STUDIES ON A HAEMOLYMPH PEPTIDE THAT REGULATES ECDYSTERONE MEDIATED LYSOSOMAL ACTIVITY AND PROTEIN PHOSPHORYLATION DURING POSTEMBRYONIC DEVELOPMENT OF RICE MOTH *CORCYRA CEPHALONICA*

Thesis submitted for the degree of

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DECLARATION

I hereby declare that the work embodied in this thesis entitled **"Studies on a haemolymph peptide that regulates ecdysterone mediated lysosomal activity and protein phosphorylation during postembryonic development of rice moth** *Corcyra cephalonica* " has been carried out by me under the supervision of Dr. Aparna Dutta Gupta and that this has not been submitted for any degree or diploma of any other university

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CERTIFICATE

This is to certify that Ms. M. Vasanthi has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D ordinance of this university. 1 recommend her thesis entitled " Studies on a haemolymph peptide that regulates ecdysterone mediated lysosomal activity and protein phosphorylation during postembryonic development of rice moth *Corcyra cephalonica* " for the degree of Doctor of Philosophy of this university.

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ABBREVIATIONS

20E	20-Hydroxyecdysone
ATP	Adenosine 5'-triphosphate
BCIP	4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
CaM kinase	Calcium/calmodulin-dependent protein kinase
CaM	Calmodulin
CK	Casein kinase
CNS	Central nervous system
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethyleneglycol-bis-(P-amino-ethyl ether)
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethane sulphonic acid)
JH	Juvenile hormone
kDa	Kilo Dalton
MES	(2-[N-Morpholino]ethane-sulphonic acid)
NBT	Nitroblue tetrazolium
nm	Nanometer
PKC	Calcium/phospholipid-dependent protein kinase
PMSF	Phenyl methyl sulphonyl flouride
POPOP	1,4-bis [5-(phenyl-2-oxazolyl)] benzene
PPO	2,5, diphenyloxazole
PTK	Protein tyrosine kinase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SP	Storage protein
SPBP	Storage protein binding protein
TBS	Tris buffered saline
TEMED	N, N, N^1, N^1, tetramethylethylene diamine
Tris	Hydroxymethyl aminomethane
v/v	Volume/volume
w/v	Weight/volume

CHAPTER 1

INTRODUCTION

Postembryonic development in insects involves growth, moulting and metamorphosis. These events are controlled by the endocrine cues that are mainly secreted by the brain, corpora cardiaca, corpora allata and prothoracic glands. A choreographic precision of the titer of the morphogenetic hormones i.e., juvenile hormones and ecdysteroids and their interaction is required for the events to occur normally. The role of hormones in the regulation of insect postembryonic development especially in the holometabolous group has received a lot of attention resulting in several studies. However, knowledge regarding the regulation of the action of hormones themselves is very limited and the field remains largely unexplored.

Several workers proposed a basic model of the endocrine control of postembryonic development (Kopec 1917, 1922; Wigglesworth 1934, 1936, 1940; Fukuda 1940a.b, 1944; Williams 1947, 1948, 1952). According to them, the neurosecretory cells in the brain, in response to appropriate environmental stimuli synthesise and release various neuropeptides including allatotropic and prothoracicotropic hormone which in turn stimulate the corpora allata (CA) and prothoracic glands (PGL) to synthesise and secrete juvenile hormones (JHs) and ecdysteroids respectively. The relative titre and interplay between JHs (sesquiterpenes) and ecdysteroids (a group of steroid hormones) orchestrates the progression of one developmental stage to the next (egg-larva, larva-larva, larva-pupa, pupa-adult) (Sehnal and Mayer, 1968; Gilbert *et al.*. 1988, 1996). The ecdysteroids regulate the onset and the timing of the moult while JHs regulate the quality of the moult (Sehnal, 1989; Riddiford, 1994, 1996). Moulting in the presence of high JH titre would result in larval-larval moult while in the presence of reduced JH titre would result in larval-pupal and in absence of JH would result in pupal-adult moult.

During larval-pupal development of insects there is a precise temporal fluctuation in the haemolymph titre of the JHs that appears to affect changes in the haemolymph titre of ecdysteroids and vice versa (Nijhout and Williams, 1974; Riddiford and Truman, 1978). This sequence of interactions begins early in the last larval instar when the JH titre is at its peak and the ecdysteroid titre at its lowest. In the presence of this high JH and low ecdysteroid titre, the neurosecretory cells

(prothroacicotrope) synthesise and release prothoracicotropic hormone (PTTH) in the haemolymph. The PTTH then activates the PGL to synthesise ecdysone resulting in an initial subtle increase in the ecdysteroid titre, which evokes wandering behaviour and pupal commitment. This ecdysteroid surge in turn stimulates the CA via the brain and corpora cardiaca to synthesise JH, leading to a second increase in the JH titre which is necessary for normal metamorphosis to the pupa (Kiguchi and Riddiford, 1978). This peak of JH titre also stimulates the PGL indirectly contributing to the second major increase in ecdysteroid titre, which elicits the pupal moult. This model suggests that the titre and action of the ecdysteroids are of critical importance in the governance of the physiological activities associated with growth, metamorphosis and reproduction (Doane, 1973).

Ecdysteroids were originally viewed as being moulting hormones in insects based on their biological activity in larval bioassays (Karlson, 1980). In the mid 1950"s the first two moulting hormones (MHs) were isolated in crystalline form from *Bombyx mori* pupa (Buttenandt and Karlson, 1954; Karlson, 1956). The two MHs were provisionally termed a and β ecdysone, which are now designated as ecdysone and 20-hydroxyecdysone (20E) respectively. In arthropods, 20E is one of the most ubiquitously distributed ecdysteroid utilised by the moult cycle and is also associated with various physiological events (Horn and Bergamasco, 1983).

Detailed studies reveal that the basal concentration of ecdysteroids in the haemolymph of various lepidopteran insects, during postembryonic development ranged from 10^{-} to 10 M, which is species specific as well as stage dependent. A major peak of the ecdysteroid during larval-larval and larval-pupal development is usually present during the latter half of each stadium, the duration of which is once again species specific. During the pupal-adult development, the major peak occurs in the first half or middle of the pupal stadium (Dean *et al,* 1980). This pupal peak is normally 1.5 to 2.0 times greater in magnitude and duration and is attributed to the accentuated need of these compounds for extensive remodelling and differentiation of different tissues. In addition to the major peaks, there are non-moulting peaks during inter-moult period which are of lower magnitude and duration. Some of the inter-moult peaks have been found to correlate temporally

with metabolic activities such as synthesis of DNA, RNA, protein and other macromolecules. The haemolymph ecdysteroid titre at any given moment is therefore a reflection of several metabolic processes, which include ecdysteroid biosynthesis, secretion, and transport to target tissues, tissue uptake, degradation and excretion. Thus the changing haemolymph titre of ecdysteroids in holometabolous insects is responsible for eliciting the change in commitment and is necessary for metamorphosis as well as for the critical sequence of behavioural, physiological and biochemical events termed moulting.

Studies that orient towards the precise temporal, quantitative and qualitative regulation of haemolymph titre of ecdysteroids and the regulation of their mode of action have gained prominence owing to its great importance in the regulation of physiological and biochemical events associated with moult and metamorphosis. Parameters such as synthesis of hormones, rates of hormone metabolism, intracellular receptor titres, receptor activity, the existence of multiple isoforms of receptor proteins, availability of other proteins that modulate tropic hormones and presence of humoral factors, collectively determine the nature and degree of hormonal response (Truss and Beato, 1993). Extensive studies have been carried out on the regulation of 20E titre at the synthesis level (Gilbert *et al.,* 1980, 1997; Gruetzmacher *et al.,* 1984a,b; Song and Gilbert. 1998). However, studies on the other regulatory mechanisms are very limited and are a major thrust area in ecdysteroid action.

In lepidopterans like in most holometabolous insects, the ecdysteroid that elicits moulting and metamorphosis is 20E, the monooxygenated product of prohormone ecdysone, the latter is synthesised and secreted by the PGLs (Gilbert *et al.,* 1980). Detailed studies reveal that the cerebral neuropeptide PTTH is the primary effector in the regulation of ecdysone biosynthesis by the PGLs (Bollenbacher and Bowen, 1983; Bollenbacher and Granger, 1985). Increasing evidences are put forth to suggest that secondary effectors also control the gland activity. These regulators include environmental signals such as photoperiod (Mizoguchi and Ishizaki, 1982) temperature (Meola and Adkisson, 1977), direct neural input (Richter and Gersch, 1983) and humoral factors such as lipoproteins

(Chino *et al.* 1974) and hormones other than PTTH (Beylon and Lafont, 1983; Safranek *et al.* 1980; Gruetzmacher *et al,* 1984 a,b).

These effectors either individually or in combinations precisely regulate the synthesis and release of ecdysone by PGLs during postembryonic development. Thus, they control the quantitative and temporal fluctuations in the haemolymph ecdysteroid titre (Bollenbacher *et al,* 1981). Of the secondary effectors thought to regulate the PGLs. JH is of particular interest since it has both stimulatory and inhibitory effects on the gland. Several workers have shown that the effect of JH in post-committed last-instar *Manduca* larvae was indirect via its stimulation of the fat body to secrete a factor, which stimulates the PGLs (Williams, 1959; Gilbert and Schneiderman, 1959; Hiruma *et al,* 1978; Hiruma, 1980; Safranek *et al,* 1980; Gruetzmacher *et al.* 1984 a,b). This factor was shown to stimulate *in vitro* synthesis of ecdysone by *Manduca* PGL and its steroidogeneic effect was additive with that of PTTH. Later. Watson *et al.* (1985), established that the biological activity of the haemolymph factor is trypsin sensitive as well as heat labile. The apparent molecular weight of this factor was around 30 kDa. They hypothesized that the chemical nature of the stimulatory protein would be to transport a sterol precursor from which ecdysone is synthesised. They further suggested that the mode of action of this 30 kDa haemolymph factor is distinctly different from that of PTTH. Their studies also revealed a direct correlation between the activity of the haemolymph factor and titre of JH during development *of Manduca* (Watson *et al,* 1988).

Ecdysone production may also be suppressed by another mechanism in which the PGLs themselves become refractory to PTTH stimulation during diapause (Agui, 1975; Browning, 1981; Bowen *et al.* 1984; Ciancio *et al,* 1986). Meola and Adkisson (1977) observed that diapausing *Heliothis zea* pupae release PTTH for pharate adult development at the onset rather at the termination of pupal diapause. Despite the release of this hormone pupae remain in diapause because an unknown mechanism prevents ecdysone synthesis. Further investigation of this diapause mechanism by Meola and Gray (1984) showed that when pupae are maintained in a diapause sustaining temperature of 19°C, implanted PGLs are able

to produce ecdysone only in non-diapausing hosts. However, when haemolymph from non-diapausing host pupae is injected into diapausing insects kept at 19°C, ecdysone synthesis is stimulated. They concluded that the temperature sensitive mechanism controls diapause by regulating the availability of a humoral factor necessary for ecdysone synthesis. It was further reported that the fat body releases a factor into the haemolymph in response to diapause terminating temperature and that the factor is neither a free or conjugated ecdysteroid nor PTTH (Gray *et al,* 1987). An interesting mechanism of regulation of ecdysteroidogenesis at the PTTH level was reported by Sakurai *et al.,* (1989). They reported that PGLs *of Manduca sexto* synthesised dehydroecdysone which is rapidly converted to ecdysone through the mediation of a haemolymph enzyme, a 3-β forming 3-keto steroid reductase. They also reported that the enzyme is heat labile, inactivated by trypsin and has a molecular weight between 20 to 30 kDa.

In recent past. studies oriented towards the regulation of ecdysteroidogenesis have taken newer dimensions. Recently, Gilbert *et al.,* (1997) reported that during the process of ecdysteroidogenesis, PTTH initiates a cascade of events, that progresses from the influx of Ca^{2+} and cAMP generation through phosphorylation of the ribosomal protein S6 and S6 dependent protein synthesis and concludes with an increase in the synthesis and export of ecdysone from the gland. Their studies further suggest that S6 phosphorylation probably controls the steroidogeneic effect of PTTH by gating the translation of selected mRNAs whose protein products are required for increased hormone synthesis. Song and Gilbert (1998) have also shown that the ecdysone produced by the PGL feeds back upon the gland by increasing the expression and phosphorylation of a specific p47 USP isoform that is a constituent of the functional ecdysone receptor. Thus, changes in the concentration and composition of the ecdysone receptor complex of the prothoracic gland could modulate the gland's potential for ecdysone synthesis.

In insects, ecdysone 20-monooxygenase catalysed hydroxylation of ecdysone to convert it into the active hormone 20-hydroxyecdysone has been defined as activation. However, during times of decreasing hormone titres, inactivation occurs by several routes including (i) 26-hydroxylation and further

oxidation to 26-oic acid (ii) formation of various conjugates (eg. phosphates) and (iii) in lepidoptera in particular, ecdysone oxidase catalysed formation of 3-dehydroecdysteroid, which is reduced to 3-epiecdysteroid, followed by phosphotransferase catalysed formation of phosphate conjugates (Williams *et al,* 1997). These results indicate that moulting hormone stimulates atleast one universal route of its own inactivation by inducing 26-hydroxylase activity, thus regulating its activity.

Ashburner *et al.,* (1974) proposed a model for the mode of action of 20E. They hypothesized that 20E after binding to its receptor directly regulates two classes of genes, a small class of early genes that are transcribed when bound by the hormone receptor complex and large class of late genes that are repressed. These early genes in turn encode for the transcription factors, which facilitate late gene transcription. Extensive studies based on this model, over the past decade have provided insights into the molecular mechanism of 20E action (Cherbas, 1993; Antonieweski *et al.,* 1993; Henrich and Brown. 1995; Thummel, 1996). The focus of these studies have been on two aspects (i) studies on the transcription factors induced by 20E and how these factors transduce and amplify the hormonal signal by co-ordinating the induction of secondary response genes, (ii) discovery, cloning, characterisation and expression of ecdysone receptor proteins (Thummel *et al.,* 1990; Segraves and Hogness, 1990; DiBello *et al.,* 1991; Palli *et al.,* 1992; Koelle *et al.,* 1992). From these studies has emerged a clearer understanding of the mechanism by which a systemic hormonal signal is refined into stage and tissue specific developmental responses.

Another important pathway that regulates steroid hormone action is the presence of binding proteins and/or carrier proteins. In vertebrate system almost all the steroid hormones have been known to be regulated by the presence of plasma binding globulins (Rosner *et al,* 1991). In insects, a wealth of information exists on carrier proteins for the JHs. These proteins appear to be present in the haemolymph of a large variety of insect orders (Goodman, 1983). However, only a few reports are available on the existence of possible haemolymph carrier proteins for ecdysteroids. Feyereisen *et al,* (1978) for the first time, demonstrated the presence of a 280 kDa ecdysteroid binding protein in the haemolymph of *Locusta migratoria.*

They further demonstrated that in this insect species the majority of circulating 20E was bound to haemolymph proteins (Feyereisen 1980). Cao *et al,* (1983) purified a weakly acidic 270 kDa protein from the haemolymph of adult females of *Locust a* and showed that it is dimer of 135 kDa subunits. Despite these preliminary studies, this area remains largely unexplored.

Another area, which is gaining prominence as far as ecdysteroid action is concerned is its possible non-genomic effects. According to the traditional theory of steroid hormone action, steroids were primarily thought to modulate nuclear transcription and subsequently protein synthesis, thus triggering genomic events that are responsible for physiological effects. Most of these effects occur after a lag phase of 10 minutes or more usually after several hours. Consequently, a steroid effect that occurs within a few minutes after steroid application is likely due to a non-genomic rather than a genomic effect. To support the designation of steroid effects as non-genomic in nature, the response should not be blocked either by transcriptional inhibitor like actinomycin D or translational inhibitor cycloheximide (Wehling 1997; Tomaschko 1999).

A major area with regard to understanding the regulation of 20E action is that of tissue specificity. The above mentioned possible points of regulation could account to some extent for the diversity of responses among cell types that typifies the action of 20E. The hormone also underscores different effects on the same tissue at different developmental stages. Thus, studies focused on molecular basis of differentiation during development and built-in regulatory mechanisms at the tissue level largely aid in understanding the regulation of 20E action and such studies call for immediate attention.

The role of ecdysteroids in postembryonic development of insects is well documented (Lanot *et al,* 1989; Sehnal, 1989; Steele and Vafopoulou, 1989; Gilbert *et al,* 1996; Gu and Chow, 1996, 1997). 20E regulates the growth of motor neurons (Prugh *et al,* 1992), controls choriogenesis (Belles *et al,* 1993), stimulates the development and differentiation of imaginal discs (Bayer *et al,* 1996), initiates the breakdown of the larval structures during metamorphosis (Truman, 1996) and promotes the deposition of new cuticle by the epidermis (Willis, 1996). In

lepidopterans, as in many other holometabolous insects various tissues that are required during both larval and imaginal stages as well as the ones which are selectively imaginal depend upon pulses of 20E, to induce transition from the juvenile to adult forms or their differentiation from precursor undifferentiated cells to functional adult structure (Granger and Bollenbacher, 1981; Riddiford, 1985; Sridevi *et al.*. 1988; Gu and Chow. 1993; Wang *et al.*. 1995; Sehnal *et al.*. 1996).

Extensive studies on lepidopteran insects from our laboratory reveal that 20E stimulates synthesis of various proteins, in different tissues during postembryonic development (Ray *et al.*. 1987 a,b; Sridevi *et al.*. 1988, 1989; Ismail and Dutta-Gupta 1990a; Dutta-Gupta *et al.*. 1996; Shanavas *et al.*. 1996). The uptake of storage proteins by the fat body (Ismail and Dutta-Gupta, 1990b, Dutta-Gupta and Ismail, 1990, 1992; KiranKumar *et al.*. 1997, 1998) as well as the male accessory reproductive gland (Ismail and Dutta-Gupta. 1990c. 1991; Dutta-Gupta and Ismail, 1992; Ismail *et al.*. 1993) was also shown to be regulated by ecdysteroids. Detailed studies from our laboratory, show that the lysosomal activity in the whole body as well in the fat body exhibits a specific pattern during postembryonic and adult development and that an increase in the lysosomal activity is governed by the elevation of 20E levels (Rao *et al.*, 1984; Ray *et al.*. 1984; Sridevi *et al.*, 1987; Ashok and Dutta-Gupta, 1988; Dutta-Gupta and Sridevi, 1991).

In holometabolous insects the larval structures degenerate at the beginning of metamorphosis (Schin and Clever, 1968; Radford and Misch, 1971; Lockshin and Beaulton, 1974). Lysosomal enzymes play an important role in histolysis of larval organs, tissue remodelling, cellular destruction and reorganisation, the metabolic fuel for which are provided by the fat body. Acid phosphatase is one of the commonly used marker enzyme to study the lysosomal activity in insects (Verkuil, 1979, 1980). Acid phosphatases (EC 3.1.3.2) have been found in every organism studied to date and they exist in multiple forms and different isozymes. In *Drosophila*, acid phosphatase has often been used as a tool for survey of genetic polymorphism since the finding that the major acid phosphatase, Acph-1 in *D. melanogaster* is encoded by a gene (MacIntyTe, 1966; Chung *et al*, 1996). At the beginning of the wandering stage in *Calliphora erythrocephala* and preceding the actual metamorphosis, a large increase in the activity of the lysosomal marker

enzyme acid phosphatase is observed (Verkuil *et al.* 1979). Transplantation experiments with *Calliphora* suggest that the induction of lysosomal activity is under hormonal control and ecdysteroids may be a factor responsible for the induction of this process (Verkuil *et al.* 1979; Verkuil, 1980). In the larval fat body of *Calpodesethlius* a relationship could be derived between some autophagic events observed in the ultra structural study (Locke and Collins. 1968) and variations in acid phosphatase activity in homogenates (Collins, 1975). In short, the rise in acid phosphatase activity in the fat body of the late-last instar larvae may be related to increasing autophagic activity leading to the elimination of certain organelles like endoplasmic reticulum and mitochondria.

Administration of exogenous 20E stimulated the lysosomal activity in the fat body of the thorax-ligated larvae of *Corcyra cephalonica* (Ashok and Dutta-Gupta, 1988). On the other hand, addition of 20E to *in vitro* cultures of larval fat body fails to alter the enzyme activity. *In vitro* studies conducted in *Manduca* also revealed that the acid phosphatase activity was not induced in response to exogenous 20E alone (Caglayan, 1990) and the reason was elusive. However, Ashok and Dutta-Gupta (1991) reported a significant stimulation in the fat body acid phosphatase activity when haemolymph from late-last instar larval stage was added to fat body cultures in the presence of 20E. This suggested that the hormone may thus require the presence of some additional factors which are present in the *in vivo* system and mediate the action, on lysosomal activity. Therefore an attempt was made in the present study to analyse the possible role of haemolymph factor(s) if any, in mediating 20E action on selected marker proteins.

The fat body in insects is an important metabolic centre and can be compared to the vertebrate liver, in view of the complex functions performed by it, i.e. intermediary metabolism, detoxification, synthesis and release of macromolecules, storage of reserve substances and hormone metabolism (Keeley, 1985) and it is also a likely source of humoral factors (Meola and Gray, 1984; Gray *et al,* 1984). The fat body undergoes growth and development along with the other tissues and its function changes in accordance with the developmental stage of the insect. The fat body is structurally organised to provide maximal exposure to the haemolymph. Because of its changing metabolic role and integral position in

maintaining metabolic homeostasis, fat body serves with increasing frequency as a model for examining endocrine regulation (Kunkel. 1981). For the present study, the visceral fat body obtained from late-last instar larvae was chosen as the experimental tissue and rice moth, *Cocyra cephalonica,* and silk worm, *Bombyx mori* were used as the experimental models. In the present study, most of the experiments were carried out on *in vitro* cultured fat bodies. Thorax ligation of the larvae for 24 h provides us with a posterior part that is free of endogenous hormone. By the end of 24 h of ligation, the circulating 20E in the posterior part is reduced to basal levels (Dutta-Gupta and Ashok. 1998). Thus, the posterior fat body obtained from 24 h thorax-ligated larvae provided us with an experimental system that could be manipulated for hormonal regulation studies.

Literature search suggested that 20E not only exerts its action through the modulation of transcription but also exerts its action at the post-translational level *e.g.*. by protein phosphorylation. CaM kinase 11 and storage protein binding protein (SPBP) were chosen as the marker proteins for the present study. Our group has done extensive studies on autophosphorylation of CaM kinase II (Shanavas *et al.,* 1998) and phosphorylation of SPBP (KiranKumar, 1998) and demonstrated their developmental regulation in lepidopteran insects. These two proteins offered an excellent system to find out if, their phosphorylation was stimulated by 20E and there was an alteration in the phosphorylation status of these proteins in the presence of haemolymph factor and 20E.

Protein phosphorylation is now recognised to be the major regulatory mechanism by which synthetic activities of various tissue/cell type are controlled by external physiological stimuli. It is one of the major post-translational event whose importance is established in hormone action (Boyer *et al.,* 1983; Cochrane and Deeley, 1984). Protein phosphorylation system consists of three primary components, a protein kinase, a protein phosphatase and a substrate protein. When a protein kinase transfers the terminal phosphate from ATP to the hydroxyl group of a serine or threonine or tyrosine residue of the substrate protein, the substrate protein gets phosphorylated. The phosphate group is to be removed by a phosphatase. Based on second messenger systems required to activate, specific kinases are classified as cAMP dependent protein kinases, cGMP dependent protein kinase

and multiple types of calcium dependent protein kinases which fall into two subclasses. One subclass activated in conjunction with calcium-binding protein calmodulin (CaM) is referred to as calcium/calmodulin dependent protein kinase (CaM kinase). The other subclass activated in conjunction with phosphatidylserine and other lipids is referred to as calcium/phospholipid dependent protein kinase (PKC).

