CHANGES IN LIPOPHORIN STRUCTURE AND FUNCTION DURING PHASE TRANSITION IN *SCHISTOCERCA GREGARIA* (Forskal) (ORTHOPTERA: ACRIDIDAE)

BY

DORINGTON OKEYO OGOYI, MSc. (NAIROBI)

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DECLARATION

I, Dorington Okeyo Ogoyi, hereby declare that this thesis is my original work and the work described here has not been presented to any other University.

DORINGTON OKEYO OGOYI

CANDIDATE

This thesis has been submitted for examination with our approval as supervisors.

DR. ELLIE O. OSIR

PROF. NORAH K. OLEMBO

PROF. DOMNIC W. MAKAWITI

CHAIRMAN,DEPARTMENT OF BIOCHEMISTRY

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ABBREVIATIONS

AKH	Adipokinetic hormone
ApoLp-I	Apolipophorin - I
ApoLp-II	Apolipophorin - II
ApoLp-III	Apolipophorin - III
BSA	Bovine serum albumin
CA	Corpus allata
CC	Corpus cardiacum
cGMP	Cyclic guanosine monophosphate
cAMP	Cyclic adenosine monophosphate
Con A	Concanavalin A
cpm	Counts per minute
DG	Diacylglyceride
dpm	Disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid
FFA	Free fatty acids
FAME	Fatty acid methyl esters
HDLp	High density lipophorin
JH	Juvenile hormone
IEF	Isoelectrofocussing
KBr	Potasium bromide
LDLp	Low density lipophorin
LTP	Lipid transfer protein
MOPS	Morpholinopropane sulfonic acid

UNIT ABBREVIATIONS

gm	Gram
h	Hour
M	Molar concentration
mA	MilliAmpheres
min	Minute
mg	Milligram
ml	Millilitre
mm	Millimetre
mM	Millimolar
M_r	Molecular weight
nmol	nanomol
nm	Nanometre
pH	- Log hydrogen ion concentration
pmol	picomol
pI	Isoelectric point
rpm	Revolutions per minute
sec	Second
μg	Microgram
μM	Micromolar
μl	Microlitre

NaCl	Sodium chloride
PAGE	Polyacrylamide gel electrophoresis
PAS	Periodic acid Schiff reagent
PBS	Phosphate buffered saline
pI	Isoelectric point
RI	Refractive Index
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TB	Tris buffer
TBS	Tris buffered saline
TG	Triacylglyceride
TG-lipase	Triacylglyceride lipase
TLC	Thin layer chromatography
VG	Ventral gland
Tris	Tris-(hydroxy methyl)-aminomethane
VHDLp-E	Egg specific, very high density lipophorin
w/w	Weight by weight

SUMMARY

The desert locust, *Schistocerca gregaria* is a major agricultural pest especially in the gregarious phase when it can cover an infestation area of approximately 20% of the total land mass. The solitary phase on the other hand, occur in low densities with discontinuous distribution of no economic importance. The two locust phases differ in their anatomy, behaviour, morphology, pigmentation, physiology and biochemistry. Swarming behaviour, a characteristic feature of the gregarious locusts, is associated with long distance flights of upto 5000 km.

Locusts mainly use carbohydrates at the initiation of flight while, lipid is the main fuel in prolonged flight. Thus, lipid metabolism is of prime importance in the maintenance of long distance flights as in locust swarms. Due to the high flight activity in swarming (gregarious) locusts, in addition to the need for adequate lipid reserve, locusts require an efficient system for lipid mobilization, transport through the haemolymph and the ultimate utilization at the flight muscles. Although lipid metabolism plays a crucial role in prolonged flight, only limited studies have been carried out to determine how phase transition in locusts affects lipid metabolism.

In order to understand how lipid metabolism is affected by phase transition in locusts, lipophorin, the major insect haemolymph lipoprotein involved in the transport of lipids from the fat body to the flight muscles, was chosen for the study. Differences between the two phases could in the long run be exploited in designing novel control strategies. Furthermore, it could also be important in distinguishing the locust phases either from field isolates or laboratory reared colonies.

High- and low density- lipophorin (HDLp and LDLp) were isolated from both the

solitary and gregarious *S. gregaria* by KBr density gradient ultracentrifugation (206,000 x*g*, 4° C, 4 h). HDLp isolated from the two phases had identical native molecular size of M_r ~ 620,000 as determined by non-denaturing-PAGE. Analysis by SDS-PAGE showed the presence of similar subunits, apolipophorin-I (apoLp-I, M_r ~ 224,00) and apolipophorin-II (apoLp-II, M_r ~ 81,000). Both apoproteins were shown to be glycosylated with mannose rich oligosaccharide chains. LDLp isolated from both phases showed the presence of an additional apoprotein, apolipophorin-III (apoLp-III, M_r ~ 20,000). The lipid percent content of the lipoproteins was determined gravimetrically in both phases. Further analysis of lipid moiety by gas chromatography indicated that the major lipid classes present in both HDLp and LDLp were phospholipids and diacylglycerides. Diacylglycerides in HDLp constituted 43.5 and 35.5% of the total lipids in gregarious and solitary locusts, respectively. On the other hand, it constituted 60 and 48% in LDLp of the gregarious and solitary locusts, respectively. Immunological cross-reactivity was demonstrated between the lipoproteins isolated from the two phases as well as with antibodies raised against HDLp from *Locusta migratoria.*

Haemolymph titres of lipophorin during the development of the locusts were estimated by single radial immunodiffusion. Starting from the third nymphal instar, there was a general increase in the levels as the insect developed, with the gregarious locusts having higher titres of the protein. Exceptions were observed during the fifth nymphal instar of the solitary locusts, when there was a large increase, which was higher than in the corresponding gregarious stage.

ApoLp-III was purified from LDLp by exploiting its solubility properties, stability to heat in addition to affinity chromatography on concanavalin-A-Sepharose. Analysis using

SDS-PAGE showed that, apoLp-III isolated from the two phases had similar molecular size (M_r ~ 20,000). However, analysis on non-denaturing PAGE showed the presence of two isoforms in each case. Immunological cross-reactivity was demonstrated between apoLp-III isolated from both phases as well as with antibodies raised against *L. migratoria* apoLp-III.

Estimation of the lipid reserve available in the locust fat body revealed that the gregarious locusts had a higher lipid reserve (79.02 ± 2.77%) as compared to the solitary ones (64.75 ± 2.55%). Analysis of the fat body lipids by gas chromatography revealed that triacylglycerides was the major lipid type and constituted 83.9% and 73.85% of the total lipids in solitary and gregarious locusts, respectively.

Lipid mobilization in response to administration of adipokinetic hormone (AKH), was demonstrated in both phases. A maximum response was observed 90 min after the hormone administration when the diacylglyceride levels reached a peak value. Furthermore, evidence of lipoprotein shift was obtained using gel permeation chromatography on an AcA_{22} column. The response in gregarious locusts, led to the formation of a larger LDLp molecule than that formed in the solitary locusts. Increase in particle size was shown to be due to increase in diacylglyceride levels as well as association with more apoLp-III molecules. The response to a range of doses of AKH showed that the gregarious locusts were more sensitive to low doses below 2 pmol and the response was more tightly controlled than in solitary locusts. Thus, whereas gregarious locusts had a maximum response from 5 pmol, the solitary locusts peaked above 10 pmol. The ED_{50} was estimated to be 6.60 and 1.53 pmol for solitary and gregarious locusts, respectively.

Activities of the fat body triacylglyceride lipase was studied using ^{14}C-labelled triolein as the substrate tracer. The resting levels of the lipases was estimated to be 1.98 ± 0.24 and

1.95 ± 0.53 nmol/h/mg protein for solitary and gregarious locusts, respectively. Evidence of the activation of the lipase in response to AKH administration was deduced. Thus, administration of 2 pmol of AKH resulted in the elevation of the levels to 2.47 ± 0.39 and 2.30 ± 0.43 nmol/h/mg for gregarious and solitary locusts, respectively. Estimation of kinetic parameters showed that the solitary locusts K_m value was 18.75 compared to 46.67 μM for the gregarious locusts. The V_{max} was estimated to be 2.52 and 10.29 nmol/h/mg for solitary and gregarious locusts, respectively. The lipase from the gregarious locusts showed higher catalytic ability but lower affinity for the substrate. These properties of the enzyme may reflect a physiological adaptation arising from their metabolic requirements.

CHAPTER 1

1 INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

The desert locust, *Schistocerca gregaria*, is a major agricultural pest with an infestation area of about 28 million square Km (20% of the total land mass). The invasion area covers Africa North of the equator, skirting Mediterranean Europe, the Middle East through to the Arabian and Indo-Pakistanian Peninsula (Uvarov, 1966; Steedman, 1988).

During recession, the solitary or isolated locusts retreat to more arid zones in the Southern Saharan and Sahelo-Saharan regions. When environmental conditions become favorable, the locust populations increase leading to the formation of hopper bands and adult swarms. The process of gregarisation can be continued from one generation to the next as long as the numbers remain high. Should the build up continue long enough, a plague results. A desert locust plague usually occurs when many gregarious bands and swarms occur at the same time, over a large area in different regions. While desert locust outbreaks are common, upsurge large enough to start a plague are rare (US congress, special report, 1990; Panos Inst., 1993).

Since 1908, there has been four major desert locust plagues ranging in duration from 7 to 13 years with short periods of recession lasting upto 6 years. The last period of major activity which was relatively continuous albeit with peaks and troughs was during 1939-1963. Since then, swarms have been reported in 1967 and 1968. The most recent plague was in 1987-1988, which receded in 1989 (Waloff and Popov, 1990). At its height, the plague covered 20% of the earths surface and affected 43 countries (Panos Inst., 1993).

Gregarious or crowded desert locusts are highly polyphagous, consuming natural vegetation and a wide range of food crops and are capable of migrating long distances of upto 5000 Km (Kevan, 1989; Richie and Pedgley, 1989). A major invasion of swarms may contain upto 50 million adults per square Km and weigh 100,000 tonnes, with the capacity of eating an equivalent weight of green vegetation each day (Steedman, 1988). In previous plagues, it has been estimated that US$ 4.5 million worth of damage was done to crops in Kenya between 1928-1929, while in Ethiopia more than 167,000 tonnes of grains were destroyed in 1958 (Panos Inst., 1993). During the three years of the last plague, the affected countries and the donor communities endeavored to suppress the plague chemically by treating more than 30 million hectares. Approximately US $ 300 million was spent towards this end with the affected countries spending US $ 100 million out of their scarce resources (SGR 1992 report; Brader, 1988). However, statistics on locusts and grasshopper damage to crops are generally hard to come by.

Locust control had depended on ultra-low volume applications of dieldrin for many years. However, the persistence of dieldrin in the environment over long periods led to its ban in many countries. Thus, to achieve the same results as formerly provided by a single application of dieldrin over a small area, repeated blanket sprays is now required (Brader, 1988). In the last plague for example, 13 million liters of malathion and fenitrothion was applied over the affected areas. Such large scale repeated applications of insecticides has an adverse effects on the environment, affecting non-target organisms including the natural enemies of the locusts and grasshoppers. The very high costs of these control measures and the adverse effects of the chemical pesticides in the environment has led to intensification of research towards alternative control strategies.

Research into the development of integrated pest management strategy for the control of locusts and grasshoppers has intensified over recent years. A number of entomopathogenic microorganisms such as viruses, protozoa and fungi are already being screened for possible applications (Odindo, 1991; Panos Inst. report, 1993). Other approaches towards this end include studies on chemical ecology of the locusts with a view preventing the formation of swarms (Krall and Nasseh, 1991; ICIPE Ann. Report, 1991). Possible use of locust hormone analogs for control has also been suggested as an alternative approach. The use of remote sensing imagery in monitoring the locust dynamics has also been perfected in recent years (Waloff and Popov, 1992). Early detection of potential epidemics and initiation of control measures in good time is therefore possible.

Phase transition from an economically harmless solitary locusts to the gregarious phase which is associated with the locust swarms, is a complex mechanism which as yet is not fully understood. Physiological manifestations of these phases may be exploited in the design of novel control strategies.

1.2 Literature review

1.2.1 Phase polymorphism in locusts

The phase theory of locusts was first put forward by Uvarov in 1921. According to the theory, locusts exists in two extreme phases referred to as solitary and gregarious, which are separated by a more or less continuous series of intermediate forms (transient phase). The intermediate forms are a product of changes from the solitarious to gregarious or the vice versa. Extreme phases with full scale differences is limited to the field, whereas, laboratory reared locusts under conditions of crowding or isolation can only approach the characteristics of the gregarious and solitary phase respectively (Uvarov, 1966). The phases of locusts differ in coloration, behavior, morphology, physiology, ecological responses and ultimately, their geographical distribution. The phase characters can, however, be shifted in either direction and the direction of shift is reversible in any stadium, except the egg stage (Uvarov, 1966; 1977; Pener, 1983). Phase polymorphism is cumulative such that a shift in an instar progresses in the following one and in the next generation. Uvarov, in his theory further stated that the periodical locusts plagues were associated with phase transition from solitary to the gregarious phase which formed the hopper bands and swarms of adults which emigrated from the breeding grounds. Even during the swarming period, most of the population are in a state of flux, some being nearer the gregarious phase in certain respects than others and only some qualify for the full phase status (Uvarov, 1966). Uvarov also postulated that phase transformation was governed by environmental factors and that in addition to increase in population density, sensory reactions of individuals to one another also had a role to play.

The current concept of phase states that locusts show density dependent changes such

that when crowded as nymphs or adults, they develop the characters of the gregarious phase and when isolated, of the solitary phase. These characters may shift in either direction in ontogeny or in successive generations and that phase transformation is reversible (Pener 1983; Waloff and Popov, 1990). Phase change however, is not the cause of locust plagues as suggested by Uvarov, for it follows and does not precede change in density (Pener, 1983; 1985; 1991, Hardie and Rees, 1985). Further evidence for this comes from the fact that phase change can occur in some populations which may not be large enough to give rise to plagues (Uvarov, 1966; Pener, 1991).

Many species of acridids exhibit tendencies of aggregation, migration and a more or less rudimentary phase polymorphism (Hardie and Rees, 1985; Jago, 1985; Uvarov, 1966). Typical locust species probably only constitute an evolutionary culmination of these tendencies. *Schistocerca gregaria* shows a highly developed gregarious potential, such that under suitable ecological conditions, phase transformation can be triggered off when densities exceed 250-500 adults per hectare and when hopper densities reach 0.5-5/m^2 (SGR report, 1992). However, environmental factors that promote gregarisation often involving several biotopes must be maintained for at least four successive generations to enable transformation from solitary to a fully expressed gregarious phase. Complete de-gregarisation is generally more rapid, occurring within one or two generations (Uvarov, 1966; 1977; SGR report, 1992).

1.2.2 Distinguishing features of the locust phases

A number of phase characters have been used to identify the locust phases. However, phase characters wherever they occur, are species specific and show a wide variation

between species (Albrecht, 1967; Uvarov, 1977). Pigmentation differences appear to be the most conspicuous. In *S.gregaria* for example, gregarious first nymphal instars are dark with little light spots which turn creamish with dark patterns during the 2nd and 3rd instars. Immature adults are bright pinkish before turning bright yellow in maturation. In contrast, the solitary locusts are uniformly pale green with the adults being sandy brown or grey (Uvarov, 1966; Steedman, 1988). The colors of the solitary locusts tend to match that of the underlying background in the field or inside the cages (Albrecht, 1985). The colors of the locusts are however, affected by other factors such as humidity and temperature (Albrecht and Lauga, 1978; 1979).

Morphometric studies has shown a keel shaped pronotum in the solitary locusts while the same is depressed and laterally constricted in the gregarious phase. Morphometric parameters such as E/F (tegmen length/femur ratio) is lower while F/C (femur/head width ratio), is higher in solitary than in the gregarious locusts (Uvarov, 1966). However, as indicated by Steedman, 1988, morphometrics do not always give a reliable indication of behavioral changes since change in behavior and appearance do not occur at the same time. Thus in *S. gregaria* some swarms consist of locusts whose morphometrics are the same as those of the solitary ones.

Gregarious phase is also associated with lower reproductive potential as well as less number of nymphal instars and higher chiasma frequency than the solitary locusts (Nolte, 1974; Rankin and Burchsted, 1992). In *S. gregaria* for example, the solitary locusts undergo six nymphal instars as compared to five in the gregarious phase. More intense motor activity and increased oxygen consumption is associated with the gregarious phase (Pener, 1965, Michel, 1980a, b).

Phase differences have been noted in the cuticular hydrocarbons as well as aliphatic ethers of the cuticular waxes in some species (Genin *et al.*, 1986; 1987). Studies on fat content of the body has shown that hatchlings originating from eggs laid by crowded females of *Locusta, Schistocerca,* and *Nomadacris* are higher than those originating from conspecific isolated females (Blackith and Howden, 1961). Further, Hunter *et al.*, 1981, showed that in the Australian locust, *Chortcetes terminifera* those with higher fat body lipid content were more prone to migration. Phase differences in isoenzyme profiles of glycolytic enzymes has also been noted by Colgan (1987) in *Locusta migratoria* . He found that aldolase phenotypes found in hatchlings of crowded locust hoppers was replaced by a novel isoenzyme in the isolated hoppers. In addition, the levels of glyceraldehyde-3-phosphate dehydrogenase isoenzyme were higher in the isolated than in the crowded hoppers. Differences have also been shown in the responses of the locust phases to adipokinetic hormone. According to Ayali and Pener (1992), injection of the hormone or corpus cardiacum extracts elicited a response in the solitary *L.migratoria* which was only 30-40% of the response in the gregarious locusts. However, Chino *et al.* (1992) reported that no response was elicited in the solitary locusts following AKH or corpus cardiacum injection, contrary to the finding of Ayali and Pener (1992).

1.2.3 Factors controlling the locust phase status

Polymorphism is generally under the strict control of the genotype, with the immediate environmental factors determining the actual morph. Such factors include; photoperiod, temperature, humidity, diet and population density. The potential to exhibit polymorphism is genetic with genotype capable of modifying the phenotypic responses to

environmental cues (Hunter-Jones, 1952; Albrecht *et al.*, 1959; Hardie and Rees, 1985; Pener, 1991). The environmental switching mechanism is thought to involve some kind of a receptor which in response to the cues, leads to the transduction of the effectors, usually the endocrine system.

The role of juvenile hormone (JH) and corpus allatum (CA) in phase transition has been investigated by several authors (Nijhout and Wheeler, 1982; Pener, 1991). Injeyan and Tobe (1981b), observed that CA volume in the penultimate and the last instar of female hoppers and adult females of *S. gragaria* were larger in isolated than crowded locusts. The biosynthetic activity of CA as well as the JH titres was also found to be higher in the solitary locusts compared to the gregarious ones. Coloration in the morphs also seems to be under the control of JH and CA. Thus, implantation of extra CA or administration of JH to crowded hoppers of *Locusta* and *Schistocerca*, leads to induction of the green coloration (Mordue, 1977). Couillaud *et al.*, (1987) further observed that even among the solitary hoppers, the green ones became greener. It has also been reported that allatectomy of the last instar hoppers or young adults of *Schistocerca* prevents the mature adult yellowing, whereas re-implantation of CA or JH administration reintroduces it (Pener, 1976; Amerasignhe, 1978b). However, implantation of extra CA into isolated locusts do not induce yellowing, implying that CA and/or JH is not the primary factor involved. Implantation of an extra CA into crowded *Locusta* females has also been shown to shift the reproductive parameters (Cassier, 1977b) and the morphometrics (Lauga, 1977b) toward those of the solitary locusts.

The role of CA or JH in locust flight activity is surrounded with a lot of controversy arising from different conditions and experimental parameters employed by various authors

(Pener, 1991). Some data showed that allatectomized males are rather sluggish (Wajc and Pener, 1969) and that CA exert stimulatory effect on the excitability of the locusts (Cassier, 1965). In a review, Nijhout and Wheeler, (1982) advocated a major role of JH in the control of locust phase polymorphism. The review focused on a model of JH gene switching mechanism as the basis of phase polymorphism. However, all evidence that contradicted the model was ignored. Pener (1991), in his review, stated that CA and JH promote solitary features in many instances but promote gregarious features or do not exert a relevant effect in many others. Thus, the physiological determination of the locust phases can not be explained on the basis of differential activity of CA. Differences between CA activity and JH titres may constitute additional physiological characteristics responding to density. Such differences may also cause appropriate change in some but not all characters. Injeyan and Tobe, (1981b) in an attempt to explain this, suggested that locust density may alter the responsiveness of the target tissues to JH. Pener, (1991) in his conclusion stated that CA activity and JH titres plays a role, but not the primary role in the chain of events and physiological causal characters which are responsible for phase transition.

The ventral glands (VG) have been shown to persist in solitary locusts whereas it degenerates in crowded locusts (Cassier and Maurel, 1969). The ecdysteroid titres have, however, been found to be similar or not markedly different between the crowded and isolated locusts (Wilson and Morgan, 1978; Jolly *et al.*, 1977). The physiological role of the persistence of VG is unknown and may be a result of rather than a cause of phase change. Michel, (1972a), observed that VG exert an inhibitory effect on locust flight activity and suggested that the persistence of VG in isolated adults may be responsible for the reduced flight activity. Such effects of VG may only be explained assuming that VG produces some

other hormone(s) besides ecdysteroid.

The role of corpus cardiacum (CC) and related hormones, adipokinetic hormones (AKHs) and octopamine in the regulation of phase polymorphism is poorly understood. Corpus cardiacum has been shown to promote sustained flight whereas, AKH controls a major metabolic event necessary for the mobilization of fuel and energy for sustained flight (Stone and Mordue, 1980). It has been reported that the maximum response elicited on AKH or CC injection into isolated *Locusta* is only 30-40% that of the crowded locusts (Ayali and Pener, 1991). Despite the central role of AKH in energy metabolism in locusts, not much is known about AKH in the solitary locusts. However, as suggested by Pener, (1991), AKH may be responsible or contribute to different flight activities between isolated and crowded locusts. Octopamine receptors in the brain were reported to be more sensitive in isolated than in crowded locusts (David and Fuzeau-Braesch, 1979). However, the implication of this finding is unknown.

Locust pheromones have also been implicated as playing an important role in phase transition. Gregarisation pheromone has been shown to be crucial in initiating gregarisation and sustaining of swarms (Nolte *et al.*, 1974; Gillete, 1983). A set of pheromones which appear to reverse some of the effects elicited by the gregarisation pheromone have been identified and may thus have the potential role in the degregarisation process (Gillet, 1983; ICIPE Annual report, 1991). The mode of action of the pheromones in phase transformation is not yet fully understood but may be two fold. Phase may affect pheromone production and/or reception or pheromones may affect the phase changes (Pener, 1991). The possible relationship between phase affecting pheromones and the endocrine factors have not been studied.

1.3 Locust flight metabolism

1.3.1 Fuel utilization during flight

Flight in insects is a high energy demanding process which is associated with 50-100 fold increase in the metabolic rate. Such an increase leads to 50-100 fold increase in enzyme activities, substrate flux, oxidative phosphorylation, as well as ATP production (Kamer, 1978; Beenakkers *et al.*, 1984). Initially, flight muscles are dependent on its own metabolic reserves starting with the ATP available. However, to maintain the supply of ATP, glycogen plus other carbohydrates in the muscle fibres are metabolised immediately. In *L. migratoria* muscles, there are large quantities of glucose, trehalose and some glycogen. These carbohydrates, plus trehalose in the blood and that from other stores, are used during the first 20-30 min of flight. Thereafter, lipids are the major fuel for sustained flights (Mayer and Candy, 1969a; Candy, 1989).

During flight in *Locusta*, the major change in carbohydrate reserves of the flight muscles takes place during the first 10 min of flight after which, the level is sustained at 50% the original value (Worm and Beenakkers, 1980). In *Schistocerca*, no more than a third of muscle glycogen remains after 10 sec of flight and after 3 min, no further decrease takes place (Rowan and Newshome, 1979).

In *Locusta*, trehalose is the major energy source during 20-30 min of flight with the concentration decreasing to 35 % within 30 min. Turnover during the steady state is much lower, but even when lipids become the major fuel, 25% of the energy utilized is still supplied by trehalose (Van der Horst *et al.*, 1978b). Haemolymph trehalose is sustained by trehalose mobilised from fat body or gut wall and to a minor extent, from glycerol released in the hydrolysis of diacylglycerides by the flight muscles (Beenakkers *et al.*, 1984).

Lipid reserves in insects are in the fat body in the form of triacylglycerides (TG). During flight, lipids have to be mobilised, transported through the haemolymph, before being taken up by the muscles (Beenakkers *et al.*, 1981). The mobilised diacylglycerides are stereospecific with sn, 1,2- configuration (Tietz and Weintraub, 1980; Lok and Van der Horst, 1980). The dominant neutral lipid class in the haemolymph is DG which during flight, or in response to adipokinetic hormone injection, is elevated to peak levels three fold the resting concentration (Mayer and Candy, 1969a; Justum and Goldswothy, 1976). Oxidation of DG in the first periods of flight increases concomitantly with the rise in haemolymph DG levels (Van der Horst *et al.*, 1980). Lipid oxidation reaches a plateau value between 30-45 min after initiation of flight. At the flight muscles, DG is converted to free fatty acids and glycerol. In *Schistocerca* and *Locusta*, most glycerol is released into the haemolymph where its concentration rises to 6-10 fold during long term flight. Glycerol is taken up by the fat body and converted to trehalose or DG (Candy *et al.*, 1976; Van der Horst *et al.*, 1983).

In addition to carbohydrates and lipids, proline and perhaps other amino acids may constitute minor substrates for flight since, their concentrations decrease considerably during flight (Mayer and Candy, 1969a). Proline has been suggested to provide intermediates of citric acid cycle necessary for the enhanced oxidation of acetylCoA, derived from flight induced increase in metabolism of carbohydrates or lipids (Worm and Beenakkers,1980).

1.3.2 Hormonal control of locust flight metabolism

Adipokinetic hormones (AKH), the lipid mobilizing factor released from corpus cardiacum was first identified simultaneously in *S. gregaria* and *L. migratoria* (Beenakkers, 1969; Mayer and Candy, 1969b). These neuropeptide hormones are characterized by N- and

C- terminally blocked peptides with a chain length of 8-10 amino acid residues (Gade, 1990; Goldsworthy and Wheeler, 1990). In addition to the lipid mobilizing function, AKH have been implicated in regulation of carbohydrate metabolism, protein synthesis, myotropic activity (Orchard, 1987), lipid synthesis (Gokuldas *et al.*, 1988), as well as activation of glycogen phosphorylase (Siegert and Ziegler, 1983; Goldsworthy and Wheeler, 1990).

In *L. migratoria*, *S. gregaria* and *S. nitans*, two AKHs designated AKH-I and AKH-II have been identified (Siegert *et al.*, 1985; Gade *et al.*, 1985). Quantitatively, more AKH-I than AKH-II is stored and released from corpus cardiacum (Orchard and Lange, 1983a, b). Both AKHs are capable of effecting lipid mobilization, lipoprotein transformation as well as activation of glycogen phosphorylase. However, AKH-II elicits only 80% of the activity of AKH-I at the maximum effect dose (Goldsworthy *et al.*, 1986; Goldsworthy and Wheeler, 1986).

During flight activity, AKH-I and AKH-II titres in the haemolymph reach a maximum value after 30 min. Thereafter, the titres decline to sustained lower levels, approximately 50% the peak value (Cheeseman and Goldsworthy, 1979; Orchard and Lange, 1983b). The release of the hormones induces mobilization of DG from the fat body resulting in a massive increase in haemolymph DG levels. In locusts, release or injection of AKH causes upto 300% increase in DG levels which peaks after 90 min (Mayer and Candy, 1969a; Jutsum and Goldsworthy, 1976; Van der Horst *et al.*, 1980). During steady state, DG mobilization matches the rapid mobilization of the lipids by the flight muscles (Van der Horst *et al.*, 1978a). The AKH-I level required for maximum response has been estimated in *Locusta* to be 3 pmol (Goldsworthy *et al.*, 1986).

The mechanisms of AKH release remains undetermined. However, indications are

that circulating metabolites may influence the release. Thus, injection of trehalose or lipids into the locust haemocoel leads to decrease in the amount of AKH released when the insects are subsequently flown (Houben and Beenakkers, 1973; Cheesman and Goldsworthy, 1979). Evidence for the involvement of other factors has been suggested since AKH release starts at the onset of flight before trehalose levels are substantially reduced.

The elevation of octopamine as a part of excitation response may also potentiate the action of specific octopaminergic neurones supplying the corpus cardicum and stimulate the initial AKH release. Decline in octopamine during 15-30 min of flight is accompanied by decrease in trehalose levels thus, the depressed trehalose levels may contribute towards the continued AKH release (Downer, 1985). In *S. americana gregaria*, octopamine levels increase in the haemolymph after 2 min of flight, reaching a maximum value (10- fold the resting level) after 10 min. When flight is extended above 10 min, the levels of octopamine fall until after 60 min when the concentration is sustained (Candy, 1978; Goosey and Candy, 1980). Studies with isolated locust flight muscle preparations have shown that octopamine stimulate oxidation of carbohydrates and lipids (Candy, 1978; Orchard, 1987). It has also been suggested that octopamine is involved in the strengthening of the contraction of locust flight muscles as well as in the mobilization of fuel reserves from the fat body during flight. Orchard (1987), further proposed that octopamine may mediate in flight response and initiate lipid mobilization during the earliest moments of flight, with only small amounts of lipids released. Such low levels of lipids are probably not used to support flight muscle contraction, but it is possible that octopamine initiates hydrolysis of the lipids to provide energy and conditions necessary for trehalose synthesis.

The lipolysis process is thought to involve cAMP mediated process, leading to the

activation of a protein kinase and thereafter, triacylglyceride lipase (Van der Horst, 1990; Beenakkers, 1984). However, as pointed by Beenakkers *et al.* (1985), the ultimate mechanism for hormonal control of the lipase is still unknown. No *in vivo* stimulation of the lipase by AKH injection has been demonstrated (Pines *et al.*, 1981).

1.4 Lipophorin structure and function

1.4.1 Structure and composition

Lipophorin is the principal haemolymph lipoprotein throughout the life cycle of many insects (Chino *et al.*, 1981; Beenakkers *et al.*, 1985; Shapiro *et al.*, 1988). Synthesis of the protein occurs in the fat body from where it is released into the haemolymph (Prasad *et al.*, 1986b; 1987; Venkatesh *et al.*, 1987). All lipophorins so far studied consists of two apoproteins, apolipophorin-I (apoLp-I; M_r ~210,000 - ~250,000) and apolipophorin-II (apoLp-II; M_r~ 70,000 - 85,000). Both apoproteins are glycosylated with mannose rich oligosaccharide chains (Ryan *et al.*, 1984; Shapiro *et al.*, 1988). The lipid moiety is predominantly composed of phospholipids and diacylglycerides. Immunological studies using antibodies raised against apoLp-I and apoLp-II has shown that the two apoproteins are not homologous (Van der Horst *et al.*, 1987). A third apoprotein, apolipophorin-III (apoLp-III, M_r ~18-20,000) associates reversibly with lipophorin of certain insects especially those that utilize lipids for flight (Ryan *et al.*, 1984). The involvement of apoLp-III in flight-related lipid mobilization from the fat body has been studied in *M. sexta* (Shapiro and Law, 1983) and in *L. migratoria* (Mwangi and Goldsworthy, 1977; Chino *et al.*, 1986). In *L. migratoria*, Goldsworthy *et al.* (1985) have reported the existence of two glycosylated apoLp-III species (Mr ~16,000 and 20,000) both of which are competent to associate with

lipophorin during AKH-induced lipid mobilization. Other authors have since, identified two or three isoforms of apoLp-III in *L. migratoria* which are indistinguishable by SDS-PAGE but separable by either non denaturing PAGE or by ion exchange chromatography (Chino and Yazawa, 1986; Van Heusden *et al.*, 1987; Van der Horst *et al.*, 1991). Chino and Yazawa, (1986) have suggested that the isoforms may be due to variations in the phosphorylation of the oligosaccharide chains.

Lipophorin occurs in the haemolymph in several forms with respect to relative lipid content and apolipoprotein composition (Beenakkers *et al.*, 1988). The high density lipophorin (HDLp) characterized by 35-50 % lipid and a density of ~1.11 g/ml is present in insects at rest whereas the low density lipophorin (LDLp, density ~1.04 g/ml) is induced in insects that utilize lipids during flight (Shapiro *et al.*, 1988). It has, however, been shown that there are some LDLp in the resting adult *Manduca sexta* (Kawooya and Law, 1988; Wells *et al.*, 1987) and in *Thasus actangulus*, an insect that does not engage in extended flight (Ryan *et al.*, 1985). LDLp differs from HDLp in that it has a higher lipid content which is stabilized by the binding of apoLp-III molecules. The binding of apoLp-III molecules confers upon lipophorin the ability to bind more diacylglycerides. In *L. migratoria* for example, HDLp has 41% lipid with a density of 1.12 g/ml whereas LDLp has 46.3% lipid and a density of 1.07 g/ml (Chino *et al.*, 1986).

ApoLp-III is generally free in the haemolymph but may associate with lipophorin of the resting adults in some species such as in *M. sexta* (Ryan *et al.*, 1984; Wells *et al.*, 1985; Gondim *et al.*, 1989a). ApoLp-III is also present in the haemolymph of *M. sexta* larvae though not associated with HDLp (Shapiro *et al.*, 1984). In an attempt to explain why adult but not larval HDLp contain apoLp-III, Cole and Wells, (1990) examined the possibility of

different processing of the precursors between larval and adult fat body or the existence of larval and adult specific transcription of apoLp-III gene. Both ideas were tested and disapproved. They then proposed that there could be differences in the assembly of HDLp in adults and larvae.

Ziegler *et al.* (1988) have identified functional apoLp-III from flightless *Barytettix psolus* and *Melanoplus differetialis*, which is not a long distance flier. A follow up study by Ryan *et al.* (1990), showed that apoLp-III from Orthopteran species: *L. migratoria*, *B. psolus* and *M. differentialis*, are similar in composition. In addition, apoLp-III from *B. psolus* and *M. differentialis* cross-reacted with polyclonal antibodies directed against *L. migratoria* apoLp-III. No cross-reaction was observed with *M. sexta* apoLp-III.

Studies on physical properties of apoLp-III from *M. sexta* and *L. migratoria* have revealed that this apoprotein is well suited to bind to lipid surfaces(Kawooya *et al.*, 1986; Cole *et al.*, 1987). *In vitro* diacylglycerol mobilization studies have further shown that *M. sexta* apoLp-III is capable of associating with locust lipophorin. Isolated locust flight muscles was shown to be capable of utilizing the hybrid LDLp (Van der Horst *et al.*, 1988).

Katagiri *et al.* (1987), have proposed a centro-symmetrical three layer model for locust and cockroach HDLp in which an outer shell contains apoLp-I and phospholipids and the middle layer apoLp-II and diacylglycerides. The core is proposed to be comprised of apolar lipids such as hydrocarbons. The apoproteins seems to be organized such that apoLp-I is more exposed to the aqueous haemolymph environment than apoLp-II (Shapiro *et al.*, 1984: Van der Horst *et al.*, 1987; Schulz *et al.*, 1987). It has further been shown that a significant amount of DG will be in the surface layer (Shapiro *et al.*, 1988). The presence of diacylglycerols in the easily accessible shell of lipophorin may allow their exchange without

degradation of the rest of the particle thus, enabling lipophorin to function as a reusable shuttle (Chino *et al.*, 1985). Ryan *et al.* (1984) have also observed that apoLp-II has a conserved structure over a wide range of insect orders. Thus, antibodies to *Manduca sexta* apoLp-II cross-reacted with eight insect species representing seven insect orders. It was suggested that the conserved structure is for the maintenance of structural integrity of lipophorin particle. However, structural studies on LDLp has shown that all three apoproteins and especially apoLp-III are still exposed in the particle (Van der Horst *et al.*, 1987; Schulz *et al.*, 1987). This may reflect the presence of a recognition site on apoLp-III. It has further been suggested that the activation of flight muscle lipoprotein lipase may involve apoLp-III (Wheeler *et al.*, 1986).

1.4.2 Functions of lipophorin

Lipophorin is involved in a number of processes, principal among which is the transport of lipids from sites of storage, absorption or synthesis to sites of utilization (Chino, 1985; Kanost *et al.*, 1990). Lipophorin loads dietary lipids at the site of absorption, the midgut and then either delivers the lipids at the growing tissues or the fat body for storage. Lipophorin also functions in the redistribution of lipids stored in the fat body, to peripheral tissues. Among the lipids transported are diacylglycerols, cholesterol, hydrocarbon and carotenoids. Lipophorin has also been shown to be capable of transporting phospholipids from the fat body to the developing oocytes in *Rhodnius prolixus* (Gondim *et al.*, 1989b). Previously, phosholipids were considered to be part of the lipophorin structure, itself not being loaded or unloaded like other lipids (Katagiri *et al.*, 1985). In contrast to the mammalian low density lipoprotein which, after delivering lipids are taken up into cells by

receptor mediated endocytosis and hydrolysed intracellularly, insect lipophorins function as reusable shuttle without any endocytosis or hydrolysis of the apoproteins (Chino and Kitazawa, 1981; Chino, 1985; Van Heusden *et al.*, 1991). Transport of lipids to the flight muscles for the provision of energy and supply of lipids to the developing oocyte are among the most studied roles of lipophorin (Wheeler *et al.*, 1984 Kawooya and Law, 1988).

Egg lipids in insects are used as a major source of energy for the developing embryo. Some of the lipids may play a major role in the construction of the embryonic cell membranes. Lipophorin has a dual role during vitellogenesis in lepidopterans namely, shuttling of precursors from the fat body to ovaries for deposition of lipid yolk droplets and in some species, becoming one of the major constituents of the yolk proteins (Telfer *et al.*, 1991; Kawooya *et al.*, 1988; Gondim *et al.*, 1989b). In *M. sexta* for example, it has been shown that lipophorin contributes upto 95% of the egg lipid (Kawooya and Law, 1988). Furthermore, *in vitro* studies by Kawooya and Law (1988) has shown that HDLp of *M. sexta* is not recycled back into the haemolymph after being sequestered by the ovarian follicles.

