

ISOLATION AND CHARACTERIZATION OF LIPOPHORIN FROM  
THE LARVAL STALK BORER, BUSSEOLA FUSCA.

BY

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A thesis submitted in partial fulfilment for the degree  
of Master of Science in the University of Nairobi

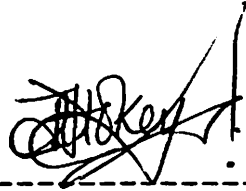
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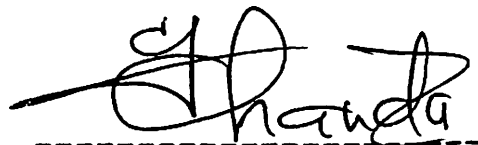
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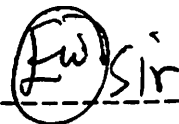


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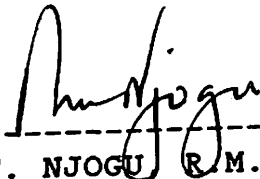
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## A C K N O W L E D G E M E N T S

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## ABBREVIATIONS

AKH	-	Adipokinetic hormone
ApoLp-I	-	Apolipoprotein - I
ApoLp-II	-	Apolipoprotein - II
ApoLp-III	-	Apolipoprotein - III
BSA	-	Bovine serum albumin
cAMP	-	Cyclic adenosine monophosphate
Con A	-	Concanavalin A
FITC-Con A	-	Fluorescein isothiocyanate conjugated - Con A
HDL	-	High density lipoprotein
HDLp	-	High density lipoprotein
HDLp-L	-	Larval, high density lipoprotein
HDLp-A	-	Adult, high density lipoprotein
IEF	-	Isoelectrofocussing
KBr	-	Potassium bromide
LDL	-	Low density lipoprotein
LDLp	-	Low density lipoprotein
LTP	-	Lipid transfer protein
NaCl	-	Sodium chloride
PAGE	-	Polyacrylamide gel electrophoresis
PAS	-	Periodic acid Schiff reagent
PBS	-	Phosphate buffered saline
PEG	-	Polyethylene glycol
PTTH	-	Prothoracicotropic hormone
RI	-	Refractive Index
SDS	-	Sodium dodecyl sulphate

SDS-PAGE - Sodium dodecyl sulphate-polyacrylamide  
gel electrophoresis

TB - Tris buffer

TBS - Tris buffered saline

Tris - Tris-(hydroxy methyl)-aminomethane

VHDL - Very high density lipoprotein

VHDLp-E - Egg specific, very high density  
lipophorin

VLDL - Very low density lipoprotein

w/w - Weight by weight

## UNIT ABBREVIATIONS

gm	-	Gram
h	-	Hour
M	-	Molar concentration
mA	-	Milli Amperes
min	-	Minute
mg	-	Milligram
ml	-	Millilitre
mm	-	Millimetre
mM	-	Millimolar
M <sub>r</sub>	-	Molecular weight
nm	-	Nanometre
pH	-	- Log hydrogen ion concentration
pI	-	Isoelectric point
rpm	-	Revolutions per minute
sec	-	Second
ug	-	Microgram
ul	-	Microlitre

## SUMMARY

In Kenya the most important and prevalent pests that attack maize and sorghum are the stalk-borers. There are three species of the stalk borer in Kenya namely, Chilo partellus, Sesamia calamistes and Busseola fusca. B. fusca which thrives in high altitude areas with cool climate, poses the greatest threat to the crops.

Lipophorin acts as a reusable shuttle in the transportation of a variety of lipid classes from sites of storage, absorption and synthesis to sites of utilization (Chino, 1985). Lipids play a vital role in insect development as components of cell membranes and cuticle, source of metabolic energy and as hormones and pheromones. Interference with lipid transport would therefore be fatal to the insects. Studies of lipophorins thus hold potential for selective control of destructive insect pests. In this study, lipophorin, the principal haemolymph lipoprotein in most insects, was isolated and characterized from B. fusca larvae.

Lipophorin was isolated from the B. fusca larvae by density gradient ultracentrifugation. The apoproteins were isolated by electroelution following SDS-PAGE. The isolated lipophorin ( $M_r \sim 700,000$ ) was a high density lipoprotein (density = 1.13 g/ml), composed of 46% lipid, 50% protein and 4% carbohydrate. Analysis by SDS-PAGE revealed that it consisted of two apoproteins, apoLp-I

( $M_r \sim 210,000$ ) and apoLp-II ( $M_r \sim 78,000$ ), which were present in a molar ratio of 1:2 (apoLp-I: apoLp-II) in the intact lipophorin molecule. Both apoproteins were glycosylated and lipidated as shown by PAS and Sudan Black staining, respectively. The presence of high mannose containing oligosaccharide chains was demonstrated by the binding of lipophorin to concanavalin A-Sepharose column. Studies on the lipid moiety of B. fusca lipophorin indicated predominance of phospholipids and diacylglycerol.

Amino acid composition analysis of lipophorin showed predominance of glutamate (9%), aspartate (13%) and glycine (9%). Methionine was only present in trace amounts.

Structural organization of the apoproteins was investigated by limited trypsin digestion and immunological studies. Limited trypsin digestion of the isolated lipophorin showed that apoLp-I was more susceptible to cleavage than apoLp-II, suggesting an interior location of apoLp-II. By immunoblotting, both apoproteins were shown to be immunoreactive towards antibodies to the isolated lipophorin. However, double radial immunodiffusion of the apoprotein against the antisera only showed precipitin line with apoLp-I. Thus in the intact lipophorin, apoLp-I is more exposed to the aqueous haemolymph environment than apoLp-II.

An investigation was carried out to determine whether there was immunological cross-reactivity between lipophorins from Eldana saccharina, Glossina mortisana, Locusta

migratoria, Galleria mellonella and Chilo partellus with antibodies to B. fusca and M. sexta lipophorins. Using double radial immunodiffusion, it was shown that lipophorins from E. saccharina, G. mellonella and C. partellus cross-reacted with both antisera. Immunoblotting with anti B. fusca lipophorin showed the same cross-reactivity. The results suggested that lipophorins from insects of the same order share antigenic determinants predominantly on apoLp-I.



## CHAPTER 1

### INTRODUCTION AND LITERATURE REVIEW:

#### 1.1 INSECT GROWTH AND DEVELOPMENT

The growth of insect larvae is restricted by the cuticle which can only allow limited expansion. Growth is therefore punctuated by a series of moults. The number of moults which occur is variable but is generally less in more advanced insects (Chapman, 1971). The last larval instar of most insects undergo metamorphosis, a process in which changes in the internal as well as external parts of the body accompany the transition from the last larval instar to adult (Wigglesworth, 1972; Chapman, 1971). In those insects with incomplete metamorphosis (hemimetabolous), the process occurs in one step with the last larval stage transforming directly into the adult. In insects with complete metamorphosis (holometabolous), the changes during metamorphosis are more extensive and a pupal stage is interposed between the last larval stage and the adult (Chapman, 1971; Wigglesworth, 1972).

The cyclic process of growth and moulting is brought about by prothoracicotropic hormone (PTTH) and prohormone, ecdysone. Secretion of PTTH stimulates the prothoracic glands to secrete prohormone, ecdysone, which is then converted to the moulting hormone ( $\beta$ -ecdysone). The moulting process is initiated by  $\beta$ -ecdysone acting on the

epithelial cells. The type of moulting is determined by the amount of juvenile hormone secreted by the corpora allata. Thus high juvenile hormone titers dictates the larval-larval moult whereas low titers, larval-pupal moults. The pupal to adult metamorphosis occurs in the absence of juvenile hormone (Gilbert et al, 1980; Riddiford and Truman, 1978).

## 1.2 THE LIFE CYCLE OF BUSSEOLA FUSCA

B. fusca is a holometabolous lepidoptera with a life cycle lasting upto sixty six days during rainy season and as long as 200 days during the dry season (Ingram, 1958). The last generation of the insect on crops (maize or sorghum) survive on stalks and stubbles after harvest as a mature diapausing larva. The diapausing stage lasts upto the onset of the rains and subsequent cropping season (Harris, 1962). In Mbita, Western Kenya, where there is only one crop of sorghum a year during the long rainy season (March - August), the second generation of B. fusca entered diapause which lasted upto 10 months (Oloo, 1985).

Post embryonic development in B. fusca maintained in the laboratory on sorghum stems lasted 40.8 days. The development, was completed without intervening larval diapause (Unnithan, 1987). It was concluded that facultative diapause in B. fusca could be prevented if larva were fed on young plants. Smithers, 1959 also reported that it was possible to rear B. fusca continuously on fresh green maize plants. Eclosion of the laboratory maintained B.

fusca (Unnithan, 1987) was followed immediately with mating and oviposition. The overall mean fecundity and egg fertility of these moths were 723 and 84%, respectively. Male and female moths survived for an average of 8.7 and 6.8 days, respectively. It was also observed that feeding the moths with sucrose, in addition to distilled water, shortened the preoviposition and oviposition periods slightly but their fecundity was not affected.

### 1.3 LIPID TRANSPORT IN INSECTS

Insect lipids function as components of cell membranes and as important sources of metabolic energy for cell maintenance, flight, reproduction, embryogenesis and metamorphosis. They also serve communication roles as pheromones and kairomones, regulatory roles as hormones and protective roles as cuticular lipids (Jackson and Arnold, 1977; Downer, 1978). Lipid transport from sites of storage and absorption to sites of utilization, is therefore a crucial process for the survival of insects.

Studies on insect lipid transport was initiated on locusts by Tietz, 1962. She observed that acylglycerol was released from the fat body into the haemolymph. Chino and Gilbert, 1964; 1965 demonstrated in Locusta migratoria, Hyalophora cecropia and Periplaneta americana that fatty acids were transported from the fat body in the form of diacylglycerol, associated with a specific lipoprotein. Many investigators also later confirmed that diacylglycerol

may serve to transport fatty acids from sites of storage and absorption to sites of utilization in almost all insects (Tietz, 1967; Gilbert and Chino, 1974; Chino and Downer, 1979; Thomas, 1979).

The specific lipoprotein was first purified from H. cecropia (Chino et al, 1969; Thomas and Gilbert, 1968). In vitro studies by Chino et al, 1969, showed that the purified lipoprotein had the capacity to take up diacylglycerol from fat body, hence the original term, diacylglycerol-carrying lipoprotein. Other terms have been used to describe the lipoprotein: high density lipoprotein (Thomas and Gilbert, 1968), lipoprotein-I (Gilbert and Chino, 1974; Gellisen and Emmerich, 1980; Chinzei et al, 1981), diacylglyceride-transporting lipoprotein (Mwangi and Goldsworthy, 1977; Van Der Horst et al, 1979), diglyceride binding lipoprotein (Gellissen and Emmerich, 1980). Chino and Kitazawa, 1981 demonstrated that the lipoprotein was involved in the transportation of several other polar and non-polar lipids. The multiplicity of functions therefore rendered the terms for the lipoprotein, inadequate. Chino et al, 1981 proposed the term "lipophorin" (from Greek, lipos, fat; phoros, bearing), as a more appropriate generic term for the lipoprotein.

#### 1.4 INSECT HAEMOLYMPH LIPOPROTEINS

Several very high density lipoproteins (VHDL) have been purified by differential density gradient

ultracentrifugation (Haunerland et al, 1987). The most studied VHDL's are vitellogenins and vitellins (Beenackers et al 1986; Hagedorn and Kunkel, 1979), which are female-specific proteins. Vitellogenins which are synthesized in the fat body, are the precursors of the egg yolk protein vitellins (Engelmann, 1979).

Vitellogenins from most insects are high molecular weight proteins that contain lipids (7-16%) and carbohydrates (1-14%) (Engelman, 1979). Manduca sexta vitellogenin ( $M_r \sim 500,000$ ) has two apoproteins: apovitellogenin-I ( $M_r \sim 180,000$ ) and apovitellogenin-II ( $M_r \sim 45,000$ ). It is composed of 13% lipids, 84% protein and 3% carbohydrate (Osir et al, 1986). One of the functions of vitellogenin is to transport lipids from the fat body to the oocyte (Chino et al, 1977).

A larval stage specific VHDL, arylphorin, has been identified in the haemolymph of several insect orders (Munn et al, 1971; Wolf et al, 1971; Tojo et al, 1980; Kramer et al, 1980; Telfer et al, 1983). Arylphorins are usually associated with (2-5%) lipid and are characterized by a hexameric structure plus an unusually high content of aromatic amino acids (Riddiford and Law, 1983). Arylphorins are synthesized during the larval instars and utilized mainly during adult development (Riddiford and Law, 1983). It is thought that arylphorins are degraded in the fat body to supply amino acids for the construction of adult tissues

and exoskeleton (Ogawa and Tojo, 1981; Roberts and Brock, 1981). Xenobiotics and ecdysteroids also appear to be bound by arylphorin (Enderle et al, 1984; Haunerland and Bowers, 1986 a).

Another larval specific VHDL (density = 1.26 g/ml) chromoprotein ( $M_r \sim 560,000$ ) has been isolated from Heliothis zea (Haunerland and Bowers, 1986). It consists of four identical subunits ( $M_r \sim 150,000$ ) and 8% lipid composition. The protein is absent during early larval, pupal and adult stages. It is, however, the major haemolymph protein during the 5th larval instar. A similar type of protein has been isolated from the larval honey bees (Shipman et al, 1987). The role of the protein in lipid transport is as yet unknown.

A diapause induced lipoprotein (density = 1.29 g/ml) has been isolated by Osir et al, (personal communication), from B. fusca diapausing larvae. The protein which appears in the haemolymph prior to the onset of diapause has two subunits of molecular weights, 88,000 and 79,000. The high aromatic amino acid composition of the protein, suggests a storage role.

Lipid transfer protein (LTP) of density = 1.23 g/ml has also been purified from M. sexta (Haunerland, et al 1987; Ryan et al 1986). Lipid transfer protein is composed of two apoproteins ( $M_r \sim 320,000$  and  $M_r \sim 85,000$ ). The lipids constitute approximately 13.8% of the total weight. It has

been suggested that LTP participates in the distribution of lipids between haemolymph lipoproteins or between membranes and lipoproteins (Ryan et al 1987).

Lipophorins are the principal lipoproteins in the haemolymph of insects at all stages of development (Chino et al, 1981). They exist in various interconvertible forms, from high to low density forms (Ryan et al, 1987). Lipophorins are involved in various lipid transport processes where they function as reusable shuttles (Chino and Kitazawa, 1981; Van Der Horst et al, 1981; Downer and Chino, 1985).

### 1.5 INSECT LIPOPHORINS

Most insects have lipophorin as the major haemolymph lipoprotein during all stages of their development (Chino et al, 1981). Lipophorin isolation and characterization has been accomplished on a number of insects including M. sexta (Prasad et al, 1986; Ryan et al, 1986; Shapiro et al, 1984; Pattnaik et al, 1979; Apis mellifera (Robbs et al, 1985); L. migratoria, H. cecropia, P. americana (Chino et al, 1981; Chino and Kitazawa, 1981), Phylosamia cynthia (Chino et al, 1969), Diaprepes abbreviatus (Shapiro, 1988), Diatraea grandiosella (Dillwith et al, 1986), and Drosophila melanogaster (Fernando-Warnakulasuriya and Wells, 1988). Ryan et al, 1984, also isolated lipophorins from eight insect species representing seven insect orders .

### 1.5.1 Composition of lipophorin

All lipophorins so far examined consist of two apoproteins, apolipophorin-I (apoLp-I) with molecular weight ( $M_r$  ~250,000) and apolipophorin-II (apoLp-II) ( $M_r$  ~80,000) (Ryan et al, 1984; Chino, 1985; Shapiro et al, 1988). The lipophorins show predominance of phospholipids and diacylglycerols (Shapiro et al 1988; Chino, 1985). The presence of covalently bound carbohydrate has also been demonstrated in lipophorins from several insects (Ryan et al, 1984).

A third apoprotein, apolipophorin-III (apoLp-III) of  $M_r$  ~ 18,000 - 20,000, appears to associate reversibly with lipophorin of certain insects (Van Der Horst et al, 1984; Van Der Horst et al, 1981; Wheeler and Goldsworthy, 1983, 1983 a). ApoLp-III has been purified from M. sexta (Kawooya et al, 1984), Thasus acutangulatus (Wells et al, 1985), L. migratoria (Goldsworthy et al, 1985; Van Der Horst et al, 1985), and Gastromargus africanus (Haunerland et al, 1985). In both M. sexta and L. migratoria, apoLp-III is present at very low concentrations in the larval haemolymph but abundant in mature adults (Kawooya et al, 1984; Wheeler and Goldsworthy, 1983). Ryan et al, 1984 has speculated that apoLp-III is present only in insect species which utilize lipid as fuel for flight.

### 1.5.2 Interconversion of lipophorin forms



Studies on lipophorin from M. sexta indicate that there are stage specific forms of lipophorin (Ryan and Law, 1984; Prasad et al, 1986; Ryan et al, 1986). The various forms have been attributed to changes in metabolic requirements which may necessitate changes in lipophorin function and composition.

