STUDIES ON CYTOSKELETAL PROTEINS AND PROTEIN PHOSPHORYLATION IN THE CENTRAL NERVOUS SYSTEM OF SILK WORM, *BOMBYXMORI* DURING POSTEMBRYONIC DEVELOPMENT

Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

BY

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I dedicate this work to the memory of my father, A. Jahabar Kutty (1944-94)

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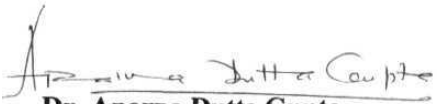
DECLARATION

I hereby declare that the work embodied in this thesis entitled **"Studies on cytoskeletal proteins and protein phosphorylation in the central nervous system of silk worm,** *Bombyx* ***mori*** **during postembryonic development"** has been carried out by me under the supervision of Dr. Aparna Dutta Gupta and Prof. Ch. R. K. Murthy and that this has not been submitted for any degree or diploma of any other university.

Shanavas A.
Candidate



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CERTIFICATE

This is to certify that Mr. Shanavas A. has carried out the research work embodied in the present thesis under our supervision and guidance for a full period prescribed under the Ph.D. ordinance of this university. We recommend his thesis entitled **"Studies on cytoskeletal proteins and protein phosphorylation in the central nervous system of silk worm,** *Bombyx* ***mori*** **during postembryonic development"** for submission for the degree of **Doctor of Philosophy** of this university.

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Shanavas

ABBREVIATIONS

ATP	:	Adenosine 5'-triphosphate
BCIP	:	4-chloro-3-indolyl phosphate
BSA	:	Bovine serum albumin
CaM	:	Calmodulin
CaM kinase	:	Calcium/calmodulin-dependent protein kinase
cAMP	:	Adenosine 3':5'-cyclic monophosphate
cGMP	:	Guanosine 3':5'-cyclic monophosphate
CK	:	Casein kinase
CNS	:	Central nervous system
DMSO	:	Dimethyl sulfoxide
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]
HRPO	:	Horse radish peroxidase
IEF	:	Isoelectric focussing
JH	:	Juvenile hormone
kDa	:	Kilo Dalton
KN-62	:	(1-[*N*, *O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine)
LTP	:	Long-term potentiation
NBT	:	Nitroblue tetrazolium
nm	:	Nanometer
NP-40	:	Octylphenoxy polyethoxyethanol
PKC	:	Calcium/phospholipid-dependent protein kinase
POPOP	:	1,4-bis [5-(phenyl-2-oxazolyl)] benzene
PP1	:	Protein phosphatase I
PP2A	:	Protein phosphatase 2A
PPO	:	2,5-diphenyloxazole
PTK	:	Protein tyrosine kinase
SDS	:	Sodium dodecyl sulphate
SDS-PAGE	:	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
TBS	:	Tris buffered saline
TCA	:	Trichloroacetic acid
TEMED	:	N, N, N^1, N^1, tetramethylethylene diamine
Tris	:	Hydroxymethyl aminomethane
v/v	:	Volume/volume
w/v	:	Weight/volume
20E	:	20-Hydroxyecdysone

PART -1

STUDIES ON PROTEIN SYNTHESIS AND THEIR HORMONAL REGULATION IN THE CENTRAL NERVOUS SYSTEM OF *BOMBYXMORI* DURING POSTEMBRYONIC DEVELOPMENT

CHAPTER 1

INTRODUCTION

Insects showing complete metamorphosis (holometabolous) undergo some of the most complex transformations seen in the animal kingdom. After a period of extensive growth during the larval stage, the animal undergoes a dramatic reorganization at pupal stage during which many of larval tissues are partially and /or completely destroyed. Adult specific structures are formed either from reorganization of larval cells / tissues or groups of precursor cells of embryonic origin retained through the larval life. These profound changes in the morphology and behaviour during metamorphosis are accompanied by equally dramatic changes in the central nervous system (CNS) of the insect. These include neurogenesis, programmed cell death and reorganization of the larval neurons to perform new functions in the adult insect (Truman and Levine, 1983; Truman, 1996). Unlike in the embryo, these processes are carried out in an animal that is capable of showing specific behavioural response to the surroundings during the entire transition. Consequently provisions must be made for restructuring parts of the CNS, while other regions remain functional. Studies on the postembryonic development of CNS of insects attracted considerable attention. These studies revealed extensive morphological, biochemical, electrophysiological and molecular changes during metamorphosis.

Earliest work on the postembryonic developmental changes in the CNS of holometabolous insects was that of Lyonet (1762) who dissected and described the differences in the organization of nervous system of larval and adult forms of the insect, *Cossus*. This work laid foundation for knowledge on the extent of reorganization and remodelling of the nervous system during larval-pupal-adult transformation of holometabolous insects. Later, Weismann (1864) described histological changes during the development of nervous system in the house fly, *Musca domestica*, a holometabolous insect. Work done since then has established that development of nervous system in holometabolous insects occurs in two distinct phases, viz. embryonic and postembryonic development.

During embryogenesis, a set of neuroblasts delaminate from the ectodermal layer and serve as pre-runners of future nervous system. These neuroblasts become arranged in a repeating pattern to form sequential ganglia (Poulson, 1956). A key

observation made by Bate (1976a, b) was that each neuroblast has a fixed position and neuroblasts are arranged in a stereotyped array Each neuroblast produces a lineage of neurons by a repeated series of unequal divisions (Schrader, 1938). The smaller product of each division, ganglion mother cell (GMC), subsequently undergoes an equal division, thereby producing two daughter cells which then differentiate into neurons. For at least the early neurons produced in each lineage, the cellular phenotype of a given cell is determined by its parent neuroblast and the order of birth by its GMC (Taghert and Goodman, 1984). The mechanism by which the two daughter cells of a given GMC may assume different fates apparently involves interactions between the two cells after their birth (Kuwada and Goodman, 1985). In addition to GMC's, each segment also has a smaller number of median precursor cells, which undergo a single symmetrical division to produce two daughter neurons (Bate and Grunewald, 1981, Thomas et al., 1984). They along with other segmental neurons, extend axons and act as central pioneer neurons to establish a characteristic pattern of longitudinal tracts and transverse commisures to form the segmental architecture of neuropil. These initial pathways are used for guiding axons of neurons that are formed later (Bastiani et al., 1985). This phase of the embryonic development of nervous system is common for both hemi- and holometabolous insects (Truman, 1996).

Subsequent postembryonic development of CNS of hemi- and holometabolous insects differ considerably. In the former group of insects, the adult nervous system is established by the end of embryogenesis (Bate, 1976a; Doe and Goodman, 1985). Many of the central neurons of the nymphs can be readily recognized because their pattern of central branching is very similar to that seen in the adult (Shankland and Goodman, 1982; Raper et al., 1983). Likewise, neuronal numbers remain quite stable after hatching (Gymer and Edwards, 1967; Sbrenna, 1971). Two notable exceptions occur in the brain : (i) the optic lobes continue to add visual interneurons to accommodate the new ommatidia that are added to the compound eye at each larval moult (Anderson, 1978) (ii) also the mushroom bodies, areas of the brain associated with learning and memory (Davis, 1993), continue to add neurons throughout larval life and even during the adult stage (Cayre et al., 1994). Nevertheless, the essence of

the adult nervous system in terms of the number of neurons and their main pattern of connections, is clearly evident in hemimetabolous nymph by the end of embryogenesis.

In contrast to that of hemimetabola, the CNS of holometabolous insects undergoes profound changes during the postembryonic life. Neurogenesis, programmed cell death and reorganization and remodelling of existing neurons transforms the larval CNS into that of the adult. Neurogenesis plays an prominent role in forming parts of the adult CNS especially the brain (Nordlander and Edwards, 1969a, b; White and Kankel, 1978; Ito and Hotta, 1992) and thoracic ganglia (Booker and Truman, 1987a; Truman and Bate, 1988). The segmental ganglia of larvae possess a stereotyped array of neuroblasts, which get mitotically activated during early larval life, and undergo a pattern of division typical of insect neuroblasts (Edwards, 1970). Each neuroblast generates a lineage of up to 100 cells. However, unlike the embryonic cells, development of the progeny is arrested soon after the young one is born. With the onset of metamorphosis, these arrested cells undergo development and differentiation and mature into functional adult neurons. During postembryonic development of *Manduca sexta* between 2000-3000 new neurons are added to each thoracic ganglion and about 50-100 cells to each unfused abdominal ganglion (Truman, 1988). Accordingly, in *Manduca* about 60-70% of the adult ventral CNS is produced postembryonically (Booker and Truman, 1987a) while in *Drosophila*, the proportion is over 90 % (Truman and Bate, 1988).

Metamorphosis is always accompanied by the programmed cell death of a subset of larval neurons throughout the CNS. This process is more pronounced in the abdominal ganglia, which is in accord with the shift in locomotor function from the abdomen of the larva, to the thorax of the adult. As a result in *Manduca*, an abdominal ganglion in the adult contains only about 350 neurons as compared to 700-800 in the larva (Taylor and Truman, 1974). The larval neurons die in two waves. The first wave of death occurs about 2 days after pupal ecdysis (Weeks and Truman, 1985; Weeks and Ernst-Utzschneider, 1989). The second wave of neuronal death takes place after the emergence of the adult. Most of the neurons that die at this time

are either involved in maintaining the behavioural pattern during the pupal-adult transition or in the performance of ecdysis and associated behaviours. Because adult is a terminal stage that does not molt again, neurons and muscles dedicated to ecdysial behaviour undergo extensive programmed degeneration after adult emergence (Truman, 1983; Kimura and Truman, 1990).

An important change which occurs during the transformation of larval nervous system to that of adult is the reduction in the length of the nerve cord and in the number of ganglia, especially in the thoracic and abdominal regions. Based on the observations made on larval and adult nervous systems of Lepidoptera, Brandt (1879) identified four different patterns of reduction in the number of ganglia. In two of these groups, there are 2 cerebral ganglia, 2 thoracic and 4 abdominal ganglia - difference between these two groups is the presence of a constriction in one of the thoracic ganglia giving the false appearance of two separate ganglia instead of one. In the third group, there are 2 cerebral, 3 thoracic and 4 abdominal ganglia, while in the fourth group, there are 2 cerebral, 3 thoracic and 5 abdominal ganglia. However, larval forms of all these groups show the presence of 2 cerebral, 3 thoracic and 8 abdominal ganglia.

Reduction in the number of ganglia during metamorphosis of various holometabolans has attracted considerable attention. Newport's (1832, 1834) concept of degeneration and disappearance as the cause for reduction in ganglionic number has been ruled out by the investigations of later workers. Further, it has been suggested that fusion of ganglia leads to the reduction in their number. In rice weevil, *Calandra oryzae*, mesothoracic, metathoracic and first abdominal ganglia fuse to form the anterior ganglionic complex and it was proposed that fusion of ganglia is due to proliferation and overgrowth of the neurons in the ganglia (Murray and Tiegs, 1935). Pipa and his colleagues (1963, 1964, 1965) have done extensive investigations on ganglionic fusion in greater wax moth, *Galleria mellonella*. In this insect during metamorphosis, interganglionic connectives between first and second thoracic ganglia are reduced by 75% while third thoracic, first and second abdominal ganglia fuse to form a single ganglionic mass. Further, they have shown that the cell number in the

ganglion remains constant, thus discarding the hypothesis of Murray and Tiegs (1935). These investigators have also demonstrated that ganglionic fusion in *Galleria mellonella* is accompanied by coiling and looping of axons of interganglionic connectives (Pipa, 1963; Pipa and Woolever, 1965). This was shown to occur within the neurilemma and glial cells that are involved in the shortening of these axons. They also suggested that the glial cells may unwrap their elaborate foldings and withdraw from the interganglionic spaces during the shortening of the ventral nerve cord axons. Shortening of interganglionic connectives and fusion of ganglia was also demonstrated in another Lepiodpteran insect, *Pieris brassicae* (Heywood, 1965). In this insect, the nerve cord gets shortened by about 30% of its original length during metamorphosis and that the process of shortening is entirely different from that of *Galleria mellonella* (Pipa and Woolever, 1965). In *Pieris brassicae,* no coiling and looping of axons was observed during the shortening process, however this process occurs few hours after the reduction in the length of the body wall. It was suggested that "resorption" of axonic material is responsible for the reduction in the length of interganglionic connectives. As a result, neuronal perikarya are pulled forwards from their original abdominal position to thoracic portion of the body. Shortening of neural lamella is passive and is attributed to its elasticity (Heywood, 1965). Beals and Berberet (1976) reported similar pattern of fusion of ganglia in, *Elasmopalpus* ***lignosellus*** (Lepidoptera).

