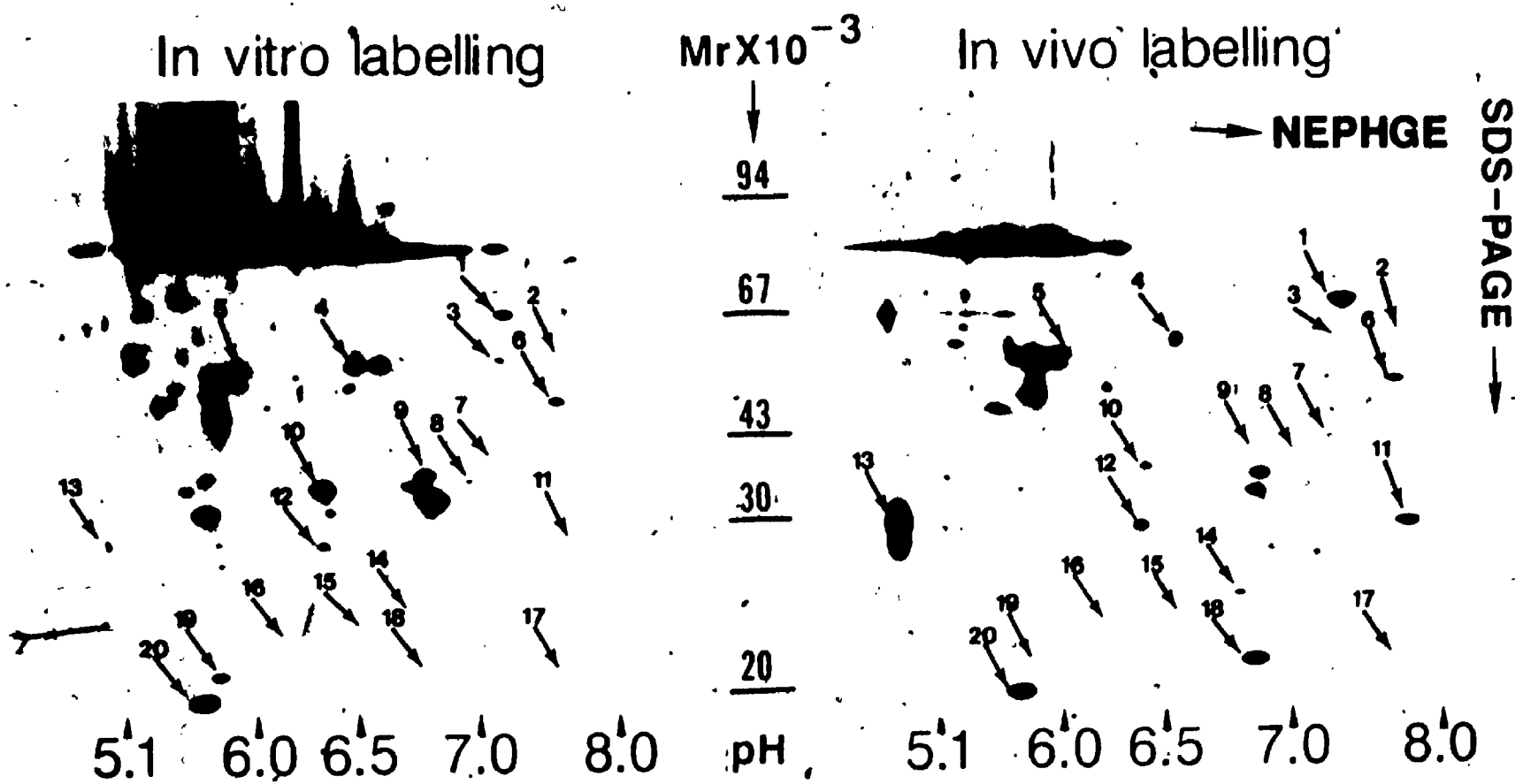


F=Fat body

M=Medium

H=Hemolymph

Fig. 16. The similarity between fat body proteins labelled *in vitro* and *in vivo*. Proteins from the fat body after [^{35}S] methionine labelling *in vitro* and from fat body labelled *in vivo* were separated by NEPHGE-SDS-PAGE employing 5-15% SDS polyacrylamide gradient gels in the second dimension. Approximately 100,000 counts/min were loaded onto each gel and the fluorograms were developed for 15 days. pH and M_r were determined as described in Materials and Methods. The arrows point to 20 of the most pronounced spots that are present in both fluorograms.


In vitro labelling
MrX10^{-3}
In vivo labelling
NEPHGE
SDS-PAGE
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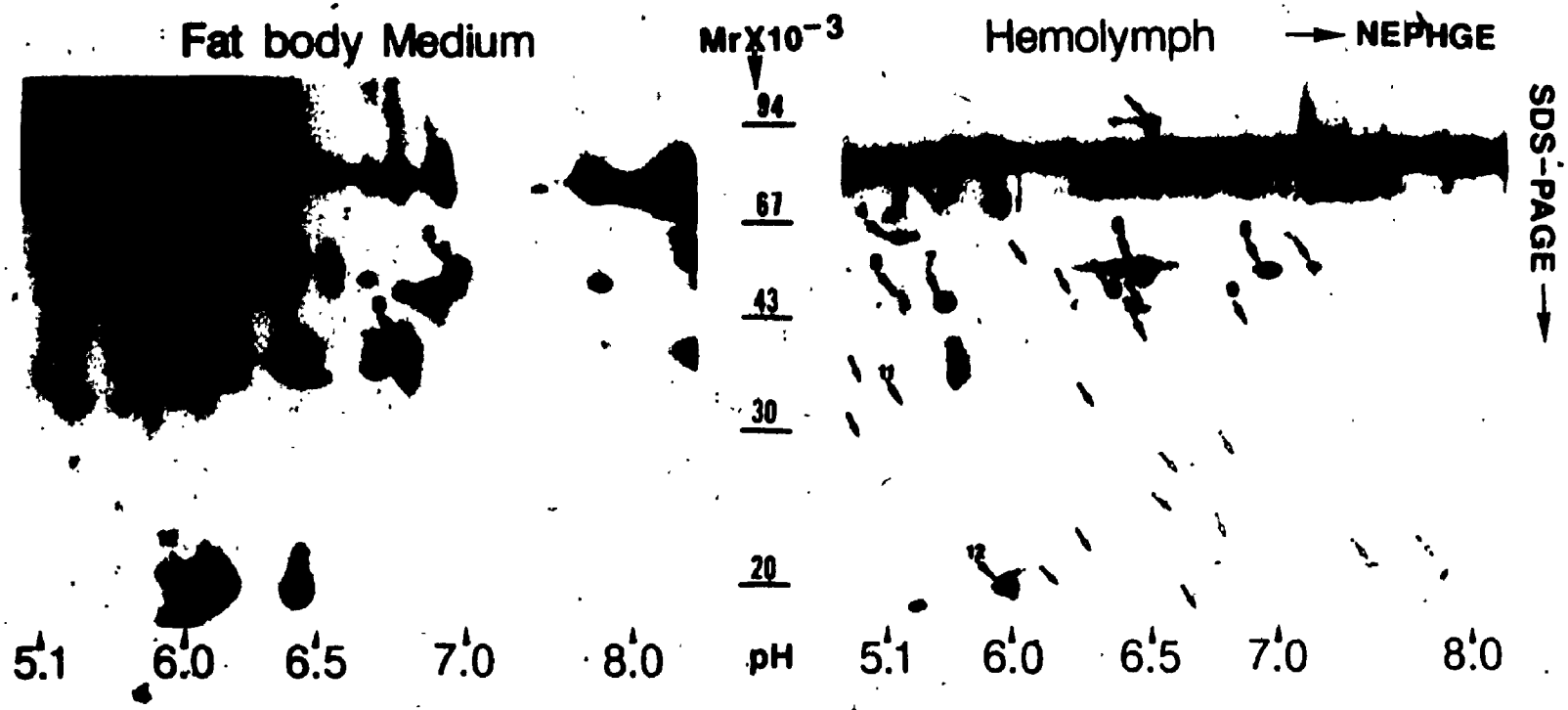
from the fat body and hemolymph and separated on SDS-PAGE and the gels processed for fluorography to identify the newly synthesized proteins. Approximately 30 polypeptides were labelled in the fat body (Fig. 15, In vivo F) and more than 25 in the hemolymph (Fig. 15, In vivo H). The polypeptides labelled in the fat body under in vivo and in vitro conditions show similar patterns on these SDS gels. There are a few quantitative differences perhaps due to shock or the absence of hormones and other controlling factors, but the overall polypeptide pattern appears similar. Two-dimensional analysis of the fat body proteins labelled in-vitro and in-vivo confirmed the similarity and showed that 45 out of 50 of the most distinct spots were common to both the gels (Fig. 16). The similar labelling of cellular proteins under in vitro and in vivo conditions suggests that the in vitro system functions normally.

5.2.3 The Similarity between Fat body Secretory proteins and Naturally Occurring Hemolymph Proteins

The fat body secretory proteins labelled in vitro and hemolymph proteins labelled in vivo were analysed by two-dimensional gel electrophoresis to determine the degree of correspondence between them. Most of the bands in the fat body medium co-migrated with similar bands from hemolymph (Fig. 15, in vitro M; in vivo H). Twelve of these polypeptides (Fig. 17; 1-12) could be identified in the hemolymph NEPHGE-SDS-PAGE gel. Additional spots in the fat body medium are probably at too low a concentration to be resolved among the background of many hemolymph polypeptides. Antibodies prepared

Fig. 17. The similarity between proteins secreted by the fat body and those occurring naturally in the hemolymph . Proteins from the medium after [^{35}S] methionine labelling *in vitro* and hemolymph labelled *in vivo* were resolved on NEPHGE-SDS-PAGE empolying 5-15% SDS polyacrylamide gradient gels in the second dimension. Approximately 100,000 counts/min were loaded onto each gel and the fluorograms were developed for 15 days. pH and M_r were determined as described in Materials and Methods. The numbered solid arrows point to spots present in both fluorograms. Unnumbered open arrows point to non-fat body hemolymph proteins.


Fat body Medium
MrX10⁻³
Hemolymph
NEPHGE
SDS-PAGE
94
67
43
30
20
5.1
6.0
6.5
7.0
8.0
pH
5.1
6.0
6.5
7.0
8.0

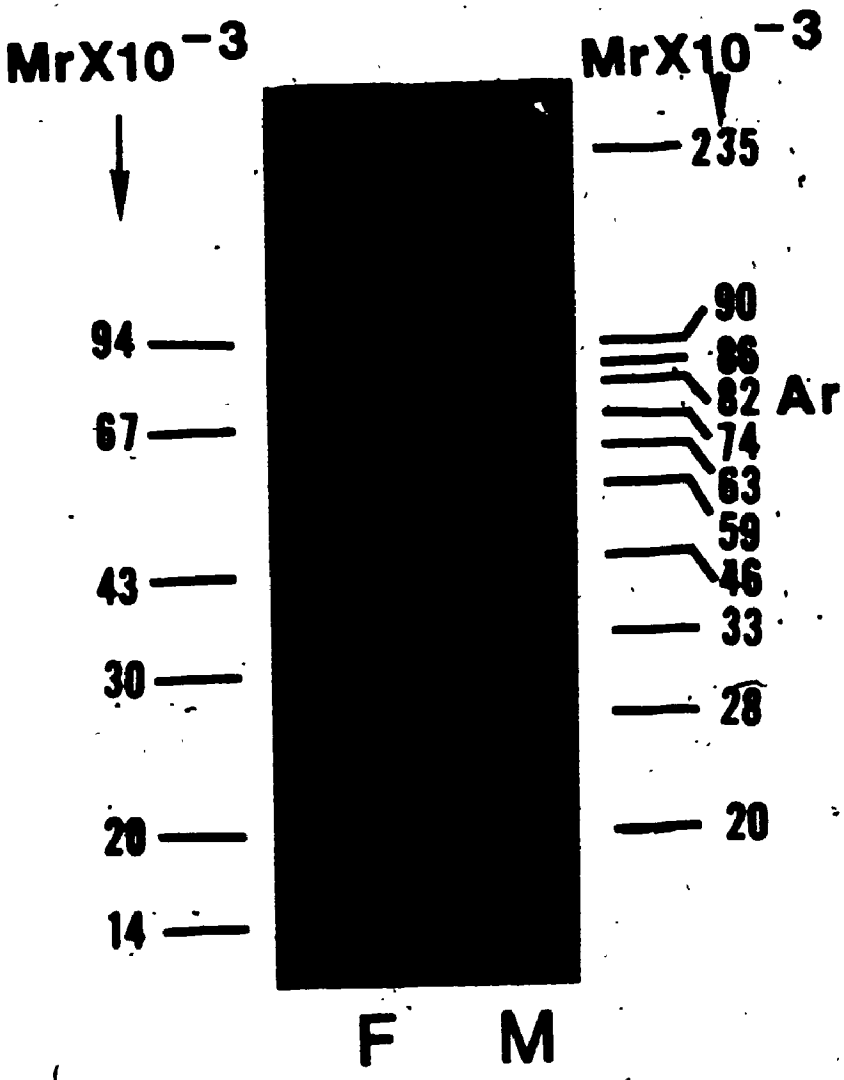
against hemolymph proteins were therefore used to identify the fat body proteins secreted into the hemolymph.

At least 12 hemolymph polypeptides (340, 210, 190, 180, 165, 53, 38, 37, 34, 30, 26 and 22 K) labelled in vivo do not have co-migrating bands in the fat body medium (Fig. 15, in vitro M; in vivo H). These polypeptides are therefore not synthesized by the fat body. Two-dimensional analysis of fat body medium and hemolymph proteins showed that there are at least 17 spots in the hemolymph that are not present in fat body medium (Fig. 17; spots indicated with unnumbered open arrows). The results show that at least half (about 10% by quantity) of the hemolymph polypeptides are not secreted by the fat body and must therefore be contributed by other tissues.

5.2.4 The Immunological Similarity between Fat body Secretory proteins and Hemolymph Proteins

In vitro labelled proteins from the fat body and medium were precipitated using antibodies made against hemolymph proteins. The resultant precipitates were separated on SDS-PAGE and the gels processed for fluorography. Antibodies made against hemolymph proteins recognized at least 11 polypeptides (235, 90, 86, 82, 74, 63, 59, 46, 33, 28 and 20 K) present both in fat body and medium (Fig. 18). Precautions like pretreating the protein sample with SAS (IgSorb) and inclusion of a non-ionic detergent (NP-40) in the assay, make it likely that the antibodies to the hemolymph proteins were recognizing these polypeptides because of their immunological similarity. The results show that the fat body contributes at least 11 polypeptides to the hemolymph protein pool.

Fig. 18. The immunological similarity between fat body secretory proteins and hemolymph proteins. The proteins from fat body (500,000 counts/min), and medium (500,000 counts/min) labelled with [^{35}S] methionine in vitro, were precipitated with antibodies to hemolymph (see Materials and Methods for details). Immunoprecipitates of fat body and medium were separated on a 5-15% SDS-PAGE gradient. The fluorogram were developed for a week at -70°C. Eleven polypeptides secreted by fat body were recognized by antibodies prepared against the hemolymph proteins. The relative mobilities of Pharmacia low molecular weight standards and the M_r of fat body secreted hemolymph polypeptides are shown on the left and right sides of the figure.