During the period of larval-pupal metamorphosis of M. sexta, four forms of lipophorin appear in the haemolymph at different times. The four forms are all of high density (HDLp) with identical apoproteins, apoLp-I ( $M_r \sim 245,000$ ) and apoLp-II ( $M_r \sim 78,000$ ) but varied lipid content. The larval lipophorin (HDLp-L) has a density of 1.15 g/ml and is composed of 37% lipid. In contrast to HDLp-L, the adult form of lipophorin (HDLp-A) has a lower density (1.08 g/ml) and a higher lipid content (51%). HDLp-A is also associated with a third apoprotein (apoLp-III) ( $M_r \sim 17,000$ ) (Shapiro and Law, 1983; Kawooya et al, 1984; Ryan et al, 1986). In the resting state HDLp-A contain apoLp-I, apoLp-II and apoLp-III in the ratio 1:1:2 (Kawooya et al, 1984; Shapiro et al 1984). HDLp-A conversion to a low density lipophorin (LDLp) occurs during prolonged flight or stimulation with adipokinetic hormone (AKH) (Shapiro and Law, 1983). LDLp (density = 1.03 g/ml) has a higher lipid: protein ratio and is associated with more apoLp-III than HDLp-A.

Egg specific very high density lipophorin (VDHLP-E;  $M_r \sim 414,000$ ,  $d = 1.238$  g/ml) has been isolated from the egg of M. sexta. VDHLP-E which is derived from HDLP-A consists of 80% protein and 20% lipid (Kawooya et al, 1988). The HDLP-A is selectively taken up by the follicles then converted to VDHLP-E. The conversion has been shown to involve stripping off of most lipids from HDLP-A and conversion of diacylglycerols to triacylglycerols. In addition, the two molecules of apoLp-III of HDLP-A are dissociated from the particle. Consequently HDLP-A decreases in size as the density is increased resulting into VDHLP-E (Kawooya et al, 1988; Kawooya and Law, 1988).

In vivo studies by injection of radiolabelled forms of lipophorin into M. sexta has demonstrated that all forms of lipophorin are interconvertable (Ryan et al, 1986). In vitro studies with radiolabelled lipophorins however, indicated the presence of an essential lipid transfer component in the haemolymph. The lipid transfer factor (LTP) which is capable of catalysing net lipid transfer between lipophorin particles, has been shown to be the essential component. (Ryan et al, 1986).

### 1.5.3 Structural organization of lipophorin

Evidence from electron microscopy shows that lipophorins are spherical particles (Chino et al, 1986; 1987; Pattnaik et al, 1979; Chino and Kitazawa, 1981). The

core of lipophorins is composed of hydrocarbons whereas the surface has phospholipids (Katagiri, 1985; Katagiri et al, 1986). In M. sexta, both HDLp and LDLp have diacylglycerol and phospholipids on the surface layer. The core on the other hand is made up of hydrocarbons, triacylglycerol, cholesterol and diacylglycerol. In L. migratoria, the core of HDLp is made up entirely of hydrocarbons and triacylglycerol, unlike LDLp which has a significant amount of diacylglycerol in the core (Shapiro et al, 1988).

The apoproteins of lipophorins seems to be organized such that apoLp-I is more exposed to aqueous haemolymph environment than apoLp-II. Evidence for this has come from limited trypsin digestion, iodination and immunological studies (Mundal et al, 1980; Pattnaik, et al, 1979; Shapiro et al, 1984; Robbs et al, 1985; Kashiwazaki and Ikai, 1985). These results have been confirmed by immunoblotting with monoclonal antibodies to the apoproteins (Van Der Horst et al, 1987).

Ryan et al, 1984 have shown that apoLp-II has a conserved structure over a wide range of insect orders. Thus antibodies to M. sexta apoLp-II cross reacted with apoLp-II's from eight insect species representing seven insect orders. At variance with this observation is a report by Van der Horst et al, 1987 in which no cross reactivity was observed between monoclonal antibodies to L.

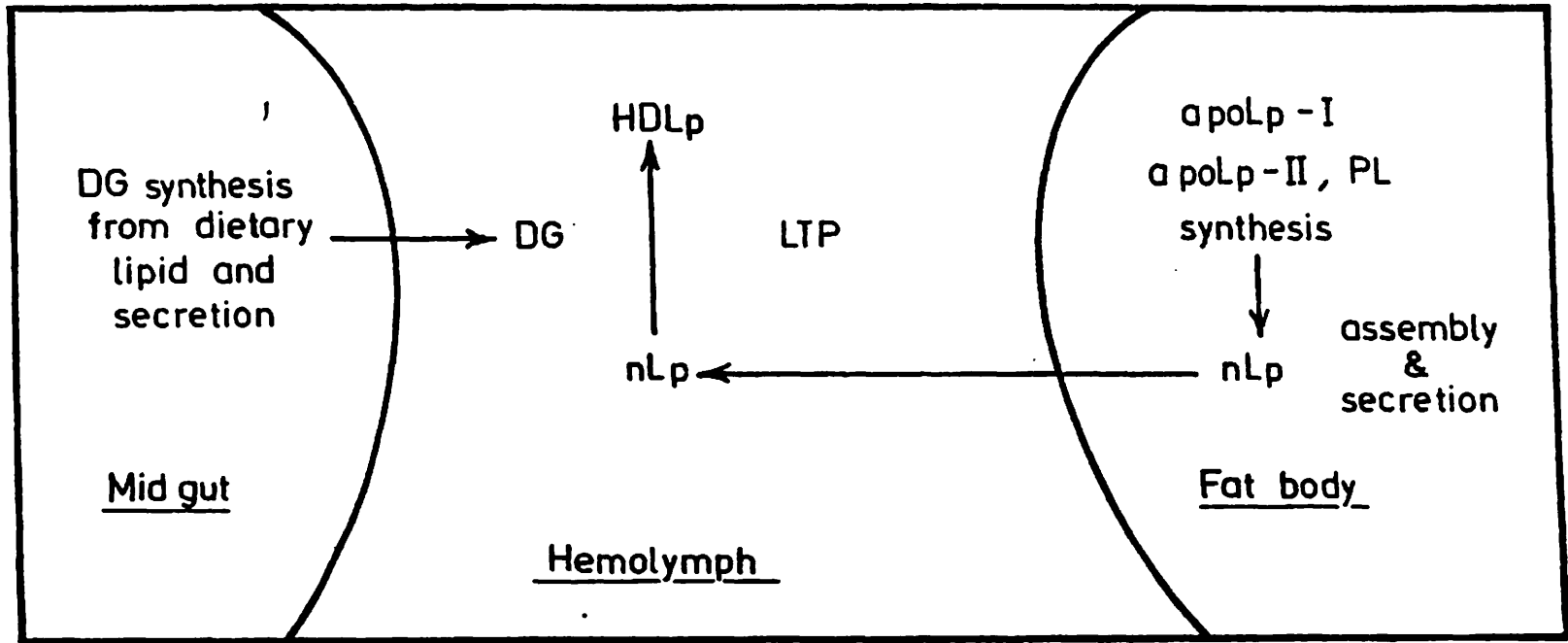
migratoria apoLp-II and apoLp-II's from P. americana,  
Leptinotarsa decemlineata and Deilephila elpenor.

#### 1.5.4 Biosynthesis of lipophorin

Biosynthesis of lipophorin occurs in the fat body (Gellissen and Wyatt, 1980; Harry et al, 1979, Peled and Tietz et al, 1973; Thomas, 1972). The process involved in the biosynthesis has been studied in details only in the feeding fifth instar larvae of M. sexta (Prasad et al, 1986a). In vitro studies with fat body from M. sexta larvae, showed that a nascent VHDLP particle that contained apoLp-I, apoLp-II and phospholipids, but very little diacylglycerol was secreted into the medium (Prasad et al, 1986). The maturation of the nascent VHDLP was shown to involve the uptake of diacylglycerol derived from the dietary lipids in the midgut (Scheme 1). Unlike in M. sexta larvae, diapausing D. grandiosella fat body secreted into the medium a nascent lipophorin similar in density and lipid composition to the circulating lipophorin (Venkatesh and Chipperndale, 1986; Venkatesh et al, 1987). The difference has been attributed to the fact that the diapausing D. grandiosella has to use fat body lipid for fuel (Turunen and Chipperndale, 1981).

The biosynthesis of lipophorin during the larval development of M. sexta takes place only during the feeding periods, from the beginning of the fourth instar to pupation (Prasad et al, 1987; Tsuchida et al, 1987).

SCHEME 1: Biosynthesis of lipophorin in M. sexta larvae  
(Shapiro et al., 1988).



Nascent lipoprotein particle..( nLp )  
Diacylglycerol.....( DG )  
Lipid transfer protein.....( LTP )

HDLp-A of M. sexta contains two molecules of apoLp-III in addition to one each of apoLp-I and apoLp-II (Kawooya et al, 1984; Shapiro and Law 1983; Wells et al, 1987). The two molecules of apoLp-III are intimately intergrated into the structure of HDLp-A, as they do not exchange with free apoLp-III (Wells et al, 1987). It has been suggested that pupal lipophorin is replaced late in development by a newly synthesized lipophorin containing apoLp-III (Wells et al, 1987). The mechanism by which this occurs is unknown at the present time.

In both L. migratoria and M. sexta, apoLp-III is found in low levels in the larval haemolymph. The rate of synthesis increases after adult ecdysis (Kanost et al, 1987; Kawooya et al, 1984). It is not clear what determines whether or not a newly synthesized apoLp-III gets incorporated into the adult lipophorin or secreted into the haemolymph.

## 1.6 PHYSIOLOGICAL ROLE OF LIPOPHORIN

Lipophorins function as reusable shuttles in a variety of lipid transport processes without being degraded or taken up (Chino, 1985; Downer and Chino, 1985). They transport digested fat from the gut to the peripheral tissues or to and from the storage sites. Lipophorins also function in the distribution of hydrocarbons, cholesterol and carotenoids (Chino, 1985; Katase and Chino, 1982; 1984). Lipophorin has also been implicated in the distribution of

hydrophobic xenobiotics (Winter et al, 1975; Skalsky and Guthrie, 1975). The binding of lipophorin to insecticides has also been reported (Haunerland and Bowers, 1986). The in vitro studies also implicated arylphorin, as being involved in the binding of insecticides. As yet, there is no evidence that the binding of haemolymph proteins to xenobiotics or insecticides enhances their intoxication or detoxication (Haunerland and Bowers, 1986 a).

Kawooya and Law, 1988, have demonstrated by in vitro studies that HDLp-A of M. sexta is not recycled back into the haemolymph after internalization by the follicles. Thus lipophorin does not seem to function as a reusable shuttle between the fat body and the ovary.

#### 1.6.1 Lipophorin metabolism during flight

The onset of flight in L. migratoria is preceded by release of AKH from the corpus cardiacum. The release of the hormone leads to the mobilization of lipids from the fat body leading to the release of diacylglycerol from triacylglycerol, via a second messenger cAMP (Wheeler and Goldsworthy, 1983, 1983 a; Van Der Horst et al, 1981; 1984; 1985; Van Heusden et al, 1984). Shapiro et al, 1988 has proposed that the released diacylglycerol accumulate in the plasma membrane of the fat body cells and then moves via fluid phase diffusion into HDLp. The involvement of LTP in the transfer has been proposed (Ryan et al, 1986; 1986a).



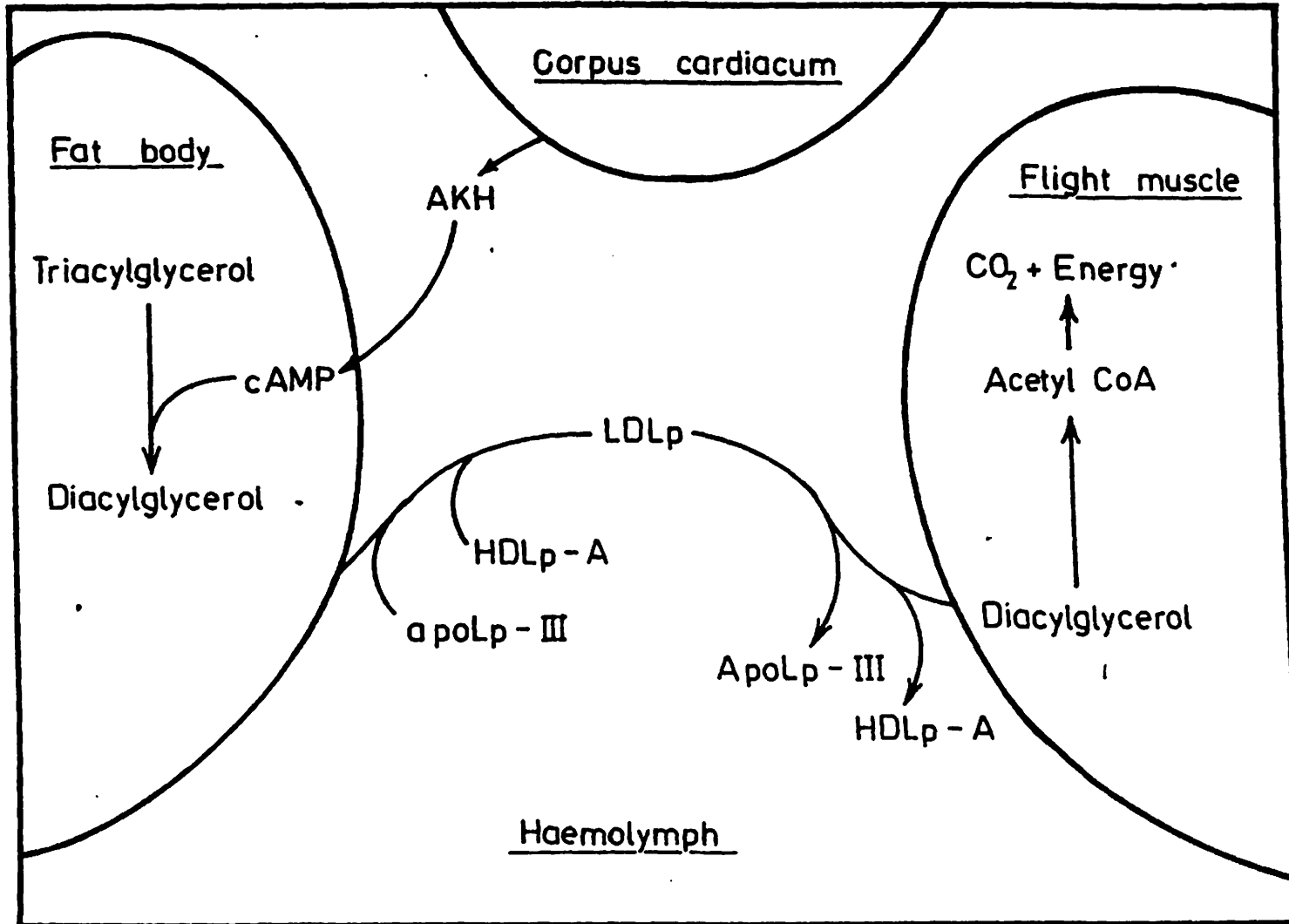
The loading of diacylglycerol onto HDLp-A results into a larger but a less denser lipoprotein (LDLp). Concomitant with diacylglycerol loading is an association of several molecules of apoLp-III with diacylglycerol rich particle (Mwangi and Goldsworthy, 1977; 1981; Van Der Horst et al, 1981; 1979; Wheeler and Goldsworthy, 1983, 1983a). Physical and surface properties of apoLp-III suggest that it is well suited to stabilize the diacylglycerol rich particle (Kawooya et al, 1986). ApoLp-III has also been proposed as a possible recognition signal or activator of the flight muscle lipoprotein lipase (Van Der Horst et al, 1987).

At the flight muscle, diacylglycerol is released from LDLp particle and excess apoLp-III dissociated. The resulting HDLp-A can then return to the fat body and bind more diacylglycerol and free apoLp-III once again forming LDLp. The delivery of diacylglycerol at the flight muscle is not well understood but a lipoprotein lipase identified in the flight muscle of L. migratoria may be involved (Van Heusden et al, 1986; Wheeler and Goldsworthy, 1985; Wheeler et al, 1985).

Similar events as observed in L. migratoria, have been shown to take place in M. sexta, following injection of synthetic locust AKH into the adult moth (Shapiro and Law, 1983; Ryan and Law, 1984) (Scheme 2).

SCHEME 2:

Effect of AKH on lipid transport in adult M.  
sexta (Ryan and Law, 1984).



## 1.7 RATIONALE FOR THE STUDY OF LIPOPHORIN FROM

### B. FUSCA LARVAE

*B. fusca* is an economically important insect of the order lepidoptera. It is the major stem-borer affecting sorghum and maize (Nye, 1980). Infestations of upto 100% during long rains and 67% during short rains has been reported on sorghum (Unnithan and Reddy, 1985). Larval feeding on either maize or sorghum causes foliar damage, stem tunnelling, pannicle and cob damage (Unnithan, 1987).

There are a wide range of both granular and dust insecticides available for the control of *B. fusca*. Soil applied insecticides such as carbofuran granules are also on trial for control of stalk borers. However, the use of chemical pesticides has side effects which are not sufficiently known. Successful management of the insect pests should be by methods which are environmentally safe and economically feasible for subsistence farming conditions in developing countries. Efforts towards such an end has been focussed mainly on the development of crops resistant to infestation and the use of biological control methods. Parasitoids that infest stem borers of sorghum and maize have been identified (Oloo, 1985). The possible use of such parasitoids in biological control of the insect pests is yet to be established.