During metamorphosis the first and second abdominal ganglia fuse with the third thoracic ganglion to form a last compound abdominal ganglion in *Philosamia cynthia* (Tsui-Yin and Fang, 1966). In the silkworm, *Bombyx mori,* the shortening of the interganglionic connectives was found to be accompanied by the formation of two ganglionic complexes - ie., anterior ganglionic complex (AGC) and posterior ganglionic complex (PGC). AGC is formed by the fusion of second and third thoracic ganglia and first and second abdominal ganglia, while the PGC is formed by the fusion of fourth, fifth, sixth, seventh and eighth abdominal ganglia (Sivaprasad, 1987).

Role of hormones in insect neurometamorphosis

In insects, the role of hormones in the development of embryonic nervous system is largely unexplored. Their effects during postembryonic life, by contrast, has received more attention and are better understood, especially in the holometabolous forms. These insects produce a larval stage with a simple CNS with few sensory systems. The growth of the nervous system and its synaptic field occurs mainly during the postembryonic development which consists of growth punctuated by a series of moults followed by metamorphosis, thereby it comes under the control of the endocrine cues which regulate the larval growth, moults and metamorphosis (Truman, 1988). These moults and metamorphosis are initiated and coordinated by morphogenetic hormones (Riddiford, 1994). It is generally accepted that the interplay of ecdysteroids, a group of steroid hormones, and juvenile hormones, sesquiterpenes, serves to orchestrate the progression from one developmental stage to the next, with ecdysteroids regulating the onset and timing of moult and JH regulating the quality of moult (Sehnal and Mayer, 1968; Gilbert et al., 1988, 1996; Sehnal, 1989, Riddiford, 1994, 1996)

Larval-pupal transition

The decline and disappearance of JH during the final larval instar development allows metamorphosis to occur (Gilbert et al., 1996). In the absence of JH, ecdysteroids are known to initiate the metamorphic moult resulting in the formation of the pupal stage (Bollenbacher et al., 1975; Riddiford, 1976). In Lepidopteran insects, there are two peaks of ecdysteroids during the larval-pupal transformation. The initial commitment peak is relatively small; it turns off the feeding behaviour and also commits larval tissues such as epidermis for a pupal response (Riddiford, 1978,1985; Dominick and Truman, 1985; Gu and Chow, 1993). This is followed by the prepupal peak that actually causes the molt to the pupal stage (Bollenbacher et al., 1981; Sehnal et al., 1986, 1996).

Calvez et al., (1976) demonstrated that in the larvae of the silk worm, *Bombyx mori*, a difference exits in baseline haemolymph ecdysteroid levels between the fourth and fifth (final) larval instars: low but significant levels of ecdysteroids (30-40 ng/ml)

were observed during the early stages of last instar (Kiguchi and Agui, 1981). The physiological significance of this difference has recently been demonstrated (Gu and Chow, 1993, 1996, 1997). It was found that very low ecdysteroid levels during early stages of last instar larvae are a prerequisite for larvae to undergo metamorphosis. In *Bombyx*, like other Lepidopteran insects, two distinct peaks of ecdysteroids have been reported, a commitment peak at prepupal stage followed by major pupal peak at mid-pupal stage (Calvez et al., 1976).

Studies on the greater wax moth, *Galleria mellonella* have provided clear cut evidence for hormonal stimulation of interganglionic connective shortening, by implanting sections of connectives from larval stages into metamorphosing hosts. The implant shortened in concert with the connectives of the host animal (Pipa, 1967). Subsequently, injections of ecdysone proved effective in causing the shortening response (Pipa, 1969). These *in vivo* studies were then complimented with *in vitro* studies in which isolated connectives from final larval stage were cultured in the presence of 20-hydroxyecdysone (20E). Interestingly, the hormone could not initiate the process *in vitro,* but it could maintain shortening in culture if the process had already begun *in vivo,* suggesting the involvement of additional factor(s) in the initiation of nerve cord shortening (Robertson and Pipa, 1973, Robertson, 1974). Similar factors which mediate the action of 20E have been reported in these insects (Caglayan, 1990; Ashok and Dutta-Gupta, 1991)

Detailed studies have demonstrated that the prepupal peak of ecdysteroids causes dendritic regression in larval motor neurons (Runion and Pipa, 1970; Weeks and Truman, 1985). The regulation of this regression has been examined in detail for the motorneuron, PPR (principal proleg retractor) which innervates an abdominal proleg retractor muscle in *Manduca sexta.* When larval abdomens were isolated prior to the prepupal peak of ecdysteroids, PPR retained its morphology. Ecdysteroid infusion into such abdomens triggered dendritic loss followed by the death of the cell a few days later (Weeks and Truman, 1985). The other hormone regulating the fate of PPR is JH, but JH can act only during the small commitment peak. The low levels of ecdysteroids normally present during this peak are not sufficient to cause neurite loss

but regression can be experimentally induced by large doses of 20E. Treatment with JH prior to 20E infusion prevents this neurite loss (Weeks and Truman, 1986). In addition to inducing dendritic regression, prepupal ecdysteroid peak also causes the death of some of the larval neurons after pupal ecdysis (Streichert and Weeks, 1994).

Larval neurons are known to respond to prepupal ecdysteroid peak by dendritic regression, while the imaginal neurons respond to this peak by initiating maturation. In *Manduca,* resumption of maturation is first evident as an increase in the cell body size (Booker and Truman, 1987b). Studies on *Drosophila* indicate that this size increase is preceded by an up-regulation of the homeotic gene *ultrabithorax* (Glicksman and Truman, 1990).

Pupal-adult transition

A prolonged release of ecdysteroids causes the transformation of the pupa into the adult (Warren and Gilbert, 1986). During this stage there is considerable dendritic sprouting of the surviving larval neurons (Prugh et al., 1992). In the motorneuron, MN-1 of *Manduca*, this outgrowth includes an extensive new arbour ipsilateral to the cell body. Neurite extension starts on day 3, after pupal ecdysis and continues through the next 8-10 days (Truman and Reiss, 1988). The adult-specific growth of MN-1 is also sensitive to JH as treatment with JH mimics up to 2-3 days after pupal edysis block adult outgrowth (Truman and Reiss, 1988; Prugh et al., 1992).

The final metamorphic change in CNS is the wave of neuronal death that occurs after adult emergence. In both *Manduca* and *Drosophila* the withdrawal of ecdysteroids at the end of metamorphosis is essential for these cells to die. Treatment with 20E either *in vivo* or to cultured ganglia delays or prevents the death of these neurons (Truman and Schwartz, 1984; Bennett and Truman, 1985; Robinow et al., 1993). Recent studies have found a strong correlation between ecdysteroid receptor expression in the CNS and the nature of the neurons response to ecdysteroids, but there is not yet any direct evidence to indicate that the relationship is casual (Rabinow et al., 1993; Truman et al., 1994).

Scope and objectives of the present study

Among the most remarkable events of CNS development and differentiation are the process of extension and the assumption of highly characteristic cellular morphology. Though there are considerable number of studies on various aspects of insect neurometamorphosis, there are no reports on the role of cytoskeletal elements in the ganglionic fusion and nerve cord shortening process during the metamorphosis of holometabolous insects. Cellular movements such as extension of neurites, coiling or looping of axons, resorption of axonic material involve the active participation of the cytoskeletal components (Hollenback, 1989; Oblinger et al., 1989; Mizobuchi et al., 1990; Warn et al., 1993; Barklow and Hartwig, 1995, Haendel et al., 1996; Caroni, 1997). The present study focuses on the changes in the synthesis and content of the two major cytoskeletal proteins - actin and tubulin in the nervous system of the silk worm, *Bombyx man* during the postembryonic development. In the present study, hormonal involvement in the regulation of expression of these proteins was also examined. Phosphorylation was shown to play a central role in dynamic remodelling of cytoskeletal architecture (Vallano et al., 1986, Nixon and Sihag, 1991; de Freitas et al., 1995). Wandosell et al., (1987) demonstrated the phosphorylation of both the subunits of tubulin (a and β). Further, phosphorylation of tubulin has been shown to prevent its incorporation into microtubules. (Yamomoto et al., 1985; Wandosell et al., 1987). Thus, an attempt was also made to find out whether these cytoskeletal proteins undergo phosphorylation during nervous system development and reorganization.

CHAPTER 2

MATERIALS AND METHODS

CHEMICALS

[^{35}S]-Methionine (1000 Ci/mmole) was obtained from Amersham, (England) and American Radiolabeled Chemicals Inc., (St. Louis, USA). Bovine serum albumin (fraction V), brilliant blue G-250, bromophenol blue, PPO (2,5 diphenyloxazole), POPOP (1,4-bis [5-(phenyl-2-oxazolyl)] benzene), ethylene glycol, urea, NP-40, Triton X-100, 4-chloro-1-naphthol and TC-100 insect culture medium and high molecular weight protein markers were obtained from Sigma Chem. Co. (St. Louis, USA). Tris, acrylamide, N-N'-methylene bis-acrylamide, 2-mercaptoethanol and glycine were purchased from Spectrochem (Bombay, India). Silver nitrate, TEMED (N, N, N^1, N^1 tetramethylethylenediamine), dimethyl sulfoxide (DMSO), glycerol and hydrogen peroxide (30%) were obtained from E. Merck (Schuchardt, Germany). Monoclonal anti-actin and anti-β-tubulin antibodies were purchased from Boehringer Mannheim (Darmstadt, Germany) Anti-mouse IgG-HRPO conjugated antibody was from Pierce (Rockford, USA). SDS and cellophane sheets were from Bio-Rad Laboratories (Richmond, USA). 20-Hydroxyecdysone (20E) was purchased from Rohto Pharmaceutical Co. (Osaka, Japan). Juvenile hormone I (cis-10, 11 epoxy-7-ethyl-3, 11-dimethyl-trans, trans-2, 6-tridecadienoic acid methyl ester), nitrocellulose membranes (Millipore, Bedford, USA) and ampholines (LKB, Sweden) were gifted by Prof. A. Krishna Kumaran of Marquette University, Milwaukee, USA. X-ray films were obtained from Kodak (Rochester, USA). All other chemicals were obtained from commercial sources in India and were of analytical grade.

METHODS

Experimental insects:

Third instar larvae of silk worm, *Bombyx mori* (pure Mysore strain) were obtained from local breeding centre and reared in an insect culture room at 26 ± 1°C, 70 ± 5% relative humidity and 14 h : 10 h light-dark period on fresh mulberry leaves. Staging of insects was done based on their age after the fourth ecdysis. One to 2 days old last

instar larvae were designated as early-last instar (ELI), 5 to 6 days old as mid-last instar (MLI), 9-10 days old as late-last instar (LLI). Larvae collected after spinning were designated as prepupa (PP), 1 day old prepupa as early-prepupa (EPP), 2 days old prepupa as mid-prepupa (MPP), 3 days old prepupa as late-prepupa (LPP), 1 to 2 days old pupa as early-pupa (EP), 4-5 days old as mid-pupa (MP) and 9-10 days old as late-pupa (LP), while freshly emerged moths (<12 h old) as adult (A).

Morphometric studies:

For studying the changes in the length of the nervous system, CNS from insects of different developmental stages were dissected and were placed on a glass microslide. Length of the CNS was measured under a binocular macroscope (Wild Heerbrug) using an ocular micrometer which was calibrated with a stage micrometer.