Lipophorin has also been shown to bind xenobiotics thereby raising the possibility of a detoxification function (Haunerland and Bowers, 1986; Shapiro, 1989). The involvement of lipophorin in haemolymph clotting has also been described in certain insects (Barwig, 1985). The function of lipophorin as a juvenile hormone (JH) carrier protein has been demonstrated in a number of insects including *Chironomus thummi* (Wisniewski and Steuernagel, 1990). In *C. thummi*, 83% of JH binding sites in larval haemolymph is represented by lipophorin.

Further support for the theory that lipophorin is a multifunctional transport protein in insects has come from the observations by Haunerland *et al.* (1992) that lipophorin is capable

of transporting the blue chromophore, biliverdin in *Podisus maculiventris*.

1.4.3 Structural changes due to physiological status

Lipophorin is a dynamic particle with a turnover of the lipid component being much faster than that of the apoprotein moieties (Downer and Chino, 1985). A remarkable flexibility thus, exists with respect to the relative lipid content and composition of lipophorin (Beenakkers *et al.*, 1988). Changes in lipophorin particle size and density may be correlated to the physiological (e.g rest vs flight) or developmental state of the animal. While there is a continuum of lipophorin species with respect to particle density, species isolated from carefully staged animals or after hormonal administration fall within a relatively narrow range. Thus, the characteristics of lipophorin subspecies present in the plasma at any given time can be considered to reflect the physiological state of the organism with respect to lipid metabolism (Van der Horst, 1990; Ryan and Law, 1984).

Biosynthesis of lipophorin during the larval development in *M. sexta* does not occur continuously but only during feeding periods. In fourth larval instars, lipophorin levels increased during the first 2 days then remained constant during larval ecdysis before another increase during the first three days of the fifth instar. No increase in levels was observed during the prepupal stage through to the pupal ecdysis (Prasad *et al.*, 1986a; Tsuchida *et al.*, 1987; Shapiro *et al.*, 1988). These changes appear to be due to the cyclic changes in mRNA presence of which, is shown during the feeding stages and is absent after cessation of feeding. Apart from the changes in the levels, Prasad *et al.* (1986) and Ryan and Law, (1984) noted that there were also changes in lipophorin composition and physical properties. Thus, at the end of the fifth instar and at the initiation of prepupal stage, there is a decrease

in lipid content which is followed 12 hours later by a large increase. The lipid rich lipophorin molecule then remains in the haemolymph until pupal ecdysis, when yet another change in lipid composition takes place. Although there is no synthesis or secretion of new lipophorin molecules from the fat body during these changes, the fat body is nevertheless the source of the lipid added to the haemolymph lipoprotein. In adult *M. sexta*, HDLp has a lower density and relatively higher diacylglyceride content compared to the larval lipophorin. Two molecules of apoLp-III also associate with the lipophorin molecule.

Lipophorin thus seems to be modelled for new functions, in a kind of molecular metamorphosis (Ryan and Law, 1984). The larval form being involved in the transport of digested fats to the storage depots, whereas, during the pupal stage, fat is transported from the storage depots to developing adult tissues. The adult form, on the other hand, is involved in the transport of the lipids to the flight muscles to support flight or to the ovaries for egg development.

In diapausing larvae the rate of respiration is low. Thus, the need for lipid transport is equally low. It is therefore not surprising that in *Hyalophora cecropia*, lipophorin density is high at this stage (Telfer *et al.*, 1991). In male insects, the density dropped just prior to eclosion, reflecting the changes in lipid transport demands by metamorphosis. However, extreme transition occurs during eclosion when 70% of lipophorin is in the heavily loaded form, LDLp (Telfer *et al.*, 1991).

Egg specific lipophorin has been isolated from mature eggs of *Philosamia cynthia* and *M. sexta* (Kawooya *et al.*, 1988; Chino *et al.*, 1977). In *M. sexta*, isolated follicles were shown to sequester HDLp which upon entering the follicle is stripped off of 80% of its lipids. The associating apoLp-III molecules are also stripped off. The resulting very high

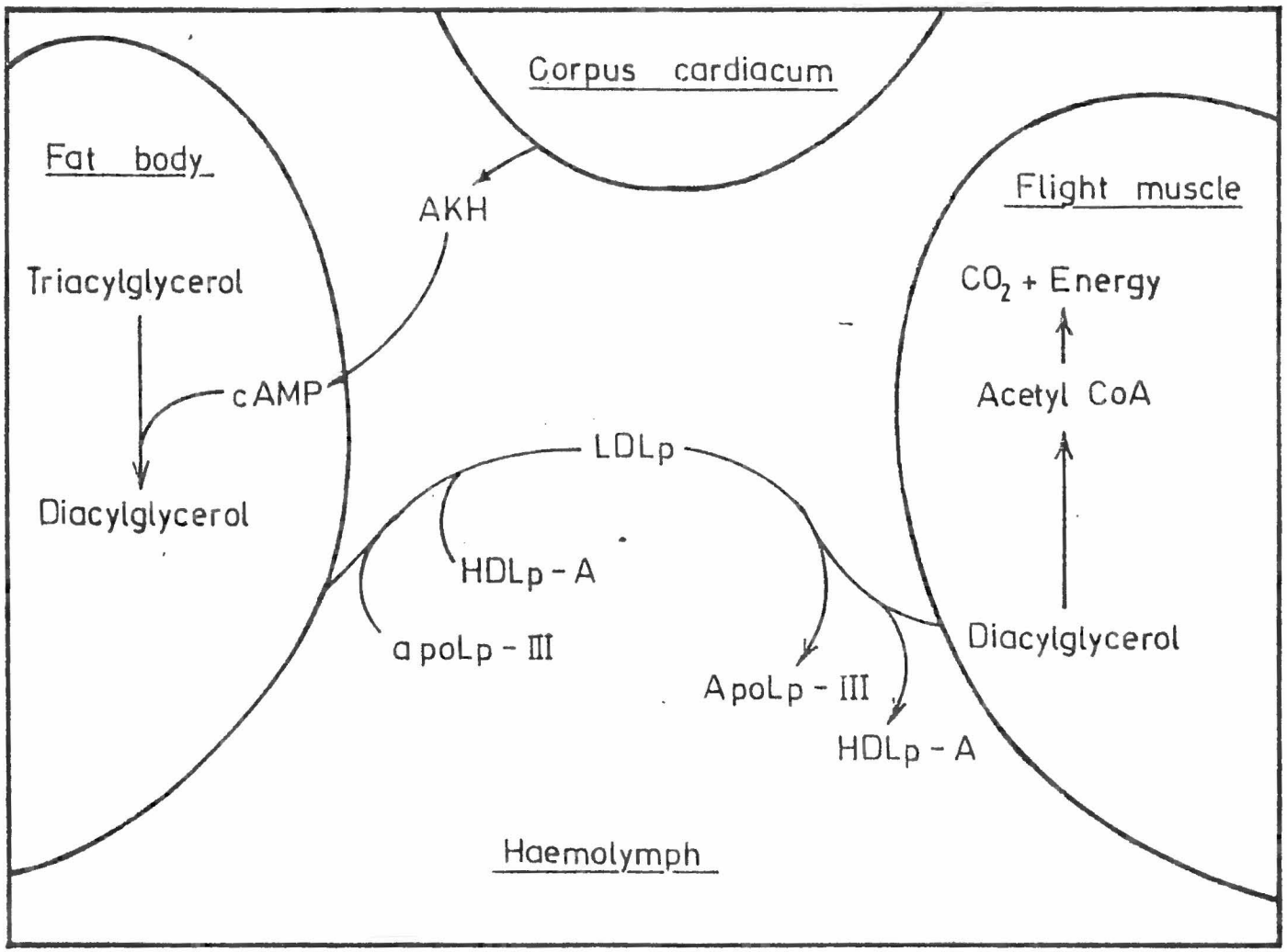
density lipoprotein (VHDLp-E) is subsequently retained in the follicles (Kawooya *et al.*, 1988).

It has recently been shown that the levels of lipophorin may also reflect on the physiological status of the insect. Gonzalez *et al.* (1991) observed in *Triatoma infestans* that the levels of HDLp and VHDL during the last nymphal stage increased followed by a decrease during the molt and at the start of the adult life. Subsequently, HDLp was the most abundant protein of the adult insect haemolymph reaching a peak level after 3 weeks which was then maintained. Furthermore, in *Hyalophora cecropia*, low levels of lipophorin was observed in the diapausing pupae while there was a steady increase in the pharate adults. In female insects, the concentration increased to a new peak value during vitellogenesis. In both sexes, however, the concentration dropped to lower levels during the last few days before eclosion (*Telfer et al., 1*991). Onset of vitellogenic function was further shown to be signalled by rise in apoLp-III titres, drop in the density of HDLp and its binding of one mole/ mol of apoLp-III as well as the presence of low levels of LDLp

1.4.4 Lipophorin metabolism during flight

The onset of flight in *L. migratoria* is preceded by the release of adipokinetic hormone (AKH) from 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 cyclic adenosine monophosphate (cAMP)(Wheeler and Goldsworthy, 1983a; Shapiro and Law, 1983)(Scheme 1). Shapiro *et al.* (1988) has proposed that the released diacylglycerol accumulates in the plasma membrane of fat body cells and then moves via fluid phase diffusion into HDLp. A lipid transfer protein (LTP) has been shown

Scheme I: Lipid mobilization, transport and utilization in *M. sexta* (From Ryan and Law, 1984)


Corpus cardiacum
Fat body
AKH
Flight muscle
Triacylglycerol
CO_2 + Energy
cAMP
Acetyl CoA
LDLp
Diacylglycerol
HDLp - A
apoLp - III
Diacylglycerol
ApoLp - III
HDLp - A
Haemolymph

to facilitate this process (Ryan *et al.*, 1986a). Although the molecular aspects of the lipid transfer process are not yet fully understood, a mechanism in which LTP first accepts lipid from a donor lipoprotein and subsequently delivers the lipid to an acceptor lipoprotein has been proposed (Ryan *et al.*, 1988). The loading of diacylglycerol onto HDLp results into a larger but less denser lipoprotein, LDLp. Concomitant with diacylglycerol loading is the association of several molecules of apoLp-III with the diacylglycerol-rich particle (Mwangi and Goldsworthy, 1977; Wheeler and Goldsworthy, 1983a). It has been shown that in response to injected AKH, diacylglyceride content of *M. sexta* lipophorin increases from 25 to 46% of the particle mass with 16 molecules of apoLp-III associating with the particle (Wells *et al.*, 1987). In *L. migratoria*, reports of 10, 14 and 28 apoLp-III molecules per LDLp particle have appeared (Chino *et al.*, 1986; Van Heusden *et al.*, 1987; Van der Horst *et al.*, 1988; Surholt *et al.*, 1992). *In vitro* studies have shown that the formation of LDLp from HDLp in response to AKH, requires the absolute presence of the fat body and the extent of loading depends on amount of apoLp-III present. Furthermore, the process of LDLp formation has been shown to be Ca^{2+}- depended (Van Heusden *et al.*, 1984; Chino *et al.*, 1989; Lum and Chino, 1990). Lipophorin receptors identified in the *L. migratoria* and *M. sexta* fat body membrane have been shown to have a 20-fold lower affinity for lipid rich LDLp as opposed to HDLp (Van Antwerpen *et al.*, 1989; Tsuchida and Wells, 1990). This could be partially responsible for the observed directional flux of diacylglycerides out of the fat body in response to AKH.

The delivery of diacylglycerol at the flight muscles is not well understood but a lipoprotein lipase identified in the flight muscles of *L. migratoria* may be involved (Wheeler *et al.*, 1984; Wheeler and Goldsworthy, 1985; 1986; Van Heusden *et al.*, 1987a). The

process also appears to be receptor mediated since HDLp high affinity receptor has been identified in the locust flight muscle membrane (Hayakawa, 1987; Van Antwerpen *et al.*, 1990). The lipase has been shown to have substrate specificity that favours LDLp over HDLp. ApoLp-III, bound to LDLp has been implicated in the activation of the flight muscle lipoprotein lipase (Van der Horst *et al.*, 1983). Wheeler *et al.*, (1986) have proposed further, that free apoLp-III may serve as an inhibitor of the flight muscle lipoprotein lipase and upon lipid loading and apoLp-III association with HDLp, lipase inhibition is relieved. At the flight muscles, diacylglycerol is released from LDLp and apoLp-III dissociated. The resulting HDLp can then return to the fat body and bind more diacylglycerol and apoLp-III, once more forming LDLp. Fatty acid binding protein, recently isolated from the locust flight muscles is thought to be involved in transfer of the released fatty acids from the extracellular matrix into the muscle cells for oxidation (Haunerland and Chisholm, 1990).

1.5 Rationale for the study

Flight in insects is accompanied by high metabolic activity which may be between 50-100 fold higher than that of the resting insect. Consequently, substrate mobilization from the fat body stores and utilization in the flight muscles must reach high values as well (Beenakkers *et al.*, 1984; Bailey, 1978).

Locusts mainly use carbohydrates at the initiation of flight while lipid is the main fuel in prolonged flight. Thus, lipid metabolism is of prime importance in the maintenance of flight in locust swarms, which can cover upto 5000 Km (Kevan, 1989; Ritchie and Pedgley, 1989). Although lipid metabolism plays a crucial role in prolonged flight, only limited studies have been carried out to show how phase transition in locusts affects lipid

metabolism.

In order to understand how lipid metabolism is affected by phase polymorphism in locusts, lipophorin, an insect lipoprotein involved in the transport of lipids from the fat body to the flight muscles, was chosen for the study. Due to the high flight activity in swarming (gregarious) locusts, it is possible that phase transition in locusts may affect lipophorin structure and function. Such an effect would most likely lead to a more efficient lipid transport system as well as the subsequent delivery at the flight muscles in the gregarious than solitary locusts.

Evidence for structural and functional adaptations related to lipid metabolism in response to a physiological state has been observed in many insects. Indeed, it has been observed that the characteristic lipophorin species in the haemolymph at any given time, reflect on the physiological state of the organism with respect to lipid metabolism (Van der Horst *et al.*, 1990; Ryan and Law, 1984; Beenakkers *et al.*, 1988; Telfer *et al.*, 1991). The biosynthetic process of lipophorin as well as the titres of lipophorin in the haemolymph also seem to correlate with the physiological status of the insect (Prasad *et al.*, 1987; Tsuchida *et al.*, 1987; Gonzalez *et al.*, 1991).

Furthermore, Ziegler *et al.*, (1988) observed that the flightless grasshopper, *Barytettix psolus*, does not show typical lipid mobilization and lipophorin interconversion upon injection with extracts of its own corpus cardicum. The extracts as well as lipophorin and apoLp-III were however, functional when injected in other species known to respond to AKH by mobilizing lipids. The lack of a full response of the fat body to AKH or absence of a haemolymph factor was suggested to be responsible for the observed response (Ziegler *et al.*, 1988). Studies on the response of immature locusts without wings to AKH further showed

the partial response observed, was due to incompetence of the fat body to respond as well as the absence of haemolymph factor (Wheeler and Goldsworthy, 1983; Van der Horst *et al.*, 1987). Responses of the immature animals to AKH was further addressed by Van der Horst *et al.* (1987) using precocene to induce adult forms from 5th nymphal instars and azadirachtin to induce over aged nymphs. In the precocene treated insects, the responses observed resembled those of the adults. In azadirachtin-treated insects, the conversion of HDLp to LDLp was less complete and produced lipophorin species intermediate in size between HDLp and LDLp. This latter result was explained by the presence of less apoLp-III than in normal adults.

The initial event in the utilization of the fat body lipids involves the hydrolysis of triacylglycerol by lipases which are activated by AKH released at the onset of flight. Thus, the two phases would probably differ in their response to AKH at the level of either the activation of lipases or in the subsequent loading diacylglycerol onto HDLp. On the other hand, the loading may be influenced by the effectiveness of apoLp-III in stabilizing LDLp structure or by the levels HDLp and apoLp-III available in the haemolymph. Finally, it is possible that phase polymorphism in locusts is characterized by differential affinities of lipophorin receptors at the flight muscles and fat body for HDLp and LDLp.

This study was therefore, initiated to establish the effect of locust phase status on lipophorin structure and function. Results from this study, will be vital in the understanding of the phenomenon of phase transition in locusts, which to date is poorly understood. Furthermore, as was noted by Beenakkers, 1991, flight specific metabolic systems could be starting points for new devices in control strategies. In addition, if aspects of lipophorin structure and function may be specific to either of the phases, then it would be possible to

identify individuals of either phases biochemically.

1.6 Aims and objectives

The overall aim of the present study is to establish differences in lipophorin structure and function that are associated with the solitarious and gregarious phases of *S. gregaria*. If established such differences would shade in more light into how phase transition affects flight related metabolism, which to date is poorly understood. Such differences could also be exploited in the introduction of new devices for control strategies. Furthermore, such differences would be important in distinguishing the two locusts phases either from field isolates or laboratory-reared insects. The specific objectives were:

(1) Isolation and characterization of lipophorin and apolipophorin-III from the gregarious and the solitarious *S. gregaria* adults.

(2) Estimation of the lipophorin titres in the haemolymph during the development of the locusts, starting from the third nymphal instar.

(3) To determine ability of the locusts to mobilize lipids from the fat body in response to administration of adipokinetic hormone (AKH). Lipoprotein shifts associated with the mobilization was also studied.

(4) To determine the kinetic parameters of the fat body triacylglycerol lipase in both locust phases. The control levels as well as activated levels in response to AKH administration was to be determined.

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); Bio-Rad, (Richmond); Serva, (Germany); Peninsula laboratories, (USA) or Amersham, (England).

2.2 Experimental insects

The insects were reared by the Insect and Animal Breeding Unit (IABU) of ICIPE. The original stock of the locusts were obtained from the Red Sea and Addis-Ababa regions. At the beginning of the study, both phases were in the ninth generation since introduction. Gregarious locusts were maintained under crowded conditions (~ 200 per cage) whereas, the solitary ones were kept singly in cages a few hours after the emergence of the first nymphal instars. The two locust phases were kept in separate rooms maintained at 28-34° C, 40-50% relative humidity and daily illumination of 12 h. The diet mainly consisted of fresh sorghum plants or wheat brans. Adult male locusts were used 3-4 weeks after maturation. For the other development stages used in the study, male insects midway in a particular stadium, were selected. Each generation of the insects used in this study were monitored at the insectary for their phase status by morphometric mesurements, pigmentation as well as the existence of an extra molt in the case of the solitary locusts.

2.3 Haemolymph collection

Chilled insects were bled according to the methods described by Chino *et al.* (1987).

In the flush method, ice-cold bleeding buffer (130 mM NaCl, 5 mM KCl, 1.9 mM $NaH_2PO_4.H_2O$, 1.7 mM K_2HPO_4, 10 mM, EDTA, 0.02% NaN_3, pH 7.5) was slowly injected into the abdominal hemocoel through an incision made by severing one of the metathoracic legs. After injection of 0.4 ml of the buffer the haemolymph was drained off into Microfuge tubes. For each insect bled, 30 μl of bleeding buffer containing: 50 mM glutathione, 1.0 mM diisopropylphosphofluoridate, 2 mM aprotinin and 5 mM phenylmethyl sulphonyl fluoride and a few crystals of phenylthiourea was added.

In the puncture method, haemolymph was collected with a microsyringe through a puncture in the ventral neck membrane or through a membrane between the hind coxa and the abdomen. The haemolymph collected was diluted with a known volume of the bleeding buffer that contained the protease inhibitors and anti oxidants described above. Haemolymph samples were subsequently centrifuged (5,000 x *g*, 4° C, 10 min) to remove the haemocytes.

2.4 Lipophorin purification

The purification involved a single step density gradient ultracentrifugation (Shapiro *et al*, 1984; Haunerland and Bowers, 1986). In the preparation of high density lipophorin (HDLp), haemolymph obtained from resting adult insects was used whereas, for low density lipophorin (LDLp), haemolymph obtained from insects injected 90 min earlier, with 10 pmol of AKH was used. Haemocyte free haemolymph 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 QuickSeal centrifuge tube (Beckman), the sample was overlayed 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 overlayed with 0.9% NaCl was fractionated into 1.0 ml fractions after centrifugation. The fractions were used to estimate the protein content by the BCA method using Beckman DU-50 spectrophotometer and refractive indices using a refractometer (Bellingham Stanley Ltd). Densities of the fractions were computed from the formula: density = 6.4786 RI - 7.6430, (where RI is the refractive index at 25° C).

Aliquots (100 μl) of each fraction was dialysed against three changes of PBS (0.15 M phosphate/0.1 M NaCl, 7.0) and then analysed by SDS-PAGE. Fractions that contained the lipophorin yellow band were subsequently pooled. The 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.

2.5 Isolation of apolipophorin-III

Apolipophorin-III (apoLp-III) was isolated from LDLp using a method modified from Wells *et al.* (1985). Lipids were extracted from lyophylized LDLp samples using the chloroform: methanol (2:1) mixture, and the resulting pellet resuspended in 10 mM ammonium carbonate solution. After centrifugation (16,000 x *g*, 30 min, 4° C), the supernatant was applied onto a concanavalin-A-Sepharose column to remove contaminating apoLp-I and II. The flow through was then heated (70° C, 5 min) and the contaminating denatured proteins, centrifuged off (500 x *g*, 10 min). The final step involved microfiltration using 3 μm millipore filters (Kanost *et al.*, 1988; Telfer *et al.*, 1991). The isolated protein was subsequently dialysed against 0.2% acetic acid and the purity assesed using native and

SDS-PAGE.

2.6 Protein estimation

Protein estimation was carried out by the 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.

2.7 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% β-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 (4-20%) 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 (0.13 M 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 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) at room temperature. Gels were also stained for protein by the silver staining method of Wray *et al.* (1981). Native molecular weights of the proteins and the molecular weights of the apoproteins were estimated from plots of log. molecular weights versus the relative migration of the standards. Non-denaturing molecular weight standards were obtained from Pharmacia whereas, the SDS-PAGE standards were from Bio-Rad.

2.8 Isoelectro- focussing

Isoelectro-focussing was performed on PhastGel IEF (3-9) using the PhastSystem (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 PhastGel 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 Analysis of the lipid moiety

2.9.1 Gravimetric determination of the lipid content

Lipids were extracted from lipophorin or the fat body using chloroform: methanol (2:1) as described by Grapes *et al.* (1989). Lyophilized sample of lipophorin ($\approx$8.0 mg) was extracted three times for lipids by centrifugation (1,300 x *g*, 15 min, 10° C). The pooled supernatant was then evaporated to dryness under nitrogen gas and then redissolved in

10 ml of extraction solvent mixture. The extract was washed successively with 2.5 ml of aqueous KCl (0.88%) and methanol: water (1:1). The lower phase was then evaporated to dryness in a pre-weighed vials under nitrogen followed by Savant, SpeedVac concentrator. Total lipids were determined gravimetrically using Mettler AE 163 balance.

In the estimation of fat body lipid content, fat body was dissected from the locusts and immediately frozen in liquid nitrogen. The fat bodies were then lyophilized and the dry weights taken before lipid extraction and quantification as described above (Ziegler *et al.*, 1988).

2.9.2 Estimation of diacylglyceride levels

Estimation of diacylglyceride levels was done using the vanillin-phosphoric acid assay method as described by Van der Horst *et al.* (1978). To estimate the levels in the locust haemolymph, 10 μl of haemolymph sample was collected from the insects and added to 1.5 ml conc H_2SO_4. After a thorough vortexing, 0.45 ml was taken for the assay. The samples and the blank (0.45 ml of conc H_2SO_4) were heated for 10 min at 95° C and after cooling under a stream of tap water, 3.0 ml of vanillin reagent was added. Vanillin reagent consisted of 0.6% vanillin solution mixed in a ratio of 1:4 with phosphoric acid. Absorbance of the samples was determined at 536 nm after incubation at 40° C for 15 min.
A standard curve constructed using oleic acid (0-120 μg) in 0.45 ml of ethanol, was used to estimate the levels of diacylglycerides.

To estimate the levels of DG in other lipid or protein samples, known amounts of the lipid extract or protein was added to 0.45 ml of conc. H_2SO_4 and then processed as described above. Fractions from gel permeation chromatography were lyophilized, reconstituted in 1.5

ml of conc. H_2SO_4 then 0.45 ml used for DG estimation.

2.9.3 Fractionation of the lipid classes

Lipid extracts were separated into various classes using Bond-Elut amino columns (Jones Chromatography, U.K.) according to the method of Grapes *et al.* (1989). The columns were equilibrated with 2 x 2 ml of hexane before sample application. The lipid sample (8 mg for lipophorin and 16 mg for the fat body), was applied to the column and sequentially eluted into various fractions. Neutral lipids were eluted with chloroform: methanol (2:1); fraction 1. The column was then eluted with diethyl ether/glacial acetic acid (98:2) 4 ml; fraction 2: free fatty acids and then with methanol (4 ml; fraction 3: phospholipids). Fraction 1 was concentrated under nitrogen gas then applied to a second column and eluted with 4 ml of hexane to yield fraction 4: steryl esters. A third column was connected below the second column and co-eluted with hexane/dichloromethane/ diethylether (8:10:1; 20 ml, fraction 5: triacylglycerols and hexane/ethylacetate (95:5; 20 ml, fraction 6: sterols. The two columns were then separated and the upper one eluted with hexane/ethylacetate (85:15, 8 ml, fraction 7: diacylglycerides and chloroform/methanol (2:1; fraction 8: monoacylglycerides. The efficiency of the fractionation was monitored by thin layer liquid chromatography.

2.9.4 Transesterification of the lipid fractions

Fractions (2-8) obtained as described in 2.9.3, were evaporated to dryness under nitrogen and each re-dissolved in 0.4 ml of chloroform. Portions (200 μl) of fractions 2-5, 7 and 8 were transesterified in preweighed vials as described by Lepage and Roy (1986).

Methanol (800 μl) and heptane (200 μl) was added to vials followed by slow addition with constant agitation of acetylchloride (100 μl). The vials were sealed and weights taken and then incubated (100° C, 1 h). After cooling, the vials were weighed again and any that showed significant weight loss discarded. Contents were neutralized with 6% potasium carbonate (2.5 ml) and stored at 4° C for subsequent analysis in the gas chromatograph.

2.9.5 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 system; 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, cholestryl oleate, oleic acid, triolein and methyl oleate (Sigma Chemical Co.). The detection of lipids was done by iodine vapour.

2.9.6 Gas chromatography of the fatty acid methyl esters

Separation of the fatty acid methyl esters (FAMES) was carried out using CP Sil 8 capillary column (length: 50 m; id: 0.22 mm, film thickness: 0.21 μm; Chrompack, Middelburg, Netherlands) in a Hewlett Packard 5890A gas chromatograph. The carrier gas was hydrogen at an average velocity of 50 cm/s. Injection block and the detector were

maintained at a constant temperature of 250° C. Samples for Separating were diluted 1:1 with hexane and 1-2 μl injected. On injection, an oven temperature programme was initiated from 120° C rising linearly at 5° C/min to 230° C. The temperature was thereafter maintained until the end of the Separation at 49 min.

FAMES were quantified by integration of peak areas using Hewlett Packard integrator, model 3393A. External reference FAME standards were obtained from Sigma, UK. Percent composition of the various lipid classes was computed by comparison of the total peak areas of the FAME from a given lipid class and corresponding to standard FAME, and the total peak area of all the lipid classes arising from a lipid extract.

2.10 Analysis of the carbohydrate moiety

2.10.1 Staining for carbohydrates

The presence of covalently-bound carbohydrates was tested by staining proteins 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. Destaining was done using 7% acetic acid.

2.10.2 Determination of carbohydrate content

Percentage carbohydrate content 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 (80% in distilled water) added. Concentrated sulphuric acid (1.0 ml) was then added to each of the

assay mixtures and absorbance measured at 480 nm after 30 min.

2.10.3 Affinity chromatography on concanavalin A Sepharose.

Concanavalin A-Sepharose column (10 x 1.0 cm, i.d) was equilibrated with concanavalin A (Con A) buffer (0.01 M Tris, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 M NaCl, 0.02% NaN_3, pH 7.5). Samples were dialysed overnight in Con A buffer and thereafter approximately 1 mg of the protein applied to the column. The column was washed with Con-A buffer then eluted with 500 mM ∝-methyl-D-mannopyranoside in Con A buffer. Fractions (1.0 ml) were obtained and absorbance monitored at 280 nm (Osir *et al.*, 1989).

2.11 Immunological studies

2.11.1 Preparation of polyclonal antibodies

Antibodies against high density lipophorin and apolipophorin-III were raised in New Zealand White rabbit a (Robbs *et al.*, 1985). Protein (≈ 1.0 mg) emulsified in Freund's complete adjuvant was injected intramuscularly into the rabbit. A booster injection of (~ 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 (1,000 x *g*, 30 min). The supernatant was stored at -70° C in 0.1% sodium azide.

2.11.2 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 or apoLp-III the anti-serum was poured in the central well and the corresponding antigen in the peripheral wells. The presence of HDLp and apoLp-III in the haemolymph of the various development stages was also tested by pouring haemolymph samples from the stages in the peripheral wells and the appropriate antiserum in the centre well. Immunological cross-reactivity was also tested between the proteins from the two locust phases. Antibodies to *L. migratoria* HDLp and apoLp-III (donated by A. Th. Beenakkers, University of Utrecht, The Netherlands), were also tested against HDLp and apoLp-III from *S. gregaria*. The immunodiffusion slides were placed in moist chambers and the proteins left to diffuse at room temperature for 24 h. The slides were then washed extensively with PBS and water to remove excess proteins, dried, stained with Coomassie Brilliant Blue and finally destained for examination.

2.11.3 Single radial immunodiffusion

Single radial immunodiffusion was carried out to estimate the levels of lipophorin in the haemolymph of the insects, from the third nymphal instar to mature adults (Mancini *et al.*,1965; Gonzalez *et al.*, 1991). Agarose (0.9%) in 0.1 M sodium phosphate buffer (0.3 M NaCl, 5 mM EDTA, 0.01% NaN_3, PH 7.2), was melted and maintained at 45° C. Antibodies to lipophorin, preheated to 45° C, was added to the agarose solution and mixed to a final concentration of 0.85%. The agarose-antibody solution was then poured onto glass

slides (5 ml per 20 cm^2) and allowed to solidify.

Diluted haemolymph samples (10 μl), were poured into the wells made on the slides and allowed to diffuse for 72 h. Standard curve was prepared using lipophorin samples (10 μl) of known concentrations (5-1500 μg/ml). The slides were then washed with several changes of PBS and water. After drying the plates, staining with Coomassie Brilliant Blue was done for 30 min followed by destaining until a clear background was obtained. Perpendicular diameter of the precipitin rings was obtained using Sylvac electric vanier callipers (Trismos, Sylvan, UK). The values obtained were multiplied to compensate for slight deviations from circularity. Values obtained with lipophorin samples were used to plot a standard curve of diameter squared (d^2) versus log. lipophorin concentration. Titers of haemolymph lipophorin was based on the comparison d^2 values obtained with the haemolymph samples and the standard curve.

2.11.4 Immunoblotting

Electrophoretic transfer of antigens from polyacrylamide gels to nitrocellulose (Hybond C, Amersham) was carried out according to Towbin *et al.*, 1979) using an LKB-NOVA BLOTT (Broma, Sweden) electrophoretic transfer kit. Electrode buffer (39 mM glycine, 48 mM Tris) was used to saturate both the anodic and cathodic graphite plates. The transfer was performed for 2 h at a constant current of 0.8 mA per cm^2. Following the transfer, the non-specific sites were blocked using 3% gelatin in Tris buffered saline (TBS)(20 mM Tris, 500 mM NaCl, pH 7.5). After washing three times with TBS-tween-20 (0.1%)(TTBS), the blot was incubated with the raised antibody diluted 300 times with TTBS containing 0.3% gelatin for 2 h. This was followed by washing as before, then the blot

incubated with biotinylated goat anti-rabbit IgG, diluted 1000 times in TTBS for 60 min. After washing off the unbound secondary antibody complex, the blot was incubated with streptavidin labelled horse radish peroxidase conjugate (diluted 3000 times with TTBS) for 30 min. Colour development was done using 0.3% 4-chloronapthol in methanol (diluted five times with Tris-HCl buffer(TB) (20 mM Tris, pH 6.8). Hydrogen peroxide was added in aliquots (2 μl) until the desired colour background was obtained. The nitrocellulose paper was rinsed in distilled water and stored for photography.

2.12 Responses to adipokinetic hormone

2.12.1 Time course studies

Varying doses of adipokinetic hormone AKH-I (2, 5 and 10 pmol) in 10 μl of BSA (1 mg/ml) was injected into groups of experimental locusts, between the first and the second abdominal segments. Control insects were injected with 10 μl of BSA (1 mg/ml) (Van der Horst *et al.*, 1979). Haemolymph samples (5 μl) were taken through a puncture in the membrane between the hind coxa and the abdomen, prior to the injection and at 30 min interval for 150 min. The samples were added into 0.75 ml of conc H_2SO_4, and after thorough mixing, 0.45 ml was used in the estimation of diacylglycerides as previously described.

2.12.2 Dose response studies

In order to determine the sensitivity of the locusts to AKH, varying doses (1-10 pmol and 10^{-14}-10^{-8} mol/insect) in 10 μl of BSA was injected into the locusts as described above. Control insects were injected with 10 μl BSA. Haemolymph samples (10 μl) was collected

after 90 min and added to 1.5 ml of conc. H_2SO_4. An aliquot (0.45 ml) was used in the estimation of diacylglycerides.

2.12.3 Lipoprotein profiles in response to AKH

Haemolymph lipoprotein profiles were determined by gel permeation chromatography on AcA_{22} column (Pharmacia, Upssala, Sweden) as described by Van der Horst *et al.* (1987, 1989). Groups of six locusts from both solitary and gregarious phases were injected with 10 μl of BSA for control groups and 10 pmol AKH-I for the experimental groups. After 90 min, haemolymph was flushed out and pooled for each group and the haemocytes removed as previously described.

The column (160 x 1.6 cm) was equilibrated with the elution buffer (130 mM NaCl, 5 mM KCl, 1.7 mM K_2HPO_4, 1.9 mM $NaH_2PO_4.2H_2O$, 5 mM EDTA, 0.02% NaN_3 pH 7.5). Calibration was carried out using molecular weight standards from Bio-Rad. Haemolymph samples (30 mg) from each group of insects was applied to the column and the eluate, at a flow rate of 12 ml/h, monitored continuously at 280 nm with a Pharmacia UV absorption detector. Fractions (3 ml) were collected and the protein and lipid contents estimated. The lipid content was estimated using the vanillin phosphoric acid method on selected lyophilized fractions.

2.13 Studies on fat body triacylglyceride lipase

2.13.1 Preparation of the enzyme

Triacylglyceride lipase was extracted from the locust fat body using a method modified from Hirayama and Chino (1987). Fat bodies were dissected out from chilled

insects, rinsed in homogenising buffer (0.25 mM EDTA, 10 mM Tris-HCl, pH 7.4 and then blotted dry with a filter paper. The tissue was then homogenized in 0.5 ml of the of the homogenizing buffer using Polytron homogenizer (Kinematica GmbH, Littar-Luzern, Switzerland) at level 5 for ten passes. The homogenate was centrifuged (14,000 x *g*, 20 min, 4° C) and the floating fat layer removed using a spatula. The infranatant was re-centrifuged at the same speed for 10 min and the supernatant kept at -20° C. Protein estimation was done on the samples before enzyme assays.

To study the activation of the lipase with AKH, fat bodies were dissected out 25 min after injection of the hormone. Control insects in these studies were injected with 10 μl of BSA (1 mg/ml).

2.13.2 Standard substrate preparation

Radiolabeled triolein (^{14}C-) was mixed with non-labeled triolein to yield about 3 x 10^{12} dpm/mol. Stock substrate solution was prepared by dissolving the triolein mixture in ethanol to a final concentration of 1 mM and containing 130 mM triton-X-100 (Hirayama and Chino, 1987). This solution was aliquoted (1 ml) and stored at -20° C in Microfuge tubes filled with nitrogen gas. The substrate samples were warmed to room temperature before use.

2.13.3 Triacylglyceride lipase assay procedure

Enzyme assay was performed as described by Hirayama and Chino, (1987). To estimate the resting levels of the lipase, stock substrate (70 μl) was transferred to each test tube followed by addition of 0.93 ml of assay buffer (0.5 M NaCl, 20 mM MOPS-NaOH, pH 6.9). The reaction was initiated by the addition of 0.1 ml of the enzyme solution. In

order to estimate the kinetic parameters of the lipase, a range of substrate concentrations (1-30 μM) with the volume made up to 100 μl with ethanol, was used. Assay buffer (0.95 ml) was added and the reaction initiated by addition of 50 μl of the enzyme.

After incubation for 90 min at 30 °C, the assay was stopped by addition of 5 ml of fatty acid extraction solution (chloroform: methanol: benzene, 2:2.4:1), containing 0.5 μmol of unlabeled oleic acid as a carrier and 0.1 ml of 1 N NaOH was then added. The mixture was vortexed and 1.0 ml of the upper aqueous phase containing free fatty acid fraction transferred to a scintillation counter containing 10 ml of aquasol as the scintillant. The radioactivity was counted using Hewlet Packard Scintillation counter. An estimated efficiency of 84% was used in the conversion of cpm to dpm. Estimation of lipase activity was based on the worked out dpm equivalent to 1 μmol of free fatty acid released and expressed as nmol free fatty acid/h/mg fat body protein.