Basic research in biochemistry, physiology and ecology of the insect pests are vital for successful pest management. Through such studies, it is possible to identify unique features of the insects, that can be selectively exploited for the control of the pests.

Lipids play a vital role in insect development as components of cell membranes, and cuticles, source of metabolic energy and as hormones and pheromones. Interference with lipids transport would therefore be fatal to the insects. Lipophorin, the major haemolymph lipoprotein in most insects is involved in a variety of lipid transport processes. Interference with the synthesis or structure and hence the physiological role of lipophorin would thus be lethal to the insect pests. Selective management is feasible through such approach as the mammalian lipoproteins involved in the transportation of lipids and xenobiotics, differ in composition and possibly mechanism of action from insect lipophorins (Newshome and Leech, 1984; Shapiro et al, 1988; Fredrickson, 1973). Unlike the insect lipophorins in which the different forms are interconvertable, four distinct forms of mammalian lipoproteins are known. Whereas insect lipophorins function as reusable shuttles (Prasad et al, 1988; Ryan and Law, 1984; Ryan et al, 1986), mammalian lipoproteins are only loaded once as some are taken up by cells and subsequently degraded (Brown and Goldstein, 1986). Lipophorin does not

seem to be degraded even when internalized by the follicles (Shapiro and Law, 1988).

To evaluate lipophorin as a possible control target, it is of paramount importance to undertake a study of lipophorins from economically important insects such as B. fusca. Understanding the basic structure is important for the elucidation of mechanisms involved in lipid transport.

1.8

#### AIMS OF THE STUDY

The aim of the present study was to;

1. Isolate lipophorin from B. fusca larvae
2. Characterize the isolated lipophorin both chemically and physically.
3. Test for immunological cross-reactivity between lipophorins from other economically important insects using anti-B. fusca lipophorin.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 CHEMICALS

The laboratory chemicals used in all experiments were obtained from BDH, England, Sigma Chemical Co., England, Pierce Chemical Co., Rockford or Bio-Rad, Richmond.

#### 2.2 INSECTS

The last larval instar of B. fusca were obtained from The International Centre of Insect Physiology and Ecology (ICIPE), Mbita Point Field Station. The larva were initially obtained from sorghum and maize farms in the farmers fields around the Centre and reared on sorghum stalks before use. Adult Glossina mortisan (Diptera) and larval forms of Chilo partellus (Lepidoptera), Galleria mellonella (Lepidoptera) and Eldana sacharina (Lepidoptera) were obtained from ICIPE, Nairobi. Adult L. migratoria (Orthoptera) were obtained from Zoology Department, University of Nairobi.

#### 2.3 HAEMOLYMPH COLLECTION

Chilled insects were bled through an incision in the proleg. The haemolymph was collected into ice-cold phosphate buffered saline (PBS) [(0.1 M sodium phosphate, 0.15 M NaCl, pH 7.0)] containing 50 mM glutathione, 1.0 mM

diisopropylphosphofluoridate, 2 mM aprotinin and 5 mM phenylmethyl- sulphonyl fluoride. A few crystals of phenylthiourea were added to the bleeding solution to prevent melanization. For every 1.0 ml of haemolymph collected, 100  $\mu$ l of the bleeding solution was used.

#### 2.4 LIPOPHORIN PURIFICATION

The purification involved a single step density gradient ultracentrifugation (Shapiro *et al*, 1984; Haunerland and Bowers, 1986). Haemolymph sample was mixed with KBr to a final concentration of 44% KBr in PBS, and a final volume of 20 ml. After transfer to a 39 ml Quick Seal centrifuge tube (Beckman), the sample was overlaid with either 0.9% NaCl (Shapiro *et al*, 1984) or 33% KBr (Haunerland and Bowers, 1986). The sealed tubes were centrifuged (50,000 rpm, 4 h, 4°C), in a VTi 50 vertical rotor using Beckman model L8-70 ultracentrifuge.

The tube overlaid with 0.9% NaCl was fractionated into 1.0 ml fractions after centrifugation. The fractions were used to estimate the protein content (Bradford, 1976) using Beckman DU-50 spectrophotometer and refractive indices using a refractometer (Bellingham Stanley Ltd). Densities of the fractions were computed from the relationship density = 6.4786 RI - 7.6430, (where RI is the refractive index at 25°C).



Aliquots (100  $\mu$ l) of each fraction was dialysed against three changes of PBS, and then analysed by SDS-PAGE. Fractions containing the lipophorin yellow band were subsequently pooled. Purity of lipophorin was ascertained by SDS-PAGE and non-denaturing-PAGE.

Centrifugation with 33% KBr as the overlaying solution, floated lipophorin at the top of the centrifuge tube. In this case, lipophorin was directly pipetted out.

A sample of the isolated lipophorin was recentrifuged (50,000 rpm, 4 h, 4°C) in a 5ml Quick Seal centrifuge tube (Beckman), with 0.9% NaCl as the overlaying solution (Shapiro *et al.*, 1984). The sample was extensively dialysed against PBS then mixed with KBr to a final concentration and volume of 44% KBr and 2.5 ml respectively in PBS. After the centrifugation, fractions (200  $\mu$ l) were collected from the centrifuge tube and protein content and refractive index determined.

## 2.5 PROTEIN ESTIMATION

Protein estimation was carried out by either the Bradford method (Bradford, 1976) or BCA protein assay method (Pierce, Co.). Lipophorin samples for the protein estimation were initially dialysed against three changes of PBS to remove KBr. Other protein samples were directly used for the assays. Bovine serum albumin (BSA) was used as the protein standard.

## 2.5 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli, 1970. The gradient gels, 4-15%, were cast using gradient maker (BRL). Lipophorin samples were extensively dialysed against PBS prior to the electrophoresis. Samples were dissolved in an equal volume of sample buffer (0.13 M Tris-HCl, 20% glycerol, 0.002% bromophenol blue, 4% SDS, 1%  $\beta$ -mercaptoethanol, pH. 6.8) and boiled for three minutes in a water bath, prior to application on to the gel. Electrophoresis was carried out at a constant current of 30 mA at room temperature.

Non-denaturing PAGE was carried out on gradient gels (4-15%) at 4°C with a constant 60V for 20 h. Samples for the electrophoresis were dissolved in an equal volume of non-denaturing sample buffer (0.13M Tris-HCl, 20% glycerol, 0.002% bromophenol blue) before application on to the gel.

After electrophoresis, the gels were stained for proteins with 0.6% Coomassie Brilliant Blue in a solution of acetic acid, methanol and distilled water (in ratios of 9.2:50:40.8, respectively) overnight. The gels were then treated with several changes of destaining solution (acetic acid, methanol, distilled water; 9.2:50:40.8) for 12 - 15 h at room temperature. Destained gels were stored in 7% acetic acid until photographed using Kodak, Panatomic-X films.

## 2.7 MOLECULAR WEIGHTS DETERMINATION

SDS-PAGE was used to determine the molecular weights of the apoproteins using protein standards from Bio-Rad. The protein standards used were: phosphorylase b ( $M_r \sim 97,400$ ), BSA ( $M_r \sim 66,200$ ), ovalbumin ( $M_r \sim 42,699$ ), Bovine carbonic anhydrase ( $M_r \sim 31,000$ ), trypsin inhibitor ( $M_r \sim 21,500$ ), lysozyme ( $M_r \sim 116,000$ ) and myosin ( $M_r \sim 200,000$ ). The native molecular weight of lipophorin was also estimated by non-denaturing - PAGE using high molecular weights standards from Pharmacia; (thyroglobulin, ( $M_r \sim 669,000$ ); ferritin ( $M_r \sim 440,000$ ); catalase ( $M_r \sim 232,000$ ); lactate dehydrogenase ( $M_r \sim 140,000$ ) and BSA ( $M_r \sim 67,000$ ). After staining the gels with Coomassie Brilliant Blue and destaining the molecular weights were determined from plots of log molecular weight versus relative migration of the standards.

The molecular weight of the native lipophorin was also estimated by gel permeation chromatography on a Bio-Gel A.I.5 m column (2.5 x 90 cm). The column was initially equilibrated with PBS, then calibrated with the following standards from Bio-Rad: thyroglobulin ( $M_r \sim 670,000$ ) globulin ( $M_r \sim 158,000$ ), ovalbumin ( $M_r \sim 45,000$ ) and myoglobin ( $M_r \sim 17,000$ ). Elution was done with PBS and the eluate monitored at 280 nm to determine the elution volumes of the standards as well as lipophorin. Blue dextran was used to determine the void volume. The molecular weight of lipophorin was estimated from the plot of elution

volume/void volume versus log molecular weights of the standards.

## 2.8 ISOELECTROFOCUSSING

Isoelectrofocussing was performed on PhastGel IEF (3-9) using the Phast system (Pharmacia). Calibration was done using broad calibration kit, (pI range 3.50 - 9.30). The Pharmalyte carrier ampholytes in the Phast IEF media were prefocussed to generate a stable linear pH gradient. The lipophorin sample and the calibration sample were then applied and focussed using instructions supplied by manufacturer. The gel was automatically stained with Phast Gel Blue R and destained in the development unit of the Phast system. The pI value for lipophorin was obtained from a plot of pI of the standards versus the distance moved from cathode.

## 2.9 AMINO ACID COMPOSITION ANALYSIS

Lipophorin sample for amino acid analysis was dialysed against three changes of PBS, then lyophilized. The lyophilized sample (2.0 mg) was used to determine the amino acid composition on a Spinco analyser (model 120B, Beckman). The sample was hydrolysed for 20 h at 115°C using 6 N HCl/0.05%  $\beta$ -mercaptoethanol. A crystal of phenol was added before the acid hydrolysis. Serine level was increased by 10% and threonine by 5% to compensate for acid destruction.

## 2.10 STOICHIOMETRIC STUDIES ON LIPOPHORIN

Stoichiometry of lipophorin apoproteins was determined by scanning for the apoproteins following SDS-PAGE of increasing amounts of lipophorin (2.5  $\mu$ g - 100  $\mu$ g). The proteins were stained with Coomassie Brilliant Blue prior to the scanning using Hoefer densitometer. The ratio of the apoproteins in a native lipophorin was estimated by comparison of peak areas corresponding to the stained apoproteins during the scan.

## 2.11 ISOLATION OF THE APOPROTEINS

The apoproteins were obtained by electroelution after separation by SDS-PAGE. After staining and destaining a vertical slice of the gel for protein, the gel was cut at parts corresponding to apoLp-I and apoLp-II. The cut gel slices were then transferred to dialysis tubings containing 25 mM Tris, 192 mM glycine buffer, pH 8.3. The apoproteins were subsequently eluted out of the gel slices using horizontal electrophoresis apparatus at 30 mA overnight. The eluted samples were concentrated using polyethylene glycol (PEG) then analysed by SDS-PAGE.

## 2.12 LIMITED TRYPSIN DIGESTION

Lipophorin was subjected to limited trypsin digestion by incubation of 50  $\mu$ g of lipophorin with a constant amount of trypsin at a trypsin:lipophorin weight ratio of 1:5, for

a period of (0-30) min. The reaction was initiated by addition of trypsin in PBS to lipophorin sample and stopped by addition of SDS-PAGE sample buffer and boiling for 5 min in a water bath (Robbs et al, 1985). Samples from various incubation media were subsequently analysed by SDS-PAGE.

## 2.13 ANALYSIS OF THE LIPID MOEITY

### 2.13.1 Staining for lipids

Staining for lipids was done on lipophorin separated by SDS-PAGE using Sudan black (Nayaran, 1975). Sudan black (500 mg) was dissolved in a mixture of 20 ml acetone, 15 ml acetic acid and 80 ml water. The supernatant obtained after centrifugation (500 rpm, 20 min) was used to stain the gel overnight. The gels were destained using acetone: acetic acid: water (30:40:130) solution.

### 2.13.2 Lipid extraction

Lipids were extracted from lipophorin (4.0 mg) by chloroform: methanol (3:1) according to the method of Bligh and Dyer, 1959. Lipophorin sample was mixed with the solvent, vortexed for 10 minutes then centrifuged (10,000 rpm, 15 min) in a Sorvall RC-SC centrifuge. The organic phase was pipetted out and the interface re-extracted. The extracted lipid was dried under N<sub>2</sub> gas then Savant, SpeedVac concentrator overnight. Total lipids were determined gravimetrically using Mettler AE 163 balance.

### 2.13.3 Total phosphorous determination

Lipids extracted from lipophorin were reconstituted in 0.5 ml of chloroform; methanol (3:1), and used to determine total phosphorous (Bartlett, 1959). Potassium dihydrogen phosphate was used as the standard phosphorous in the range 0.5 to 5.0  $\mu\text{g}$ . Samples for the assay were made upto 2.0 ml with distilled water followed by addition of 0.5 ml of 10 N  $\text{H}_2\text{SO}_4$ , then incubation at  $150^\circ\text{C}$  for 3 h. The samples were further incubated for 1.5 h at  $150^\circ\text{C}$  following addition of 2 drops of 30% hydrogen peroxide. After the incubations, 4.6 ml of 0.22% ammonium molybdate and 0.2 ml of Fiske Subba Row reagent (Sigma) was added and samples boiled in water bath for 7 min. Optical densities of the cooled samples were obtained at 830 nm using DU-50 spectrophotometer. The amount of phospholipid was calculated assuming its phosphorous content to be 4% w/w.

### 2.13.4 Thin layer chromatography (TLC)

Neutral lipids were separated from the lipid extract using TLC on polygram Sil G/UV 254 plastic sheets precoated with 0.25 mm Silica gel. A two solvent system was employed for the separation (Skipski and Barclay, 1959). The first solvent system consisting of diethyl ether, benzene, ethanol and acetic acid (40:50:20:0.2) was used to develop the TLC sheets upto three quarters to the top, from the point of application. The sheets were then dried and developed in a

second solvent, diethyl ether: hexane (6:94) upto 0.2 cm from the top.

To identify the various lipids separated, lipid standards were applied onto the sheets together with the samples. The lipid standards used included: monopalmitin, dipalmitin, tripalmitin, cholesterol, cholesteryl oleate, oleic acid, triolein and methyl oleate (Sigma). The detection of lipids was done by iodine vapour.

## 2.14 ANALYSIS OF THE CARBOHYDRATE MOEITY

### 2.14.1 Staining for carbohydrates

The presence of covalently bound carbohydrate was tested by staining lipophorin separated by SDS-PAGE with periodic acid Schiff reagent (PAS) (Kapitany and Zebrowski, 1973). Prior to the staining, the electrophoregram was fixed in 12.5% trichloroacetic acid for 1 h followed by immersion in 1% periodic acid for 2 h. The gel was destained with 7 % acetic acid.

### 2.14.2 Determination of carbohydrate content

Percentage carbohydrate in lipophorin was determined according to the method of Dubois et al, 1956). D-mannose was used as the standard carbohydrate. Samples for the assay were made upto 0.4 ml with water and 10 µl of phenol (2.0 gm in 0.5 ml of distilled water) added. Concentrated



H<sub>2</sub>SO<sub>4</sub> (1.0 ml) was then added to each of the assay mixtures and absorbance determined at 480 nm after 30 min.

### 2.14.3 Affinity chromatography on concanavalin A Sepharose (Con A - Sepharose) column

Con A-Sepharose column (10 x 1.0 cm) was equilibrated with concanavalin A (Con A) buffer (0.01 M Tris, 1 mM MgCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, 0.15M NaCl, 0.02% NaN<sub>3</sub>, pH 7.5). After application of sample (1.0 mg), the column was washed with Con-A-buffer then eluted with 500 mM  $\alpha$ -methyl-D-mannopyranoside in Con A buffer. Fractions (1.0 ml) were obtained and absorbance monitored at 280 nm.

## 2.15 IMMUNOLOGICAL STUDIES

### 2.15.1 Raising of antibodies against lipophorin

Antibodies against lipophorin were raised in Zealand White rabbit. Lipophorin (1.0 mg) emulsified in Freund's complete adjuvant was injected intramuscularly into the rabbit. A booster injection of lipophorin (0.5 mg) in incomplete Freund's adjuvant, was given after four weeks. The animal was bled two weeks later through the main ear artery. The fresh blood was left to stand at room temperature for 1 h for clot formation, then kept overnight at 4°C. The serum was decanted then centrifuged (1000 xg, 30 min). The supernatant was stored at -70°C in 0.1% sodium azide.

### 2.15.2 Double radial immunodiffusion

Double radial immunodiffusion was carried out using 1% agarose in PBS on glass slides (Ouchterlony, 1958). A well was punched at the centre of the glass slides and other wells punched circumferentially around the central well. To detect the presence of antibodies to lipophorin and the apoproteins, the anti-serum was poured in the central well and haemolymph, lipophorin and the apoproteins in the peripheral wells. Haemolymph samples from L. migratoria, E. saccharina, C. partellus, G. morsitan and G. mellonella, were also poured in the peripheral wells to test for cross reactivity with anti-B. fusca lipophorin and anti-M. sexta lipophorin. The slides were placed in moist chamber at room temperature for 24 h. The slides were then washed extensively to remove excess proteins, dried, stained with Coomassie Brilliant Blue and then destained for examination.