Preparation of tissue sample:

Intact CNS (brain + ventral nerve cord) were rapidly dissected from animals of different developmental stages, frozen in liquid nitrogen and stored at -70° C. Frozen tissue was homogenized (4 CNS/50 µl) by hand in 10 mM Tris buffer (pH 7.1), containing 0.1 % Triton X-100, using an all glass microhomogenizer (Kontes). These homogenates were centrifuged at 1000 x g for 5 min to remove large debris. An aliquot of the supernatant was used for protein estimation and electrophoresis.

Protein estimation:

Protein content was estimated according to the microprotein assay method of Bradford (1976).

(i) ***Preparation of protein reagent:*** 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 Whatman No.1 filter paper and stored in an amber coloured bottle at 4° C.

(ii) ***Protein estimation:*** An aliquot of the sample was pipetted out into a 1.5 ml microfuge tube. The volume of the sample was adjusted to 0.1 ml with 10 mM Tris

buffer (pH 7.1). One ml of protein reagent was added and 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 ml of protein reagent Protein content of the sample was calculated using a standard curve drawn using bovine serum albumin (fraction V).

Sample preparation for electrophoresis:

An aliquot of the sample for sodium dodecylsulfate-polyacrylamide electrophoresis (SDS-PAGE) was mixed with an equal volume of 2x 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. For two-dimensional gel electrophoresis, protein in the homogenate was precipitated overnight with cold acetone at - 80° C. The protein precipitate was pelleted by centrifugation at 10,000 x g for 15 min at 4°C. The pellet was then dissolved in 100 ul IEF sample buffer containing 9.5 M urea, 2% LKB carrier ampholytes (comprised of 1.6% 5-7 pH and 3.5-10 pH), 2% NP-40 and 5% 2-mercaptoethanol.

***In vitro* studies on protein synthesis - effect of hormones:**

For studying *in vitro* incorporation of [^{35}S]-methionine into CNS proteins, intact CNS was dissected under aseptic conditions from different developmental stages of insects. Tissue was cultured in TC-100 insect culture medium (2 CNS/100 ul) for 12 h in presence of 20 µCi of [^{35}S]-methionine at 25° C under sterile conditions. All manipulations were done in a sterile laminar hood. At the end of incubation period, tissue was removed and rinsed extensively with insect Ringer (NaCl 7.5 g, KC1 0.35 g, $CaCl_2$ 0.21 g in 1 litre of distilled water). The tissue was homogenized in 10 mM Tris buffer (pH 7.1) containing 0.1% Triton X-100, using an all glass microhomogenizer (Kontes) and centrifuged at 1000 x g for 5 min to remove large debris. Small aliquots of the supernatants were used for radiolabel quantitation and electrophoresis.

For studying the effect of JH I and 20E on protein synthesis, the dissected CNS was incubated in TC-100 culture medium initially for 4 h to deplete the endogenous hormones and were treated with the respective hormone (JH I was used

at final concentration of 7 x 10^{-7}M and 20E at a final concentration of 5 x 10^{-6} M) for 4 h. Subsequently, 20 u.Ci of [^{35}S]-methionine was added and incubation was carried out for another 4 h

In vivo JH treatment of the larvae:

To study the effect of JH on the CNS protein profile, the mid-last instar larvae were treated with 5 µg of JH-1 dissolved in 5 µl of acetone by topical application. The hormone treatment was repeated after 3 days. Under these conditions, last larval stadium was extended by an additional 5-6 days. When the larvae entered into the prepupal stage, CNS were dissected and the tissue was processed for two-dimensional gel electrophoresis. Stage matched untreated larvae were used as controls.

Preparation of sample for radiolabel quantitation:

Proteins in the homogenate were precipitated in an 1.5 ml microfuge tube by adding an equal volume of 20% chilled TCA for 30 min at 4° C. The mixture was centrifuged at 10,000 x g for 15 min at 4° C. The resultant pellet was washed twice with 1 ml of 5% TCA and then with ethanol-ether mixture (3:1). The final pellet was air dried and dissolved in 100 ul of 0.1 N sodium hydroxide. An aliquot (50 ul) of the sample was added to 5 ml of Bray's mixture (PPO 4 g, POPOP 200 mg, naphthalene 60 g, ethylene glycol 20 ml, methanol 100 ml and volume made up to 1 litre with 1,4-dioxan). The radioactivity in the samples was quantified using a Beckman liquid scintillation spectrophotometer (Model LS 1800). An aliquot (10 µl) of the same sample was used for protein estimation.

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 15 cm 10 % resolving gel (pH 8.8). 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.

Two-dimensional gel electrophoresis:

Two-dimensional electrophoresis was performed as described by O'Farrell (1975). Ampholyte polyacrylamide tubes were prefocused for 1 h at 200 V to set-up the pH gradient. The gels were run for a total of 10,000 V h. The anolyte used was 0.01 M H_3PO_4, and the catholyte used was 0.02 M NaOH The gels were subsequently transferred to 5 ml equilibration buffer containing 10% glycerol, 5% 2-mercapto-ethanol, 2.3% SDS and 0.0625 M Tris-HCl (pH 6.8) and stored frozen at -20°C till use. The second-dimension separation was carried out using 3.3% stacking gel and 10% separating gel. Sigma high molecular weight standards were run at the acidic end of gels. The gels were run at 25mA/gel until the bromophenol blue dye reached the bottom of the gel. The gels were fixed and silver stained. To determine the pH gradient of IEF gels, parallel gels were cut into pieces of 0.5 cm length and incubated for 1 h in 0.5 ml degassed distilled water. The pH was measured electrometrically. In all these studies, equal quantity of protein samples were loaded and comparisons were made.

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, 50 ul of 37% formaldehyde/ 100 ml) for 1 h and were then treated with 50% ethanol for 30 min (3 changes). This was followed by pre-treatment with sodium thiosulphate (20 mg/ 100ml) for 1 min. The gels were rinsed thrice with double distilled water (20 sec each) and impregnated with silver nitrate (0.5% silver nitrate, 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), 0.05% 37% formaldehyde (v/v). Finally, stained gels were thoroughly rinsed with double distilled water and stored in 50% methanol.

Fluorography:

This was employed for the detection of [^{35}S]-methionine labelled polypeptides on SDS-PAGE and was done according to the procedure of Bonner and Laskey (1974). The gel was immersed first in DMSO for 2 h and then in a fresh solution of the same for 2 h. The gel was transferred to a solution of PPO in DMSO (24.8 g PPO in 100 ml DMSO) for 2 h. The gel was washed with distilled water till there was no floating PPO and transferred onto a Whatman No.3 filter paper. It was sandwiched between cellophane sheets and was dried under vaccum at 80° C using a Hoeffer Gel Dryer. The dried gel was exposed to Kodak X-OMAT X-ray film at - 80° C.

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 (BioRad) according to the procedure of Towbin et al., (1979). After the transfer, membrane was air dried and incubated for 1 h at room temperature with 3% (w/v) bovine serum albumin (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 (BCIP).

Molecular weight determinations:

Molecular weight of electrophoretically separated polypeptides was determined by co-electrophoresing high molecular weight marker proteins using a UVP gel documentation system (Ultra Violet Products). They included myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97 4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and α-lactalbumin (14 kDa).

Laser scanning densitometry:

Quantitation of immunoblots was done with a computed laser scanning densitometer (Molecular Devices, USA) using the Image Quant software programme.

Statistical analysis:

Statistical analysis was performed by one way ANOVA followed by comparisons of the means by Student-Newman-Keuls multiple comparison test using the Sigma Stat software. $p<0.05$ was defined as the criterion for statistical significance. The data are represented as mean + S D

CHAPTER 3

RESULTS

Morphometric changes

Results presented in figure 1 show the changes in the length of the nervous system of *Bombyx mori* during the larval-pupal-adult transformation. The length of the nervous system reached its highest in the late-last instar larvae and remained more or less the same in early-prepupa. Thereafter, it declined gradually through the prepupal and pupal stages and reached its minimal in 10-12 h old adult moths. However, the process of shortening was more pronounced during the prepupal stage.

Changes in the total protein content of the CNS

Results presented in figure 2 show the changes in the total protein of the *Bombyx mori* nervous system during postembryonic development. Protein content was very low in the early-last instar larvae and increased gradually through mid-last, late-last larval, prepupal and pupal stages and reached a high value in late-pupa and remained more or less the same in 10-12 h old moths.

Studies on *in vitro* protein synthesis

Figure 3 summarizes the results of the *in vitro* incorporation of [^{35}S]-methionine into *Bombyx* CNS proteins. For this study CNS from different developmental stages were dissected out and short-term cultures were carried out in presence of [^{35}S]-methionine. Protein synthetic activity was found to be fairly high in the CNS of mid-last instar larvae among the larval stages and in late-pupa during the pupal period. It is interesting to note that the adult CNS showed the lowest rate of protein synthesis of all the stages used in the present study.

The above experiments were followed up with electrophoretic and autoradiographic analysis of [^{35}S]-methionine labelled polypeptides during different developmental stages and the results are presented in figure 4 This study also indicated high degree of radiolabel incorporation into the CNS proteins during mid-last instar larval and late-pupal stage. Further, high degree of radiolabel incorporation into two major polypeptides with molecular weights of 45 kDa and 55 kDa, which were later identified as actin (45 kDa) and tubulin (55 kDa) was observed

Developmental profile of actin content of the CNS

SDS-PAGE analysis of the CNS proteins from various developmental stages revealed a quantitative change in the 45 kDa actin protein (Fig. 5a). This was further confirmed using immunoblotting with monoclonal actin antibody and the results are presented in figure 5b. Quantitative analysis of the immunoblot revealed that the actin concentration of the CNS remained more or less the same during the larval stadium but it significantly increased from early-pupa and reached a peak value in adult stage (Fig. 5c).

Developmental profile of β-tubulin content of the CNS

SDS-PAGE (Fig. 6a) and immunoblotting analysis of CNS proteins from different developmental stages revealed quantitative and qualitative changes in the 55 kDa P-tubulin protein expression (Fig. 6b). Figure 6c shows the results obtained by the quantitative analysis of the immunoblot (Fig. 6b). Fairly large quantity of β-tubulin was found to be present in the CNS of early-last instar larvae (lane 1), it increased during the final instar larval development and reached its maximum in late-last instar larvae (lane 2). Thereafter, the content declined during prepupal (lane 3) and pupal stages of development (lanes 4, 5 and 6). However, it increased once again and adult CNS showed a higher content of tubulin (lane 7).

An interesting observation was the expression of a new p-tubulin isoform, migrating just above the major P-tubulin band in the samples from the pupal stages (Fig. 6b). The expression of this isoform begins in the early-pupal CNS (lane 4) and reaches a maximum in mid-pupa (lane 5) and then decreases in late-pupa (lane 6). This isoform was found to be absent in the in the CNS protein extracts from larval and adult stages.

Effect of hormones on *in vitro* protein synthesis

To study the effect of hormones on protein synthesis, the dissected intact CNS from mid-last instar larvae were cultured in presence of either JH 1 (7×10^{-7} M) or 20E (5×10^{-6} M)and [^{35}S]-methionine. After the labelling period, CNS proteins were separated by SDS-PAGE and the gel was subjected to fluorography. Result of this

study revealed the stimulatory role of 20E on the synthesis of cytoskeletal proteins actin (A) and tubulin (T) (Fig. 7b, lane 2). In addition, synthesis of few other polypeptides was also stimulated by 20E treatment. Short-term JH treatment (8 h) in this experiment did not exert any significant effect on the synthesis of proteins (Fig 7b, lane 3).