Although extensive studies on protein phosphorylation systems have been carried out in vertebrates, very little information is available on insects. However, in the last decade, several studies have been initiated on this aspect and cyclic nucleotide dependent protein kinases like cAMP-PK (Muller and Spatz, 1989; Jiang and Struhl, 1995; Lepage *et al,* 1995; Smith *et al,* 1996; Muller, 1997a) and cGMP-PK (Foster *et al.,* 1996; Muller, 1997b) have been well characterised from various tissues of different species of insects.

Regulation of protein phosphorylation is one of the several mechanisms by which calcium exerts its intracellular action. In recent years, it has become evident that many of the cellular actions of calcium are mediated by its binding to specific protein kinsases resulting in their activation (Nestler and Greengard, 1984). Two classes of calcium dependent protein kinases are known till date and they are calcium/phosphatidylserine-dependent protein kinase(PKC) and calcium/ calmodulin dependent protein kinase (CaM kinase).

Studies on the mechanism of action of calmodulin, an ubiquitous low molecular weight protein, indicated that calcium binds to it inducing structural changes. The Ca^{+}/CaM complex then binds and alters the function of other cellular proteins (Cheung 1980; Carafoli, 1987; Heizmann and Hunziker, 1990). Several studies done over the last two decades established that some of the second messenger actions of calcium in a variety of tissues might be achieved by the activation of Ca^{2+}/CaM dependent protein kinases (Hanson and Schulman, 1992). Protein phosphorylation has been considered as one of the important routes by which Ca^{2+}/CaM signal transduction regulates cellular function. In the mammalian neural and non-neural tissues multiple types of CaM kinases have been demonstrated and characterized. They include CaM kinase I (Nairn and Greengard, 1987), II (Kennedy and Greengard, 1981), III (Nairn *et al,* 1985), IV (Kato *et al,*

1992), myosin light chain kinase (Hagiwara *et al,* 1989) and phosphorylase kinase (Cohen *et al.* 1978).

CaM kinase II is the most abundant type among the CaM kinases and has been well characterised in the neural tissue. It was first identified in rat brain as a calcium-dependent protein kinase that catalyses the phosphorylation of site 2 and 3 of Synapsin 1 (Kennedy and Greengard. 1981). Rat brain CaM kinase II comprises of several related isozymes (Hanson and Schulman. 1992). The rat isozymes consist of a catalytic domain, an autorcgulatory domain containing a calmodulin-binding site and a C-terminal "association domain that mediates holoenzyme formation" (Bennett and Kennedy, 1987; Lin *et al.* 1987; Tobimatsu *et al,* 1988). A distinct property of CaM kinase II is the autophosphorylation of a threonine residue near its calmodulin binding domain which converts the enzyme to Ca^{2+} independent form (Miller and Kennedy, 1986; Wang *et al.* 1994). It has been postulated that this autophosphorylation may induce changes in the subcellular distribution of the enzyme in *Aplysia* and *Drosophila* cells (Saitoh and Schwartz, 1985; Willmund *et al,* 1986) and it may also be involved in prolonging the effects triggered by a transient calcium signal (Miller and Kennedy, 1986).

There are only a few reports on the characterisation of CaM kinase II in insects (Cho *et al,* 1991; Ohsako *et al,* 1993; Shanavas *et al,* 1998). Adult *Drosophila* head contains three species of CaM kinase II with molecular masses of 54/55, 58 and 60 kDa (Cho *et al.* 1991, Ohsako *et al,* 1993). Both amino acid sequence and tissue specificity of the rat kinase are highly conserved in *Drosophila.* Extensive studies have been carried out in our laboratory on the identification, characterisation and localisation of CaM kinase II from *Bombyx mori* CNS (Shanavas, 1997; Shanavas *et al,* 1998). The *Bombyx* CNS consists of two species of CaM kinase II with molecular weight of 59/60 kDa, which cross react with anti-rat CaM kinase II a monoclonal antibody and show a high degree of autophosphorylation in neural tissue (Shanavas *et al,* 1998). It was further reported, that two peaks of enzyme activity occur in the CNS of *Bombyx* during the postembryonic development, the first peak at late-larval stage and the next peak at late-pupal stage (Shanavas *et al,* 1998) which coincided with the reported parallel

changes in the ecdysteroid titre in the haemolymph *of Bombyx mori* (Calvez *et al,* 1976).

Transformed strains of *Drosophila* expressing a transgene inhibitor of CaM kinase II have been shown to be deficient in an associative conditioning behavioural paradigm (Griffith *et al,* 1993; Wang *et al.* 1994). CaM kinase 11 was also shown to be invoked in the phosphorylation of *Drosophila* visual phosrestin thereby regulating photoreceptor light adaptation (Kahn and Matsumoto, 1997; Kahn *et al,* 1998).

Protein tyrosine kinases (PTKs) are relatively recently discovered class of enzymes which especially catalyse the phosphorylation of proteins at tyrosine residues. Several receptor tyrosine kinases (RTKs) have been identified in *Drosophila* (Yamamoto. 1994; Freeman. 1996; Raabe *et al,* 1996). The *Drosophila* homologue of the mammalian EGF receptor has been identified as an RTK, involved in many stages of development (Doyle and Bishop, 1993; Duffy and Perrimon, 1994; Schweitzer and Shilo, 1997). Insulin receptor like tyrosine kinase activity has been reported in PGLs of *Manduca sexta* (Smith *et al, 19*97). Recent studies from our laboratory, revealed the involvement of protein tyrosine kinases in the phosphorylation of a 48 kDa protein in neural tissues of *Bombyx mori* (Shanavas, 1997) and a 120 kDa storage protein binding protein in the fat body of *Corcyra cephalonica* (KiranKumar, 1998).

The insect storage proteins (SPs) are high molecular weight multimers, usuallly hexamers, composed of subunits in the molecular weight range of 70-90 kDa (Telfer and Kunkel, 1991; Haunerland, 1996). In holometabolous insects, including lepidoptera, during the feeding phase, these proteins are synthesised by the fat body cells and released into the haemolymph. The haemolymph concentration of SPs reaches its maximum during the final larval instar, then declines during the prepupal and early-pupal development, concurrently with their accumulation in the fat body. Several studies on lepidopteran insects have shown that ecdysteroids stimulate the SPs uptake by the fat body (Tojo *et al, 19*82; Webb and Riddiford, 1988a,b; Ismail and Dutta-Gupta, 1990b; KiranKumar *et al,* 1997, 1998). Recent studies from our laboratory showed that the process of SPs uptake in

Corcyra cephalonica is mediated by binding proteins (SPBP) present in the plasma membrane of fat body with molecular weight of 120 and 125 kDa. (KiranKumar *et al.*. 1997). SPBP-120 showed phosphorylation which is Ca^{2+} independent and this protein cross reacted with phosphotyrosine antibody suggesting that phosphorylation was most likely mediated by a tyrosine kinase. Further, phosphorylation of this protein is also developmentally regulated (KiranKumar, 1998).

The phosphorylation of specific substrates by a variety of protein kinases appears to be a general mechanism by which many hormones, neurotransmitters and other extra cellular signals produce their physiological responses in specific target cells (Greengard, 1978). Only a few reports are available regarding the regulation of protein kinase activity by insect hormones. 20E has been shown to stimulate *in vitro* phosphorylation of few fat body proteins of *Mammestra brassicae* (Sass, 1988) and *Sarcophaga peregrina* (Itoh *et al.,* 1985). CK 11 activity in the brain of *Acheta domesticus* was shown to be inhibited by the injection of 20E (Degrelle *et al.,* 1997). Juvenile hormone was shown to be involved in the activation of a specific Na K ATPase via PKC in the follicle cell membrane of *Rhodnius prolixus* (Sevala and Davey. 1989). JH was also shown to stimulate protein synthesis in male accessory glands of *Drosophila* through the activation of PKC (Yamamoto *et al,* 1988). cGMP-PK activity in the CNS of *Manduca sexta* has been shown to be stimulated by eclosion hormone (Morton and Truman, 1986; 1988). PTTH has been shown to stimulate cAMP-PK activity in the prothoracic glands *of Manduca sexta* (Smith *et al,* 1996).

Scope and objectives of the present study

In the present study, an attempt has been made to determine the role of haemolymph factor(s) in the ecdysteroid mediated stimulation of acid phosphatase activity and protein phosphorylation. The specific objectives of the dissertation work are given below:

1. To identify the haemolymph factor which is present in the *in vivo* system and not present in the *in vitro* system

2. To identify marker proteins other than the fat body acid phosphatase activity that require the additional presence of haemolymph factor(s) when their activity is mediated by 20E
3. To further study the physiological role / function of the haemolymph factor(s) in the regulation of 20E action

CHAPTER 2

MATERIALS AND METHODS

CHEMICALS

[γ^{32}P] ATP (3000 Ci/m mole) was purchased from Bhaba Atomic Research Centre, Trombay, India. Ethylene diamine tetra acetic acid (EDTA), ethylene glycol-bis-(P-amino-ethyl ether) (EGTA), dithiothreitol (DTT), calmodulin, cAMP, cGMP, phosphatydylserine, diacylglycerol, syntide-2 (CaM kinase II substrate peptide), HEPES buffer, bovine serum albumin (BSA), Comassie brilliant blue G-250, bromophenol blue, PPO (2,5 diphenyloxazole), POPOP (1,4-bis [5-(phenyl-2-oxazolyl)] benzene), Sephadex G-25-50 matrix, Triton X-100, TC-100 insect culture medium, *para* nitrophenol, *p*-nitrophenyl phosphate bisodium salt, N-N'-N-N' tetra ethyl methylene diamine (TEMED), phenyl methyl Sulfonyl fluoride (PMSF), leupeptin, actinomycin-D, cycloheximide, phenylthiourea, high and low molecular weight protein markers were obtained from Sigma Chemical Co. (St. Louis, MO USA). Biotin protein labelling kit, chemiluminescence detection kit and monoclonal rat anti-CaM kinase II α antibody were purchased from Boehringer Mannheim (Darmstadt, Germany). Alkaline phosphatase-conjugated anti mouse secondary antibody, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were obtained from Bangalore Genei, India. P-81 phosphocellulose sheets were purchased from Whatman (Maidstone, UK). Acryiamidc, N-N'-methylene:bis-acrylamide, 2-mercaptoethanol and glycine were purchased from Spectrochem (Bombay, India). Silver nitrate and glycerol were obtained from E. Merck (Schuchardt, Germany). Sodium dodecyl sulphate (SDS) was procured from Bio-Rad Laboratories (Richmond, USA). 20-Hydroxyecdysone (20E) was purchased from Rohto Pharmaceutical Co. (Osaka, Japan) and was also gifted by Prof. Frantisek Sehnal, Institute of Entomology, Czech Republic. Nitrocellulose membranes (Millipore, Bedford, USA) and Amicon membrane filter (Model YM-100, YM-30 and YM-10 from Amicon Grace and Co.) were gifted by Prof. A. Krishna Kumaran, Marquette University, Milwaukee, USA and Dr. A. Shanavas, University of Texas, USA. X-ray films were obtained either from Kodak (USA) or Indu (India). All other chemicals used were obtained from commercial sources in India and were of analytical grade.

EXPERIMENTAL INSECTS

(A) *Corcyra cephalonica* (Stainton) :

It belongs to the order Lepidoptera and family Galleridae. It is commonly known as rice moth and is a serious pest of stored cereals, oil seeds and legumes in the tropical and sub-tropical regions of the world (Freeman 1976).

Rearing method :

The insects were reared in culture troughs which contained coarsely crushed sorghum seeds. Equal number of female and male moths were introduced into the troughs for egg laying. The cultures were maintained in insect culture room at 26 ± 1°C temperature, 60 ± 5% relative humidity (RH) and 14:10 h light dark (LD) period.

Life cycle :

Under the above mentioned conditions, the larvae emerge from 4-5 days old eggs. The larval development proceeds through five instars and is completed in about 25-30 days. The last larval instar (V instar) is followed by the non-feeding and prepupal stages which extends over 4-5 days followed by the pupal stage which lasts for 7-8 days. The adult moths normally survive for 8-10 days.

Stages used for experiments :

Early-last instar (ELI), mid-last instar (MLI), late-last instar (LLI) larvae and prepupae (PP) were used in the study The larval stages were classified based on their body weight and head capsule size. The larvae weighing 56-65 mg with head capsule size of 0.8 to 0.95 mm were categorised as ELI. Larvae weighing 66-75 mg and head capsule size of 0.96-1.03 mm were classified as MLI and those weighing 76-85 mg and head capsule size of 1.03-1.08 mm were classified as LLI. Insects collected during the wandering stage were designated as prepupae (PP). Fat body was collected from all stages and used for various studies. Haemolymph from ELI, MLI, LLI and PP was collected and used for various studies.

(B) *Bombyx mori*

It is commonly known as silk worm and belongs to the order Lepidoptera and family Bombycidae. Fourth instar larvae of pure Mysore strain were obtained from ocal breeding centre and were reared on fresh mulberry leaves under sterile ;onditions in insect culture room maintained at 26 ± 1°C temperature, 60 ± 5% RH and 14:10 h LD period. Staging of larvae was done based on their age after the fourth ecdysis. One to two days old last instar larvae were designated as early-last instar (ELI), 5 to 6 days old as mid-last instar (ML1) and 9 to 10 days old as late-last instar (LLI). Insects collected after spinning were designated as prepupa (PP), one day old prepupa as early-prepupa (EPP), 2 days old prepupa as mid-prepupa (MPP) and 3 days old prepupa as late-prepupa (LPP). For the present study pupal and adult stages were also used and they were designated as early-pupa (EP) 1-2 days old, mid-pupa (MP) 4-5 days old and late pupa (LP) 9-10 days old, and freshly emerged moths (< 12 h old) as adult (A). Fat body was collected from all stages and used for acid phosphatase assay. Haemolymph from ELI, ML1, LLI and EP was collected and used for various studies.

(C) *Papilio demolius*

It is commonly known as lemon butterfly and belongs to the order Lepidoptera and family Papilionidae. The larval forms were collected from local citrus fields and maintained on fresh citrus leaves in insect culture room maintained at 26 ± 1°C temperature, 60 ± 5% RH and 14:10 h LD period. Once again the last instar larvae were categorised as early-last instar (ELI - 1 to 2 days old), mid-last instar (MLI - 4 to 5 days old) and late-last instar (LLI – 7 to 8 days old). For certain studies prepupae (PP) were also used. Haemolymph from ELI, MLI, LLI and PP was collected and used for various studies.

(D) *Achoea Janata*

It is commonly known as castor semilooper and belongs to order Lepidoptera and family Noctuidae. The larval forms were collected from local gardens and maintained on fresh castor leaves in insect culture room maintained at 26 ± 1°C temperature, 60 ± 5% RH and 14:10 h LD period. Based on their age after the fourth ecdysis they were categorised as early-last instar (ELI - 1 - 2 days old), mid-

last instar (MLI - 5 - 6 days old) and late-last instar (LLI - 8 - 9 days old). For few experiments prepupae (PP) were also used. Haemolymph from ELI, MLI, LLI and PP was collected and used for various studies.

METHODS

Preparation of tissue sample :

The ribbon shaped visceral fat body was dissected out in cold insect Ringer and weighed after blotting onto a filter paper. A 2% homogenate of the fat body was made in cold 10 mM Tris-HCl (pH 7.4) containing 0.1% Triton X-100 using a glass-glass homogenizer (Kontes). The homogenate was centrifuged at 1,000 x g for 5 min to remove large debris and aliquots of the supernatant were used for protein estimation, assay of acid phosphatase activity, polyacrylamide gel electrophoresis, western blotting, ligand blotting and phosphorylation studies.

Thorax ligation :

The last instar larvae were used for this experiment. Ligation was done behind the first pair of prolegs by slipping a loop of silk thread (Ethicon, NJ, USA) around the head of the larvae. The loop was adjusted behind first pair of prolegs and then gradually tightened. The ligated larvae were kept in petridishes covered with moist filter paper to maintain humidity and to prevent dessication.

Hormone injection :

A stock solution of 20E was prepared by dissolving 1 mg of hormone in 100 µl of ethanol which was finally diluted to 1 ml with distilled water. This was aliquoted into small volume and stored at -20°C till use. This stock solution was further diluted with 10% ethanol as per requirement. Thorax-ligated, late-last instar larvae were injected with 8×10^{-8} M 20E (in 2 µl) after 24 h of ligation. Control insects received equal volume of carrier (2 µl *10*% ethanol). The insects were sacrificed 24 h after hormone treatment and the visceral fat body was dissected out in cold insect Ringer. Homogenates were prepared as mentioned above and were used for protein estimation and for the determination of acid phosphatase activity.

Protein estimation :

Protein content in various samples were estimated according to the micro protein assay method of Bradford (1976).

Preparation of protein reagent : Coomassie brilliant blue G-250 (10 mg) was dissolved in 5 ml of 95% ethanol. To this solution, 10 ml of 85% w/v phosphoric acid was added. The resulting solution was diluted to a final volume of 100 ml with double distilled water and filtered through Whatmann No. 1 filter paper and stored in an amber coloured bottle at 4°C.

Protein estimation : An aliquot of sample was pipetted into a 1.5 ml microfuge tube. The volume of the sample was adjusted to 0.1 ml with 10 mM Tris buffer (pH 7.4). One ml of protein reagent was added and the contents were mixed by gentle inversion. After 10 min, absorbance at 595 nm was measured spectrophotometrically against a reagent blank prepared from 0.1 ml of Tris buffer and 1.0 ml of protein reagent. Protein content of the sample was calculated using a standard curve prepared using bovine serum albumin (fraction V).

Assay of acid phosphatase enzyme activity :

The assay was carried out according to the method of Henrickson and Clever (1972). The reaction mixture had 190 µl of 0.2 M sodium acetate buffer (pH 5.0) and 10 µl of protein homogenate. The enzyme sample with the buffer was incubated at 37°C for 10 min to exclude glucose-6-phosphatase activity (Beaufay *et al*, 1954). At the end of 10 min, 50 µl of substrate, *p*-nitrophenyl bisodium phosphate (5 µ moles), was added to the assay mixture and the tubes were incubated for 1 h at 37°C. The reaction was terminated by the addition of 0.5 ml of 0.1 N NaOH. The yellow colour thus developed was measured at 420 nm against a substrate blank. *p*-Nitrophenol was used for the preparation of standard curve. The activity of the enzyme was expressed as n moles of PNP released / h / µg protein.

In vitro culture studies :

In vitro studies were carried out under sterile conditions, in a laminar flow chamber. The posterior fat body from two 24 h ligated late-last instar larvae was dissected

out in cold insect Ringer and transferred into microfuge tube, containing 100 µl of TC-100 insect culture medium with trace amounts of antibiotic streptomycin sulphate. After thorough rinsing, the tissue was transferred into a fresh microfuge tube containing 200 ul of the culture medium. To the experimental set, 8×10^{-8} M 20E (in 2 µl of 10% ethanol) was added while equal volume of 10% ethanol was added to the control tubes. The tubes were incubated for 4 h at 25°C. At the end of the incubation period the tissue was removed and rinsed in ice cold insect Ringer, homogenised as mentioned before and was used for the various studies.

In vitro studies using haemolymph :

Haemolymph was collected from different stages of the insects : early, mid, late-last instar larvae and prepupae (unligated and thorax-ligated) into microfuge tubes precoated with 0.025% phenylthiourea. The haemolymph samples were diluted 1 : 20 with 10 mM Tris buffer (pH 7.4) and were used for *in vitro* studies. Diluted haemolymph (10 ul) obtained from the above mentioned stages was added to 200 ul of culture medium containing 8 x 10^{-} M 20E (in 10 µl of 10% ethanol), while to control tubes equal amount of carrier was added. Fat body from 24 h ligated late-last instar larvae was dissected out, rinsed and incubated in these tubes at 25°C for a period of 4 h. At the end of the incubation period, the tissue was removed from the medium, thoroughly rinsed in ice cold insect Ringer, homogenised as mentioned before and used for various studies.

Fractionation of *Corcyra* haemolymph :

Haemolymph was collected from the posterior half of the late-last instar larvae, after 24 h of ligation into microfuge tubes precoated with 0.025% phenylthiourea. About 100 larvae were used for the experiment. The haemolymph was diluted (1:20) with 10 mM Tis-HCl (pH 7.4) and was spun at 3000 rpm for 3 min in a Kubota centrifuge to remove haemocytes and other suspensions. The supernatant was transferred into Amicon Centricon concentrators (30 kDa membrane filter) which had the capacity to cut off fractions of molecular weights above 100 kDa. Generally, to obtain the required molecular weight cut-off, a filter size that is around 3 times the lower range is used. Thus, 30 kDa cut off membrane would give in the filtrate, proteins with molecular weight less than 100 kDa (Please refer -

Amicon catalogue). The concentrators containing the haemolymph samples were centrifuged at 4000 x g for 20 min in J-2 centrifuge (Beckman) at 4°C. The filtrate and the contents remaining in the filter were collected separately. The filtrate which had molecules whose molecular weights were below 100 kDa was again subjected to filtration in Amicon filtration unit (10 kDa membrane filter) with a membrane filter which gives a 30 kDa molecular weight cut off. Both filtrate and retentate were saved. Three fractions having molecular weights above 100 kDa, 30-100 kDa and below 30 kDa, thus obtained were used for in *vitro* studies.

Purification of protein :

Haemolymph was collected from late-last instar larvae. Around 500 larvae were used for this experiment. Haemolymph was diluted (1:20) with 10 mM Tris-HCl (pH 7.4). The diluted haemolymph was spun at around 3000 rpm for 5 min to remove haemocytes. The supernatant was treated with 0.01% phenylthiourea to prevent melanisation. The supernatant was transferred into Amicon Centricon concentrators (30 kDa membrane filter) which had the capacity to cut off protein fractions of molecular weight above 100 kDa. Less than 30 kDa fraction which was obtained using a 10 kDa membrane filter in the earlier experiment was available only in limited volume. This limited volume was not sufficient for proceeding with purification. Hence, less than 100 kDa fraction which was essentially free of very high molecular weight proteins was used as the starting material for purification on the column. The filters containing the sample were centrifuged at 4000 x g for 20 min in J-2 centrifuge at 4°C. The filtrate was collected and passed on a Sephadex G- 25-50 matrix which was equilibriated with 10 mM Tris-HCl (pH 7.4). The protein fractions were eluted with the same buffer and 500 al fractions were collected. An aliquot of each fraction was added to *in vitro* cultured fat body in the presence of 20E (8 x 10" M) and its potentiating effect on acid phosphatase activity was assayed. Peak fractions that stimulated acid phosphatase activity were pooled for further studies. The purity of the peak fraction was checked by SDS-PAGE.

Polyacrylamide gel electrophoresis :

Sample preparation for electrophoresis : An aliquot of the sample for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was mixed with an

equal volume of 2 x sample buffer [containing 0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenol blue] and incubated at 100°C for 1 min.

One-dimensional gel electrophoresis : SDS-PAGE was carried out according to the procedure of Laemmli (1970). on a 1 cm, 2.1 % stacking gel (pH 6.8) followed by a 7.5 or 15 cm, 12% resolving gel (pH 8.8) for purification studies and 10 % resolving gel (pH 8.8) for phosphorylation studies. Tris-glycine (25 mM, pH 8.3) with 0.1 % SDS was used as the electrode buffer. Electrophoresis was carried out at 120 V until the tracking dye reached 1 cm above the base of the resolving gel.

Silver staining of polyacrylamide gels : Silver staining of proteins separated on polyacrylamide gels was carried out according to the procedure of Blum *et al.*, (1987) with minor modifications. The gel was incubated in fixative (50% methanol, 12% acetic acid and 50 µl of 37% formaldehyde/100 ml) for 1 h and was then treated with 50% ethanol for 30 min (3 changes). This was followed by pre-treatment with sodium thiosulphate (20 mg/100 ml) for 1 min. The gel was rinsed thrice with double distilled water (20 sec each) and impregnated with silver nitrate (100 ml of 0.2% silver nitrate containing 187 ul of 37% formaldehyde) with gentle agitation on a mechanical shaker for 30 min. The gels were rinsed with double distilled water and developed with a solution containing 6% sodium carbonate (w/v) and 50 ul of 37% formaldehyde (v/v). Finally, the stained gels were thoroughly rinsed with double distilled water and stored in 50% methanol.