### 2.15.3 Immunoblotting

The lipophorin apoproteins were tested for immunological reactivity towards anti-B. fusca lipophorin by immunoblotting (Towbin et al, 1979; Burnette, 1981). Haemolymph samples from L. migratoria, G. mortisan, C. partellus, E. saccharina and G. mellonella were also tested for cross reactivity with anti-B. fusca lipophorin.

Protein samples to be tested, were separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose paper using a transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol) at a constant voltage of 30 volts for 12 h. Following the transfer, the nitrocellulose paper was washed with Tris buffered saline (TBS) [20 mM Tris, 500 mM NaCl, pH 7.5], then blocked overnight with 5% fat-less milk powder in TBS (TBS-milk-5%), to block non specific binding sites. The blot was then incubated with anti-*B. fusca* lipophorin diluted x200 with TBS-milk-1% overnight. The unbound antibodies were washed off with TBS-milk-5% and TBS. Bound antibodies were detected by either horseradish peroxidase labelled goat anti-IgG or gold labelled protein-A (Bio-Rad). Detection of bound antibodies with horseradish peroxidase labelled goat anti-IgG, involved incubation of the blot with the secondary antibody (diluted x2000 with TBS-milk-1%) for 2 h. After washing off the unbound secondary antibodies with TBS-milk-5% and Tris-HCl buffer (2 mM Tris, pH 6.8) [TB], the colour was developed using 0.3% 4-chloro-1-naphthol in methanol (diluted five folds with TB) and hydrogen peroxide. Hydrogen peroxide was added in aliquots (2  $\mu$ l) until the desired colour background was obtained. Detection of the bound antibodies with protein-A gold involved incubation of the blot with protein-A-gold for 2 h.

After the detection of the bound antibodies, the nitrocellulose papers were rinsed with distilled water and kept between filter papers.

## CHAPTER 3

### RESULTS

#### 3.1 PURIFICATION OF LIOPHORIN

The larval B. fusca lipophorin was purified by a single step density gradient ultracentrifugation.

Ultracentrifugation of haemolymph in a KBr gradient (1.03 - 1.30 g/ml) (Shapiro et al, 1984) floated lipophorin on the upper part of the tube. The lipophorin yellow band was fractionated as a peak with a density of 1.13 g/ml (Fig. 1 a). SDS-PAGE profile (Fig. 1 b) of fractions from ultracentrifugation confirmed the fractions containing lipophorin to be 13-21. In the altered KBr gradient (Haunerland and Bowers, 1986), lipophorin floated on top of the tube and was subsequently pipetted out. Lipophorin fractions were pooled then concentrated using polyethylene glycol. Recentrifugation of the isolated lipophorin showed a single protein peak of density 1.13 g/ml (Fig. 2).

The purity of the isolated lipophorin was ascertained by SDS-PAGE and non-denaturing PAGE. In the non-denaturing PAGE (Fig. 3 a) a single protein band was observed whereas in SDS-PAGE (Fig. 4 a), two protein bands were observed indicating that the isolated lipophorin consisted of two apoproteins.

Fig. 1a Density gradient ultracentrifugation profile of haemolymph. Haemolymph was collected and centrifuged as described in Materials and Methods. Fractions (1.0 ml) were collected from the top of the tube and used to determine refractive indices and protein concentration

Density (g/ml):            o—o—o

Protein concentration:    ●—●—o

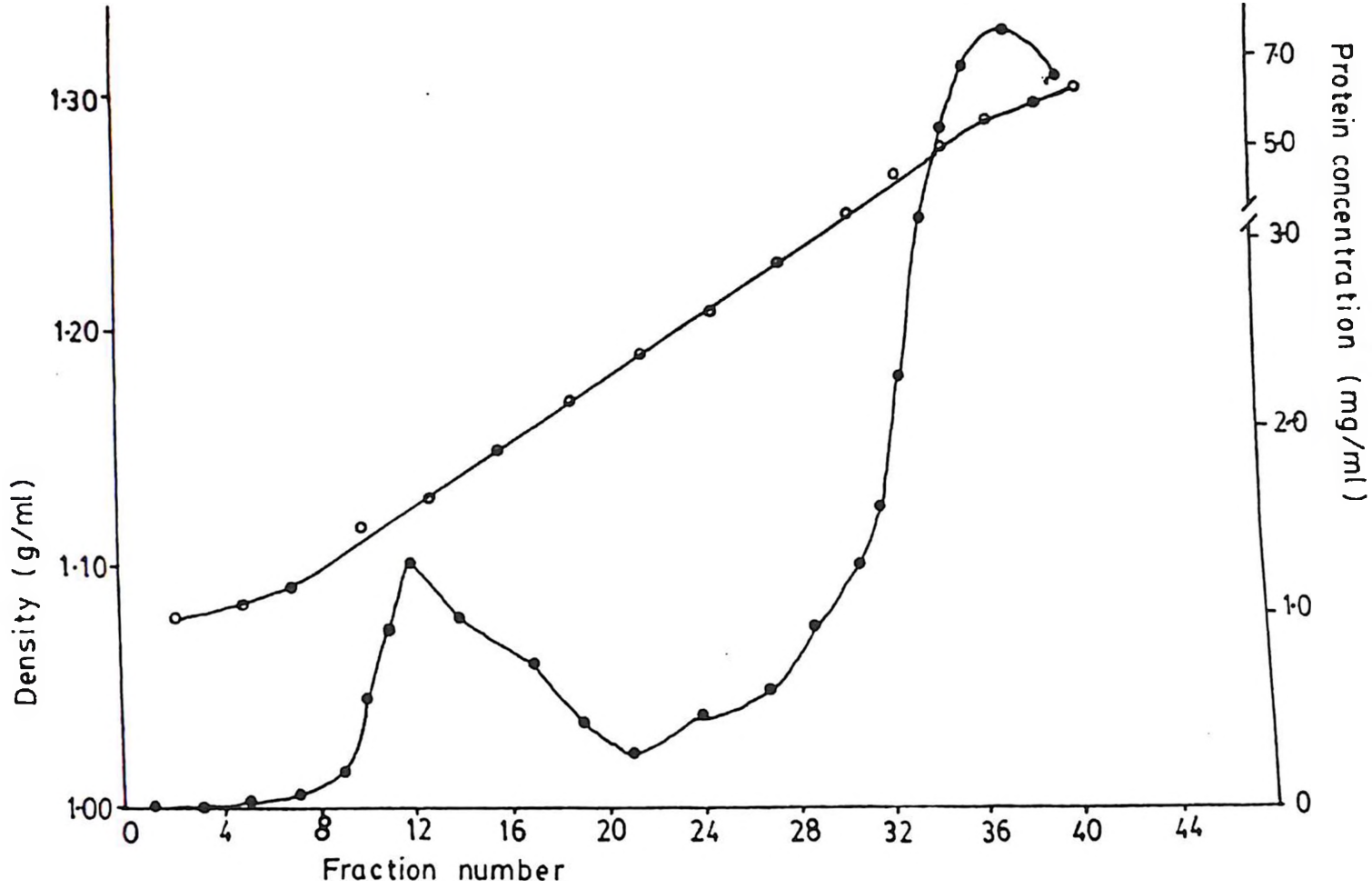


Fig 1b SDS-PAGE of density gradient fractions 100  $\mu$ l of the fractions obtained after the ultracentrifugation of haemolymph was dialysed against PBS and 20  $\mu$ l applied for SDS-PAGE. Numbers on top indicate mls from the top of the centrifuge tube.

3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39





Fig 3a Non-denaturing-PAGE of isolated lipophorin. Lane  
1, Pharmacia high molecular weight standards; lane  
2, isolated lipophorin (20  $\mu$ g).

Fig. 3b Standard curve of log molecular weight against  
relative mobility on (4-15%) non-denaturing  
polyacrylamide gel.

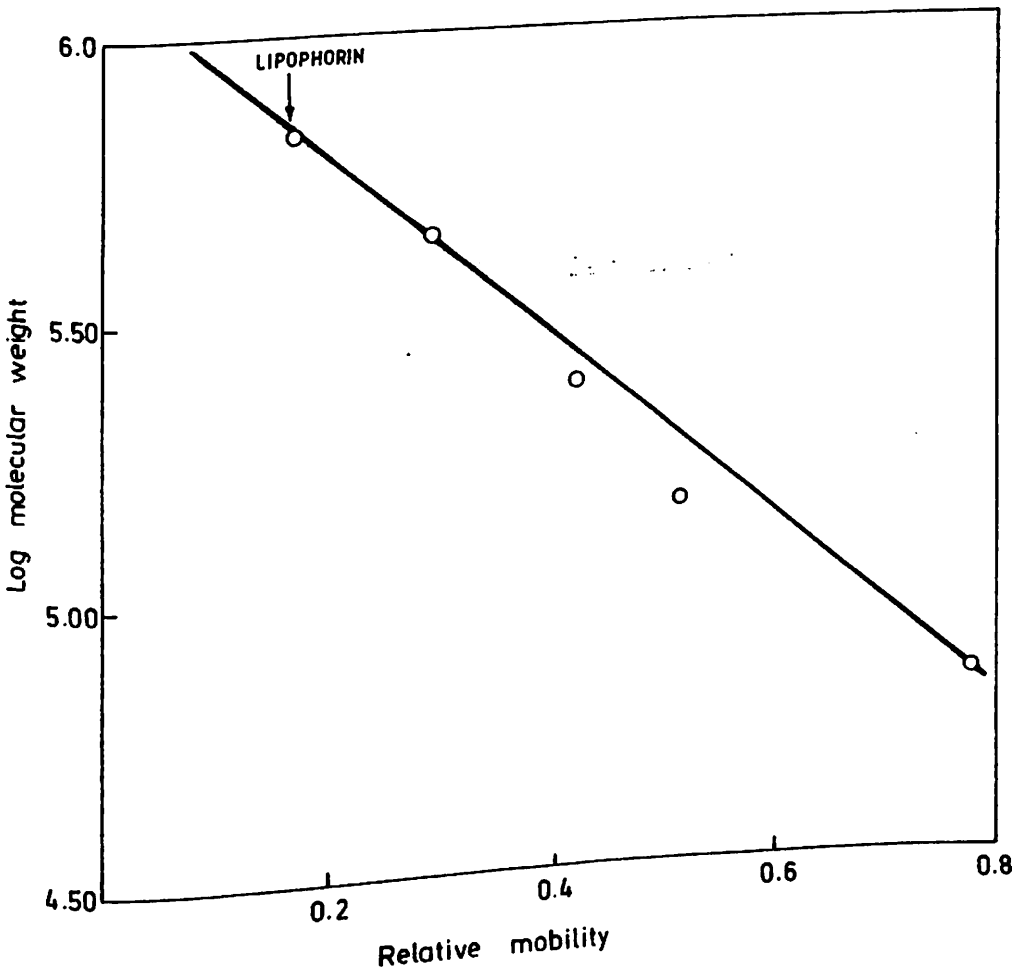
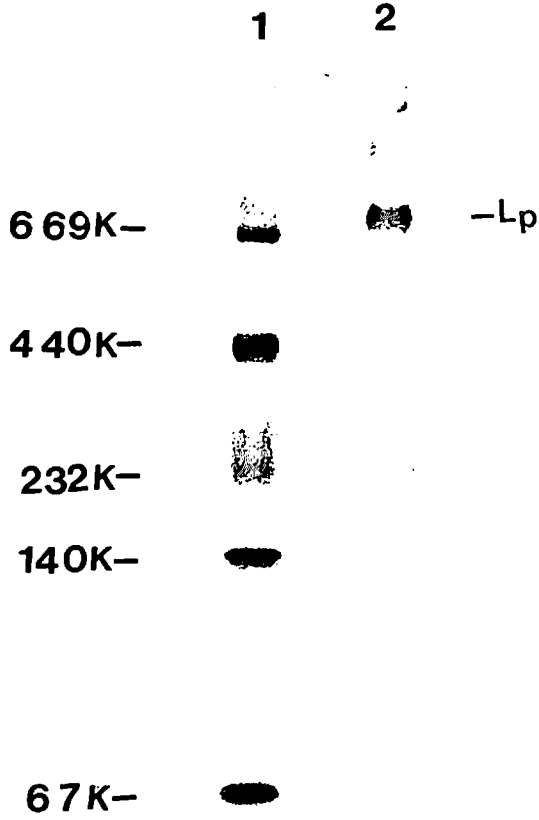
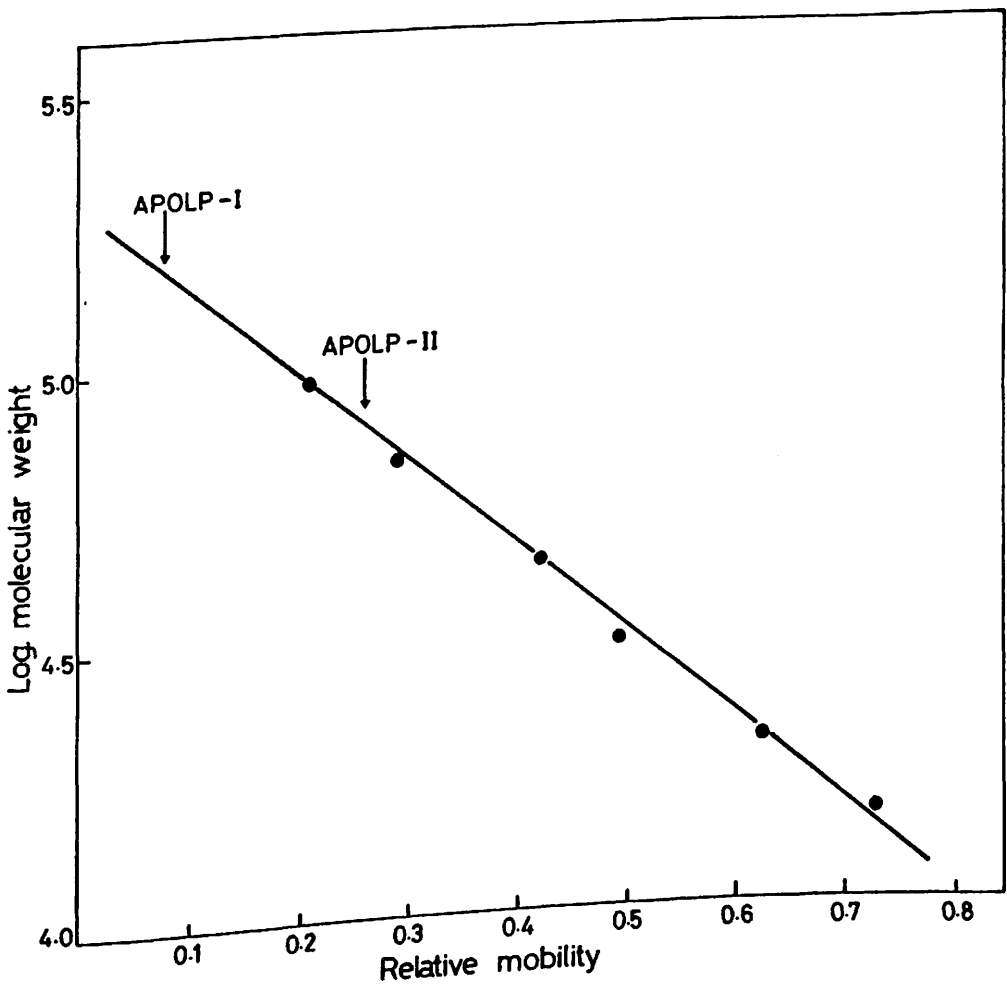
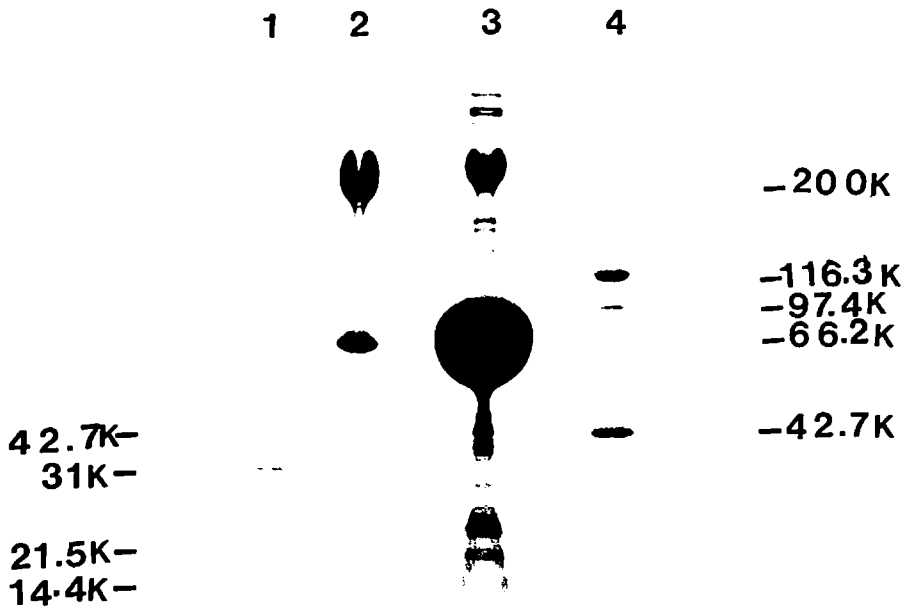


Fig. 4a SDS-PAGE of haemolymph and isolated lipophorin. Samples were subjected to electrophoresis on a 4-15% gradient gels. Lane 1, Bio-Rad low molecular weight standards; Lane 2, Isolated lipophorin (10  $\mu$ g); lane 3, B. fusca haemolymph (20  $\mu$ g); Lane 4, Bio-Rad high molecular weight standards.