Effect of *in* *vivo* JH 1 treatment on CNS **protein profile:**

Two-dimensional gel electrophoresis analysis of CNS proteins of control and JH 1 treated larvae revealed major qualitative as well as quantitative changes (Fig. 8a and b). JH 1 treatment induced the qualitative expression of following polypeptides (Fig. 8b):

Spot No.	Molecular Mass (kDa)	**Iso-electric** point (p*I*)
1	**48.8**	5.5
2	**40.9**	5.4
3	**39.0**	5.5
4	**36.8**	4.9
5	**32.0**	5.6
6	**31.5**	5.7
7	**30.2**	5.7
8	**24.9**	5.2
9	**24.1**	6.2

In vitro **phosphorylation of endogenous proteins of *Bombyx*** CNS

/// *vitro* phosphorylation of CNS proteins from late-last instar larvae in presence of EGTA revealed the phosphorylation of few proteins of which a 48 kDa band was the major protein labeled with [^{32}P] (Fig. 9, lane 1). Inclusion of 1 mM $CaCl_2$ stimulated the phosphorylation of several proteins, particularly 59/60 kDa (Fig. 9, lane 2) and this stimulation was further enhanced by the addition of 2 uM calmodulin (Fig. 9,

lane 3). However, this study revealed that neither actin nor tubulin were phosphorylated under these conditions

Phosphorylation of CNS proteins of *Bombyx* were investigated in detail and these studies form the subject matter of part-II of this thesis.

Stage specific expression of a 235 kDa protein during pupal-adult development

Detailed electrophoretic studies carried out on the CNS proteins of *Bombyx* during pupal-adult transformation revealed the appearance of a new polypeptide with an apparent molecular weight of 235 kDa in late-pupa (pharate adults) (Figs. 10 and 11). This polypeptide was absent in the late-last instar larval, prepupal, early and mid-pupal stages (Fig. 10, lanes 1 and 2; Fig. 11, lanes 1 and 2). Laser scanning densitometry of dried gels indicated that this polypeptide was present in low concentration in late-pupa (Fig. 11, lane 3) and its content gradually increased during adult development and reached highest in 48 h old moths (Fig. 11, lane 6).

Subsequent analysis of CNS proteins by two-dimensional electrophoresis clearly showed that this 235 kDa polypeptide was expressed only during the late-pupal and adult development (Fig. 12c) and it was absent during the larval (Fig. 12a) and early-pupal development (Fig. 12b). The p/ value of the polypeptide ranged between 6-6.2.

Fig. 1 - Changes in the total length of the CNS **of *Bombyx mori* during different stages of postembryonic development.** Values are mean of 12 independent measurements

Fig. 2 - Changes in the total protein content of *Bombyx mori* CNS during the various stages of postembryonic development. ELI - early- last instar, MLI - mid-last instar, LLI - late-last instar, PP - prepupa, EP- early pupa, MP - mid-pupa, LP - late-pupa, A - adult. (Protein is expressed as ug protein/CNS). Values are mean of 10 independent determinations.

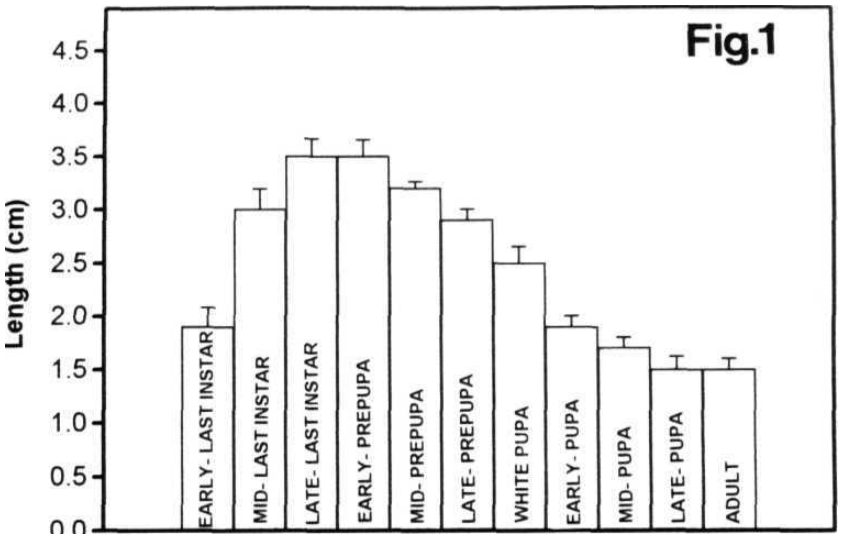
Fig.1
Length (cm)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
EARLY- LAST INSTAR
MID- LAST INSTAR
LATE- LAST INSTAR
EARLY- PREPUPA
MID- PREPUPA
LATE- PREPUPA
WHITE PUPA
EARLY- PUPA
MID- PUPA
LATE- PUPA
ADULT

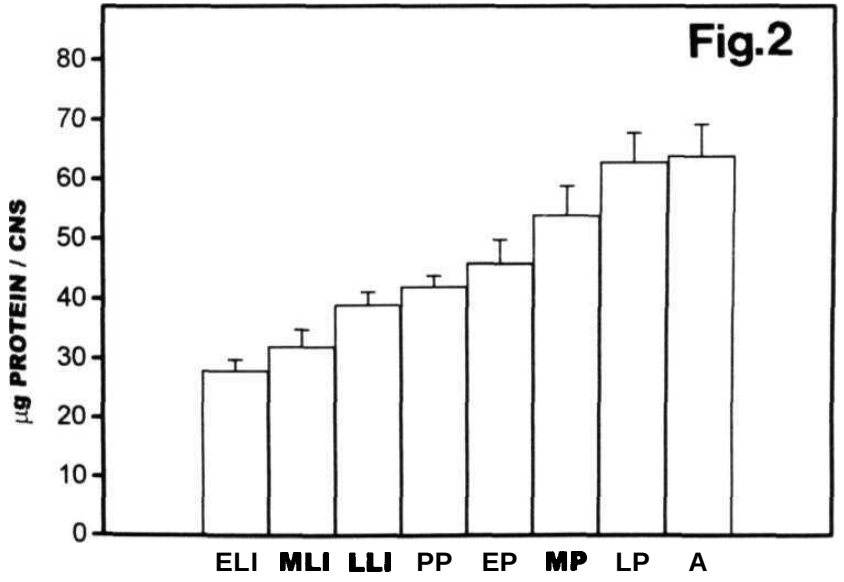
Fig.2
µg PROTEIN / CNS
80
70
60
50
40
30
20
10
0
ELI
MLI
LLI
PP
EP
MP
LP
A

Fig. 3 - Changes in the *in vitro* [^{35}S]-methionine incorporation into CNS proteins at different stages of development. Radiolabeling was done for 8 h. ELI - early-last instar, MLI - mid-last instar, LL1 - late-last instar, PP - prepupa, MP - mid-pupa, LP - late-pupa and A - adult. * indicates $p<0.05$.

Fig. 4 - Autoradiograph of the profile of *in vitro* [^{35}S]-methionine labelled CNS polypeptides at different stages of development. Lane 1 - early-last instar, lane 2 - mid-last instar, lane 3 - late-last instar, lane 4 - prepupa, lane 5 - mid-pupa, lane 6 - late-pupa and lane 7 - adult. Note the high degree of synthesis of actin (◄46 kDa) and tubulin (◄55 kDa) proteins in the CNS of *Bombyx* 15 µg protein was loaded in each lane.

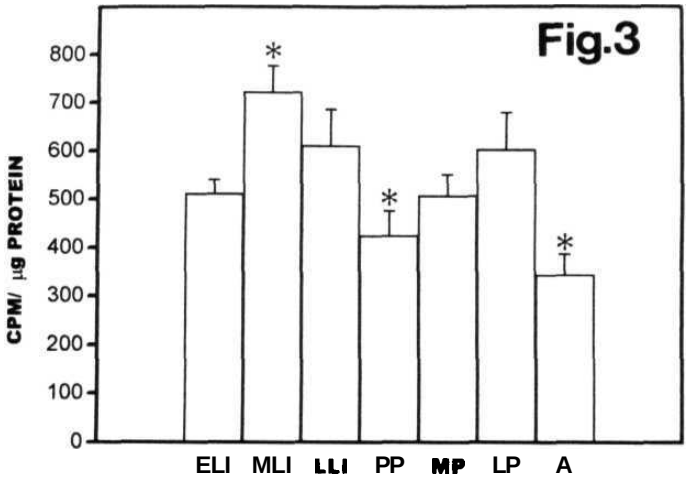
Fig.3
CPM/ µg PROTEIN
800
700
600
500
400
300
200
100
0
ELI
MLI
LLI
PP
MP
LP
A

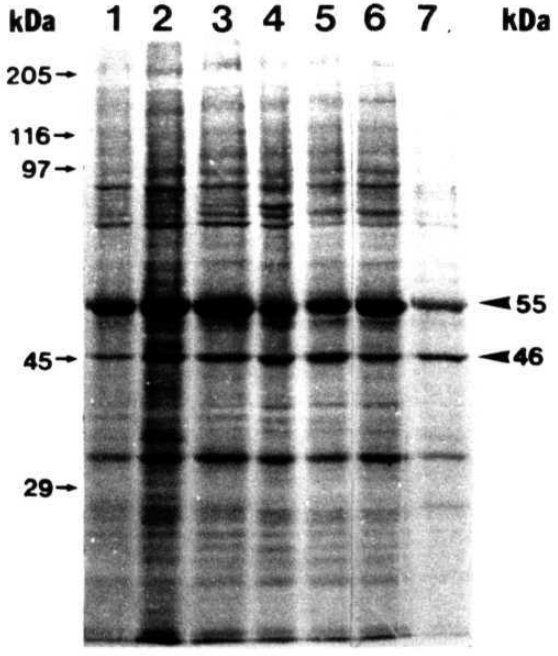
kDa
1
2
3
4
5
6
7
kDa
205
116
97
45
29
55
46

Fig.4

Fig. 5 - Developmental profile of actin (◄ 45 kDa) in the CNS of *Bombyx mori*. **a** - SDS-PAGE, b - immunoblot probed with actin monoclonal antibody, **c** - quantitative representation of the data obtained by laser scanning densitometry of the immunoblot. ELI - early-last instar, MLI - mid-last instar, LLI - late-last instar, PP - prepupa, EP- early-pupa, MP - mid-pupa, LP - late-pupa and A - adult. 10 μg protein was loaded in each lane.

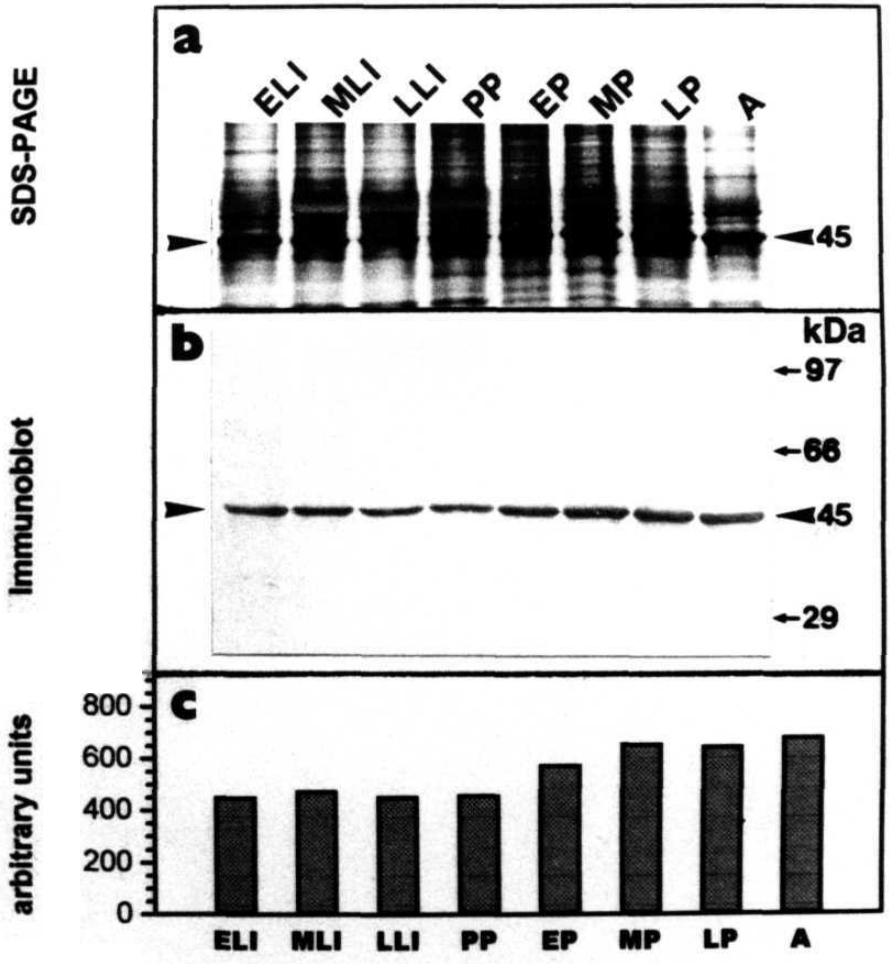
a
ELI
MLI
LLI
PP
EP
MP
LP
A
45
SDS-PAGE
b
kDa
97
66
45
29
Immunoblot
c
800
600
400
200
0
arbitrary units
ELI
MLI
LLI
PP
EP
MP
LP
A

Fig.5

Fig. 6 - Developmental profile of β-tubulin (◄ 55 kDa) in the CNS of ***Bombyx*** *mori*. **a** - SDS-PAGE, b - immunoblot probed with β-tubulin monoclonal antibody, c - quantitative representation of the data obtained by laser scanning densitometry of the immunoblot. ELI - early-last instar, LLI - late-last instar, PP - prepupa, EP- early-pupa, MP - mid-pupa, LP - late-pupa and A - adult. Note the presence of a new isoform of tubulin in EP, MP and LP stages (◄).