***In vitro* phosphorylation of endogenous proteins in *Corcyra* fat body homogenates** :

Incubations were carried out in 1.5 ml microfuge tubes in a total volume of 40 ul, containing 20 u.g of fat body homogenate protein. The reaction mixture consisted of 50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 10 mM ATP, 1 mM EGTA or 0.1 mM EGTA and 1 mM $CaCl_2$. Reaction mixture was preincubated for 5 min at 30°C for temperature equilibration. Reaction was initiated by the addition of 4 µ Ci of [γ^{32}P] ATP. After 1 min, the reaction was terminated by the addition of 20 ul of 3 x SDS sample buffer [0.188 M Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 15%

2-mercaptoethanol and 0.003% bromophenol blue] and immersion in boiling water for 2 min. Following centrifugation at 10,000 x **g** for 3 min to remove insoluble material, 30 µl of the supernatant (10 µg protein) was subjected to SDS-PAGE (Laemmli, 1970). The gels were silver stained (Blum *et al,* 1987) as mentioned before. Phosphorylation reactions were also carried out under similar conditions to study the effect of phophatydylserine (10 µM) + diacylglycerol (100 µM) and 20E (8×10^{-8} M).

Autoradiography :

The silver stained gels were dried under vacuum, sandwiched between cellophane sheets at 80°C for 1 h using a Hoeffer gel drier. They were exposed for 1-3 days to Kodak X-Omat or Indu X-ray film using DuPont Cronex intensifying screens at - 70°C, developed and fixed as per standard procedure.

Ligand blotting :

Ligand blotting was done according to the procedure of KiranKumar *et al,* (1997). As a preliminary step towards ligand blotting, storage protein was purified from the haemolymph of LLI larvae of *Corcyra* using a rapid purification protocol (Arif *et al,* 1999). The purified protein was biotinylated using a biotin labelling kit according to the procedure of KiranKumar *et al,* (1997). Fat body proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Towbin *et al,* 1979). After blotting, the membrane was washed (5 min x 3 changes) with 10 ml of TBS [50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl]. Then the membrane was immersed in the same buffer containing 2% BSA and 0.2% gelatin [blocking buffer] and was washed again (5 min x 3 changes) with binding buffer [5 mM Mes (pH 6.5), 100 mM NaCl, 8 mM $CaCl_2$, 24 mM $MgCl_2$, 0.5% Tween 20 (v/v) and 5 mg/ml BSA] and incubated in 5 ml of binding buffer, containing 100 µg of biotinylated SP for 4 h at 22°C. This was followed by a thorough wash with blocking buffer (5 min x 3 changes). Fat body proteins, which were bound to the biotinylated SP, were visualised with a streptavidin-horseradish peroxidase complex using chemiluminescence detection kit (Boehringer Mannheim).

Western blotting and immunostaining :

Proteins were electrophoresed by SDS-PAGE and were electroblotted at 70 V **for** 3 h on to **a** nitrocellulose membrane using Trans Blot apparatus (Bio-Rad) according to the procedure of Towbin *et al.,* (1979). After transfer, the membrane was air dried and incubated for 1 h at room temperature with 3% (w/v) BSA in TBS (10 mM Tris, 150 mM NaCl, pH 7.5) to block non-specific binding sites. The blot was then incubated overnight with the primary antibody in TBS containing 3% BSA. This was followed by a thorough wash in TBS (5 min x 6 changes). Thereafter, the blot was incubated with the alkaline phosphatase conjugated anti-mouse IgG (goat) for 1 h. Once again, the blot was washed in TBS (5 min x 5 changes) and stained in 10 ml ALP buffer (10 mM Tris, 5 mM $MgCl_2$ and 100 mM NaCl, pH 9.5) containing 0.033% nitroblue tetrazolium (NBT) and 0.0165% 5-bromo-4-chloro-3-indolyl phosphate (BC1P).

Back phosphorylation :

Back phosphorylation was carried out as described by Forn and Greengard (1978). The posterior fat body from two 24 h ligated late-last instar larvae was dissected out in cold insect Ringer and transferred into microfuge tube, containing 200 ul of TC - 100 insect culture medium (Sigma Chem. Co.) with trace amounts of antibiotic streptomycin sulphate. After thorough rinsing, the tissue was transferred into fresh microfuge tube containing 200 ul of the culture medium. To the experimental set 8 x 10^{-8} M 20E (in 10 ul of 10% ethanol) was added, while equal volume of 10% ethanol was added to the control tubes. The tubes were incubated for 4 h at 25°C. At the end of the incubation period, fat body was removed from the culture vials, rinsed thoroughly with insect Ringer, homogenized and subjected to *in vitro* phosphorylation.

Assay of CaM kinase II in *Corcyra* fat body homogenates :

CaM Kinase II activity was assayed by following the phosphorylation of synthetic substrate syntide-2 according to the method of Fukunaga *et al.,* (1989). Reaction mixture contained 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 0.1 mM [γ^{32}P] ATP (2000-4000 cpm/p mol), 30 μMsyntide, 2 μM calmodulin, 1 mM $CaCl_2$, 0.1 mM EGTA **and** suitable amounts of homogenate protein in **a total** volume of 50 μl. A

control reaction was carried out in the presence of 1 mM EGTA. After incubation at 30°C for 1 min, the reaction was terminated by adding 10 µ 1 of 0.4 M EDTA and a 50 µl aliquot was spotted on to 2 x 2 cm phosphocellulose strips. Radioactivity was determined as described by Roskoski (1983). The strips were immersed in 75 mM phosphoric acid (10 ml per strip) and swirled gently for 2 min. The phosphoric acid was decanted and the phosphocellulose strips were washed twice (1 min each) in 75 mM phosphoric acid with gentle agitation. After drying in an oven (100°C, 5 min), the radioactivity was measured by liquid scintillation spectrophotocounter using toluene scintillation fluid (0.5 gm POPOP, 5 gm PPO/litre of toluene). Assay conditions were standardized using fat body homogenates from late-last instar larvae.

Statistical method used :

All the data obtained in the present study were subjected to statistical analysis and the significance of the difference between any two values was calculated according to one way analysis of variance using Sigmastat software.

CHAPTER 3

RESULTS

Studies on *Corcyra cephalonica* fat body acid phosphatase activity:

Developmental profile of acid phosphatase activity in the fat body :

Acid phosphatase activity was determined in the fat body of different developmental stages and the results are presented in figure 1. The specific activity of the enzyme was found to be low at penultimate larval stage and it increased gradually through the early, mid and late-last instar larval stages and attained a maximum value at the prepupal stage.

Effect of ligation and injection of exogenous 20E on LLI fat body acid phosphatase activity :

Figure 2 a and b show the *in vivo* effect of ligation and injection of exogenous 20E in the fat body acid phosphatase activity in ligated LLI. Specific activity of acid phosphatase was assayed in the posterior fat body of late-last instar larvae after a period of 24, 48 and 72 h of ligation. Normal fed larvae were used as controls. The results presented in figure 2a, show that the activity of the enzyme, declined gradually from 24 to 72 h of ligation. To study the *in vivo* effect of 20E, 24 h ligated LLI larvae were injected with 8 x 10 M of 20E. Larvae injected with exogenous hormone were maintained either for 24 or 48 h . There was a significant increase in the enzyme activity in the fat body of hormone treated larvae when compared to the control ligated larvae (Fig. 2b).

Effect of hormone and/or haemolymph factor(s) on *in vitro* cultured LLI fat body acid phosphatase activity :

For these experiments fat body was dissected out from 24 h ligated LLI and cultured *in vitro* for 4 h in presence of 8 x 10" M 20E and/or haemolymph obtained either from *Corcyra* or other insects. To the control cultures equal volume of carrier was added.

a) Effect of 20E :

The results presented in figure 3 show that administration of exogenous 20E to the fat body cultures did not alter the enzyme activity, when compared with controls.

b) Effect of 20E in the presence of haemolymph factor(s) from *Corcyra* :

From the results presented in figure 4, it can be inferred that addition of haemolymph obtained from the posterior part (henceforth it will be referred to as posterior haemolymph) of either ligated or unligated late-last instar larvae along with 20E to the fat body culture caused a significant increase in the specific activity of the enzyme in the tissue. It was further observed, that haemolymph obtained from anterior part (henceforth it will be referred to as anterior haemolymph) of 24 h ligated larvae failed to potentiate 20E mediated acid phosphatase activity in LLI fat body cultures.

c) Effect of 20E in the presence of haemolymph from different developmental stages of *Corcyra* :

Results presented in figure 5 show that addition of posterior haemolymph obtained from ligated early-last, mid-last instar larval and prepupal stages to the fat body culture along with 20E, did not alter the enzyme activity significantly when compared with controls (20E alone). On the other hand, there was a significant increase in the specific activity of fat body acid phosphatase activity when posterior haemolymph from late-last instar larvae was added to the fat body cultures in the presence of 20E.

d) Effect of 20E in the presence of haemolymph from other Lepidopteran insects :

The results (Fig. 6-8) indicate that the addition of haemolymph from the late-last instar larvae of *Papilio* (Fig.6), *Bombyx* (Fig.7) and *Achoea* (Fig.8) along with 20E to the LLI fat body cultures of *Corcyra* caused a significant stimulation of acid phosphatase activity. However, haemolymph from the early and mid-last instar larval as well as prepupal stages of the above mentioned insects did not have any stimulatory effect on the enzyme activity.

Possible site of synthesis of haemolymph factor(s) which mediate the effect of 20E on fat body acid phosphatase activity :

In the present study an attempt was made to locate the source of the factor(s) which potentiate 20E mediated effect on acid phosphatase activity. Result obtained from this experiment (Fig.9) clearly show that co-culture of only hind gut associated

lobular fat body with LLI fat body culture **in the** presence of 20E caused **a** significant **stimulation in the** enzyme activity. On the other hand, other tissues **like** carcass, gut and Malphigian tubules and carcass associated peripheral fat body failed to stimulate the specific activity of acid phosphatase in **LLI** fat body cultures even in the presence of 20E.

Effect of different pre-treatments on the activity of posterior haemolymph factor(s) in mediating the acid phosphatase activity of *in vitro* cultured LLI fat body :

It can be inferred from the result presented in figure 10, that either physical or chemical treatment of the haemolymph factor destroys its ability to potentiate acid phosphatase activity in the presence of 20E. The treatment of posterior haemolymph with acid, alkali and protease V_8 enzyme blocked the *in vitro* stimulation of enzyme activity in the presence of 20E. The addition of alcohol precipitated haemolymph preparation also failed to stimulate the 20E mediated acid phosphatase activity in the fat body cultures.

Effect of different concentrations of haemolymph factor on 20E mediated fat **body acid phosphatase activity** :

The results presented in figure 11a show that 10 ul of 1 : 20 diluted posterior haemolymph was sufficient to stimulate the acid phosphatase activity in *in vitro* cultures of fat body in the presence of 8 x 10" M of 20E. The increase in activity was more significant with an increase in haemolymph volume up to 20 al. However, a further increase in the concentration of haemolymph did not cause further stimulation in fat body enzyme activity.

Effect of different concentrations of 20E on fat body acid phosphatase activity in the presence of haemolymph factor(s):

The result (Fig. 11b) indicates that 8 x 10^{-8} M of 20E **was sufficient to stimulate the acid phosphatase activity in** *in vitro* cultured **LLI** fat body. Any further increase in the concentration of **20E did** not show any increase **in the acid phosphatase activity. The values remained more or** less the same upto a concentration of 16 x 10'

M of 20 E. However, the activity of the enzyme declined significantly in the presence of higher concentrations of 20E ranging between 20-40 x 10^{-6} M.

Effect of incubation period on *in vitro* cultured LLI fat body acid phosphatase activity in the presence of optimum concentrations of 20E and haemolymph factor(s) :

From the results presented in figure 12a, it can be inferred that a period of 4 h is required for the haemolymph factor to exhibit its optimal potentiating effect on fat body acid phosphatase activity in the presence of 20E. However, when the fat body samples were cultured for longer durations of 6-12 h, the haemolymph factor started losing its potentiating effect as is evident from decreased fat body acid phosphatase activity (Fig. 12a). From figure 12b it can be inferred that shorter incubation periods of 5, 15 and 30 min, were not sufficient for the haemolymph factor to exhibit its potentiating effect on fat body acid phosphatase activity in the presence of 20E, however the increase was noticeable with 1 and 2 h of incubation periods.

Effect of different haemolymph fractions of *Corcyra* **in the** presence **of 20E on** *in vitro* **cultured LLI fat body acid phosphatase activity** :

The results presented in figure 13a, show the effect of different fractions of haemolymph obtained using Amicon membrane filters. This protocol was designed and developed during the course of present study. Addition of the fraction below 30 kDa to the fat body cultures along with 20E caused a significant increase in the activity of the enzyme as compared to the control. On the other hand, addition of either 30-100 kDa or >100 kDa fraction failed to stimulate the enzyme activity in the fat body cultures.

Protein profile of haemolymph fractions :

SDS-PAGE analysis of various haemolymph fractions obtained using Amicon membrane filters is shown in figure 13b. The <30 kDa haemolymph fraction of *Corcyra* revealed the presence of two major protein bands.

haemolymph was added to the cultures and the enzyme assay was carried out after h of incubation. The results presented in figure 16 reveal that administration of physiological concentration of exogenous 20E to the fat body cultures did not alter the enzyme activity. However, a significant increase in the enzyme activity was observed when haemolymph from LL1 was added to the fat body cultures in the presence of 20E.

Effect of different haemolymph fractions of *Bombyx* along with 20E on *in vitro* cultured LLI fat body acid phosphatase activity :

The haemolymph was collected from LLI of *Bombyx* and fractionated using Amicon membrane filters. The results presented in figure 17a, clearly show that the addition of the haemolymph fraction with molecular weight of <30 kDa to the LLI fat body cultures along with 20E caused a significant increase in the activity of the enzyme when compared to the control. On the other hand, addition of either 30-100 kDa fraction or >100 kDa fraction failed to stimulate the enzyme activity in the fat body.

Protein profile of various haemolymph fractions of *Bombyx* :

Various fractions obtained from Amicon membrane filtrations were seperated on SDS-PAGE and the results are presented in figure 17b. The <30 kDa fraction of haemolymph revealed the presence of only three to four bands of proteins.

Effect of haemolymph peptide on the phosphorylation pattern of CaM Kinase II and Storage protein binding protein in *Corcyra* fat body :

***In vitro* phosphorylation of endogenous proteins *of Corcyra* fat body :**

In vitro phosphorylation studies with the fat body proteins from LLI in either the absence or the presence of EGTA revealed the phosphorylation of few proteins in the molecular weight range of 30-120 kDa (Fig.18b). Inclusion of 20E stimulated the phosphorylation of several proteins, particularly of a 59/60 kDa protein (lane 2). However, addition of either PKC activators, phosphatidylserine + diacylglycerol or EGTA did not have any effect on the phosphorylation of this 59/60 kDa protein

(lanes 3 and 5). The phosphorylation of 120 kDa protein was unaffected by Ca^{2+} as well as phosphatidylserine + diacylglycerol. However, the phosphorylation of this protein was significantly enhanced by the presence of 20E in the reaction mixture (lane 4). The present study also revealed the phosphorylation of 32 kDa protein which was significantly enhanced by the addition of phosphatidylserine + diacylglycerol (lane 5).

Identification of 120 kDa protein as storage protein binding protein (SPBP) :
Fat body proteins from early, late-last instar larvae and prepupae were subjected to SDS-PAGE and ligand blotting (Figs. 19a and b). Results indicate that biotinylated SP binds to two major proteins (Fig. 19b) and the intensity of binding was highest at the prepupal stage. The apparent molecular weights of these SP binding proteins were 125 and 120 kDa.

Phosphorylation profile of SPBP during various stages of development of *Corcyra* :
For this study fat body proteins from ELI, LLI and PP were *in vitro* phosphorylated either in the presence or absence of Ca^{2+} and the results are presented in figure 20 . At the ELI stage of development this protein did not get phosphorylated (lanes 1 and 2) and the protein from LLI stage was moderately phosphorylated (lanes 3 and 4). However, maximum phosphorylation was observed at prepupal stage (lanes 5 and 6). The phosphorylation status of the protein was not affected either by the presence or absence of Ca^{2+} in the medium.

Identification of the 59/60 kDa protein as CaM kinase II :
Monoclonal rat CaM kinase II a antibody was used to identify the CaM kinase II in the fat body homogenates of *Corcyra* and the results are presented in figure 21. In rat brain homogenate, this antibody showed cross reactivity with the 50 kDa a subunit of the kinase. In addition, it also showed non-specific cross reactivity with a 97 kDa protein (lane 4). In *Corcyra* fat body homogenates two proteins of molecular weight of 59/60 kDa and 70 kDa cross reacted with the antibody (lane 1,2 and 3). No other protein in the fat body homogenate of *Corcyra* cross reacted with the antibody.

Back phosphorylation studies - effect of haemolymph peptide :

Back phosphorylation studies were carried out to verify whether the 120 and 59/60 kDa proteins were also phosphorylated under *in vivo* conditions and whether 20E had any stimulatory effect on this process. The rationale in this experiment is as follows. If the protein is already phosphorylated under *in vivo* conditions, in response to different concentrations of 20E treatment, then, the phosphorylation sites in the proteins would be occupied by endogenous unlabelled phosphate and they would be less available/unavailable for accepting labelled phosphate during *in vitro* phosphorylation reactions. For this experiment, the fat body tissue from 24 h ligated LLI of *Corcyra* was organ cultured for 4 h either in the presence of 20E or carrier and in the presence or absence of haemolymph peptide. At the end of the incubation period, the fat body was homogenised and subjected to *in vitro* phosphorylation. The pattern of phosphorylation obtained with the homogenates of fat body treated with 8 x 10^{-8} M and 16 x 10^{-8} M 20E for 4 h (Fig. 22, lanes 2 and 3) shows considerably less incorporation of [γ^{32}P] ATP into the 120 kDa protein as well as 59/60 kDa protein when compared with carrier treated controls. Only these two proteins consistently showed this change in response to exposure to 20E. However, when haemolymph peptide was added to the fat body culture in the presence of 20E, there was maximum incorporation of [γ^{32}P] ATP into the 120 kDa and 59/60 kDa protein after *in vitro* phosphorylation.

Effect of different time incubations on the phosphorylation status of fat body CaM kinase II and SPBP in the presence of 20E and haemolymph peptide :

The results presented in figure 23 clearly show that the incubation period ranging between 5 min to 2 h was not sufficient for the haemolymph peptide to exhibit its inhibitory effect on phosphorylation of CaM kinase II (lane 4 in Fig.23 a, b, c and d) and SPBP (lane 4 in Fig.23 f, g, h and i) of LLI fat body in the presence of 20E. However, with an incubation period of 4 h the haemolymph factor inhibited the *in vivo* phosphorylation of both these proteins and hence the sites were available for the incorporation of radiolabelled phosphate during *in vitro* phosphorylation and hence increased incorporation of [γ^{32}P] ATP in CaM kinase II (Fig. 23e, lane 4) and SPBP (Fig. 23j, lane 4). On the other hand, no signal was detected in the samples

which were *in vivo* incubated with 20E alone for 4 h either for CaM kinase II (Fig.23e, lane 3) or SPBP (Fig.23j, lane 3).

Changes in the fat body CaM kinase **II activity during larval-prepupal development of** *Corcyra* :

CaM kinase II activity was assayed in the fat body homogenates from different developmental stages and the results are presented in figure 24. A comparison of the CaM kinase II activity revealed significant differences at different stages of development. The enzyme exhibited highest activity at late larval stage and lower activity during the early-last instar larval and prepupal stages.

Catalytic properties of *Corcyra* fat body CaM kinase II **activity** :

The degree of phosphorylation of syntide-2 was linear upto 15 μg of homogenate protein (Fig. 25). The rate of phosphorylation of syntide-2 by *Corcyra* fat body CaM kinase II showed normal Michaelis-Menten kinetics with respect to the concentration of syntide-2. In the presence of saturating amount of syntide-2, the K_m value for syntide-2 was 5.5 μM with a V_{max} of 1 n mol/min/mg protein (Fig. 26).

Possible mechanism of action :

In vitro effect of 20E on **LLI** fat body CaM kinase **II** activity in the presence of haemolymph peptide :

CaM kinase II activity was assayed in the fat body homogenate from late-last instar larva either in the presence of 20E and/or haemolymph peptide and the results are presented in figure 27. Addition of 8×10^{-8} M 20E to fat body homogenates caused a significant increase in the activity of CaM kinase II when compared with control. On the other hand, when haemolymph peptide was added along with 20E, there was a drastic decrease in fat body CaM kinase II activity. When haemolymph peptide was added alone the activity was more or less same as that of the control.

***In vitro* effect of 20E on LLI fat body acid phosphatase activity in the presence of haemolymph peptide** :

Acid phosphatase activity was assayed in fat body homogenates from late last instar larva in the presence of 20E and/or haemolymph peptide and the results are presented in figure 28. Addition of 20E alone to fat body homogenate did not alter the enzyme activity. The activity recorded was on par with that of control and carrier treated control. However, when haemolymph peptide was added along with 20E there was a significant increase in the fat body acid phosphatase activity. Addition of haemolymph peptide alone, to the fat body homogenate also failed to stimulate the enzyme and the activity remained close to that of control samples.

Effect of actinomycin D and cycloheximide on acid phosphatase activity of **LLI** fat body in the presence of 20E and haemolymph peptide:

It can be inferred from the results presented in figure 29 that actinomycin D as well as cycloheximide failed to inhibit the haemolymph peptide's enzyme potentiating activity in the presence of 20E. When actinomycin D or cycloheximide were added to the fat body homogenate in the presence of 20E and haemolymph peptide, the haemolymph peptide mediated the stimulation of fat body acid phosphatase activity and the values were comparable to the values obtained with 20E and haemolymph peptide treated fat body homogenates.

Effect of protease inhibitors on the haemolymph **peptide's** potentiating effect on fat body acid phosphatase activity in the presence of 20E :

Results presented in figure 30. indicate that when the haemolymph peptide was treated with either PMSF or leupeptin prior to its addition to fat body homogenates, the peptide lost its potentiating effect on fat body acid phosphatase activity. However, when untreated haemolymph peptide was added to the homogenate, the fat body acid phosphatase activity increased significantly as compared to that of control.

Fig. 1 - Developmental profile of acid phosphatase activity in the fat body of *Corcyra* - Each value is the mean ± S.D. of four independent determinations. For each determination fat body from 2-3 insects were pooled. The stages used were PEN, ELI, MLI, LLI and PP. * The value at one stage is significantly different from the previous developmental stage ($p<0.005$) as determined by one-way analysis of variance with Sigma Stat. Software.