2.14 Statistical analysis

Statistical analysis were performed using the statistical Analysis System (SAS, Institute, 1985). Means were tested for significance using either by the Students *t-test* or ANOVA (Analysis of variance). Unless stated otherwise, rejection of the null hypothesis was based 0.05 % confidence interval . Straight line graphs were drawn by linear regression.

CHAPTER 3

3 ISOLATION AND CHARACTERIZATION OF LIPOPHORIN AND APOLIPOPHORIN-III.

3.1 Isolation of lipophorin

Lipophorin was isolated from the haemolymph of the solitary and gregarious locust using a single step KBr density gradient ultracentrifugation. In the preparation of HDLp, haemolymph was obtained from the resting insects whereas for LDLp, haemolymph was obtained from insects which had been injected with AKH, 90 min earlier. In the case of HDLp, the lipoprotein separated out in the upper portion of the QuickSeal tube with a characteristic yellow banding (Fig. 1a). The fractions monitored at 450 nm peaked up at densities of 1.114 and 1.105 g/ml for solitary and gregarious locusts, respectively. On the other hand, preparation of LDLp resulted into yellow banding of lower densities of 1.074 and 1.066 g/ml for solitary and gregarious locusts respectively (Fig. 1b). Thus, the administration of AKH resulted into a lipoprotein molecule of lower density. In the preparation of LDLp, there was evidence of yellow banding at positions corresponding to HDLp which suggested that administration of the hormone did not result into total conversion of HDLp to LDLp. This was also evident in the ultracentrifugation profiles in LDLp separation, especially with the solitary locusts (Fig. 1b). Fractions containing HDLp or LDLp were confirmed by SDS-PAGE profiles and the appropriate lipoprotein peaks pooled and dialysed (Fig. 2a, b). From the SDS-PAGE of the ultracentrifugation fractions, there was no evidence of contamination from the subphase.

Fig. 1a **KBr density gradient ultracentrifugation profile of haemolymph**. Fractions (1.0 ml) were taken from the top of the tube and absorbance at 450 nm and refractive indices monitored for odd numbered fractions:

——·—— absorbance for solitary locusts

——— absorbance for gregarious locusts

- - - - - density (g/ml)

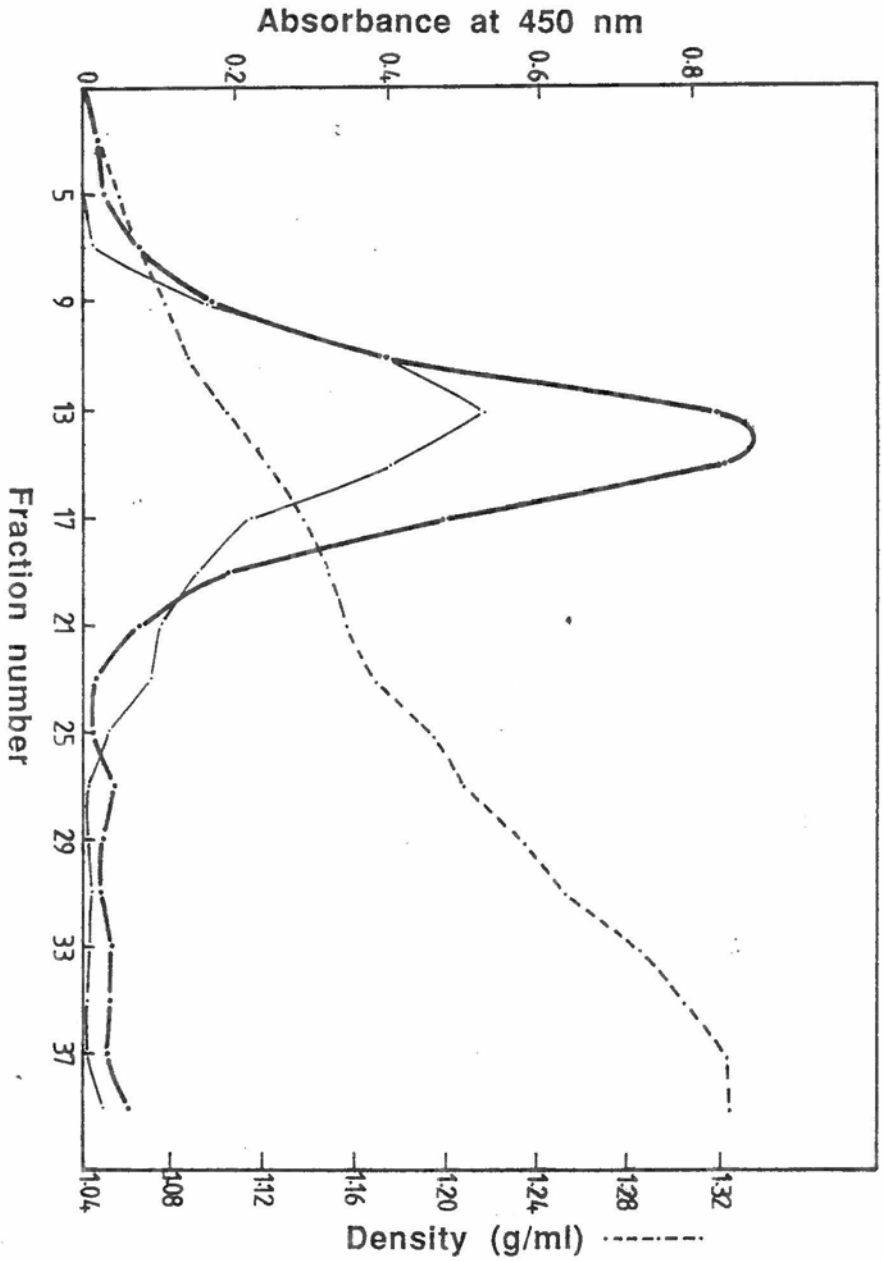

Absorbance at 450 nm
0
0.2
0.4
0.6
0.8
Fraction number
5
9
13
17
21
25
29
33
37
Density (g/ml)
1.04
1.08
1.12
1.16
1.20
1.24
1.28
1.32

Fig. 1b **KBr density gradient ultracentrifugation profile of haemolymph following injection of AKH.** Details as in (a) above.

—△— absorbance for solitary locusts

····□··· absorbance for gregarious locusts

—⊟— density (mg/ml)

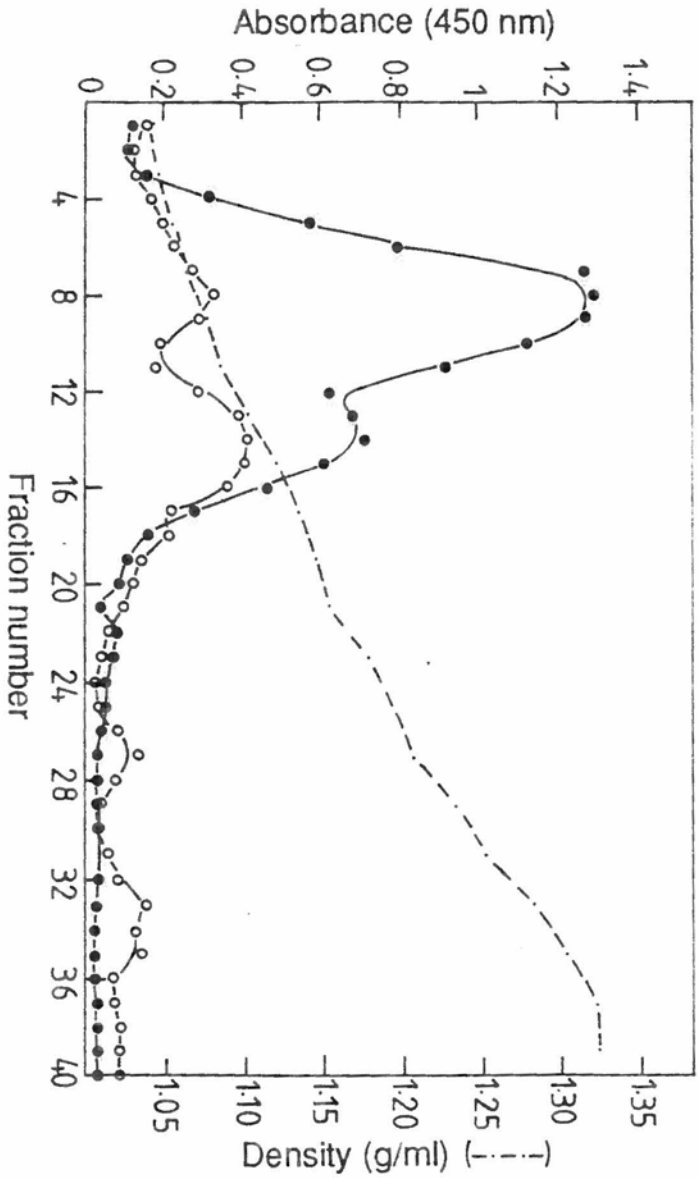

Absorbance (450 nm)
0
0·2
0·4
0·6
0·8
1
1·2
1·4
Fraction number
4
8
12
16
20
24
28
32
36
40
Density (g/ml) (-·-·-)
1·05
1·10
1·15
1·20
1·25
1·30
1·35

Fig. 2a **SDS-PAGE of haemolymph proteins of gregarious locusts following density gradient ultracentrifugation.** Fractions (100 μl) were dialysed against PBS and 30 μl applied onto the gels. Numbers at the top show fraction numbers from the top of the tube. L (Low molecular weight standard) and H (High molecular weight standard)

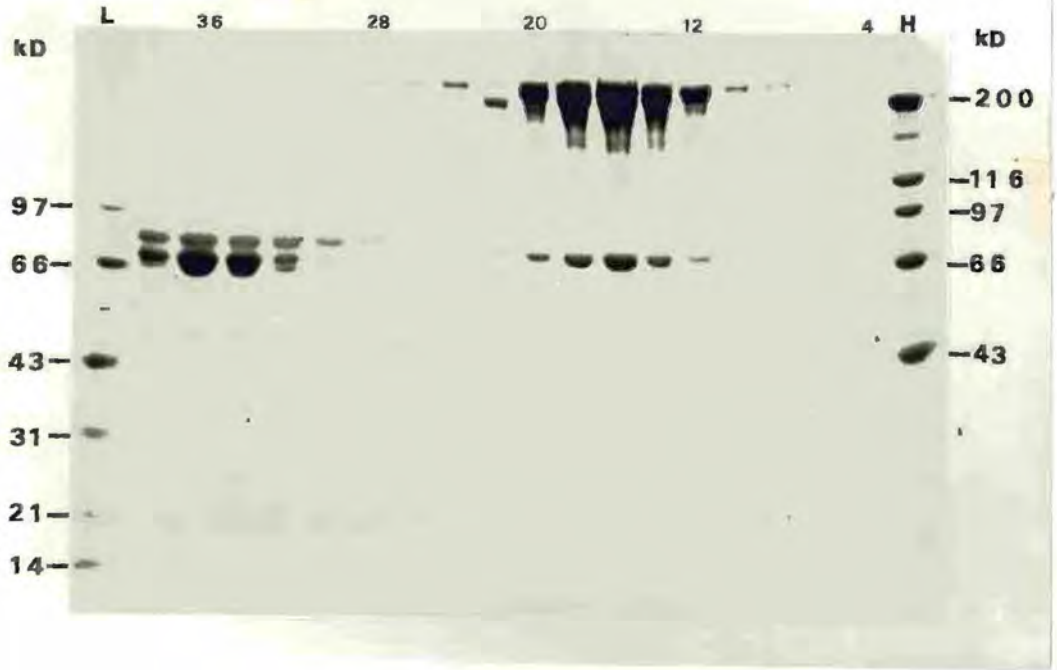
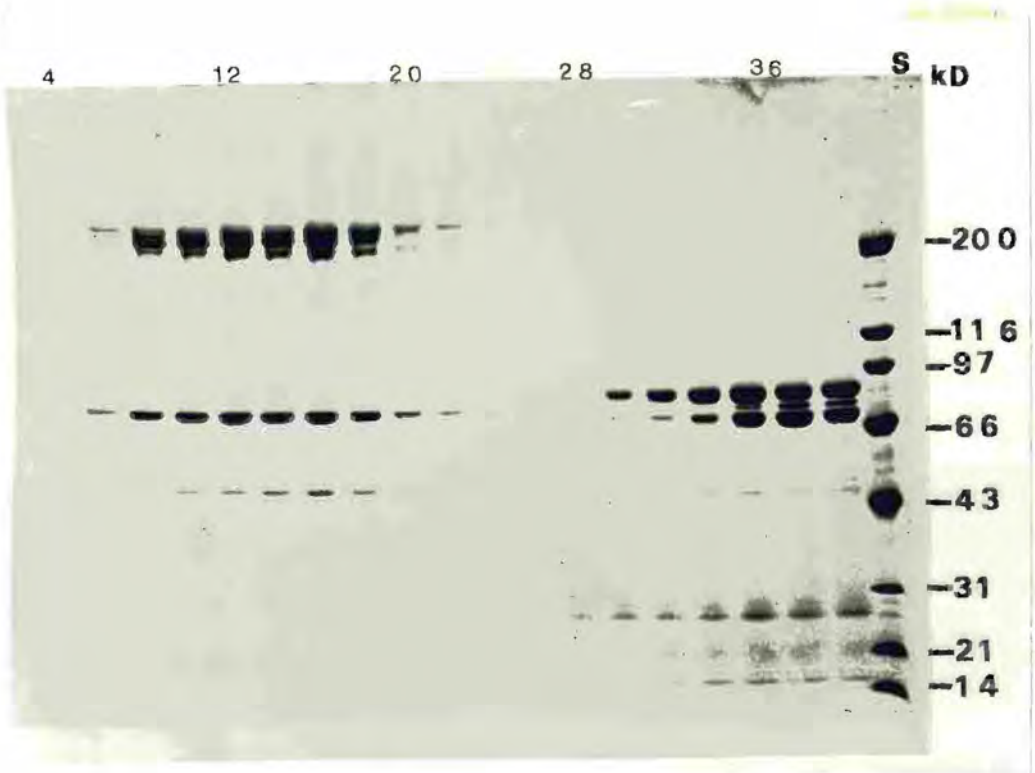

L
36
28
20
12
4
H
kD
kD
200
116
97
66
43
97
66
43
31
21
14

Fig. 2b **SDS-PAGE of haemolymph proteins of solitary locusts following density gradient ultracentrifugation.** Fractions obtained were processed as described in Fig. 2a. S (Mixture of high and low molecular weight standards)


4
12
20
28
36
S
kD
200
116
97
66
43
31
21
14

HDLp was shown to correspond to fractions 9-19, with a peak concentration observed on fraction number 13. On the other hand, LDLp was obtained between fractions (4-9) with a peak concentration on fraction number 8. The isolated lipoproteins were shown to be pure using both non-denaturing (Fig. 3a) and SDS-PAGE (Fig. 4a).

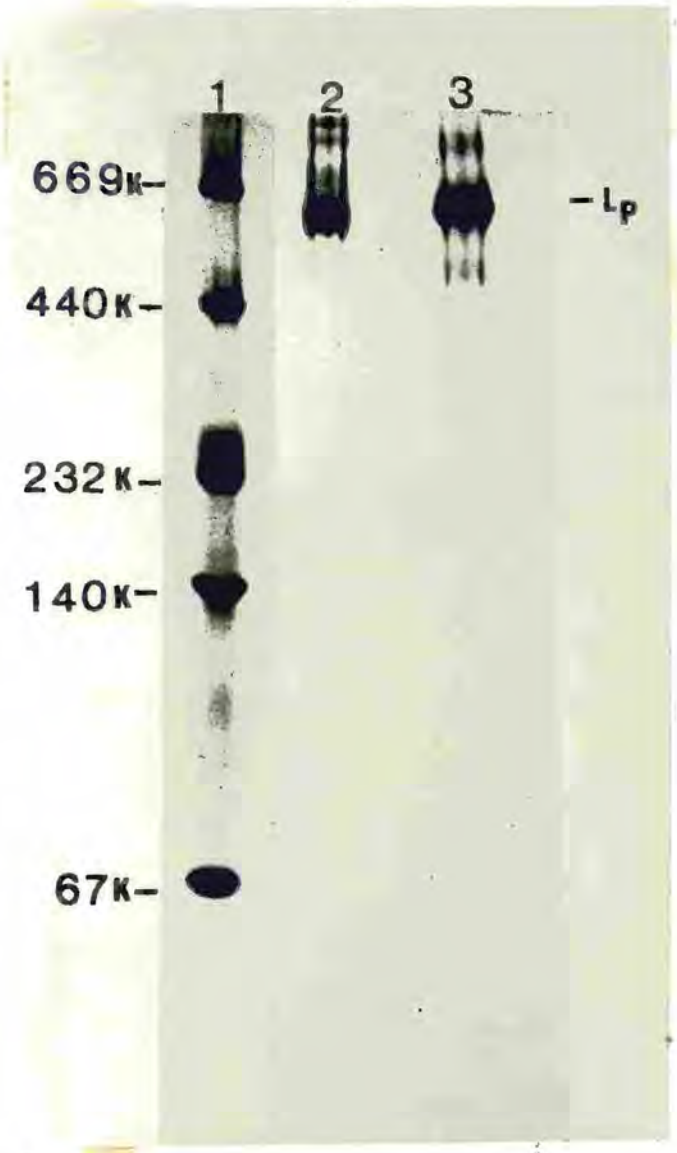
3.2 Physical-chemical characterization of lipophorin

3. 2. 1 Apoprotein composition

The HDLp isolated from the two phases of *S. gregaria* showed structural similarity in both the native form and in apoprotein composition. Non-denaturing PAGE revealed a native molecular size of $M_r \sim$ 620,000)(Fig. 3a, b), while the SDS-PAGE showed the presence of two apoproteins (apoLp-I, $M_r \sim$ 224,000, and apoLp-II, $M_r \sim$ 81,000) (Fig. 4a, b). A third apoprotein (apoLp-III) was present only in trace amounts which could be detected with silver but not with Coomassie Brilliant Blue staining. LDLp isolated from both solitary and gregarious locusts were shown to have the same apoprotein composition. The apoproteins, apoLp-I and apoLp-II, were of the same molecular size and intensity on staining as in HDLp described above. However, the third apoprotein, apoLp-III ($M_r \sim$ 20,000) was more intense even on staining with Coomassie Brilliant Blue (Fig. 4a, b). The native molecular weight of LDLp as estimated by gel permeation chromatography on AcA_{22} column was $M_r \sim 1.7 \times 10^6$ for solitary (Fig. 5). On the other hand, the LDLp from the gregarious locusts was totally excluded in this column. Thus, the gregarious locusts LDLp had a higher molecular weight compared to the solitary locusts. Analysis of LDLp by non-denaturing-PAGE was not possible due to its large size. In most preparations of HDLp and LDLp, apoLp-I appeared as a doublet. A degradation product of molecular weight $M_r \sim$ 50,000 was also observed in

Fig. 3a **Non-denaturing PAGE (4-20%) of isolated lipophorin.** Lane 1, Pharmacia molecular weight standards; lane 2, HDLp (gregarious) (20 μg); lane 3, HDLp (solitary) (20 μg)

Fig. 3b **Standard curve for native molecular weight determination.** Relative mobility on non-denaturing PAGE (4-20%) was plotted against log. molecular weights of Pharmacia standards. Molecular weights corresponding to HDLp were as indicated.


1
2
3
669K-
440K-
232K-
140K-
67K-
-Lp

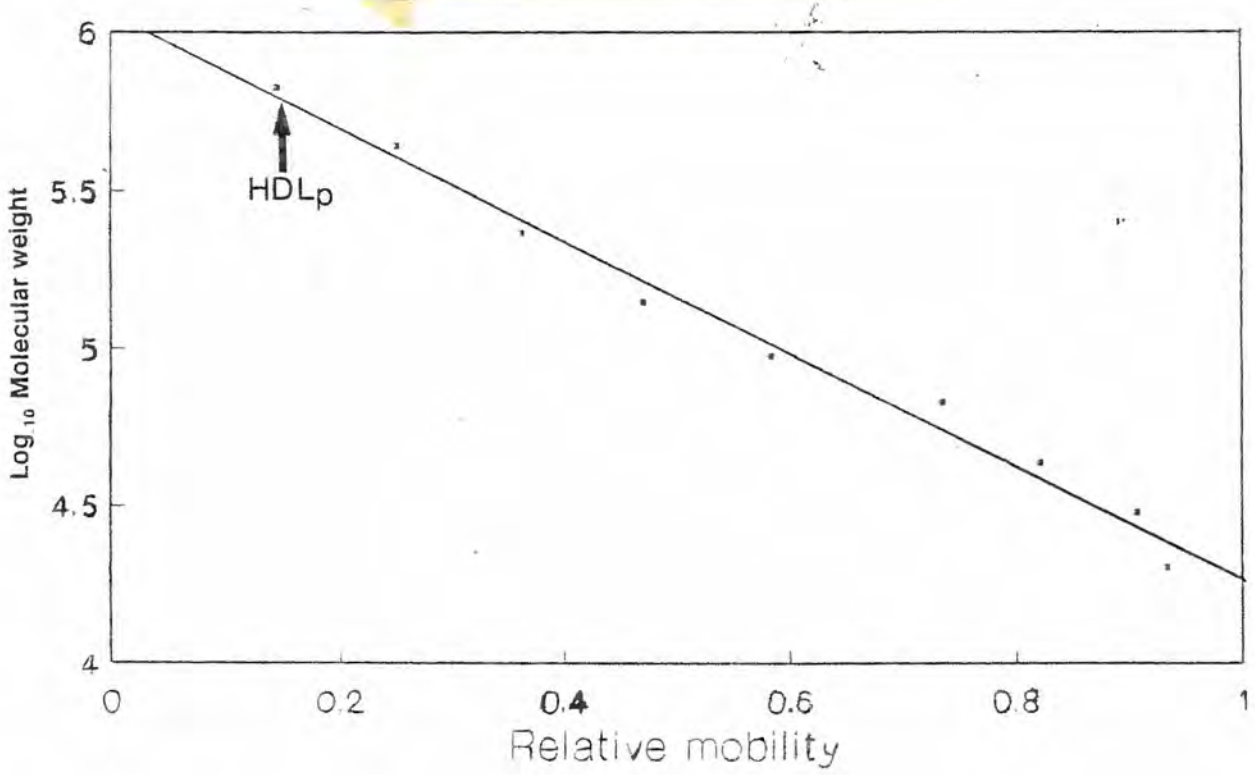
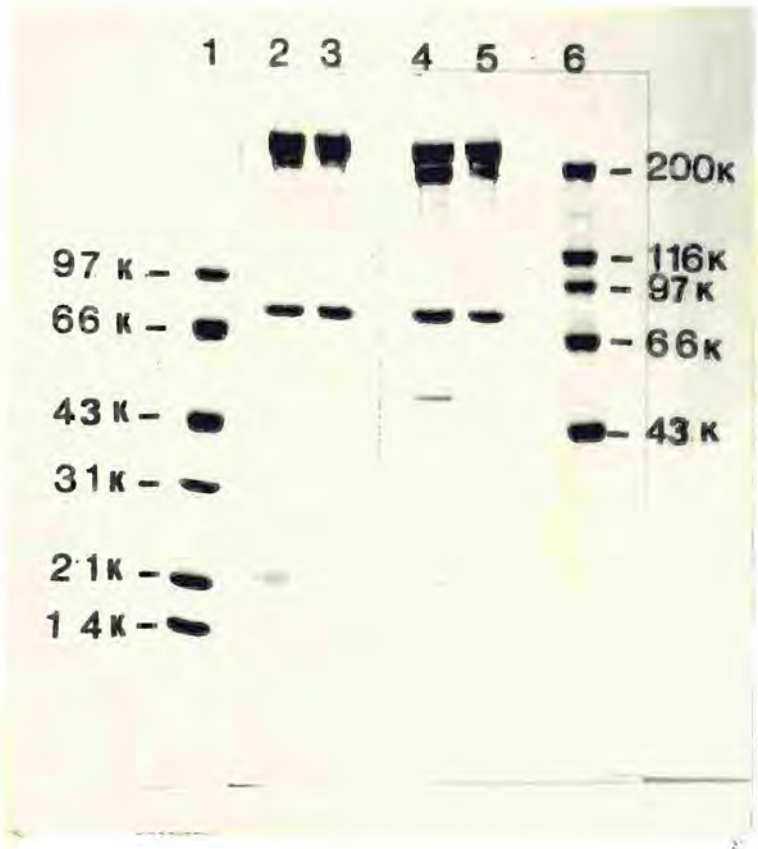

6
5.5
5
4.5
4
HDLp
Log10 Molecular weight
0
0.2
0.4
0.6
0.8
1
Relative mobility

Fig. 4a **SDS-PAGE (4-15%) of isolated low- and high- density lipophorin**. Lanes 1 and 6, molecular weight standards; lane 2, LDLp (gregarious) (20 μg); lane 3, HDLp (gregarious) (20 μg); lane 4, LDLp (solitary) (20 μg); lane 5, HDLp (solitary) (20 μg).

Fig. 4b **Standard curve for molecular weight determination. on SDS-PAGE (4-15%).** Relative mobility was plotted against log. molecular weight of the standards. Molecular weights of the apoproteins were as shown.


1 2 3 4 5 6
200K
116K
97K
66K
43K
97 K
66 K
43 K
31K
21K
14K

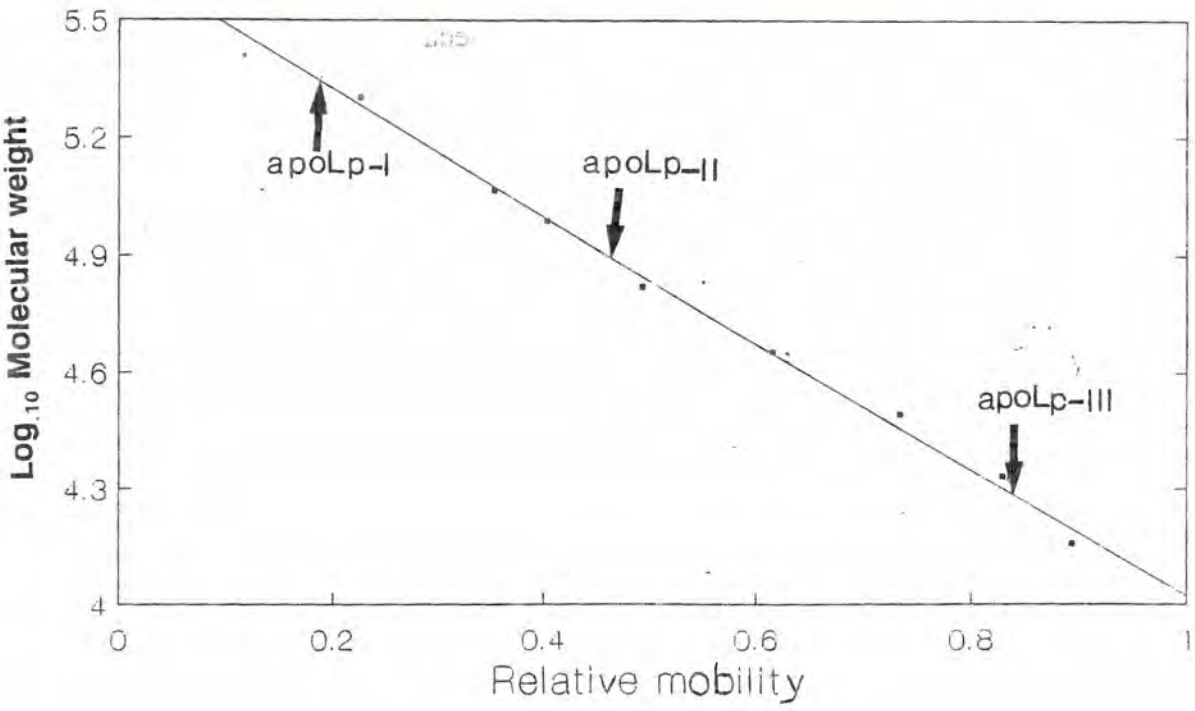

Log10 Molecular weight
5.5
5.2
4.9
4.6
4.3
4
0
0.2
0.4
0.6
0.8
1
Relative mobility
apoLp-I
apoLp-II
apoLp-III

Fig. 5 **Callibration curve for gel permeation chromatography on AcA_{22} column.** Kav values were plotted against the log. of molecular weight of the standards. The Bio-Rad standards included: thyroglobulin (670 Kd), gamma globulin (158 Kd), ovalbumin (44 Kd), myoglobulin (17 Kd), cyanocobalamin (1,350). The molecular weights of the lipoproteins were as shown by arrows.

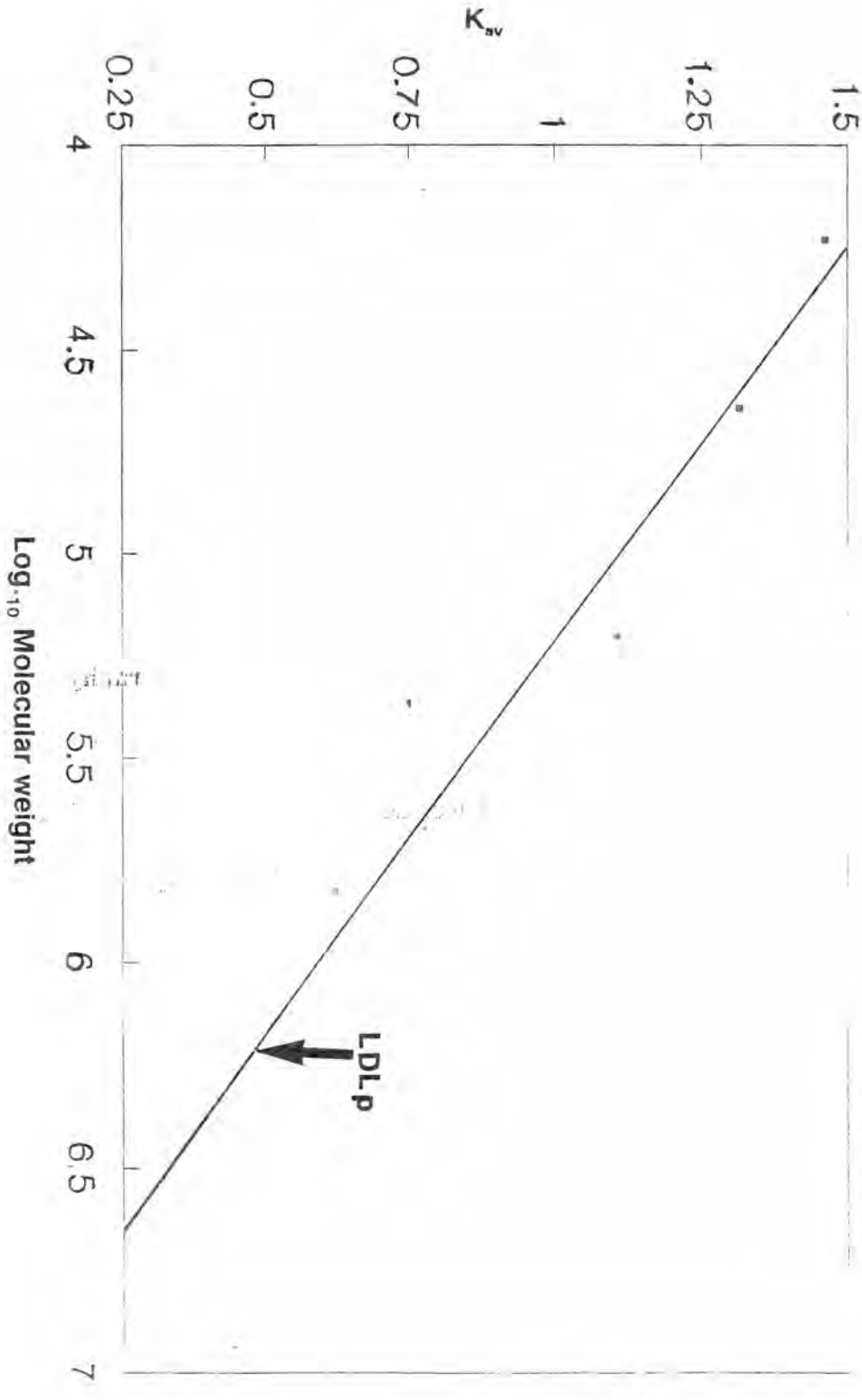
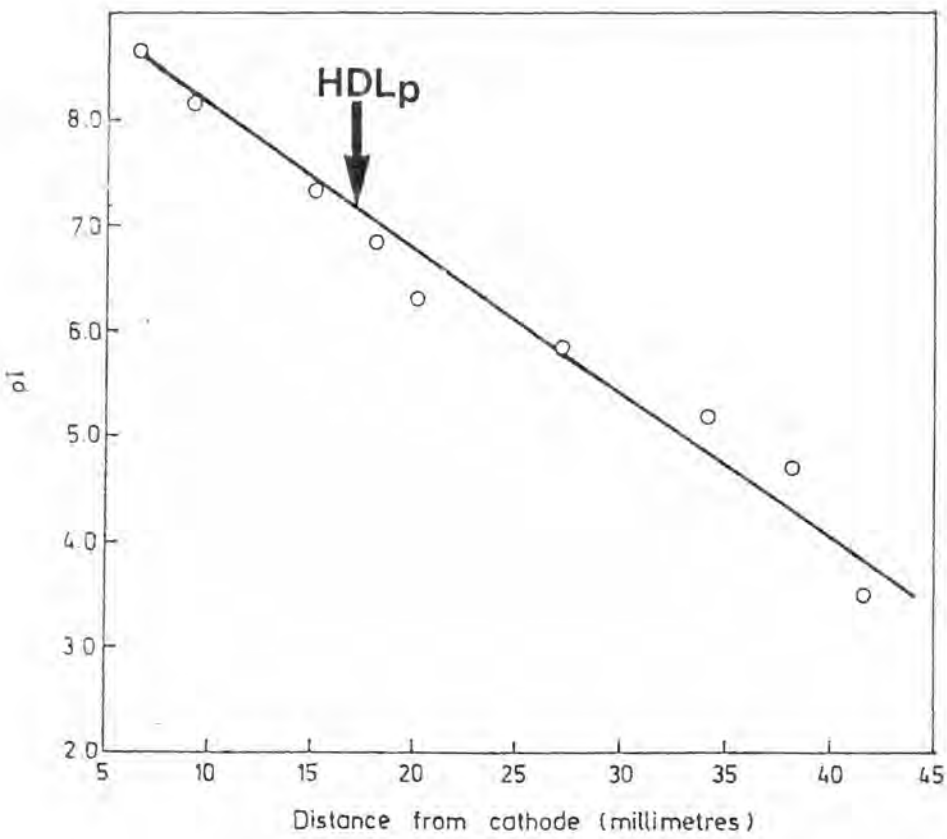

K_{av}
0.25
0.5
0.75
1
1.25
1.5
4
4.5
5
5.5
6
6.5
7
Log_{10} Molecular weight
LDLp

Fig. 6 **Isoelectric focusing of S. gregaria lipophorin on PhastGel IEF medium (3-9).** The migration distances were measured from the cathode in millimetres. The calibration sample contained: lentil lectin (pI ~ 8.65); lentil lectin (pI ~ 8.45); lentil lectin (pI ~ 8.15); horse myoglobin (pI ~ 7.35); horse myoglobin (pI ~ 6.85); human carbonic anhydrase (pI ~ 6.55); bovine carbonic anhydrase (pI ~ 5.85); β-lactoglobulin (pI ~ 5.20); soya bean trypsin inhibitor (pI ~ 4.55); amyloglucosidase (pI ~ 3.5). The pI of HDLp from solitary and gregarious locusts were as shown by arrow.


HDLp
8.0
7.0
6.0
5.0
4.0
3.0
2.0
pI
5
10
15
20
25
30
35
40
45
Distance from cathode (millimetres)

product of molecular weight $M_r \sim$ 50,000 was also observed in SDS-PAGE of some preparations of HDLp or LDLp(Fig. 4a). The isolelectric points for the isolated HDLp were estimated at 6.20 for both for solitary and gregarious locusts(Fig. 6a, b).

3. 2. 2 Analysis of the carbohydrate moiety

The presence of carbohydrate moiety on lipophorin was demonstrated by PAS staining. LDLp isolated from both solitary and gregarious locusts was separated by SDS-PAGE and stained for carbohydrates. All the three apoproteins, apoLp-I, -II and -III were shown to be glycosylated (Fig. 7). However, the staining of apoLp-III with PAS was faint and not evident after storage of the gels. Gravimetric determination of the carbohydrate content was carried out using the phenol-sulphuric acid method. A standard curve was constructed with D- mannose as the standard (Fig. 8). The carbohydrate content of HDLp expressed as a percentage of the protein was estimated to be 3.43 and 3.12% for solitary and gregarious locusts, respectively (Table 1). The percent content in LDLp was estimated to be 5.10 and 7.99% for gregarious and solitary locusts, respectively. Further analysis by concanavalin A-Sepharose showed that the carbohydrate moiety had mannose rich oligosaccharide chains (Fig. 9).

3. 2. 3 Analysis of lipophorin lipid moiety

The percent lipid composition of the isolated HDLp and LDLp was gravimetrically determined in both phases of *Schistocerca gregaria* (Table 1). Lipids constituted 51.8 and 48.4% (wt%) of HDLp isolated from gregarious and solitary locusts, respectively. In the case of LDLp, lipids constituted 57.1 and 59.8%(wt%) for solitary and gregarious locusts,

Fig. 7 **Periodate Schiff (PAS) staining of LDLp isolated from *S. gregaria*.** LDLp samples from solitary (S) and gregarious (G) locusts were separated by SDS-PAGE and stained as described in Materials and Methods. Lane 1, LDLp (G) (50 μg); lane 2, LDLp (S) (50 μg).


1
2

Fig. 8 **Standard curve for gravimetric determination of carbohydrate content.** The samples for the determination were hydrolysed with H_2SO_4 (10 N), followed by incubation with 80% phenol for 30 min. Absorbance was determined at 480 nm.