F=Fat body

M=Medium

Ar=Arylphorin

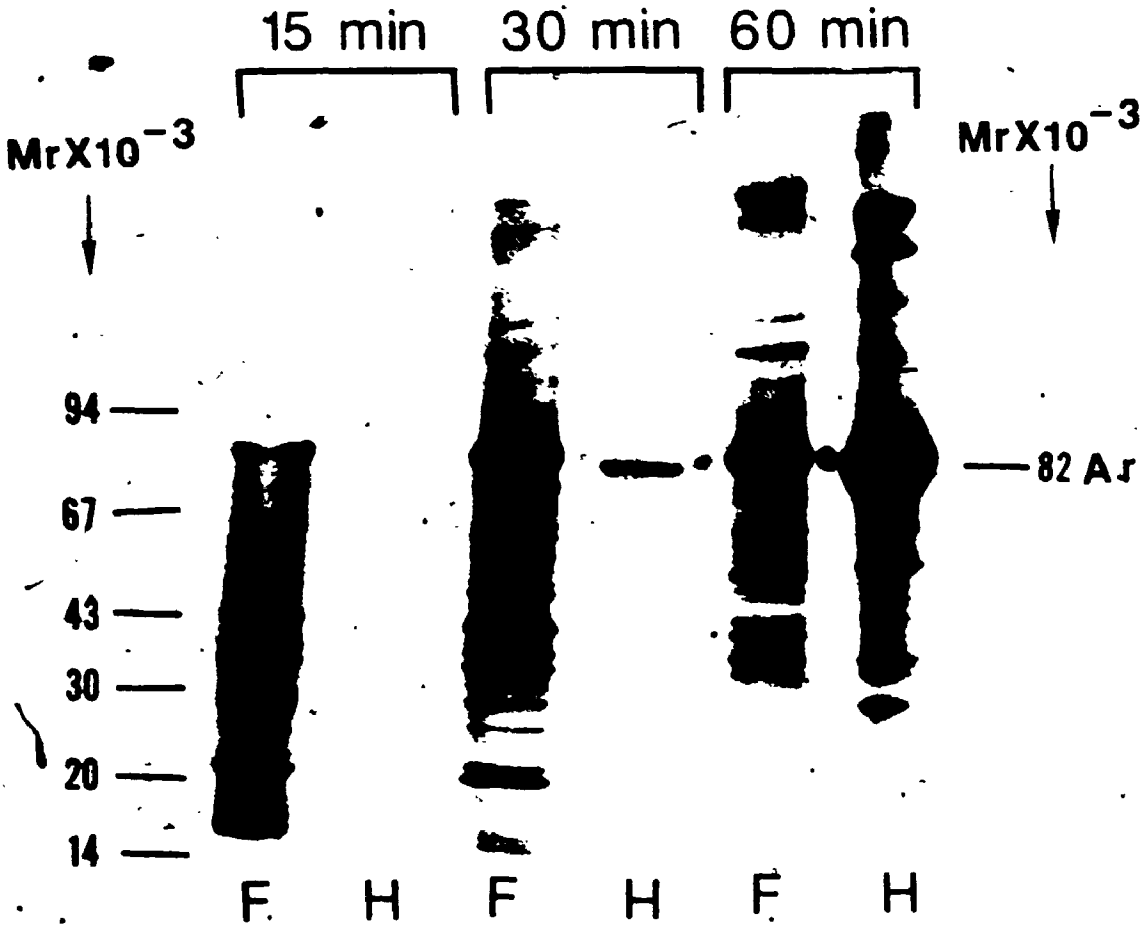
5.2.5 The Sequential Appearance of Secretory Proteins in the Fat body Prior to their Appearance in the Hemolymph

To verify whether the fat body might have taken up proteins newly synthesised by other tissues during the 2 hr of *in vivo* labelling, the tissues were exposed to [^{35}S] methionine *in vivo* for 15, 30 and 60 min, proteins solubilized, separated on gels and the gels processed for fluorography. The labelled polypeptides appeared in the fat body after 15 min of labelling at a time when the hemolymph is not labelled (Fig. 19). By 30 min labelled polypeptides started appearing in the hemolymph and by 60 min the hemolymph is strongly labelled. Since labelled polypeptides (which includes Ar) appeared in the fat body within 15 min after the beginning of labelling, at a time when nothing was labelled in the hemolymph, the fat body is probably the source of such polypeptides.

5.2.6 The Secretion of Arylphorin by the Fat body

The 470 K protein present in larval hemolymph of *Calpodes* is a lipoglycoprotein that dissociates into six similar subunits each with a molecular weight of 82 K in the presence of SDS (Locke et al., 1982). This protein now called as arylphorin (Ar, Chapter 7) was present in fat body (Fig. 15, *in vitro* F), medium (Fig. 15, *in vitro* M) and hemolymph (Fig. 15, *in vivo* H). Experiments in which fifth instar larvae were exposed to [^{35}S] methionine for 15, 30 and 60 min showed that Ar occurred in the fat body by 15 min, prior to its appearance in the hemolymph (Fig. 19). It was also labelled *in vitro* in fat body and medium in the absence of other tissues (Fig. 15).

Fig. 19. The sequential appearance of secretory proteins in the fat body prior to their appearance in the hemolymph. Fat body was labelled in vivo by injecting [^{35}S] methionine into mid fifth instar larvae. After incorporation for 15, 30 and 60 min proteins were solubilized from fat body and hemolymph. Approximately 20,000 counts/min were loaded onto each well (except in the case of H at 15 min where the incorporation was so low that, 100 ul of sample were loaded which is at least 5X the volume loaded for 30 and 60 min labelling) and the fluorograms were developed for a week at -70°C. Labelled polypeptides appear first in the fat body (15 min) before they appear in the hemolymph (30 min). The relative mobility of Pharmacia low molecular weight standards is shown on the left side of the figure.

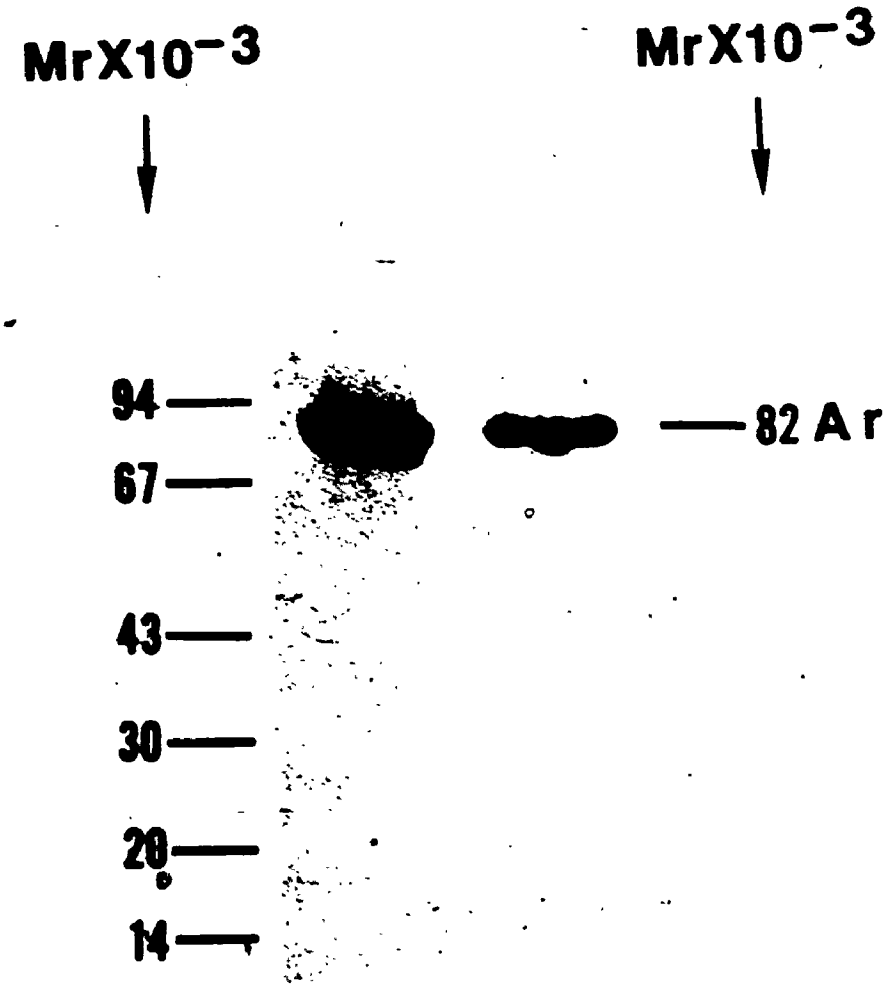


F=Fat body

H=Hemolymph

Ar=Arylphorin

Fig. 20. The immunological evidence that Ar is secreted by the Fat body. $[^{35}S]^-$ methionine labelled proteins from fat body (500,000 counts/min) and medium (500,000 counts/min) were precipitated with antibodies to hemolymph Ar. Immunoprecipitates of fat body and medium were separated on a 5-15% SDS-PAGE gradient. Fluorograms were developed for a week at -70°C. The Ar present in the fat body and medium has been precipitated by antibodies prepared against hemolymph Ar. The relative mobility of Pharmacia low molecular weight standards is shown on the left side of the figure.



F=Fat body

M=Medium

Ar=Arylphorin

Antibodies prepared against hemolymph proteins recognized Ar present in the fat body and medium (Fig. 18). Antibodies made against hemolymph Ar itself (purified by electrophoretic separation from hemolymph, Chapter 2) recognized the Ar from both fat body and medium (Fig. 20). These findings confirm that the fat body synthesizes and secretes Ar.

5.3 Discussion

5.3.1 Hemolymph Protein Secretion by the Fat body

The main finding is that Calpodes fat body secretes more than 11 hemolymph polypeptides. Since the fat body normally releases these proteins into the bathing medium it is reasonable to assume that they contribute directly to the hemolymph. Sources of error such as cell breakdown or membrane blebbing are unlikely, since: (a) protein synthesis is similar in vivo and in vitro, (b) at least 12 fat body secretory polypeptides occur naturally in the hemolymph, (c) there are few bands in the medium compared to many in the tissue, and (d) at least 11 polypeptides secreted into the medium are immunologically similar to naturally occuring hemolymph proteins. These results strongly suggest that the protein secretion observed in the in vitro system is natural. The contribution of a few specific proteins to the hemolymph by the fat body has been reported in several insects (Dean et al., 1985; Keeley, 1985). The three major hemolyph polypeptides and the 235 K polypeptide together make up approximately 90% of the hemolymph protein pool and are all synthesized and secreted by the fat body. Quantitatively the fat body is the major source of hemolymph

proteins. The new findings presented here show that fat body contributes at least 11 polypeptides to the hemolymph. Qualitatively also the fat body is a major hemolymph contributor of hemolymph proteins.

5.3.2 Synthesis and Secretion of Arylphorin by the Fat body

The second finding confirms that the fat body secretes Ar. Arylphorin is present in the fat body and medium (Fig. 15) and is precipitated by antibodies to hemolymph Ar (Fig. 18). Since polypeptides are labelled in the fat body isolated *in vitro* the possibility that Ar has been taken up by the fat body is excluded. Fat body synthesizes and secretes Ar in many insects (Riddiford and Law, 1983). The synthesis and secretion of Ar by epidermis (Chapter 3), midgut (Chapter 4) and pericardial cells in *Calpodes* (Fife et al., 1987) has therefore not evolved in conjunction with a loss of synthesis by the fat body. Hemolymph Ar is made by several tissues. Although Ar synthesized by different tissues react with antibodies to hemolymph Ar, this does not prove identity, since antibodies were made against the mixture of all Ar in the hemolymph. The central question yet to be answered concerns the significance of Ar synthesis and secretion by so many tissues.

5.3.3 Non-Fat body Hemolymph Proteins

The third finding is that the fat body does not contribute all hemolymph proteins. Approximately half (12 polypeptides; about 10% by quantity) of the hemolymph polypeptides labelled in the whole animal are not made by the fat body. These polypeptides are probably

secreted by other tissues such as epidermis, midgut, hemcytes, pericardial cells etc. Some of the non-fat body hemolymph polypeptides can be correlated with polypeptides secreted by the epidermis (53, 38, 26 and 22 K, Chapter 3), midgut (30 K, Chapter 4) and hemocytes (37, 34 and 26 K; Chapter 6).

CHAPTER 6

HEMOLYMPH PROTEIN SYNTHESIS BY THE HEMOCYTES

6.1 Introduction

Insect hematologists studying the structure and function of hemocytes and insect biochemists studying hemolymph proteins, have paid relatively little attention to hemocytes as sites for hemolymph protein synthesis. There is therefore little information about the role of hemocytes, if any, in the origin of hemolymph proteins (Gupta, 1985). In Diatraea grandiosella Chippendale and Kilby (1970) measured the relative protein synthetic capability of larval midgut, fat body and hemocytes. The rate of [^{14}C] leucine incorporation into the hemocyte proteins was only one-tenth of that in either midgut or fat body. They concluded that hemocytes are of minor importance for hemolymph protein synthesis. However, more recently, several workers have reported that the hemocytes are active sites of protein synthesis (reviewed in Crossley, 1979). Periplaneta americana hemocytes incorporate [^{14}C] leucine injected into the hemocoel into hemocyte proteins. Labelled hemocyte proteins reinjected into the hemocoel reach the cuticle in one to two hours (Gieger et al., 1977). Immunological similarity between hemolymph proteins and cuticular proteins has been reported in Periplaneta americana (Fox et al., 1972) and Manduca sexta (Koeppe and Gilbert, 1973). We have observed that Ar is widely distributed and present in tissues such as fat body (Chapter 5), midgut (Chapter 4), epidermis (Chapter 3), cuticle (Chapter 3) and pericardial cells (Fife et al., 1987). These

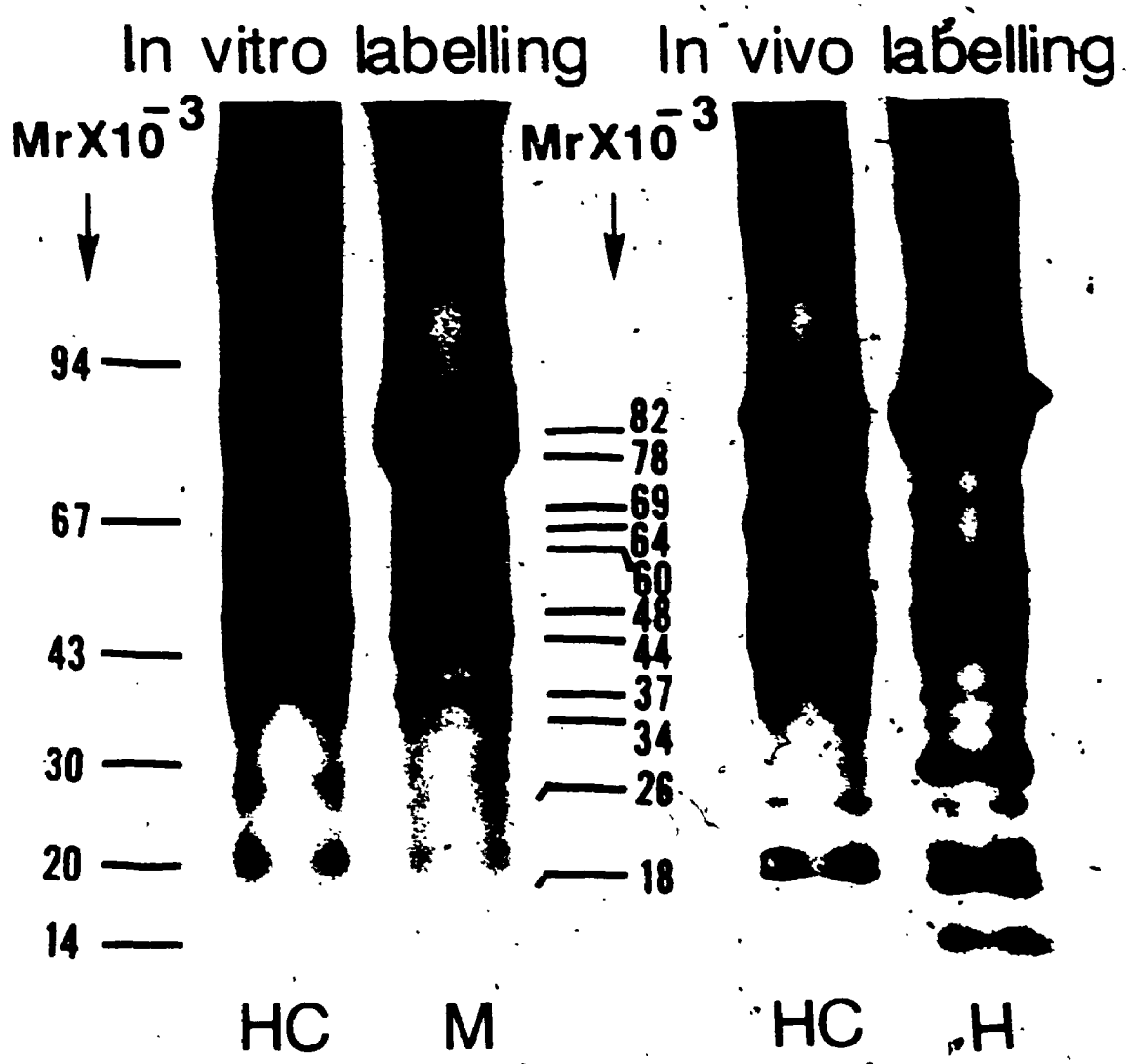
observations suggest that the similarity between hemolymph/hemocyte proteins and cuticular proteins could probably be due to the presence of the same protein (Ar) in both compartments. To test the above hypothesis and to clarify the role of the hemocytes in hemolymph protein synthesis, protein synthesis by Calpodes hemocytes was investigated under in vivo and in vitro conditions.

6.2 Results

6.2.1 Protein Synthesis In vitro

Hemocytes were maintained in vitro in dilute hemolymph (hemolymph was diluted 10X with artificial hemolymph) in the presence of [^{35}S] methionine for 2 hr as described in Materials and Methods. At the end of the incubation period the hemocytes were centrifuged down and the proteins solubilized in non-denaturing solubilization solution. The proteins were separated on SDS-PAGE and the gels processed for fluorography to identify the newly synthesized polypeptides. Under these conditions the hemocytes synthesize approximately 20 [^{35}S] methionine labelled polypeptides (Fig. 21, in vitro HC). At least 11 of those polypeptides (82, 78, 69, 64, 60, 48, 44, 37, 34, 26 and 18 K) are synthesized for secretion and released into the incubation medium (Fig. 21, in vitro M). Since the polypeptides present in the medium do not include the main polypeptides labelled in the cells themselves but do include a known secretory protein (Ar), it is most likely that there is no contamination from tissue breakdown or cell death. Since these polypeptides are secreted into the bathing medium, it is reasonable

Fig. 21. Synthesis and secretion of hemolymph proteins by the hemocytes. Hemocytes were incubated in dilute hemolymph in the presence of 0.05 mCi of [^{35}S] methionine for 2 hr. The proteins present in the hemocytes and medium were separated on a 5-15% SDS-PAGE gradient. Approximately 20,000 counts/min were loaded in each well and the fluorograms were developed for a week at -70^{0}C. Approximately 20 [^{35}S] methionine labelled polypeptides were retained in the tissue and 11 of them were secreted into the medium. Since these polypeptides are labelled <u>in vitro</u> and there is no evidence for cell breakdown, these are probably normally secreted by hemocytes into the hemolymph. Whole larvae were also injected with [^{35}S] methionine and after 2 hr of incorporation <u>in vivo</u> the proteins labelled in hemocytes and hemolymph were electrophoresed. Note the correspondence between polypeptides labelled <u>in vitro</u> and <u>in vivo</u> in their respective compartments. The relative mobilities of Pharmacia low molecular weight standards and the M_r of hemocyte secretory polypeptides are shown on the left and right sides of the figure.