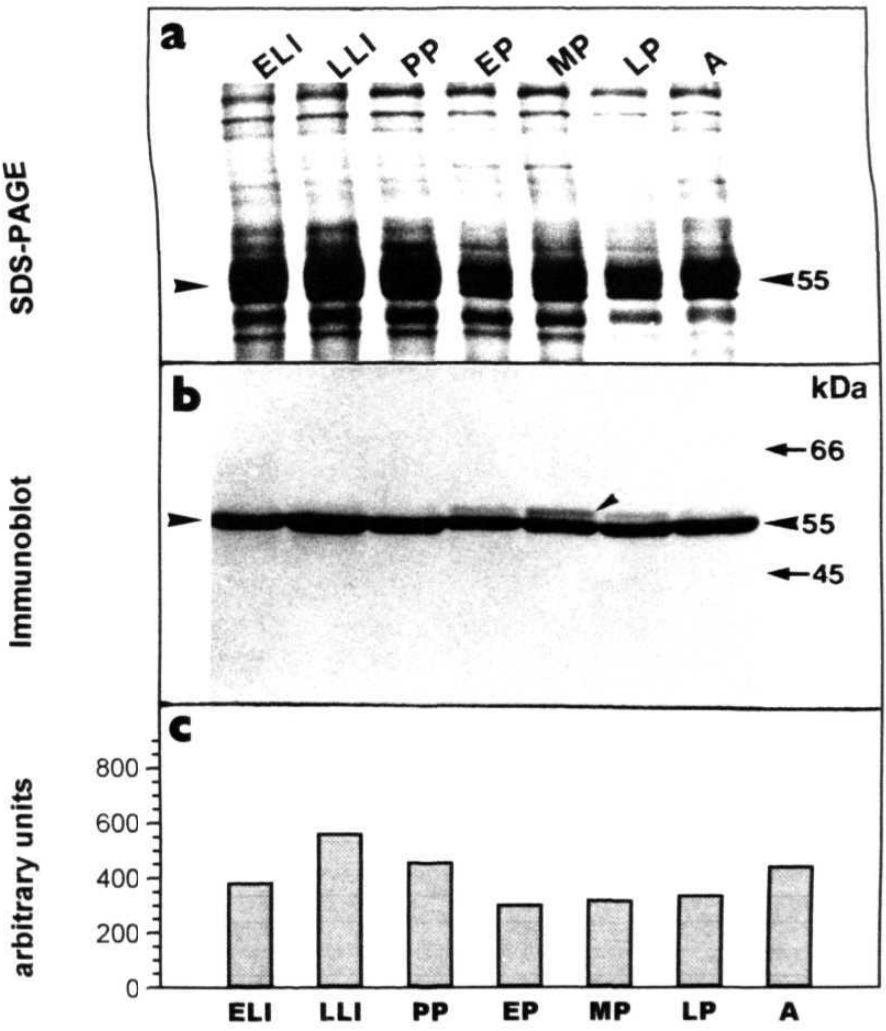
a
ELI
LLI
PP
EP
MP
LP
A
55
SDS-PAGE
b
kDa
66
55
45
Immunoblot
c
800
600
400
200
0
arbitrary units
ELI
LLI
PP
EP
MP
LP
A

Fig.6

Fig. 7 - Effect of hormones on CNS protein synthesis *in vitro*. **a** - SDS-PAGE, b - autoradiograph. Lane 1 - control, lane 2 - 20E treated and lane 3 - JH 1 treated. (◄T) indicates the tubulin band and (◄A) indicates the actin band.

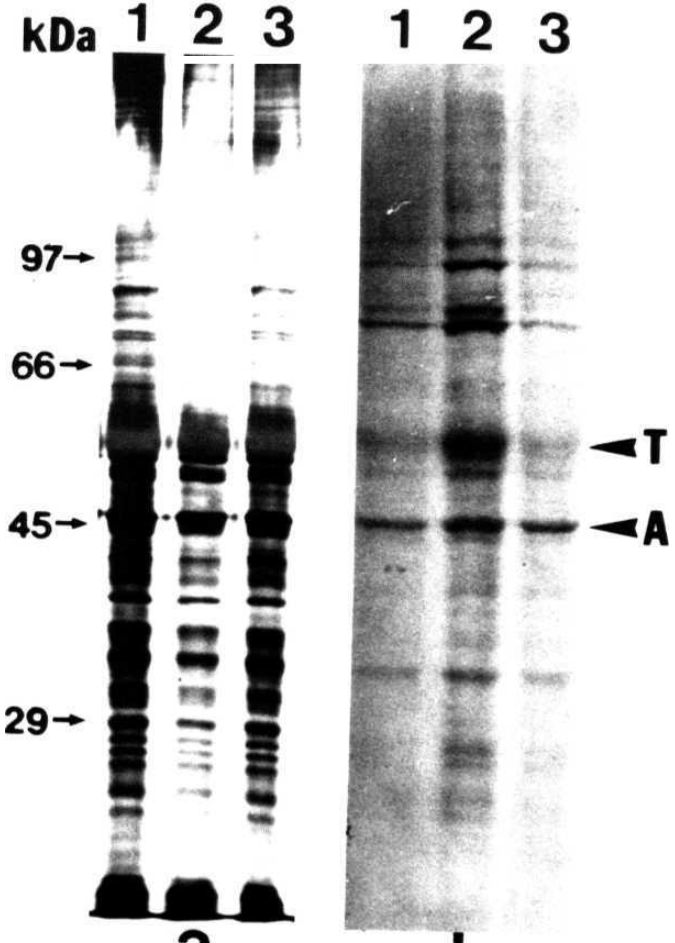

Fig.7

Fig. 8 - Effect of *in vivo* JH I treatment on CNS protein profile of last instar larvae using two-dimensional gel electrophoresis. a - stage matched control, b - JH 1 treated Equal quantity of protein (75 μg) was loaded in both the gels. Note the presence of few additional polypeptides in JH I treated insects (spots 1-9).

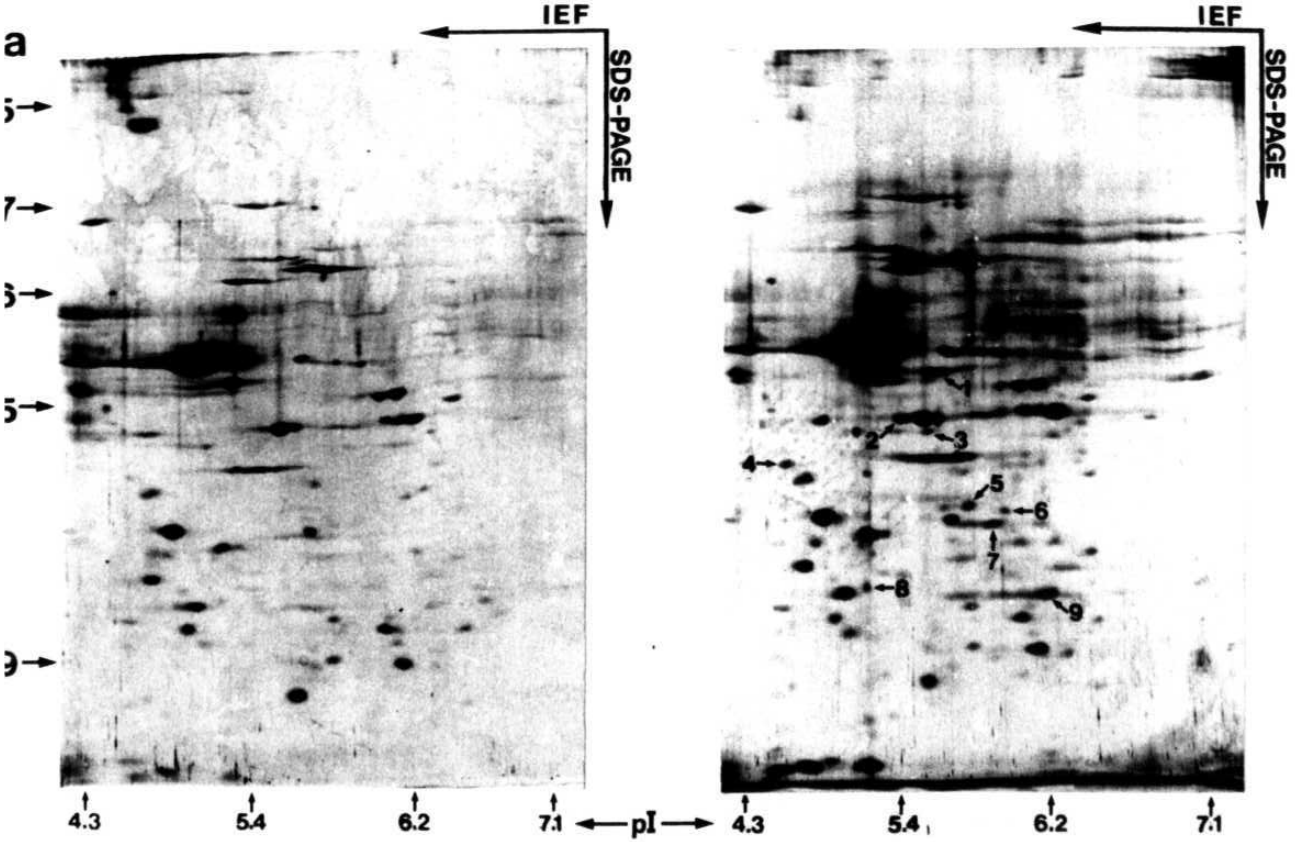

Fig.8a

Fig.8b

Fig. 9 - Autoradiograph showing *in vitro* phosphorylation of *Bombyx* CNS proteins. following incubation of homogenates with [$^{32}\gamma$-P] ATP under phosphorylating conditions (see materials and methods - Part II): Incubations were carried out under the following conditions: lane 1 : 1 mM EGTA + 2 uM calmodulin, lane 2 : 1 mM $CaCl_2$, and lane 3 : 1 mM $CaCl_2$ + 2 uM calmodulin. Note that the phosphorylation of the 59/60 kDa proteins (◄) was significantly stimulated in presence of calcium and calmodulin. Equal amount of protein was loaded in all the lanes.

Fig. 10 - SDS-PAGE showing the expression of a 235 kDa protein in the CNS: Lane 1 - late-last instar, lane 2 - mid-pupa and lane 3- adult. The proteins were separated on 10% gel and in each lane 10 μg protein was loaded. Note the presence of a 235 kDa (◄) protein in adult sample (lane 3).