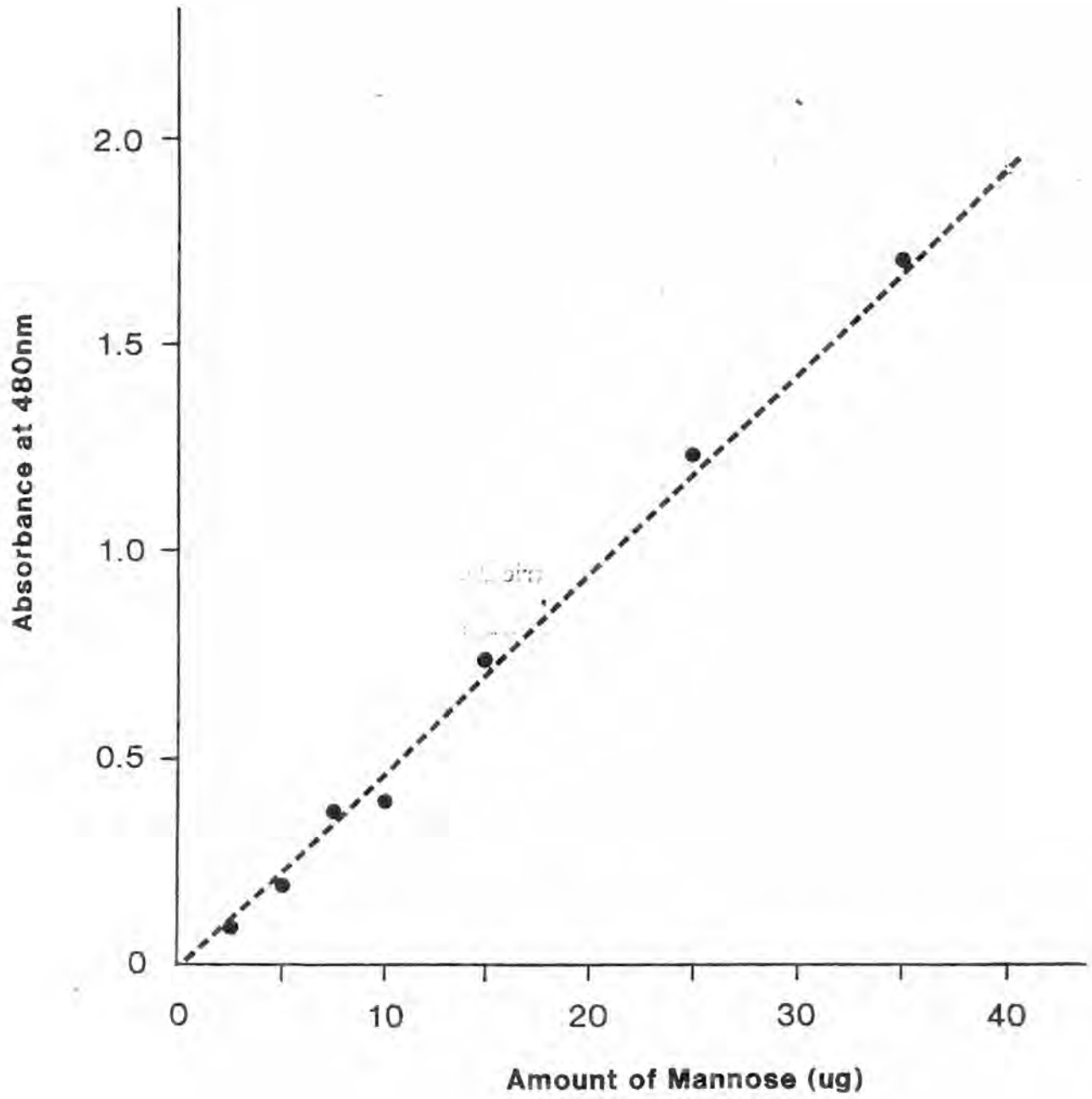

Absorbance at 480nm
2.0
1.5
1.0
0.5
0
0
10
20
30
40
Amount of Mannose (ug)

Fig. 9 **Elution profile of HDLp from gregarious locusts on Concanavalin A-Sepharose**. The column (1.0 x 10 cm) was equilibrated with concanavalin- A buffer prior to sample application. Elution was done with 500 mM methyl ∝-D-mannopyranoside and fractions (2 ml) monitored at 280 nm.

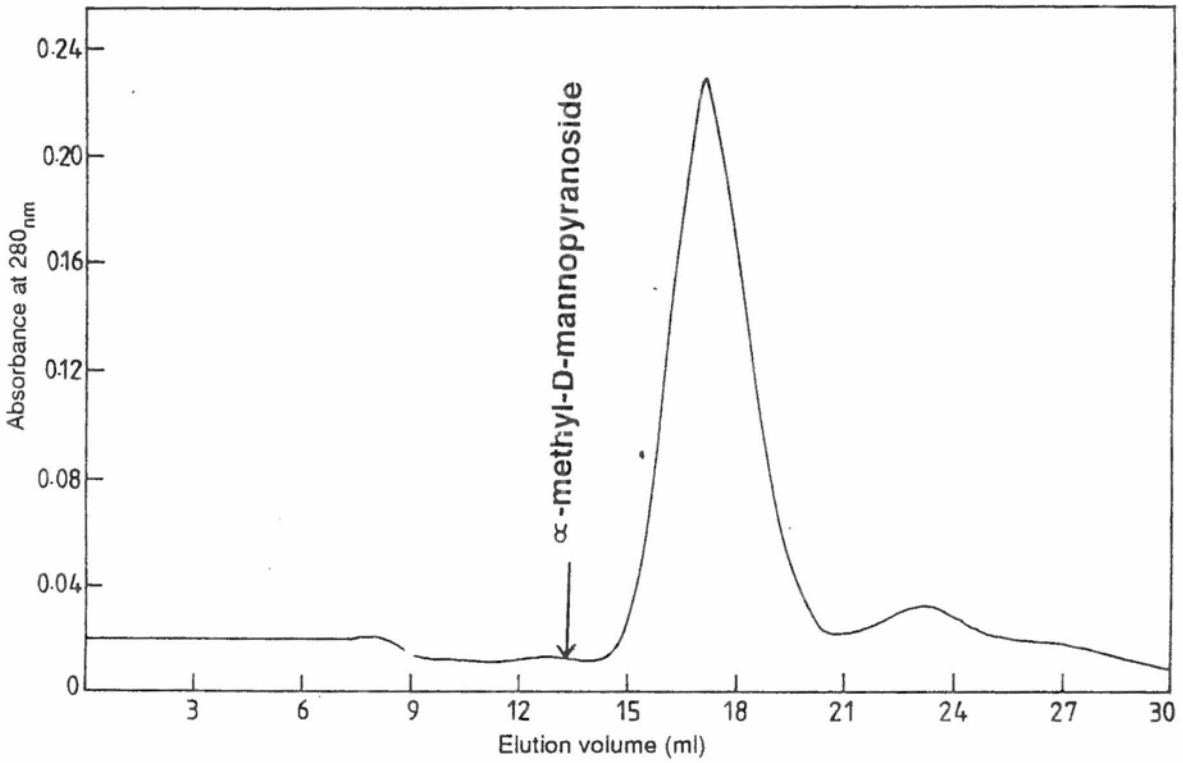

0·24
0·20
0·16
0·12
0·08
0·04
0
Absorbance at 280nm
α-methyl-D-mannopyranoside
3
6
9
12
15
18
21
24
27
30
Elution volume (ml)

Table 1 **Proportional compostion (wt%) and densities of lipophorin from solitary and gregarious *S. gregaria***

		Protein	Lipid	Carbohydrate	Density (g/ml)
HDLp	GREG.	48.22	51.78	3.12	1.105
	SOL.	51.64	48.36	3.43	1.114
LDLp	GREG.	40.20	59.80	7.99	1.066
	SOL.	42.93	57.07	5.10	1.074

Lipid content was gravimetrically determined on lyophilized sample of lipophorin following thorough dialysis. The carbohydrate content is expressed as a percentage of the protein content. GREG (gregarious locusts), SOL (solitary locusts).

respectively. The results showed a higher lipid content in LDLps than in HDLps indicating that the administration of AKH resulted in more lipid associating with the lipoprotein molecule, hence the decrease in lipoprotein density.

Further analysis of the lipid components was done by gas chromatography. The lipid extracts were separated into various lipid classes using Bond-Elut amino columns using different solvent mixtures. The efficiency of the separation of the lipid classes was monitored by TLC as explained in Materials and methods. The relative percentage (wt%) composition of lipid classes present in HDLp and LDLp from both locusts phases is shown in Table 2. In both HDLp and LDLp, the major lipid classes present were shown to be phospholipids and diacylglycerides. The other lipid types present included triacylgycerides, monoacylglycerides, steryl esters and free fatty acids. In the gregarious locusts, the percent composition of phospholipids and diacylglycerides in HDLp was 26.7% and 43.1% , respectively, whereas in LDLp, percent phospholipids and diacylglycerides was estimated to be 22 and 60%, respectively. In the case of the solitary locusts, the percent composition of phospholipids and diacylglycerides in HDLp was 25.8 and 35.5%, respectively, while in LDLp, the percent PL and DG was 31.4 and 48%, respectively. Evidently, the major lipid mobilized from the fat body was DG resulting into LDLp particles with a much higher level of DG as compared to that in HDLp. The DG percent composition in LDLp is much more increased in gregarious than solitary locusts. Minor differences were also evident between the phases for other lipid classes. Gas chromatograph analysis of methylated fatty acids from the lipid extracts showed the presence of palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic (18:2) and linolenic acid (18:3) among other fatty acids (Table 3).

Table 2 Relative percentage (wt%) composition of lipid lasses in LDLp and HDLp from solitary an gregarious locusts

	Gregarious		Solitary	
Component	HDLp	LDLp	HDLp	LDLp
Triacylglycerol	2.1	3.7	9.1	0.4
Diacylglycerol	43.1	60.0	35.5	48.0
Monoacylglycerol	2.7	6.8	9.2	13.4
Steryl ester	13.3	5.5	11.2	0.8
Phospholipids	26.7	22.0	25.8	31.4
Free fatty acids	12.1	2.0	9.2	6.0
Total	100	100	100	100

The lipid clases were separated and the fatty acids derivatised as explained in Materials and Methods. The percent composition was computed by comparison of intergrated peak areas from gas chromatograph profiles.

Table 3 Fatty acid percent composition (wt%) of HDLp and LDLp from solitary and gregarious locusts

	Gregarious		Solitary	
Fatty acid	HDLp	LDLp	HDLp	LDLp
Palmitic (16:0)	60.2	10.2	28.6	7.6
Stearic (18:0)	7.2	6.6	19.1	17.6
Oleic (18:1)	15.9	22.0	31.2	39.8
Linoleic (18:2)	5.6	46.2	11.4	23.4
Linolenic (18:3)	11.4	15.0	9.7	11.6
Total	100	100	100	100

The percentages were determined from the total peak areas corresponding to the external fatty acid methyl esters standards run under the same condition.

3.3 Immunological characterization of lipophorin

3.3.1 Detection of antibodies

Antibodies raised against HDLp from gregarious locusts were detected by double radial immunodiffusion and immunoblotting. The antibodies were tested against haemolymph, LDLp and HDLp samples from gregarious locusts. Using immunodiffusion, the antibodies were shown to be specific with continuous precipitin bands observed with haemolymph, HDLp and LDLp samples (Fig. 10). Immunoblot test with the antibodies, showed specificity to bands corresponding to apoLp-I and apoLp-II (Fig. 11a, b). The banding pattern was the same for haemolymph, HDLp and LDLp. Although the presence of apoLp-III had been shown by silver staining on HDLp, no band corresponding to this apoprotein was observed.

3.3.2 Immunological cross-reactivity

Immunological cross-reactivity of lipophorin from the two locusts phases was tested by immunobloting and double radial immunodiffusion (section 2.11). Cross-reactivity was observed with the antibodies raised against HDLp from gregarious locusts reacting with samples from the solitary locusts (Fig. 12). Single continuous precipitin bands were observed with HDLp, LDLp and haemolymph samples. Evidence for cross-reactivity was also observed using immunoblot tests in which samples from solitary locusts reacted with antibodies raised against HDLp from gregarious locusts, with both apoproteins showing positive reactions (Fig. 13). Using double radial immunodiffusion, antibodies raised against *L. Migratoria* HDLp, were also shown to cross-react with HDLp, LDLp and haemolymph samples from *S. gregaria* (Fig. 14). The precipitin bands were continuous, indicating

Fig. 10 **Double radial immunodiffusion for the detection of antibodies raised against HDLp (gregarious).** The central well (Ab) contained 10 μl of the antibody while the peripheral well had samples from gregarious locusts: 1, HDLp; 2, LDLp; 3, haemolymph

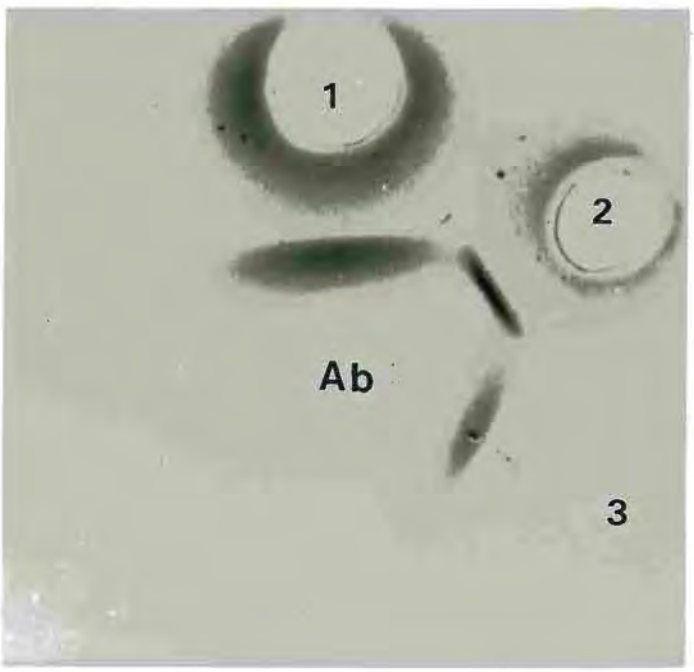

1
2
Ab
3

Fig. 11a, b **SDS-PAGE and immunoblot of isolated lipophorin and haemolymph.** Lipophorin and haemolymph samples from gregarious locusts were subjected to SDS-PAGE then electrophoretically transferred onto nitrocellulose paper. The blot was incubated with antibodies to HDLp of gregarious and processed as described in Materials and Methods. **a: SDS-PAGE**, lane 1, HDLp (10 μg); lane 2, LDLp (10 μg); lane 3, haemolymph (20 μg); lane 4, molecular weight standards **b: Immunoblot**, lane 1, HDLp (5 μg); lane 2, LDLp (5 μg); lane 3, Haemolymph (10 μg).

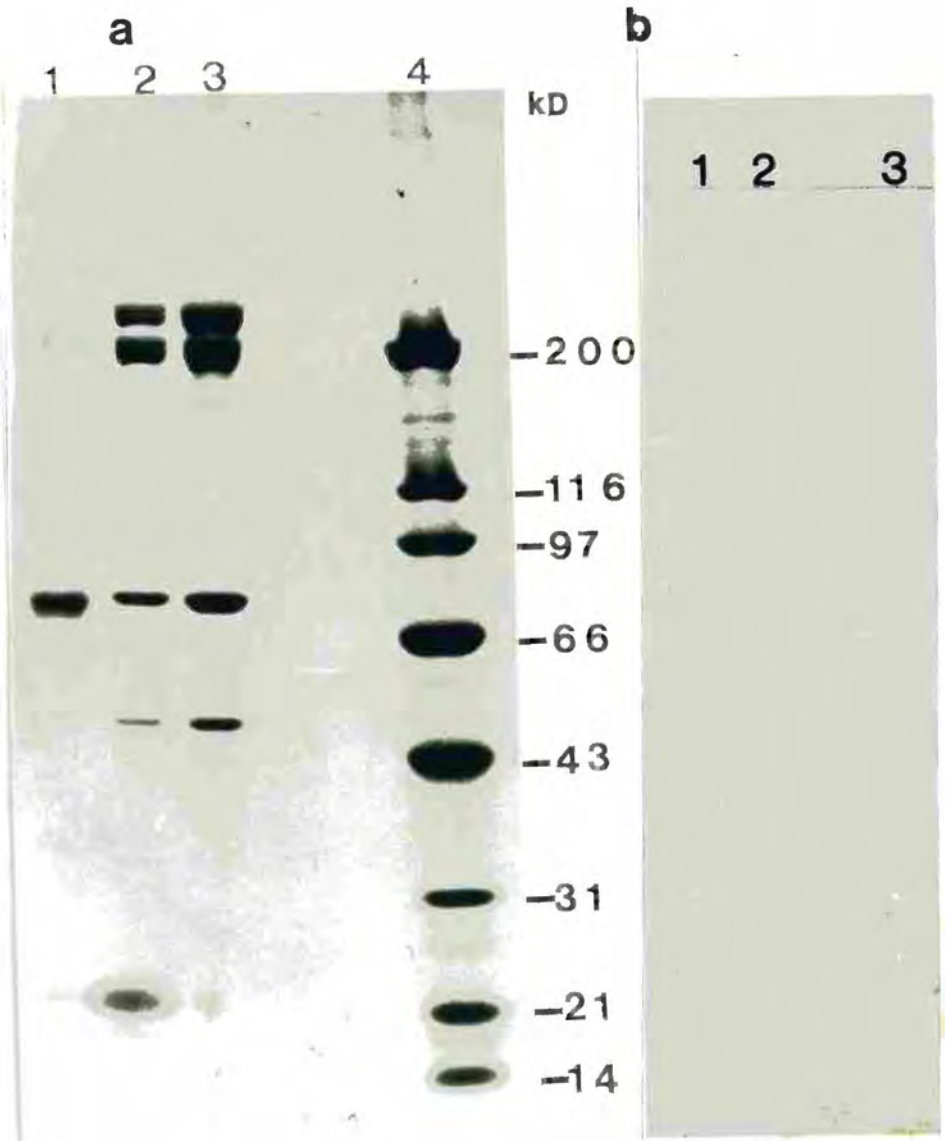

a
1
2
3
4
kD
200
116
97
66
43
31
21
14
b
1
2
3

Fig. 12 **Double radial immunodiffusion for cross-reactivity test with antibodies raised against HDLp.** Samples from solitary locusts were tested against antibodies to HDLp of gregarious locusts. The central well had the antibody while the peripheral wells had the following samples: 1, haemolymph; 2, HDLp; 3, LDLp.


1
Ab
2
3

Fig. 13 **SDS-PAGE and immunoblot of haemolymph and lipophorin samples.** Lipophorin and haemolymph samples from solitary locusts were subjected to SDS-PAGE, electrophoretically transfered onto nitrocellulose paper then incubated with antibodies to HDLp of gregarious locusts. **a: SDS-PAGE**, lane 1, haemolymph (20 μg); lane 2, LDLp (10 μg); lane 3, HDLp (10 μg). **b: Immunoblot**, lane 1, haemolymph (10 μg); lane 2, LDLp (5 μg); lane 3, HDLp (5 μg).

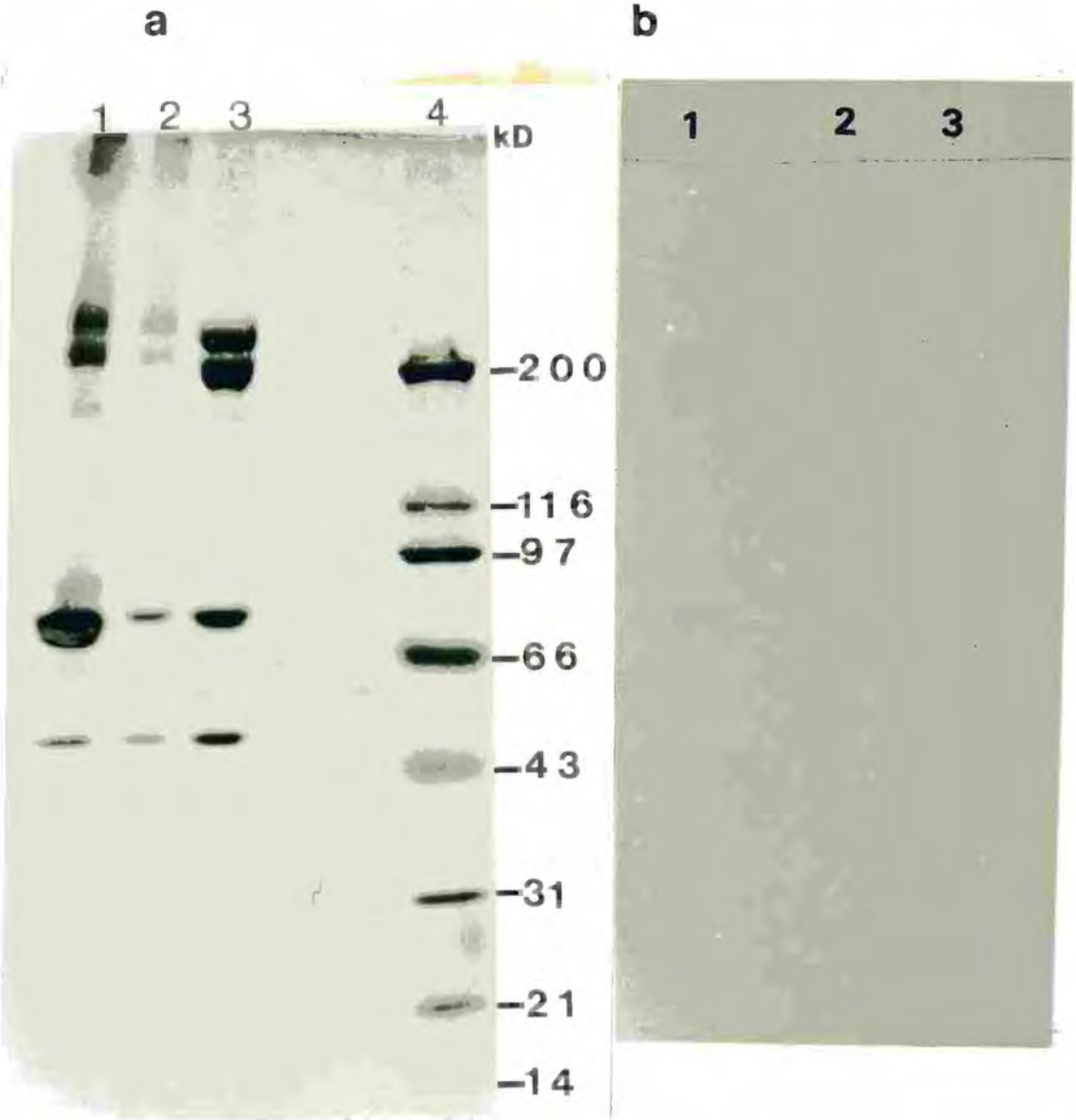
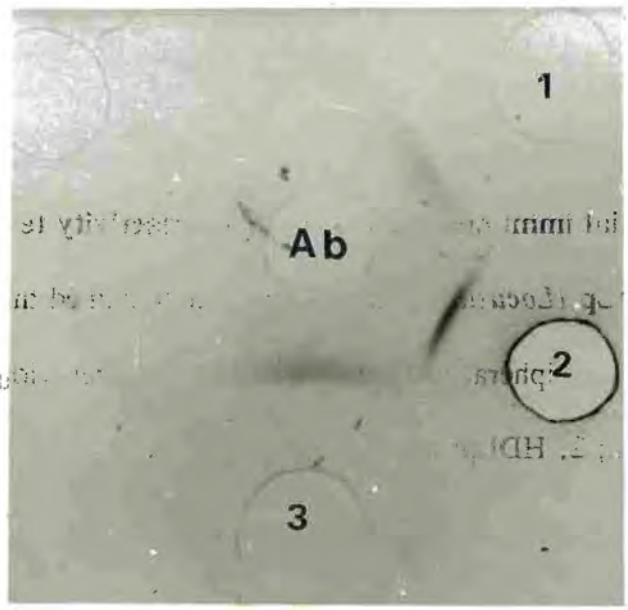

a
b
1
2
3
4
kD
200
116
97
66
43
31
21
14
1
2
3

Fig. 14 **Double radial immunodiffusion for cross-reactivity test with antibodies raised against HDLp *(Locusta)*.** The central well contained the antibodies to Locusta HDLp. The peripheral wells had samples from gregarious locusts: 1, haemolymph; 2, HDLp; and 3, LDLp


1
Ab
2
3

reaction towards the same antigenic determinants. However, the precipitin bands were faint when compared to those obtained with antibodies raised against *S. gregaria*, HDLp.

3.4 Isolation and characterization of apolipophorin-III

3.4.1 Isolation of apolipophorin-III

Apolipophorin-III (apoLp-III) was isolated from LDLp using a method modified from Wells *et al.* (1985). Preparation of apoLp-III by the method of Wells *et al.* (1985) exploited the insolubility of apoLp-I and II following lipid extraction from LDLp and lyophilization of the remaining apoproteins. The contaminating apoLp-I and apoLp-II were subsequently removed using affinity chromatography on concanavalin A-Sepharose in which apoLp-III was eluted in the unbound fraction (Fig. 15). This method of preparation always resulted in the contamination of apoLp-III with additional bands ($M_r \sim$ 34,000 and 17,000). In some preparations only the $M_r \sim$ 34,000 protein appeared (Fig. 16). These bands could have resulted from degradation and/or aggregation of more than one molecule of the apoprotein. Thus, additional steps involving heat denaturation (70° C, 10 min), centrifugation to remove denatured proteins and microfiltration (2 μm filters) had to be incorporated. Analysis of the resulting apoLp-III preparation was shown to consist of a single band ($M_r \sim$ 20,000) by SDS-PAGE (Fig. 17). Non-denaturing PAGE showed the presence of two isoforms, ($M_r \sim$ 20,000 and 16,000)(Fig. 18). Based on the intensity of the staining, the $M_r \sim$ 20,000 isoform appeared to be the major one. In both locust phases, the molecular size of apoLp-III was similar.

Fig. 15 **Elution profile of partially purified apoLp-III from solitary locusts on concanavalin-A Sepharose.** The column was equilibrated with concanavalin-A buffer. Elution was then done with 500 mM methyl-α-D-mannosepyranoside in con-A buffer.

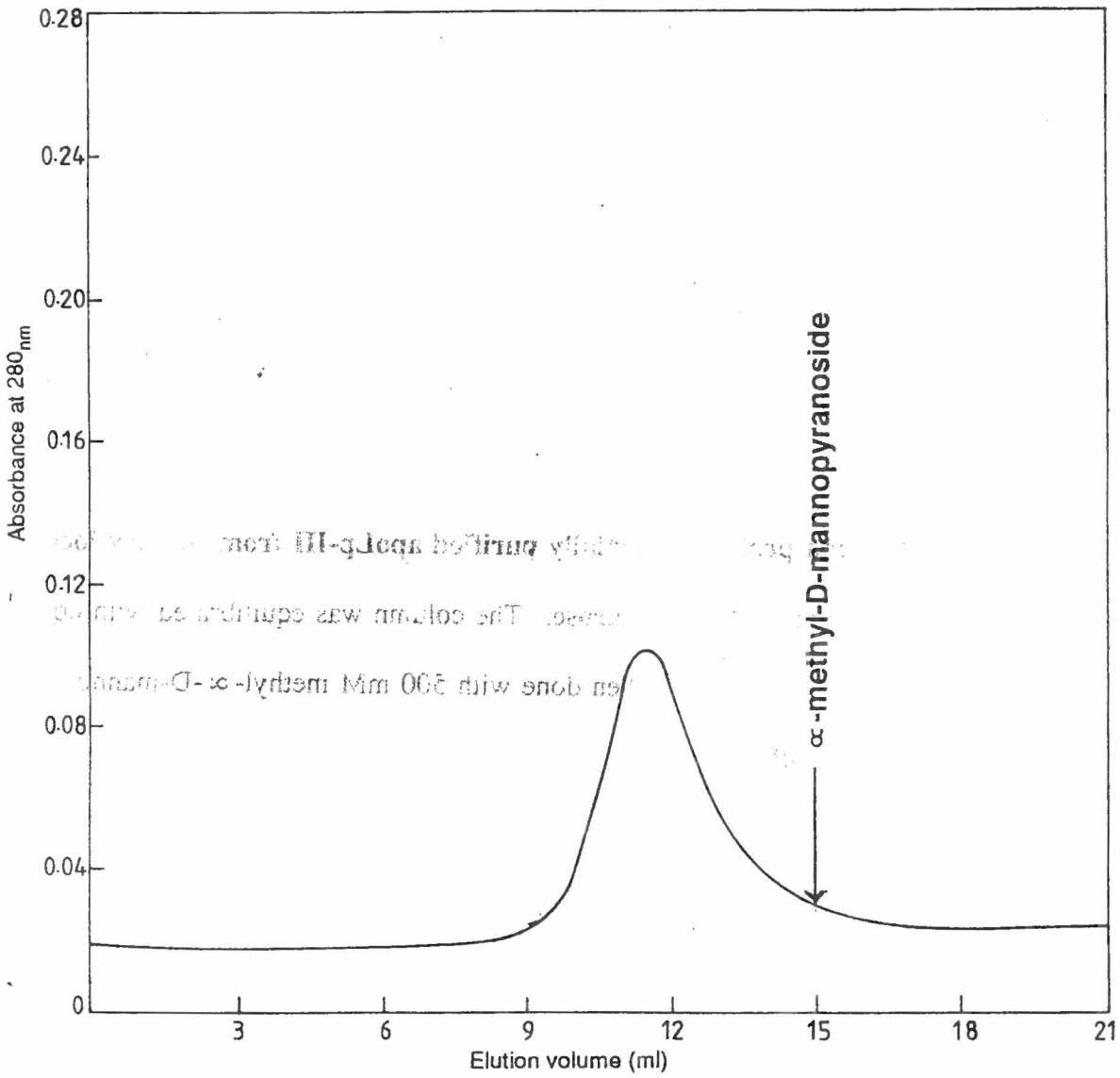

0·28
0·24
0·20
0·16
0·12
0·08
0·04
0
Absorbance at 280nm
3
6
9
12
15
18
21
Elution volume (ml)
α-methyl-D-mannopyranoside

Fig. 16 **SDS-PAGE (4-15%) of haemolymph and partially purified apoLp-III.** Lanes 1, LDLp (10 μg); lane 2, apoLp-III (gregarious)(20 μg); lane 3, apoLp-III (solitary)(20 μg); lane 4, LDLp (10 μg); lane 5, molecular weight standards

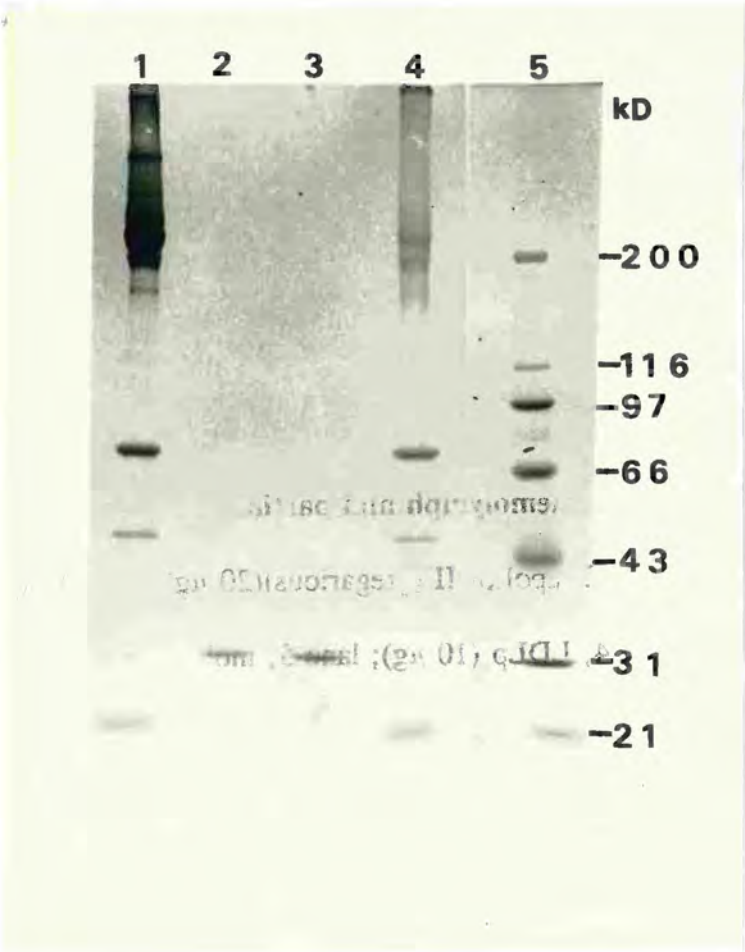
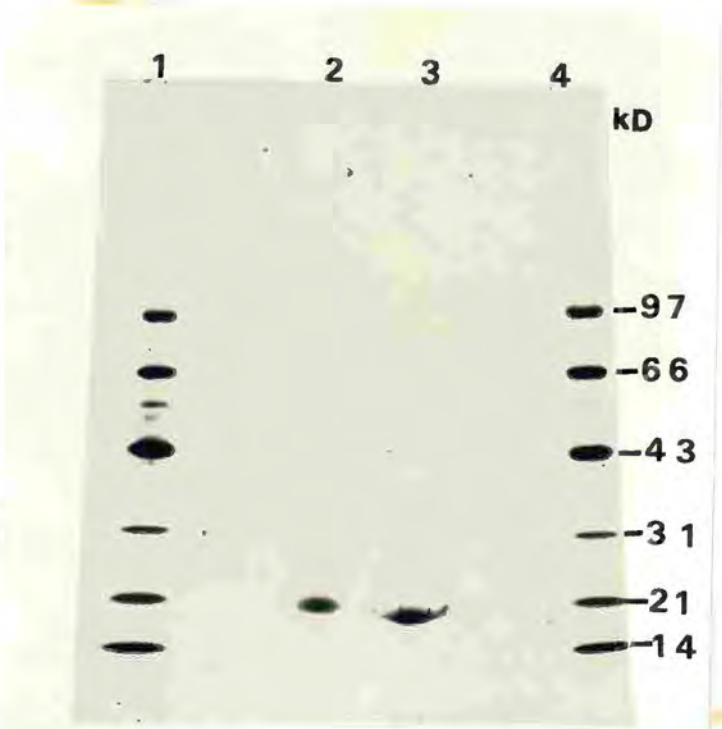
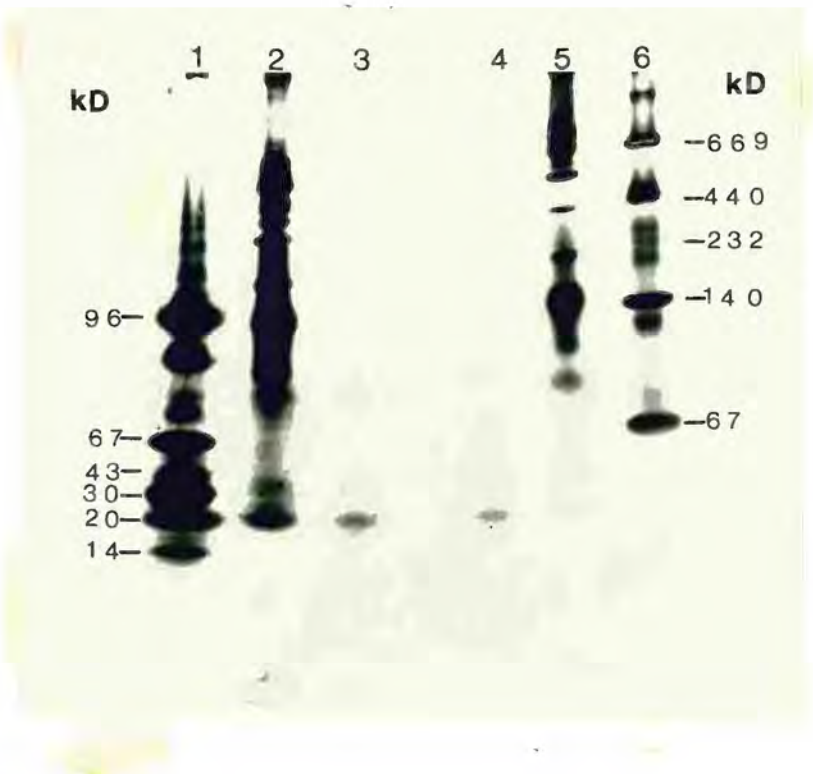

1
2
3
4
5
kD
200
116
97
66
43
31
21

Fig. 17 SDS-PAGE (4-15%) of isolated apolp-III. Lane 1 and 4 molecular weight standards; lane 2, apoLp-III (gregarious); lane 3, apoLp-III (solitary).


1
2
3
4
kD
97
66
43
31
21
14


1
2
3
4
5
6
kD
96
67
43
30
20
14
kD
669
440
232
140
67

3.4.2 Properties of apolipohorin-III

Isoelectric-focusing of apoLp-III isolated from either solitary or gregarious locusts showed the presence of a single band. The isoelectric points were estimated at 6.10 and 5.95 for gregarious and solitary locusts, respectively (Fig. 19). Despite the silver staining of the of the PhastGels, only single isoforms were evident for the isolated apoLp-III.

The apoLp-III molecule was shown to be glycosylated since it stained with PAS stain (Fig. 7). Further analysis of the carbohydrate moiety was carried out using affinity chromatography on concanavalin A-Sepharose, which showed the absence of mannose rich oligosaccharide chains (Fig. 15).