HC=Hemocytes

M=Medium

H=Hemolymph

to assume that these are normally secreted by the hemocytes into the hemocoel. The results show that Calpodes hemocytes secrete at least 11 polypeptides into the medium in vitro.

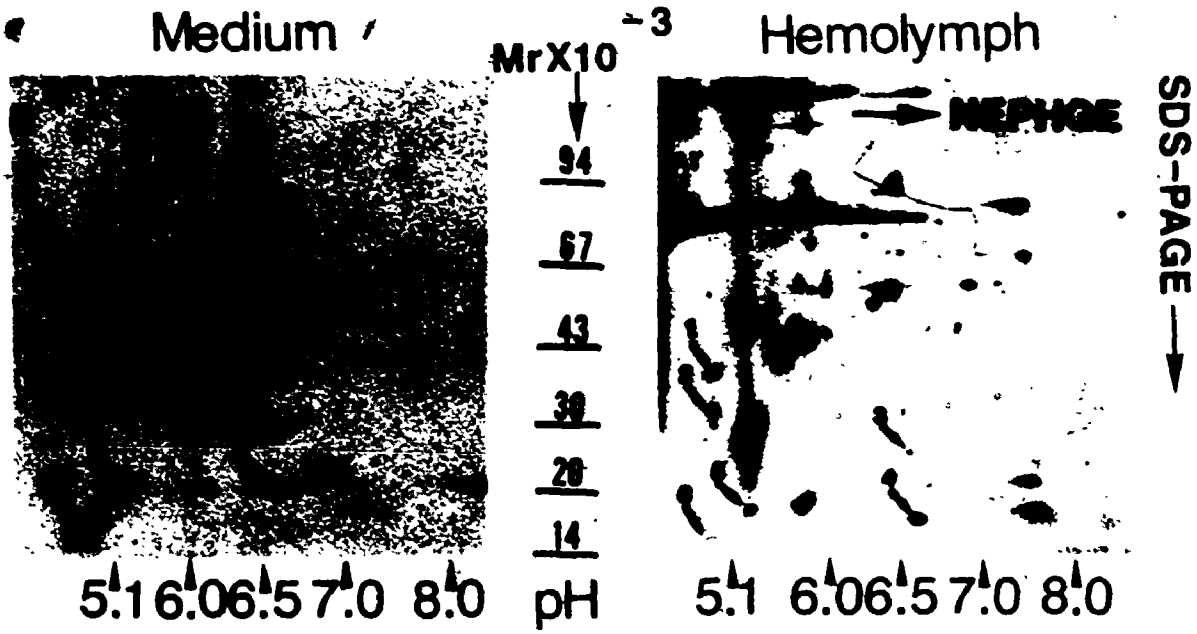
6.2.2 Protein Synthesis In vivo

To study protein synthesis and secretion by the hemocytes in vivo [^{35}S] methionine was injected into mid fifth instar (approximately 90 hr after ecdysis) Calpodes larvae and allowed to incorporate for 2 hr. Proteins from the hemocytes and hemolymph were solubilized, separated on SDS-PAGE and the gels processed for fluorography to identify the newly synthesized polypeptides. Approximately 20 polypeptides were labelled in the hemocytes (Fig. 21, In vivo HC) and more than 25 in the hemolymph (Fig. 21, In vivo H). The polypeptides labelled in the hemocytes under in vivo and in vitro conditions show similar patterns on these SDS gels. There are a few quantitative differences, probably due to the absence of hormones and other controlling factors, but the general polypeptide patterns are similar. The similar labelling of cellular proteins under in vitro and in vivo conditions suggests that the in vitro system functions normally.

6.2.3 The Similarity between Hemocyte Secretory proteins and naturally occurring hemolymph proteins

To identify the hemocyte secreted proteins in hemolymph the hemocyte secretory proteins labelled in vitro and hemolymph labelled in vivo were analysed by one-dimensional and two-dimensional gel electrophoresis. Most of the bands in the hemocyte medium (Fig. 21,

Fig. 22. The similarity between proteins secreted by the hemocytes and those occurring naturally in the hemolymph. Proteins from the medium after [^{35}S] methionine labelling *in vitro* and hemolymph labelled *in vivo* were resolved on NEPHGE-SDS-PAGE employing 5-15% SDS-PAGE gradient in the second dimension. Approximately 100,000 counts/min were loaded onto each gel and the fluorograms were developed for 30 days. pH and M_r were determined as described in Materials and Methods. The arrows point to the Ar and six other spots present in both medium and hemolymph fluorograms.


Medium
MrX10 -3
Hemolymph
NEPHGE
SDS-PAGE
94
67
43
30
20
14
5.1 6.0 6.5 7.0 8.0
pH
5.1 6.0 6.5 7.0 8.0

in vitro M) had corresponding bands in the hemolymph (Fig. 21, *in vivo* H). Arylphorin and six other polypeptides (Fig. 22) could be identified in the hemolymph NEPHGE-SDS-PAGE gel. The identification of hemolymph proteins secreted by hemocytes received further support from the immunological studies described below.

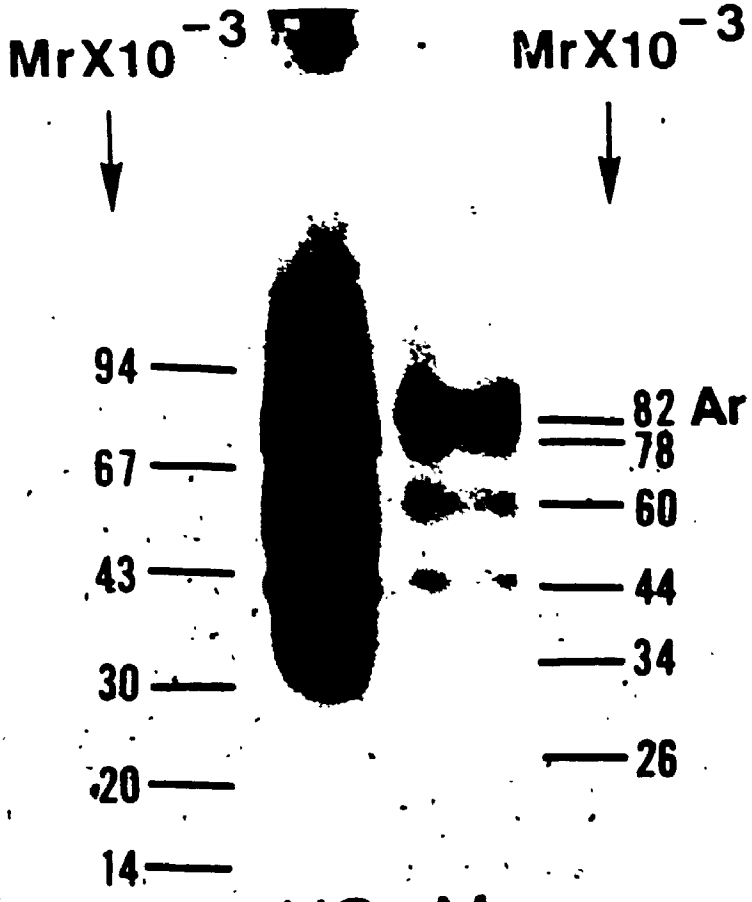
6.2.4 The Immunological Similarity between Hemocyte Secretions and Naturally Occurring Hemolymph Proteins

Hemocyte secretory proteins labelled *in vitro* were precipitated with antibodies made against hemolymph. The resultant precipitates were separated on SDS-PAGE and the gels processed for fluorography. Antibodies made against hemolymph proteins recognized at least six polypeptides including Ar (82, 78, 60, 44, 34 and 26 K) present both in the hemocytes and medium (Fig. 23). Precautions such as pretreating the protein sample with SAS (IgSorb) and the inclusion of a non-ionic detergent (NP-40) in the assay to reduce the background, make it likely that the hemolymph antibodies were recognizing these polypeptides because of their immunological similarity. The results establish that hemocytes contribute at least six polypeptides present in the hemolymph.

6.2.5 The Sequential Appearance of Secretory Proteins in the Hemocytes Prior to their Appearance in the Hemolymph

To verify whether hemocytes might have taken up proteins newly synthesised by other tissues during the 2 hr of labelling, hemocytes were exposed to [^{35}S] methionine *in vivo* for 20 and 60 min, the proteins from hemocytes and hemolymph solubilized and separated on

Fig. 23. The immunological similarity between hemocyte secretory proteins and hemolymph proteins. The proteins from hemocytes (500,000 counts/min), and medium (500,000 counts/min) labelled with [^{35}S] methionine *in vitro*, were precipitated with antibodies to hemolymph. Immunoprecipitates were separated on a 5-15% SDS-PAGE gradient. The fluorogram was developed for seven days at -70^{o}C. Six hemocyte secretory polypeptides were recognized by antibodies prepared against the hemolymph proteins. The relative mobilities of Pharmacia low molecular weight standards and the M_r of hemocyte secretory polypeptides are given on the left and right side of the figure.

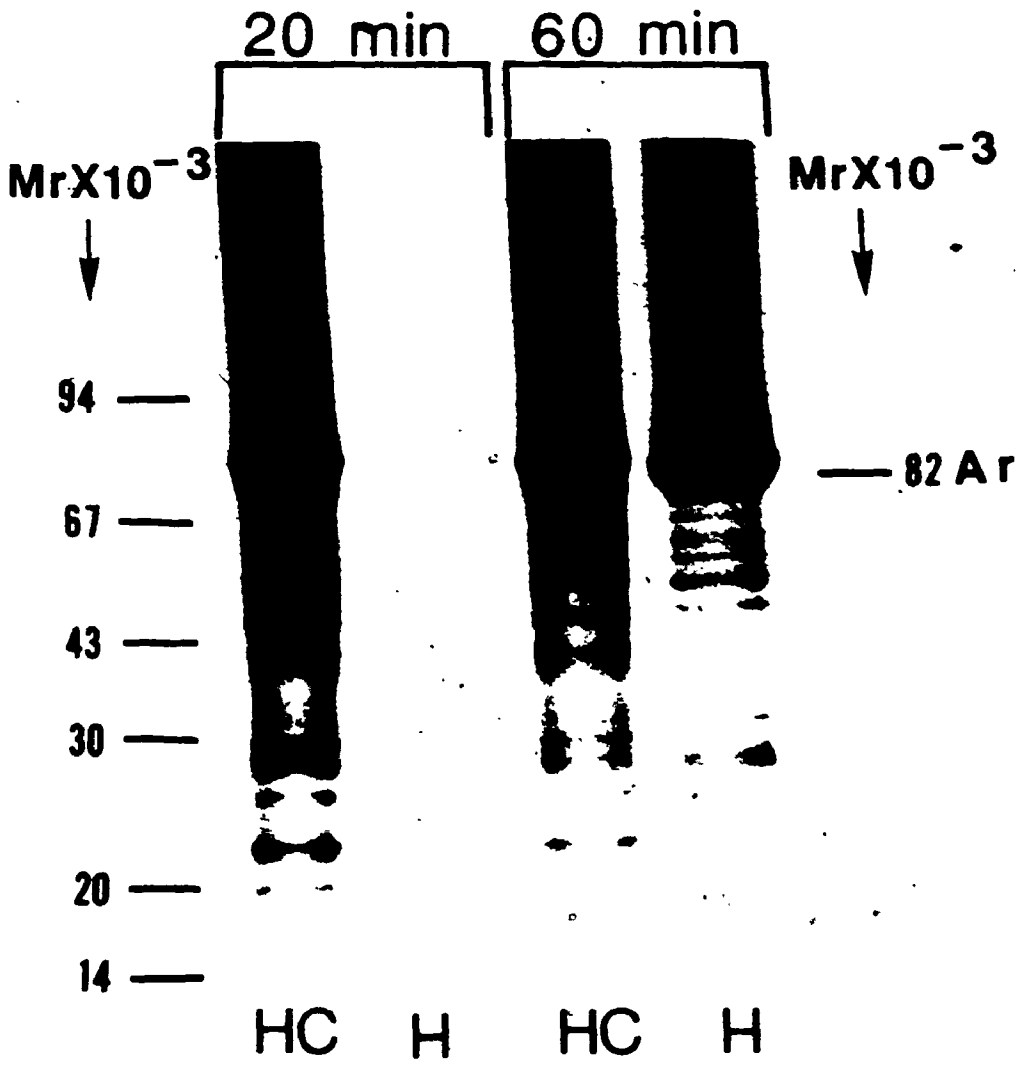


HC=Hemocytes

M=Medium

Ar=Arylphorin

Fig. 24. The sequential appearance of secretory proteins first in the hemocytes and later in the hemolymph. Hemocytes were labelled *in vivo* by injecting [^{35}S] methionine into mid fifth instar larvae. After incorporation for 20 and 60 min proteins were solubilized from hemocytes and hemolymph. Approximately 20,000 counts/min were loaded onto each well (except in the case of H at 20 min where the incorporation was so low that, 100 ul of sample was loaded which is at least 5X the volume loaded for 60 min labelling) and the fluorogram was developed for a week at -70°C. Labelled polypeptides appear first in the hemocytes (20 min) before they appear in the hemolymph (60 min). The relative mobility of Pharmacia low molecular weight standards is given on the left side of the figure.



HC=Hemocytes

H=Hemolymph

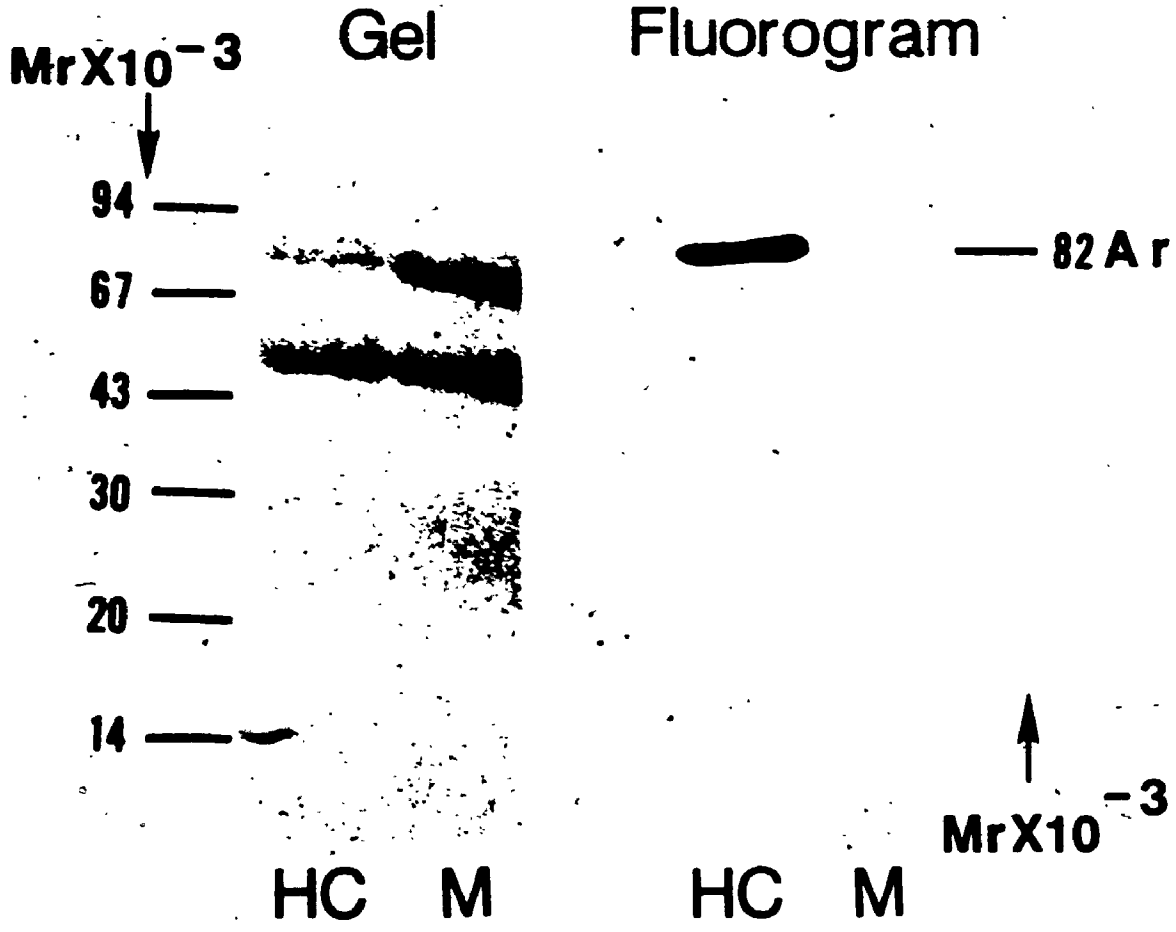
Ar=Arylphorin

SDS-PAGE and the gels processed for fluorography. Labelled polypeptides appeared in the hemocytes after 20 min of labelling at a time when the hemolymph is not labelled (Fig. 24). By 60 min the hemolymph is completely labelled. Since labelled polypeptides (which includes Ar) appeared in the hemocytes 20 minutes after the beginning of labelling, when nothing was labelled in the hemolymph, the hemocytes must be the source of such polypeptides.