Fig. 4b Standard curve of log molecular weight against relative mobility on 4-15% SDS-PAGE polyacrylamide gel.



### 3.2 PROPERTIES AND COMPOSITION OF LIPOPHORIN

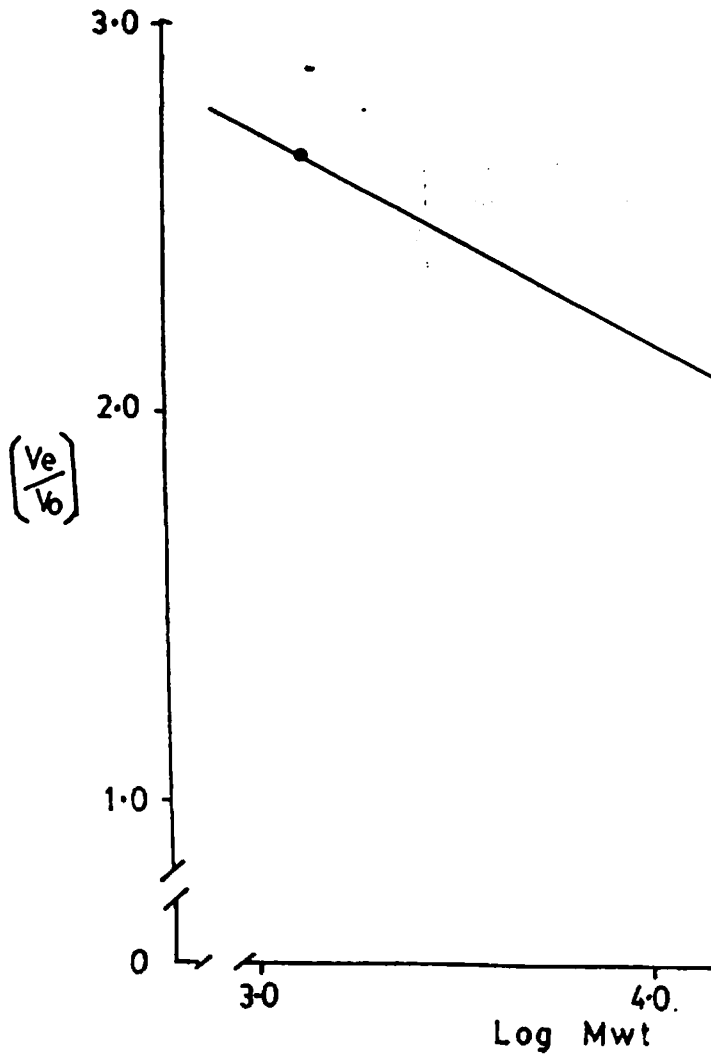
The native molecular weight of lipophorin was estimated by both gel permeation chromatography and non-denaturing PAGE. Gel permeation chromatography on Bio-Gel A.I.5 m column gave an estimate of  $M_r \sim 525,000$  (Fig. 5). Non-denaturing PAGE on the other hand, gave molecular weight estimate of  $M_r \sim 700,000$  (Fig. 3b). Gel permeation chromatography has however been observed to give low molecular weight estimates for some insect proteins (Telfer *et al.*, 1983). Thus the non-denaturing PAGE estimate is more reliable. The pI of the isolated lipophorin was estimated to be 6.3 using Phast-gel IEF-(3-9) (Fig. 6 a,b). The single band observed during isoelectrofocussing further indicated the homogeneity of the isolated lipophorin.

Analysis of lipophorin by SDS-PAGE revealed the presence of two apoproteins apoLp-I ( $M_r \sim 210,000$ ) and apoLp-II ( $M_r \sim 78,000$ ) (Fig. 4 a,b). Densitometric scanning of the apoproteins after SDS-PAGE (Fig. 7) suggested that apoLp-II/apoLp-I weight ratio was (1:1.4), corresponding to a molar ratio of approximately (2:1). If the intact lipophorin had only one apoLp-I and two apoLp-II molecules, the minimum molecular weight would be  $M_r \sim 732,000$ . Thus each lipophorin molecule ( $M_r \sim 700,000$ ) contains two copies of apoLp-II and one copy of apoLp-I.

Fig. 5 Molecular weight determination of lipophorin by gel permeation chromatography on Bio-Gel 1.5 A column. The column was calibrated with protein standards and the eluents monitored at 280 nm.

Lp - lipophorin

Protein standards - 



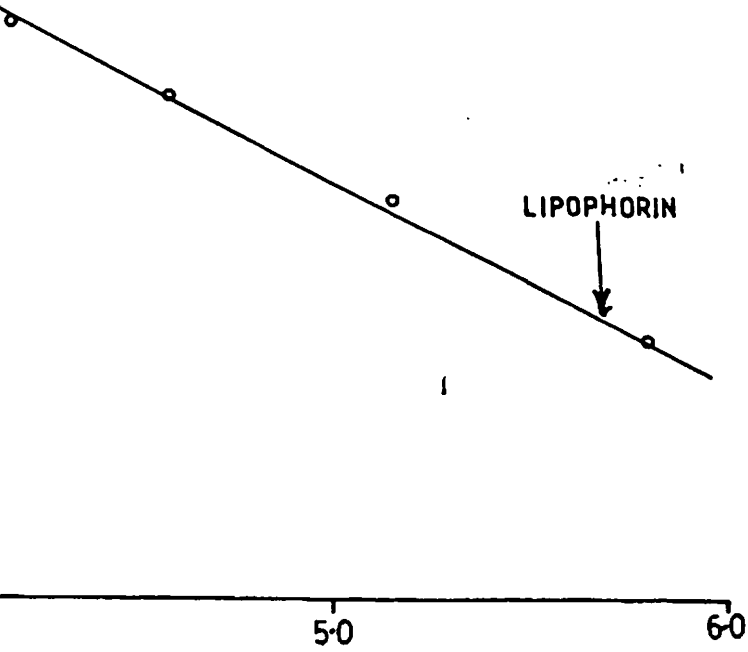
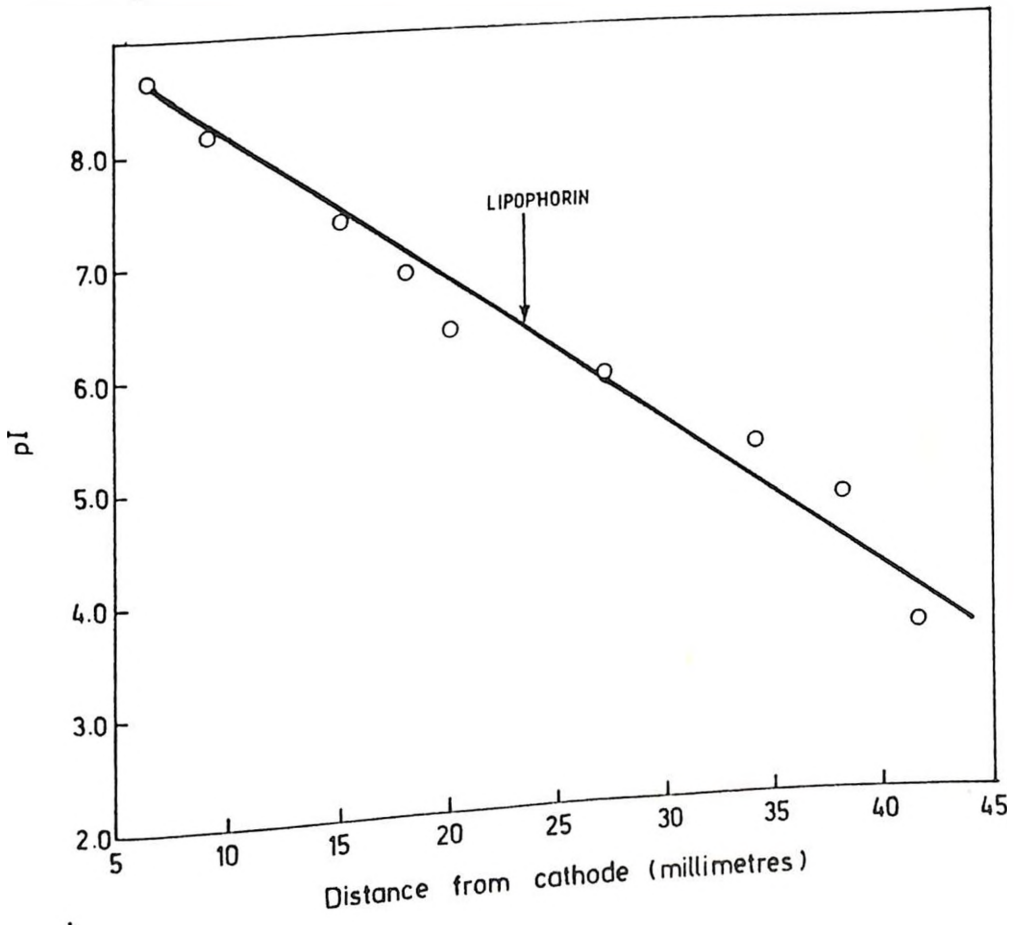




Fig. 6a Isoelectrofocussing pattern of B. fusca lipophorin. Lane 1, IEF calibration sample (2  $\mu$ l), lane 2, lipophorin (2  $\mu$ l). The calibration sample contained: Lentil lectin (basic) (pI  $\sim$ 8.65), lentil lectin (middle) (pI  $\sim$ 8.45), lectil lectin (acidic) (pI  $\sim$ 8.15), horse myoglobin (basic) (pI  $\sim$ 7.35), horse myoglobin (acidic) (pI  $\sim$ 6.85), human carbonic anhydrase (b) (pI  $\sim$ 6.55), bovine carbonic (B) (pI  $\sim$ 5.85), B. lactoglobulin (A) (pI  $\sim$ 5.20), soya bean trypsin inhibitor (pI  $\sim$ 4.55), amylocosidase (pI  $\sim$ 3.50).

Fig. 6b Standard curve of pI against migration distance on Phastgel IEF (3-9). The migration distances were measured from the cathode in millimetres.




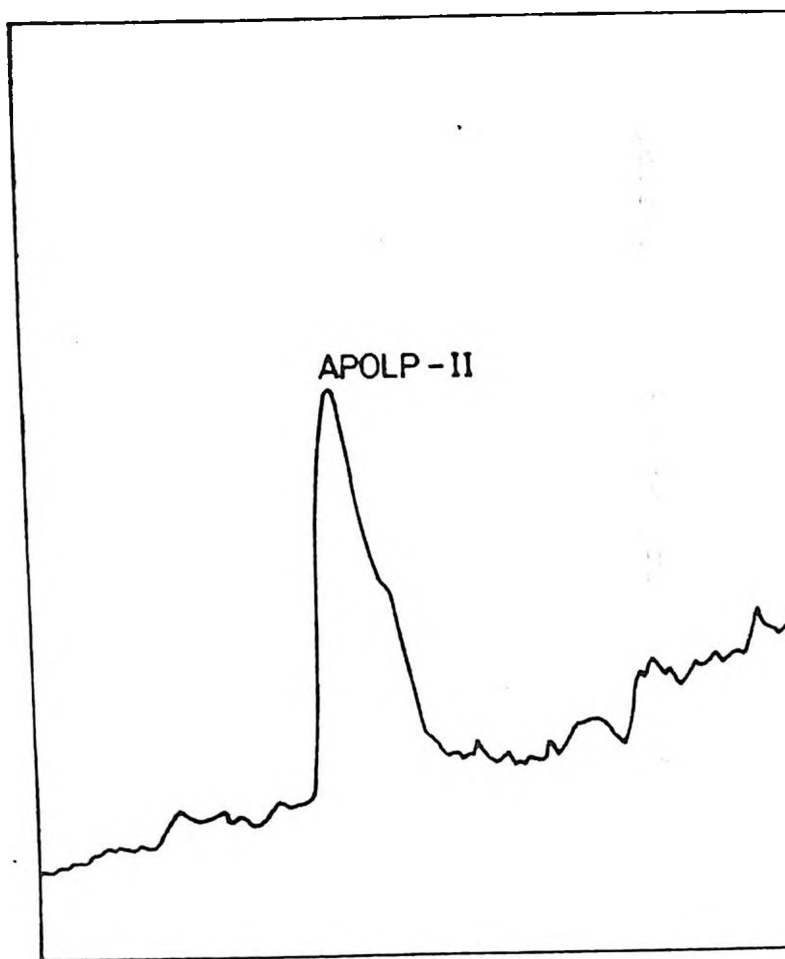
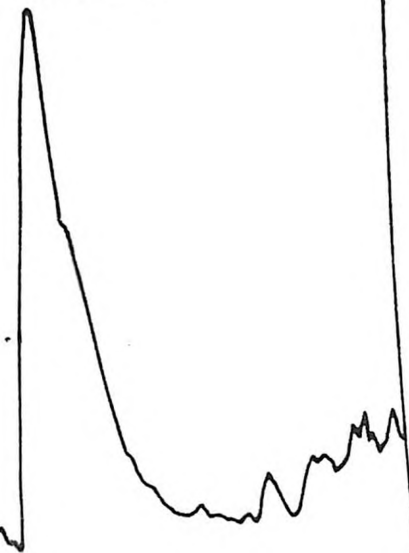
The image shows a faint, high-contrast scan of a gel. There are several horizontal bands of varying intensity, with the most prominent ones appearing as dark, slightly curved lines. The background is light and grainy, typical of a scanned document. The bands are distributed across the upper and middle portions of the page.

Fig. 7 Densitometric scan of lipophorin apoproteins separated by SDS-PAGE.



APOLP-I



The apoproteins were isolated by SDS-PAGE followed by electroelution and their purity ascertained by SDS-PAGE (Fig. 8). No change in molecular weight was observed with the isolated apoproteins.

### 3.2.1 Amino acid composition

Table 1 gives the amino acid composition of B. fusca lipophorin in mole percentages. The results showed a predominance of aspartate (13%), glutamate (9%) and glycine (8.95%). Methionine was only present in trace amounts (0.73%). The aromatic amino acids constituted (7.8%). These results compare favourably with those of larval lipophorins from other insects, M. sexta (Pattnaik et al, 1979); D. abbreviatus (Shapiro, 1988); honey bee, A. mellifera (Robbs et al, 1985).

### 3.2.2 Limited trypsin digestion

Incubation of native lipophorin with bovine trypsin showed that apoLp-I was more accessible to digestion than apoLp-II. However, as apoLp-I was progressively digested, apoLp-II became accessible to the enzyme. The results was as shown in Fig.9.

### 3.2.3 Lipid composition

Lipophorin apoproteins separated by SDS-PAGE both stained with Sudan Black (Fig. 10) indicating presence of lipids on both apoproteins. The extracted lipids from

Fig. 8 SDS-PAGE of apoLp-I and apoLp-II following their isolation. The apoproteins were isolated by SDS-PAGE followed by electroelution. Lane 1, B. fusca haemolymph (3  $\mu$ l); lane 2, isolated lipophorin (10  $\mu$ g); lane 3, apoLp-I (10  $\mu$ g); lane 4, apoLp-II (10  $\mu$ g).

1



2



3



4





TABLE 1 : AMINO ACID COMPOSITION OF LIPOPHORIN

Amino Acid	Mole %
Ala	7.36
Arg	4.27
Asp	13.00
Cys	ND*
Glu	9.03
Gly	8.93
His	2.45
Ile	4.53
Leu	8.34
Lys	8.78
Met	0.73
Phl	4.05
Pro	4.73
Ser	7.57
Thr	5.70
Trp	ND*
Tyr	3.52
Val	7.00
	99.99

\*ND - Not determined.

Fig. 9 SDS-PAGE of lipophorin following limited trypsin digestion in PBS. Lipophorin was incubated with trypsin at a concentration of 1:5 w/w (trypsin:lipophorin) for: lane 1, 30 min; lane 2, 20 min; lane 3, 15 min; lane 4, 10 min; lane 5, 5 min; lane 6, 0 min.