Fig.1

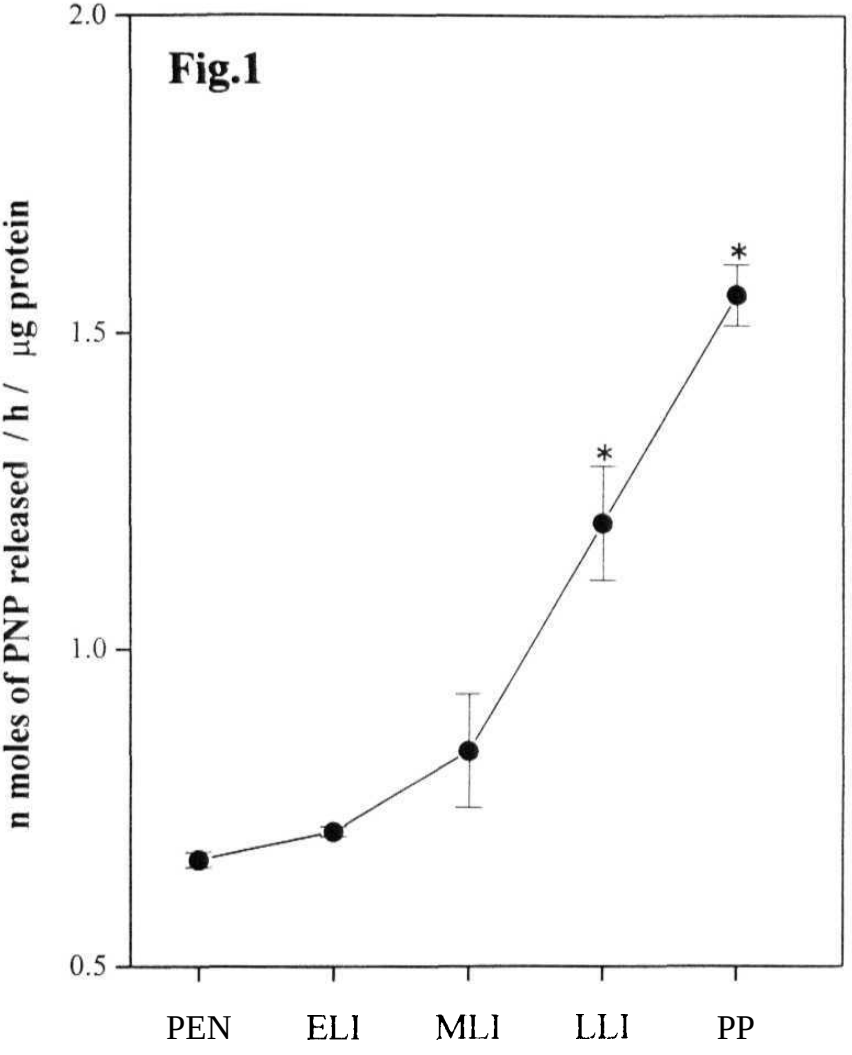
2.0
1.5
1.0
0.5
n moles of PNP released / h / µg protein
*
*
PEN
ELI
MLI
LLI
PP

Fig. 2a - Effect of ligation on LLI fat body acid phosphatase activity - Each value is the mean ± S.D. of four independent determinations. For each determination fat body from 2-3 insects were pooled. The insects were used after 24, 48 and 72 h ligation and the fat body was dissected out, homogenised and assayed for acid phosphatase activity.

Fig. 2b - Effect of injection of exogenous 20E on ligated LLI fat body acid phosphatase activity - Each value is the mean ± S.D. of four independent determinations. For each determination fat body from 2-3 ligated larvae were pooled. After 24 and 48 h of ligation experimental insects were injected with 8×10^{-8} M 20E (in 2 µl of 10% ethanol) while control insects received equal volume of carrier . After 24 h of incubation, fat body was dissected out homogenised and assayed for acid phosphatase activity.

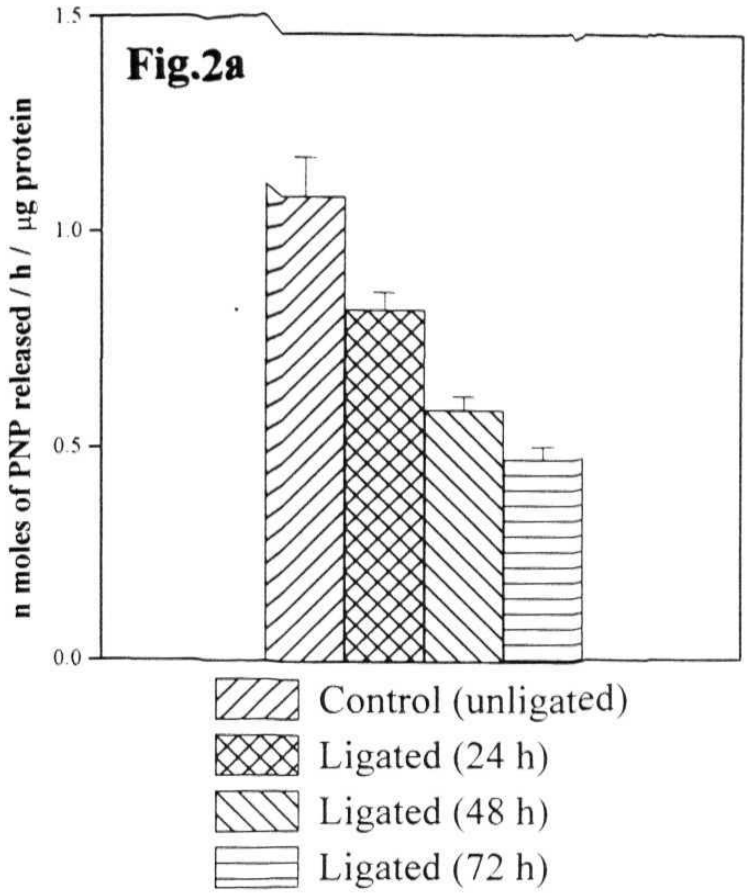
Fig.2a
n moles of PNP released / h / µg protein
1.5
1.0
0.5
0.0
Control (unligated)
Ligated (24 h)
Ligated (48 h)
Ligated (72 h)

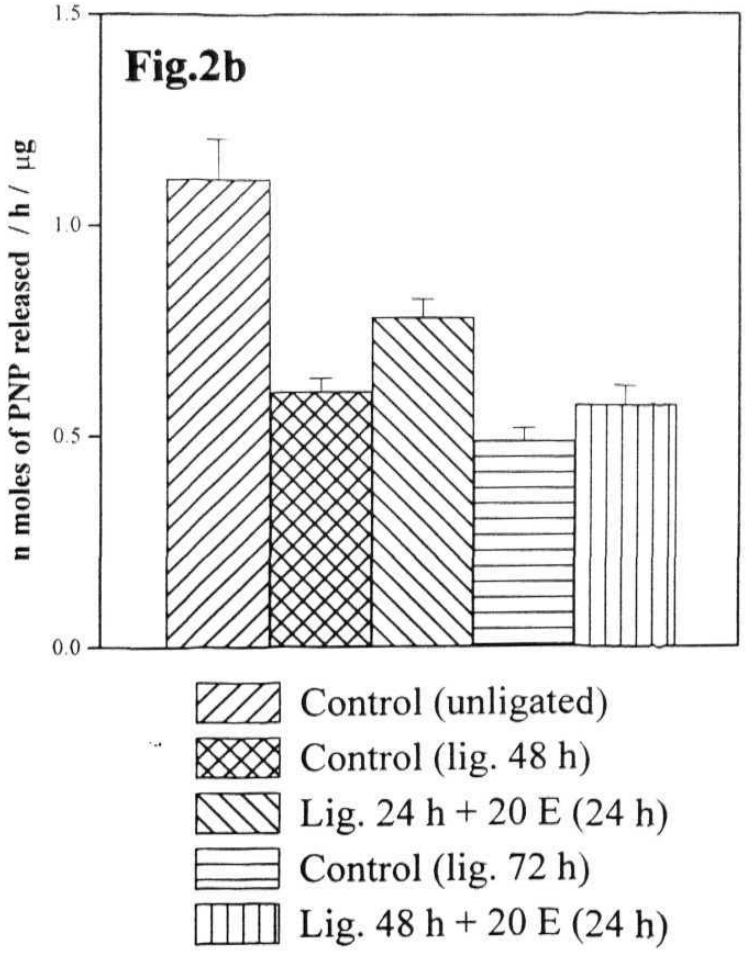
Fig.2b
n moles of PNP released / h / µg
1.5
1.0
0.5
0.0
Control (unligated)
Control (lig. 48 h)
Lig. 24 h + 20 E (24 h)
Control (lig. 72 h)
Lig. 48 h + 20 E (24 h)

Fig. 3 - Effect of 20E on *in vitro* cultured LLI fat body acid phosphatase activity Each value is the mean ± S.D. of four independent determinations. For each determination fat body from two insects were used after 24 h of ligation. The tissue was incubated in 200 ul of TC-100 insect culture medium with 8 x 10 M 20E (in 10 ul of 10% ethanol) for 4 h. To the control cultures equal volume of carrier was added. At the end of the incubation period fat body was removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenised and assayed for acid phosphatase activity.

Fig. 4 - Effect on 20E on *in vitro* cultured LLI fat body acid phosphatase activity in the presence of haemolymph factors from *Corcyra* Each value is the mean ± S.D. of four independent determinations. For each determination fat body from two insects were used after 24 h of ligation. The tissue was incubated in 200 ul of TC-100 insect culture medium with 8 x 10^{-8} M 20E (in 10 ul of 10% ethanol) and 10 ul of 1: 20 diluted haemolymph obtained either from the anterior and posterior part of 24 h ligated LLI larvae or unligated LLI larvae for 4 h. To the control cultures equal volume of insect Ringer was added. At the end of the incubation period fat body was removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenised and assayed for acid phosphatase activity.

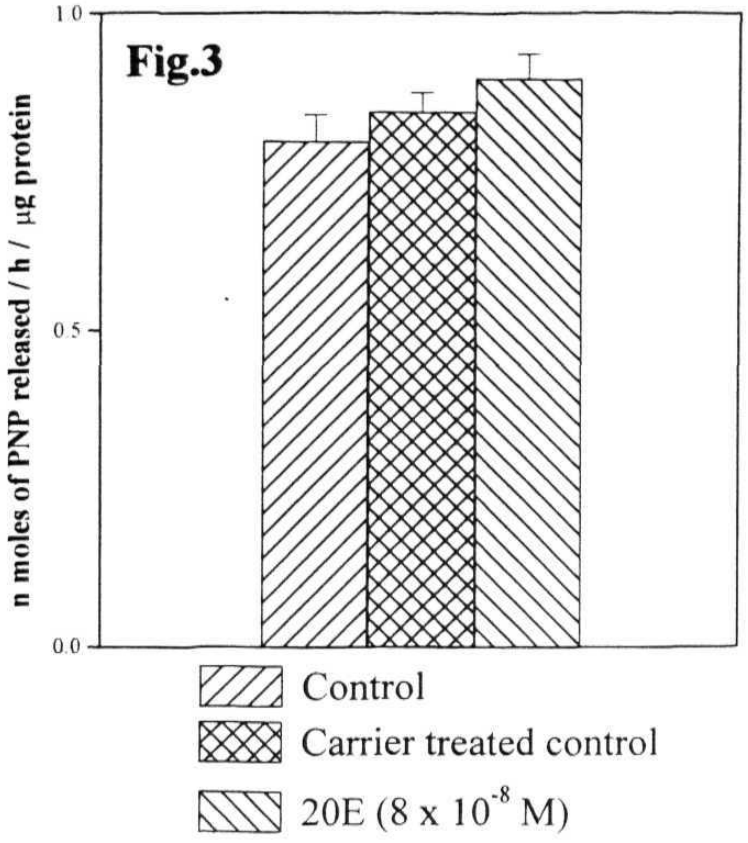
Fig.3
n moles of PNP released / h / µg protein
1.0
0.5
0.0
Control
Carrier treated control
20E (8 x 10-8 M)

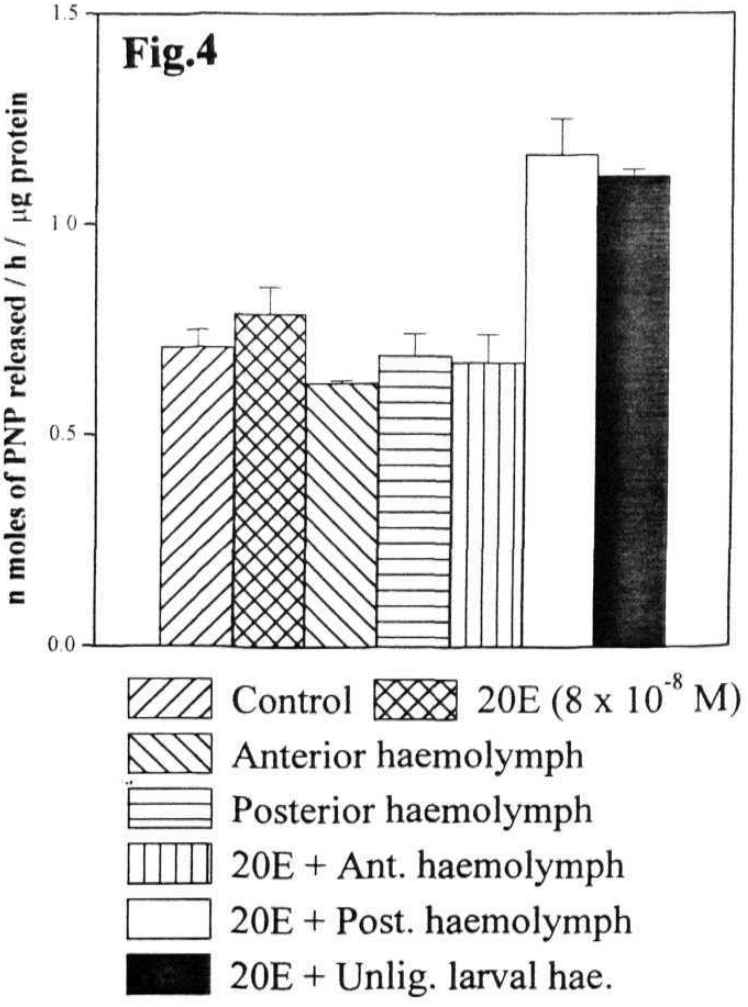
Fig.4
n moles of PNP released / h / µg protein
1.5
1.0
0.5
0.0
Control
20E (8 x 10-8 M)
Anterior haemolymph
Posterior haemolymph
20E + Ant. haemolymph
20E + Post. haemolymph
20E + Unlig. larval hae.

Fig. 5 - Effect of 20E **on** ***in*** *vitro* **cultured LLI fat** body **acid phosphatase activity in the presence of haemolymph from different developmental stages of** ***Corcyra***
Each value is the mean ± S.D. of four independent determinations. For each determination fat body from two insects were used after 24 h of ligation. The tissue was incubated in 200 ul of TC-100 insect culture medium with 8 x 10^{-8} M 20E (in 10 µl of 10% ethanol) and 10 ul of 1: 20 diluted haemolymph obtained from the posterior part of 24 h ligated ELI, MLI, LLI larval and PP stages for 4 h. To the control cultures equal volume of carrier was added. At the end of the incubation period fat body was removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenised and assayed for acid phosphatase activity.

Fig. 6 - Effect of 20E on *in vitro* cultured **LLI** fat body acid phosphatase activity in the presence of haemolymph from *Papilio* ***demoleus*** - Each value is the mean ± S.D. of four independent determinations. For each determination fat body from two insects were used after 24 h of ligation. The tissue was incubated in 200 µl of TC-100 insect culture medium with 8 x 10^{-8} M 20E (in 10 µl of 10% ethanol) and 10 µl of 1: 20 diluted haemolymph obtained from ELI, MLI, LLI larval stages and PP stage of *Papilio demoleus* (lemon butterfly) for 4 h. To the control cultures equal volume of carrier was added. At the end of the incubation period fat body was removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenised and assayed for acid phosphatase activity.

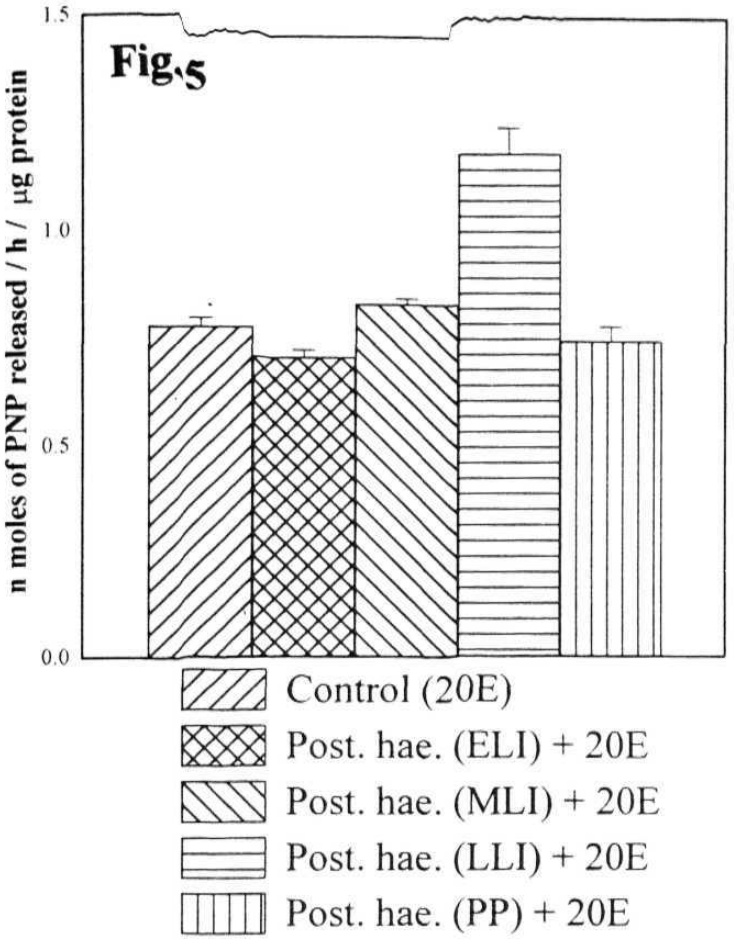
Fig.5
n moles of PNP released / h / µg protein
1.5
1.0
0.5
0.0
Control (20E)
Post. hae. (ELI) + 20E
Post. hae. (MLI) + 20E
Post. hae. (LLI) + 20E
Post. hae. (PP) + 20E

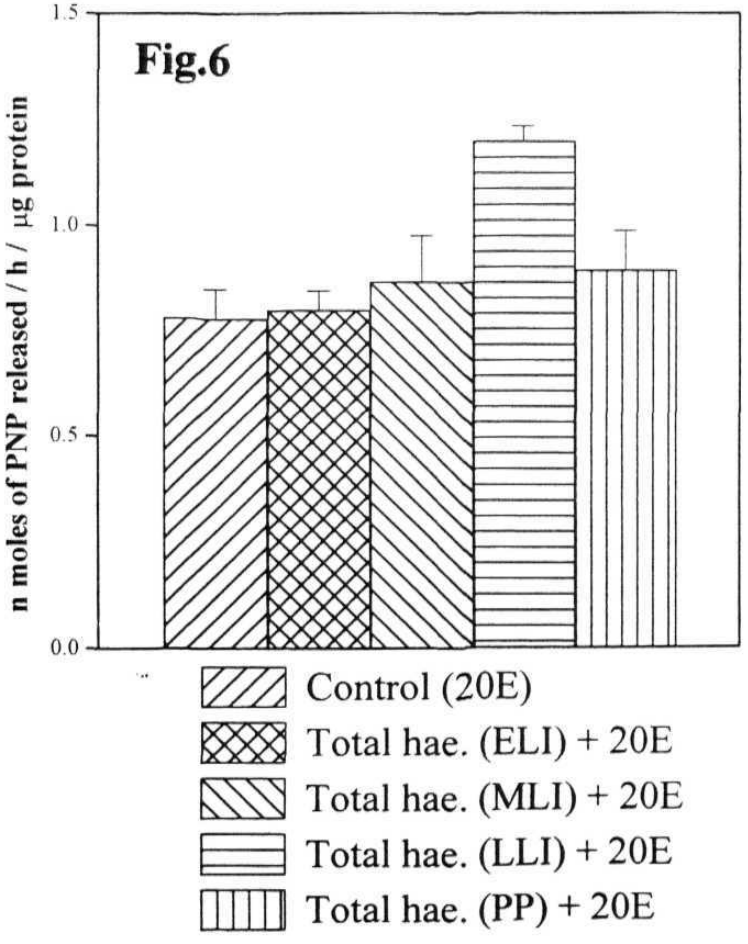
Fig.6
n moles of PNP released / h / µg protein
1.5
1.0
0.5
0.0
Control (20E)
Total hae. (ELI) + 20E
Total hae. (MLI) + 20E
Total hae. (LLI) + 20E
Total hae. (PP) + 20E

Fig. 7- **Effect of** 20E on *in vitro* **cultured LLI fat** body **acid phosphatase activity in the** presence **of** haemolymph **from** *Bombyx mori* - Each value is the mean ± S.D. of four independent determinations. For each determination fat body from two insects were used after 24 h of ligation. The tissue was incubated in 200 µl of TC-100 insect culture medium with 8 x 10^{-8} M 20E (in 10 µl of 10% ethanol) and 10 ul of 1: 20 diluted haemolymph obtained from ELI, MLI, LLI larval stages and PP stage of *Bombyx mori* (silk worm) for 4 h. To the control cultures equal volume of carrier was added. At the end of the incubation period fat body was removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenised and assayed for acid phosphatase activity.

Fig. 8 - Effect of 20E on *in vitro* cultured **LLI** fat body acid phosphatase activity in the presence of haemolymph from ***Achoea janata*** - Each value is the mean ± S.D. of four independent determinations. For each determination fat body from two insects were used after 24 h of ligation. The tissue was incubated in 200 ul of TC-100 insect culture medium with 8 x 10^{-8} M 20E (in 10 ul of 10% ethanol) and 10 ul of 1: 20 diluted haemolymph obtained from ELI, MLI, LLI larval stages and PP stage *of Achoea Janata* (castor semilooper) for 4 h. To the control cultures equal volume of carrier was added. At the end of the incubation period fat body was removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenised and assayed for acid phosphatase activity.

Fig.7

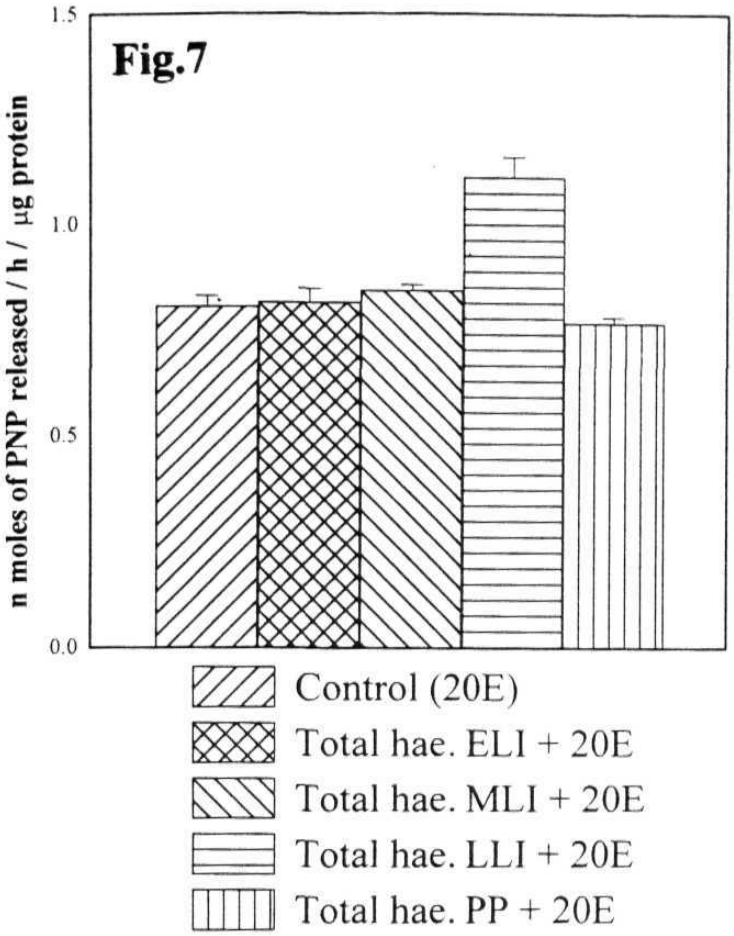
n moles of PNP released / h / µg protein
1.5
1.0
0.5
0.0
Control (20E)
Total hae. ELI + 20E
Total hae. MLI + 20E
Total hae. LLI + 20E
Total hae. PP + 20E

Fig.8

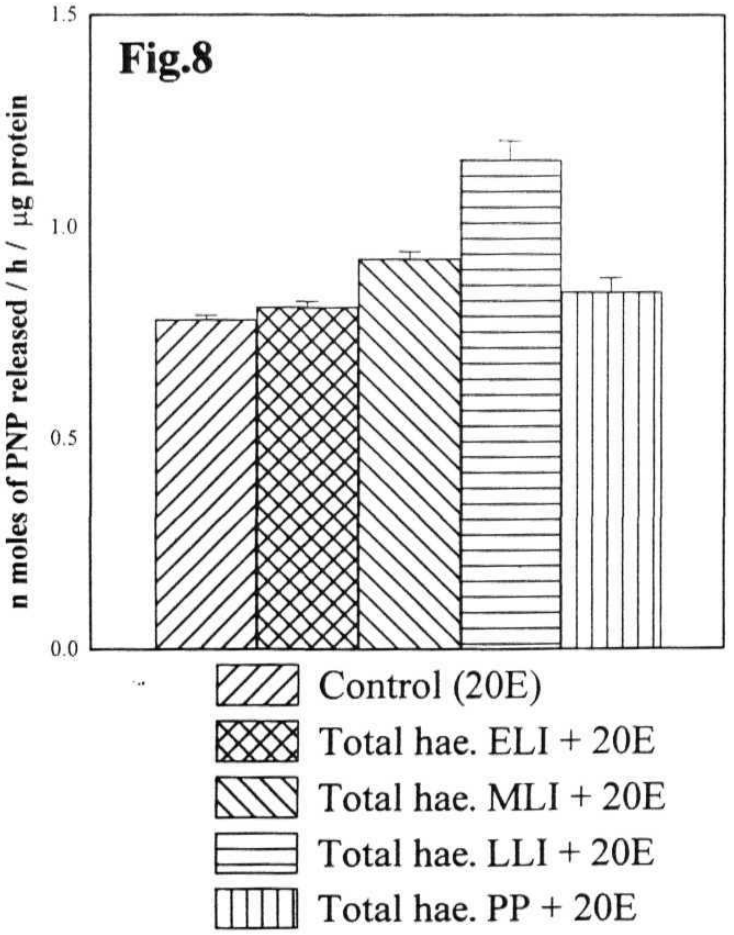
n moles of PNP released / h / µg protein
1.5
1.0
0.5
0.0
Control (20E)
Total hae. ELI + 20E
Total hae. MLI + 20E
Total hae. LLI + 20E
Total hae. PP + 20E

Fig. 9 - Possible site of synthesis of haemolymph factor(s) which mediate the effect of 20E on fat body acid phosphatase activity Each value is the mean ± S.D. of four independent determinations. For each determination fat body from two insects were used after 24 h of ligation. The tissue was incubated in 200 µl of TC-100 insect culture medium with $8 \times 10^{-}$ M 20E (in 10 µl of 10% ethanol) for 4 h. To the control cultures equal volume of carrier was added. The fat body was co-cultured with different tissues dissected out from posterior part of two late-last instar larvae after 24 h of ligation. At the end of the incubation period fat body was removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenised and assayed for acid phosphatase activity. *Significantly different from all other values ($p < 0.005$) as determined by one way analysis of Sigma Stat Software.