6.2.6 The Secretion of Arylphorin by the Hemocytes

A polypeptide with M_r similar to that of Ar [470 K dissociating into six similar subunits each with a molecular weight of 82 K in the presence of SDS (Chapter 7)] was present in hemocytes (Fig. 21, *in vitro* HC) and medium (Fig. 21, *in vitro* M) labelled *in vitro* and in hemocytes (Fig. 21, *in vivo* HC) and hemolymph (Fig. 21, *in vivo* H) labelled *in vivo*. The experiments in which fifth instar larvae were exposed to [^{35}S] methionine for 20 and 60 min showed that Ar occurred in the hemocytes (by 20 min), prior to its appearance in the hemolymph (Fig. 24). The synthesis and secretion of Ar by hemocytes was confirmed by immunological studies. Antibodies made against hemolymph proteins recognized Ar present in the hemocytes and medium (Fig. 23). Antibodies made against purified hemolymph Ar recognized Ar present in the hemocytes and medium (Fig. 25). Due to the competition between unlabelled Ar present in the incubation medium (hemocytes were incubated in dilute hemolymph for *in vitro* studies) and labelled Ar released by hemocytes, it becomes very difficult to precipitate the hemocyte Ar separately. Usually a light band is seen

Fig. 25. The immunological evidence that Ar is secreted by the hemocytes. The proteins from hemocytes (500,000 counts/min) and medium (500,000 counts/min) labelled with [^{35}S] methionine in vitro were precipitated with antibodies made against purified hemolymph Ar. Immunoprecipitates of hemocytes and medium were separated on a 5-15% SDS-PAGE gradient. The gel was stained with Coomassie blue R250. The fluorogram was developed for 15 days at -70^{0}C. The Ar present in the hemocytes and medium has been precipitated by antibodies prepared against hemolymph Ar. Due to the competition between unlabelled Ar present in the incubation medium (hemocytes were incubated in dilute hemolymph) and labelled Ar secreted by hemocytes, it becomes difficult to precipitate the hemocyte Ar. Usually a light band is seen in the medium lane of the fluorogram when compared to a dense band in the tissue. However, both lanes are stained well in the gel indicating that there is more unlabelled than labelled Ar in the medium. The relative mobility of Pharmacia low molecular weight standards is given on the left side of the figure.



HC=Hemocytes

M=Medium

Ar=Arylphorin

in the medium lane of the fluorogram when compared to a dense band in the tissue. However, both bands stained well in the gel (Fig. 25) indicating that there is more unlabelled than labelled Ar in the medium. The above evidence suggest that hemocytes can synthesize and secrete Ar.

6.3 Discussion

6.3.1 Hemolymph Protein Secretion by the Hemocytes

The main finding is that Calpodes hemocytes secrete at least six hemolymph polypeptides. Observations like similar in vivo and in vitro labelling, the presence of at least seven hemocyte secretory polypeptides in the hemolymph and precipitation of six hemocyte secretory polypeptides by hemolymph antibodies suggest that the protein secretion observed in the in vitro system is natural and is not due to the breakdown of cells or membrane blebbing. There is no direct evidence for hemocyte protein contribution to the hemolymph protein pool (Gupta 1985). These are the first direct experiments to show that hemocytes make hemolymph proteins. Although the contribution of hemocytes may be insignificant quantitatively (only about 5% that of the fat body) they could be functionally important, perhaps enzymes involved in various metabolic activities (Hughes and Price, 1976). Hemocytes now join an ever lengthening list of tissues (fat body, epidermis, midgut and pericardial cells) that make hemolymph proteins.

6.3.2 Synthesis and Secretion of Arylphorin by the Hemocytes

Immunoprecipitation of in vitro labelled proteins present in

hemocytes and medium with Ar antibodies showed that Ar is synthesized by the hemocytes. Only a light band in the medium lane of fluorogram raised some doubt about Ar secretion. However, other observations like the presence of Ar both in the hemocytes and medium labelled in vitro (Figs. 21, 22) and its precipitation by hemolymph antibodies (Fig. 23) suggest that hemocytes secrete Ar. These results along with the presence of a dense band in the stained gel support the interpretation that the light band in the medium lane of the fluorogram is due to the competition between labelled and unlabelled Ar (Fig. 25). The results show that hemocytes synthesize and may secrete Ar. The synthesis and secretion of Ar has already been demonstrated in Calpodes epidermis, midgut, fat body and pericardial cells.

6.3.3 Transport of Hemolymph/Hemocyte Proteins to the Cuticle

The suggestion that some hemolymph/hemocyte proteins are transported to the cuticle was based on two observations.(1) there is an immunological similarity between certain cuticular proteins and certain hemolymph proteins (Fox et al., 1972; Koeppe and Gilbert, 1973; Durliat et al., 1980). (2) labelled hemolymph/hemocyte proteins injected into the hemocoel reach the cuticle in one or two hours (Fox et al., 1972; Koeppe and Gilbert, 1973; Gieger et al., 1978). The presence of Ar in cuticle, epidermis (Chapter 3), hemocytes and hemolymph of Calpodes ethlius as well as cuticle, epidermis and hemolymph of Manduca sexta (Riddiford and Hice, 1985) suggest that the immunological similarity between cuticular proteins and hemolymph or

hemocyte proteins (Fox et al., 1972 and Roeppe and Gilbert, 1973) was probably due to the presence of the same protein (Ar) in both compartments, rather than the transport of hemolymph or hemocyte proteins to the cuticle.

CHAPTER 7

PURIFICATION AND CHARACTERIZATION OF THE THREE MAJOR HEMOLYMPH PROTEINS

7.1 Introduction

The hemolymph of holometabolous insects contains 2-4 abundant proteins called storage proteins, whose concentration increases with time during the last larval stadium, becoming 80-90% of the total soluble protein. Storage proteins were first described in Calliphora erythrocephala (Munn et al., 1967). Although the storage proteins have been characterized from numerous species (reviewed in Wyatt and Pan 1978, Riddiford and Law, 1983; Levenbook 1985), there is little information on their function. Most authors suggest that they are amino acid stores (eg. Levenbook and Bauer, 1984) others speculate that they may function in transport (eg. Miller and Silhacek, 1982b).

Calpodes hemolymph has three major hemolymph proteins. Locke et al (1982) identified one of them as lipoprotein (470 K on non-denaturing gels) and the other two as storage proteins (580 K and 720 K on non-denaturing gels). The 470 K protein is widely distributed and synthesized by many tissues including fat body midgut, epidermis, hemocytes and pericardial cells and is also present in the cuticle. The two storage proteins, on the other hand, are synthesized only by the fat body. To find out whether any of these proteins fall into the class of arylphorins described by Telfer et al (1983), I have purified and characterized them.

7.2 Results

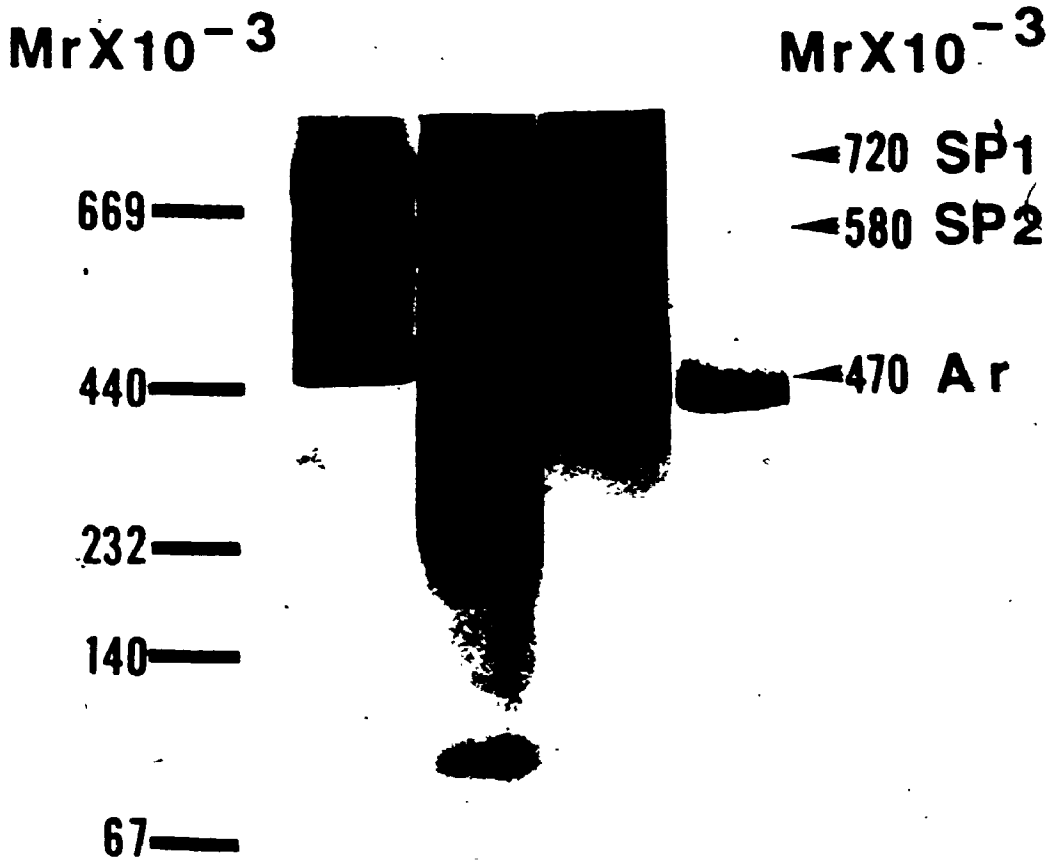
7.2.1 The Three Major Hemolymph Proteins

The protein components of Calpodes larval hemolymph were resolved into approximately 15 bands by 3-15% N-PAGE (Fig. 26, C; S). Three of these 15 proteins make up more than 80% of the total. Their molecular masses are 470, 580 and 720 K. All three proteins stained with the PAS for carbohydrates with 1:2 glycol groups (Fig. 26, PAS). Only the 470 K band stained with SB (SB) for lipids (Fig. 26, SB). Analysis of hemolymph by SDS-PAGE resulted in approximately 25 bands with major bands in the 80-90K region (Fig. 27, C; S). Previous studies (Locke et al., 1982) resolved the 720 K protein on SDS gels as a doublet with molecular weights of 86 and 90 K. The 580 and 470 K proteins were resolved into single bands of 86 and 82 K respectively. The major bands between 80 and 90 K consist of the subunits for all three major hemolymph proteins. All stained with PAS (Fig. 27, PAS) but only the 82 K band stained with SB (Fig. 27, SB). To justify calling the 470 K protein Ar according to the criteria of Telfer et al (1983) and the other two proteins as storage proteins (SP1, 720 K; SP2, 580 K) the three proteins were further purified and characterized.

7.2.2 Purification of Three Hemolymph Proteins

In initial experiments column chromatography using Sepharose 6B (Gellissen and Wyatt, 1981) and low ionic strength precipitation of hemolymph (Chino and Downer, 1982) did not give encouraging results. We therefore turned to preparative N-PAGE and SDS-PAGE as methods to

Fig. 26. The three major proteins of Calpodes larval hemolymph. Approximately 10 ul of hemolymph containing 50 ug of protein were loaded into each well of a 3-15% N-PAGE gradient. After electrophoresis the gel was cut into four pieces longitudinally and stained with Coomassie blue R250 (C), silver nitrate (S), peripdic acid-Schiff method (PAS) and Sudan black B (SB). The hemolymph separated into at least 15 bands and three of them (Ar, SP1 and SP2) constitute most of the protein. Arylphorin is stained both with PAS and SB. Storage protein 1 and SP2 are stained only with PAS. The relative mobility of co-electrophoresed Pharmacia high molecular weight standards is given on the left side of the figure.



C S PAS SB

C=Coomassie blue S=Silver

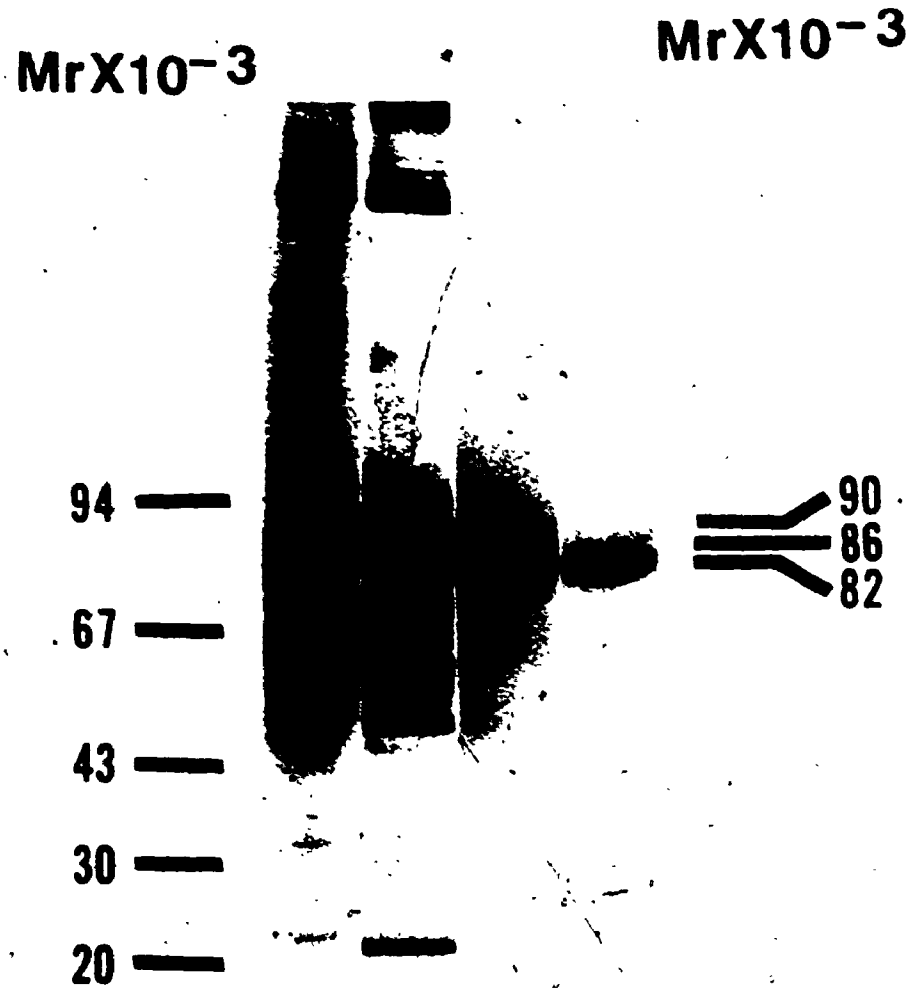
PAS=Periodic acid Schiff reagent

SB=Sudan black B Ar=Arylphorin

SP1=Storage protein 1

SP2=Storage protein 2

Fig. 27. The subunits of major hemolymph proteins. Approximately 10 ul of hemolymph containing 50 ug protein were loaded into each well of a 3-15% SDS-PAGE gradient. After electrophoresis the gel was cut into four pieces longitudinally and stained with Coomassie blue R250 (C), silver nitrate (S), periodic acid-Schiff method (PAS) and Sudan black B (SB). The hemolymph resolved into at least 30 bands with major bands between 80-90 K which probably includes all subunits (82, 86 and 90 K) of three major hemolymph proteins (Locke et al., 1982). PAS stains all bands whereas SB stains only the 82 K band. M_r on the left side of the figure represent the relative mobility of Pharmacia low molecular weight standards.