Fig. 11 - SDS-PAGE showing the stage specific expression of the 235 kDa protein during metamorphosis. Proteins were separated on a 5 % gel. The sample loaded in lane 1 is from prepupa, lane 2 - early-pupa, lane 3 - late-pupa, lane 4 - 12 h old adult, lane 5 - 24 h old adult, lane 6 - 48 h old adult. Note that the concentration of 235 kDa polypeptide (◄) increased in the CNS from late-pupal to adult development.

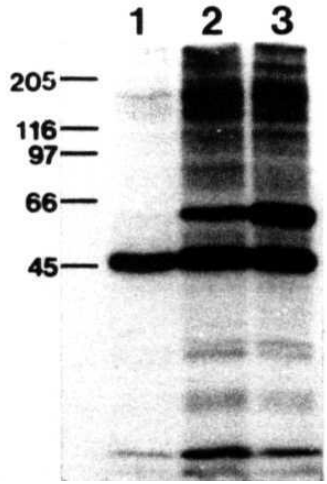

Fig.9

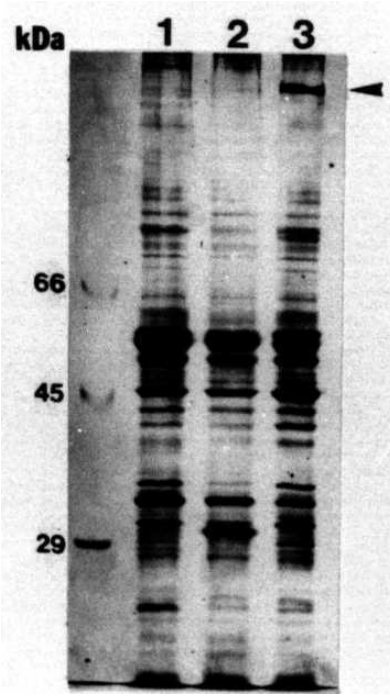

Fig.10

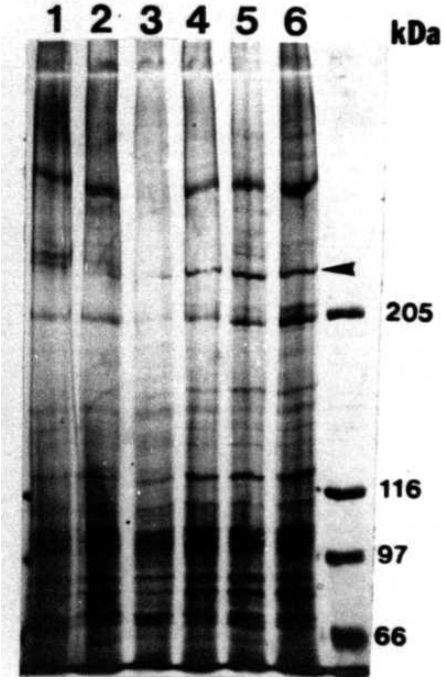

Fig.11

Fig. 12 - Two-dimensional gel analysis of the 235 kDa protein. Proteins were separated between 20 to 260 kDa on vertical axis and 4.7 to 7 2 p/ on horizontal axis. **a** - late-last instar, b - early-pupa and c - adult. Note the presence of 235 kDa protein (◄) in the CNS of the adult moths.

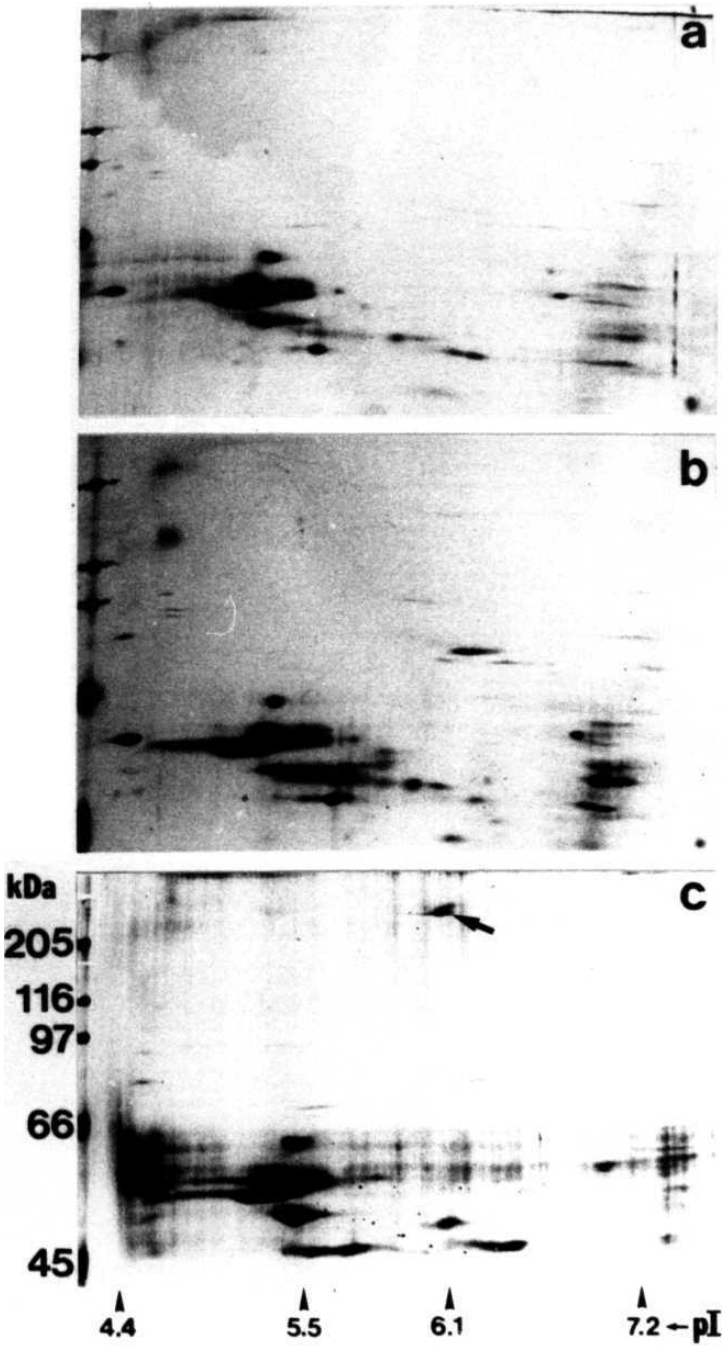

Fig.12

CHAPTER 4

DISCUSSION

Results of the present investigation, in brief, revealed an overall increase in the length and weight of the body of the larva in the last (fifth) instar. Consequent to this, the length of the nervous system almost doubled. Most of this increase was observed to be due to the elongation of the interganglionic connectives (nerve cord). During pupal stage the length of the body shortens and thus the length of the nervous system was half of that in late-last instar larval stage. These results are in agreement with those of earlier investigators (Heywood, 1965; Pipa and Woolever, 1965; Sivaprasad, 1987). Increase in the length of the nervous system might be due to elongation of the connectives without the addition of new tissue material or elongation associated with the addition of tissue material. Similarly, increase in the size of the ganglia might be due to addition of new cells or due to enlargement of existing cells. The reduction in the length of nervous system and number of ganglia might be interpreted along the same lines.

The present investigation revealed a gradual but continuos increase in the total protein content of the CNS of *Bombyx mori* during larval-pupal-adult transformation. Radiolabelling and fluorographic studies further substantiate that the synthesis of protein continues uninterrupted throughout postembryonic development of the nervous system. Considerable number of studies carried out on the developmental changes in the protein synthesis in various insects, reveal a similar pattern. Mansingh and Smallman (1967a, b) observed a marked increase of both soluble and total proteins in the brain of five species of Lepidoptera viz. *Hyalophora cecropia*, *Antheria polyphemus*, *Antheria pernyi*, *Malacosoma americanum*, *Galleria mellonella* and ***Ostrinia*** *nubia/is* during larval-pupal-adult transformation. Qualitatively, the increase in the protein content during metamorphosis reflects an overall synthesis of specific proteins such as choline acetylase, acetylcholine esterase and other proteins coincident with an increase in the amount of nervous tissue during the development of the adult nervous system (Smallman and Mansingh, 1969). Brain of adults of *Hyalophora* and *Antheria* showed five times more protein than that of pupae and the pattern of increase was remarkably synchronous with the increase in choline acetylase and acetylcholinesterase (Mansingh and Smallman, 1967a). An interesting correlation between acetylcholinesterase and total body protein at different

developmental stages was also demonstrated in *Musca domestica* (Casida, 1956). Hence it is evident that, a disproportionately high amount of newly synthesized protein is directed towards the production of the hydrolytic enzymes during larval-pupal-adult transformation (Smallman and Mansingh, 1969). Studies on honey bee, *Apis mellifera* revealed that increase in acetylcholinesterase activity and levels during pupal development was associated with neurogenesis, differentiation and maturation of the nervous system (Sanes and Hildebrand, 1976; Masson and Arnold, 1987; Kreissl and Bicker, 1989; Lenoir-Rousseaux et al., 1994; Polyzou et al., 1997). An accelerated growth of optic lobe associated with compound eye formation occurs during the metamorphosis in many insects (Sehnal, 1985). The increase in the total protein content of the CNS of *Bombyx* observed in the present study can be correlated with the growth and development of the nervous system during larval-pupal-adult transformation.

Most cell types change their shape and may move relative to one another during different phases of postembryonic development particularly at metamorphosis in insects (Locke, 1985). In the CNS of holometabolous insects, postembryonic development is characterized by profound increase in the size of the ventral ganglia and length of interganglionic connectives during the larval stages. During metamorphosis, there is a reduction in the number of ganglia and length of the connectives between the ganglia undergoing fusion. The mechanical explanation for these observations clearly lies in the assembly and movement of the cytoskeleton component. The cytoskeleton participates in both, maintenance of cell shape and dynamics of cell motility. Two major structural elements involved in these functions are tubulin-containing microtubules and actin-containing microfilaments (Lazarides and Revel, 1979; Vitek et al., 1984; Warn et al., 1993., Haendel et al., 1996; Carlier and Pantaloni, 1997).

Actin is a major structural and contractile protein found in every eukaryotic cell. In muscle cells, it is the most abundant constituent of the thin filaments on which the thick filament slide to produce muscle contraction. While in non-muscle cells, it is a major component of microfilaments which are implicated in cytoskeletal

morphology, cell motility, phagocytosis, endo- and exocytosis (Mounier and Prudhomme, 1991; Shelterline and Sparrow, 1994). Actin genes represent a class of ubiquitous eukaryotic gene families and these are differentially expressed in different tissues. *Drosophila melanogaster* genome contains six actin genes: two of them code for cytoplasmic proteins and the remaining four code for muscle actin isoforms (Fyrberg et al., 1980, 1983). In *Bombyx mori*, three actin genes - A1, A2 and A3, have been cloned and of these A1 and A2 codes for muscle actins and A3 codes for cytoplasmic actin (Mounier and Prudhomme, 1986, 1991; Mounier et al., 1987). Comparative studies on the pattern of accumulation of actin transcripts during development of *Drosophila melanogaster* and *Bombyx mori* indicated that three classes of actin genes can be distinguished in these two species: adult muscle actin genes, larval-adult muscle actin genes and cytoplasmic actin genes (Mounier and Prudhomme, 1991).

Expression of cytoplasmic actin genes was shown to play an active role in cell motility, migration, neurogenesis and axonal sprouting and outgrowth (Mizobuchi et al., 1990, Barkalow and Hartwig, 1995; Haendel et al., 1996; Mogilner and Oster, 1996; Kislauskis et al., 1997). Mizobuchi et al., (1990) reported a significant increase in the actin mRN A in the neuronal growth cones of the regenerating optic nerve of frog retinas following axotomy. They correlated this increase in actin message to axonal outgrowth. Microfilament disruption by cytochalasin D during neurogenesis in the cultures of avian neural crest cells, arrested the movement of these cells and caused them to round up (Haendel et al., 1996). Studies on the cultured *Drosophila* cells revealed a marked increase in the synthesis and accumulation of cytoplasmic actin coincident with morphological transformation induced by ecdysterone (Vitek et al., 1984). Insect embryogenesis and pupation are characterized by high mitotic activity and cell movements which require large amounts of cytoplasmic actin (Mounier and Prudhomme, 1991). Mounier and Prudhomme, (1991) reported a precise developmental regulation of the expression of actin genes in *Bombyx mori*. These genes are principally expressed in embryo, silk gland and gut of larvae and whole body pupa. In the present study, a marked increase was observed in the P-actin content of CNS during pupal development as compared to the larval stages. This

increase in actin content might be due to the addition of microfilaments during the reorganization and development of the adult nervous system.