Fig. 10 - Effect of different pre-treatments on the activity of posterior haemolymph factor in mediating the acid phosphatase activity **of** *in vitro* **cultured LLI fat body** Each value is the mean ± S.D. of four independent determinations. For each determination fat body from two insects were used after 24 h of ligation. The tissue was incubated in 200 ul of TC-100 insect culture medium with 8×10^{-8} M 20E (in 10 ul of 10% ethanol) together with 10 µl of 1 : 20 diluted haemolymph which was previously subjected to different treatments, for 4 h. To the control cultures equal volume of carrier and insect Ringer was added. At the end of the incubation period fat body was removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenised and assayed for acid phosphatase activity. *Significantly different from all other values ($p < 0.005$) as determined by one way analysis of Sigma Stat Software.

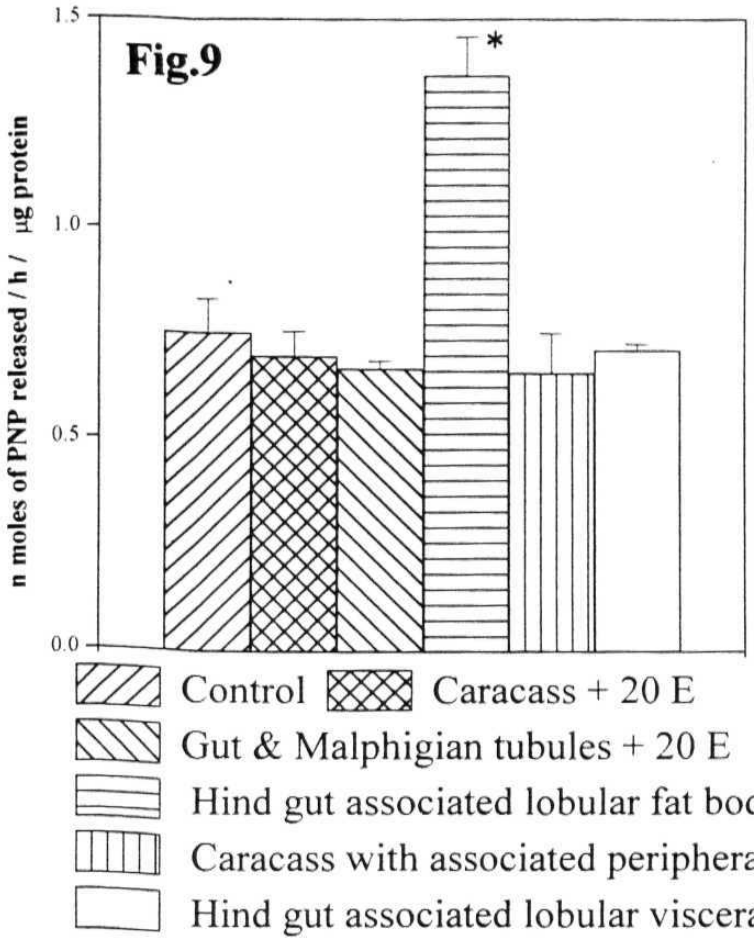
Fig.9
n moles of PNP released / h / µg protein
1.5
1.0
0.5
0.0
*
Control
Caracass + 20 E
Gut & Malphigian tubules + 20 E
Hind gut associated lobular fat body + 20 E
Caracass with associated peripheral fat body + 20 E
Hind gut associated lobular visceral fat body

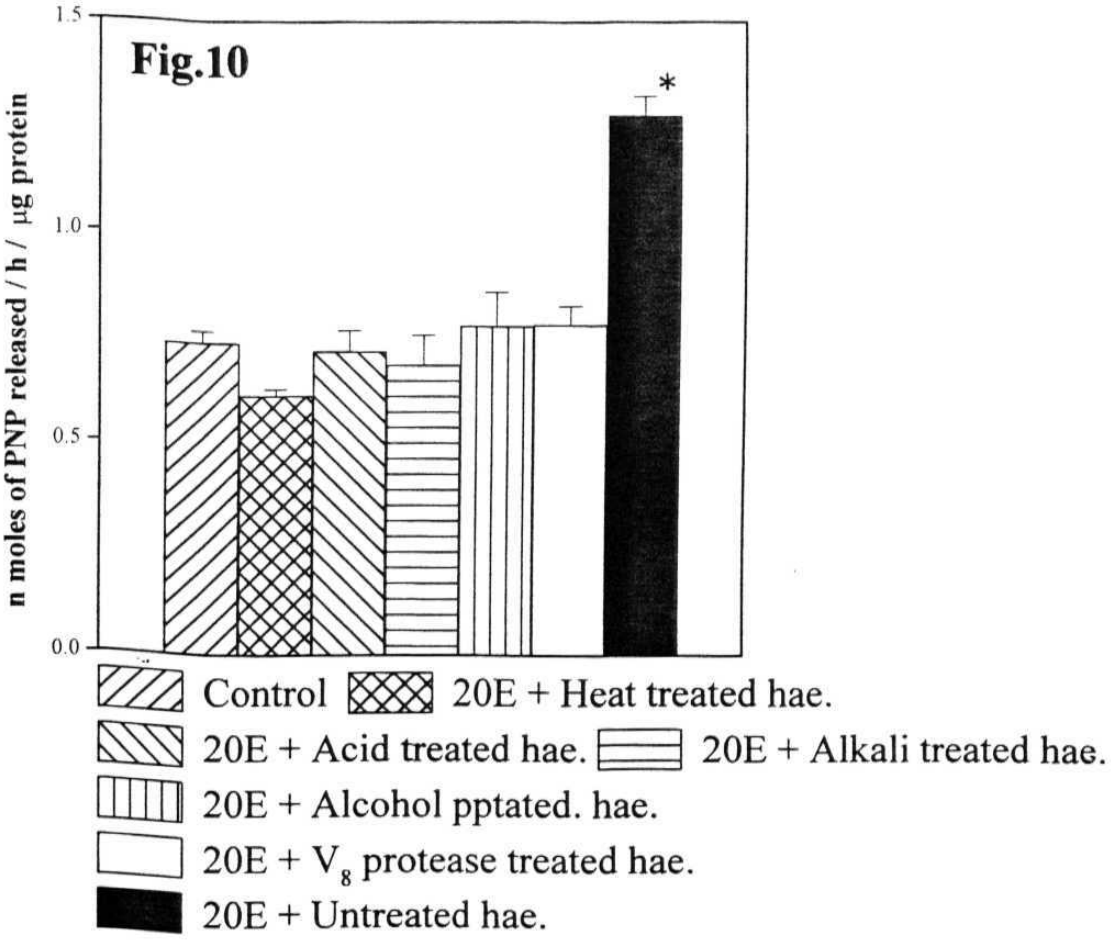
Fig.10
n moles of PNP released / h / µg protein
1.5
1.0
0.5
0.0
*
Control
20E + Heat treated hae.
20E + Acid treated hae.
20E + Alkali treated hae.
20E + Alcohol pptated. hae.
20E + V_g protease treated hae.
20E + Untreated hae.

Fig. 11a - Effect of different concentration of haemolymph factor on 20E mediated fat body acid phosphatase activity Each value is the mean ± S.D. of four independent determinations. For each determination fat body from two insects were used after 24 h of ligation. The tissue was incubated in 200 ul of TC-100 insect culture medium with 8 x 10^{-8} M 20E (in 10 ul of 10% ethanol) together with different concentrations of 1 : 20 diluted haemolymph obtained from posterior part of 24 h ligated LLI larvae for 4 h. To the control cultures equal volume of carrier and insect Ringer was added. At the end of the incubation period fat body was removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenised and assayed for acid phosphatase activity.

Fig. 11b - Effect of different concentrations of 20E on fat body acid phosphatase activity in **the** presence of haemolymph factor - Each value is the mean ± S.D. of four independent determinations. For each determination fat body from two insects were used after 24 h of ligation. The tissue was incubated in 200 µl of TC-100 insect culture medium with different concentrations of 20E (in 10 ul of 10% ethanol) together with 10 µl of 1 : 20 diluted haemolymph obtained from posterior part of 24 h ligated LLI larvae for 4 h. To the control cultures equal volume of carrier and insect Ringer was added. At the end of the incubation period fat body was removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenised and assayed for acid phosphatase activity.

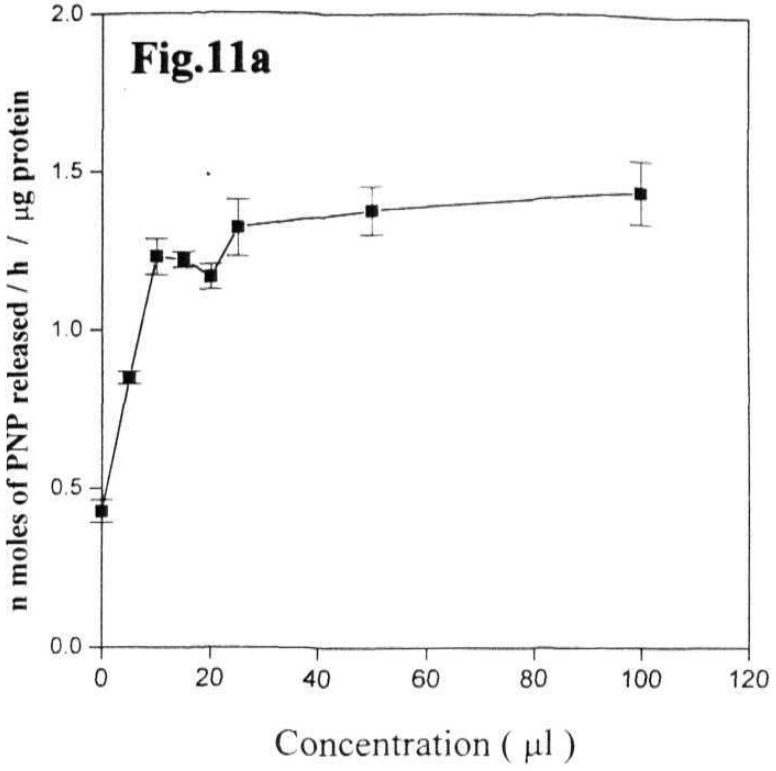
Fig.11a
n moles of PNP released / h / µg protein
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
120
Concentration (µl)

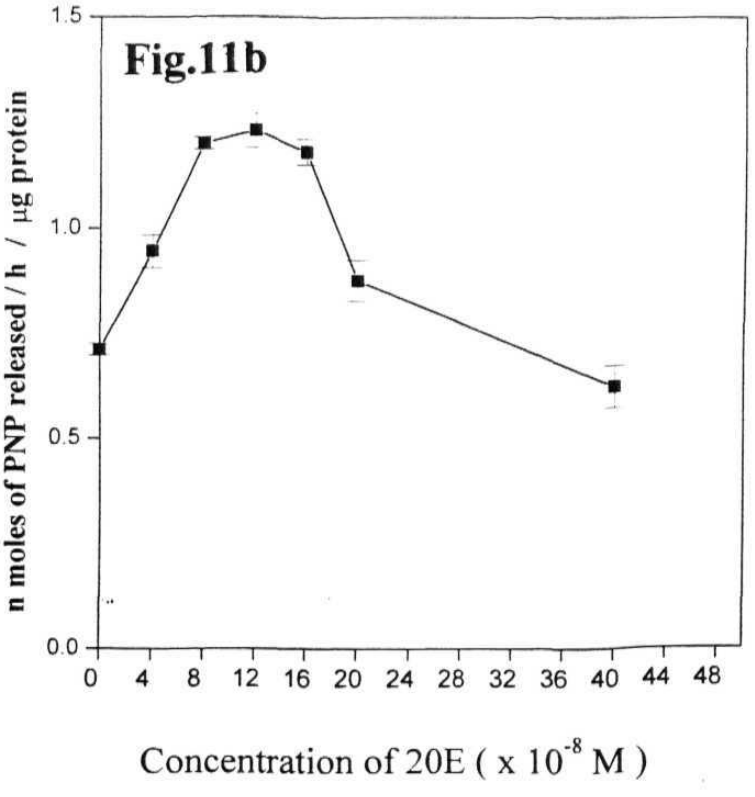
Fig.11b
n moles of PNP released / h / µg protein
1.5
1.0
0.5
0.0
0
4
8
12
16
20
24
28
32
36
40
44
48
Concentration of 20E (x 10-8 M)

Fig. 12a - Effect of longer incubation period on *in vitro* cultured LLI fat body acid phosphatase activity in the presence of optimum concentrations of 20E and haemolymph factor - Each value is the mean ± S.D. of four independent determinations. For each determination fat body from two insects were used after 24 h of ligation. The tissue was incubated in 200 ul of TC-100 insect culture medium with 8 X 10^{-8} M 20E (in 10 ul of 10% ethanol) together with 10 ul of 1 : 20 diluted haemolymph obtained from posterior part of 24 h ligated LLI larvae for longer incubation periods (4, 6, 12 and 24 h). To the control cultures equal volume of carrier and insect Ringer was added. At the end of the incubation period fat body was removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenised and assayed for acid phosphatase activity.

Fig. 12b - Effect of shorter incubation period on *in vitro* cultured LLI fat body acid phosphatase activity in the presence of optimum concentrations of 20E and haemolymph factor - Each value is the mean + S.D. of four independent determinations. For each determination fat body from two insects were used after 24 h of ligation. The tissue was incubated in 200 ul of TC-100 insect culture medium with 8 x 10^{-8} M 20E (in 10 ul of 10% ethanol) together with 10 ul of 1 : 20 diluted haemolymph obtained from posterior part of 24 h ligated LLI larvae for shorter incubation periods (5 min, 15 min, 30 min, 1 h , 2 h and 4 h). To the control cultures equal volume of carrier and insect Ringer was added. At the end of the incubation period fat body was removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenised and assayed for acid phosphatase activity.

Fig. 13a - Effect of different haemolymph fractions of *Corcyra* in the presence of 20E on *in vitro* cultured LLI fat body acid phosphatase activity - Each value is the mean ± S.D. of four independent determinations. For each determination fat body from two insects were used after 24 h of ligation. The tissue was incubated in 200 ul of TC-100 insect culture medium containing 8 x 10^{-8} M 20E (in 10 ul of 10% ethanol) and 10 ul of fractionated haemolymph (>100 kDa, 30-100 kDa or <30 kDa fraction) for 4 h. To the control cultures equal volume of carrier and insect Ringer was added. At the end of the incubation period fat body was removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenised and assayed for acid phosphatase activity. *Significantly different from all other values ($p < 0.005$) as determined by one way analysis of Sigma Stat Software.

Fig. **13b** - SDS-PAGE showing **the** protein profile of **various** haemolymph fraction Lane 1 - Crude haemolymph, lane 2- > 100 kDa fraction, lane 3 - 30-100 kDa fraction, lane 4 - > 30 kDa fraction, lane 5 - < 30 kDa fraction, and M-molecular weight markers. The proteins were separated on a 12% gel. In lane 1-4, 10 ug protein and in lane 5, 3 ug protein was loaded.

Fig.13a

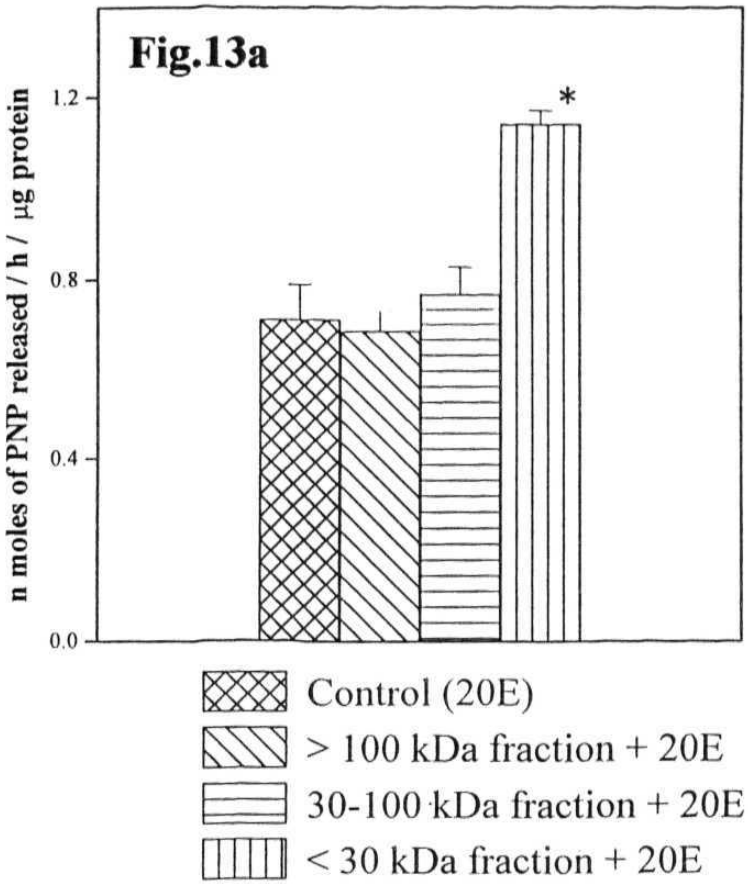

Fig.13b

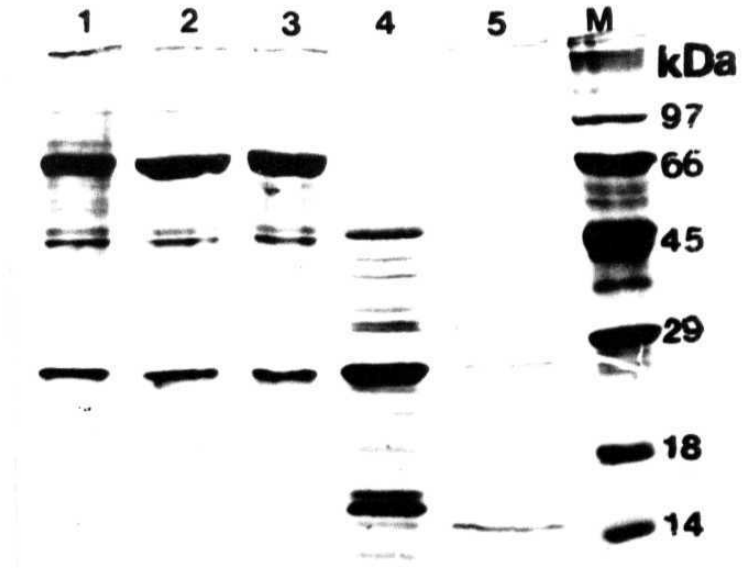

Fig. 14a - Elution profile of haemolymph peptide on Sephadex G-25-50 matrix

Elution profile of the haemolymph peptide fractions obtained from Sephadex G-25-50 matrix when the protein was eluted with 10 mM Tris-HCl (pH 7.4).

Fig. **14b** - SDS-PAGE **profile** of **different** fractions **from** Sephadex **G-25-50 matrix** Lane 1 - < 100 kDa fraction, lane 2- 23rd fraction elute, lane 3 – 24th fraction elute , lane 4 - 33rd fraction elute , lane 5 - 34th fraction elute and M - molecular weight markers. The proteins were separated on a 12% gel and in lane 1-3 5 μg protein was loaded. In lanes 4 and 5 , the 33rd and 34th fraction elutes were completely lyophilised and loaded on to the respective well.

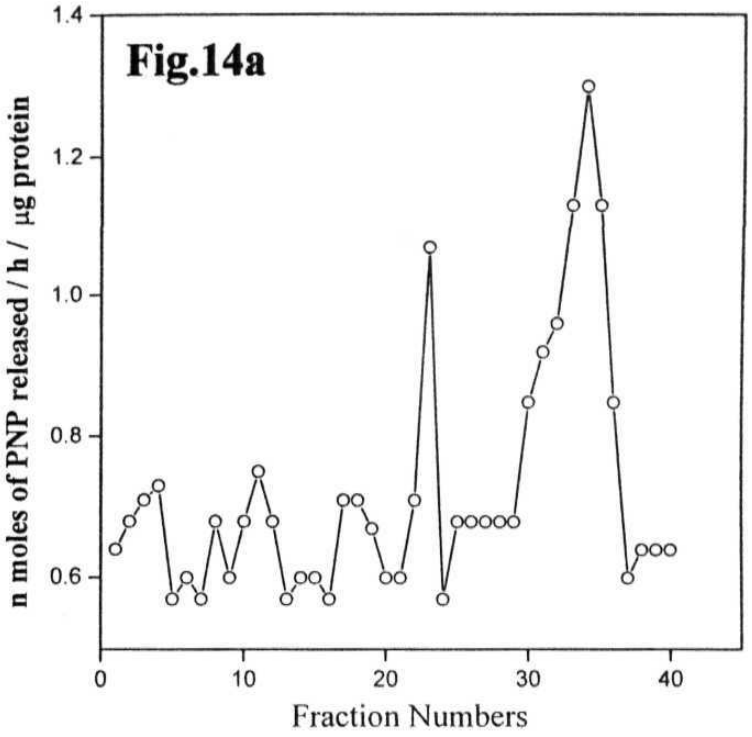
Fig.14a
n moles of PNP released / h / µg protein
1.4
1.2
1.0
0.8
0.6
0
10
20
30
40
Fraction Numbers

Fig.l4b

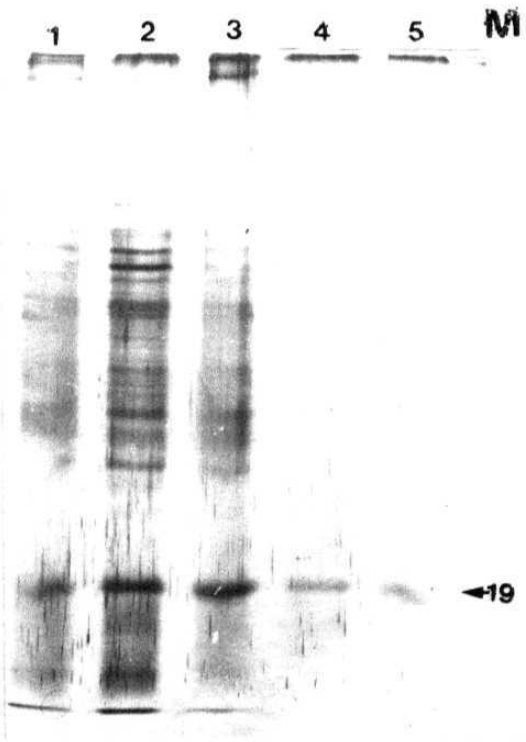
1
2
3
4
5
M
19

Fig. 15 - Developmental profile of fat body acid phosphatase activity of *Bombyx*
Each value is the mean ± S.D. of four independent determinations. For each determination fat body from 2-3 insects were pooled. The stages used were ELI, MLI, LL1, EPP, LPP, EP, LP and A.