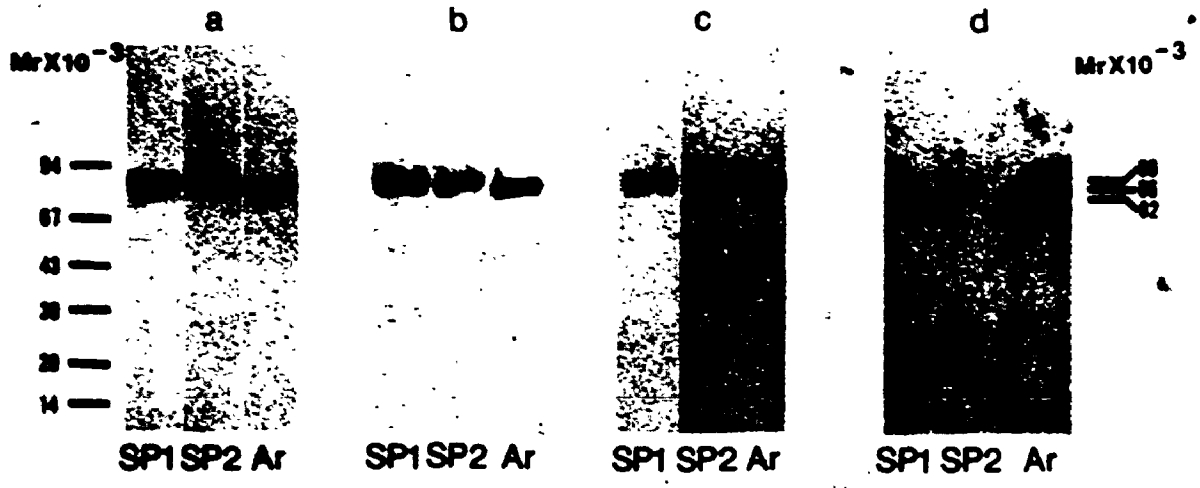
C=Coomassie blue S=Silver

PAS=Periodic acid Schiff reagent

SB=Sudan black B

obtain pure fractions of Ar, SP1 and SP2. About 0.5 ml of mid fifth instar larval hemolymph containing 5-10 mg protein was loaded on 3-15% N-PAGE into a long stacking well made by inverting the regular comb. After electrophoresis the gel was stained for 10 min (0.1 % Coomassie blue R250) and destained for 10 min each in primary and secondary destain. The three bands at 720, 580 and 470 K were identified and excised from the gel viewed over a light box. Each band was equilibrated in SBA for 15 min, boiled for 3 min and loaded into long wells of three separate 3-15% SDS-PAGE gels. After electrophoresis the gels were stained with ice cold solution containing 0.25 % KCl and 1 mM DTT (Hager and Burgess, 1980) and destained in 1 mM DTT. The stained areas of the gel (86-90 for 720 K, 86 for 580 K and 82 for 470 K) were excised and homogenized in 2 ml elution buffer [0.1 % SDS, 50 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 5 mM DTT and 0.2 M NaCl]. The homogenate was mixed on a rotary shaker for 10-15 hr at 4°C and centrifuged in an Eppendorf microfuge (15,000g) for 5 min. The supernatant was then concentrated to 200 ul (Minicon concentrator, type-B 15). Protein concentrations were estimated and the purity was checked by SDS-PAGE. Gels showed purified proteins in the same discrete bands as before, a single band at 82 K (Ar), a doublet at 86 and 90 K (SP1) and a single band at 86 K (SP2; Fig. 28a; b). All three purified proteins continued to contain carbohydrate (PAS stain Fig. 28c) but only Ar contained lipid (SB stain Fig. 28d). This identification received further support from the amino acid analysis below.

Fig. 28. The purity of isolated major hemolymph proteins. Approximately 10 ug of purified Ar, SP1 and SP2 were separated on a 3-15% SDS-PAGE gradient which was divided into four pieces longitudinally. Each piece was stained with Coomassie blue R250 (a), silver nitrate (b), periodic acid-Schiff method (c) and sudan black B (d). All three proteins are resolved into single bands at appropriate positions indicating the purity of the sample. Periodic acid-Schiff method stained subunits of all three proteins whereas SB only stains subunits of Ar. The relative mobility of Pharmacia low molecular weight standards is given on the left side of the figure.



SP1-Storage protein 1

SP2-Storage protein 2

Ar-Arylphorin

7.2.3 Amino Acid Composition of the Three Major Hemolymph Proteins

The amino acid composition of the three major hemolymph proteins is given in Table 2. Arylphorin (470 K) is rich in aromatic amino acids (21.1 % compared to 8.1 and 13.7% for SP1 and SP2). Storage protein 1 and SP2 are rich in glycine (23.2 and 18.1% compared to 7.3% for Ar). Other amino acids (for example isoleucine and leucine) are similar in SP1 and SP2 but different from Ar. The 470 K protein falls into the class of Ar described by Telfer et al (1983) for the following reasons: (a) it is a hexamer dissociating into similar subunits with a molecular weight of 82 K in the presence of SDS [Figs. 27; 28 and Locke et al (1982)]; (b) its aromatic amino acids account for 21% of the total (Table 2); (c) it has lipid and carbohydrate like Manduca Ar [Figs. 26; 27 and Kramer et al (1980)].

7.2.4 Subunits of the Three Major Hemolymph Proteins

To characterize these three major hemolymph proteins further and to know the ionic forms of their subunits, purified proteins were analyzed by two-dimensional gel electrophoresis. Initial attempts to separate the proteins by isoelectric focussing (IEF) were unsuccessful, since they did not enter the gel from the basic end. We therefore used NEPHGE (O'Farrell, 1975) with the modifications suggested by Jones (1980). Even tnough the polypeptides in the NEPHGE gels are not at equilibrium, the pH profiles are consistent and the polypeptides running at a given distance and time along the gel are reproducible. Two-dimensional analysis (NEPHGE in the first dimension and 3-15% SDS-PAGE gradient in the second dimension)

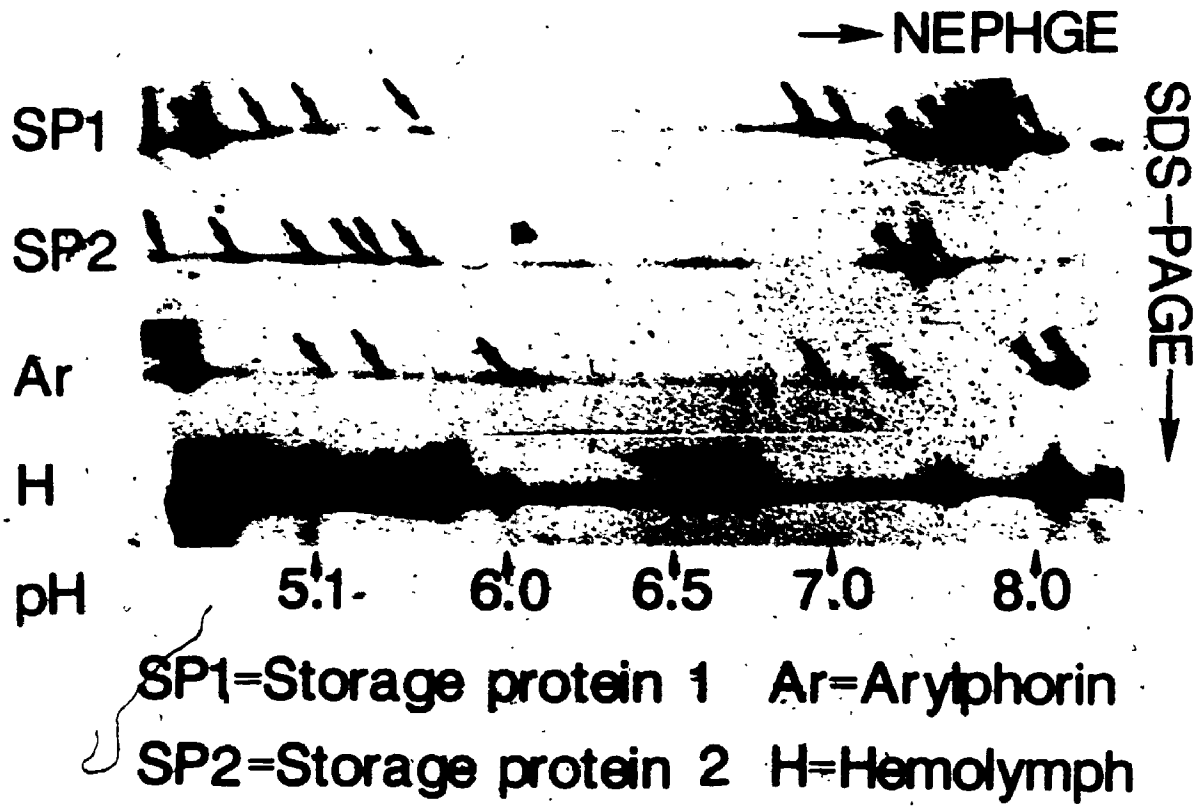
Table 2

Amino acid composition of arylphorin and storage proteins 1 and 2

Amino acid	% composition*		
	SP1	SP2	Ar
Alanine	3.3	3.2	3.9
Arginine	4.8	4.2	3.2
Aspargine/Aspartic acid	11.5	11.4	10.0
Cysteine	1.3	0.8	N.D.
Glutamine/Glutamic acid	6.9	2.5	7.2
Glycine	23.2	18.1	7.3
Histidine	2.0	3.3	2.5
Isoleucine	4.5	4.6	3.2
Leucine	8.0	8.5	7.0
Lysine	5.0	6.6	9.8
Methionine	4.0	2.6	1.5
Phenylalanine	3.9	5.8	8.4
Proline	2.4	3.7	5.8
Serine	5.0	4.9	4.9
Threonine	5.3	5.2	4.9
Tryptophan	N.D.	N.D.	N.D.
Tyrosine	4.2	7.9	12.7
Valine	4.7	6.7	7.7
Total	100.0	100.0	100.0

N.D.= Not detectable. * Each value is average of three determinations.

Fig. 29. The Subunits of isolated major hemolymph proteins. Purified Ar, SP1, SP2 (20 ug) and whole hemolymph (50ug) are analysed by NEPHGE-SDS-PAGE employing 3-15% SDS-PAGE gradient in the second dimension. pH gradients were determined as explained in Materials and Methods. All three major hemolymph proteins show multiple subunits across the pH gradient. Major isoforms are indicated by arrowheads and several minor isoforms by arrows.



SP1=Storage protein 1 Ar=Arylphorin

SP2=Storage protein 2 H=Hemolymph

resolved purified Ar, SP1 and SP2 into multiple minor spots and two or more major spots (Fig. 29). Two dimensional analysis of whole hemolymph gives a big band across the gel in the 80-90 K region. This was at first thought to be an artifact, but many repetitions with various pH gradients and running times gave results similar to that shown in figure 29. The two-dimensional gels of purified proteins explain the result for whole hemolymph. Multiple spots for each protein overlap into the smear shown in figure 29. The results suggest that Ar, SP1 and SP2 are composed of multiple subunits of similar size but different charge.

7.2.5 The Structure of the Larval Storage Proteins and Arylphorin

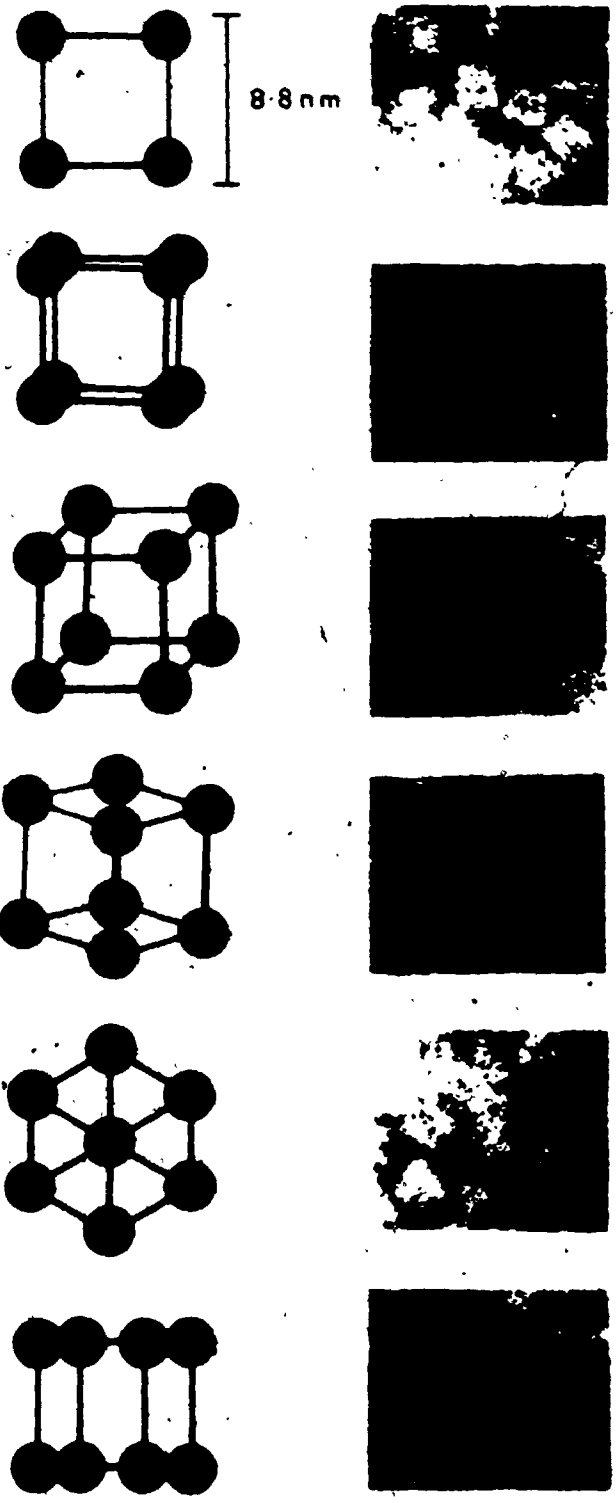
7.2.5.1 Storage Protein 1

The larger storage protein (SP1) has a molecular mass of about 720 K (Fig. 26) and it is made from two kinds of subunit of mass 86 and 90 K (Fig. 28). If these occur in equal proportions then eight of them would have a combined mass of 704 K, within two percent of the estimate for the polymer. This suggested that SP1 might be an octamer. I therefore prepared negatively stained samples of the purified protein for transmission electron microscopy, looking for profiles that would be expected for an octamer.

Figure 30 shows that the profiles of many molecules are square or rectangular. More rarely, others are roughly circular. The most probable three dimensional structure to give these profiles is a cube, which would give a square as the most frequently seen profile. Where the structure is tilted on an edge it would appear rectangular.

Fig. 30. A cubic structure for SP1 deduced from the profiles seen in negatively stained preparations. The diagrams show the appearance of a cube seen in profile from various directions and the electron micrographs show some of the shapes actually observed. The survey picture shows that the most common profile is a square, as would be expected if eight subunits form the vertices of a cube. Scale marker = 10 nm.

SP 1



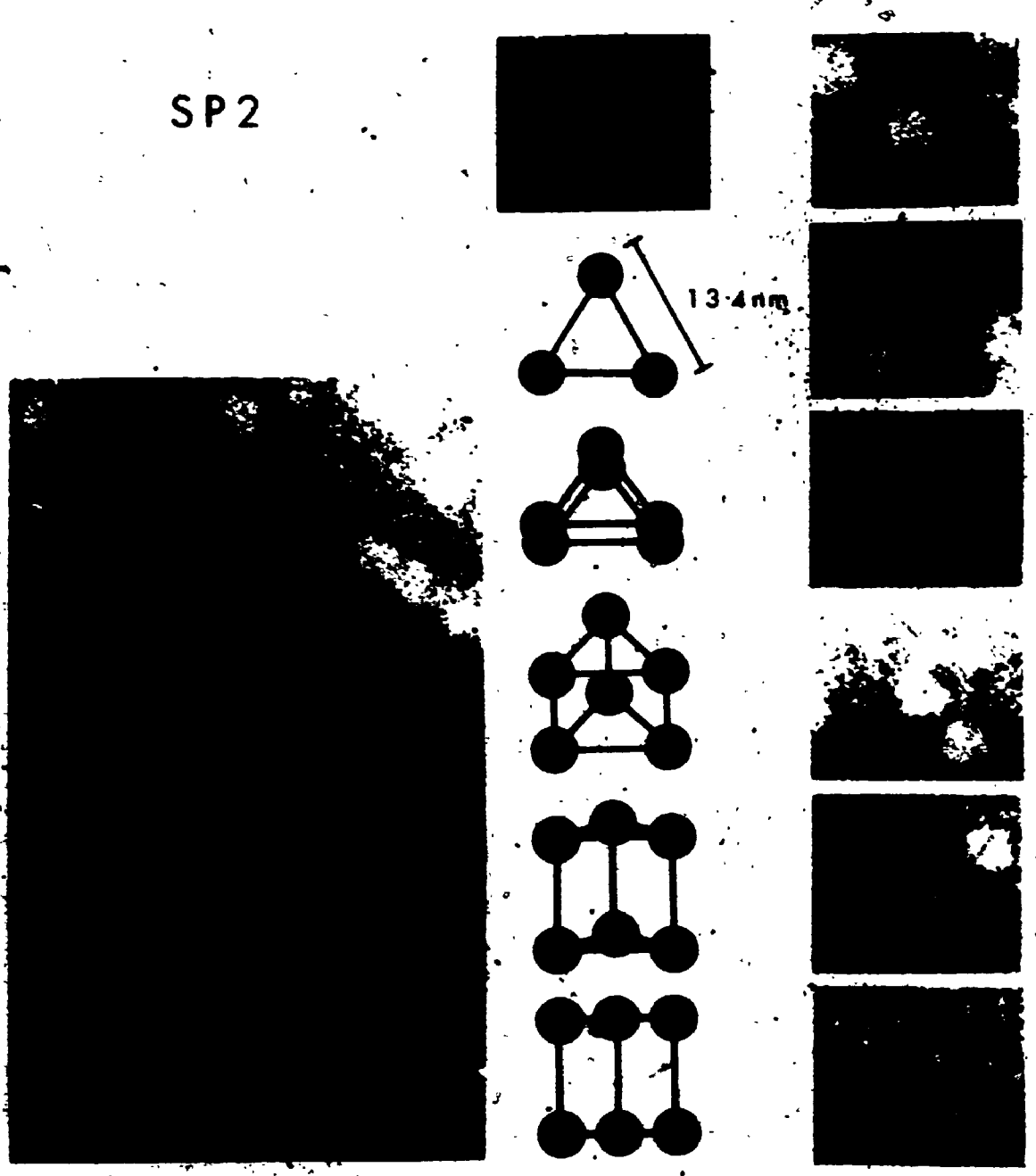
More rarely, tilting on a vertex would give a six-sided figure tending to become symmetrical and roughly circular. Measurements of the long and short dimensions of squares tending to rectangles gave the unit dimension of the cube as 8.8±0.8 nm (S.D.). The long dimensions were all values between 8.8 and 2(8.8 cos 45°) = 12.4 nm, as would be expected if the rectangular profiles are due to the tilting of a cube on an edge. The diameter of the roughly circular profiles was measured to be 11.3 nm. This is close to the value of 12.4 nm calculated for a cube of unit dimension 8.8 nm viewed in the direction of edge to most distant edge. No other geometrical figures give profiles with these appearances and dimensions. I conclude that the most probable arrangement of polypeptides to make SP1 is that of a cube with eight subunits forming the eight vertices. This would allow touching subunits to be about 4.2 nm in diameter.