I - apoLp-I

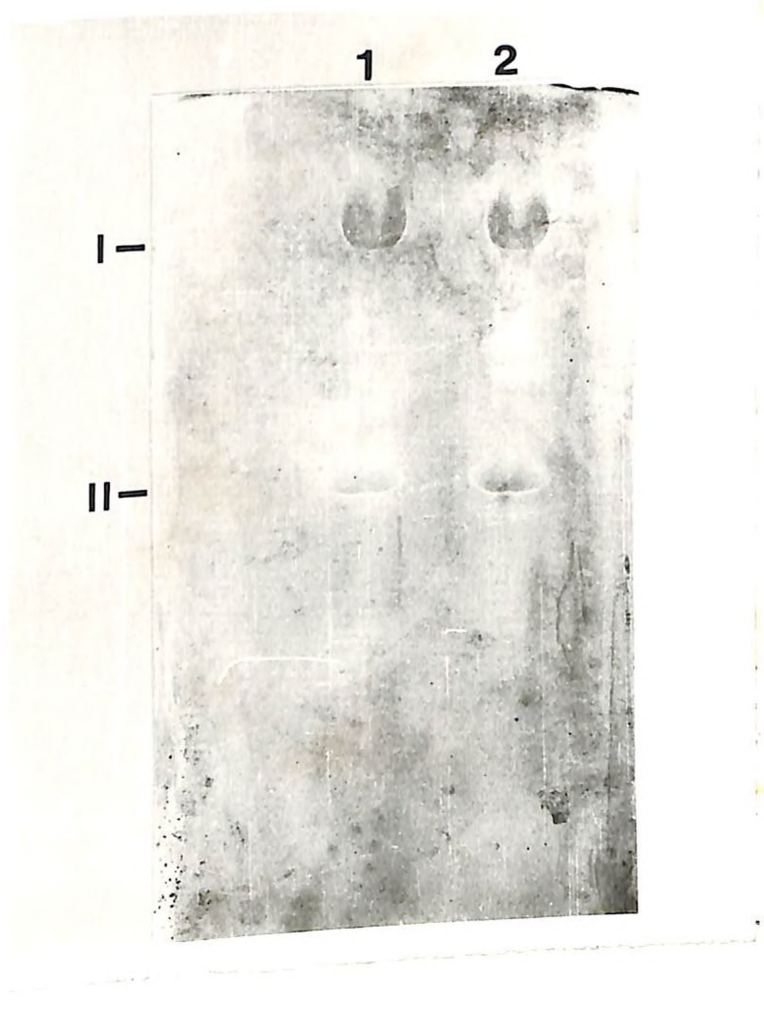
II - apoLp-II



Fig. 10 SDS-PAGE of isolated B. fusca larval lipophorin stained with Sudan Black B for lipids. Lane 1, lipophorin (10  $\mu$ g); lane 2, lipophorin (20  $\mu$ g).

I - apoLp-I

II - apoLp-II



lipophorin according to the method of Bligh and Dyer, 1959 constituted 46% of its dry weight. The phospholipids represented 36.5% of the total lipids as estimated from total inorganic phosphate (Bartlett, 1959) (Fig. 11).

Further analysis of the extracted lipids by TLC revealed the presence of diacylglycerols, cholesterol, triacylglycerols, hydrocarbons and cholesterol esters (Fig. 12). The relative percentages of these lipid classes was not determined. Diacylglycerides were however the most heavily stained by iodine vapour, suggesting predominance among the neutral lipids.

#### 3.2.4 Carbohydrate composition

Periodate Schiff staining of B. fusca lipophorin apoproteins separated by SDS-PAGE (Fig. 13) indicated the presence of covalently bound carbohydrate on both apoproteins (Kapitany and Zebrowski, 1973). Lipophorin was further shown to bind to Con-A-Sepharose (Fig. 14). This binding showed the presence of mannose rich oligosaccharide chains on the protein.

Percentage of carbohydrate on lipophorin was estimated to be 4% according to the phenol-sulphuric acid method of Dubois et al, 1956 (Fig. 15).

Fig. 11 Standard curve for the estimation of inorganic phosphate.

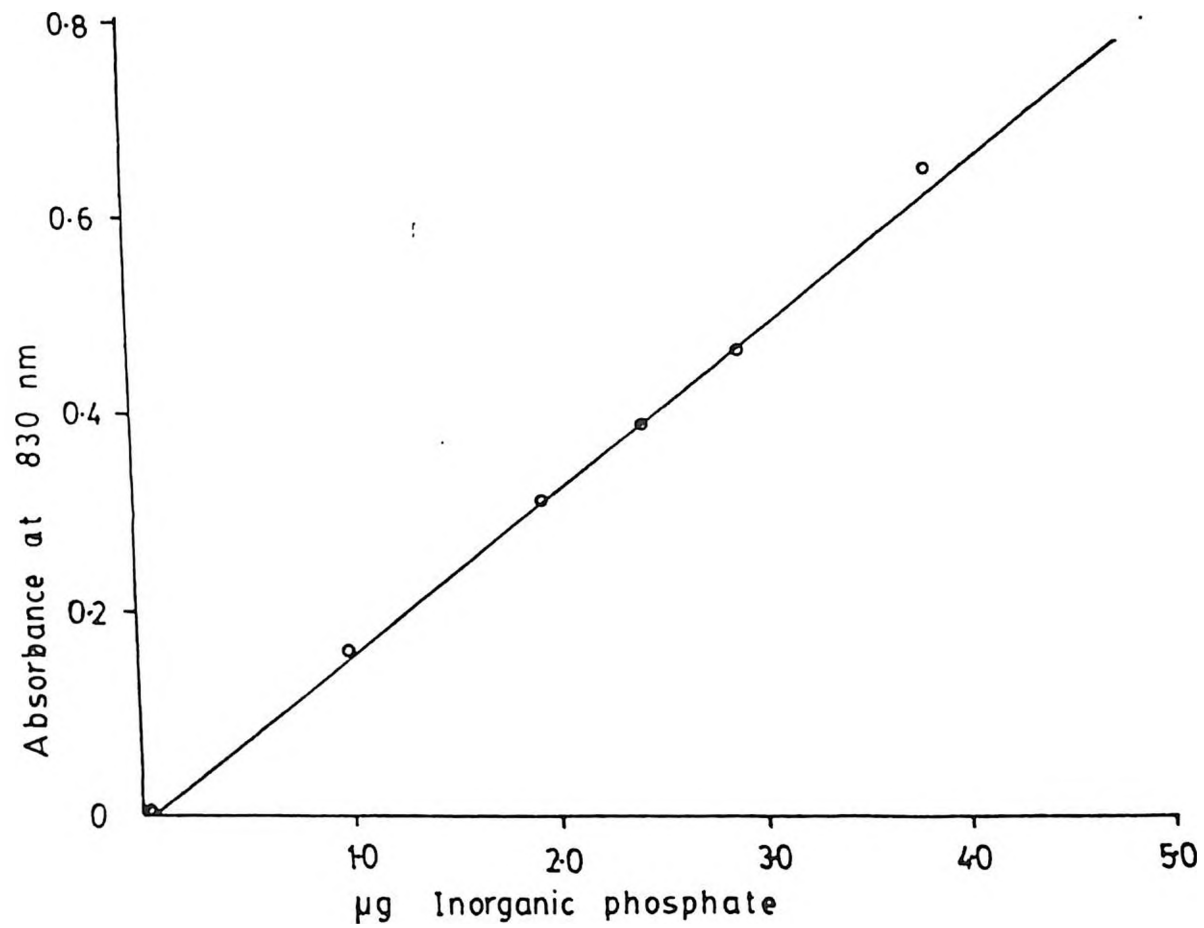




Fig. 12 Separation of neutral lipids extracted from *B. fusca* lipophorin by thin layer chromatography. Lane 1 and 4 reference compounds:MG (monopalmitin), TG (tripalmitin), TRI (triolein), MO (methyl oleate), CE (cholesterol ester), C(Cholesterol), DG(diapalmitin); lane 2, lipid extract; ;lane 3, lipid extract plus the reference compounds.

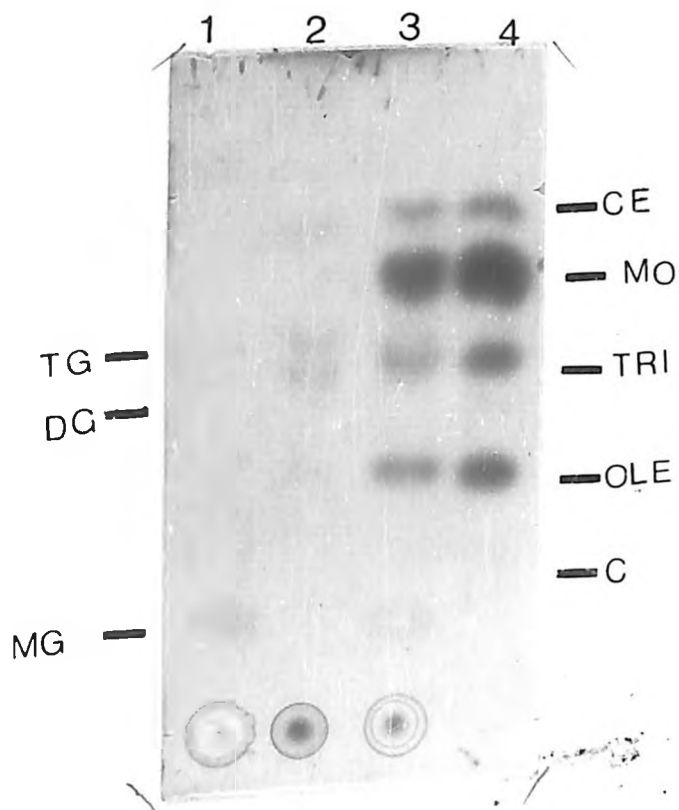


Fig. 13 SDS-PAGE of isolated B. fusca larval lipophorin stained with periodate Schiff reagent for carbohydrates. Lane 1, lipophorin (10  $\mu$ g); lane 2, lipophorin (20  $\mu$ g).

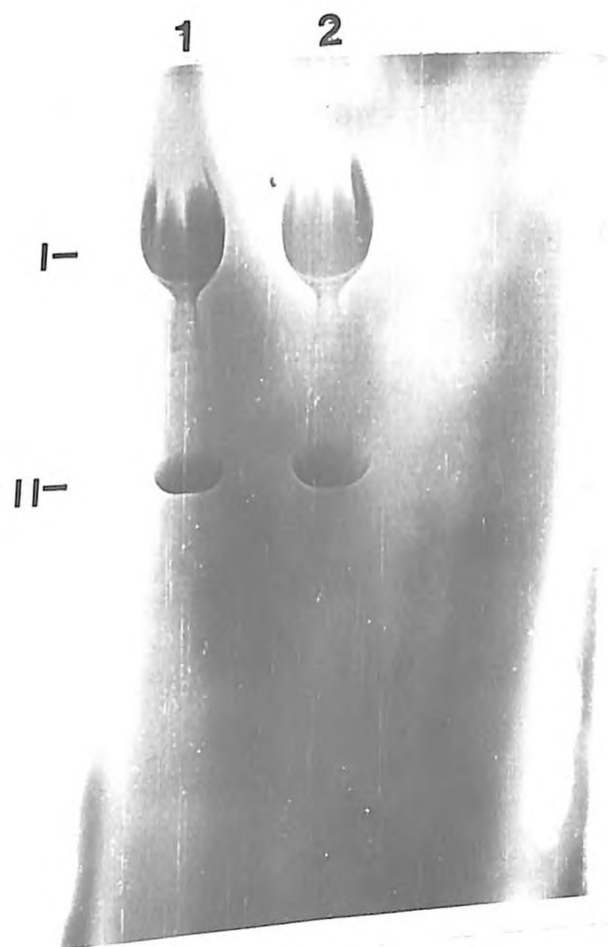


Fig. 14 Elution profile of lipophorin from concanavalin A-Sepharose column. The column (1.0 x 15 cm) was equilibrated with concanavalin A buffer prior to the sample application. Elution was done with 500 mM methyl- $\alpha$ -D-mannopyranoside and fractions (2 ml) monitored at 280 nm.

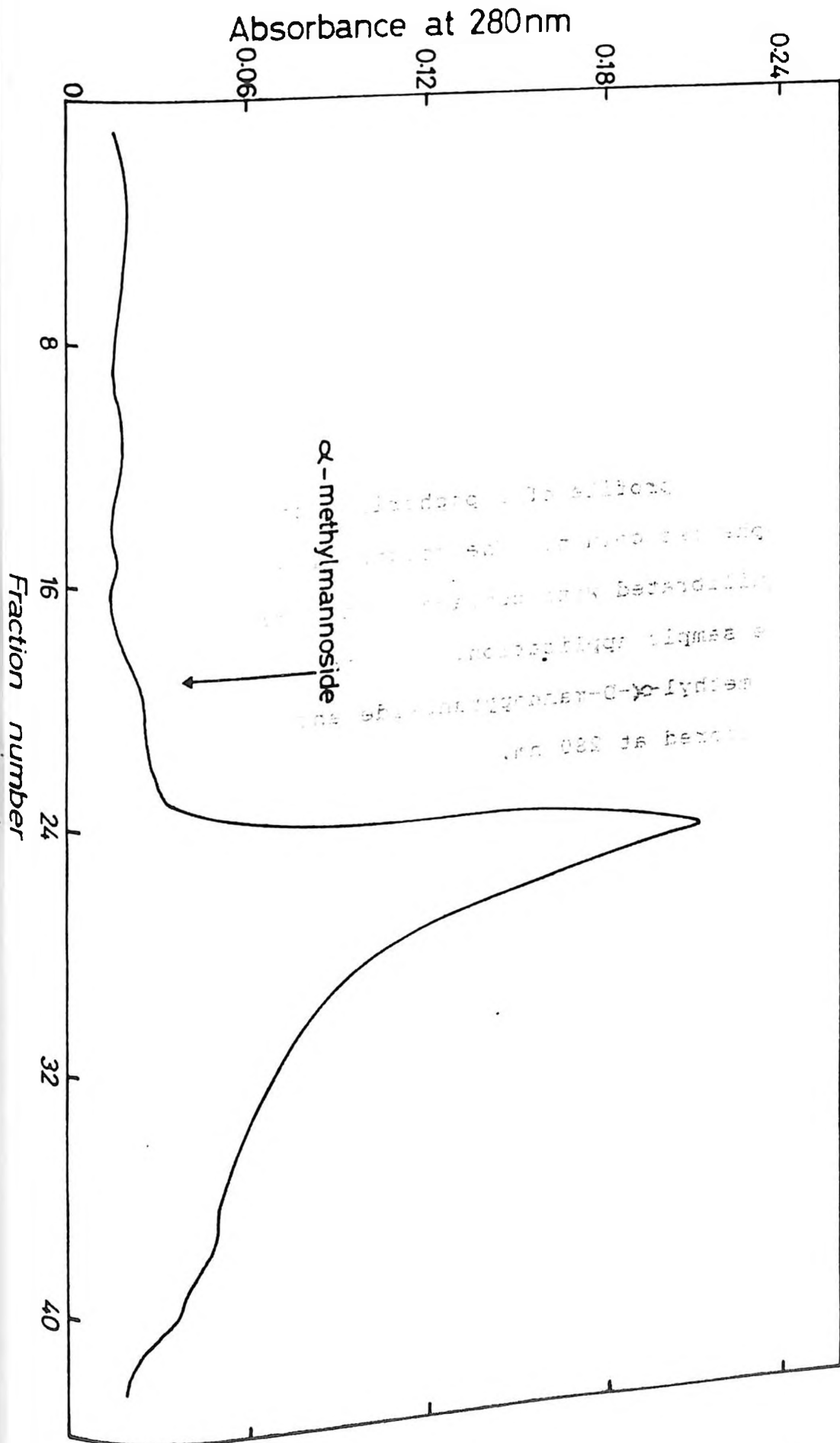
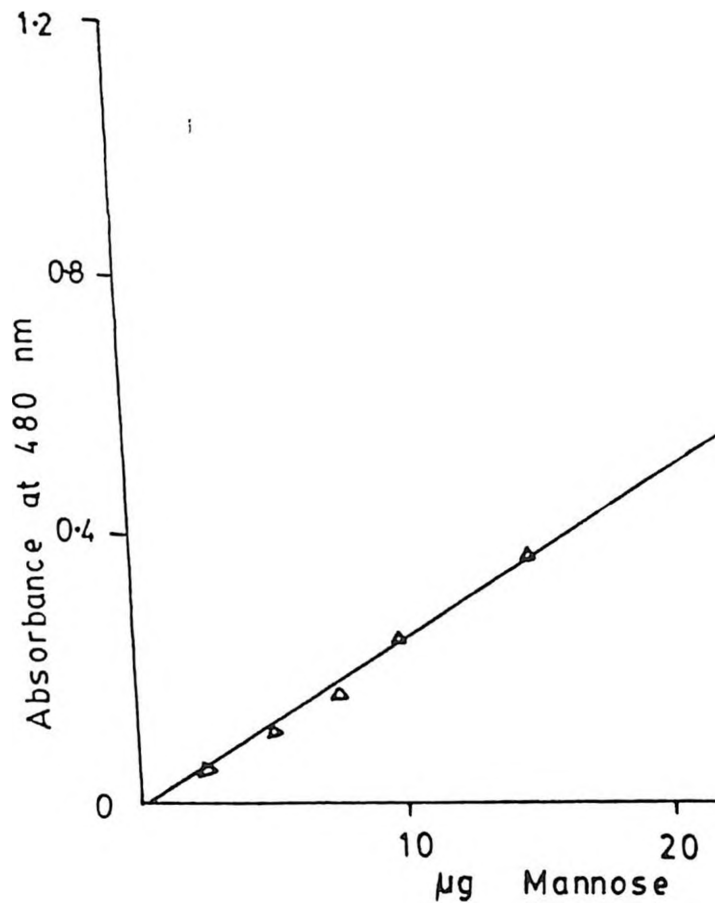
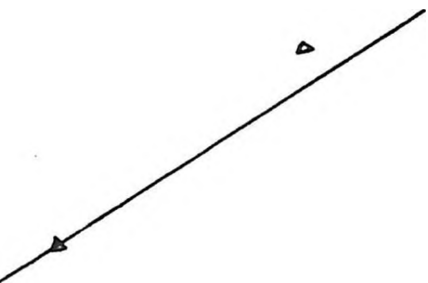


Fig. 15 Standard curve for the estimation of carbohydrate percentage in lipophorin. D-mannose was used the carbohydrate standard.