Fig. 16 - Effect of 20E on *in vitro* cultured LLI fat body acid phosphatase activity in the presence of haemolymph from *Bombyx* Each value is the mean ± S.D. of four independent determinations. For each determination fat body from two insects were used. The tissue was incubated in 200 µl of TC-100 insect culture medium for 4 h to deplete the endogenous hormone. After 4 h, the tissue was removed from the medium, rinsed in fresh culture medium and again incubated for 4 h in 200 µl of TC-100 insect culture medium containing 5 X 10^{-6} M 20E (in 10 µl of 10% ethanol) and 10 µl of 1: 20 diluted haemolymph obtained from LLI larvae. To the control cultures equal volume of carrier and insect Ringer was added. At the end of the incubation period fat body was removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenised and assayed for acid phosphatase activity. * Significantly different from all other values ($p < 0.005$) as determined by one way analysis of Sigma Stat Software.

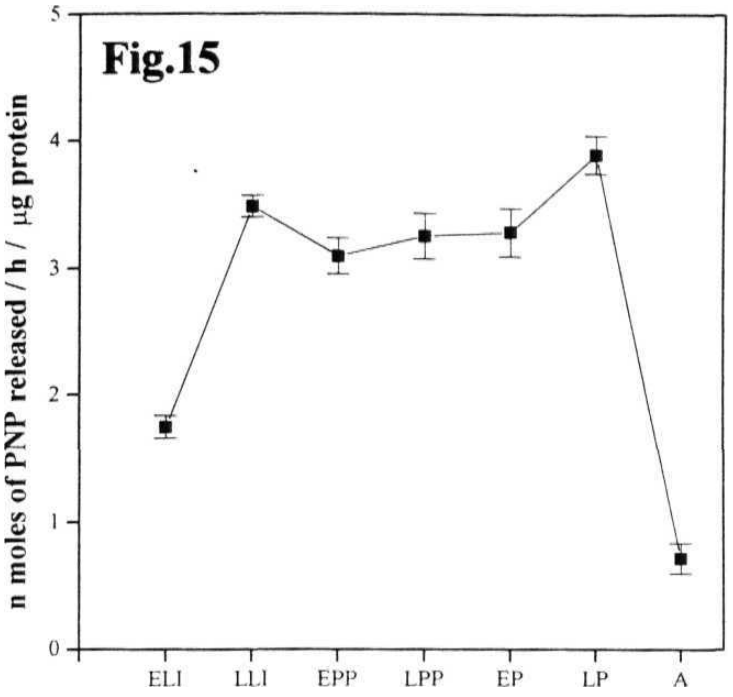
Fig.15
n moles of PNP released / h / µg protein
0
1
2
3
4
5
ELI
LLI
EPP
LPP
EP
LP
A

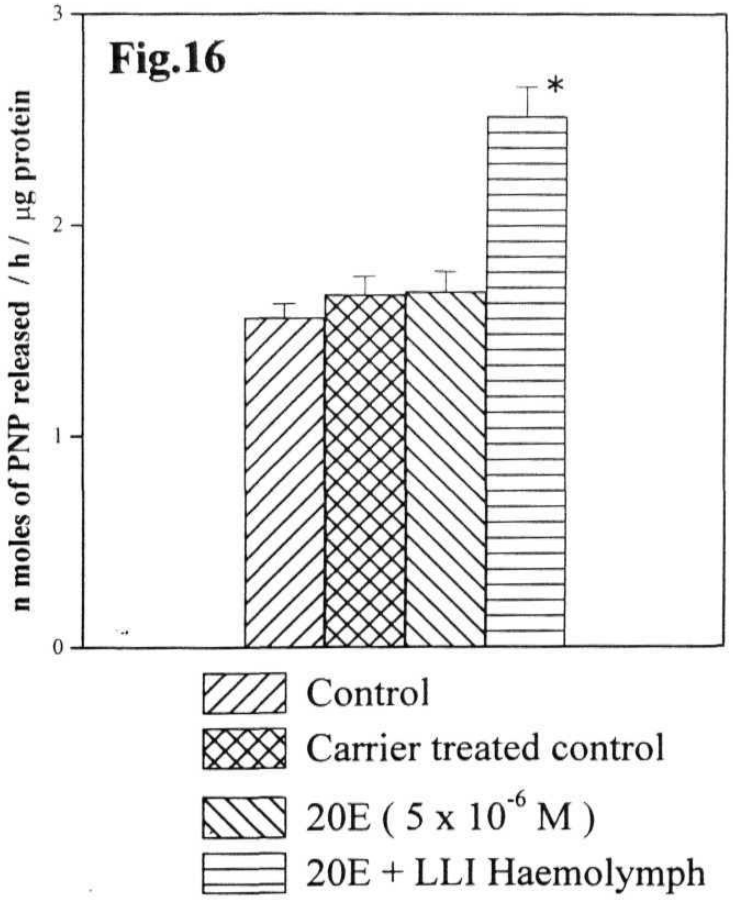
Fig.16
n moles of PNP released / h / µg protein
0
1
2
3
*
Control
Carrier treated control
20E (5 x 10-6 M)
20E + LLI Haemolymph

Fig. 17a - Effect of different haemolymph fractions of *Bombyx* along with 20E on *in vitro* cultured LLI fat body acid phosphatase activity - Each value is the mean ± S.D. of four independent determinations. For each determination fat body from **two** insects were used. The tissue was incubated in 200 ul of TC-100 insect culture medium for 4 h to deplete the endogenous hormone. After 4 h the tissue was removed from the medium, rinsed in fresh culture medium and again incubated for 4 h in 200 ul of TC-100 insect culture medium containing 5 x 10^{-6} M 20E (in 10 µl of 10% ethanol) and 10 ul of fractionated haemolymph (>100 kDa, 30-100 kDa or <30 kDa fraction) for 4 h. To the control cultures equal volume of carrier and insect Ringer was added. At the end of the incubation period fat body was removed from the culture medium, rinsed thoroughly in ice cold insect Ringer, homogenised and assayed for acid phosphatase activity.

Fig. 17b - SDS-PAGE showing the protein profile of various haemolymph fraction Lane 1 - Crude haemolymph, lane 1- > 100 kDa fraction, lane 3 - 30-100 kDa fraction, lane 4 - < 30 kDa fraction and M- molecular weight markers. The proteins were separated on a 12% gel. In lanes 1-3, 5 ug and in lane 4, 2 µg protein was loaded.

Fig.17a

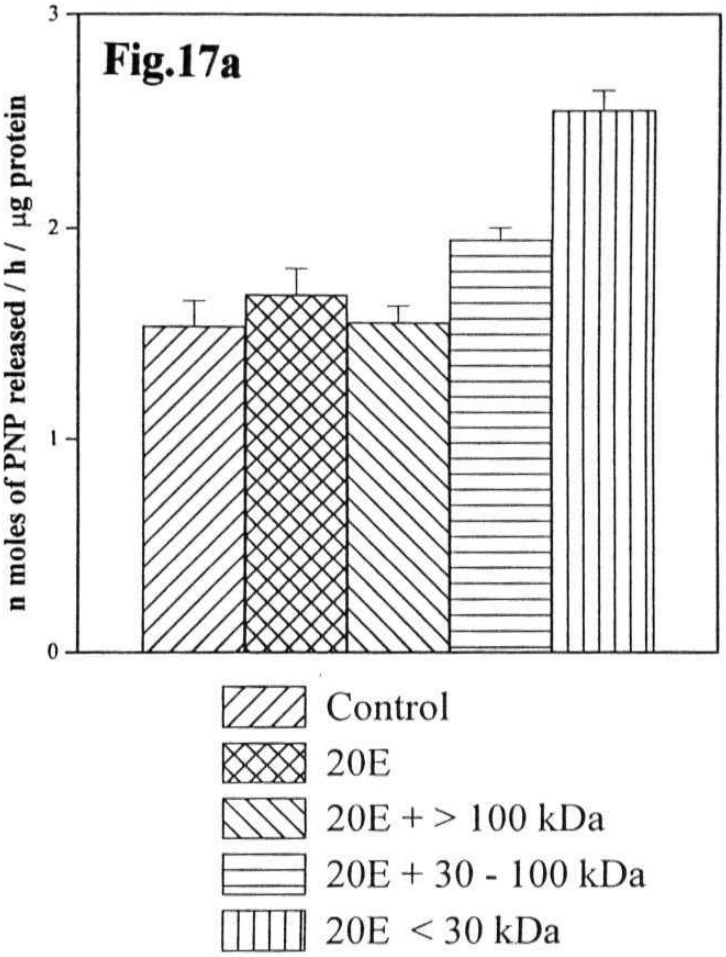

Fig.17b

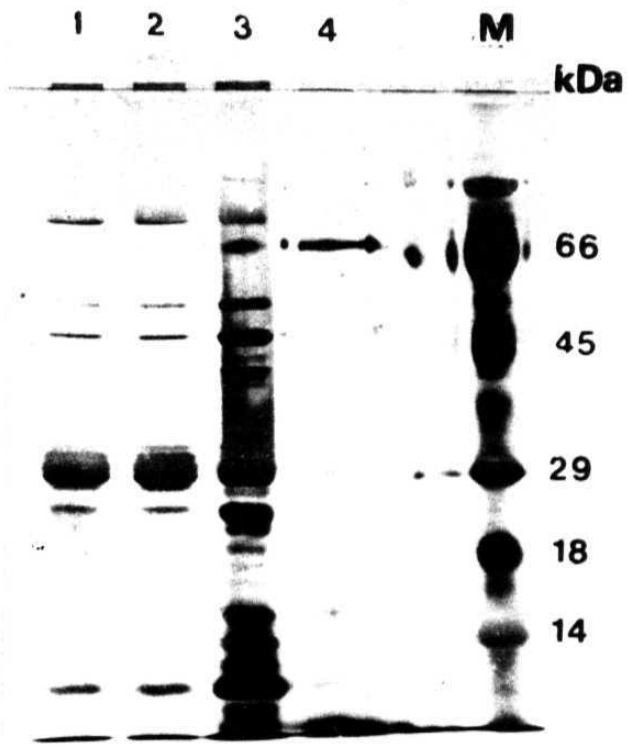

Fig. 18 - *In vitro* phosphorylation of *Corcyra* fat body proteins Following incubation of homogenate with [γ^{32}P] ATP under phosphorylating conditions (see materials and methods). (a) - SDS-PAGE, (b) autoradiograph of the same gel. The incubations were under the following conditions : lane 1: 1 mM $CaCl_2$, lane 2: 1 mM $CaCl_2$ + 8 x 10^{-8} M 20E, lane 3 : 1 mM EGTA, lane 4 : 1 mM EGTA + 8 x 10^{-8} M 20E, lane 5 : 1 mM $CaCl_2$ + 10 mM phosphatidylserine + 100 mM diacylglycerol. Note the phosphorylation (⟵) of the 59/60 kDa and (⟵) 120 kDa protein which was significantly stimulated in the presence of 20E.

Fig.l8a

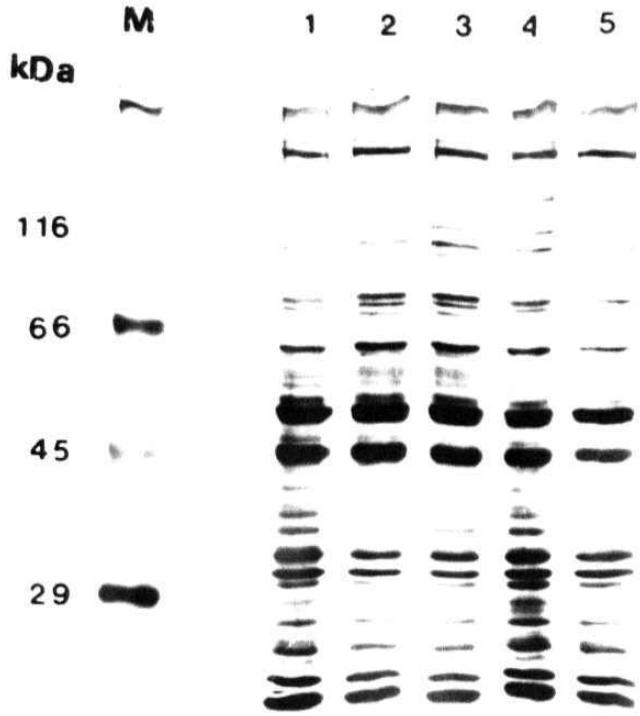

Fig.l8b

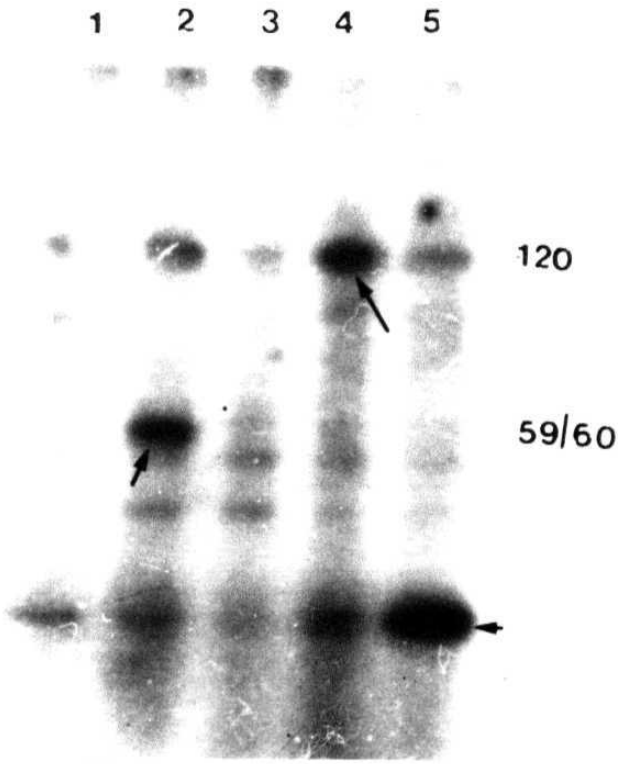

Fig. 19 - Developmental profile of fat body proteins and identification of 120 kDa protein as storage protein binding protein by ligand blotting

19a : SDS-PAGE profile. An equal quantity of fat body proteins (10 ug) was loaded in each lane. Lane 1-ELI, lane 2 –LLI , lane 3 - PP and M- Molecular weight markers.

19b : Shows ligand blot of corresponding gel. Fat body proteins (10 ug) were electrophoresed and blotted on to nitrocellulose membrane and incubated with 100 ug of biotinylated SP. Note the presence of two SP binding proteins with molecular weights of 125(→)and 120 kDa (→).

Fig. 20 - Phosphorylation profile of storage protein binding protein during various stages of development of *Corcyra* - Autoradiogram showing the phosphorylation status of the 120 kDa protein (→). The reaction was carried out for 1 min either in the presence of Ca^{+} (lanes 1, 3 and 5) or in the absence of Ca^{2+} (lanes 2, 4 and 6). Lanes 1 and 2 - ELI, lanes 3 and 4 –LLI, lanes 5 and 6 PP. 10 ug protein was loaded in each lane.

Fig.l9a **Fig.l9b**

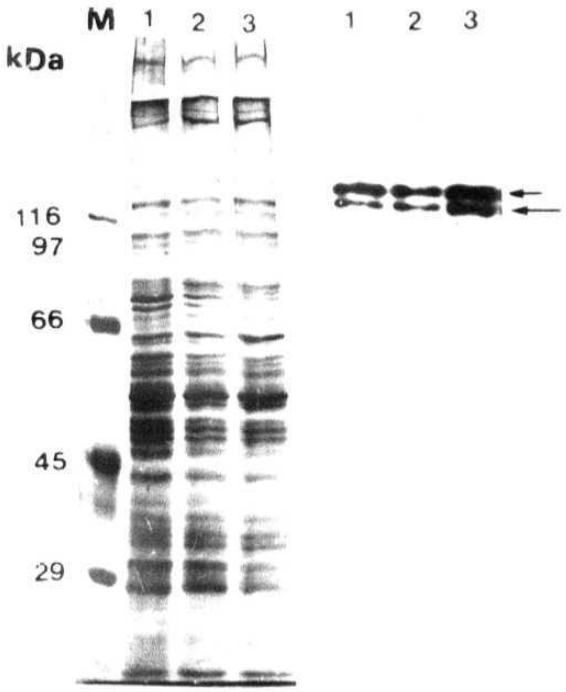

Fig.20

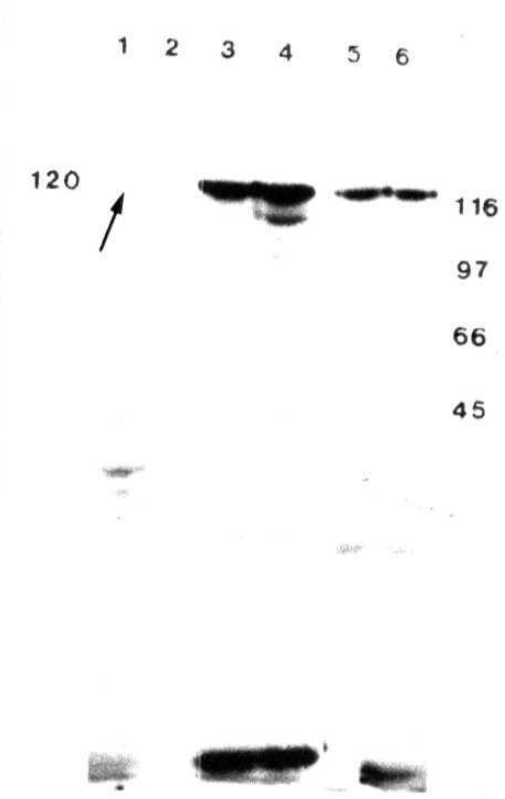

Fig. 21 - Identification of the 59/60 kDa protein as CaM kinase II in the fat body- Equal amount of homogenate protein (50 μg) from different developmental stages, were electrophoretically separated on a 10 % gel, transferred onto a nitrocellulose membrane and immunodetected using an antibody raised against a subunit of rat brain CaM kinase II . Note the presence of 59/60 kDa (⟶) protein band in lanes 1-3. Lane 1 - ELI, lane 2 - LLI , lane 3 - PP and lane 4 - rat brain homogenate (20 μg).

Fig. 22 - Effect of 20E and haemolymph peptide on phosphorylation of 59/60 and 120 kDa protein - Autoradiograph of the gel obtained from back phosphorylation experiment showing the effect of 20E alone and 20E + haemolymph peptide on the phosphorylation of 59/60 and 120 kDa protein. For this experiment the fat body tissue from 24 h ligated LLI of *Corcyra* was organ cultured for 4 h either in the presence of 20E and /or carrier and in the presence or absence of haemolymph peptide. At the end of incubation period the fat body was homogenised and subjected to *in vitro* phosphorylation. 10 μg protein was loaded in each lane. Lane 1- Carrier treated control, lane 2 - 8 x 10^{-8} M 20E, lane 3 - 16 x 10^{-8} M 20E, lane 4 - 8 x 10^{-8} M 20E + haemolymph peptide.

Fig.21

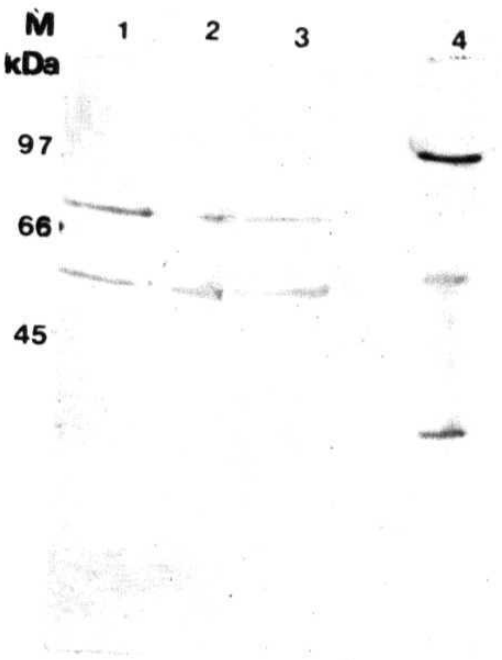

Fig.22

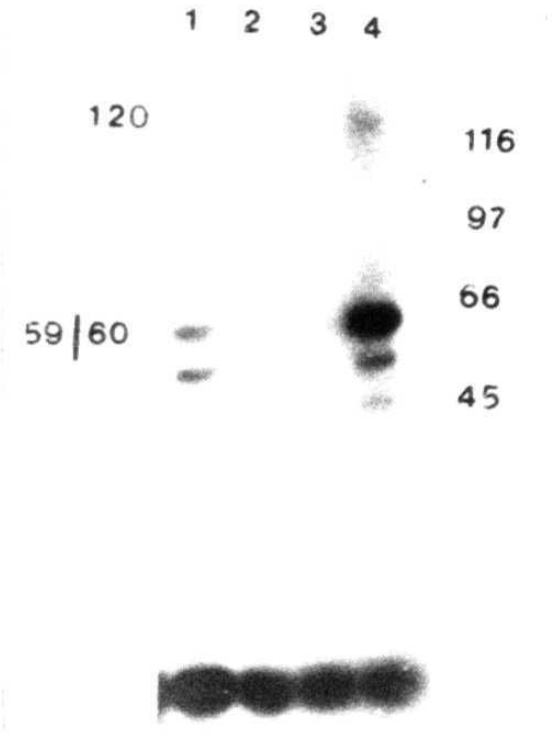

Fig. 23 - Effect of different time incubations on the phosphorylation status of fat body CaM kinase II and SPBP in the presence of 20E and haemolymph peptide - Autoradiograph of the gel obtained from back phosphorylation experiment showing the effect of different time incubations on the phosphorylation of 59/60 kDa protein (panel a- 5 min, b- 30 min, c- 1 h, d –2 h and e - 4 h) and 120 kDa protein (panel f- 5 min, g- 30 min, h- 1 h, i - 2 h and j - 4 h) in the presence of 20E alone and 20E + haemolymph peptide. For this experiment the fat body tissue from 24 h ligated LLI of *Corcyra* was organ cultured for 4 h either in the presence of 20E and/or haemolymph peptide. At the end of incubation period the fat body was homogenised and subjected to *in vitro* phosphorylation. 10 μg protein was loaded in each lane. Lane 1 - carrier treated control, lane 2 - 8 x 10^{-8} M 20E, lane 3 - 8 x 10^{*8} M 20E + haemolymph peptide , lane 4 - haemolymph peptide.