7.2.5.2 Storage Protein 2

The smaller storage protein (SP2) has a molecular mass of 580 K (Fig. 26) and is made from a single size of polypeptide of molecular mass 86 K (Fig. 28). Six subunits would give a combined mass of 516 K, 11% less than that estimated for the polymer from its position on the non-denaturing gel, a close enough match to suggest that the native protein might be a hexamer.

Electron microscopy of the negatively stained molecule shows triangular, square and rectangular profiles with an occasional pentagonal trapezoid. These profiles are most easily explained if the polymer is a pentahedral prism with three square and two equilateral

Fig. 31. A pentahedral prism structure for SP2 deduced from the profiles seen in negatively stained preparations. The diagrams show the appearance of the prism seen in profile from various directions and the electron micrographs show some of the shapes observed. The most common profiles are triangular and square as would be expected if six subunits form the vertices of a prism with three square and two triangular faces. Scale marker = 10 nm.


SP2
13·4nm

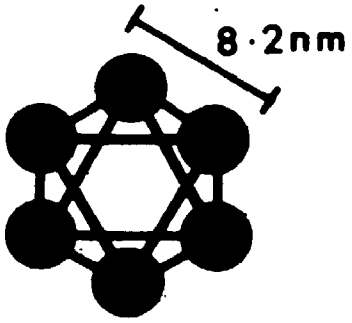
triangular faces. The profiles expected for this geometrical shape are all found in the negatively stained images of SP2 (Fig. 31). In agreement with this interpretation the length of the sides of the triangle were measured to be 13.6±1.1 nm (S.D.), indistinguishable from those of the square, 13.2±1.5 nm (S.D.). It can be concluded that the six subunits of SP2 are arranged at the vertices of a pentahedral prism. The distance between vertices is greater than in SP1 although the subunits have a similar mass, suggesting that SP2 subunits are asymmetrical, with a dimension of about 6.7 nm. This observation could account for the 11% greater estimate of the mass of the polymer than expected from the sum of the subunits.

7.2.5.3 Arylphorin

Arylphorin has a molecular mass of about 470 K (Fig. 26) and is made from a single kind of subunit of molecular mass 82 K (Fig. 28). Six subunits would give a mass of 492 K, 5% more than that observed for the polymer. Electron microscopy of the polymer gave profiles quite different from those of the storage proteins. The images lacked angular outlines. They were all roughly circular or slightly elongated with a suggestion of sixfold symmetry. A possible interpretation would suppose that the structure is, that of a triangular antiprism, that is an octahedron with eight identical triangular faces (Fig. 32). Such a figure would tend to rest on one of the faces giving identical views for all, that of a six-sided figure difficult to separate from a rough circle. Tilting of such a figure shortens the profile in one direction but common orientations

Fig. 32. An octahedral structure for Ar deduced from the profiles seen in negatively stained preparations. The diagram shows the appearance of the octahedron viewed through any of its eight triangular faces. The profiles seen in electron micrographs have no sharply angled outines as would be expected for this geometrical figure with six subunits at the vertices of a triangular antiprism (i.e. like SP2 but with one triangular face rotated through 60°). Some preparations have slightly separated subunits suggesting a six-fold symmetry. Scale marker = 10 nm.

ARYLPHORIN



give no easily resolvable angular images different from the most common one. The mean diameter of the profiles was 9.5±0.6 nm (S.D.), giving a unit dimension for each triangular side of 8.2 nm. This would allow touching subunits to be about 4.1 nm in diameter. I conclude that Ar may have its six subunits arranged at the vertices of an octahedron.

7.3 Discussion

7.3.1 Identification of Arylphorin and Storage Proteins 1 and 2

There are three major proteins in Calpodes larval hemolymph with molecular weights of 470, 580 and 720 K as estimated by 3-15 % N-PAGE. Other Lepidoptera such as Hyalophora cecropia (Riddiford and Law, 1983), Bombyx mori (Tojo et al., 1980) and Manduca sexta (Riddiford and Law, 1983) also have 2-3 major hemolymph proteins with apparent molecular weights between 480-530 K. The 470 K protein stains both with PAS and SB indicating that it contains lipid and carbohydrate like Manduca sexta Ar [Figs. 26;27;28 and Kramer et al., (1980)]. The 470 K protein has a high aromatic amino acid content (21%) similar to the Ar of Hyalophora cecropia [18.8%; Telfer et al (1983)]. This 470 K protein is therefore similar to the Ar described in other insects (Telfer et al., 1983). The 580 and 720 K proteins stain with PAS but not with SB indicating that they have only carbohyrate attached to them. Locke et al (1982) and Collins (1974) also found the three PAS stainable bands in Calpodes hemolymph. The 580 and 720 K proteins are rich in the amino acid glycine (18.1, 23.2%). No other major hemolymph proteins have been reported to be so high in glycine

(Levenbook, 1985). Calpodes hemolymph is also high in glycine [23.7% of free amino acids (Irvine, 1969)]. The possibility of contamination of these proteins with glycine from the electrophoresis running buffer has been eliminated, since all three proteins were washed thoroughly with TCA and ether prior to amino acid estimation and the 470 K protein contained very little glycine (7.3%). The 580 and 720 K proteins of Calpodes larval hemolymph fit into the class of storage proteins described by Roberts and Brock (1981) and Levenbook (1985). Roberts and Brock (1981) suggested that hemolymph storage proteins are (a) few in number and occur only in the larval stages, (b) synthesized predominantly by fat body and (c) increase in concentration enormously in the last larval instar (Locke and Collins, 1968). Levenbook (1985) added three more criteria, (d) they should have a molecular weight of about 500,000, (e) be composed of six subunits and (f) should contain high proportions of phenylalanine and tyrosine. The results presented here and in Locke et al., 1982 indicate that the 580 and 720 K proteins have all the characteristics suggested by Roberts and Brock (1981) but not criteria e and f of Levenbook (1985). SP1 has a molecular weight of 720,000 and is composed of eight subunits. They also have aromatic amino acid contents of only 8.1 and 13.3%. The general similarity between Calpodes SP1 and SP2 and other insect storage proteins suggests that the definition of this class of proteins should be less exclusive rather than that Calpodes is exceptional.

7.3.2 Purification of Arylphorin and Two Storage Proteins

Several methods for purification of Ar and storage proteins are suggested in the literature (Levenbook 1985, Ryan et al., 1985). In almost all procedures the purification is limited to only one protein. The main problem with the purification of these proteins, especially Ar, is that once they are precipitated it is very difficult to redissolve them. The procedure we developed with preparative N-PAGE and SDS-PAGE does not have this problem and all three major hemolymph proteins can be purified in a single procedure. In a typical purification experiment 0.5 ml of hemolymph from mid fifth instar larvae containing 5-10 mg protein yielded about 0.5 mg of Ar and 0.2 mg each of the two storage proteins. Figure 28 shows the purity obtained with little effort and high yield. Since the procedure involves breaking down the protein in the presence of SDS and isolating the subunits, one might suppose that some of the protein would be lost. However when the 720, 580 and 470 K bands from non-denaturing gels are re-electrophoresed in the presence of SDS, more than 90 % of the protein is recovered in the subunit bands (Locke et al., 1982).

7.3.3 Shape, Size and Subunit Composition

Two-dimensional analysis of purified Ar, SP1 and SP2 indicate that these proteins are composed of multiple subunits having the same molecular weight but with different charge. Levenbook (1985) reported at least nine subunits with the same molecular weight but differing charge for LHP1 of Calliphora.

The common structural feature shown by these three proteins is that their subunit arrangement could enclose a central space. This could enable them to be vessels for the transport of other molecules shielded from the hemolymph in the manner that ferritin sequesters iron. It may be significant that the largest molecules accumulating in the hemolymph are all in the same size range of 8-15 nm in diameter. If the function were to be purely storage, one might have expected giant macromolecules restricted in their hemolymph movement. The size range observed may reflect the functional need to move across the basal lamina, which can certainly be a barrier to large particles. The term storage protein may be an inappropriately restrictive description for such easily transportable molecules. The reconstruction of polymer shape from negatively stained whole mounts may be another tool for establishing homology between molecules. It will be interesting to see if the putative Ar and storage proteins from other insects also have these geometrical arrangements.

CHAPTER 8

DEVELOPMENTAL CHANGES OF HEMOLYMPH PROTEINS

8.1 Introduction

In preparation for the non-feeding pupal stadium, the larvae of holometabolous insects accumulate a variety of proteins in the hemolymph (see Wyatt and Pan, 1978; Riddiford and Law, 1983; Levenbook, 1985 for reviews). In Calpodes, the hemolymph concentration increases after the first critical period, 66 hr after ecdysis, when the prothoracic glands no longer need the brain to initiate pupal development. This time marks the end of intermoult preparation and the beginning of intermoult syntheses. Hemolymph protein concentration reaches a maximum by the second critical period at 156 hr after ecdysis, when the prothoracic glands are no longer needed for pupation and there is a switch from larval syntheses to pupation (Locke, 1970). Hemolymph proteins then decline due to their sequestration by the prepupal fat body (Locke and Collins, 1968; Dean et al., 1985). Similar gross hemolymph protein changes have been reported for several Lepidoptera (Tojo et al., 1978; Kramer et al., 1980; Miller and Silhacek, 1982b; Locke et al., 1982; Telfer et al., 1983; Riddiford and Hice, 1985) and Diptera (Roberts et al., 1977; Levenbook and Bauer, 1980). In most of these studies only one or two major hemolymph proteins were observed. Quantitative changes in many other proteins during development were ignored. I therefore examined the hemolymph of Calpodes ethlius (Lepidoptera: Hesperiidae) during the fifth larval and pupal stadia, looking for stage specific

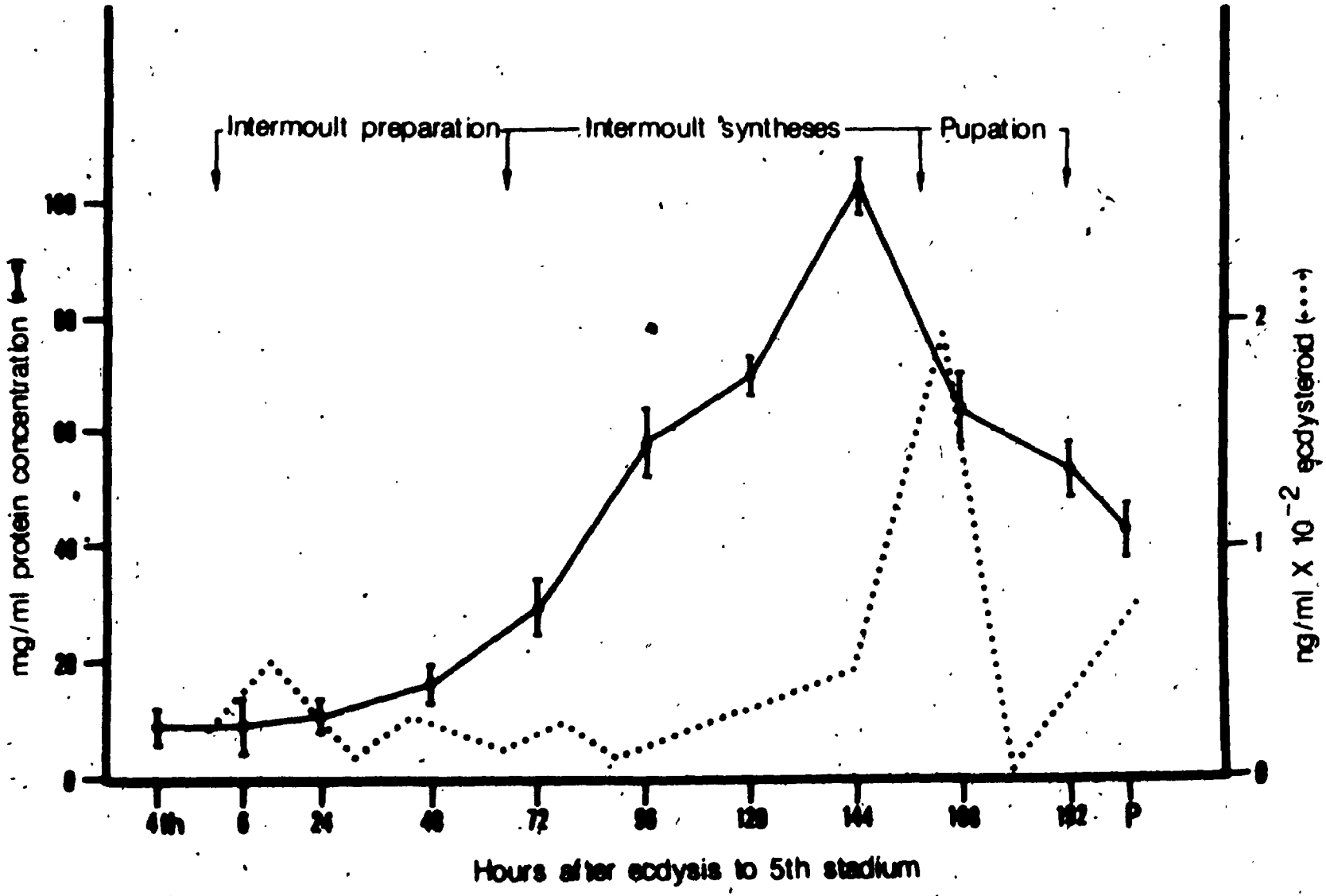
proteins as a preliminary to relating them functionally to the many precisely staged activities in development that have already been recorded. Hemolymph samples collected from timed fourth, fifth larval and pupal stadia were analysed by N-PAGE and NEPHGE-SDS-PAGE to study the changes in abundance of particular proteins, looking for their appearance and loss with phases of development during the larval-pupal transformation. I have found that out of 56 hemolymph polypeptides only ten are present throughout the fourth, fifth and pupal stadia. The others can be grouped into nine categories according to their presence or absence during intermoult preparation, intermoult syntheses, pupation and the pupal stage. The origin of some of these has been determined by correlation with proteins synthesized by particular tissues in vitro.

8.2 Results

8.2.1 Changes in Total Hemolymph Protein Concentration During Development

The total protein concentration was measured in hemolymph collected from stages between the fourth larval and pupal stadia as in Figure 33. The protein concentration was low (9-16 ug/ul) until 48 hr. It then increased linearly until 144 hr (105 ug/ul). By 168 hr the protein concentration had declined to 64 ug/ul and decreased further to reach 43 ug/ul in pupal hemolymph. The results show that the increase in hemolymph proteins (secretion/dilution and destruction) begins at the end of the phase of intermoult preparation and is maintained throughout the phase of intermoult syntheses.

Fig. 33. Changes in the total hemolymph protein concentration during development. Total protein concentration was measured in the hemolymph collected from all stages between fourth larval and pupal stadia. Each point represents the average of five determinations. Dotted line shows the ecdysteroid concentration measured by Dean et al ., 1980. Like many other secretions the hemolymph protein secretion switched to a faster rate at 66 hr after ecdysis to fifth stadium.


Intermoult preparation
Intermoult syntheses
Pupation
mg/ml protein concentration
ng/ml X 10⁻² ecdysteroid (•••)
4th
6
24
48
72
96
120
144
168
192
P
Hours after ecdysis to 5th stadium

Protein concentration then declines during pupation when the fat body is known to sequester proteins. Hemolymph protein secretion is thus like many other secretions (wax, lamellate cuticle, fat body protein, lipid, glycogen etc, Dean et al 1980) in suddenly switching to a faster rate at 66 hr.