Antibodies raised against apoLp-III from the gregarious locusts were detected by double radial immunodiffusion. The antibodies were allowed to diffuse for at least 3 h before the addition of the antigen. A continuous precipitin band was observed with haemolymph, LDLp and apoLp-III samples of gregarious locusts (Fig. 20). No precipitin band was observed with HDLp samples. This could have been due to the low levels of apoLp-III as was shown by staining procedure in which apoLp-III in HDLp was only detectable by silver staining. Antibodies raised against gregarious *S. gregaria* apoLp-III was shown to cross-react with haemolymph, LDLp and apoLp-III samples from the solitary locusts (Fig. 21). Furthermore, anti- *Locusta* apoLp-III was shown to cross-react with haemolymph, LDLp and apoLp-III samples from *S. gregaria* (Fig. 22). The precipitin bands were rather faint. This could be due to the low molecular size of apoLp-III which on complexing with the antibodies may not result into a highly precipitable molecule. The precipitin bands were also very close to the central wells despite allowing the antibodies to diffuse for three hours before the addition of the haemolymph samples. The delay in the addition of the haemolymph samples was to allow for the faster diffusion of the apoLp-III molecules.

Fig. 19 **Isoelectric focusing of *S. gregaria* apoLp-III by PhastSystem**. The proteins were seperated on a PhastGel IEF medium 3-9. Lane 1, apoLp-III (gregarious); lane 2, apoLp-III(solitary) and lane 3, pI standards

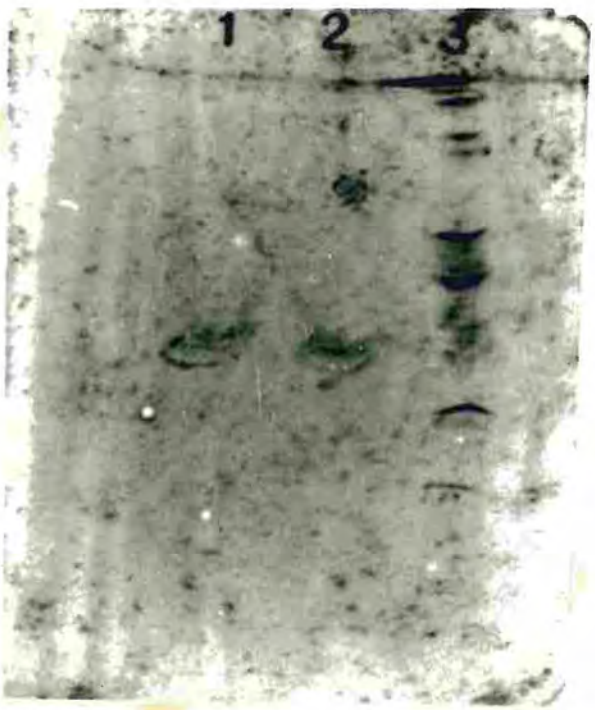

1
2
3

Fig. 20 **Double radial immunodiffussion for the detection of antibodies raised against apoLp-III.** The central well contained antibodies raised against apoLp-III from gregarious locusts while the peripheral wells contained samples from gregarious locusts: 1, haemolymph; 2, LDLp; 3, apoLp-III; 4, HDLp

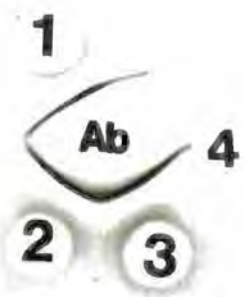

1
Ab
4
2
3

Fig. 21 **Double radial immunodiffusion for cross-reactivity test with antibodies raised against apoLp-III.** The central well contained antibodies raised against apoLp-III from gregarious locusts while the peripheral wells contained samples from the solitary locusts: 1, HDLp; 3, Haemolymph; 4, LDLp; 5, apoLp-III.

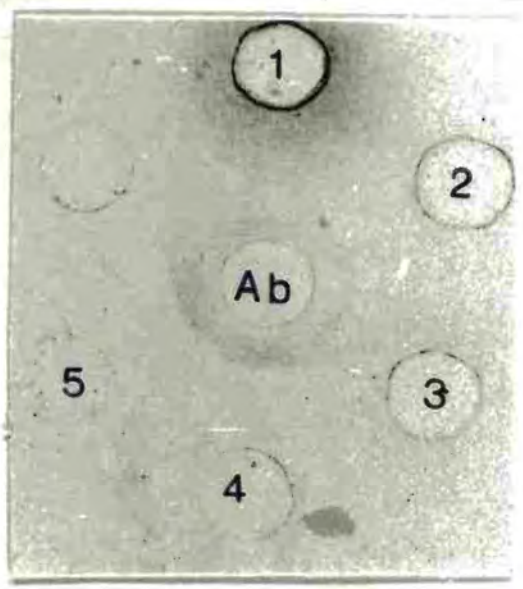
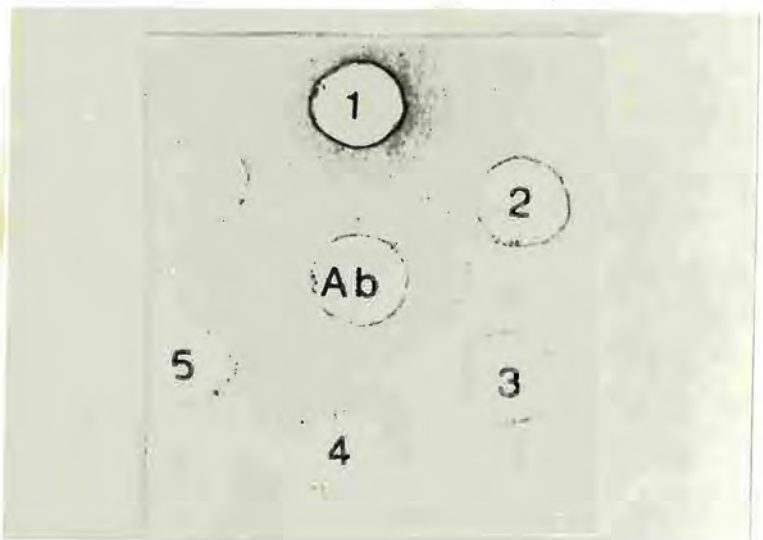

1
2
Ab
5
3
4

Fig. 22 **Double radial immunodiffusion for cross-reactivity test with antibodies raised against apoLp-III *(Locusta)*.** The central well contained the antibodies to HDLp isolated from *Locusta*. The peripheral wells contained samples from gregarious *S. gregaria*: 1, HDLp; 3, Haemolymph; 4, LDLp; 5, apoLp-III


1
2
Ab
5
3
4

3.5 Levels of lipophorin during the development of the locusts

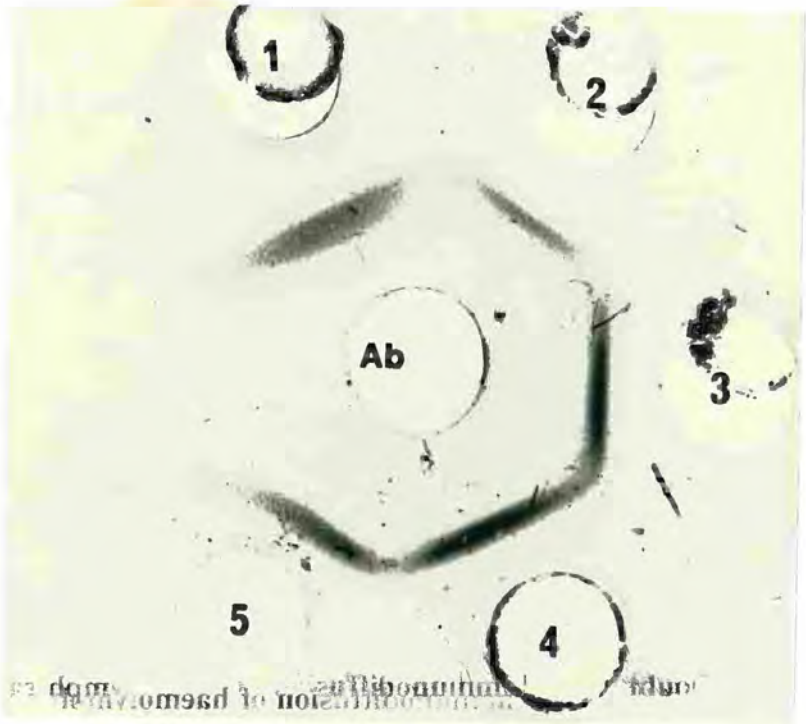
3.5.1 Detection of lipophorin and apoLp-III during the development

The presence of lipophorin and apoLp-III in the locust haemolymph was assessed using antibodies raised against HDLp and apoLp-III, respectively. Heamolymph samples from the various developmental stages starting from the third nymphal instar were taken and applied to the wells in the Ouchterlony plates. The presence of lipophorin was demonstrated in all the stages tested in both locust phases (Fig. 23a, b). The precipitin bands formed were continuous indicating that the antigens were reacting to same antigenic determinants. More intense staining of the precipitin bands were observed with the gregarious locusts samples, suggesting higher lipophorin titres.

The presence of apoLp-III was also demonstrated in all the stages of the locust tested in both phases (Fig. 24a, b). As was the case in the preceding section, the precipitin bands formed were faint and very close to the central wells despite the delay in the addition of the antigen. Since the amount of apoLp-III associating with HDLp was shown to be low in the previous studies, and also due to the apparent absence of LDLp in resting locusts, the apoLp-III detected in the haemolymph were most likely free apoLp-III molecules in the haemolymph.

Fig. 23a, b **Double radial immunodiffusion of haemolymph samples from development stages of *S. gregaria*.** The central wells contained antibodies raised against HDLp from *S. gregaria*. The peripheral wells had the following: **A: (gregarious locust stages)**, 1, 3rd; 2, 4th; 3, 5th; 4, immature; 5, mature. **B: (solitary locusts stages)**, 1, 3rd; 2, 4th; 3, 5th; 4, 6th; 5, immature; 6, mature.

a



b

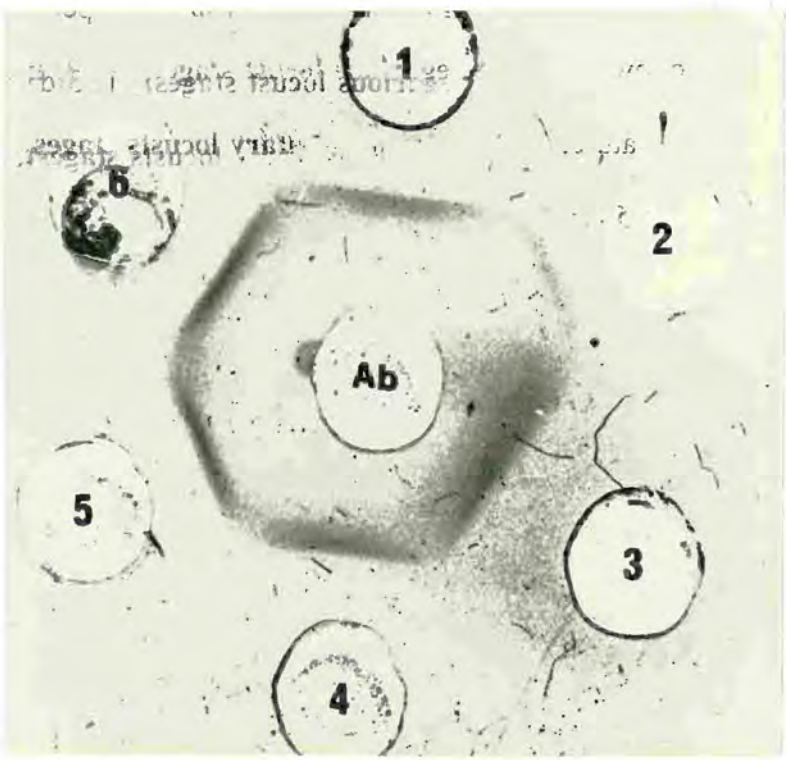
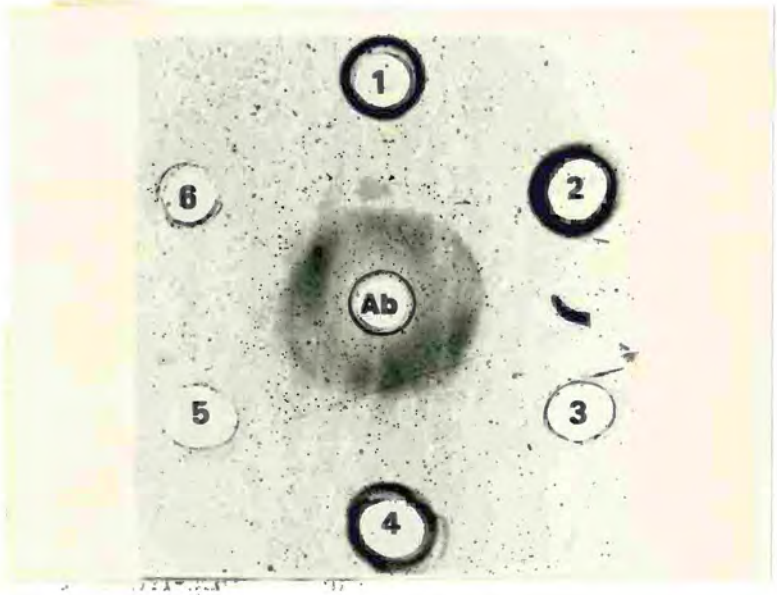
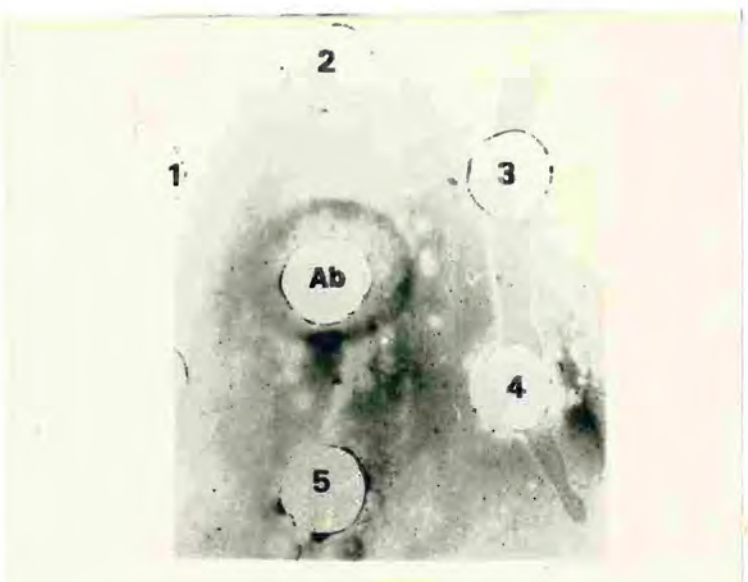


Fig. 24a, b **Double radial immunodiffusion of heamolymph from development stages of *S. gregaria* against anti- apoLp-III.** The central wells contained antibodies raised against apoLp-III from *S. gregaria*(gregarious). The peripheral wells contained the following: **b: (gregarious locust stages)**, 1, 3rd; 2, 4th; 3, 5th; 4, immature; 5, mature. **a: (solitary locusts stages)**, 1, 3rd; 2, 4th; 3, 5th; 4, 6th; 5, immature; 6, mature.

a



b



3.5.2 Lipophorin haemolymph titres during locust development

Single radial immunodiffusion was used in the estimation of lipophorin levels in the haemolymph. A standard curve was constructed using the diameter squared (D^2) versus log. the concentration of HDLp between 5-1500 μg/ml (Fig. 25). Haemolymph samples of known dilutions were applied to the wells and the D^2 of the precipitin rings compared with the standard curve to obtain the corresponding lipophorin titres. The precipitin rings obtained with the standard protein as well as the haemolymph samples were regular in shape with distinct boundaries (Fig. 26a, b). The lipophorin titres were obtained for 3rd nymphal instars to mature adults in both locust phases (Fig. 27).

In the gregarious locusts, lipophorin level in the 3rd nymphal instar was estimated to be 6.690 $\pm$ 0.572 mg/ml which increased to 7.533 $\pm$ 0.349 mg/ml in the 4th nymphal instar. The levels then decreased slightly to 6.888 $\pm$ 0.789 in 5th nymphal instar followed by increases to 8.834 $\pm$ 0.784 and 14.417 $\pm$ 2.038, respectively. Lipophorin levels in the solitary locusts was estimated to be 3.325 $\pm$ 0.595 mg/ml in the third nymphal instar. This decreased to 2.688 $\pm$ 0.254 in the fourth nymphal instar followed by a large increase in the fifth nymphal instar to 8.749 $\pm$ 0.871, then a decrease to 3.542 $\pm$ 0.295 in the sixth nymphal instar. The immature and mature stages maintained titres of 8.267 $\pm$ 1.210 and 8.438 $\pm$ 0.667 mg/ml, respectively.

Comparison of the levels between corresponding stages of the two phases, showed that the gregarious locusts had significantly ($P < 0.05$) higher titres than the solitary ones in the 3rd, 4th and mature stages. In the 5th nymphal instar, there was no significant difference between the phases although the solitary locusts had higher titres. Although the gregarious

Fig. 25 **Standard curve for the estimation of lipophorin levels.** Single radial immunodiffusion using antibodies raised against HDLp from grgarious *S. gregaria* was done over a range of lipophorin concentrations (5- 1500 μg/ml). The diameter squared (d^2) of the precipitin rings was plotted agains the log lipophorin concentration

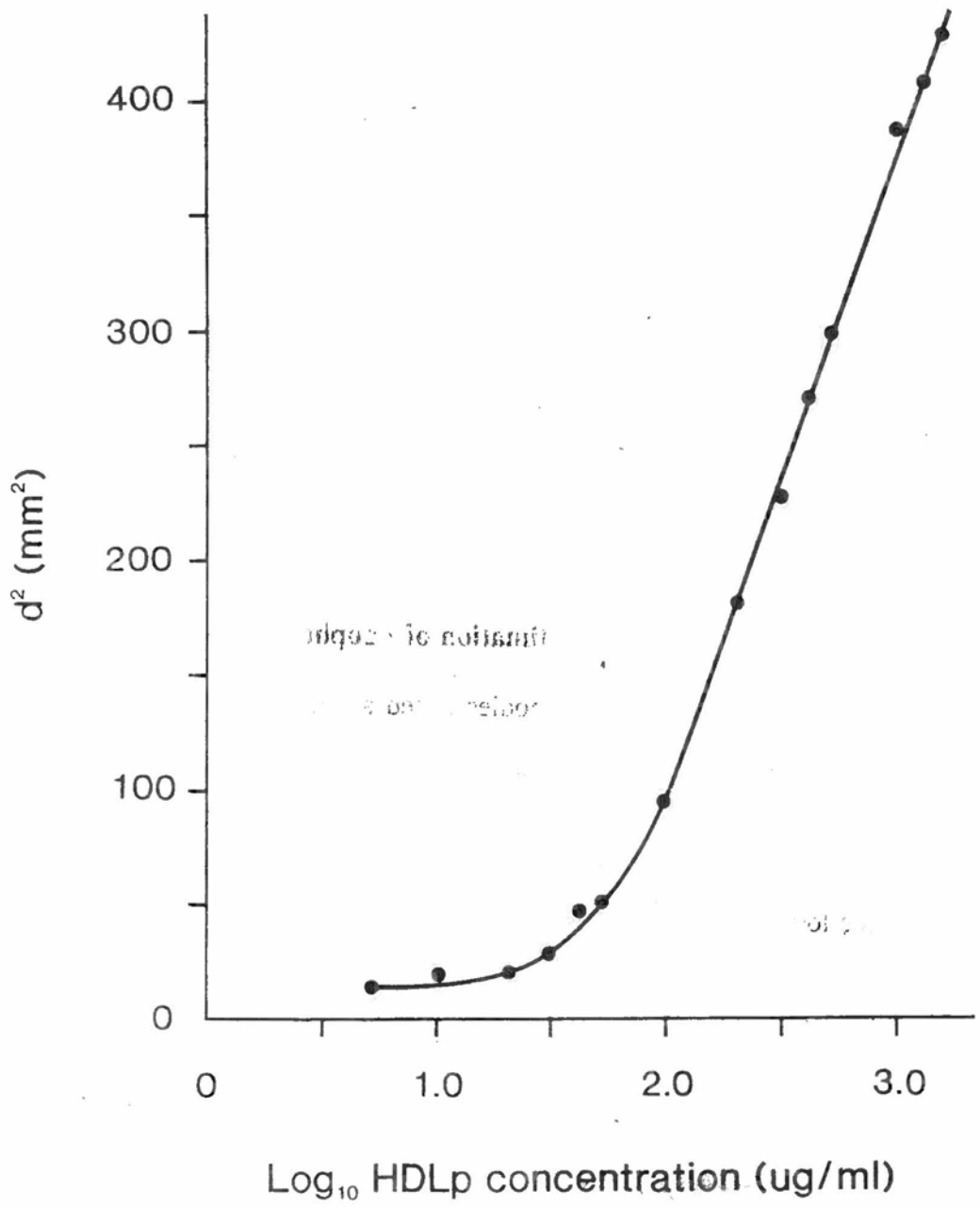
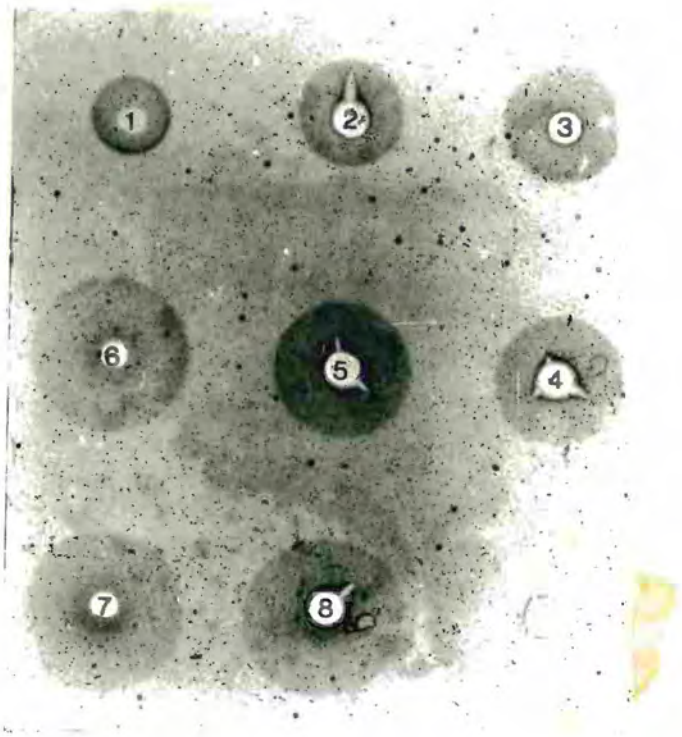

400
300
200
100
0
d^2 (mm^2)
0
1.0
2.0
3.0
Log_{10} HDLp concentration (ug/ml)

Fig. 26a, b **Precipitin rings obtained by single radial immunodiffusion.** (a): Increasing concentrations (1 - 8) of lipophorin for the standard curve. (b): precipitin rings obtained following the application of samples from the 4th nymphal instars (gregarious locusts)

a



b

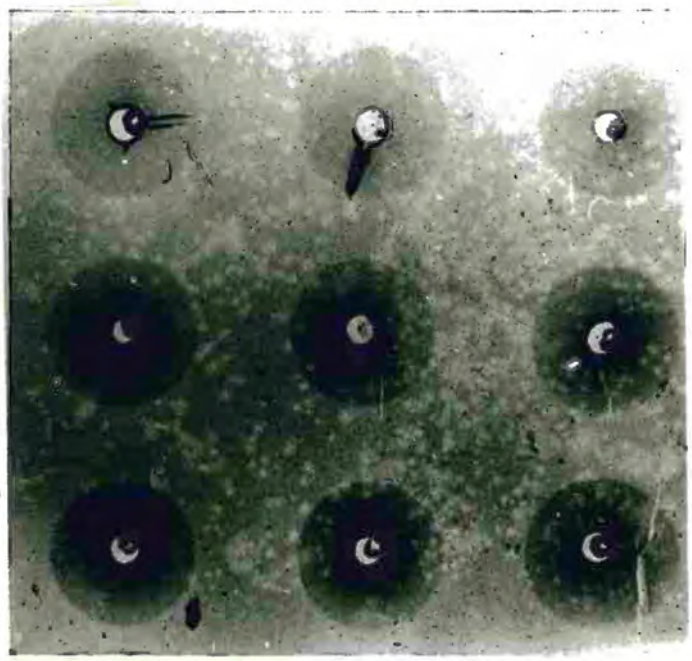
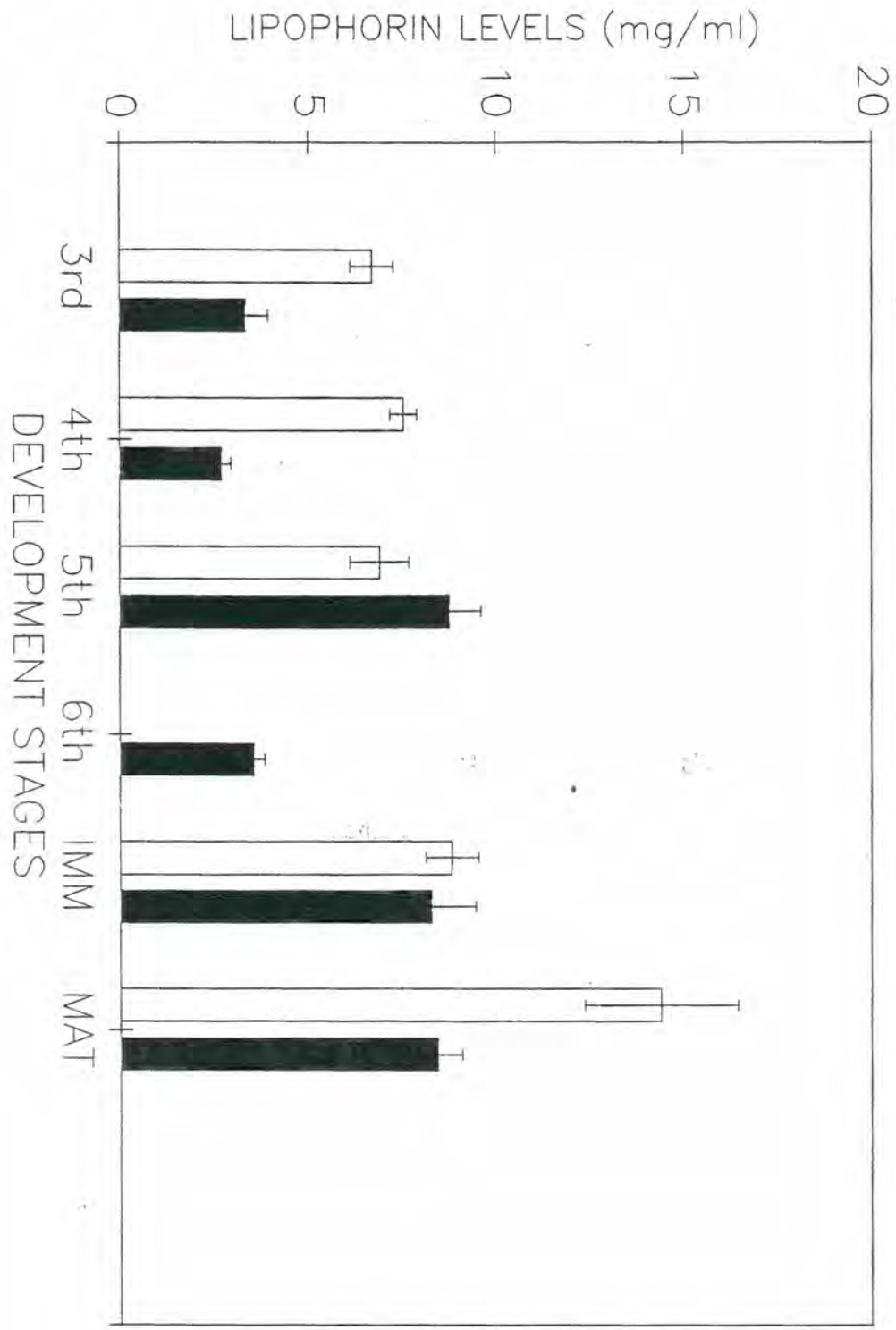


Fig. 27 **Lipophorin levels during the development of S. gregaria.** The values represe
means for 5-10 insects from each stage. Standard error bars were included.
gregarious locusts ☐ solitary locusts ■


LIPOPHORIN LEVELS (mg/ml)
0
5
10
15
20
3rd
4th
5th
6th
IMM
MAT
DEVELOPMENT STAGES

locusts had higher titres in the immature stage, the difference was not significant During the mature stages, lipophorin level in the solitary locusts was only 60% that in the gregarious locusts.

3.5.3 Estimation of haemolymph protein and lipid levels during the development of the locusts.

The haemolymph lipids and protein levels were estimated during the development of the locusts, to evaluate how this correlates to lipophorin levels. Lipid estimation was carried out using the vanillin-phosphoric acid method. The standard curve for the estimation of the lipids was constructed using oleic acid as the standard (Fig. 28). During the locust development, the protein levels showed a general increase in concentration from 233.4 $\pm$ 7.903 mg/ml in third nymphal instar to 279.84 $\pm$ 4.09 mg/ml in the gregarious locusts, whereas for the solitary locusts, the levels increased from 237 $\pm$ 10.99 in the third nymphal instar to 283 $\pm$ 9.278 mg/ml in the mature stages (Fig. 29). The results showed that the solitary locusts had significantly ($P < 0.05$) higher protein levels in the 4th and 5th nymphal stages. In the other stages, the solitary locusts had higher protein levels which were not significantly different from that of the gregarious locusts.

The haemolymph lipid levels lipid level in the gregarious locusts showed a general increase upto the immature stage, when there was a peak value of 12.152 $\pm$ 0.723 mg/ml. There was then a decline to 5.214 $\pm$ 0.723 in the mature stages. On the other hand, the levels in the solitary locusts peaked in the 6th nymphal instar before declining to 5.432 $\pm$ 0.211 in the mature stages (Fig. 30b). In the 3rd and 4th nymphal instars and mature stages, solitary locusts had higher lipid levels in the haemolymph than the gregarious ones.

Fig. 28 **Standard curve for the estimation of of lipid levels by the vanillin-phosphoric acid assay method**. Varying concentrations of oleic acid were digested in sulphuric acid then treated with vanillin- phosphoric acid reagent as described in Materials and Methods. Absorbance was then read at 536 nm.

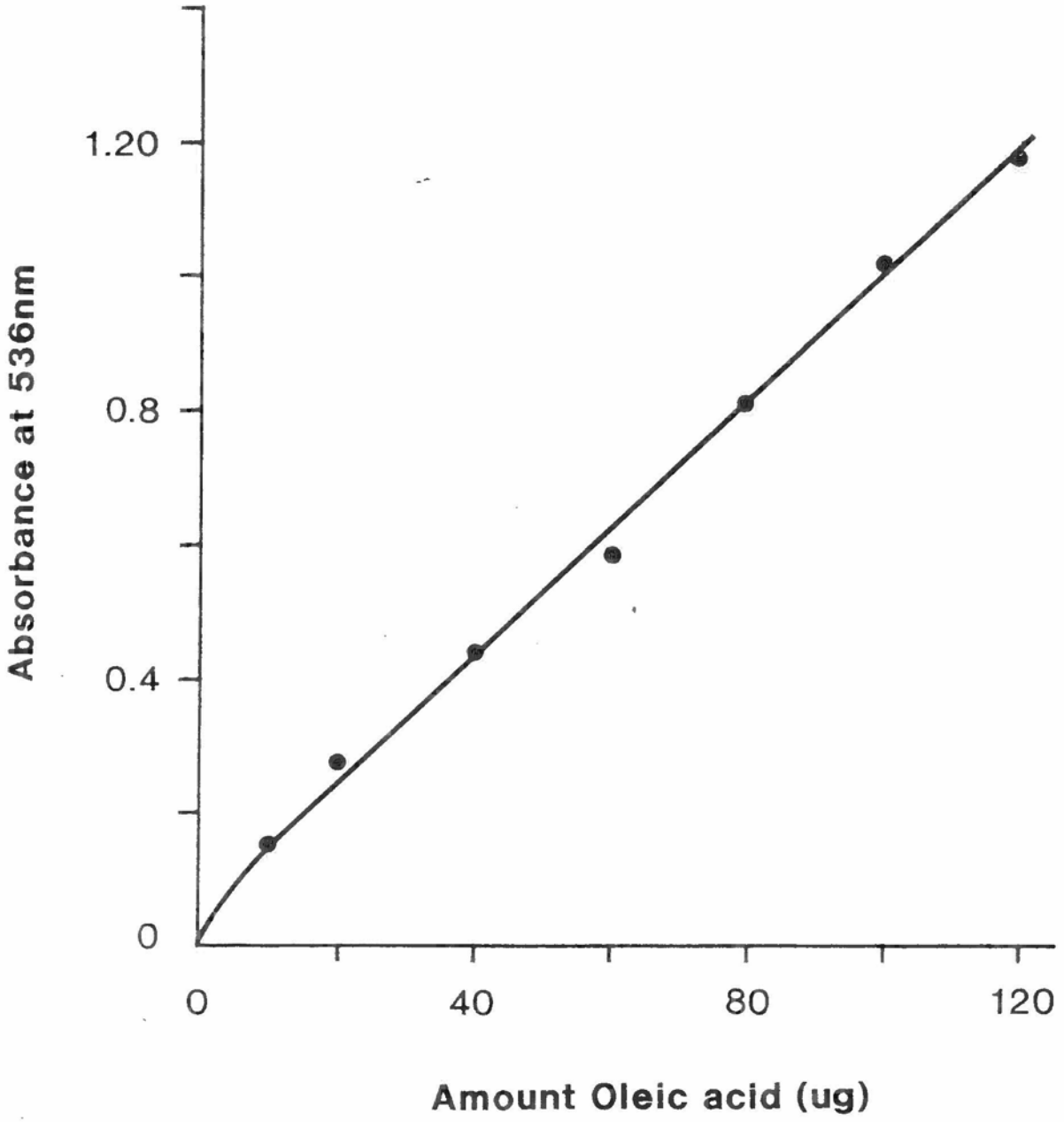

1.20
0.8
0.4
0
Absorbance at 536nm
0
40
80
120
Amount Oleic acid (ug)

Fig. 29 **Haemolymph protein levels during the development of the locusts.** Protein levels were estimated by the BCA protein assay method as explained in Material and Methods.

solitary locusts ▬ gregarious locusts ▭

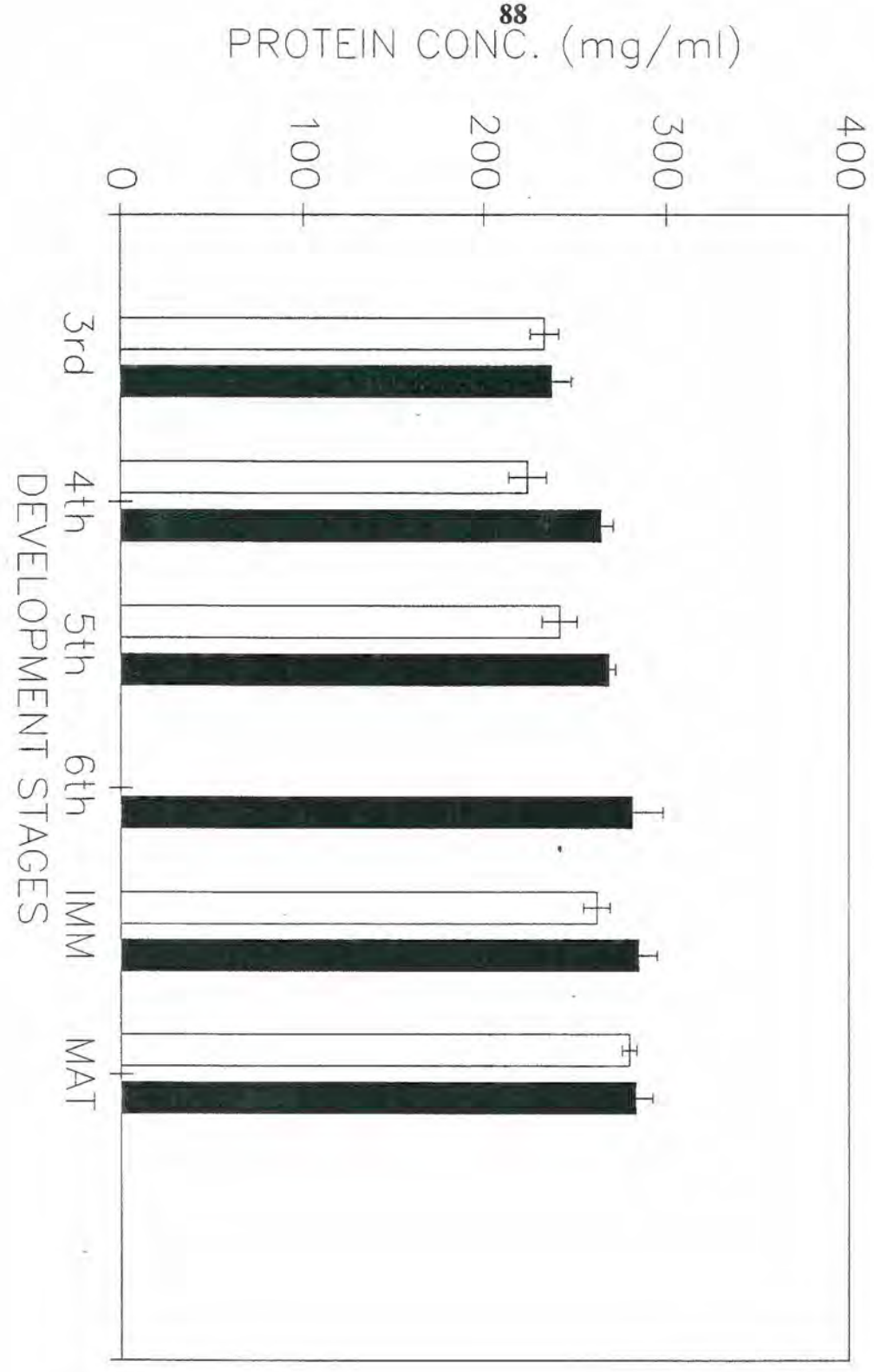

PROTEIN CONC. (mg/ml)
0
100
200
300
400
3rd
4th
5th
6th
IMM
MAT
DEVELOPMENT STAGES

Fig. 30 **Haemolymph lipid levels during the development of the locusts.** Lipid levels were estimated using the vanillin- phosphoric acid method as explained in Materials and Methods.