Studies on *Drosophila* cell lines and tissues from other insects, demonstrated that actin gene expression is regulated/modulated by the ecdysteroid - 20E (Couderc et al., 1982, 1983, 1987; Berger and Morganelli, 1984; Shanavas et al., 1996). Courgeon (1972) showed that when *Drosophila* cell cultures are exposed to 20E, they rapidly undergo cell shape changes, acquire motility and aggregate. Subsequently, Vitek et al., (1984) demonstrated that 20E induced morphological transformation in *Drosophila* S3 cells is accompanied by a 5-fold increase in rate of cytoplasmic actin synthesis and a 2-fold increase in actin content. Further, it was reported that the transcription rate for the cytoplasmic genes increased up to five fold upon 20E treatment, demonstrating a direct effect of the steroid hormone at the transcriptional level of these genes (Couderc et al., 1987). Observed increase in actin synthesis in the cultured CNS of *Bombyx* in the present study, in response to 20E treatment is in agreement with the above reports. Thus, it is tempting to speculate that the increase in the actin content of the CNS during pupal development might be due to the increased transcription of actin genes induced by the major reported pupal peak of 20E (Calvez et al., 1976).

Tubulin, as the major component of filamentous cellular system of microtubules, plays an important role in many aspects of eukaryotic cellular growth and activity. Functions such as maintenance of cell shape and asymmetry by cytoskeleton, mitosis, meiosis, flagellar and ciliary activity, intracellular transport and secretion depend on the microtubule system (Roberts and Hyams, 1979). In nervous system, microtubules are rather abundant and form the major structural component of axons (Buttgereit et al., 1991). The general structure of tubulin polymers consists of heterodimer of a and P-tubulins. Genes for a and β-tubulins have been characterized from several species (Cleveland, 1985). By cross hybridization with chicken cDNA clones, Sanchez et al., (1980) showed that there are at least four members of each tubulin gene family in *Drosophila melanogaster* Extensive studies on protein profiles and genetic analysis with *Drosophila* revealed that at least two members of the β-

tubulin gene family (i.e., β1 and β3) have distinct developmental specificities. Further, it was demonstrated that the pl-tubulin is the more commonly expressed form (Raff et al., 1982; Kimble et al., 1989). Hence, we assume that the major P-tubulin band cross reacted with P-tubulin monoclonal antibody in the present study is most likely the β1 isoform of *Bombyxmori* CNS.

Several studies have indicated that microtubules play a more active role in the generation and orientation of the growing axons (Tsui et al., 1984; Cheng and Reese 1985; Bridgman and Dailey, 1989) Although actin is undoubtedly important for growth cone structure, neurites continued to grow in presence of cytochalasin B (actin depolymerizing drug), suggested that microtubule polymerization or extension may itself be able to drive axonal growth (Marsh and Letourneau, 1984; Bently and Toroian-Raymond, 1986). Further, a direct correlation was demonstrated between longitudinal growth of axons in developing neurons with that of high levels of tubulin expression (Hoffmann, 1988). In the light of these results, the increase in the P-tubulin content observed in the CNS of *Bombyx* from early to late-last instar can be correlated to the dramatic increase in the length of the interganglionic connectives during the last instar larval growth. Similarly decrease in the p-tubulin content during pupation might be due to resorption of some of the connectives between the ventral ganglion undergoing fusion during this period.

An interesting observation in the present study, was the expression of a specific tubulin isoform cross-reacting with the antibody in the pupal CNS. Based on the earlier reports on the characterization of p-tubulin isoforms and their pattern of expression in *Drosophila*, we suggest this isoform to be β3 tubulin (Wandosell and Avila, 1985; Montpied et al., 1988; Kimble et al., 1989; Buttgeriet et al., 1991). Expression of this isoform begins in the early-pupal stage and reaches a maximum in mid-pupa and declines in late-pupa before disappearing in the adult. A similar pattern was observed during pupal development in *Drosophila* but this study was carried out with the whole body pupal extracts and information on the pattern of tissue specific expression is not available (Buttgereit et al, 1991). As suggested in the ' multi-tubulin

hypothesis ' of Fulton and Simpson (1976), appearance of new isoforms could be correlated with different microtubule functions. Based on the extensive studies on the timing and distribution of p3-tubulin expression in *Drosophila*, Kimble et al., (1989) proposed that this isoform may be important for the assembly and functioning of cytoplasmic microtubule arrays in cells undergoing rapid changes in cell shape and/or tissue organization. Specific expression of this isoform in the CNS of *Bombyx mori* seen in the present study during pupation, a stage characterized by drastic reorganization and remodelling lends, support to this proposal.

Studies on the hormonal regulation of P-tubulin synthesis in Kc cell lines indicate that at least one of the tubulin gene ((33) is regulated by 20E (Berger et al., 1980; Sobrier et al., 1986, 1989; Montpied et al., 1988; Chapel et al., 1993). However, recently Rybczynski and Gilbert (1995) reported that prothoracicotropic hormone (PTTH) stimulates the synthesis of 3-tubulin in the prothoracic glands of *Manduca sexta* and suggested that P-tubulin levels may play an important role in ecdysteroidogenesis, by influencing the dynamics of microtubule-dependent secretion or interorganelle movement of ecdysteroid precursors. Hormonal studies with the cultured CNS of *Bombyx* in the present study also demonstrate a stimulatory role for 20E but its not clear whether all the isoforms of P-tubulin are stimulated or not. Further studies are required to address this aspect However, it is interesting to note that the developmental expression of p3-tubulin isoform observed in the present study correlates with the reported 20E levels in the haemolymph *of Bombyx mori* (Calvez et al., 1976).

Phosphorylation has been suggested to play an important role in regulating neuronal cytoskeleton dynamics (Julien and Mushynski, 1983; Vallano et al., 1986, Wandosell et al., 1987; de Freitas et al., 1995). Gard and Kirschner (1985) described the phosphorylation of a P-tubulin isoform in mouse neuroblastoma cell lines. Further studies revealed that expression of this isoform was associated with neuronal commitment, where as its phosphorylation occurred during neurite outgrowth (Edde et al., 1987; Diaz-Nido et al., 1991). Yamamoto et al., (1985), for the first time

reported that phosphorylation of tubulin inhibited microtubule assembly. So far investigations on the protein phosphorylation systems in insects has not identified tubulin as a substrate for any of the kinases and in the present study also we did not notice any phosphorylation of tubulin in the CNS protein extracts of *Bombyx mori*

Investigations on the hormonal control of changes in CNS during postembryonic development in insects have been primarily concerned with effects of ecdysteroids and very little work has been done on the role of JH (Riddiford, 1994). In the larval stages JH appears to have no action on its own on the CNS but only when paired with ecdysteroids. Many of the processes - changes in the form of sensory and motoneurons, maturation of immature neurons, and some aspects of neuronal death - were prevented if JH was given along with ecdysteroids (Truman, 1988). Thus in a very real sense JH is indeed a "status quo" hormone for the nervous system (Williams, 1961) It has been proposed that JH is most likely involved in early determinative events that occur during the cell's response to ecdysteroids (Truman, 1988, 1996). These determinative events might involve synthesis of specific proteins resulting from the activation of JH inducible genes. Some of the proteins induced by JH treatment in late-larval stage of *Bombyx* CNS identified in the present study might play a role in nervous system functioning and development but further work is needed to establish and evaluate their precise roles.

Naturally occurring cell death or programmed cell death is an important feature of developing and metamorphosing nervous system of insects which regulates the neuronal population (Truman, 1984; Booker and Truman, 1987a, Fahrbach and Truman, 1987). The steroid hormone, 20E plays an important role in controlling post-metamorphic neuronal cell death in *Manduca sexta* (Truman and Schwartz, 1984). *In vivo* and *in vitro* studies using RNA and protein synthesis inhibitors revealed that *de novo* synthesis was essential for postembryonic neuronal death (Fahrbach and Truman, 1987). During pupal-adult transformation, the CNS contains a mixture of neurons that will survive during adult life, neurons that are already degenerating, and neurons that are committed to die but have not yet begun the process of degeneration (Truman, 1983; Truman and Schwartz, 1984). Thus, any changes in protein pattern

detected at this time could reflect changes in protein expression in any of these cell populations. Montemayor et al., (1990) have identified a 40 kDa protein in *Manduca sexta* during pupal-adult transformation and they have implicated its appearance to neuronal death. In the present study we have identified a novel 235 kDa acidic polypeptide in the CNS of *Bombyx mori*, which shows a precise developmental regulation. The synthesis of this polypeptide begins at pharate adult stage and it may play a role in programmed cell death. However, the apparent molecular weight of the newly synthesized polypeptide in the CNS of *Bombyx* seems to be nearly six times greater than that reported for *Manduca* (Montemayor et al., 1990) Further studies on the identification of this protein and its cellular distribution should provide an insight into whether its expression is actually related to the commencement of neuronal death.

SUMMARY AND CONCLUSIONS

1. Rate of protein synthesis was found to be high during the late-last instar and late-pupal stages of postembryonic development.

2. β-tubulin content of the CNS was found to be high during larval stages and low during pupation. Decreased content of β-tubulin in pupal stages might be due to the resorption of the interganglionic connectives between the ventral ganglia undergoing fusion.

3. A specific β-tubulin isoform (β3) expression has been found in the CNS during the pupal stages. The pattern of its expression correlates with circulating 20E titer in the haemolymph of *Bombyx*. This isoform might play an important role in organization and orientation of the microtubules during CNS reorganization and remodelling in the development of the adult nervous system.

4. The increased accumulation of P-actin in the CNS during pupal stages suggest an active role for microfilaments in cell shape changes and nerve cord shortening during neurometamorphosis.

5. Studies on the hormonal regulation reveals that 20E exerts a stimulatory role in the synthesis of actin and tubulin proteins.

6. Studies on the 235 kDa protein revealed a precise stage specific expression. This protein might play an active role in programmed cell death.

PART - II

STUDIES ON PROTEIN PHOSPHORYLATION SYSTEMS IN THE CENTRAL NERVOUS SYSTEM OF *BOMBYX MORI* DURING POSTEMBRYONIC DEVELOPMENT

CHAPTER 1

INTRODUCTION

The presence of phosphorus in proteins has been known for almost 100 years, but the importance has only been realized since the discovery of enzyme regulation by reversible protein phosphorylation. The current excitement stems from the independent work of Krebs ; and Larner's group over the period 1955-70, who discovered that the neural and hormonal control of glycogen metabolism in skeletal muscle was mediated by changes in the phosphorylation state of glycogen phosphorylase (Krebs and Fischer, 1956), phosphorylase kinase (Krebs et al., 1959) and glycogen synthase (Friedman and Larner, 1963). These three enzymes remained as the only examples of this phenomenon until late 1960s, but the situation changed rapidly following the discovery of cAMP-dependent protein kinase (Walsh et al., 1968). The past twenty years have witnessed an extraordinary and still accelerating growth in this area.