Fig.23

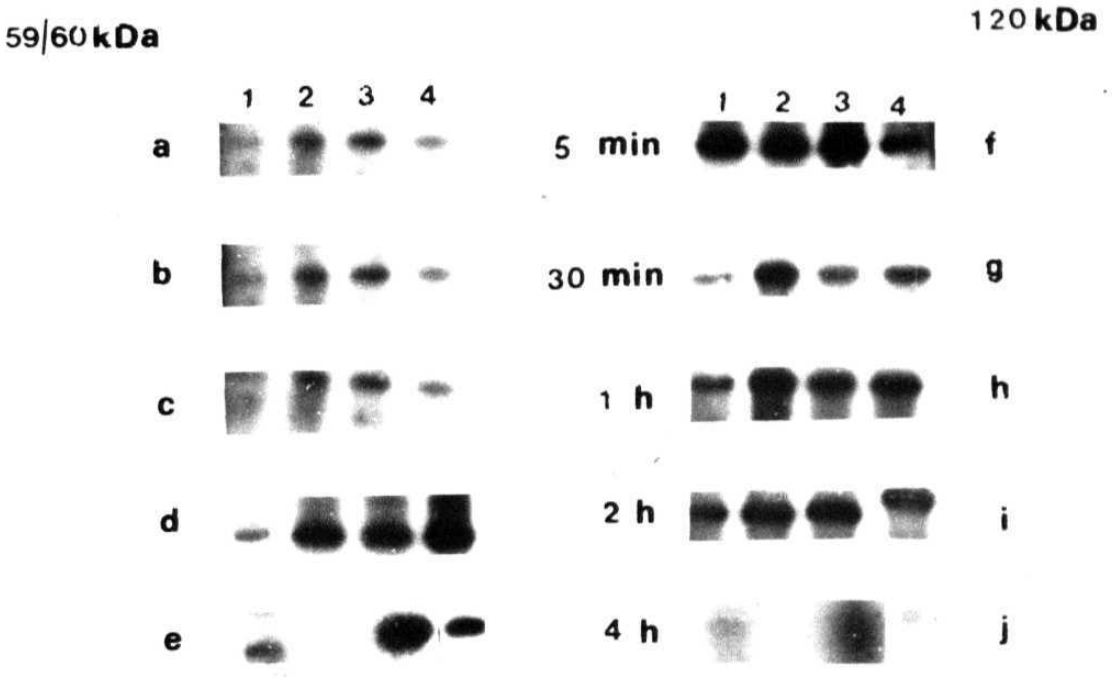

Fig. 24 - Changes in the fat body CaM kinase II activity during larval-prepupal development of *Corcyra* Each value is the mean ± S.D. of four independent determinations. For each determination fat body from 2-3 insects were pooled. The stages used were ELI, LLI and PP. * Value obtained for LLI was significantly higher ($p<0.005$) than ELI as well as PP as determined by one-way analysis of variance with Sigma Stat Software.

Fig. 25 - Graph showing the incorporation of [γ^{32}P] into Syntide-2 as a function of homogenate protein concentration. The values are average of two independent experiments.

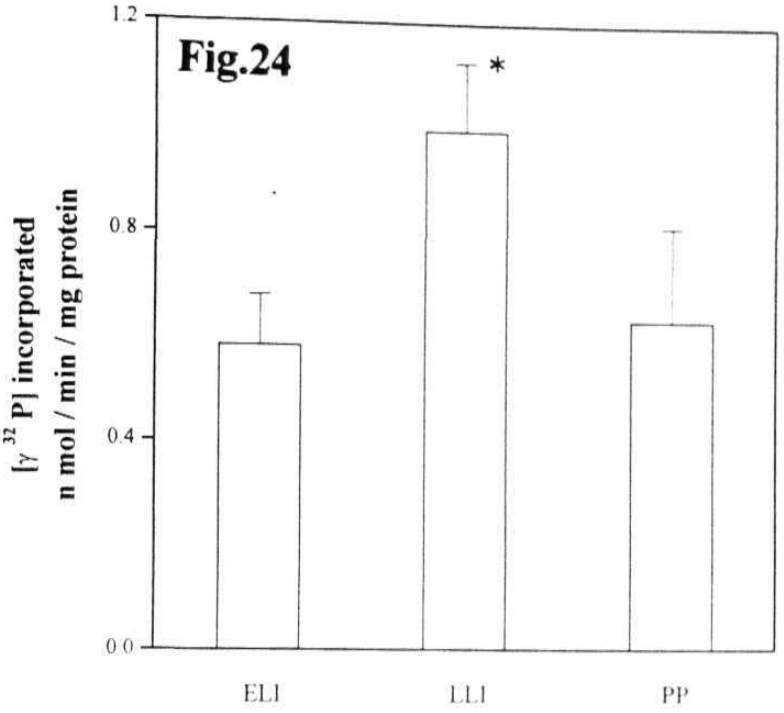
Fig.24
1.2
0.8
0.4
0.0
*
[γ 32 P] incorporated
n mol / min / mg protein
ELI
LLI
PP

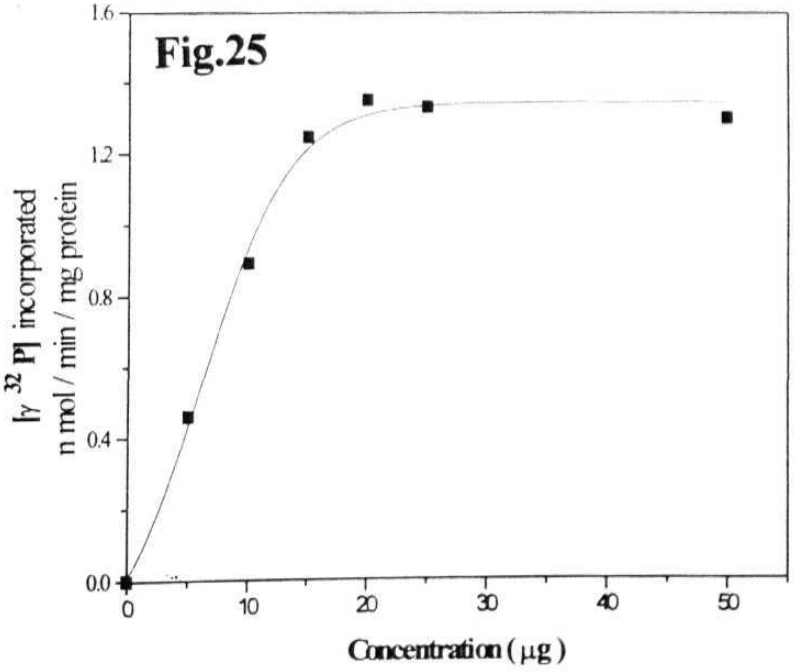
Fig.25
1.6
1.2
0.8
0.4
0.0
[γ 32 P] incorporated
n mol / min / mg protein
0
10
20
30
40
50
Concentration (μg)

Fig. 26 - Lineweaver- Burk plot of the phosphorylation of syntide 2 by the endogenous CaM kinase II of *Corcyra* fat body - Initial rates **were** measured under standard conditions for 1 min, using filter paper assay. Syntide 2 concentration was varied as indicated.

Fig.26

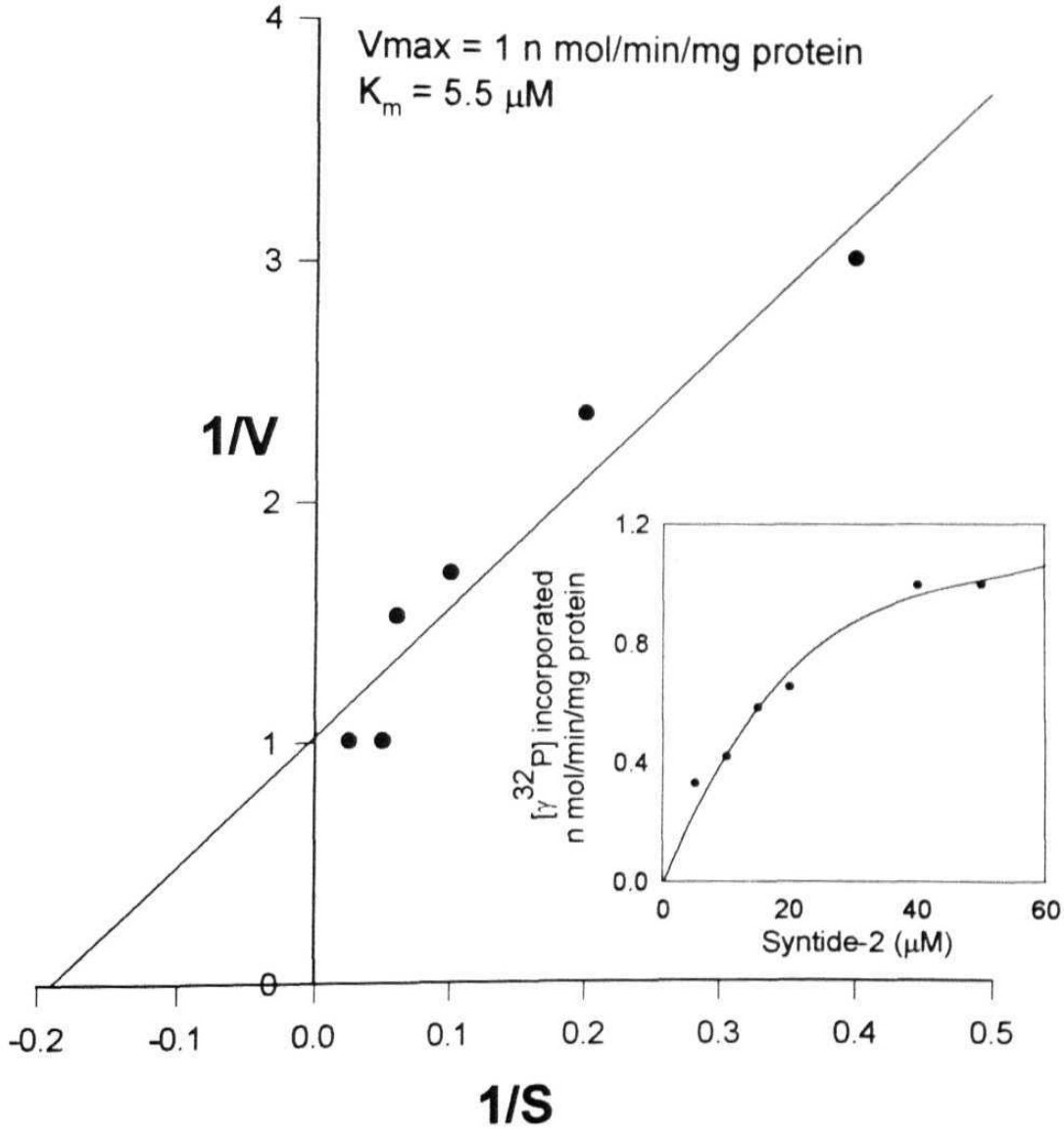
Vmax = 1 n mol/min/mg protein
K_m = 5.5 μM
1/V
1/S
4
3
2
1
0
-0.2
-0.1
0.0
0.1
0.2
0.3
0.4
0.5
[γ 32P] incorporated
n mol/min/mg protein
1.2
0.8
0.4
0.0
0
20
40
60
Syntide-2 (μM)

Fig. 27 - *In vitro* effect of 20E on LLI fat body CaM kinase II activity in the presence of haemolymph peptide - Each value is the mean ± S.D. of four independent determinations. For each determination fat body from 2-3 insects were pooled. To the assay medium 8×10^{-8} M 20E was added either alone , in the presence of 10 ul of haemolymph peptide or haemolymph peptide alone. * This value is significantly lower ($p<0.005$) than the value obtained for 20E treated sample.

Fig. 28 - ***In vitro*** effect of **20E** on **LLI fat** body acid phosphatase **activity in the** presence **of haemolymph peptide** - Each value is the mean ± S.D. of four independent determinations. For each determination fat body from 2-3 insects were pooled. To the assay medium 8×10^{-8} M 20E was added either alone, or in the presence of 10 ul of haemolymph peptide or haemolymph peptide alone. * This value is significantly higher ($p<0.005$) than the value obtained for 20E alone

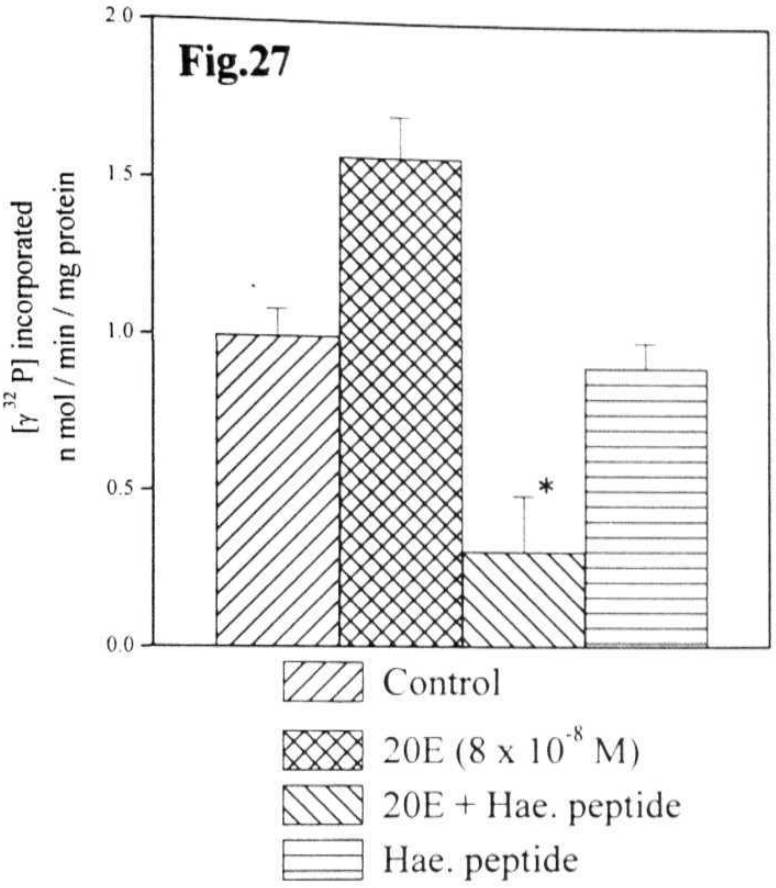
Fig.27
[γ 32 P] incorporated
n mol / min / mg protein
2.0
1.5
1.0
0.5
0.0
*
Control
20E (8 x 10-8 M)
20E + Hae. peptide
Hae. peptide

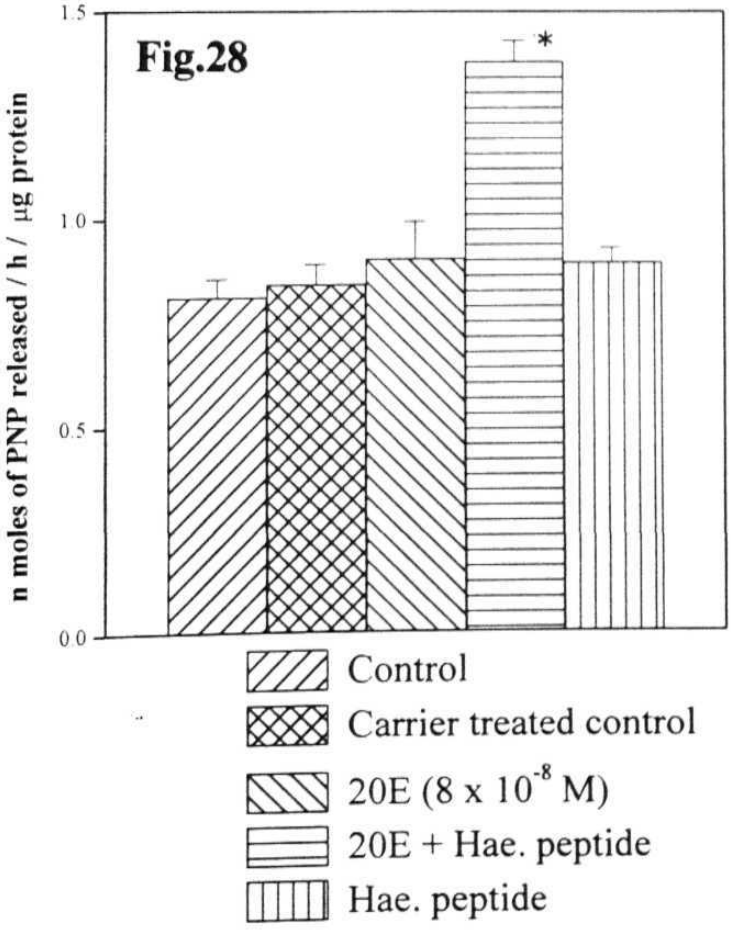
Fig.28
n moles of PNP released / h / µg protein
1.5
1.0
0.5
0.0
*
Control
Carrier treated control
20E (8 x 10-8 M)
20E + Hae. peptide
Hae. peptide

Fig. 29 - Effect of actinomycin D or cycloheximide on acid phosphatase activity of LLI fat body in the presence of 20E and haemolymph peptide - Each value is the mean ± S.D. of four independent determinations. For each determination fat body from two insects were pooled and used. To the assay medium 8 x 10^{-} M 20E was added either alone, or in the presence of 10 μl of haemolymph peptide, haemolymph peptide + actinomycin D (0.25 μg/ml) and haemolymph peptide + cycloheximide (0.5μg/ml). * These values are significantly higher ($p<0.005$) than the value obtained for 20E alone.

Fig. 30 - Effect of protease inhibitors on the haemolymph peptide's potentiating effect on **fat** body acid phosphatase activity in the presence of 20E - Each value is the mean ± S.D. of four independent determinations. For each determination fat body from two insects were pooled and used. To the assay medium 8 x 10^{-8} M 20E was added either alone, or in the presence of 10 ul of haemolymph peptide, PMSF treated haemolymph peptide and leupeptin treated haemolymph peptide. 10 ul haemolymph peptide was treated with 1 mM PMSF and 1 mM leupeptin and were allowed to stand for 30 min at 4°C. At the end of 30 min, the treated haemolymph peptide was added to the assay medium. * These values are significantly lower ($p<0.005$) than the value obtained for 20E and haemolymph peptide.

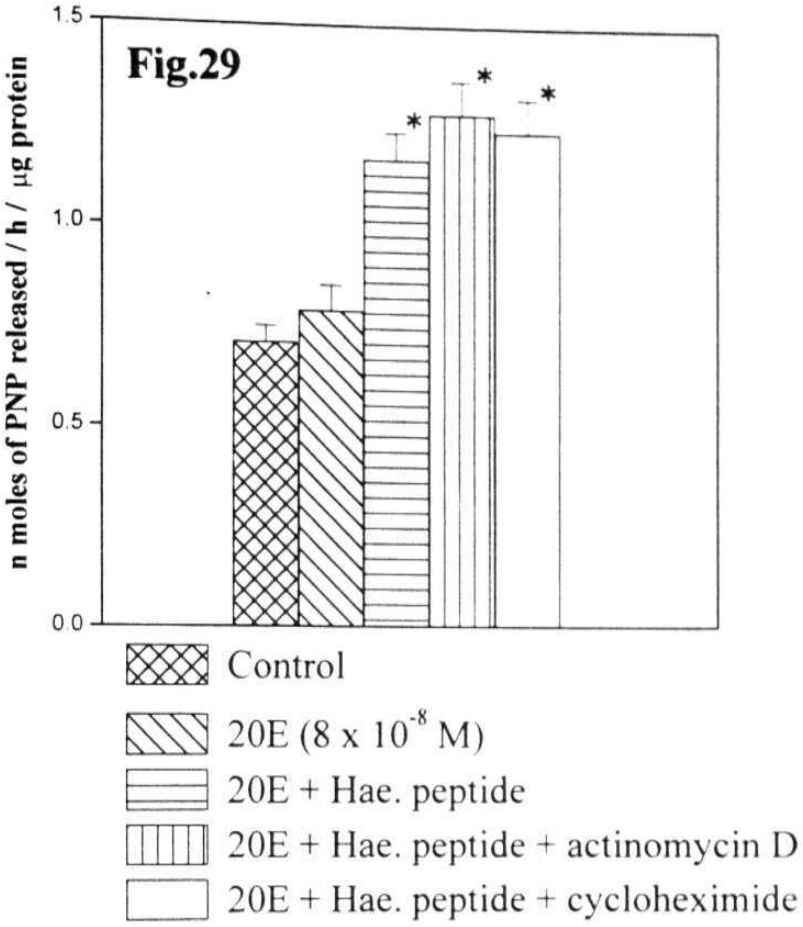
Fig.29
n moles of PNP released / h / µg protein
1.5
1.0
0.5
0.0
*
*
*
Control
20E (8 x 10-8 M)
20E + Hae. peptide
20E + Hae. peptide + actinomycin D
20E + Hae. peptide + cycloheximide

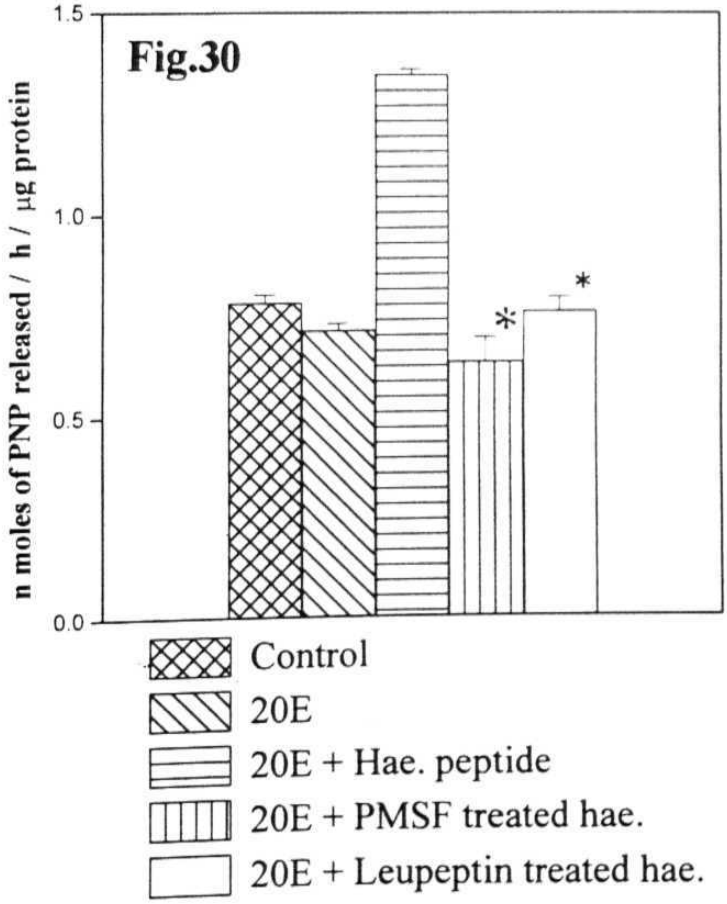
Fig.30
n moles of PNP released / h / µg protein
1.5
1.0
0.5
0.0
*
*
Control
20E
20E + Hae. peptide
20E + PMSF treated hae.
20E + Leupeptin treated hae.

CHAPTER 4

DISCUSSION

One of the major events occurring during postembryonic development of holometabolous insects, including lepidopterans, is the histolysis of larval organs. During the process, the cellular destruction and/or remodelling is mediated among others by lysosomal activity. Acid phosphatase is one of the most commonly used marker enzymes for studying the lysosomal activity. A gradual increase in the acid phosphatase activity can be observed from the penultimate to prepupal stage, which reaches a maximum during the pupal stage (Ray *et al,* 1984; Sridevi *et al.*. 1987). The present study revealed a slow and gradual increase in the fat body acid phosphatase activity during the penultimate, early-last and mid-last instar larval development and the increase in the activity was more pronounced during the late-last instar larval and prepupal stage of development *of Corcyra.* These results are consistent with the earlier reports for other lepidopteran insects (Ray *et al.,* 1984; Sridevi *et al.,* 1987).

Ecdysteroids are known to have wide ranged effects on the function of fat body cells during last larval stage development of holometabolous insects including lepidoptera. It is well known, that they stimulate the formation of autophagic vacuoles (Sass and Kovacs, 1975, 1980; Dean, 1978) and increase the activity of lysosomal enzymes (Rao *et al.,* 1984; Sridevi *et al.,* 1987; Ashok and Dutta-Gupta, 1988; Sass, 1988; Sass *et al,* 1989; Caglayan, 1990; Dutta-Gupta and Sridevi, 1991).