8.2.2 Changes in the Abundance of the Three Major Hemolymph Proteins During Development

Hemolymph collected from all stages between the fourth larval and pupal stadia was analysed by N-PAGE. The protein components were resolved into approximately 15 bands. Three of these made up more than 80 % of the total. The molecular masses are 470 K (Ar), 580 K (SP2) and 720 K (SP1; Chapter 7). Except during the first few hours, Ar (470 K) was present throughout the fifth stadium and into the pupa (Fig. 34). Storage protein 1 appeared by 72 hr and was increasingly abundant until its disappearance by 168 hr. Storage protein 2 appeared after SP1 and was present from 120 hr, declining at the same time as SP1. Both storage proteins disappeared completely by the end of pupation and were absent in the pupa. The results show that the secretion and/or appearance of these proteins in the hemolymph is non-coordinate. Arylphorin particularly differs from the other two storage proteins. The appearance of the 580 K and 720 K proteins after commitment (66 hr), the initiation of their disappearance at the time of pupation (156 hr) and their absence in pupae, are characteristics of larval storage proteins as defined by Roberts and Brock (1981) and Levenbook (1985).

Fig. 34. Changes in the abundance of three major hemolymph proteins during development. Approximately 50 ug of protein from all stages between fourth larval and pupal stadia were separated on a 3-15 % N-PAGE gradient and stained with Coomassie blue R250. Arylphorin is present through the final larval stadium except during first few hours. Storage protein 1 appears 72 hr after ecdysis and starts to decline 168 hr after ecdysis, SP2 also starts to decline at the same time but appears only 120 hr after ecdysis. The relative mobility of proteins with known M_r is shown on the left side of the figure.

4th 6 24 48 72 96 120 144 168 192 Pupa

Hours after ecdysis to 5th stadium

Ar=Aryiphorin SP1=Storage protein 1 SP2=Storage protein 2

8.2.3 Changes in the Abundance of Minor Hemolymph Proteins During Development

To study the qualitative changes in the abundance of minor hemolymph proteins during development, I used high resolution two-dimensional gel electrophoresis followed by highly sensitive silver staining. Hemolymph samples collected between the fourth larval and pupal stadia were analysed by two-dimensional gel electrophoresis to resolve the proteins into approximately 60 spots (Figs. 35a; b; c). Developmental events such as ecdysteroid titre, wax secretion, laemellate cuticle deposition etc. divide the fifth larval stadium into three phases of development (intermoult preparation, intermoult syntheses and pupation, Locke, 1970). I attempted to classify the hemolymph proteins resolved on two-dimensional gels according to their presence or absence during these developmental phases (Fig. 36). There were no differences in the hemolymph proteins (resolved by two-dimensional gel electrophoresis) between the late fourth stage and early fifth stage. The late fourth was therefore included with the intermoult preparation in the fifth. This gave four developmental stages differing in the composition of their hemolymph (fourth+intermoult preparation in the fifth, intermoult syntheses, pupation, early pupa). As shown in figure 36 the hemolymph proteins separated by two-dimensional gel electrophoresis can be divided into four groups based on their presence at the beginning of each of the four developmental phases. The groups can be further divided into 11 possible categories

depending on the disappearance of polypeptides at the end of each developmental phase. Detectable polypeptides were present in nine of these 11 possible categories (Fig. 36).

The first group includes 31 polypeptides present at the beginning of fourth+intermoult preparation in the fifth stage. Ten of these polypeptides including Ar were present in all stages studied (category 1, Fig. 35a; b; c, fourth-pupa), 13 of them disappeared at the end of pupation and were absent in pupal hemolymph (category 2, Fig. 35a; b; c, fourth-192 hr), four of them disappeared after the phase of intermoult syntheses (category 3, Fig. 35a; b, fourth-144 hr) and four other polypeptides were present only during fourth+intermoult preparation stage (category 4, Fig. 35a, fourth-48 hr). The second group includes three polypeptides which appeared at the beginning of the phase of intermoult syntheses. One of them disappeared at the end of pupation (category 6, Fig. 35b; c, 72-192 hr) and two of them (SP1 and SP2) occurred during the phase of intermoult syntheses and disappeared during pupation (category 7, Fig. 34). Eighteen polypeptides which appeared at the beginning of pupation were included in the third group. One polypeptide occurred during pupation and remained in the pupa (category 9, Fig. 35c, 168 hr-pupa) and the other 17 were present only during pupation in the last two days of the fifth larval stadium (category 10, Fig. 35c, 168 and 192 hr). The fourth group includes four polypeptides that were present only in the pupa (category 11, Fig. 35c, pupa). The results show that some hemolymph polypeptides are present throughout larval and pupal life but most are

Fig. 35a. Changes in the abundance of minor hemolymph proteins during development. Approximately 100 ug of hemolymph proteins from fourth and upto 48 hr into fifth stadium were separated by two-dimensional gel electrophoresis (NEPHGE in the first dimension and 3-15% SDS-PAGE gradient in the second dimension) and stained with silver nitrate. Ten polypeptides are continously present throughout larval and pupal life and 46 other polypeptides are stage specific. See Fig. 35c for explanation of numbers.

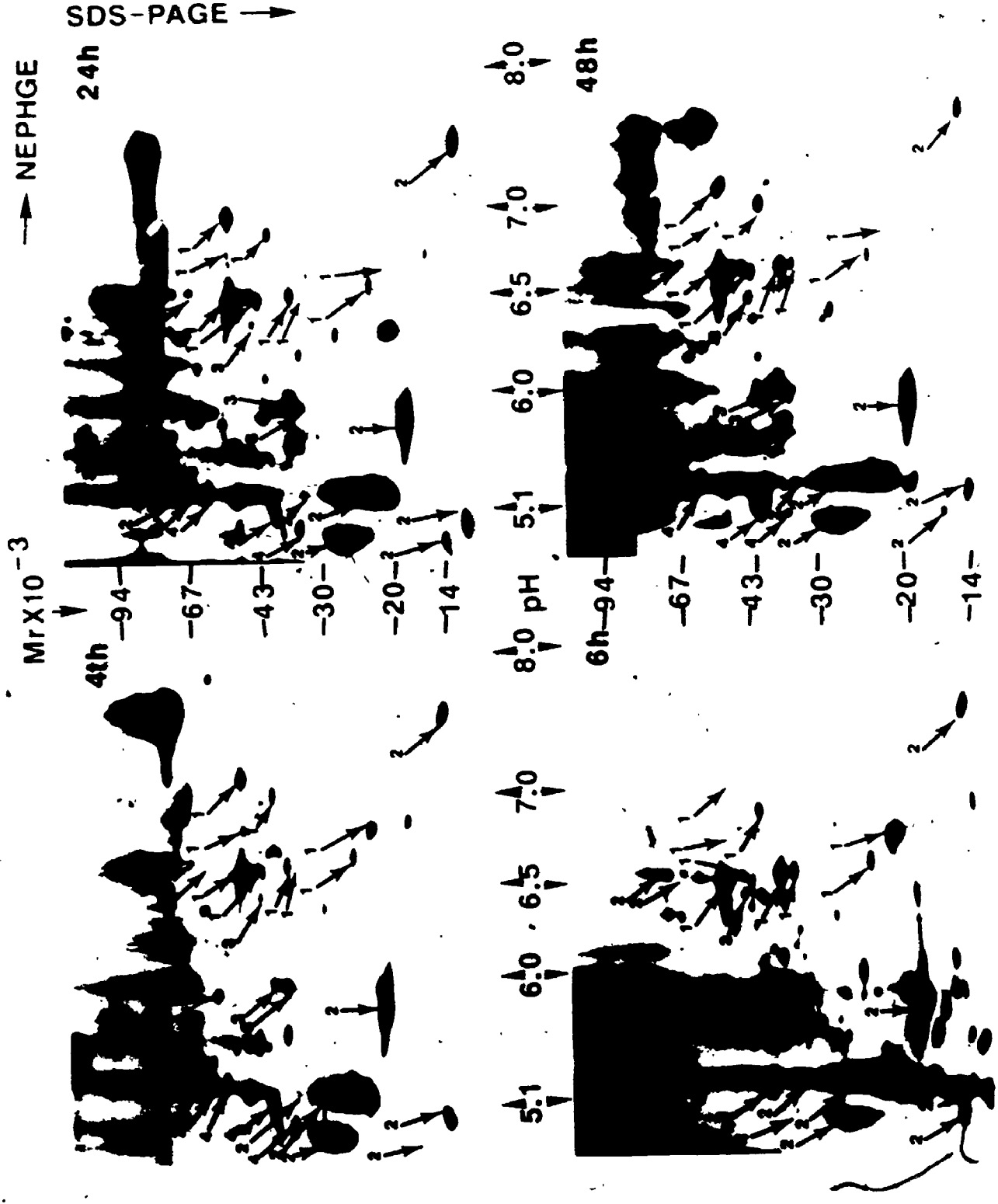

SDS-PAGE →
← NEPHGE
24h
48h
4th
6h
MrX10⁻³
94
67
43
30
20
14
8.0 pH
7.0
6.5
6.0
5.1

Fig. 35b. Changes in the abundance of minor hemolymph proteins during development. Approximately 100 ug of hemolymph proteins from larvae 72-144 hr into fifth stadium were separated by two-dimensional gel electrophoresis (NEPHGE in the first dimension and 3-15% SDS-PAGE gradient in the second dimension) and stained with silver nitrate. Ten polypeptides are continously present throughout larval and pupal life and 46 other polypeptides are stage specific. See Fig. 35c for explanation of numbers.

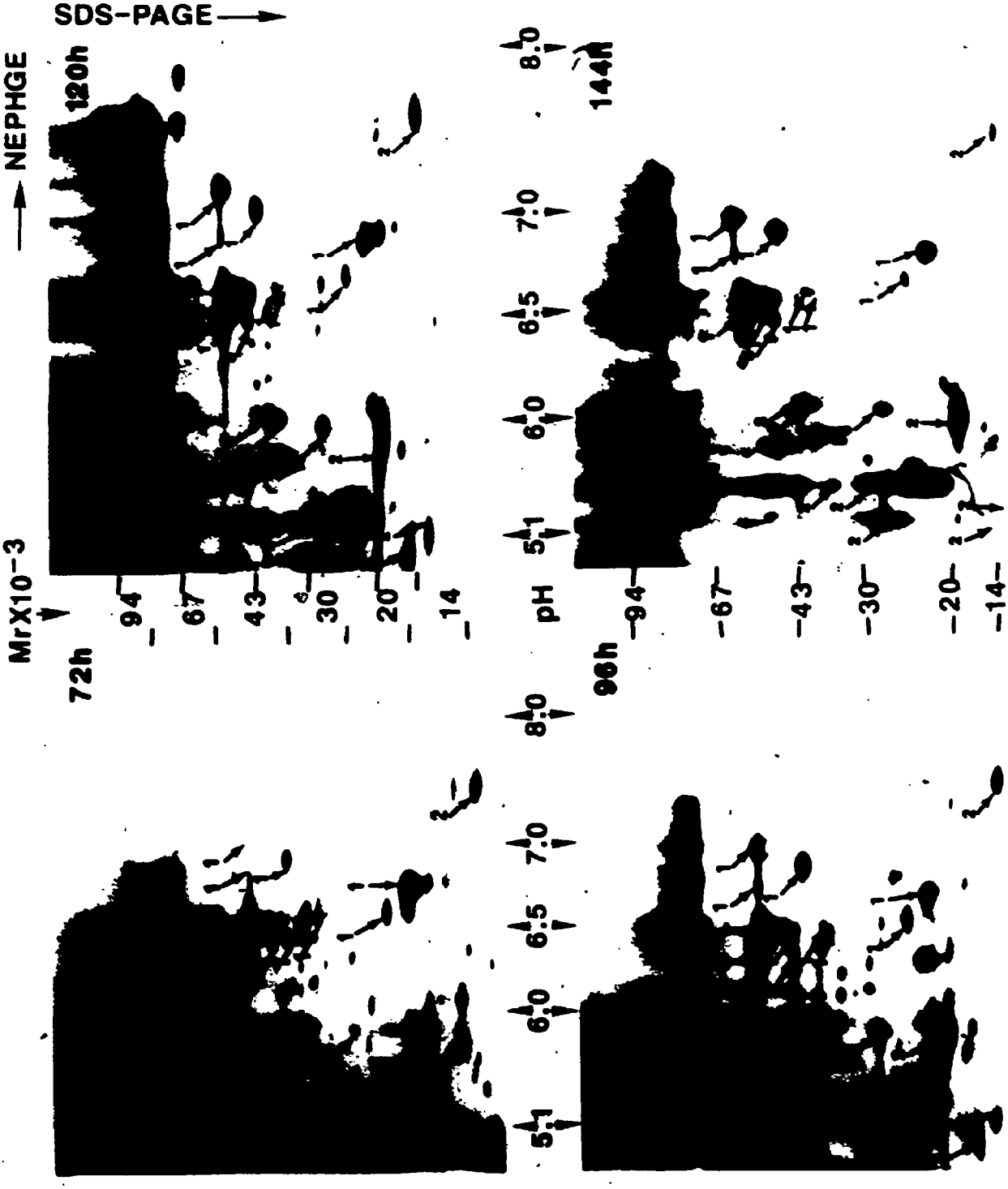

SDS-PAGE
NEPHGE
120h
144h
72h
96h
MrX10⁻³
94
67
43
30
20
14
pH
5.1
6.0
6.5
7.0
8.0

Fig. 35c. Changes in the abundance of minor hemolymph proteins during development. Approximately 100 ug of hemolymph proteins from larvvae 168 and 192 hr into fifth stadium and pupa were separated by two-dimensional gel electrophoresis (NEPHGE in the first dimension and 3-15% SDS-PAGE gradient in the second dimension) and stained with silver nitrate. Ten polypeptides are continously present throughout larval and pupal life and 46 other polypeptides are stage specific.

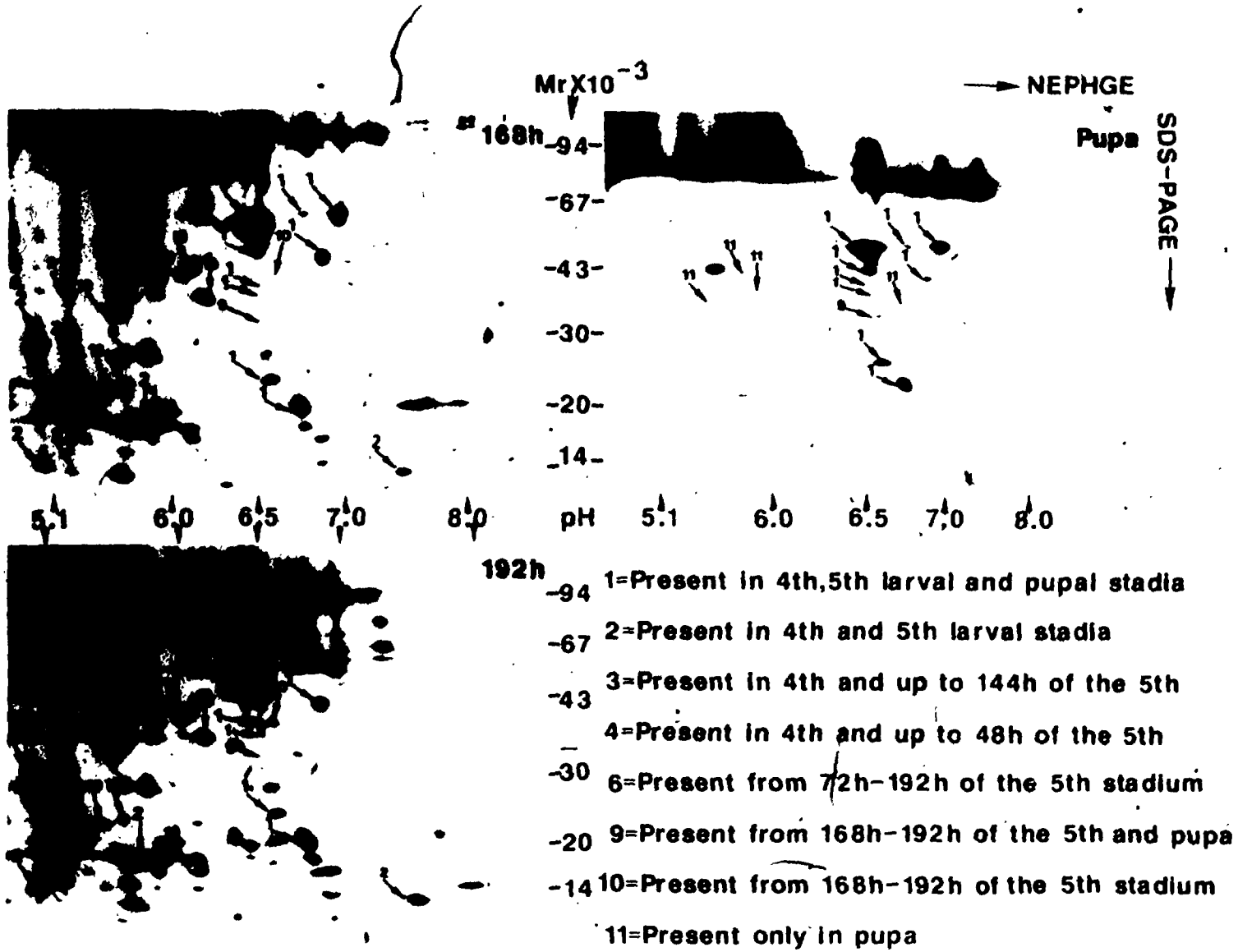
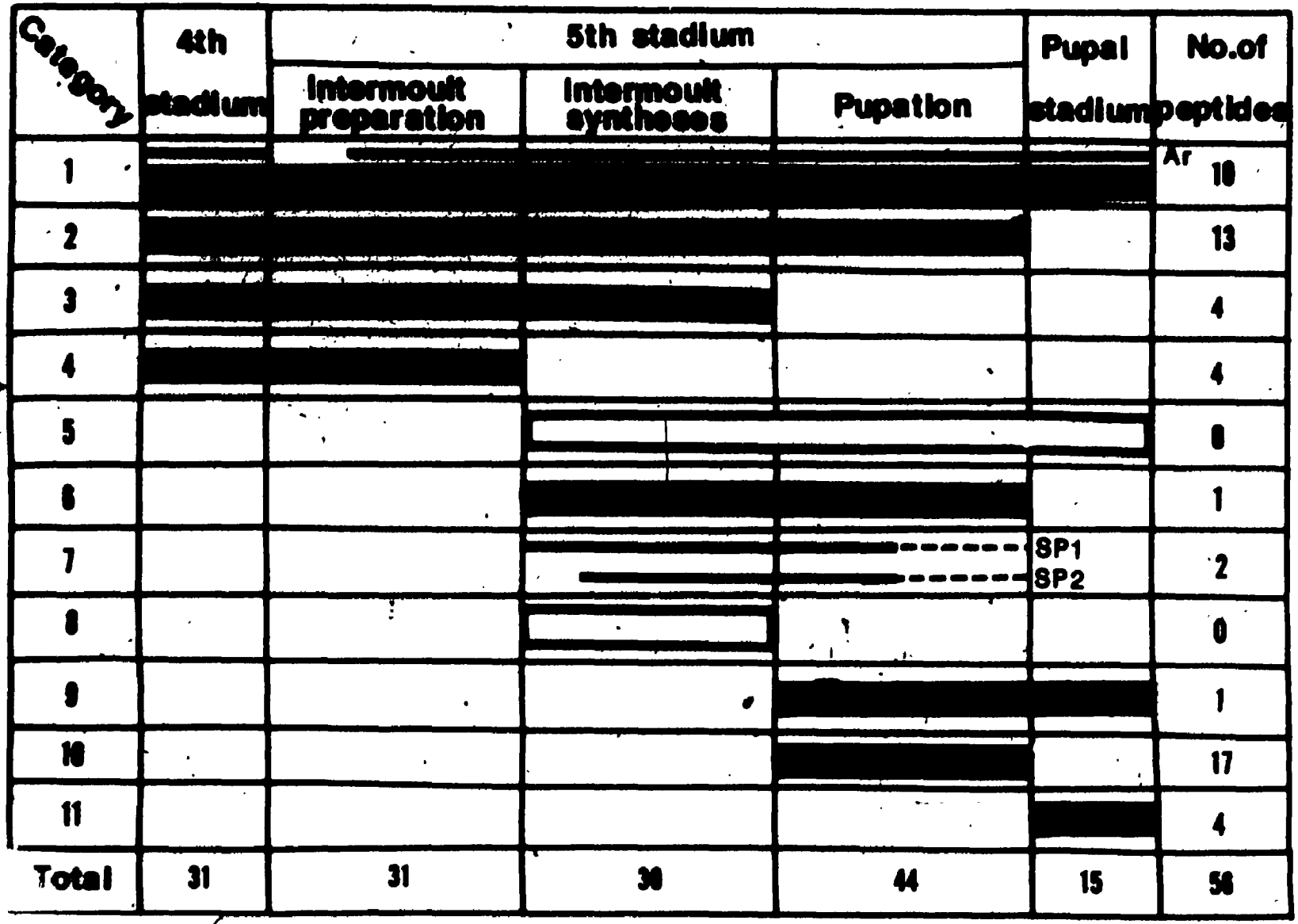