Solitary locusts ■ gregarious locusts □

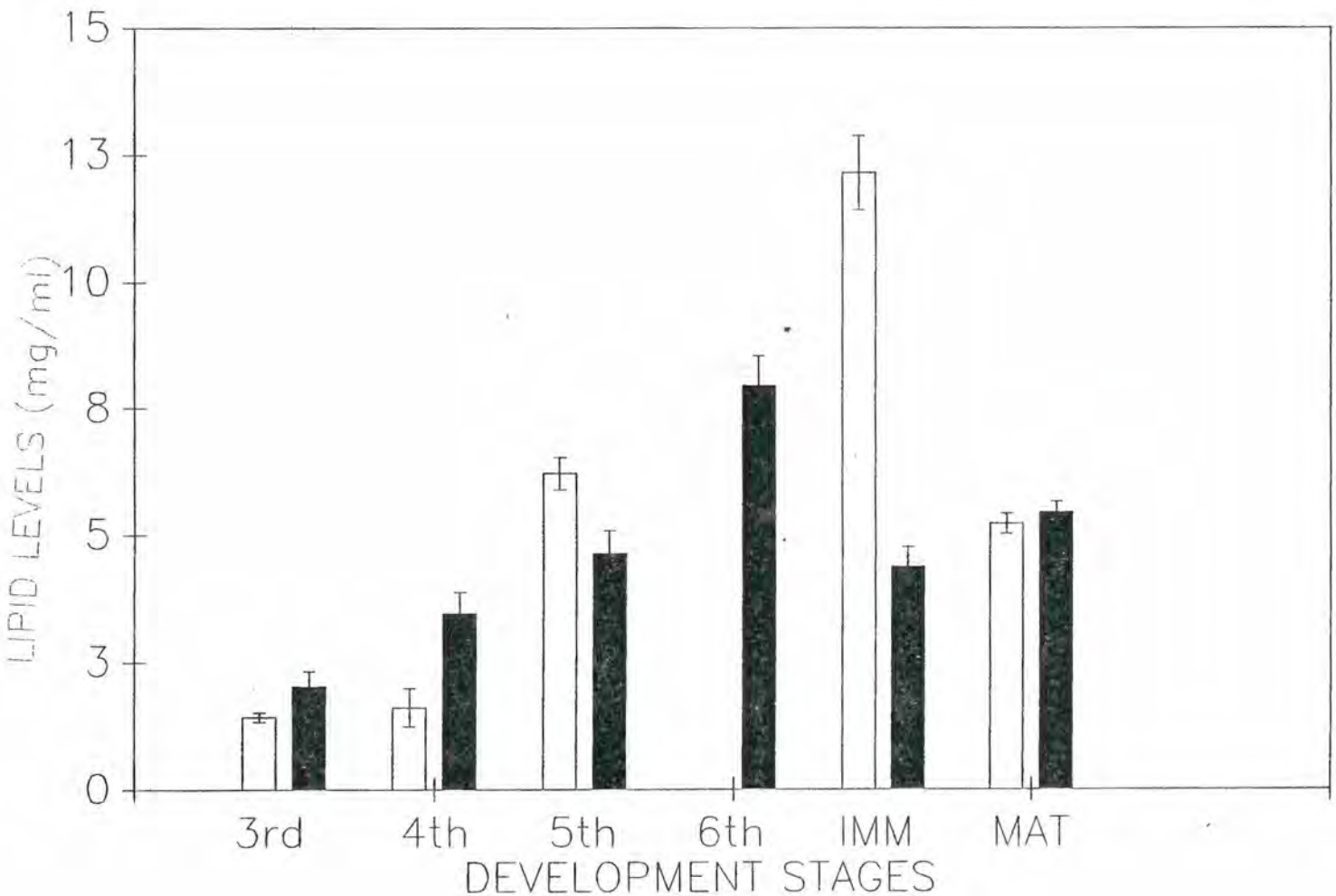

15
13
10
8
5
3
0
LIPID LEVELS (mg/ml)
3rd
4th
5th
6th
IMM
MAT
DEVELOPMENT STAGES

Table 4 Lipophorin levels expressed as a percentage (wt%) of the total haemolymph protein.

Development Stage	Solitary	Gregarious
3rd	1.40	2.80
4th	1.02	3.36
5th	3.26	2.85
6th	1.26	---
Immature	2.90	3.37
Mature	2.98	5.16

Haemolymph lipophorin and protein concentrations were determined on the same sample as explained in Materials and methods. Lipophorin content was then expressed as percentage of the total protein.

However, significant difference was only observed in the 4th nymphal instar. On the other hand, significantly higher lipid levels were observed in the gregarious locusts in the fifth and immature stages.

In order to correlate lipophorin levels with that of the total haemolymph proteins, lipophorin levels was expressed as a percentage of the haemolymph protein (Table 4). The results indicated that lipophorin in the gregarious locusts represented a higher haemolymph component than the solitary locusts, in all stages except for the fifth nymphal instar of the solitary locusts. The lowest percent composition observed was 2.8% (3rd instar) and 1.02% (4th instar) for gregarious and solitary locusts, respectively. The highest percent composition was observed in the mature (5.16%) and 5th nymphal instar (3.26 %) of solitary and gregarious locusts, respectively.

3.6 Discussion

3.6.1 Isolation of lipophorin and apolipophorin-III

High and low density lipophorins (HDLp and LDLp) were isolated from the locust haemolymph by single step density gradient ultracentrifugation. The homogeneity of the proteins was ascertained by both SDS- and non-denaturing PAGE (Fig. 2a, b). Preparations of HDLp from resting insects resulted into a single lipophorin band suggesting the absence of LDLp in resting insects. Studies on some insects such as *Manduca sexta* and *Thasus actangulas* have shown the presence of LDLp in resting insects (Kawooya and Law, 1988; Wells *et al.*, 1987; Ryan *et al.*, 1984). In most other insects, there has been no evidence of LDLp (Chino *et al.*, 1986; Shapiro *et al.*, 1988). In most preparations of LDLp, there was evidence of a banding corresponding to HDLp implying that not all HDLp molecules were

converted to LDLp following the administration of AKH. The partial conversion of HDLp to LDLp is particularly evident in the case of the solitary locusts in which HDLp still appears to be the major lipoprotein type (Fig. 1b). Despite the distinctive banding for LDLp and remnant HDLp, monitoring of the peaks at 450 nm did not result into clear distinctive peaks (Fig. 1b). This could have been due to the bands being rather broad in addition to the size of fractions taken.

ApoLp-III was isolated from the two locust phases using a method adapted from Wells *et al.* (1985). The procedure adopted mainly exploited the solubility properties of the apoproteins following lipid extraction and the stability of apoLp-III to heat denaturation (section 2.5). The apoLp-III preparations were shown to be homogeneous by SDS- and native- PAGE (section 3.4.1). ApoLp-III preparations after the affinity chromatography stage consistently showed the presence of contaminants (M_r ~ 34,000 and 17,000) or a single contaminant (M_r ~ 34,000). The presence of M_r ~ 34,000 contaminant in apoLp-III preparations has been reported by other authors (Kawooya *et al.*, 1984; Burks *et al.*, 1992). While Kawooya *et al.* (1984) attributed this to the formation of apoLp-III homodimers, Burks *et al.* (1992) suggested that this could be a product of an oxidation process during the isolation or on prolonged storage. However, in both cases, it was established by immunological methods that the higher molecular weight protein was derived from apoLp-III. It is possible that the conditions used during the affinity chromatography favoured an aggregation of apoLp-III molecules hence the contaminant (M_r ~ 34,000) could be a dimer while the M_r ~ 17,000 a degradation product.

Several other techniques have been used in the preparation of apoLp-III from haemolymph, subphase, or LDLp. Ammonium sulphate precipitation has been widely used

either in isolation or in combination with other chromatographic techniques (Cole and Wells, 1990; Burks *et al.*, 1992). Isolation of apoLp-III has also been achieved in some insects by a combination of gel permeation chromatography and/ or reverse phase HPLC (Strobel *et al.*, 1990; Ryan *et al.*, 1990; Ziegler *et al.*, 1988; Kanost *et al.*, 1987). Telfer *et al.* (1991), isolated apoLp-III from *H. cecropia* by a combination of techniques involving heat denaturation and microfiltration. In most of these studies, the isoforms were separated by cation exchange chromatography (Van der Horst *et al.*, 1991; Ryan *et al.*, 1990).

3.6.2 Properties of lipophorin and apolipophorin-III

The HDLp isolated from the two locust phases showed similarity in both the native structure (M_r~ 620,00) and apoprotein composition (apoLp-I, M_r~ 224,000 and apoLp-II, M_r~ 81,000)(Fig. 4a). A third apoprotein (M_r~ 20,000) was detectable on silver staining. The results obtained in this study showed a similar basic lipophorin structure as observed in other insect species (Shapiro *et al.*, 1988; Beenakkers *et al.*, 1985 and Chino, 1985). The presence of apoLp-III, albeit in low levels in resting lipophorin, was not a unique feature as similar observations have been made in other insect species (Ryan *et al.*, 1984; Wells *et al.*, 1985; Gondim *et al.*, 1989a; Surholt *et al.*, 1992)

Administration of AKH (10 pmol) resulted in the formation of LDLp in phases of *S. gregaria* (Fig. 4a). The resultant lipoprotein had a larger molecular size with increase in lipid content, but less denser and associated with more apoLp-III molecules. Furthermore there was evidence that the LDLp resulting from gregarious locusts was larger in size than that from the solitary locusts (Fig. 5). This results were in contrast to the findings by Chino

et al. (1992) who reported that the solitary locusts were unable to form LDLp in response to AKH injection.

In previous studies, the density, particle size as well as the composition of lipophorin has been shown to correlate with the physiological state of the insect (Ryan and Law, 1984; Beenakkers *et al.*, 1988). Whereas the apoprotein composition of HDLp and LDLp from both solitary and gregarious phase were similar, there were minor differences in the lipid content with the corresponding lipoproteins from gregarious locusts having a higher lipid content than the solitary locusts. This was further emphasized by the densities of the lipoproteins which were also lower for gregarious locusts. Significant differences were observed on the levels of diacylglycerides which constituted 43.1 and 35.1% of the total lipids in HDLp from gregarious and solitary locusts, respectively (Table 2). The increase in lipid content in LDLp was also shown to be mainly due to diacylglycerides, which constituted 60 and 48% of the total lipids for gregarious and solitary locusts, respectively (Table 2). This is consistent with the principal role of LDLp of transporting DG to the flight muscles of adult insects (Chino, 1985).

Double radial immunodiffusion and immunoblot studies showed cross-reactivity between the two locust phases (section 3.4.1). Furthermore, there was evidence of cross-reactivity with antibodies raised against *Locusta* HDLp. In immunoblot tests, both apoproteins cross-reacted indicating that there were common antigenic determinants in both apoproteins. Earlier studies by Ryan *et al.* (1984) had indicated that apoLp-II had a conserved structure among insect species hence the cross-reactivity among the orders of insects studied. It has also been shown that apoLp-I from insects of the same order are immunologically cross-reactive (Ryan *et al.*, 1984; Ogoyi *et al.*, 1993).

Fig. 18 **Non-denaturing PAGE of isolated apoLp-III.** Lane 1 and 6, molecular weight standard; lane 2, haemolymph (gregarious)(10 μg); lane 3, apoLp-III (gregarious)(20 μg), lane 4, apoLp-III (solitary)(20 μg); lane 5, haemolymph (solitary)(10 μg).

ApoLp-III isolated from both phases of *S. gregaria* showed the presence of two isoforms of molecular weights M_r~ 20,000 and 16,000. The isolated apoLp-III was further shown to be glycosylated but lacked mannose rich oligosaccharide chains. It was also demonstrated that there was cross-reactivity between the two phases and between apoLp-III from *S. gregaria* and antibodies raised against apoLp-III from *L. migratoria*. Goldsworthy *et al.* (1985) reported the presence of two glycosylated apoLp-III types of molecular weights M_r 16,000 and 20,000 which were separable by SDS-PAGE. Both forms were shown to be capable of associating with lipophorin during AKH induced lipid mobilization. For the same insect, other authors have shown the presence of two (Van Heusden *et al.*, 1987; Van der Horst *et al.*, 1991) and three isoforms (Chino and Yazawa, 1986). These apoLp-III isoforms were indistinguishable by SDS-PAGE but separable by ion- exchange and non-denaturing PAGE. The isoforms differ in amino acid compositions, N-terminal sequences and pI (Van der Horst *et al.*, 1991). In this study, the presence of two apoLp-III isoforms separable by non- denaturing PAGE, was demonstrated in both phases of *S. gregaria*. The difference between the isoforms is likely to be due to the charge rather than molecular size. However, this is not reflected in isoelectric focussing (Fig. 19) probably due to the low levels of one of the isoforms.

The primary structure of apoLp-III has shown the absence or low levels of cystein, tyrosine, methionine and tryptophan residues (Van de Horst *et al.*, 1991; Cole and Wells, 1990). This accounts for the low absorbance of apoLp-III at 280 nm and the apparent low affinity of apoLp-III for Coomassie blue staining (Kawooya *et al.*, 1984; Van der Horst *et al.*, 1991). Although the amino acid composition of *S. gregaria* apoLp-III was not determined, there was evidence of low absorbance at 280 nm, low affinity for Coomassie

brilliant blue, as well as low absorbance with the BCA protein assay reagent. Thus, the staining of apoLp-III on the gels, had to be enhanced either by silver staining or using the improved Coomassie staining. Glycosylation of apoLp-III has been observed in most orthopteran species studied so far (Ryan *et al.*, 1990). However, the presence of high mannose oligosaccharide chains has only been demonstrated in some cases. In *S. gregaria*, both phases showed the presence of covalently-bound oligosaccharide chains which lacked high mannose (Fig. 17).

Immunological tests using antibodies raised against *L. migratoria* apoLp-III has shown cross-reactivity with apoLp-III from other orthopteran species (Ryan *et al.*, 1990). No cross reaction was observed against *M. sexta* apoLp-III. Ryan *et al.* (1990), further showed that there was 50% homology on the N-terminal sequences among the orthopteran species compared to only 9-18% homology with the lepidopteran species. Among the lepidopteran species, 53% homology has recently been reported (Burks *et al.*, 1992). This may explain the immunological cross-reactivity observed between the two phases of *S. gregaria* and between apoLp-III isolated from *S. gregaria* and antibodies to *L. migratoria* HDLp.

3.6.3 Developmental changes in haemolymph lipophorin titres

The presence of lipophorin and apoLp-III in the haemolymph was immunologically detected in all developmental stages of both phases of *S. gregaria* (section 3.5.1). Prasad *et al.* (1986) working with *M. sexta* noted that lipophorin from the various developmental stages had identical apoprotein but differed in lipid contents and compositions. Van der Horst *et al.* (1987) also showed by immunoblotting and ELISA that the larval lipoprotein closely resembled the adult form. Thus, the observation in this study that lipophorin in the haemolymph of various developmental stages cross-reacted with antibodies raised against

adult HDLp, supports this view.

The presence of apoLp-III in immature stages of insects has been demonstrated in a number of insects. In *M. sexta*, the apoLp-III present in the caterpillars were free in the haemolymph and at a lower concentration of 0.46 mg/ml as compared to the concentration in the adults (17 mg/ml) (Kawooya *et al.*, 1984). In *D. grandiosella*, apoLp-III was found associated with lipophorin in the larval stages just as was the case in the adults (Burks *et al.*, 1992). However, 95% of apoLp-III was found free in the haemolymph. Studies on the fifth nymphal instars of *L. migratoria* has also shown lower titres of apoLp-III (3.85 mg/ml) which was only 20% the concentration in adults. However, adult maturation leads to increase in the concentrations of apoLp-III (Van der Horst *et al.*, 1987b; Kanost *et al.*, 1987; Izumi *et al.*, 1987). Similarly, in *Bombyx mori*, only small amounts of apoLp-III is present in the larval and pupal stages with the levels rapidly increased in the pharate moths (Miura and Shimizu, 1989). The detection of apoLp-III in all the nymphal stages of both phases of *S. gregaria* was therefore in conformity with previous studies.

Few studies have so far examined the total amounts of lipophorin present in insect haemolymph or how the amounts change in relation to developmental stage or physiological state of the insects. Studies by Prasad *et al.* (1987) indicated that in *M. sexta*, lipophorin mRNA and fat body secretion of lipophorin peaked 2 days after the onset of 5th instar. This was followed by a drastic reduction before the initiation of the wandering stage, while in the pupal stage, lipophorin mRNA were undetectable as the secretion from the fat body also ceased. In *Hyalophora cecropia,* low levels of lipophorin was evident in the diapausing pupae with a steady increase in the pharate adults (Telfer *et al.*, 1991). In contrast, increase in lipophorin content coincided with the end of active feeding period of the larvae and just

after the transition to diapause in *D. grandiosella* (Popham and Chippendale, 1993). According to Shelby and Chippendale (1990), the rate of lipophorin synthesis in non-diapausing *D. grandiosella*, peaked during the early phase of the 5th nymphal instar followed by a steady increase in content during the 5th and 6th instars while the titre remained constant. There was also a peak rate of synthesis during the 6th instar which then declined to undetectable levels. According to the Porpham and Chippendale (1993), the peak lipophorin content observed during diapause when there was no synthesis may be due to the long half life of apoLp-I and II. Telfer *et al.* (1991) also observed that in *H. cecropia*, lipophorin concentration reached peak values during vitellogenesis with the levels reduced just prior to eclosion. The higher titres of lipophorin in the gregarious compared to the solitary locusts in most of the nymphal instars may reflect a physiological adaptations by these locusts to cope with the need for high lipid reserve. The higher titres observed in the adult stages may further indicate that lipophorin of the gregarious locusts are capable of transporting more lipids as compared to the solitary. Further support for this can be based on the personal observation that the haemolymph volume in the gregarious locusts were generally higher than in solitary thus, the difference in lipophorin amounts between the two phases is likely to be wider. Expression of lipophorin as a percent of total haemolymph protein also suggests a higher lipid load in most of the stages of gregarious locusts (Table 4). The differences observed in the titres may suggest differences in the control of the biosynthetic processes in both locust phases. The reason for the observed higher titre of lipophorin in the solitary fifth nympal instar is unclear but may be due to some unique physiological process at this stage or differences in dietary intake.

Studies on haemolymph lipids has shown that diacylglycerides is the major lipid

present and may account for upto 80% of the neutral lipids (Beenakkers *et al.*, 1985). In *O. fasciatus* for example, neutral lipids constituted 80% of the total lipids with the acylglcerols accounting for 65%. Similarly, in *Galleria mellonella*, neutral lipids represented 55% of the total lipids (Wodawer and Wisniewski, 1965).

Wide variations in haemolymph lipids has been reported during development, metamorphosis and flight (Downer and Mathews, 1976). Age dependent factor has also been observed in both *M. sexta* and *L. migratoria* (Beenakkers, 1985; Ziegler, 1984). Recently Ayali and Pener (1992), observed that there was a significantly higher lipid level in crowded than isolated *Locusta* males. The haemolymph lipid levels were age depended with younger adults containing higher levels. The decrease with age was more marked in gregarious locusts.

In this study, the lipid level was significantly higher in the gregarious locusts in 5th and immature stages, while in the third and fourth stages the solitary locusts had higher levels. In mature adults, haemolymph lipid was sustained at same level in both phases. The levels compare well with those recorded for *Schistocerca americana* (Ziegler *et al.*, 1988). Lipid levels obtained for the gregarious fifth nymphal instar closely agree with those of Mwangi and Goldsworthy (1977), for the same stage in *Locusta*. The reason for the observed differences between the various stages is unclear but may be related to some stage specific developmental process. It is also worth noting that the pattern obtained does not correlate with the lipophorin titres despite the fact that it is the major lipid carrier. This could be due to the fact that the vanillin-phosphoric acid assay method is not sensitive to all lipophorin bound lipids but only to free or glyceride bound unsaturated fatty acids. Additionally, some of the sensitive lipids are bound to other haemolymph proteins.

CHAPTER 4

4 EFFECT OF PHASE STATUS ON RESPONSES TO ADIPOKINETIC HORMONE

4.1 Studies on lipid reserves in the fat body

The amount of lipid reserve in the locust fat body was estimated by gravimetric determination of the lipid content as a percentage (wt%) of the fat body dry weight (Table 5). Gregarious locusts had a significantly higher lipid content of 79.02 $\pm$ 2.77 as compared to 64.75 $\pm$ 2.55 in the solitary locusts. This result indicated that the gregarious locusts maintained a significantly (1 %) higher lipid reserve than the solitary locusts, despite the fact that both phases were maintained on the same diet. Further analysis of the fat body lipids by gas chromatographic techniques showed that triacylglycerides (TG) were the major lipid type present in the fat body of both phases (Table 6). The TG levels expressed as a percentage of the lipids was 83.9% for gregarious locusts and 73.8% for solitary locusts. Other lipid classes present included monoacylglycerides, cholesterol, cholesterol esters, DG and phospholipids. Fatty acids present in the fat body lipid extracts included palmitic acid, stearic acid, oleic acid, linoleic and linolenic acid (Table 7). While palmitic acid, linoleic and linolenic acids were present in the same proportion in both locust phase, variations were evident in the other fatty acids.

Table 5 **Fat body lipid expressed as a percentage of the fat body dry weight for solitary and gregarious locusts.**

Locust status	Lipid percent (wt%)
Solitary	64.75 ± 2.55 (8)
Gregarious	79.02 ± 2.77 (5)

Total lipids was extracted from lyophilized fat body and the dried extract expressed as a percentage of the fat body dry weight. Values shown are mean ± SE with N in the parentheses

Table 6 **Relative percentage (wt%) of lipid classes in the fat bodies of solitary and gregarious locusts.**

Componet	Solitary	Gregarious
Triacylglycerol	73.8	83.9
Diacylglycerol	15.1	0.7
Monoacylglycerol	1.6	0.9
Steryl ester	0.0	9.3
Phospholipids	9.2	3.2
Free fatty acids	0.3	2.0
Total	100	100

The lipid classes were separated and the fatty acids derivatised as explained in Materials and Methods. The percent composition was computed from comparisons of intergrated peak areas from gas chromatograph profiles.

Table 7 **Fatty acid percent (wt%) composition of fat body lipid extracts from solitary and gregarious locusts**

Fatty acids	Gregarious	Solitary
Palmitic (16:0)	30.0	33.8
Stearic (18:0)	9.4	6.1
Oleic acid (18:1)	27.9	10.7
Linoleic (18:2)	26.9	30.6
Linolenic (18:3)	5.8	18.8
Total	100	100

The percentages were determined from the total peak areas corresponding to external fatty acid methyl esters standards run under the same condition.

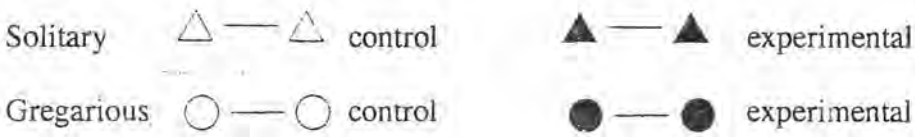
4.2 Lipid mobilization from the locust fat body.

4.2.1 Time course studies on responses to adipokinetic hormone

Doses of adipokinetic hormone (AKH I) of 2, 5, and 10 pmol/insect were administered to the insects and the responses monitored by estimating the change in DG levels at various time intervals using the vanillin-phosphoric acid assay method. There was evidence that AKH elicits responses in both locust phases with a maximum response after 90 min of administration of the hormone at all the doses tested (Fig. 31a, b, c). At peak response, there was 232%, 277% and 335% increase in DG levels for the gregarious locusts for 2, 5 and 10 pmol AKH, respectively. On the other hand, the percent increase in DG in solitary locusts was 212%, 294% and 352% for 2, 5 and 10 pmol AKH, respectively. At the end of the study period (150 min), the haemolymph lipid levels were still higher than that expected for the resting locusts.

Responses at other time intervals were expressed as a percentage of the maximum response (Table 8). The gregarious locusts showed a much faster response than the solitary. This was particularly evident after 60 min of hormonal administration and more pronounced at 2 pmol dose. Thus, after 30 min 2 pmol elicited 73.3% and 45% of the maximum response in gregarious and solitary locusts, respectively. On the other hand, at 60 min the percent response was 95% and 63% for gregarious and solitary locusts, respectively.

Fig. 31a **Time course responses to AKH (2 pmol) by the solitary and gregarious *S. gregaria*.** Following the administration of AKH or BSA as a control, DG level were estimated at 30 min interval for 150 min.



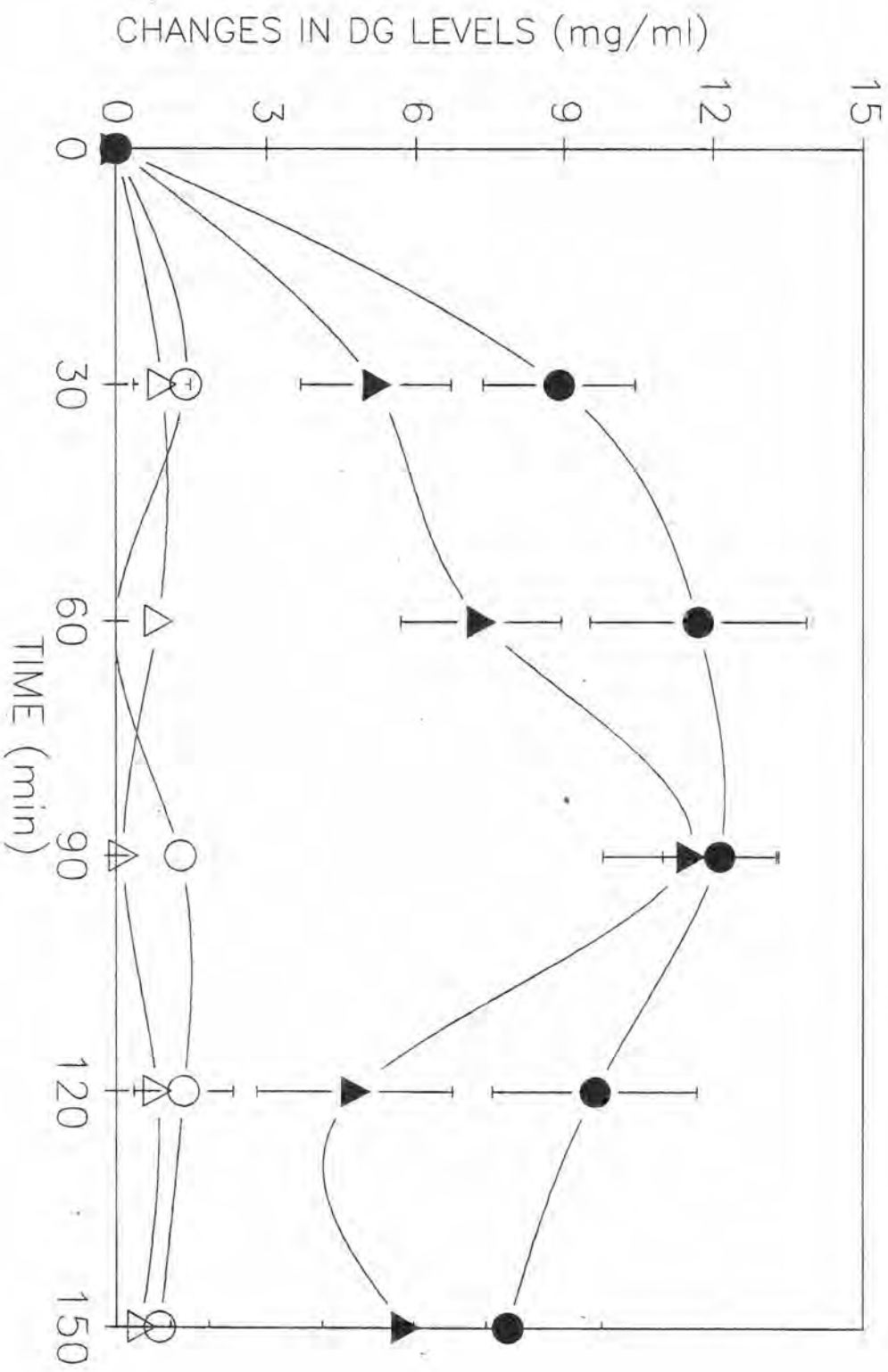

CHANGES IN DG LEVELS (mg/ml)
0
3
6
9
12
15
TIME (min)
0
30
60
90
120
150

Fig. 31b **Time course responses to AKH (5 pmol) by the solitary and gregarious** *S.gregaria*. The details were as in Fig. 31a.

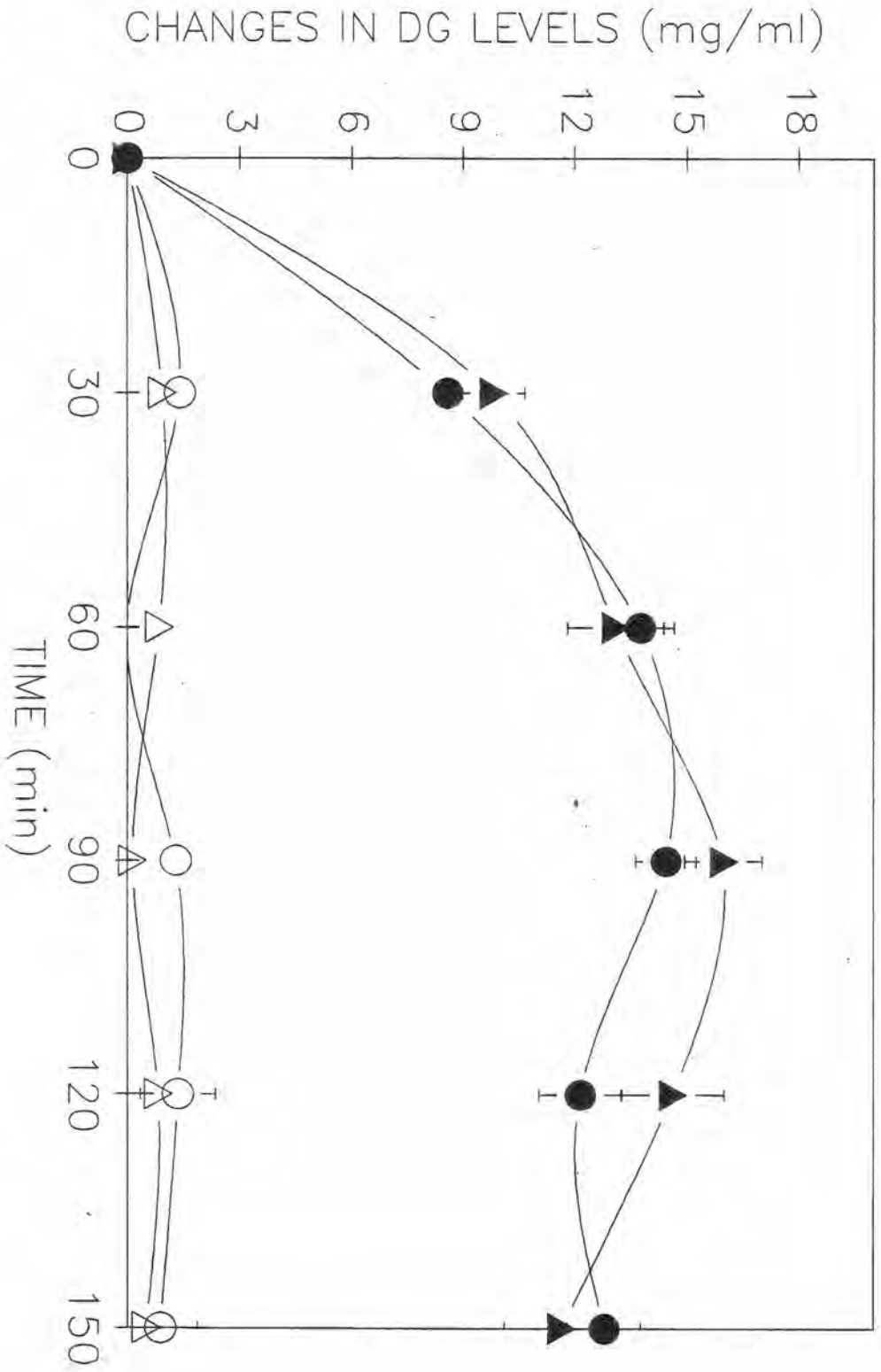

CHANGES IN DG LEVELS (mg/ml)
0
3
6
9
12
15
18
TIME (min)
0
30
60
90
120
150

Fig. 31c **Time course responses to AKH (10 pmol) by the solitary and gregarious *S. gregaria*.** Details were as in Fig. 31a

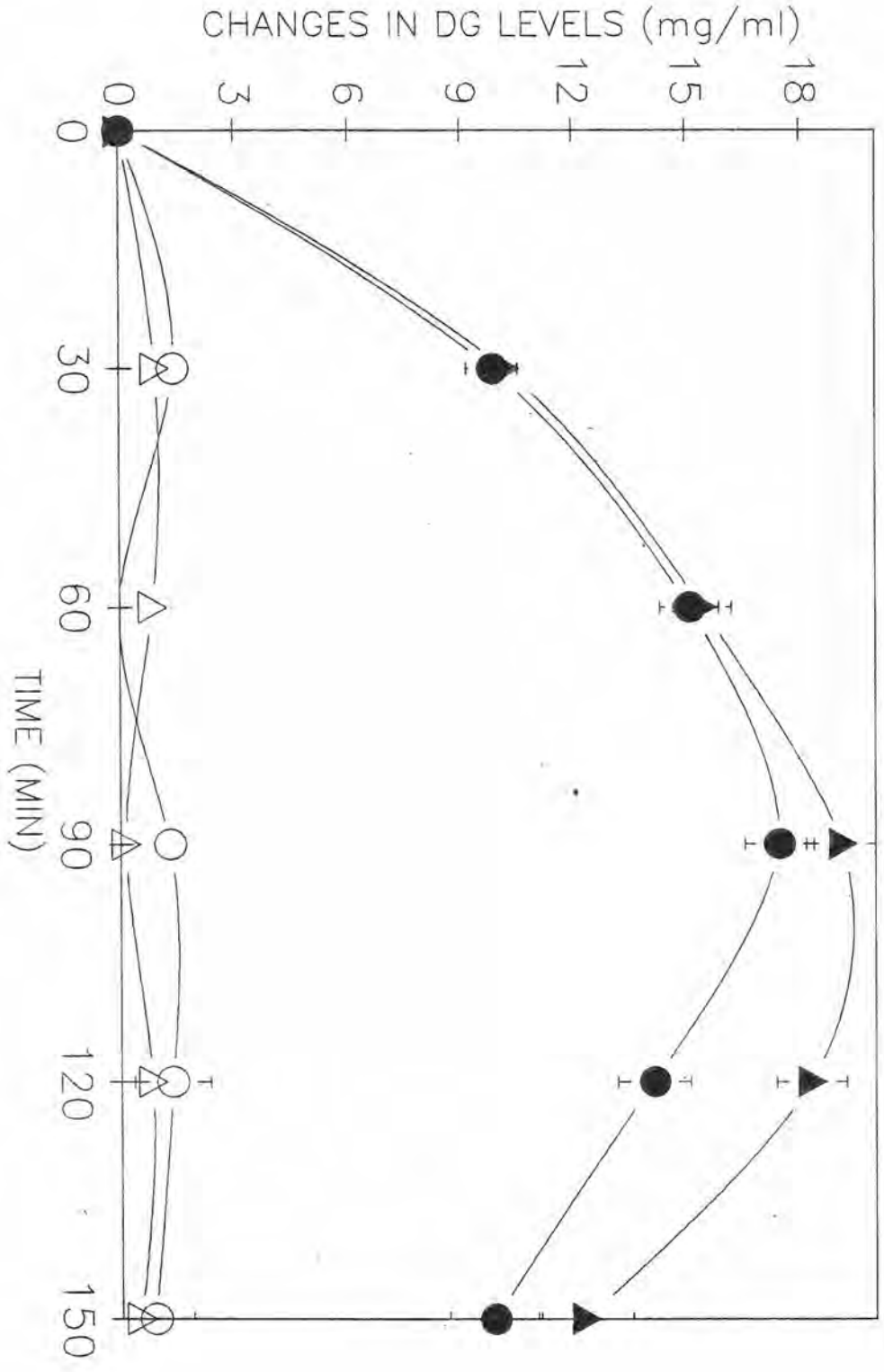

CHANGES IN DG LEVELS (mg/ml)
0
3
6
9
12
15
18
TIME (MIN)
0
30
60
90
120
150

Table 8 **Time course responses to AKH expressed as a percentage of the maximum response.**

		Time (min)		
AKH dose (pmol)	Locust status	30	60	150
2	Greg	73.3	96.5	64.8
	Sol	45.0	63.3	49.9
5	Greg	59.4	95.3	73.9
	Sol	61.6	81.9	72.8
10	Greg	56.5	86.5	56.6
	Sol	53.4	80.7	64.3

Increase in haemolymph lipid levels following the injection of AKH was determined as described in Materials and Methods. The values obtained at the above time intervals were expressed as a percentage of the maximum increase observed over the study period.