30

40

### 3.3 IMMUNOLOGICAL STUDIES

#### 3.3.1 Double radial immunodiffusion

Immunodiffusion analysis was carried out using 1% agarose in PBS on glass slides as described in 2.15.2. A positive antibody-antigen reaction was indicated by a precipitin line. Antisera to B. fusca lipophorin reacted with B. fusca haemolymph, the isolated lipophorin and apoLp-I (Fig. 16). The single precipitin line observed for each of the antigens confirmed the specificity of the antisera and hence the purity of the isolated lipophorin. The results also showed that the precipitin lines were lines of identity between haemolymph, lipophorin and apoLp-I indicating the existence of common antigenic determinants. ApoLp-II never showed any detectable reactivity towards the antisera (Fig. 16).

Haemolymph samples from L. migratoria, C. partellus, E. saccharina, G. mellonella and G. mortisan were tested for cross-reactivity with antisera to B. fusca lipophorin by immunodiffusion. Precipitin lines were observed with haemolymph samples from C. partellus, E. saccharina, and G. mellonella. Locusta migratoria and G. mortisan never showed any cross-reactivity (Fig. 17). Similar results were obtained when antisera to M. sexta lipophorin was used (Fig. 18). The precipitin lines were continuous indicating the

Fig. 16 Double radial immunodiffusion of B. fusca lipophorin and the isolated apoproteins against antisera to B. fusca lipophorin. The central well contained antiserum (Ab) whereas the peripheral wells contained: ( 1) lipophorin (10  $\mu$ g), (2) B. fusca haemolymph (5  $\mu$ l), 3) apoLp-I (20  $\mu$ g), 4) apoLp-II (20  $\mu$ g).

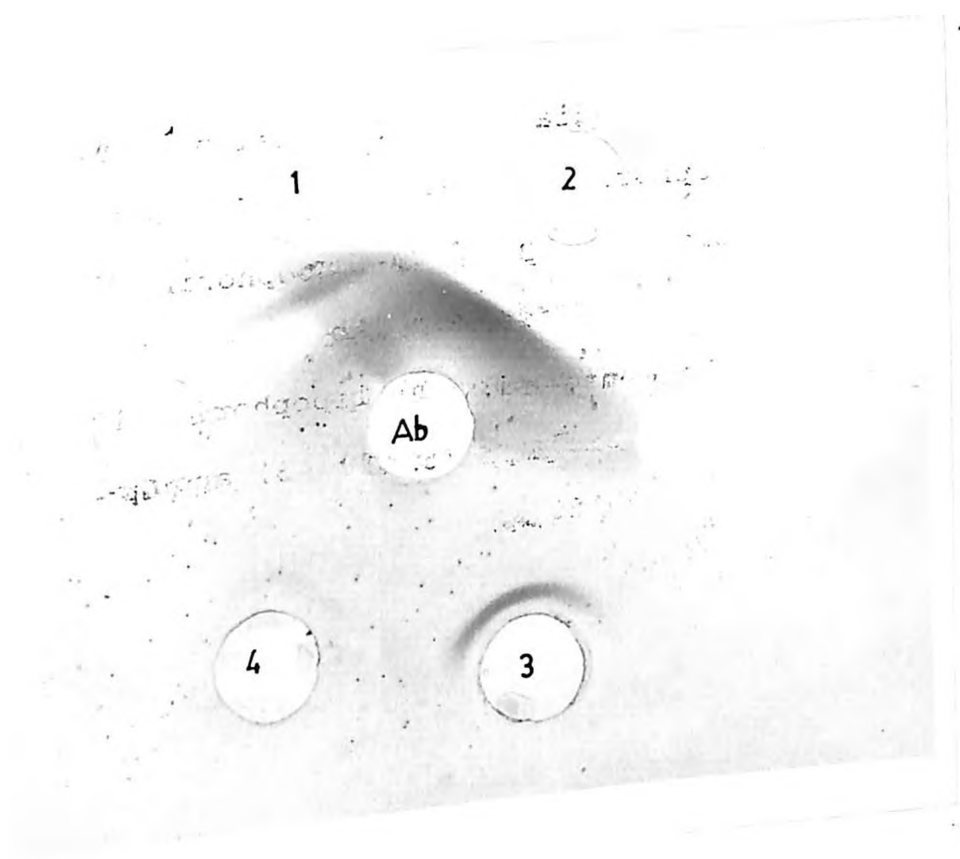
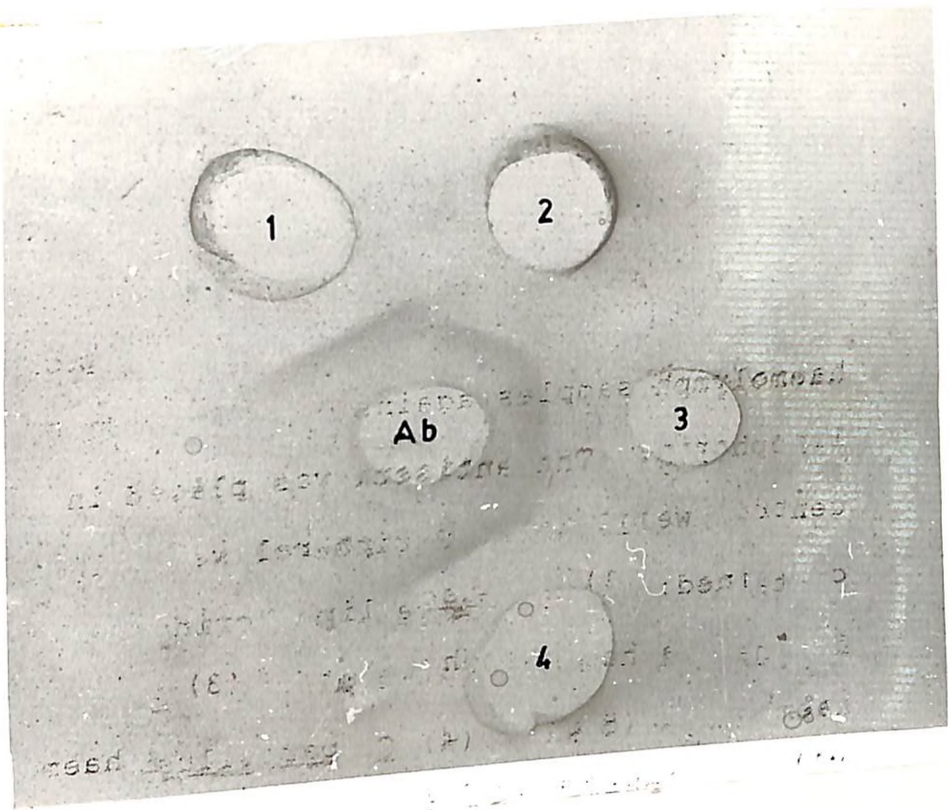


Fig. 17 Double radial immunodiffusion of lipophorin and haemolymph samples against antisera to B. fusca lipophorin. The antisera was placed in the central wells (Ab). Peripheral wells in A contained: (1) B. fusca lipophorin (10  $\mu\text{g}$ ), (2) saccharina haemolymph (5  $\mu\text{l}$ ), (3) G. mellonella haemolymph (5  $\mu\text{l}$ ), (4) C. partellus haemolymph (5  $\mu\text{l}$ ). Peripheral wells in B contained: (1) B. fusca lipophorin (10  $\mu\text{g}$ ), (2) G. mortisan haemolymph (5  $\mu\text{l}$ ), (3) L. migratoria haemolymph (5  $\mu\text{l}$ ).

A



B

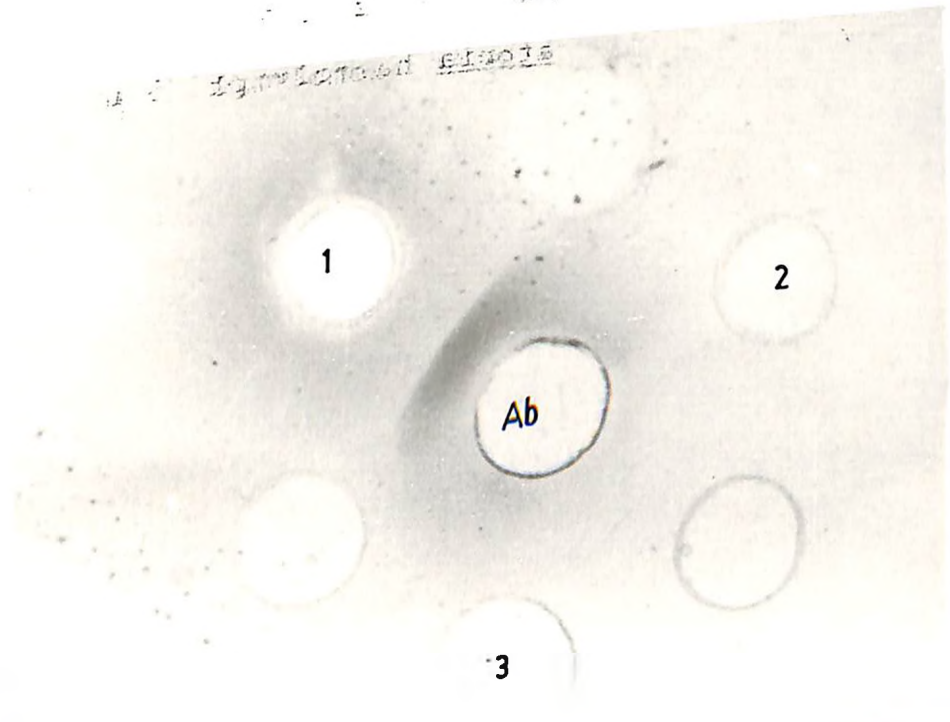
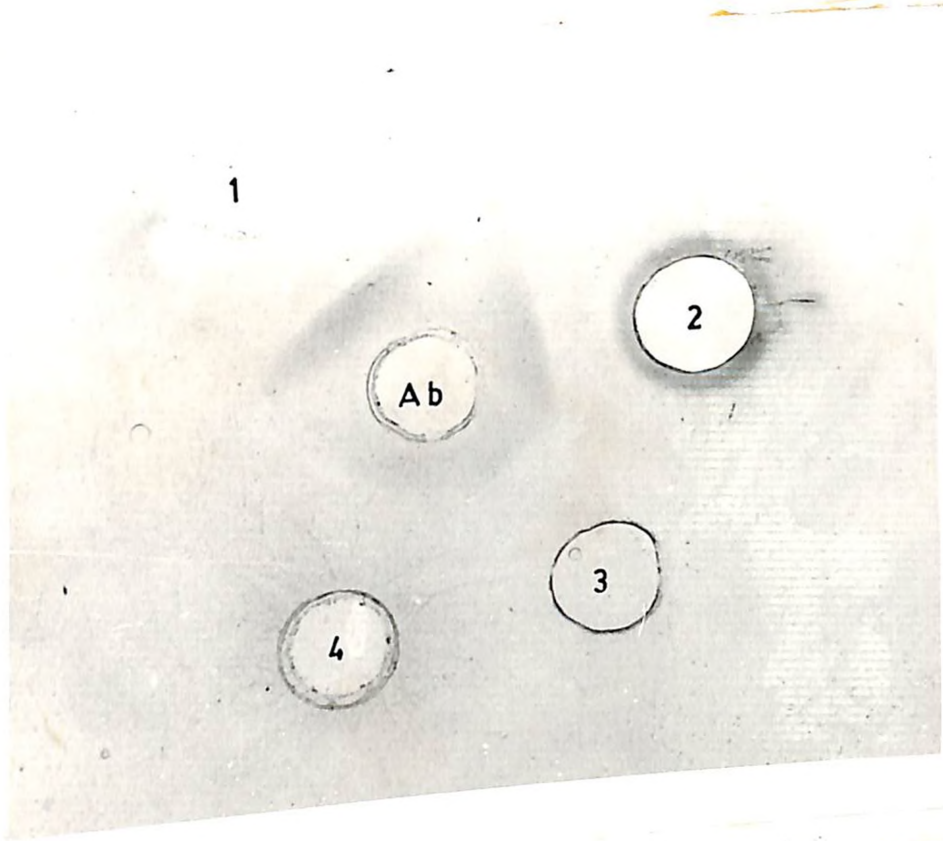


Fig. 18 Double radial immunodiffusion of lipophorin and haemolymph samples against antisera to M. sexta lipophorin. The antisera was placed in the central wells (Ab). Peripheral wells in A contained: (1) B. fusca lipophorin (10  $\mu$ g) (2) partellus haemolymph (5  $\mu$ l), (3) E. saccharina haemolymph (5  $\mu$ l), (4) G. mellonella haemolymph (5  $\mu$ l). Peripheral wells in B contained: (1) B. fusca lipophorin (10  $\mu$ g) (2) G. mortisan haemolymph (5  $\mu$ l) (3) L. migratoria haemolymph (5  $\mu$ l).

A



B:





presence of common antigenic determinants in C. paterillus, E. saccharina; B. fusca and G. mellonella.

### 3.3.2 Immunoblotting studies

The immunological reactivity of apoproteins separated by SDS-PAGE was tested by immunoblotting. The separated apoproteins were transferred onto nitrocellulose paper and then incubated with antisera against lipophorin. Bound antibodies were detected using protein-A gold (Pierce) (Fig. 19). The results showed that both apoproteins were immunoreactive towards anti-lipophorin.

Haemolymph samples from L. migratoria, G. mortisan, C. partellus, E. saccharina and G. mellonella were separated by SDS-PAGE then transferred onto nitrocellulose. The transferred proteins were tested for cross-reactivity with antibodies to B. fusca lipophorin. The nitrocellulose paper was incubated with the antisera, and the bound antibodies detected using radish peroxidase labelled goat anti-rabbit IgG (Fig. 20). Haemolymph samples from C. partellus, E. saccharina, and G. mellonella cross reacted with anti-lipophorin. Samples from L. migratoria, and G. mortisan did not show any cross-reactivity.

The **CROSS** reactivity was observed with protein bands corresponding to apoLp-I and apoLp-II of B. fusca lipophorin. The protein bands corresponding to apoLp-II however, showed a much lower reactivity as compared to

Fig. 19 Immunoblot test on B. fusca haemolymph and lipophorin separated by SDS-PAGE using antisera to B. fusca lipophorin. The separated proteins were electrophoretically transferred onto nitrocellulose paper then incubated with antisera to B. fusca lipophorin. The bound antibodies were detected by protein-A-gold. Lane 1, B. fusca haemolymph (10  $\mu$ g); lane 2, lipophorin (5  $\mu$ g).