MrX10⁻³
NEPHGE
SDS-PAGE
168h
Pupa
-94-
-67-
-43-
-30-
-20-
-14-
5.1
6.0
6.5
7.0
8.0
pH
192h
1=Present in 4th,5th larval and pupal stadia
2=Present in 4th and 5th larval stadia
3=Present in 4th and up to 144h of the 5th
4=Present in 4th and up to 48h of the 5th
6=Present from 72h-192h of the 5th stadium
9=Present from 168h-192h of the 5th and pupa
10=Present from 168h-192h of the 5th stadium
11=Present only in pupa

Fig. 36. Changes in the abundance of hemolymph proteins during development. The hemolymph polypeptides separated by one-dimensional (Fig. 34) and two-dimensional (Fig. 35) gel electrophoresis were classified based on their appearance and loss with development. No polypeptides declined to reappear later, this gave 11 possible categories of polypeptides during the four developmental stages (fourth + early fifth, larval syntheses, pupation and pupal). Only two of the 11 categories were absent. Detectable polypeptides occurred in all the nine other categories.


Category
4th stadium
5th stadium
Intermoult preparation
Intermoult synthesis
Pupation
Pupal stadium
No.of peptides
1
2
3
4
5
6
7
8
9
10
11
Ar
SP1
SP2
10
13
4
4
8
1
2
0
1
17
4
Total
31
31
36
44
15
58

present only for limited periods. Many hemolymph polypeptides are therefore stage specific, the four developmental phases characterized by other developmental events each have polypeptides that are absent at other times. There is a particularly striking increase in relation to pupation (Fig. 36).

8.2.4 The Origin of Hemolymph Proteins

A determination of the origin of some hemolymph proteins has been attempted by comparing hemolypmh proteins separated by NEPHGE-SDS-PAGE with fluorograms of media in which isolated fat body, midgut, epidermis, pericardial cells+heart and hemocytes had been incubated in the presence of [^{35}S] methionine. The results are summarised in Table 3. Fat body secretes at least 14 polypeptides, 12 of them occur naturally in the hemolymph (Fig. 17) two of them are always present (category 1, M_r 52 K; pH 6.5, M_r 52 K; pH 7.0) and three of them are present in the larval hemolymph (category 2, M_r 69 K; pH 5.2, M_r 63 K; pH 5.2, M_r 14 K; pH 7.8). Fat body is the only source of the two storage proteins that are present during the phase of larval syntheses (Fig. 34, SP1; SP2, category 7). Fat body as well as other tissues such as epidermis, midgut, pericardial cells and hemocytes secrete Ar which is almost always present in the hemolymph (Fig. 34, Ar, category 1). The epidermis secretes at least 13 polypeptides on the basal surface of which four occur in the hemolymph (Fig. 4) and one of them is present in the hemolymph at all stages (category 1, M_r 22; pH 6.7). Out of eight polypeptides secreted by the midgut, two are detectable in the hemolymph (Fig. 11).

Table 3 The origin of hemolymph proteins

Tissue	No.polypeptides secreted into-medium	No.polypeptides identified on hemolymph fluorogram	No.polypeptides asigned to categories			Reference
			Always present (category 1)	Present in larva (category 2)	Intermoult syntheses specific (category 7)	
Fat body	14	12	2	3	2	Chapter 5
Epidermis	13	4	1			Chapter 3
Midgut	8	2				Chapter 4
Percardial cells+heart	6	5		1		Fife et al., 1987
Hemocytes	11	7		2		Chapter 6

Pericardial cells+heart secrete six polypeptides, five of which occur in the hemolymph (Fig. 9, Fife et al., 1987) and one polypeptide is present only in the larval hemolymph (category 2, M_r 26 K; pH 4.9). Out of 11 polypeptides secreted by hemocytes, seven occur naturally in the hemolymph (Fig. 22) two are present in the larval hemolymph (category 2, M_r 37; pH 5.1, M_r 18; pH 5.0). At least 11 polypeptides that are always present or present in all larval hemolymph originate in fat body, epidermis, pericardial cells+heart and hemocytes. Fat body is the only source of two hemolymph storage proteins present during larval syntheses.

8.3 Discussion

8.3.1 Stage Specific Proteins in the Hemolymph

The results confirm the expectation that there are stage specific proteins in the hemolymph. This work is a first step in analysing hemolymph protein composition in relation to pupal metamorphosis. It confirms the validity of separating the fifth larval stadium into three phases of development and shows a greater complexity than might have been expected. Only spots that could be identified with fair certainity have been included in the analysis (which include at least half of the resolvable spots). The end of intermoult preparation marks the appearance of four new polypeptides that disappear at the end of intermoult syntheses together with the four polypeptides present since ecdysis. The work also underlines the intense new activity in relation to pupation, when no fewer than 17 polypeptides are seen for the first time although none of them survive

into the pupa. The pupa also differs from the larva not just in the loss of 25 polypeptides but also by the presence of four new ones (Figs. 35; 36). Since there is little or no difference in the polypeptide pattern between the fourth and fifth larval hemolymph, the polypeptide changes between the fifth larval and pupal hemolymph are presumably related to metamorphosis rather than to moulting. Such changes and more would be expected in tissue proteins, as observed for the developmental profiles of epidermal proteins in Manduca sexta (Kiely and Riddiford, 1985). Secretion into the hemolymph is presumably in relation to activities occurring during each stage. The results in figure 36 only indicate presence or absence. This may be achieved by changed synthesis and release, by secretion of stored proteins, and/or by changed degradation. The two storage proteins are probably typical in their presence being due to enhanced synthesis with secretion and their absence due to endocytosis, but these events need to be established for each of the 46 polypeptides. Hemolymph polypeptides that are not stage specific but present through several stages are presumably related to continuing functions such as transport.

8.3.2 The Three Major Hemolymph Proteins Follow Different Temporal Patterns of Appearance and Disappearance

Another finding is that the three major hemolymph proteins are independent in their time of synthesis during development. The major hemolymph proteins of Lepidopteran larvae are made up of subunits with relative molecular masses between 80-90 K (82, 86 and 90 K in

Calpodes) that may be difficult to resolve into single bands on SDS gels. Different storage proteins may even have subunits with similar molecular masses that resolve as single bands on SDS gels as in the 86 K polypeptides forming SP1 and SP2 in Calpodes (Chapter 7). Developmental studies that have used SDS-PAGE concluded that both storage proteins appeared after the first critical period and disappeared after the second critical period (Locke et al., 1982). Resolution of native proteins by N-PAGE into distinct bands (Fig. 34) shows that Ar is present through the larval and pupal periods like the Ar of other Lepidopteran insects (Kramer et al., 1980; Telfer et al., 1983). Storage protein 1 appears immediately after the first critical period and SP2 two days later. Both start to decline at the second critical period and disappear by the end of pupation. The appearance of these proteins may be under the influence of declining JH titre and the disappearance the increasing ecdysone titre as in Manduca sexta (Riddiford and Hice, 1985) and Bombyx mori (Tojo et al., 1981).

8.3.3 The Origin of Hemolymph Proteins

Fat body, epidermis, midgut, hemocytes and pericardial cells+heart together secrete 50 polypeptides and half of them were detected in the hemolymph. Of these 11 polypeptides are always present or present in all larval hemolymph. Most of the polypeptides whose origin could be determined are present in all stages (category 1) or present throughout the larval stages (category 2). None present only during one developmental stage (catagories 3-11) could be identified in the media of the tissues. There are several limitations

to these observations. Briefly present polypeptides are the most likely ones to need hormonal induction. The tissues used to study secretion were all isolated from mid-fifth instar larvae. The comparision between silver stained gels and fluorograms might be another limitation. Most of the secretory polypeptides could be identified in the hemolymph fluorograms but only a few are present in silver stained gels. Some of the polypeptides are not clear enough to be included in the analysis. Future studies should correlate labelled secretory polypeptides of the tissues at all stages with the labelled hemolymph polypeptides at all stages.

CHAPTER 9

SUMMARY AND CONCLUSIONS

9.1 Bi-directional Secretion in the Epidermis

1. Epidermis secretes at least 13 polypeptides basally and 15 apically.
2. All polypeptides secreted apically *in vitro* occur in normal cuticle.
3. At least four polypeptides secreted basally can be detected in hemolymph labelled *in vivo*.
4. Five basally secreted polypeptides and one apically secreted polypeptide are immunologically similar to hemolymph polypeptides.
5. Epidermis secretes Ar immunologically similar to that in hemolymph from both surfaces.
6. *Calpodes* larval epidermis has bi-directional secretion. Cuticular proteins are carried to the apical face and a different set of proteins are carried basally to the hemolymph.

9.2 Bi-directional Secretion in the Midgut

1. Midgut secretes at least 8 polypeptides basally and 7 apically.
2. All polypeptides secreted apically *in vitro* occur naturally in the midgut lumen.

3. At least two polypeptides secreted basally can be detected in hemolymph labelled *in vivo*.
4. All basally secreted polypeptides are immunologically similar to hemolymph polypeptides.
5. Midgut secretes Ar that is immunologically similar to hemolymph Ar.
6. *Calpodes* midgut has bi-directional secretion. Hemolymph proteins are carried to the basal face and a different set of proteins are carried to the luminal face.

9.3 Hemolymph Protein Synthesis by the Fat body

1. Fat body secretes at least 14 polypeptides into the incubation medium.
2. At least 12 fat body secretory polypeptides occur naturally in the hemolymph.
3. At least half (about 10% by quantity) of the hemolymph polypeptides are not secreted by fat body.
4. Antibodies made against hemolymph proteins recognize ten fat body secretory polypeptides.
5. Fat body synthesizes and secretes Ar.

9.4 Hemolymph Protein Synthesis by the Hemocytes

1. Hemocytes secrete at least 11 polypeptides into the incubation medium.
2. Arylphorin and six other polypeptides secreted by hemocytes occur in normal hemolymph.
3. Antibodies made against hemolymph proteins recognize six polypeptides secreted by hemocytes.
4. Hemocytes synthesize and secrete Ar.

9.5 Purification and Characterization of the Three Major Hemolymph Proteins

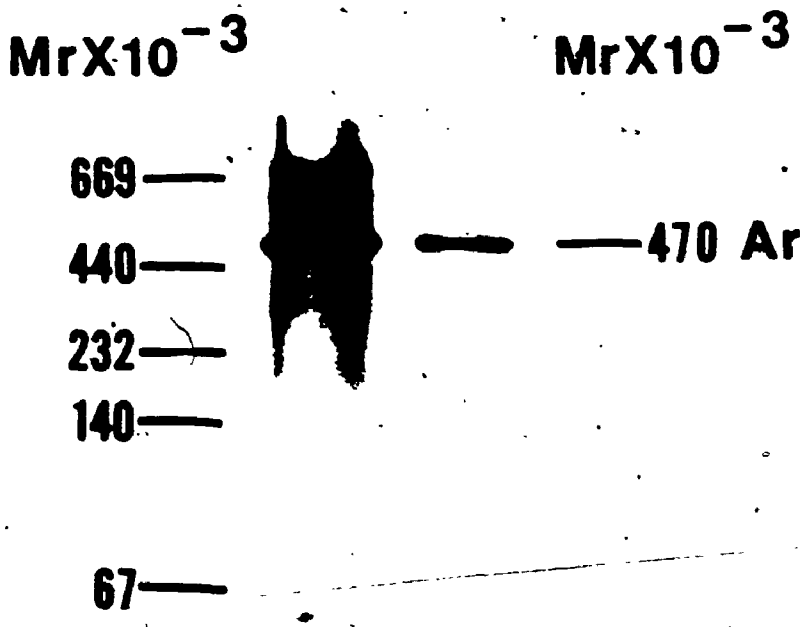
1. Fifth stage Calpodes hemolymph has three major proteins with Mr 470, 580 and 720 K.
2. All three major hemolymph proteins are associated with carbohydrate but only arylphorin (470 K) has lipid.
3. The three major hemolymph proteins have been purified by preparative N-PAGE and SDS-PAGE.
4. 470 K protein is rich in aromatic amino acids and is identified as Ar. 580 and 720 K proteins are rich in glycine and are identified as storage proteins SP1 (720 K) and SP2 (580 K).
5. Arylphorin dissociates into 82 K subunits, SP2 into 86 K subunits and SP1 into both 86 K and 90 K subunits.

6. Electron microscopy of negatively stained preparations shows that each protein has a different geometrical arrangement of subunits. Arylphorin is an octahedron made from six subunits. Storage protein 1 is a cube made from eight subunits and SP2 is a hexamer in the form of a pentahedral prism.

9.6 Developmental Changes of Hemolymph Proteins

1. The fifth larval stadium of Calpodes can be divided into three phases of development; larval growth, larval syntheses and pupation.
2. Ten hemolymph polypeptides are present throughout fourth, fifth stage larval and pupal stadia and 46 other polypeptides are stage - specific.
3. Each growth phase is characterized by the presence of many polypeptides that are either absent at all other times or present during only one other stage.
4. Major hemolymph proteins also change with development. Arylphorin is present from early in the stadium and continues into the pupa. Storage protein 1 is synthesized from 66 hr and SP2 appears two days later, but both start to disappear at the beginning of pupation and are absent in the pupa.

Appendix 1. The antibodies made against hemolymph proteins as well as arylphorin subunits recognize native arylphorin (470 K). [^{35}S] methionine labelled hemolymph proteins were precipitated with antibodies made against hemolymph (He) and arylphorin (Ar). Immunoprecipitates were separated on a 3-15% N-PAGE. The fluorogram was developed for two weeks at -70^{0}C. The relative mobility of Pharmacia high molecular weight standards are shown on the left side of the figure.



He Ar

He=Hemolymph

Ar=Arylphorin

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