Ligation behind first pair of prolegs, resulted in a time dependent decrease of the acid phosphatase activity, in the posterior fat body of late-last instar larvae of *Corcyra.* This might be due to the absence of factors / hormones produced in the anterior part of the insect which seem to be responsible for regulating the lysosomal enzyme activity in *Corcyra.* Further, Dutta-Gupta and Ashok (1998) have recently reported that thorax ligation in the late-last instar larvae of *Corcyra* siginificantly lowered the ecdysteroid titre in time dependent fashion. In the present investigation, lysosomal activity in the fat body of ligated late-last instar larvae increased significantly by injection of exogenous 20E, suggesting that ecdysteroids have a stimulatory effect on acid phosphatase activity. The results obtained in the present

study are in accordance with those of other authors as well as earlier reports from our laboratory (Verkuil, 1979, 1980; Sridevi *et al*., 1987; Ashok and Dutta-Gupta, 1988).

In vitro studies on *Corcyra* revealed that the addition of 20E alone had no stimulatory effect on acid phosphatase activity of the fat body. This result was consistent with the results of Caglayan (1990) who showed that when *Manduca* fat body was *in vitro* cultured in the presence of different concentrations of 20E, the acid phosphatase activity of the experimental sets were the same as that of the control. Surprisingly, however the activity of acid phosphatase in the *in vitro* cultured fat body was significantly stimulated when 20E treatment was given in the presence of haemolymph. These results are in agreement with the results of earlier reports from our laboratory (Ashok and Dutta-Gupta, 1991) and others. Injections of 20E to greater wax moth, *Galleria mellonella* proved to be effective in causing the shortening of interganglionic connectives (Pipa, 1969). However, when the *in vivo* study was complimented with *in vitro* studies in which isolated connectives from final larval stage were cultured in the presence of 20E, the hormone could not initiate the process. But the hormone could maintain the shortening in culture if the process had already begun *in vivo* (Robertson and Pipa, 1973; Robertson, 1974) suggesting the involvement of additional factor(s) in the initiation of nerve cord shortening. Injection of 20E under *in vivo* conditions to the larval salivary glands of *Drosphila* caused alterations in the ultrastructure of the gland finally leading to its degeneration. However, 20E could not simulate the same effect on the glands under *in vitro* conditions and lack of factors present in the haemolymph was cited as a reason for the incomplete degeneration under *in vitro* conditions (Farkas and Sutakova, 1998).

It was found that 20E could elicit its effect in the presence of the total haemolymph from the unligated late-last instar larvae, as well as the posterior haemolymph of ligated late-last instar larvae and the fat body acid phosphatase activity increased considerably in the experimental sets when compared to the controls. However, the addition of anterior haemolymph did not alter the enzyme activity. This observation suggested that there was some factor(s) in the posterior haemolymph which potentiated the 20E mediated stimulation of acid phosphatase

activity in the larval fat body of *Corcyra* during postembryonic development. Furthermore, the effect of 20E was seen only with the haemolymph of the late-last instar larvae and not with haemolymph from the early-last, mid-last larval and prepupal stages which suggested that the haemolymph factor(s) are active and/or functional in a stage dependent manner.

In order to ascertain, whether the factors which potentiated the effect of 20E are species specific, *Corcyra* fat body was *in vitro* cultured with the haemolymph from other lepidopteran insects, *Bombyx mori* (Family, Bombycidade), *Papilio dcmolius* (Family, Papilionidae) and *Achoea janata* (Family, Noctuidae). The results once again showed an increase in the fat body acid phosphatase activity only with the haemolymph of late-last instar larvae of the above mentioned insects, which suggested that similar haemolymph factor(s) might be present in different insects. However, further studies with insects belonging to different orders and families are necessary to substantiate the present finding.

Present study already demonstrated that only the haemolymph from the posterior part of the late-last instar larvae could potentiate the effect of 20E on fat body acid phosphatase activity and presumably it was synthesised by some tissue/organ present in the posterior part of insect body and released into the haemolymph. Present co-culture studies, provided direct evidence that the hind gut associated lobular fat body could be the possible site of synthesis for haemolymph factor(s). The results of the present study are in agreement with Oberlander *et al.*, (1973) and Dutkowski and Oberlander (1974) who reported that a factor from larval fat body was important for cuticle deposition in lepidopteran imaginal disks incubated *in vitro* with 20E. Furthermore, the fat body has already been well documented as a source of humoral factors (Meola and Gray, 1984; Gray *et al*, 1984).

Several lines of evidence suggest that the regulatory molecules present in haemolymph are generally peptides / proteins (Stone and Mordue, 1980; Candy, 1981; Keeley *et al*, 1985). Present study revealed that the haemolymph factor(s) of *Corcyra* was heat labile and was also sensitive to acid and alkali treatment. The factor also lost its activity after treatment with proteolytic enzyme V_8 protease.

Alcohol precipitated samples of haemolymph failed to promote the stimulatory effect of 20E on enzyme activity. This suggested that the haemolymph factor(s) might be protein and/or peptide.

With the objective of further characterising the haemolymph factor, Amicon filtration studies were carried out. Results of these studies revealed that < 30 kDa fraction of the haemolymph potentiated 20E mediated fat body acid phosphatase activity in *Corcyra*. Further purification results showed that the haemolymph peptide, which could stimulate fat body acid phosphatase activity in the presence of 20E was a low molecular weight peptide of 19 kDa.

Characteristic feature of regulatory molecules are (i) to be present in very low amounts and (ii) effective at very low concentration (Gilbert, 1994). Present study revealed that very low concentration of haemolymph peptide in the presence of physiological concentration of 20E (8 x 10" M) was sufficient to stimulate the fat body acid phosphatase activity. However, the haemolymph peptide required a time frame of minimum of 4 h to potentiate the optimal effect in *in vitro* cultured fat body in the presence of 20E. Further, the factor lost its activity and/or potentiation effect gradually from 6-12 h. In addition, the haemolymph collected from anterior part of 24 h ligated late last instar larvae could not stimulate fat body acid phosphatase activity in fat body cultures and this once again substantiates the fact that the factor has a short half life.

To know more about the haemolymph peptide similar studies were carried out in, another lepidopteran insect *Bombyx mori*. The major difference between the *Corcyra* and *Bombyx* is that the former is a dry feeding stored grain pest while the latter is a wet feeding insect. Studies with *Bombyx* fat body acid phosphatase activity revealed a slow and gradual increase in the activity of the enzyme during larval-pupal development. The activity of the enzyme was found to be high during the late-last instar larval stage and reached a maximum during the late-pupal stage. It is interesting to note that these developmental stages correspond to the physiological activities like cellular destruction and/or remodelling. Further, parallel changes have been reported in ecdysteroid titre in the haemolymph of *Bombyx mori* during postembryonic development (Calvez *et al*, 1976).

Results obtained with *in vitro* cultured *Bombyx* LLI fat body once again ascertained the fact that 20E alone could not potentiate fat body acid phosphatase activity. However, stimulation of enzyme activity was observed, when haemolymph from LLI larva was added along with 20E to *in vitro* cultured fat body.

Amicon filtration studies with *Bombyx* haemolymph once again showed that < 30 kDa fraction of haemolymph was responsible for the stimulation of *in vitro* cultured acid phosphatase activity in the presence of 5×10^{-6} M 20E (physiological concentration reported for *Bombyx*, Calvez *et al,* 1976). These results suggested that similar in-built regulatory mechanisms operate in different lepidopteran insects. However, further studies using wide variety of insects have to be carried out to establish the fact.

Another objective of the present study was to identify marker proteins other than fat body acid phosphatase that require the additional presence of haemolymph factor when their activity is mediated by 20E. As explained earlier, CaM kinase 11 and SPBP were the obvious choices. Thus in the present study, CaM kinase II and SPBP have been identified from *Corcyra* larval fat body.

The pleiotropic effects of hormones are due to several distinct processes. In eukaryotes, the modulation of transcription is not the only level of gene regulation and the multiple possibilities of post-transcriptional and post-translational events should not be forgotten (Perronnet *et al,* 1986). Protein phosphorylation is now recognised as the major regulatory mechanism by which the activity of various tissue/cell types are controlled by external physiological stimuli (Browning *et al,* 1985). Recently, Rauch *et al,* (1998) demonstrated that ecdysteroid receptor (EcR) and ultra spiracle (USP) protein from *Chironomus tentans* are phosphoproteins and are differentially regulated by ecdysteroids. Further, 20E treatment does not alter the extent and status of phosphorylation of EcR, although the concentration of EcR increases. In contrast, the phosphorylation of USP is significantly enhanced but the concentration remains more or less the same

In vitro phosphorylation of endogenous proteins of *Corcyra* fat body revealed the phosphorylation of several proteins in the molecular weight range of 30-120 kDa. Inclusion of 20E significantly stimulated the phosphorylation of 59/60 kDa and 120 kDa protein. This is in agreement with the results reported for *Mamestra brassica* (Sass, 1988) where 20E induced the phosphorylation of several fat body proteins and caused a marked elevation of cAMP content.

In the present study, the 120 kDa protein was identified as SPBP using a very sensitive ligand blotting method. This result is in agreement with the results of earlier study from our laboratory (KiranKumar *et al,* 1997). Ueno and co-workers reported the presence of an SP inactive receptor in the larval fat body cells of *Sarcophaga peregrina*, which was activated in the presence of 20E (Ueno *et al.,* 1983; Ueno and Natori, 1984). Burmester and Scheller (1992) had initially reported the presence of three distinct polypeptides 130, 96 and 65 kDa in the fat body cells *of Calliphora vicina* , while in more recent publications they suggested that all the binding proteins were derived from a single translational product having a molecular weight of 130 kDa (Burmester and Scheller, 1995, 1997). They have also suggested that 20E plays an important role in the transformation of these binding proteins. However, in *Heliothis zea*, a single receptor protein with an apparent molecular weight of 80 kDa was isolated (Wang and Haunerland, 1993, 1994). KiranKumar *et al,* (1997) reported the presence of two SPBPs (SPBP 125 and SPBP 120) in the fat body membranes *of Corcyra* during the last larval as well as prepupal stages of development. They also reported that the sequestration of SPs in *Corcyra* began only during the prepupal stage where the concentration of SPBP 120 was much higher than that of SPBP 125 and that might be due to modification and/or activation of SPBP which was mediated by 20E (KiranKumar *et al,* 1997, 1998). Further studies from our laboratory revealed that the activation process involved a phosphorylation reaction and 20E induced the same probably by activating a kinase involved in the phosphorylation (KiranKumar, 1998). Results of the present study also confirm the presence of activated SPBP (SPBP 120) and also show that the phosphorylation status of the SPBP is developmentally regulated.

In the present study, the 59/60 kDa protein of the fat body has been identified as autophosphorylated CaM kinase II as was evident from its cross

reactivity with anti CaM kinase II a subunit antibody. The kinase has a molecular weight of 59/60 kDa and exhibited Ca^{2+} and calmodulin dependent autophosphorylation. In addition to this, the *Corcyra* fat body also showed the presence of a 70 kDa protein which cross reacted with the antibody. However, this 70 kDa protein did not get phosphorylated in our phosphorylaion studies. Studies on *Drosophila* revealed the presence of three major species of CaM kinase II with molecular masses of 54/55, 58 and 60 kDa in the head tissue (Cho *et al,* 1991; Ohsako *et al,* 1993). In *Bombyx* central nervous system only two species of CaM kinase II with molecular masses of 59/60 kDa were reported (Shanavas *et al,* 1998). Griffith and Greenspan (1993) reported the presence of CaM kinase II mRNA in both, neural and non-neural tissue like fat body. Autophosphorylation of this kinase observed in non-neural tissue like fat body and muscles of *Bombyx* supports the view that these kinases might be involved in a variety of Ca^{2+} mediated processes, as expected for a kinase with a broad range of functions (Hanson and Schulman, 1992).

Preliminary studies on catalytic properties *of Corcyra* fat body CaM kinase II revealed that the K_m value for syntide-2 was much lower than the values reported for neural tissues of *Drosophila* and *Bombyx* (Oshako *et al.* 1993; Shanavas *et al,* 1998). Developmental changes in CaM kinase II activity in CNS have been well documented in *Bombyx* (Shanavas *et al,* 1998). An interesting, observation in the present study was the presence of a high peak of enzyme activity at late-last larval stage which correlated with the reported increase in haemolymph ecdysteroids of *Corcyra* (Dutta-Gupta and Ashok, 1998). This suggests a developmental regulation of CaM kinase II in the fat body of *Corcyra* during larval-pupal transformation.

Results of back phosphorylation experiments clearly indicated that 20E stimulated the phosphorylation of CaM kinase II as well as SPBP in isolated intact fat body culture of *Corcyra.* However, addition of haemolymph peptide along with 20E to fat body cultures, significantly inhibited the phosphorylation of both these proteins.

The physiological significance of the inhibition of 20E mediated phosphorylation of these proteins can be explained by the example of SPBP. The

pattern of synthesis of SPs in a variety of holometabolous insects including lepidopterans exhibit a precise developmental sequence (Levenbook, 1985; Telfer and Kunkel, 1991). In lepidopternas, SPs are synthesised both in the early larval instars as well as in the ultimate (final) larval instar (Ray *et al.* 1987a; Webb and Riddiford, 1988a; Izumi *et al.* 1988; Bean and Silhacek, 1989; Karpells *et al,* 1990; Kunkel *et al,* 1990). The rate of SP synthesis was shown to be much higher in the final instar and could be correlated with the mRNA levels of fat body (Kumaran *et al,* 1987; Ray *et al.* 1987 a,b). However, during larval pupal transformation the fat body diminishes its protein synthetic activity and acts mainly as an organ of storage for protein and other reserve materials which are used during the adult development (Keeley, 1985; Kanost *et al.* 1990; Telfer and Kunkel, 1991). It is evident from the present study that SPBP, which facilitates the uptake of storage proteins from the haemolymph into the fat body is present at the stage when the fat body is engaged in active protein synthesis i.e., late-last instar larval stage. Earlier studies from our laboratory have already shown that 20E brings about the phosphorylation of SPBP, a process which is known to activate the binding protein and initiate its physiological function of uptake (KiranKumar *et al,* 1997). Hormone titre studies reported for *Corcyra* revealed the presence of high 20E during this stage, i.e., late-last instar (Dutta-Gupta and Ashok, 1998). In this backdrop, it is expected that due to increased 20E level, the SPBP is likely to get phosphorylated and begin the precocious uptake of SPs back into the fat body. This would lead to physiological imbalance as the larva is expected only to synthesize SP and release them into the haemolymph at this stage of development. Thus the presence of haemolymph peptide during the LLI stage and its inhibition of 20E mediated phosphorylation of SPBP is physiologically as well as developmentally justified.

Present study demonstrated that CaM kinase II activity in *Corcyra* fat body is developmentally regulated and it was highest at late-last instar larval stage. This study further elucidated that the enzyme activity was stimulated by 20E as was evident from the increased autophosphorylation of this kinase in the presence of the hormone. Further, from the present study it is evident that at this stage of development active/functional haemolymph peptide is also present, which would inhibit the activity and autophosphorylation of CaM kinase II in the insect system.

One obviously expects a physiological significance to this phenomenon, which has to be confirmed through several studies.

From the results of the back phosphorylation studies it was evident that the concerted action of 20E and haemolymph peptide in bringing about the inhibition of phosphorylation of CaM kinase II and SPBP required a minimum period of 4 h. This latency in the expression of action of 20E in mediating signal transduction process, which are generally very fast was a question to be addressed. The objective of the present study was to suggest a possible mechanism of action of the haemolymph peptide.

To address the objective, the activity of CaM kinase II and acid phosphatase was assayed at the homogenate level, a point at which practically the cell's architecture is disturbed. The geneic level action of 20E, a steroid hormone is impossible at this given situation. Results of the present study with LLI fat body suggested that at the homogenate level, *in vitro* addition of 20E brought about the stimulation of CaM kinase II activity rapidly (within minutes of addition of physiological concentration of 20E) which was statistically significant when compared with the control. However, it is already demonstrated that addition of 20E alone to fat body homogenates failed to stimulate acid phosphatase activity.

Addition of very low concentrations of haemolymph peptide (40 ng / 10 µl) along with physiological concentration of 20E to fat body homogenates, caused a significant decline in CaM kinase II activity (120% inhibition with respect to 20E alone treated sample) and stimulation of acid phosphatase activity (64% stimulation with respect to 20E alone treated sample). These results clearly demonstrate that haemolymph peptide has a kinase inhibition property and is responsible for the inhibition of autophosphorylation of CaM kinase II as well as phosphorylation of SPBP in *in vitro* culture studies.

Recent studies from our laboratory suggested that SPBP in *Corcyra* fat body membrane is a phosphotyrosin containing protein and the phosphorylation of this protein is most likely mediated by a tyrosine kinase (KiranKuamr, 1998). Present

investigation demonstrated that the phosphorylation of 120 kDa protein was independent of Ca^{2+}, calmodulin and phosphatidylserine + diacylglycerol, but was stimulated by 20E. It can be inferred from these studies that 20E, by stimulating a tyrosine kinase brings about the increased phosphorylation of SPBP 120 which acts as a substrate protein. Further, the present study also suggests that the haemolymph peptide inhibits the phosphorylation of SPBP 120 by inhibiting the kinase involved, similar to the inhibition of CaM kinase II. Thus, the haemolymph peptide acts as an in-built regulatory molecule regulating several signal transduction pathways by selectively inhibiting the action of different kinases in the presence of 20E.

It is interesting to note that similar low molecular weight proteins with kinase inhibitory activity have been well documented in mammalian system (Fraser and Walsh, 1991; Eyster *et al.,* 1993). Stathmin, also known as P 19, Prosolin or OP 18, is a 19 kDa ubiquitous, cytoplasmic phosphoprotein widely conserved among vertebrates (Sobel, 1991). Its expression and phosphorylation are regulated throughout development and in response to extracellular signals regulate cell proliferation, differentiation and functions (Camoletto *et al.,* 1997; Ozon *et al.,* 1997; Lawler *et al.,* 1998). The overall pattern of its molecular forms reflects the activation of corresponding second messenger pathways. This phosphoprotein is therefore a good candidate as a general relay in signal transduction, possibly integrating diverse signals of the cell's environment. Although such a regulatory molecule has not been reported yet in insect system, the possible presence of a similar factor however cannot be ruled out. From the results of the present study on haemolymph peptide and its action, it could be related to one such relay regulatory molecule. Further studies on the identification, structural and functional characterisation should provide an insight into whether it is actually a related molecule.

Steroid effects that occur within a few minutes after steroid application is likely due to a nongenomic rather than a genomic effect (Tomaschko, 1999). In the field of vertebrate steroids, nongenomic effects have been widely recognised (Wehling, 1994, 1995, 1997). Many of these nongenomic effects of vertebrate steroids appear to involve calcium, protein kinase and tyrosine kinase (Wehling, 1997). To support the non-genomic nature of steroid effects, inhibitors of DNA

transcription or protein synthesis (actinomycin D or cycloheximide) should not block the response. Inclusion of actinomycin D (transcriptional inhibitor) and cycloheximide (translational inhibitor) along with haemolymph peptide and 20E in the present investigation did not inhibit the haemolymph peptide's potentiating activity on 20E mediated acid phosphatase activity in the fat body homogenates. This suggests the possibility of a non-genomic action of 20E, a steroid hormone, which is generally known to exert a genomic level action on its target proteins. It is also suggested that whenever 20E mediates the function of functional proteins (like enzymes), it probably opts for a non-genomic pathway of action and such an action of 20E requires the additional presence of haemolymph peptide. The presence of the regulatory molecule in the haemolymph, a tissue that is practically exposed to all other tissue suggests a possibility of similar action of 20E on other tissues too (Chaing *et al.,* 1997). Studies that would orient towards answering such questions could help us in understanding this intricate regulatory mechanism.

Non-genomic effects of 20E on selective protein phosphorylation have been demonstrated in *Sarcophaga* (Ueno *et al,* 1983; Ueno and Natori, 1984). The activation of cryptic larval receptor protein involved its phosphorylation which was induced by 20E. Further, the activation of the receptor was shown to be independent of accompanying protein synthesis. Itoh *et al.,* (1985) demonstrated 20E induced phosphorylation of a larval fat body protein (30 kDa) under *in vivo* condition, which was not accompanied with synthesis of new proteins. Burmester and Scheller (1997) reported the developmentally controlled cleavage of the *Calliphora* arylphorin receptor, an activation process to be induced by 20E *in vivo* and *in vitro* in the absence of protein synthesis.

The kinase inhibitory action of the haemolymph peptide could not possibly explain the activation of acid phosphatase activity observed in the present study. This suggested that the haemolymph peptide might exhibit other properties too. A marked increase in the acid phosphatase activity is observed during the larval-pupal tranformation, a time when the synthetic apparatus of the cells is already practically decomposed due to autophagocytosis. In this backdrop, the *de novo* synthesis of a large quantity of enzyme protein seems to be unlikely. Thus, the rapid increase of acid phosphatase activity may be due to the activation of a pool of inactive enzyme

rather than *de novo* synthesis. Csikos and Sass (1997) have reported that acid phosphatase is synthesised and secreted by fat body cells into the haemolymph during the larval stage, where it is stored in an inactive form and the increase in the 20E titre at the end of the last larval stage reverses this process and the enzyme is sequestered into the fat body cells, where it gets activated. Results of the present study with protease inhibitors reveal that the haemolymph peptide has a protease like activity. This suggests that probably acid phosphatase is present in an inactive form during the last larval stage and the haemolymph factor in the presence of 20E converts it into a an active form which shows an increased activity. Hence, the present study clearly suggests the existence of an in-built regulatory mechanism which operates during the postembryonic development for the regulation of 20E mediated actions.

SUMMARY AND CONCLUSIONS

1. There is a precise developmental regulation of fat body acid phosphatase activity during larval-pupal development of *Corcyra.* Under *in vivo* condition, exogenous supply of 20E stimulates acid phosphatase activity in fat body.

2. However, under *in vitro* conditions, only the addition of haemolymph factor(s) with 20E and not 20E alone elicited a maximum increase in the activity of acid phosphatase.

3. The effect of 20E was only seen with the haemolymph of the late-last instar larval stage when compared with the haemolymph from other stages. *In vitro* studies using haemolymph from other lepidopteran insects, ascertain the fact that these haemolymph factors are stage specific and not species specific.

4. Co-culture studies reveal that these factor(s) are synthesised by the hind-gut associated globular fat body and are released into the haemolymph.

5. Chemical alteration of haemolymph factor(s) inhibited its stimulatory effect on the acid phosphatase activity suggesting that these factor(s) might be peptides.

6. The haemolymph fraction below 30 kDa caused a significant increase in the activity of the enzyme when compared with other fractions. The peptide was found to have a molecular weight of 19 kDa.

7. Haemolymph of late-last instar larvae of *Bombyx mori* could also stimulate the 20E mediated acid phosphatase activity in *Bombyx* fat body cultures suggesting that similar regulatory mechanisms operate in different insects of the order lepidoptera.

8. CaM kinase II activity in fat body of *Corcyra* is developmentally regulated.

9. 20E stimulated the phosphorylation of 120 kDa storage protein binding protein (SPBP) and autophosphorylation of CaM kinase 11 in fat body of *Corcyra*.

10. Haemolymph peptide in the presence of 20E inhibited the phosphorylation of SPBP and CaM kinase II suggesting that the peptide might be a kinase inhibitor.

11. Haemolymph peptide's regulatory role on 20E requires a minimum of 4 hours time period when the tissues are *in vitro* cultured. With shorter incubation times haemolymph peptide failed to regulate the 20E effect on selected marker proteins.

12. At the homogenate level, haemolymph peptide in the presence of 20E brings about the inhibition of CaM kinase 11 activity and stimulation of acid phosphatase activity, suggesting that it might be a non-genomic action of 20E.

13. Addition of transcriptional inhibitor (actinomycin D) and translational inhibitor (cycloheximide) to fat body homogenate failed to inhibit the haemolymph peptide potentiated effect of 20E on acid phosphatase activity suggesting that the increase in enzyme activity need not necessarily be attributed to increased transcript synthesis and / or increased protein synthesis.

14. Treatment of the haemolymph peptide with protease inhibitor inhibited its 20E potentiating activity suggesting that the peptide might be a protease.

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