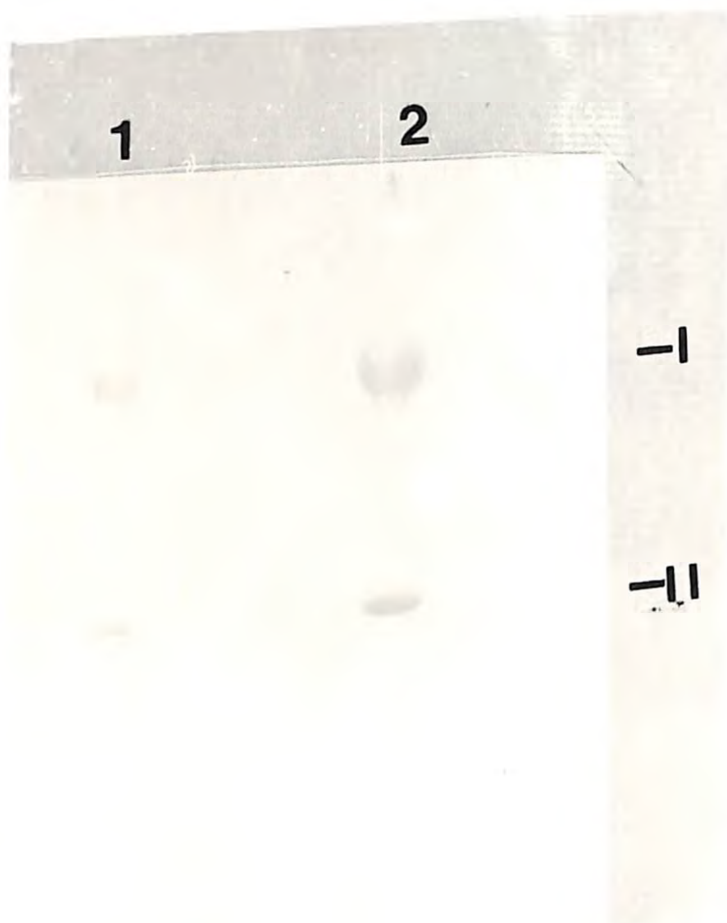
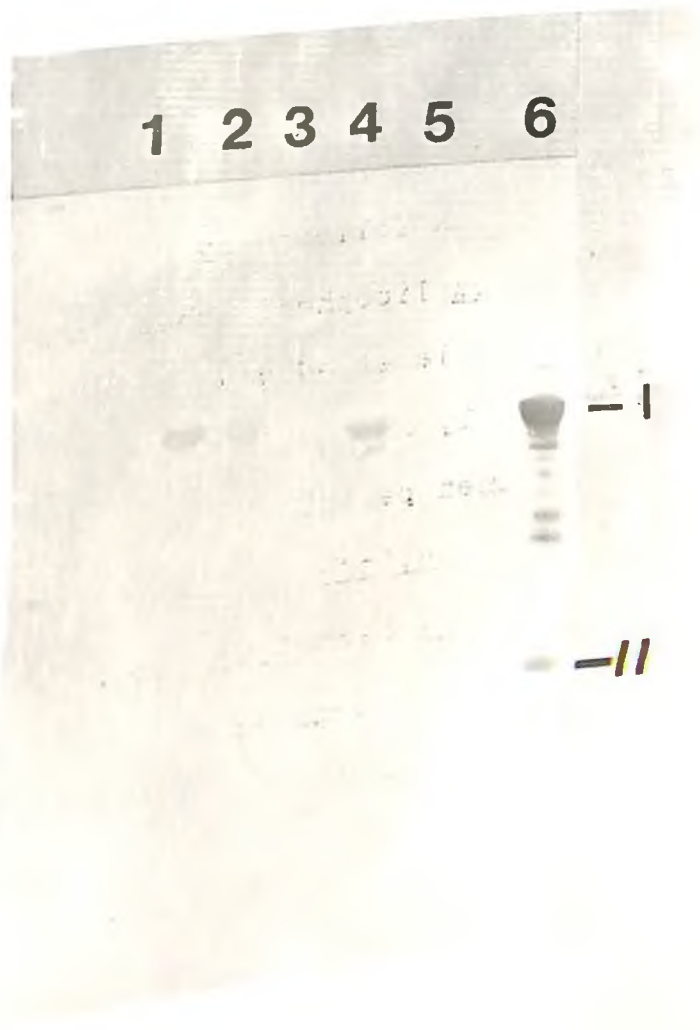


Fig. 20

Immunoblot tests on haemolymph samples separated by SDS-PAGE using antisera to B. fusca lipophorin. The separated proteins were electrophoretically transferred onto nitrocellulose paper. The transfer was followed by incubation with antisera to B. fusca lipophorin then horse radish peroxidase labelled goat anti-IgG. The blots were developed by incubation with 4-chloro-1-naphthol and hydrogen peroxide.

Lane 1, G. mellonella haemolymph (5  $\mu\text{g}$ ); lane 2, E. saccharina haemolymph (5  $\mu\text{g}$ ); lane 3, G. mortisan haemolymph (5  $\mu\text{g}$ ); lane 4, C. partellus haemolymph (5  $\mu\text{g}$ ); lane 5, L. migratoria haemolymph (5  $\mu\text{g}$ ); lane 6, B. fusca lipophorin (5  $\mu\text{g}$ ).



apoLp-I. The results suggested that insects of the same order share antigenic determinants on both apoproteins, but predominantly on apoLp-I. The bands which appear between apoLp-I and apoLp-II in lane 6 of figure 20 are presumably degradation products of apoLp-I, since the B. fusca lipophorin was used after a long time of storage.

## CHAPTER 4

DISCUSSION

Lipophorin is the major haemolymph lipoprotein found in all developmental stages of most insects (Chino et al, 1981). The lipoprotein is composed of a basic structure consisting of apoLp-I ( $M_r$  250,000) apoLp-II ( $M_r$  80,000) and lipids (Chino, 1985, Shapiro et al, 1988). Various distinct interconvertible forms of lipophorin, from HDLp to LDLp occur in the haemolymph. The various forms arise from alteration in lipid content and composition of the basic structure (Ryan et al, 1987).

In vitro studies with larvae of M. sexta (Prasad et al, 1986 a) and D. grandiosella (Venkatesh and Chipperndale, 1986; Venkatesh et al, 1987) has demonstrated that lipophorin is biosynthesized in the fat body. In M. sexta larvae, the fat body secretes into the haemolymph, a nascent VHDLp particle containing apoLp-I, apoLp-II, phospholipids and very little diacylglycerols. Formation of mature lipophorin involves the uptake of diacylglycerol derived from dietary lipids in the mid gut (Ryan et al, 1986 a).

Lipophorin functions as a reusable shuttle in the transportation of polar and non polar lipids from sites of storage, absorption or synthesis to sites of utilization (Chino, 1985, Chino et al, 1985; Katase and Chino, 1982; 1984). A lipid transfer protein (LTP) has been implicated

as enhancing transfer of lipids between the tissues (Ryan et al, 1987). In adult L. migratoria, AKH released at the onset of flight stimulate the mobilization of diacylglycerol from the fat body (Goldsworthy et al, 1985; Wheeler and Goldsworthy 1983,a; Van Heusden et al, 1984). The released diacylglycerol is loaded onto HDLp-A, which is subsequently transformed into LDLp. More apoLp-III molecules also associate with LDLp (Van der Horst et al, 1981; 1984; Wheeler and Goldsworthy, 1983a). At flight muscle, diacylglycerols are released and excess apoLp-III dissociated, resulting into HDLp-A. HDLp-A can then bind more diacylglycerols from the fat body (Van der Horst et al, 1979; Mwangi and Goldsworthy, 1977; Van Heusden et al 1987). Synthetic AKH injected into adult M. sexta has been shown to evoke similar effects as in L. migratoria (Shapiro and Law, 1983).

Several methods have been employed in the purification of insect lipophorins. The methods include: affinity chromatography (Gellissen and Emmerich, 1980), repeated gel filtrations (Mwangi and Goldsworthy 1977; Van Der Horst, 1979), precipitation by cold ethanol and sucrose density centrifugation (Thomas and Gilbert, 1968; Pattnaik et al, 1979; Thomas K, 1979). A technique involving DEAE Cellulose column chromatography has also been used in the purification (Chino et al, 1969; 1981; Chino and Kitazawa, 1981) Ultracentrifugation has long been used for preparative purification of lipoproteins and has been used in the



isolation of mammalian serum lipoproteins including, chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) (Havel et al, 1980; Hamilton and Kayden, 1975). Several insect lipophorins have also been purified by density gradient ultracentrifugation (Ryan et al, 1984; Shapiro et al, 1984; Haunerland and Bowers, 1986; Shapiro et al, 1988). Haunerland et al, 1987 have also shown that ultracentrifugation can be used to separate VHDL from HDL. The separation requires a two step density gradient ultracentrifugation employing different gradient systems. The purification of lipoproteins by ultracentrifugation has been greatly enhanced by use of vertical rotors, which require a much shorter time to reach the equilibrium unlike the swinging bucket rotors.

In this study, *B. fusca* larval lipophorin, has been purified by potassium bromide density gradient ultracentrifugation using a vertical rotor (See Section 3.1). The purification procedure is efficient and fairly rapid (4 h). The homogeneity of the purified lipophorin was demonstrated by polyacrylamide gel electrophoresis, isoelectrofocussing and by immunodiffusion analysis.

The results presented in sections 3.1 and 3.2 clearly show that the isolated larval *B. fusca* lipophorin ( $M_r \sim 700,000$ ) is a high density ( $d = 1.13 \text{ g/ml}$ ) glycolipoprotein, consisting of 46% lipid, 4% carbohydrate

and 50% protein. It is composed of two apoproteins, apoLp-I ( $M_r \sim 210,000$ ) and apoLp-II ( $M_r \sim 78,000$ ) which are both glycosylated with mannose rich oligosaccharide chains. In the intact lipophorin, one molecule of apoLp-I and two molecules of apoLp-II are present in the basic matrix structure. Amino acid composition analysis of the lipophorin showed predominance of glutamate, aspartate and glycine, with methionine present only in trace amounts. The lipid moiety was mainly composed of phospholipids (36% of total lipids) and diacylglycerol.

Limited trypsin digestion followed by SDS-PAGE revealed that apoLp-I of *B. fusca* lipophorin was more susceptible to digestion than apoLp-II. Thus apoLp-I appears to be on the surface of the lipoprotein in contrast to apoLp-II in the interior.

The above properties of *B. fusca* lipophorin indicated that the larval lipophorin shared several properties with other lipophorins so far studied (Shapiro *et al.*, 1988; Chino, 1985). The native molecular weights of insect lipophorins range from ( $M_r \sim 500,000$  to  $M_r \sim 800,000$ ) (Chino, 1985, Pattnaik *et al.*, 1979), whereas the apoprotein average molecular weights are  $M_r \sim 250,000$ ,  $M_r \sim 80,000$ ,  $M_r \sim 20,000$  for apoLp-I, apoLp-II and apoLp-III respectively (Shapiro *et al.*, 1988). Shapiro *et al.*, 1984, estimated a molar ratio of 1:1 for apoLp-I: apoLp-II in *M. sexta* lipophorin. The larval *B. fusca* lipophorin ( $M_r \sim 700,000$ ) thus has a native molecular

weight comparable to other insect lipophorins. The apoproteins apoLp-I ( $M_r \sim 210,000$ ) and apoLp-II ( $M_r \sim 78,000$ ), present in the intact lipophorin in a molar ratio of (1:2), however have much lower molecular weights than those of other insects lipophorins. Lipophorin isolated from D. abbrevialis has also been shown to possess apoproteins with comparatively low molecular weights: apoLp-I ( $M_r \sim 226,000$ ), apoLp-II ( $M_r \sim 72,000$ ) (Shapiro, 1988).

The isolated high density lipophorin from B. fusca exhibited a density of 1.13 g/ml, comparable to other larval lipophorins from M. sexta (1.15 g/ml) (Shapiro et al, 1984), Apis mellifera (1.13) (Robbs et al, 1985), South western corn borer (1.11 g/ml) (Dillwith et al, 1986), and D. abbreviatus (1.08 g/ml) (Shapiro, 1988).

As with other lipophorins, B. fusca lipophorin is a glycoprotein. The carbohydrate moiety (4%) showed presence of mannose rich oligosaccharide chains with the glycosylation being uniformly distributed between apoLp-I and apoLp-II, as judged by PAS-staining of apoproteins separated by SDS-PAGE. A study of lipophorins from eight insect species representing seven insect orders showed that all species possessed fluorescein isothiocyanate-conjugated concanavalin A (FITC-ConA) sensitive carbohydrate residue in at least one of the apoproteins (Ryan et al, 1984). In L. migratoria and P. americana, the glycosylation has been shown to involve mannose and glucosamine (Chino et al, 1981;

Chino and Kitazawa, 1981). Pattnaik et al, 1979, has also reported the presence of galactosamine in addition to mannose and glucosamine in M. sexta lipophorin.

In this study, the presence of mannose rich carbohydrate was shown by the binding of the lipophorin onto Con A-Sepharose column. The mannose rich carbohydrate could either be on apoLp-I, apoLp-II or both (See Section 3.2.4).

The lipid moiety of insect lipophorins have been studied in several insects. In HDLp lipid constitute between (52 - 35%) by weight, whereas in LDLp, it constitutes upto 60% (Shapiro et al, 1988; Chino, 1985). Phospholipids and diacylglycerols are the predominant lipids with triacylglycerols present only in trace amounts. Hydrocarbons and cholesterol are also present (Shapiro et al, 1988; Chino, 1985). In B. fusca lipophorin, the predominant lipids were phospholipids and diacylglycerols. Other lipids present included: cholesterol, triacylglycerol, hydrocarbons and cholesterol ester. Deep yellow colouration of B. fusca lipophorin also suggested presence of carotenoid pigments. Similar pigmentation has been observed in lipophorins from silkworm, locust (Chino et al, 1969), Peled and Tietz, 1975) and M. sexta (Pattnaik et al, 1979).

Structural organization of lipophorin has been investigated in several insects (Mundall et al, 1980); Pattnaik et al, 1979; Shapiro et al, 1984; Robbs et al, 1985). The results seems to suggest an interior location of

apoLp-II as apoLp-I coats the surface of lipophorin particle. Evidence for the proposed structural organization has come from: limited trypsin digestion, iodination and immunological studies. In the native lipophorin molecule, apoLp-I is more readily cleaved by trypsin and iodinated than apoLp-II. Antibodies against lipophorin also react with apoLp-I, but only mildly with apoLp-II. Limited trypsin digestion of *B. fusca* lipophorin indicated that apoLp-I was more susceptible to trypsin cleavage than apoLp-II. Thus it appears that the structural organization of lipophorin is conserved among the insects.

Immunological studies of insect lipophorins have shown that the apoproteins, apoLp-I, II and III are immunologically distinct, hence are non homologous (Shapiro et al, 1984; Kawooya et al, 1984). Immunoblotting with monoclonal antibodies raised against locust lipophorin apoproteins has also demonstrated that the apoproteins are non homologous (Van der Horst et al, 1987).

Immunological cross-reactivity of antibodies to lipophorins and the apoproteins with lipophorins from other insects has previously been studied. Chino and Kitazawa, 1981 have shown by immunodiffusion that lipophorins from *H. cecropia*, *P. americana* and *L. migratoria* are immunologically distinguishable. Anti *M. sexta* lipophorin has also been shown not to cross react with lipophorin from *A. mellifera* (Robbs et al, 1985). Immunoblotting with antibodies to



apoproteins from *M. sexta* lipophorin, has clearly demonstrated that anti-apoLp-II cross reacts with apoLp-II in all seven insect orders tested (Ryan et al, 1984). However, no cross reactivity has been observed between monoclonal antibodies to locust apoproteins and lipophorin from *P. americana*, *Deilephila alpenor* or *Leptinotarsa decemlineata* (Van der Horst et al, 1987).

A precipitin line was observed in immunodiffusion between anti-*M. sexta* apoLp-III and *G. africanus* apoLp-III (Haunerland et al, 1986). The authors have attributed the positive reaction to structural similarity between apoLp-III from *M. sexta* (Lepidoptera) and *G. africanus* (Orthoptera) as evidenced by the amino acid composition.

In this study, anti-*B. fusca* lipophorin was shown to react with both apoLp-I and apoLp-II by immunoblotting. Double radial immunodiffusion however, showed reactivity only towards apoLp-I. Cross reactivity test by immunoblotting and double radial immunodiffusion using anti-*B. fusca* lipophorin showed reaction with haemolymph samples from *C. partellus*, *E. saccharina*, and *G. mellonella*. Haemolymph samples from *L. migratoria* and *G. mortisani* never cross reacted with anti *B. fusca* lipophorin. Similar cross reactivity results were noted when anti-*M. sexta* lipophorin was used in double radial immunodiffusion. (See Section 3.3).

Immunological studies involving *B. fusca* lipophorin thus reveal that, despite the interior location of apoLp-II as deduced from limited trypsin digestion, some of its antigenic determinants are exposed in the intact lipophorin. Due to the shielding off of apoLp-II from the aqueous haemolymph environment, its antigenic determinants are only detectable by immunoblotting. The cross reactivity tests strongly suggest that lipophorin from insects of the same order share antigenic determinants on both apoproteins but predominantly on apoLp-I. Thus anti-*M. sexta* lipophorin and anti-*B. fusca* lipophorin cross reacted with lipophorin from insects of the same order (Lepidoptera).

*L. migratoria* and *G. mortisan* which belong to the insect orders Orthoptera and Diptera respectively never cross reacted with either of the anti-lepidopteran lipophorins. Ryan *et al*, 1984, showed cross reactivity between anti-*M. sexta* apoLp-II with apoLp-II's from eight insect species representing seven insect orders. It was concluded that apoLp-II had to be conserved for structural integrity of the lipophorin particle. In this study however, it was not possible to detect any reaction between anti-lipophorin and apoLp-II's of *G. mortisan* and *L. migratoria*. This is because anti-*B. fusca* lipophorin was used in this study rather than anti-apoLp-II as in Ryan *et al*, 1984. The interior location of apoLp-II in the lipophorin particle implies that antibodies can only be

produced against limited exposed antigenic determinants on apoLp-II.

The study of lipophorin from the larval stalk borer *Busseola fusca* has demonstrated a remarkable constancy in lipophorins from diverse insect species. Lipophorins from different insects however, have variations in their composition and molecular properties. It has also been shown in this study, that insects of the same order, share antigenic determinants on their apoproteins. The exploitation of lipophorin for selective insect pest control will only be realized after understanding fully how lipophorin structure relates to its function. Future research should therefore concentrate on structure versus functional studies.

Several important areas of lipophorin research remain unexplored. Among these are: mechanism by which lipids are loaded and unloaded at the cell surface, intracellular assembly, endocrine control of lipophorin metabolism and control of apolipoprotein gene expression. Another function of lipophorin that may prove fruitful for further research is in defense of the insect against threatening organisms and toxins. The ability of lipophorin to bind biologically active xenobiotics such as drugs and insecticides has been demonstrated (Haunerland and Bowers, 1986). As yet it has not been shown whether the binding enhances detoxication or intoxication of the xenobiotics.



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