4.2.2 Dose response studies

Responses to AKH doses was monitored in the locusts 90 min after the administration of the hormone. In the range of 10^{-14} - 10^{-8} mol/insect (Fig. 32), the insects showed a negligible response between 10^{-14} - 10^{-13} mol/insect. Above 10^{-13}, there was a gradual increase which subsequently started peaking off above 10^{-11} mol/insect. The response by the gregarious locusts was evidently higher than in the solitary locusts upto 2.3 pmol/insect, when there was a cross over with the responses in solitary locusts being higher. The peaking off in the gregarious locusts occurred after 10 pmol AKH while for the solitary locusts, the peaking off was rather gradual and prolonged. Thus, even at 10^{-10} mol/insect there was still evidence of increase in response.

The responses to AKH was then monitored using a narrow range of 1 - 10 pmol/insect (Fig. 33). Within this range there was evidence that the solitary locusts were more sensitive to doses above 2 pmol. Thus, in the solitary locusts, doses above 2 pmol elicited a higher response than in the gregarious locusts. Whereas in the gregarious locusts the peaking off started after 4 pmol with no significant difference thereafter, the peaking off in the solitary locusts was less obvious such that even at 8 pmol there was still more response being elicited. Statistical analysis of the responses at 1, 2, 5, 10 pmol and 10^{-8} was performed using student *t- test* (Table 9). There was a significant difference in the responses at 1 pmol with the gregarious locusts having a greater response. On the other hand, responses at the other doses showed no significant difference although the solitary locusts showed higher lipid mobilization at 5 and 10 pmol. At 10^{-8} mol/insect, the solitary locusts had a significantly higher lipid mobilized than the gregarious locusts. Estimation of ED_{50} for

Fig. 32 **Dose response curves for lipid mobilization by AKH-I in solitary and gregarious *S. gregaria*.** Varying doses of AKH (10^{-14} - 10^{-8} mol/insect) or BSA for controls were injected into the insects and the change in haemolymph lipid concentration monitored after 90 min.

——— solitary locusts

—·—·— gregarious locusts

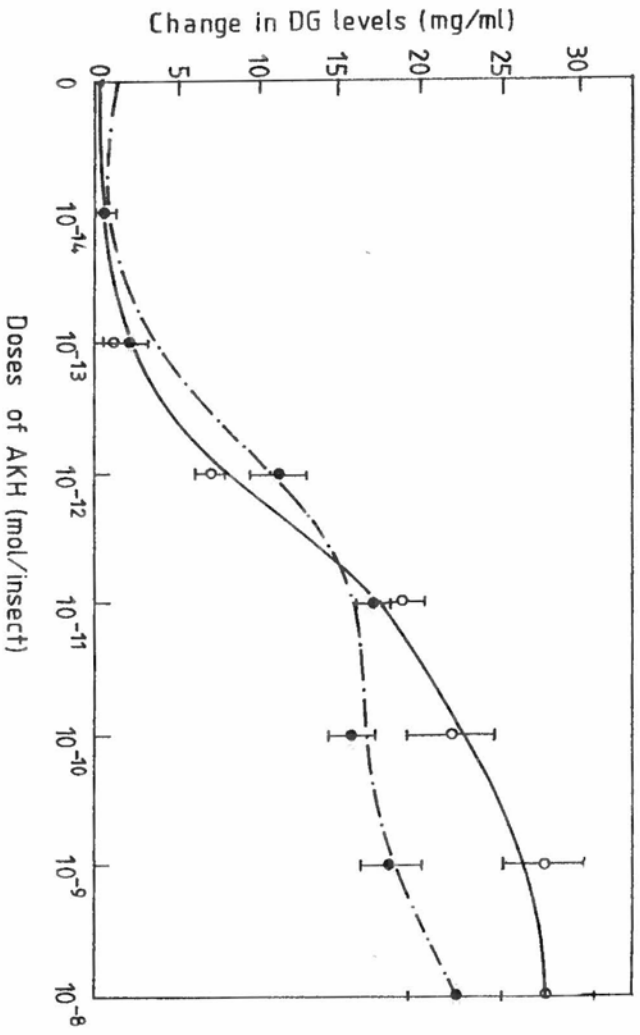

Change in DG levels (mg/ml)
0
5
10
15
20
25
30
Doses of AKH (mol/insect)
0
10^{-14}
10^{-13}
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}

Fig. 33 **Dose response curves for lipid mobilization by AKH-I in solitary and gregarious *S. gregaria*.** Varying AKH doses (1 - 10 pmol/insect) or BSA for controls were injected into the insects and change in haemlymph lipid concentration monitored after 90 min.

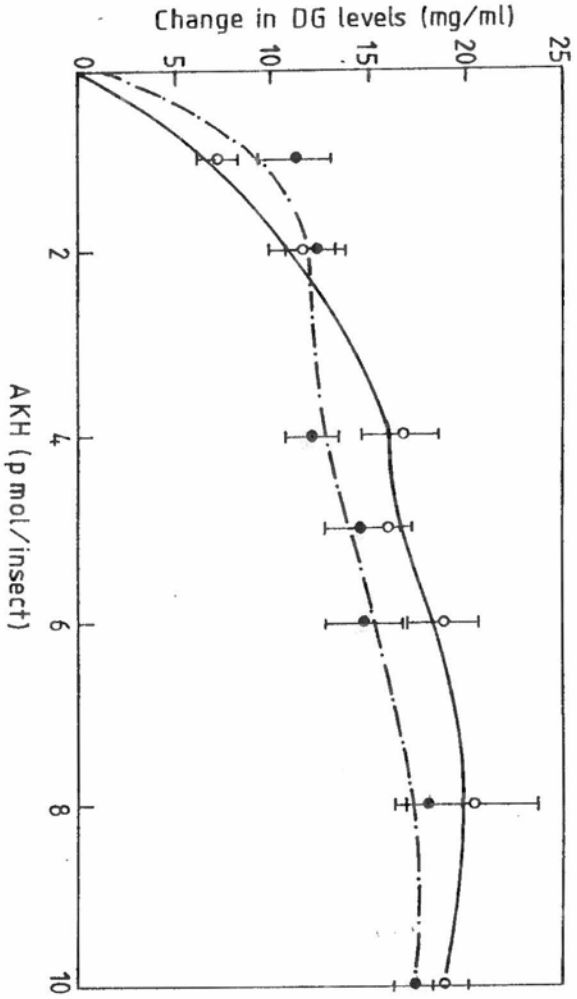

Change in DG levels (mg/ml)
0
5
10
15
20
25
AKH (p mol/insect)
2
4
6
8
10

Table 9 **Comparisons of resting and AKH-I activated haemolymph lipid levels in solitary and gregarious *S. gregaria*.**

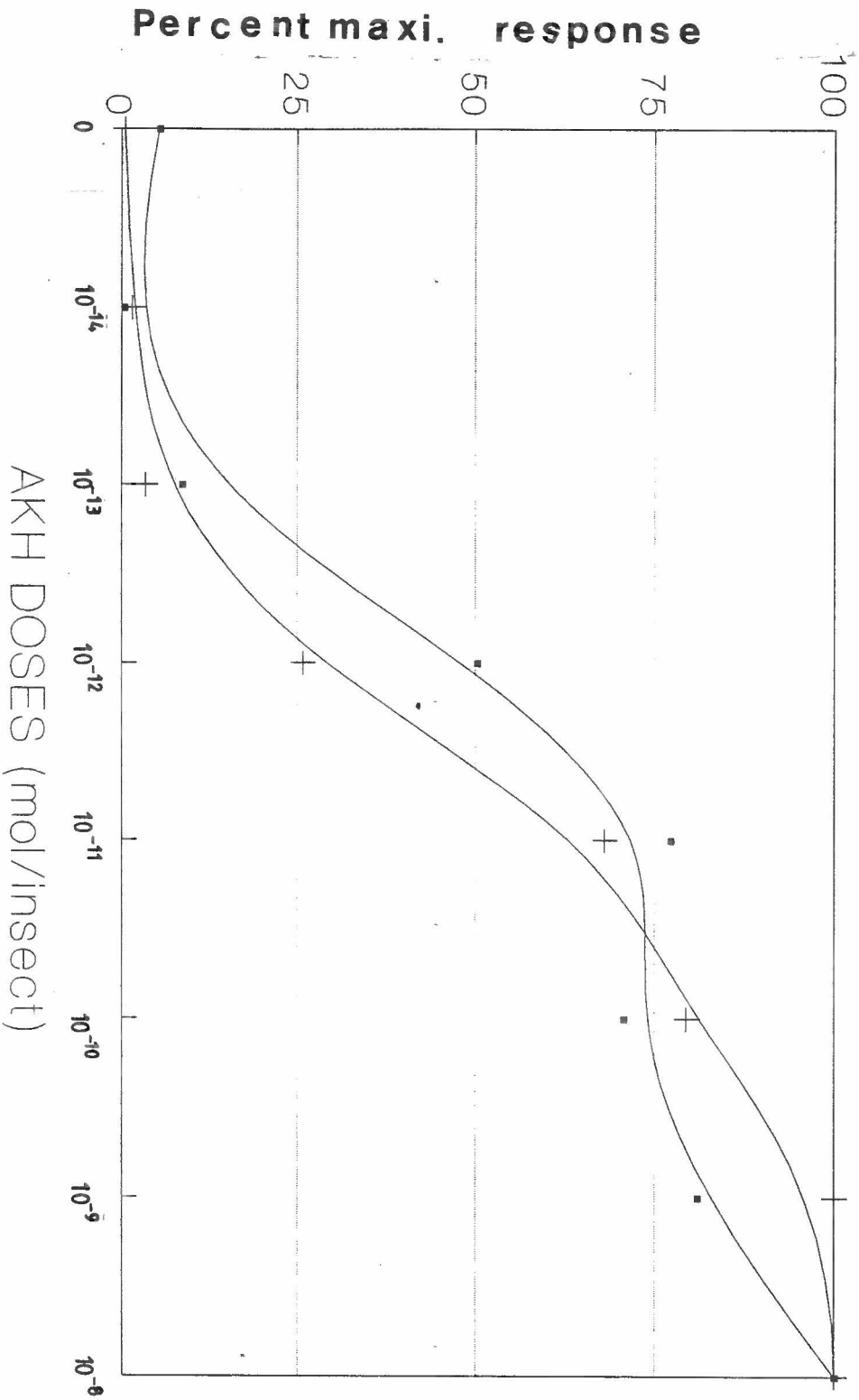
AKH Dose pmol/insect	Phase status		*P
	Gregarious	Solitary	
Control	1.204 ± 0.479(13)	0.139 ± 0.212(8)	NS
1	11.269 ± 1.894(11)	7.975 ± 0.886(10)	P<0.05
2	12.127 ± 1.144(17)	15.589 ± 1.769(15)	NS
5	14.440 ± 1.174(14)	15.987 ± 1.504(14)	NS
10	17.319 ± 0.985(21)	18.915 ± 1.318(26)	NS

Increase in haemolymph lipid levels was estimated after 90 min following the injection of AKH at the above doses or BSA for control. Values shown are mean ± SE with N in the parentheses. *P (probabilities that the values for different phases at a given dose are significantly different. Tests of significance based on two-tailed *t-tests*.

Fig. 34 **Estimation of AKH-I ED_{50} dossage for lipid mobilization**. Increase in DG levels were expressed as a percent of the maximum hyperlipaemic effect in each of the phases over the AKH dose range of 10^{-14} - 10^{-8}. The dose evoking 50% the maximum response (ED_{50}) was then determined.

+ solitary locusts

⊡ gregarious locusts


Percent maxi. response
0
25
50
75
100
AKH DOSES (mol/insect)
0
10-14
10-13
10-12
10-11
10-10
10-9
10-8

responses to AKH-I dose range of 10^{-14} -10^{-8} mol/insect, showed a higher value for the solitary locusts (6.5 pmol) as compared to the gregarious locusts (1.53 pmol) (Fig. 34).

4.2.3 Locust haemolymph lipoprotein profiles.

Haemolymph lipoprotein profiles of the locusts was obtained in both solitary and gregarious locusts by gel permeation chromatography on an AcA_{22} column. The profiles for control insects was obtained on haemolyph samples of insects injected with 10 μl of BSA (1mg/ml) 90 min prior to bleeding. On the other hand, profiles for locusts administered with AKH was obtained on haemolymph samples of insects injected with 10 pmol of AKH, 90 min before bleeding. Fractions from the column were monitored at 280 nm and by vanillin - phosphoric acid method for the estimation of the lipids.

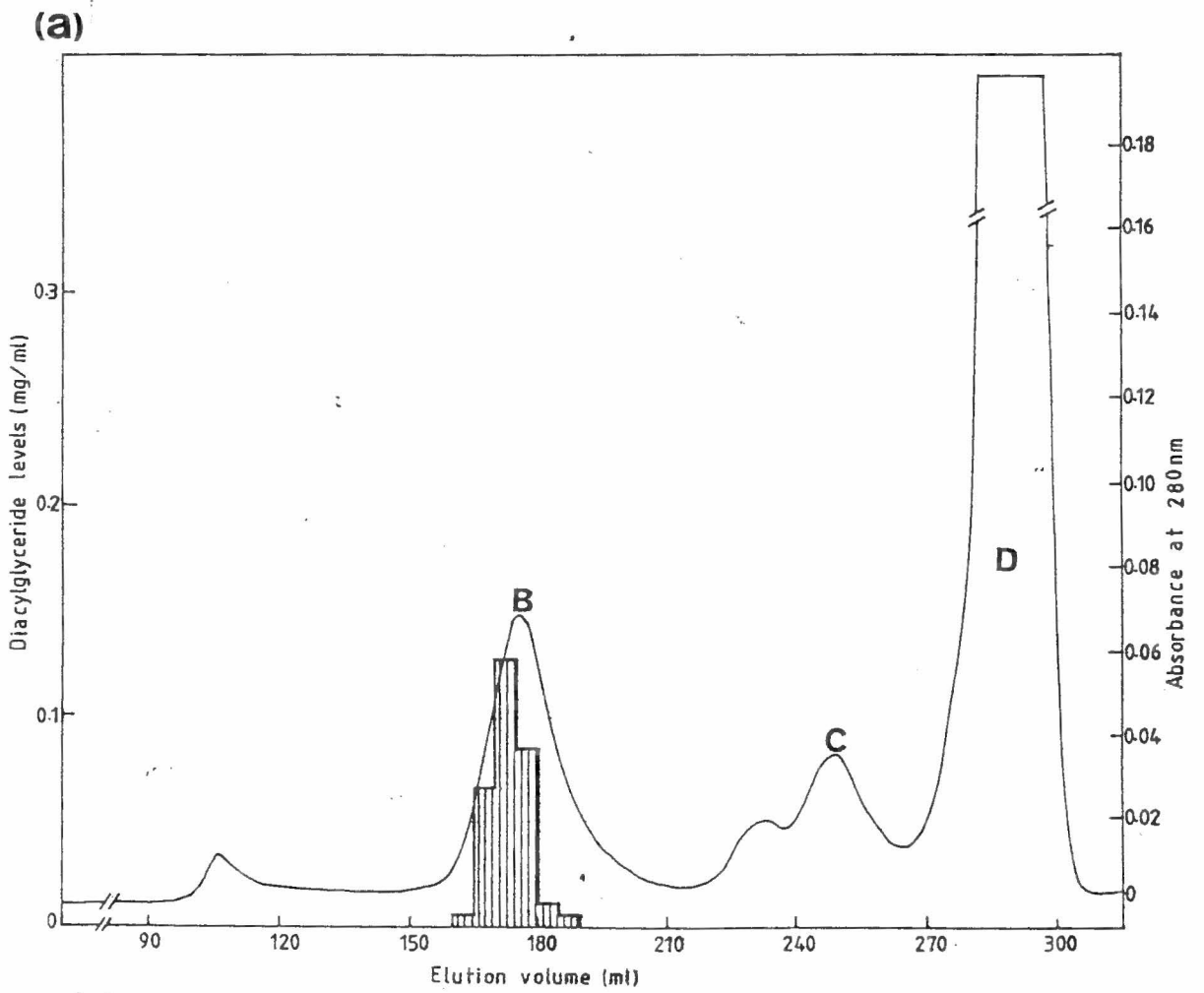
The profiles from the resting insects (control) showed three main peaks (B, C and D) in both locust phases when monitored at 280 nm (Fig. 35a, b). Additional minor peaks were observed immediately after peak B and just before peak C. The minor peak after peak B was not observed in the case of gregarious locusts. The fractions from peak B showed a yellow tint, a characteristic feature of isolated lipophorin. The fractions from this peak also showed the presence of lipids unlike the other peaks. Furthermore, this peak coincided with that of isolated lipophorin from the resting locusts. Peak D which appeared to be the most abundant was a totally included fraction with no trace of protein. The other fractions had proteins.

Injection of AKH into the locusts resulted into an additional peak (A) with a yellow colouration. This peak was eluted much earlier than any of those found in the resting locusts. In addition, there was evidently a decrease in both the quantities of peaks B and C. No difference was observed in peak D (Fig. 36a, b).

Fig. 35a,b **Elution profiles on Ultrogel AcA_{22} of solitary and gregarious locust haemolymph.** Haemolymph samples (30 mg) were taken from groups of control insects injected 90 min earlier with BSA. Elution fractions were monitored at 280 nm and assayed for lipids using the vanillin- phosphoric acid assay. (a) gregarious locusts (b) solitary locusts

Absorbance at 280 nm ——

Diacylglyceride levels 


(a)
Diacylglyceride levels (mg/ml)
0.3
0.2
0.1
0
B
C
D
90
120
150
180
210
240
270
300
Elution volume (ml)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
Absorbance at 280nm

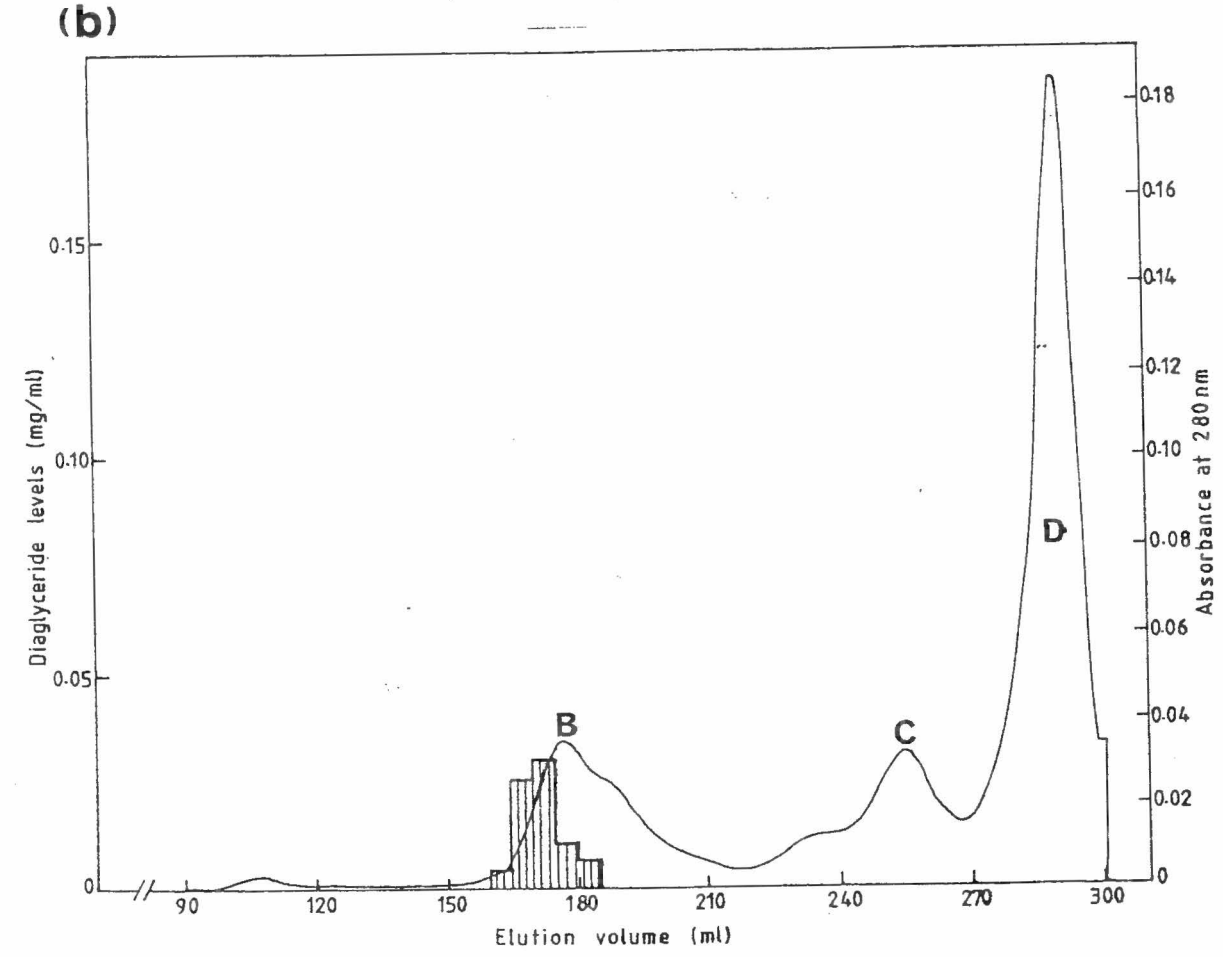
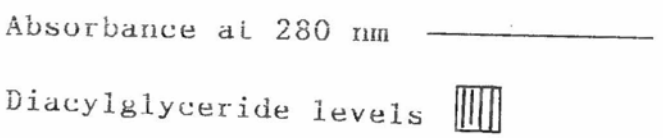
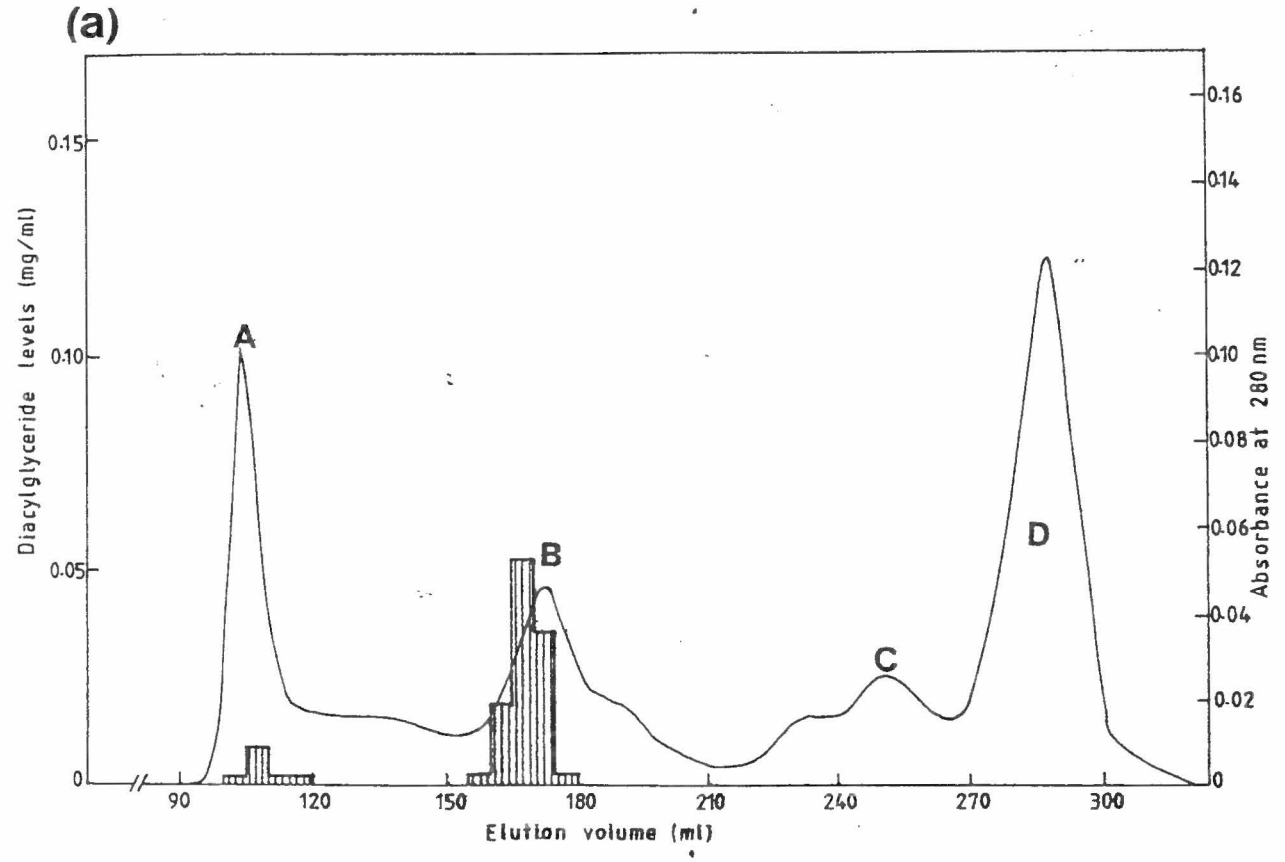

(b)
Diglyceride levels (mg/ml)
0.15
0.10
0.05
0
B
C
D
90
120
150
180
210
240
270
300
Elution volume (ml)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
Absorbance at 280 nm

Fig. 36a, b **Elution profiles on ultrogel AcA_{22} column of locust haemolymph followi AKH injection.** Haemolymph samples (30 mg) were taken from groups of insects injected 90 min earlier, with 10 pmol AKH. Elution fractions were processed as in Fig. 35. **(a)** gregarious locusts **(b)** solitary locusts




(a)
Diacylglyceride levels (mg/ml)
Absorbance at 280 nm
Elution volume (ml)
A
B
C
D
0
0.05
0.10
0.15
0.02
0.04
0.06
0.08
0.12
0.14
0.16
90
120
150
180
210
240
270
300

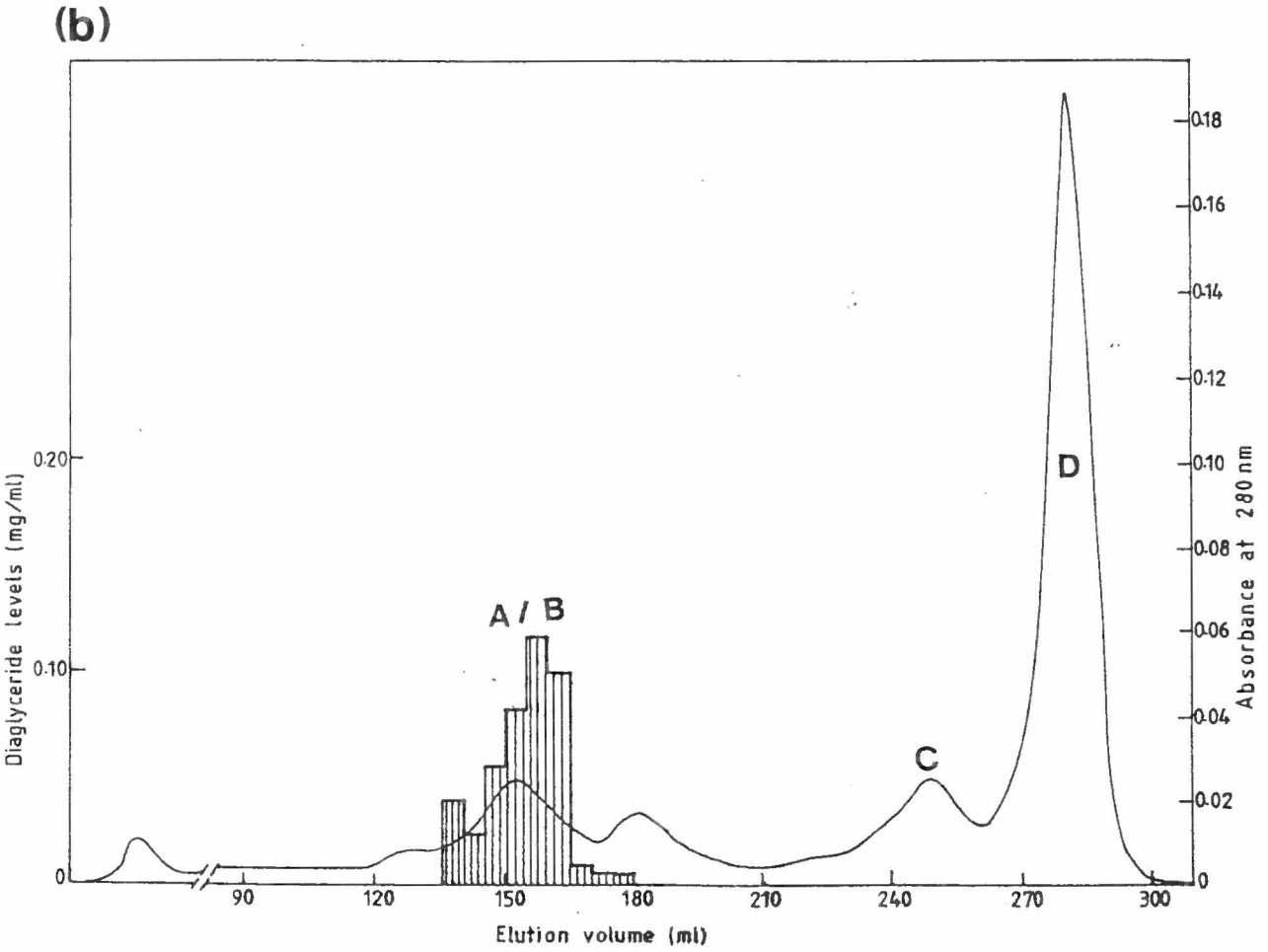

(b)
Diaglyceride levels (mg/ml)
Absorbance at 280 nm
Elution volume (ml)
A / B
C
D
0
0.10
0.20
0.02
0.04
0.06
0.08
0.12
0.14
0.16
0.18
90
120
150
180
210
240
270
300

Table 10 Percent (wt%) lipoprotein and apoLp-III shifts following AKH injection in gregarious and solitary *S. gregaria*.

	Percent reduction	
Phase	HDLp	apoLp-III
Gregarious	35.9	12.2
Solitary	43.6	13.9

The peak areas corresponding to HDLp and apoLp-III were estimated following gel permeation chromatography of the haemolymph. The percent reduction was computed by comparison of the corresponding peak areas of control and insects injected with AKH.

Estimation of the lipid content in the various fractions showed that peaks A and B contained lipids, implying that these were lipoprotein peaks. Fractions C and D were non-lipidated. Thus, in response to adipokinetic hormones, there was a shift in the lipoprotein profiles in which an a additional lipoprotein with a higher molecular weight (peak A) was formed. In addition, there was a concomitant decrease in the levels of the resting lipoprotein (peak B) as well as the non- lipidated fraction (peak C). Peak A formed in the gregarious locusts had a larger molecular size and thus appeared in the void volume of the column, compared to peak A in the solitary locusts with a molecular weight of $M_r \sim 1.7 \times 10^6$. A comparison of the percent decrease in peaks B and C , showed that the weight percent decrease in apoLp-III was 12.1 and 13.9% for gregarious and solitary locusts, respectively. On the other hand the percent decrease in peak B was 35.9% for gregarious and 43.6% for solitary locusts (Table 10).

4.3 Studies on fat body triacylglyceride lipase

4.3.1 Activation of the lipases with AKH

Preliminary studies were carried out to find out if there was any activation of the lipase in response to administration of AKH. Thus, 10 pmol of AKH was injected into groups of insects and the levels of the enzyme monitored by measuring the counts per min of the product formed from a radiolabelled substrate over a 90 min period. The results indicated that there was activation of the lipase with a maximum response observed after 30 min (Fig. 37). Locusts from both phases showed a maximum response at the same time with the activation subsequently leveling off. By the end of the 90 min study period, the levels of the lipase had decreased to that at the onset of the experiment. In subsequent experiments, the effect of AKH was monitored 30 min after injection of either the hormone or BSA for controls.

The activation of the lipase was further monitored by estimation of the enzyme levels following administration of 2 and 10 pmol AKH. Resting levels were also obtained in insects not injected at all (Table 11). The resting levels of the lipases was estimated to be 1.98 ± 0.24 and 1.95 ± 0.53 for solitary and gregarious locusts, respectively. On injection of BSA, there was a a slight reduction in the levels to 1.72 ± 0.29 and 1.52 ± 0.14 for solitary and gregarious locusts, respectively. There was evidence of activation of the lipase at the two levels of AKH studied. Thus, the levels in solitary locusts were 2.30 ± 0.43 and 2.28 ± 0.16 following injection of 2 and 10 pmol of AKH respectively. On the other hand, levels in the gregarious locusts were 2.47 ± 0.39 and 3.17 ± 0.66 for 2 and 10 pmol AKH, respectively. Significant difference from the control was only observed in the gregarious locusts. Furthermore, while there was no significant difference on the level activation of the

Fig. 37 **Time course activation of solitary and gregarious *S. gregaria* fat body triacylglyceride lipase by AKH.** The fat bodies were processed as decribed in Materials and Methods before incubation with the substrate for 90 min at 33 °C Following administration of 10 pmol AKH, the lipase levels in the fat body were estimated at varying time intervals as described in Materials and Methods.

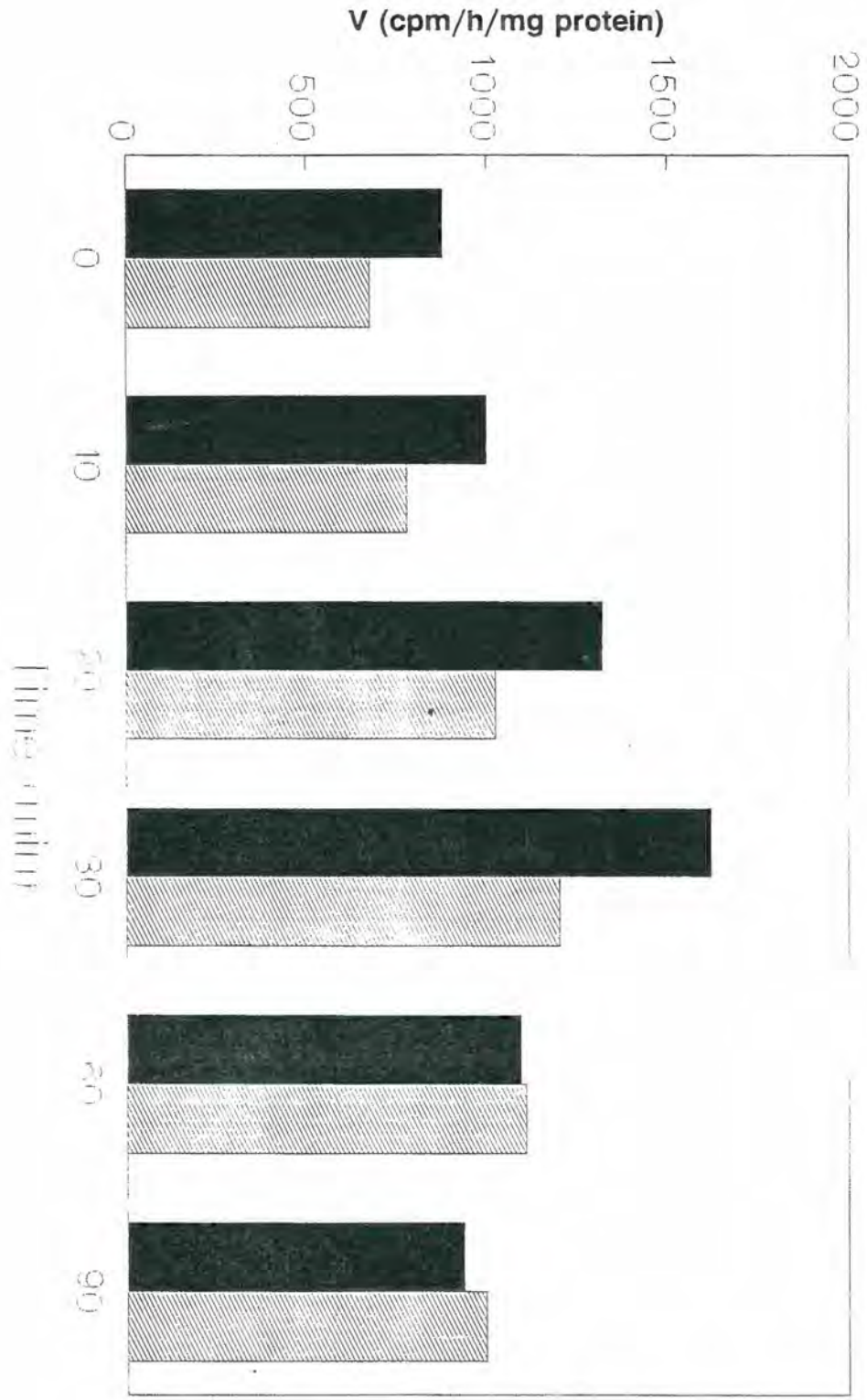

V (cpm/h/mg protein)
0
500
1000
1500
2000
time (min)
0
10
20
30
60
90

Table 11 The effect of AKH-I on the levels of triacylglyceride lipase in solitary and gregarious *S. gregaria*.

	Solitary	Gregarious
Resting levels	1.98 ± 0.24 (4)	1.95 ± 0.53 (4)
Control (BSA)	1.72 ± 0.29 (4)	1.52 ± 0.14 (5)
AKH (2 pmol)	2.30 ± 0.43 (4)	2.47 ± 0.39 (5)
AKH (10 pmol)	2.28 ± 0.16 (4)	3.17 ± 0.66 (5)

The fat bodies were dissected out from the resting insects or insects injected 30 min earlier with BSA(control) or AKH and processed as described in Materials and Methods. Values shown are mean ± SE with N in the parentheses

lipase in the solitary locusts at the AKH doses tested, there was a significant difference on the activation in gregarious locusts. Comparison of the activation between the phases at the two doses, showed no significant difference.

4.3.2 Estimation of kinetic parameters of the fat body triacylglyceride lipase

To estimate the kinetic parameters of the lipases, groups of insects were injected with 10 pmol of AKH and the fat bodies dissected out after 30 min. The pooled fat bodies were processed as described above and used as the enzyme source. A range of substrate concentration of 0 - 50 μM was initially used in the assay. However, there was evidence of substrate inhibition above 24 μM for solitary while 28.7 μM for gregarious locusts (Fig. 38a, b). Thus, a range of 0 - 30 μM was used in subsequent experiments.

A plot of V versus substrate concentration showed a generalised enzyme kinetics curve, with the value of V increasing with the substrate concentration initially before reaching a saturation stage at 30 μM (Fig. 39a, 40a). Estimation of the kinetic parameters was carried out using Lineweaver- Burk plots (1/V versus 1/[S]), Hanes plots ([S]/V versus [S]) or Eadie - Hoffstee plots(V versus V/[S]). In gregarious locusts, using the Line Weaver Burk plot, Vmax was estimated at 10.286 nmol/h/mg protein while Km was estimated to be 46.667 μM. While using the Eadie-Hoffstee plot, V_{max} and K_m were estimated at 6.800 and 22.05, respectively. The Line Weaver Burk plots however gave the best regression plot through the points (Fig. 39b). In the case of the solitary locusts, the V_{max} and K_m were estimated to be 2.521 nmol/h/mg protein and 18.750 μM, respectively, using the Hanes plot. The Eadie-Hoffsttee plot on the other hand gave values of 3.109 and 14.6312 for V_{max} and K_m, respectively. The Hanes plot gave the best approximation to the points (Fig. 40b), while

Fig. 38 The kinetics of fat body triacylglyceride lipase for solitary and gregarious *S*

***gregaria*.** Fat bodies were dissected out from insects injected 30 min earlier w
AKH and processed as in Fig. 37. The kinetics was followed over a range of
substrate concentration (0 - 50 μM) while the enzyme level was kept constant.
The rate ofproduct formation (V) was plotted against substrate concentration [S]

⊡ gregarious locusts

+ solitary locusts.

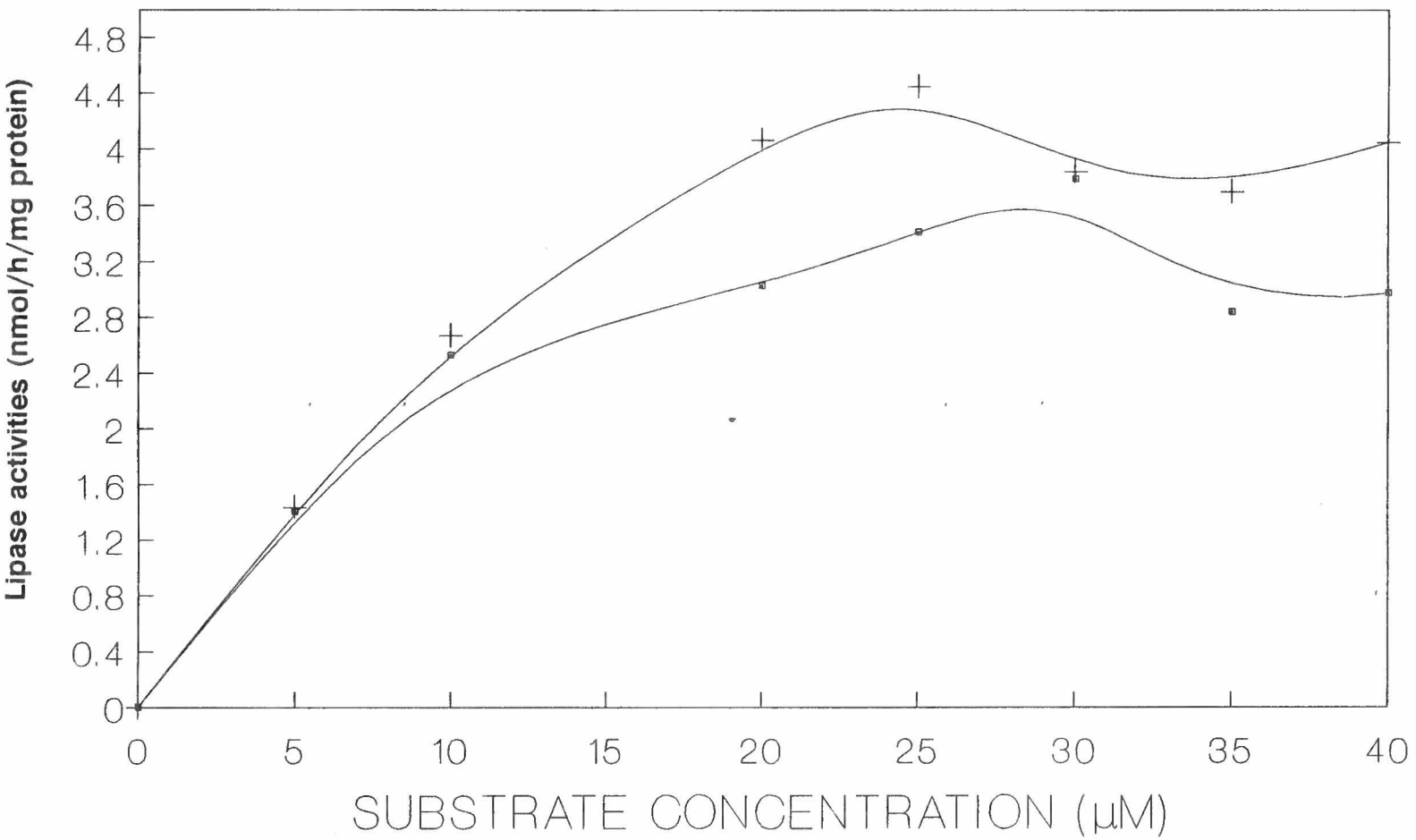
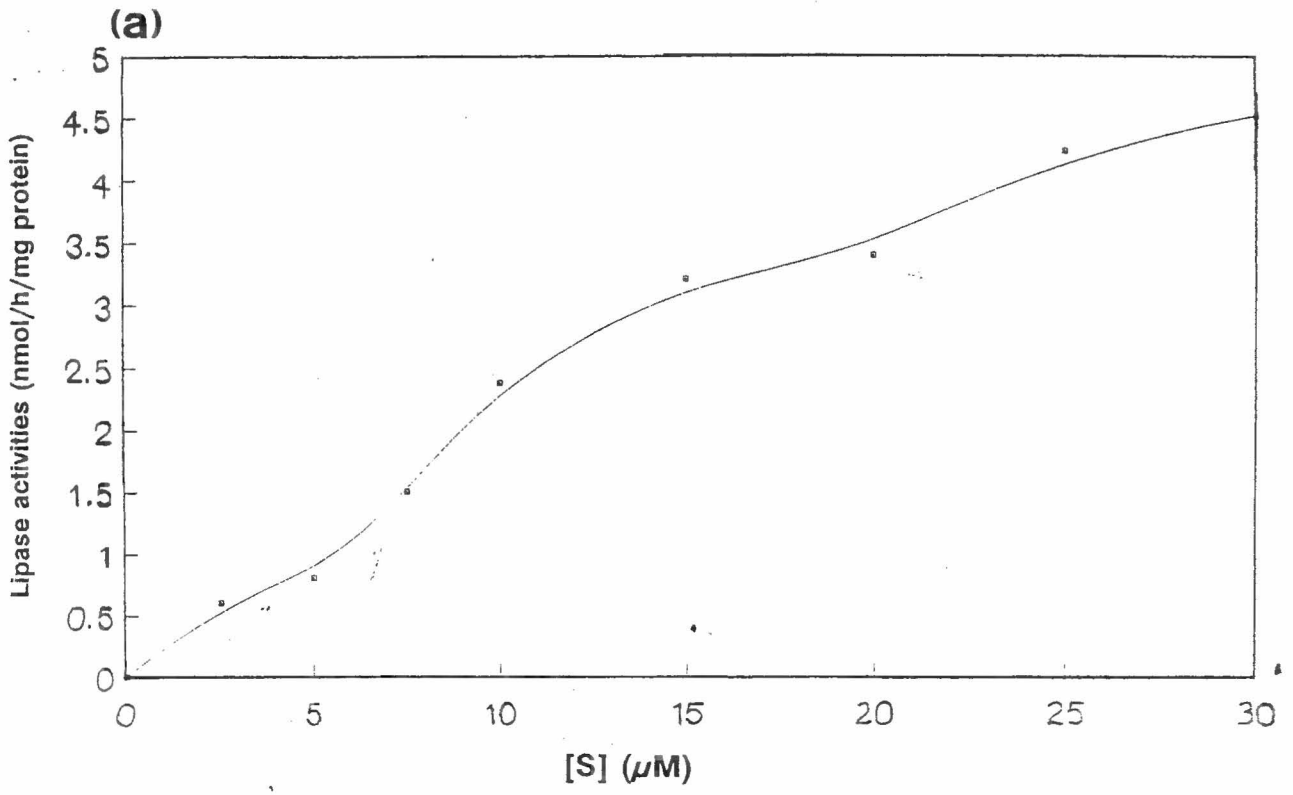

Lipase activities (nmol/h/mg protein)
4.8
4.4
4
3.6
3.2
2.8
2.4
2
1.6
1.2
0.8
0.4
0
0
5
10
15
20
25
30
35
40
SUBSTRATE CONCENTRATION (μM)

Fig. 39a, b **Determination of the Michaelis constant (Km) and maximum velocity (Vmax) for triacylglyceride lipase of gregarious *S. gregaria*.** Km and Vmax were estimated from the Lineweaver-Burk plots. Fat bodies were dissected out from insects injected 30 min earlier with AKH and processed in Fig. 37. **(a)** kinetics of the lipase (V versus [S]. **(b)** Lineweaver-Burk plot (1/V) versus 1/[S].


(a)
Lipase activities (nmol/h/mg protein)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
30
[S] (μM)

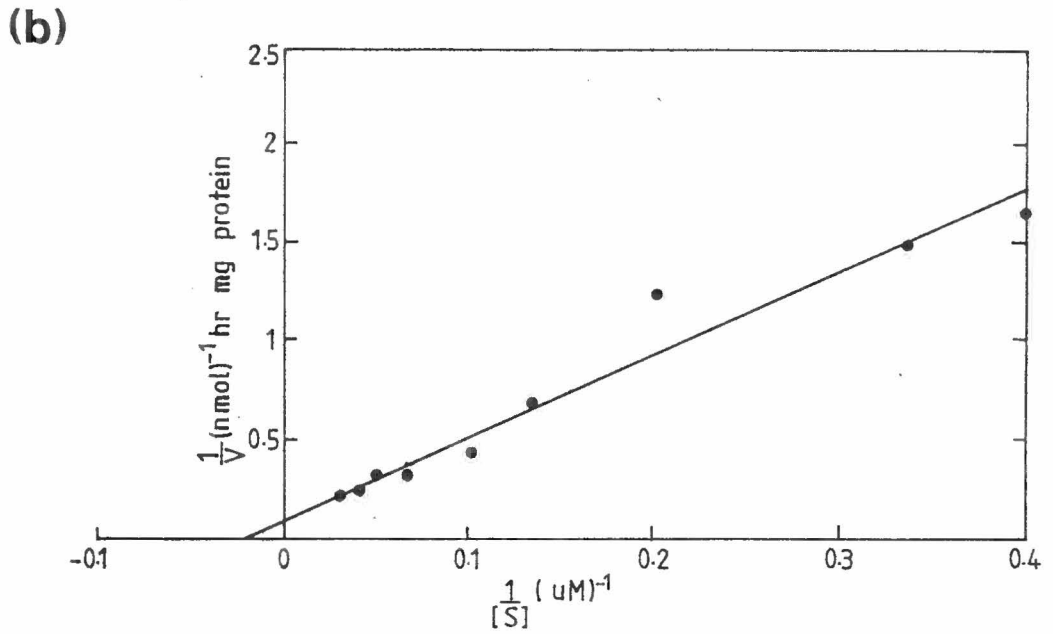
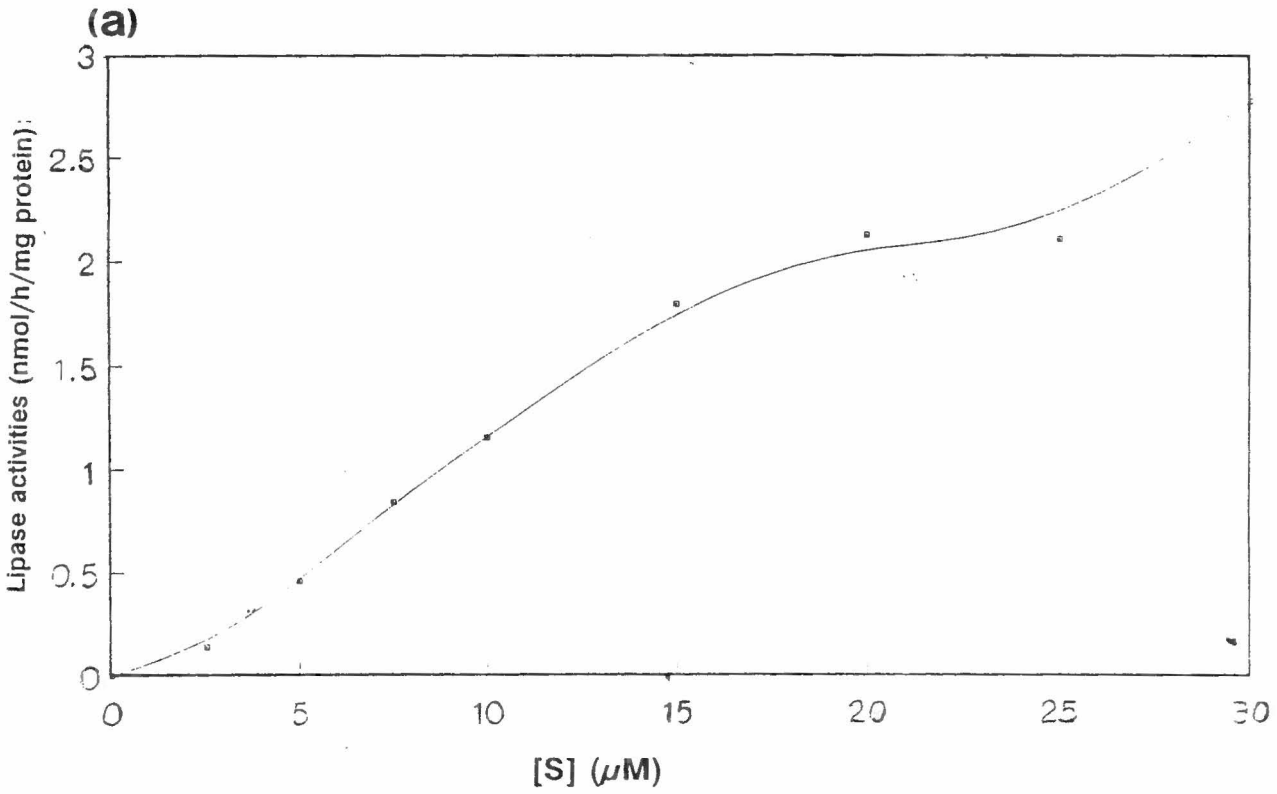
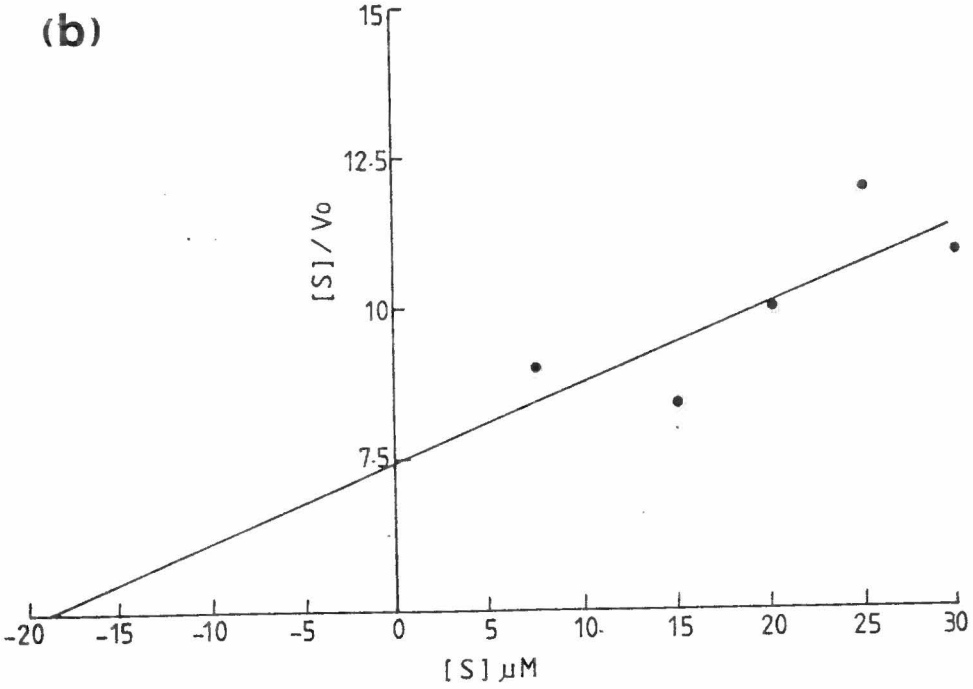

(b)
2·5
2
1·5
1
0·5
1/V (nmol)⁻¹ hr mg protein
-0·1
0
0·1
0·2
0·3
0·4
1/[S] (uM)⁻¹

Fig.40a, b **Determination of the Michaelis constant (Km) and maximum velocity (Vm for triacylglyceride lipase of solitary locusts.** The fat bodies were processed explained in Fig. 37. Km and Vmax were estimated from the Hanes plot. **(a)** kinetics of the lipase (V versus [S]). **(b)** Hanes plot [S]/V versus [S].


(a)
Lipase activities (nmol/h/mg protein)
3
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
30
[S] (μM)


(b)
15
12.5
10
7.5
[S] / Vo
-20
-15
-10
-5
0
5
10
15
20
25
30
[S] μM

the Line Weaver Burk plots could not be used for the estimation since the points of intersection were not meaningfull. Using the Line Weaver Burk plots for the gregarious locusts and the Hanes plot for the solitary locusts, the gregarious locusts had a higher V_{max} as well as a higher K_m. Thus, the fat body triacylglyceride lipase in the gregarious locusts have a higher catalytic ability but a lower affinity for the substrate than the solitary locusts. The high catalytic ability may reflect the physiological strain during long distance flights as in migration, while the high Km value may be an adaptation to cope with the high TG levels in the locusts fat body especially in the gregarious phase.

4.4 Discussion

4.4.1 Fat body lipid reserves

Accumulation of fat body lipids has been shown to correlate to a number of physiological processes in insects. Thus, reproducing female locusts accumulates large amounts of lipids (Orr, 1964; Hill *et al.*, 1968). Male insects have also been shown to contain large deposits of lipids as in *S. gregaria* (10-30% fat body wet weight) or *L. migratoria* (23% wet weight or 76% dry weight)(Beenakkers *et al.*, 1985). After adult emergence, locusts undergo a period of intense feeding activity and lipid accumulation prior to sexual maturation (Hill *et al.*, 1968; Walker and Bailey, 1970). Positive correlation has also been observed between lipid reserves in the fat body and inclination to flight in Australian plague locust, *C. terminifera* (Hunter *et al.*, 1981). Furthermore, Chino *et al.* (1992) recently reported that while the solitary *L. migratoria* fat body was only 7-8 mg, the tissue was 50-60 mg in the gregarious locusts.

In this study, a higher fat body lipid content was observed in the gregarious locusts

(79.02 ± 2.77%) as compared to 64.75 ± 2.55%) for solitary locusts (Table 5). The high lipid reserve observed in gregarious locusts may reflect a physiological adaptation for their capacity for long distance flights. The triacylglycerides were the major lipid class in the fat body constituting 83.9 and 73.9% of the total lipids for gregarious and solitary locusts, respectively (Table 6). In many insects, TG constitutes upto 90% of the total fat body lipids (Beenakkers *et al.*, 1985). The composition of the fat body lipids has been shown to be age, sex, and diet dependent (Walker *et al.*, 1970; Bailey *et al.*, 1975; Beenakkers *et al.*, 1985). Insects used in this study were of the same age and sex and were maintained on the same diet, thus limiting the impact of these factors. Comparative study of the fat body of *L. migratoria* and *Periplaneta americana*, which lacks capacity for long distance flight, showed that the amount of DG in the cockroach was extremely low compared to that in the locust (Chino *et al.*, 1992). However, the TG levels in the fat body of both insects were comparable. Comparisons of the solitary and gregarious *L. migratoria* showed that the fat body TG level in the solitary locusts was only 5% that in the gregarious locusts, while the DG levels was undetectable in solitary locusts. It was thus, concluded that the solitary locusts inability for long distance flight was due to insufficient pool of DG and TG in the fat body (Chino *et al.*, 1992). In contrast, Ziegler *et al.* (1988) observed that despite the inability of *B. psolus* to engage in long distance flights, the fat body tissue (4.7 ± 2 mg) with 50% lipid, was sufficient for a much stronger adipokinetic response. The results obtained in this study showed that in both phases of *S.gregaria*, TG was the major fat body lipid, which is consistent with previous studies (Beenakkers *et al.*, 1985). In contrast, Chino *et al.* (1992) showed that DG was the major lipid present. While, the gregarious locusts were shown to have a higher TG level, the solitary locusts had comparable DG and fatty acid

levels.

4.4.2 Mobilization of lipids from the locust fat body

Qualitative differences in haemolymph lipoprotein during flight and at rest was first demonstrated in *Schistocerca gregaria* by Mayer and Candy (1967). Since then, the mechanisms of lipoprotein conversions has only been adequately studied in *L. migratoria* and *M. sexta*, where a similar mechanism is operational (Shapiro *et al.*, 1988). During rapid lipid mobilization as in flight or upon injection of AKH, HDLp is loaded with more lipid, leading to the formation of LDLp. Concomitant with the loading is an association with non-lipid containing apoprotein, apoLp-III. The conversion of HDLp to LDLp also results into a decrease in haemolymph HDLp and apoLp-III levels as well as an increase in haemolymph lipid (Jutsum and Goldsworthy, 1976; Van der Horst *et al.*, 1980).

Lipid mobilization from the fat body in response to AKH-I, has been demonstrated in both phases of *S. gregaria.* The ultracentrifugation of heamolymph proteins before and after administration of the hormone showed that the administration led to the conversion of a high density lipophorin (HDLp) to a low density lipophorin (LDLp)(section 3.1). Thus, there was evidence of more lipid loading onto HDLp as well as association with more apoLp-III molecules resulting into a larger but a less denser LDLp. Haemolymph lipid levels also increased such that at peak response to AKH doses of 2, 5 and 10 pmol, the percent DG increase for both locusts phases was dose dependent, ranging between 200-300% (section 4.2.1). The lipoprotein shifts was also evident following the administration of the hormone (section 4.2.3)

The resulting LDLp has further been shown to exhibit a lower electrophoretic

mobility, higher molecular weight as well as a higher particle size (Chino *et al.*, 1986; Mwangi and Goldsworthy, 1977; Van der Horst *et al.*, 1979). LDLp isolated from both phases of *S. gregaria* had a much higher molecular weight. Thus, it was not possible to separate on non-denaturing-PAGE (4-20%). Gel permeation chromatography on AcA_{22} column showed that the conversion resulted into lipoprotein particles of much larger molecular weights (section 4.2.3). In addition, there was evidence from these studies that LDLp derived from gregarious locusts had a much larger molecular size in comparison to that from the solitary locusts. This could have arisen from either more lipid loading and/ or more of the apoLp-III molecules associating. The differences in lipid content between LDLp from the two phases would not account fully for the observed differences in molecular size. Thus, the differences in the levels of apoLp-III associating with LDLp may be contributing significantly to the observed difference in lipoprotein profiles between the two phases.

The formation of LDLp in response to AKH administration has been shown to correlate with the ability of insects to fly. Ziegler *et al.* (1988) observed that in *B. psolus*, an insect that has lost the ability to fly, no LDLp was formed in response to AKH injection. It has also been shown that in 5th nymphal instar of *Locusta*, the lipoprotein fraction resulting from AKH injection was of intermediate density and size between LDLp and HDLp (Van der Horst *et al.*, 1987; Van Marrewijk *et al.*, 1980; Mwangi, 1977). However, in both *Locusta* (5th nymphal instar) and *B. psolus*, there was a slight increase in haemolymph lipid.

Recent studies by Chino *et al.* (1992), indicated that the solitary *L. migratoria* were unable to form LDLp when injected with AKH. However, it was not clear from their studies how the presence of LDLp was checked or how the status of the insects used was determined. In contrast to their results, a response to AKH has been demonstrated in both

phases of *S. gregaria*. Similarly, hyperlipaemic and LDLp formation in response to corpus cardiacum extracts or AKH has been demonstrated by Ayali and Pener (1992) in both solitary and gregarious phases of *Locusta*.

Time course studies on responses to AKH indicated that there was a maximum response elicited in both locust phases at all the three dose of AKH (2, 5, and 10 pmol) tested after 90 min of administration (section 4.2.1). At the three doses of AKH tested, there was evidence of a faster response in the gregarious than solitary locusts. The results further indicated that there was an inverse relationship between the doses and the rate of responses. Thus, at 30 min there was a higher percent increase when 2 pmol was injected followed by 5 and 10 pmol. The same trend was observed at other time intervals.

Peak response to AKH has been shown to occur in several insect species after 90 min (Wheeler, 1989). Thus, the observation of a peak response after 90 min at all the doses tested in *S. gregaria*, was not a unique feature. It has further been shown that LDLp formation proceeds at a steady rate after AKH injection, with significant quantities being formed after 20 min (Wheeler and Goldsworthy, 1983a, b; Mwangi and Goldsworthy, 1977). Both apoLp-III and lipids have been shown to start associating with the HDLp immediately after injection of AKH (Goldsworthy, 1983). The responses observed in *S. gregaria* after 30 min, may thus indicate the immediate response leading to the mobilization of lipids and the subsequent association of such lipids and apoLp-III with LDLp molecule. A faster response may be an additional advantage to the gregarious locusts as it would ensure rapid mobilization of the lipids when required.

Peak responses to ranges of AKH (1-10 pmol/insect) and 10^{-14}-10^{-8} mol/insect),

revealed that the gregarious locusts were more sensitive to doses below 2 pmol (Fig. 33, 34). Above this dose, the solitary locusts elicited higher responses. There was evidence that the responses in solitary locusts were not as tightly controlled as those in gregarious locusts. The ED_{50} for the action of AKH was shown to be much higher for the solitary than gregarious locusts. These results were in contrast to those by Ayali and Pener (1992) who found that the gregarious *Locusta* had higher responses at all the doses of AKH as well as corpus cardiacum extracts tested. Chino *et al.* (1992) on the other hand, did not show whether or not there was a change in haemolymph lipids, although they showed that no LDLp was formed. This may suggest that there was no DG increase associated with AKH injection. However, this would appear unusual since hyperlipaemic effect of AKH has been shown on non flying insects as well as in nymphal instars (Ziegler *et al.*, 1988; Van der Horst *et al.*, 1987).

Responses to adipokinetic hormones among the acridids has been shown to be not only species but also intraspecific phase dependent (Chino *et al.*, 1992; Ayali and Pener, 1992; Ziegler *et al.*, 1988). The inability to respond to the hormone has been attributed to several factors such as inadequate levels of apoLp-III, haemolymph factor(s), or a defect in one or more of the lipid mobilizing factors (Ziegler *et al.*, 1988; Van der Horst *et al.*, 1987). The inability of the solitary *L. migratoria* to fly long distances has also been attributed to the nature of the fat body, specifically the content of triacylglycerides and diacylglycerides (Chino *et al.*, 1992).

Ayali and Pener (1992) and Chino *et al.* (1992) attributed their observations in *Locusta* to physiological adaptations for long distance flights. *S. gregaria* also seems to have adopted various strategies to cope with long distance flights. Although the gregarious locusts

have higher fat body lipid levels, the solitary locusts have adequate lipid reserves to maintain long distance flights. However, it appears likely that the gregarious locusts have an efficient system for the mobilization of the lipids unlike the solitary locusts. Thus, in addition to a faster response, the gregarious are also more sensitive to lower levels of AKH than solitary locusts. Furthermore, it is possible that most of the mobilized lipid ends up bound by the lipoprotein unlike in solitary locusts. The levels of apoLp-III do not appear to be significantly different in the haemolymph or in association with LDLp. However, there is the possibility that more apoLp-III associating, leading to a larger LDLp molecule.

4.4.3 Studies on fat body triacylglyceride lipase

Studies on fat body triacylglyceride lipase demonstrated that administration of AKH resulted in the activation of the lipase with the maximum activation observed in both phases after 30 min (section 4.3.1). Resting levels of the lipases was estimated to be 1.98 $\pm$ 0.24 and 1.95 $\pm$ 0.53 nmol/h/ mg protein for solitary and gregarious locusts, respectively. Injection of 2 and 10 pmol AKH showed an elevation of the lipase activities in both phases. At the two doses of AKH there was no significant difference in the activation of the solitary locusts lipase, whereas for the gregarious locusts , 10 pmol elicited a higher activation than 2 pmol. However, between the two phases, there was no significant difference in the activation of the lipase at either of the doses.

The kinetics of the lipase at varying substrate concentrations followed the general Michaelis-Menten kinetics. Estimation of the kinetic parameters on crude enzyme preparation showed that the gregarious locusts had a higher V_{max} and K_m (Fig 39, 40). This suggested that the lipases in gregarious locusts had a higher catalytic ability but a lower

affinity for the substrate. Substrate inhibition was also evident above 24 and 28.7 μM for solitary and gregarious locusts, respectively.

Lipase activities have been demonstrated among several insect orders and in a number of insect tissues including: fat body, haemolymph, egg, and the gut (Downer, 1985). Male and Storey (1981) have further demonstrated the presence of electrophoretically separable proteins with lipolytic activities specific for tri-, di- and mono- acylglycerides in *P. americana*, *L. migratoria* and *Polia adjunca*. The three types of lipases were detectable in all tissues studied namely, fat body, muscles and the gut. Studies on fat body lipases has been hampered by a number of constraints. The substrates for these studies require full emulsification since the hydrolysis can only proceed at the interface between emulsified substrate and the aqueous media. The interpretation of the results is also complicated by the unknown extent of activation or inactivation of the enzyme that occurs when the molecule is removed from its location *in situ*. Lack of standardized procedures for demonstration of lipolytic activity in insects combined with the multifarous nature of the substrates and the emulsifiers employed, renders comparative assessment of the insect lipases difficult.

Previous studies have been unable to demonstrate activation of lipases with AKH (Spencer and Candy, 1976; Pines *et al.*, 1981; Van der Horst, 1991). However, the lipases have been shown to be activated by cyclic nucleotides such as cAMP, cGMP as well as the red pigment concentrating hormone (RPCH)(Pines *et al.*, 1981). In this study, the activation of triacylglyceride lipase by AKH was demonstrated. Peak activation was observed after 30 min in both locust phases. The demonstration of the activation was probably possible due to the method used as described by Hirayama and Chino (1987). However, in their study no attempt was made to demonstrate the activation. The activation of fat body protein kinase

due to AKH, cAMP, cGMP and RPCH has been shown in *Locusta* (Pines *et al.*, 1981; Pines and Applebaum, 1978). *In vitro* activation of fat body protein kinase demonstrated by Pines and Applebaum (1978) was shown to peak after 90 sec for RPCH and 3.5 min for AKH extracts. *In vitro* activation of fat body lipase by 50% was also demonstrated 10 min after exposure of the tissue to cAMP and cGMP (Pines *et al.*, 1981). Thus, the peak activation obtained in this study after 30 min of administration, correlates well with the study by Pines *et al.*, (1981) who observed a 50 % *in vitro* activation of the lipase with cAMP and cGMP after 10 min.

Comparisons of the levels of the fat body lipase activities as well as the kinetic parameters has further complications arising from the differences in units used. Spencer and Candy (1976) obtained activities of 2.3-4.6 nmol/h/FB for *L. migratoria*, while for the same insect, Male and Storey (1981) obtained the TG-lipase activities in various tissues to be; fat body (0.03 $\pm$ 0.1) and muscles (0.09 $\pm$ 0.1) μmol/min/g wet weight. Pines *et al.* (1981) also obtained values of 2.2 nmol/mg/min for *Locusta*. In this study, the resting levels of lipase activities was estimated at 1.98 $\pm$ 0.24 and 1.95 $\pm$ 0.53 nmol/h/mg protein for solitary and gregarious locusts, respectively.

Hirayama and Chino (1987), estimated K_m and V_{max} values for *Locusta* TG-lipase to be 56 μM and 2.6 μmol FFA/h/g fat body. The K_m value is in close agreement with that obtained for the gregarious locusts, while the value for solitary locusts lipase is about 30% of the value. On the other hand, Tietz and Weintraub (1978) obtained K_m values of 0.14 nmol for the supernatant fraction and 0.33 for the microsomal fraction. Both estimates were much lower than those obtained in this study and as well as the study by Hirayama and Chino (1987). Tietz and Weintraub (1978) also estimated the V_{max} value for *Locusta* TG-lipase to

be 74 and 83 nmol/h/mg protein for the supernatant and microsomal forms, respectively. Compared to the values obtained in this study, they were rather too high. The V_{max} values obtained for gregarious *S. gregaria*, however compares well to that obtained by Hirayama and Chino (1987) for *Locusta* of 2.6 μmol FFA/h/g fat body. Based on the assumption that the *Locusta* fat body lipid constitute 80% of the dry weight and that the fat body dry weight is only 50% of the wet weight, the V_{max} for *Locusta* corresponds to an activity of 7.5 nmol FFA/h/mg protein, which is in close agreement to the value estimated for gregarious locusts but higher than that for the solitary locusts. The kinetics parameters estimated in this study and in the previous studies however, need to be interpreted with caution as all have been based on crude preparations of the enzyme.

Activities of the fat body lipases have been shown to correlate with the use of lipids as fuel. Thus, in a comparative study, the lipase activity was highest in the moth which uses lipids entirely for their flights followed by the locusts which relies on both carbohydrates and lipids. The lowest activity was registered in the cockroach which relies entirely on carbohydrates for the fuel (Male and Storey, 1981). Thus, the observation that the gregarious locusts TG-lipase had a higher catalytic capacity but a lower affinity for the substrate than in solitary locusts, may reflect a physiological adaptation to suit their migratory behaviour. Substrate inhibition observed in this study is as yet unexplained. However, it would be of interest to find out how fat body TG reserves, affect the activities of the lipases.

4.5 General conclusions and recommendations

From this study, the following conclusions can be made;

(1) High density lipophorin isolated from both locust phases showed similarity in the native form ($M_r \sim$ 620,000) and in apoprotein composition (apoLp-I; $M_r \sim$ 224,000 and apoLp-II; $M_r \sim$ 81,000). Immunological cross-reactivity was also demonstrated between the lipoproteins.

(2) Apolipophorin-III(apoLp-III) isolated from both phases of *S. gregaria* were glycosylated and showed the presence of two isoforms on non-denaturing PAGE. SDS-PAGE showed the presence of a single subunit ($M_r \sim$ 20,000). Immunological cross-reactivity was demonstrated between apoLp-III from both phases.

(3) The presence of lipophorin and apoLp-III was immunologically detected in the haemolymph of all development stages, starting from the third nymphal instar.

(4) Comparisons of the levels of lipophorin in the corresponding stages of the two phases indicated that there was a higher titer in the gregarious locusts than the solitary ones except in the fifth nymphal instar.

(5) In both locust phases, administration of AKH (10 pmol) resulted into the formation of a larger but a less dense low density lipophorin (LDLp). In addition to a higher lipid content, LDLp was associated with more apoLp-III molecules.

(6) The LDLp resulting from the gregarious locusts was shown to be larger than that from the solitary locusts by gel permeation chromatography on $Ac\Lambda_{22}$ column.

(7) Analysis of lipoprotein profiles following AKH injection showed that concomitant with the conversion of HDLp to LDLp, was a decrease in haemolymph levels of HDLp and apoLp-III.

(8) A higher fat body lipid content was obtained in the gregarious (79.02 ± 2.77%) than in the solitary locusts 64.75 ± 2.55%). In both phases, triacylglycerides were the major lipids.

(9) Peak hyperlipaemic response to AKH was demonstrated at the three dose tested (2, 5 and 10 pmol) after 90 min. There was evidence that the gregarious locusts responded faster than the solitary.

(10) At peak response, gregarious locusts were more sensitive to doses below 2 pmol while, above this the solitary locust were more sensitive. ED_{50} estimated for the gregarious locusts (1.5 pmol) was lower than that for the solitary locusts (6.6 pmol).

(11) Activities of fat body triacylglyceride lipase was demonstrated in resting insects from both phase. Activation of the lipases with AKH (2 and 10 pmol) was further demonstrated in both phases.

(12) Estimation of kinetic parameters for the lipases further suggested that the lipase from the gregarious locust had a higher catalytic ability but a lower affinity for the substrate.

From the results in this study, it is evident that the locust phase status affects some aspects of lipophorin structure and function. However, further work remains to be done to elucidated fully the impact of phase status and how such effects could be used in the design of novel control strategies. Further studies need to be carried out on the dynamics of LDLp formation from HDLp especially in relation to the loading of lipids mobilized from the fat body and the association lipophorin with apoLp-III molecules. The higher responses observed with the solitary locusts above 2 pmol of AKH also require further investigations as

Further studies on the biosynthesis of lipophorin in both locust phases is required. This could explain the higher titres observed in most of the stages of the gregarious as compared to the solitary locusts. Furthermore, the functional role of lipophorin at the nymphal stages may also explain the higher lipid reserve observed in the adult fat body of the gregarious locusts. The observation that there is substrate inhibition of the triacylglyceride lipase is very intriguing and it would be interesting to establish the nature of the inhibition and how it relates to the *in vivo* levels of the substrate. The effects of accumulating hydrolytic products also require investigation.

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