Protein phosphorylation systems consists of three primary components: a protein kinase, a protein phosphatase, and a substrate protein. The substrate protein is phosphorylated 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 addition of charged phosphate group to the protein changes its function, presumably by changing its structure. The phosphate group is to be removed by a protein phosphatase. Protein phosphorylation is now recognized to be a major regulatory mechanism by which neural activities are controlled by external physiological stimuli (Cohen, 1982; Nestler and Greengard, 1984; Browning et al., 1985). Considerable evidence now suggests that most and possibly all of the effects of second messenger actions of cAMP, cGMP, as well as many of the second messenger actions of calcium on neuronal functions are achieved through the activation of specific cAMP or cGMP-dependent or calcium-dependent protein kinases. Vertebrate brain contains two types of cAMP-dependent protein kinases (Miyamoto et al., 1969; Walter and Greengard, 1981) and one type of cGMP-dependent protein kinase (Kuo and Greengard, 1970, Kuo and Shoji, 1982), but has 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). Multiple Ca^{2+}/calmodulin-dependent protein kinases have been identified in the brain tissue of different species (Rosen and Krebs, 1981; Kikkawa et al., 1982; Kennedy et al., 1983; Nestler and Greengard, 1984; Nairn et al., 1985a, Palfrey et al., 1985; Hidaka and Ishikawa, 1992).

Although extensive investigations on protein phosphorylation systems have been carried out with the vertebrate nervous system, very little information is available for the insect nervous system. However, in the last decade, several studies have been initiated on this aspect and cyclic nucleotide-dependent protein kinases have been well characterized in insects. The cAMP-dependent protein kinases (cAMP-PKs) are ubiquitous proteins and the highly conserved structure of these kinases has been demonstrated in various species (Beebe and Corbin, 1986; Taylor et al., 1990). Properties of cAMP-dependent protein kinases of insects appear to be very similar to those of vertebrates (Foster et al., 1984; Combest and Gilbert, 1986a, 1989, Altfelder and Muller, 1991, Inoue and Yoshioka, 1997).

cAMP and cGMP-dependent protein kinases have been partially purified and characterized from various tissues and species of Arthropoda (Kuo et al., 1971). On the other hand, cAMP-PK activity has been characterized in the brain of several insects like, *Ceratitis capitata* (Haro et al., 1982), *Manduca sexta* (Combest and Gilbert, 1986a; 1989), *Apis mellifera* (Altfelder and Muller, 1991; Muller, 1997a), *Schistocerca gregaria* (Rotondo et al., 1987) and *Drosophila melanogaster* (Foster et al., 1984, Hesse and Marme, 1985, Adam and Friedrich, 1988; Inoue and Yoshioka, 1997). cAMP-PK purified from the whole body of *Bombyx mori* pupae was shown to be capable of phosphorylating rabbit skeletal muscle glycogen phosphorylase kinase and glycogen synthase, resulting in the activation and inactivation of respective enzymes (Nishiyama et al., 1975).

Genetic, biochemical and pharmacological evidences indicate an important role of cAMP-PK mediated phosphorylation in processes of learning and memory in

Drosophila (Aceves-Pina et al., 1983; Folkers and Spatz, 1984; Muller and Spatz, 1989; Drain et al., 1991; Muller, 1997a) and honeybee, *Apis mellifera* (Menzel et al., 1989; Sugawa et al., 1989; Muller, 1997a). Induced expression of a peptide inhibitor of cAMP-PK in transgenic *Drosophila* disrupted the ability of flies to learn an odour discrimination task (Drain et al., 1991). Immunocytochemical studies in *Drosophila* and *Apis* brain showed a 3-4 fold increase in cAMP-PK activity in mushroom bodies, when compared to other neuropile structures, in associative olfactory learning and memory (Muller, 1997a). In the sea mollusc, *Aplysia*, cAMP-PK was shown to modulate the function of ion channels during sensitization (Castellucci et al., 1982; Byrne, 1985; Byrne et al., 1991). In *Hermissenda*, microinjection of cAMP-PK into type B photoreceptors caused an increase in membrane resistance and this enhancement of light induced depolarization was found to be similar to the effects elicited by behavioural training (Alkon et al., 1983).

In addition to its role on neural function, cAMP-PK has also been reported to play essential role in insect development. Mutational analysis in *Drosophila* has shown that the adult female heterozygous for a strong and weak cAMP-PK allele fails to lay eggs and show a novel defect in oogenesis. Individuals zygotically null for cAMP-PK die as morphologically normal first-instar larva, implying that maternally encoded protein, which perdures for at least 12 h suffices for embryogenesis (Lane and Kalderon, 1993). Elevation of cAMP and the resultant activation of cAMP-PK has been reported to be a trigger for glial-to-neural cell-fate transition within the median neuroblast lineage in grasshopper (Condron and Zinn, 1995). Recent studies on *Drosophila* suggested that cAMP-PK is essential for limb development (Jiang and Struhl, 1995; Lepage et al., 1995). The prothoracic glands of *Manduca sexta* have been an advantageous model for investigating the cellular mechanisms underlying hormone-stimulated ecdysteroid secretion in insects (Rybczynski and Gilbert, 1995). The cerebral neuropeptide, prothoracicotropic hormone (PTTH) is currently thought to activate the prothoracic gland via calcium-dependent increase in cAMP synthesis, activation of cAMP-PK and protein phosphorylation (Smith et al., 1986; Gilbert et al., 1988; Smith and Gilbert, 1989; Keightley et al., 1990; Combest and Gilbert, 1992). PTTH enhances cytoplasmic cAMP content and appears to increase the amount of

cAMP bound to the regulatory subunit of cAMP-PK. This, in turn stimulates protein phosphorylation and ecdysteroid secretion by the glands and this is blocked by cAMP-PK inhibitors (Smith et al., 1996).

Among insects, cGMP-dependent protein kinase (cGMP-PK) has been well characterized in *Drosophila* and was found to be a dimer with an amino-terminal dimerization domain (Foster et al., 1996). Two *Drosophila* genes encoding products related to cGMP-PK have been isolated and the conserved position of the introns on these genes strongly suggest a common progenitor for these two genes (Kalderon and Rubin, 1989). Activity of this kinase was highest in heads of the flies and lowest in the embryos (Foster et al., 1996). cGMP-PK has also been partially purified from *Manduca* brain (Morton and Truman, 1986). Fewer biological roles have been established for cGMP. However, marked tissue variation in the concentration of cGMP-PK indicates that cGMP and its protein kinase have a more limited role in the regulation of cell function (Nestler and Greengard, 1984; Wang and Robinson, 1995). Recent experiments established a clear role for cGMP-PK in nitric oxide (NO) signalling in the nervous system of both mammals and insects (Desole et al., 1994; Bicker et al., 1996; Rodriguez-Pascual et al., 1996; Muller, 1997b; Wang and Robinson, 1997). In the honeybee, the NO/cGMP system in the antennal lobes has been implicated in the adaptive mechanisms during chemosensory processing (Muller and Hildebrandt, 1995). Experiments have also demonstrated that the action of eclosion hormone on the nervous system during the pupal ecdysis in *Manduca sexta* was mediated by cGMP (Truman, 1971) and the hormone-stimulated increase in cGMP resulted in the phosphorylation of two CNS proteins of molecular mass of 54 kDa (Morton and Truman, 1986, 1988).

Although for many years calcium has been known to be an intracellular second messenger for a wide variety of physiological processes, intracellular effectors of calcium activation remained obscure In recent years, it has become apparent that many of the cellular actions of calcium are mediated by its binding to specific protein kinases resulting in their activation (Nestler and Greengard, 1984). Mechanism of action of calcium differs in several respects from the mechanism of action of cAMP

and of cGMP cAMP and cGMP act through one type of binding protein. In contrast, calcium acts through any one of the several intracellular binding proteins or in conjunction with various lipids. Furthermore, the interaction of cAMP or cGMP to its binding protein results in one effect (i.e., activation of respective protein kinase) whereas the binding of calcium to its receptor proteins results in many effects, including the activation of several enzymes (such as protein kinases). cAMP and cGMP activate only one species of respective protein kinase, whereas calcium activates several distinct species of protein kinases (Takai et al., 1979; Nairn et al., 1985b; Hidaka and Ishikawa, 1992). These observations suggested that calcium plays a more general or widespread role in cell function than do cAMP or cGMP. Multiple pathways available for calcium activation presumably enable the cell to regulate each of these pathways separately (Nestler and Greengard, 1984). Thus, regulation of protein phosphorylation is one of several mechanisms by which calcium exerts its intracellular actions. Two classes of calcium-dependent protein kinases are calcium/phosphatidylserine-dependent protein kinase (PKC) and calcium/calmodulin -dependent protein kinase (CaM kinase).

Nishizuka and colleagues (1979, 1988) discovered a new species of calcium-dependent protein kinase that is activated in conjunction with lipids. This enzyme, referred to as PKC, has been found in a wide variety of animal tissues and phyla (Kuo et al., 1980; Minakuchi et al., 1981). It phosphorylates an array of tissue specific endogenous substrate proteins (Wu et al., 1982; Nestler and Greengard, 1984; Choi et al., 1991). Total activation of PKC requires the presence of calcium and two types of lipid molecules, namely diacylglycerol (DAG) and phospholipid, such as phosphatidylserine (Kikkawa et al., 1982). Several isoforms of PKC have been identified in mammals (Nishizuka, 1988, 1995). Though in insects PKC has been characterized in *Drosophila*, details on isoforms are not available (Devay et al., 1989; Schaeffer et al., 1989). Three different genes of PKC have been identified in *Drosophila* and all are expressed in head tissue (Rosenthal et al., 1987; Schaeffer et al., 1989) and deduced amino acid sequences are quite similar to each other and to the mammalian PKC family (Schaeffer et al., 1989).

PKC's have been extensively characterized biochemically in mammals. However, the specific pathways in which they function are poorly understood and also the reason for the existence of so many different isozymes. Examples of biological processes mediated and or modulated by PKC in CNS include (1) release of neurotransmitters (Tanaka et al., 1984), (2) long-term potentiation (Malinow et al., 1988) and (3) cell growth & proliferation (Persons et al., 1988). PKC activation induces conductance changes in the photoreceptors like those seen in associative learning in the sea mollusc, *Hermissenda* (Farley and Auerbach, 1986). The *Drosophila* learning mutant, *turnip* was reported to have significant reduction in PKC activity and was also deficient in the phosphorylation of a 76 kDa membrane protein in head, which is a major endogenous substrate for PKC in wild type flies (Choi et al., 1991). Recent experiments with transgenic flies specifically inhibited for PKC resulted in the disassociation of acquisition of learning and memory from the performance of task (Kane et al., 1997). PKC has also been reported to be very essential for light adaptation in *Drosophila* photoreceptors (Hardie et al., 1993) and for outgrowth of type I and II processes of cultured mature neurons (Broughton et al., 1996a). Recently, a 86 kDa PKC specific F-actin binding substrate protein has been purified from the neural tissue of *Apis mellifera* and was shown to be closely related to myristoylated alanine-rich C kinase substrate (MARCKS) of bovine brain (Muller, 1997c).

Calmodulin (CaM) is a ubiquitous low molecular weight protein present through out the animal kingdom. Studies on the mechanism of its action indicated that calcium binds and thereby induces structural changes in CaM. The Ca^{2+}/CaM complex then binds and alters the function of other cellular proteins (Carafoli, 1987; Cheung, 1980; Heizmann and Hunziker, 1990). CaM in effect, confers calcium sensitivity on other proteins which in the absence of CaM are unresponsive to calcium. 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). Several Ca^{2+}/CaM-dependent protein kinases (CaM kinases) have been demonstrated in the neural and non-neural tissues and protein phosphorylation has been considered

as one of the important routes by which Ca^{2+}/CaM signal transduction regulates cellular function. Multiple types of CaM kinases have been characterized in mammalian systems. They include CaM kinase 1 (Nairn and Greengard, 1987), II (Kennedy and Greengard, 1981), III (Nairn et al., 1985a), 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 characterized 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 I (Kennedy and Greengard, 1981). It is highly concentrated in forebrain neurons including neurons in hippocampus, where it constitutes approximately two percent of the total protein (Erondu and Kennedy, 1985). Rat brain CaM kinase II comprises of several related isozymes (Hanson and Schulman, 1992). The rat isozymes consist of a catalytic domain, an autoregulatory 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). These isozymes are encoded by separate genes and differ mainly in a region between the regulatory and association domains (Hanson and Schulman, 1992). 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). It was shown that CaM-dependent autophosphorylation of CaM kinase 11 induces a conformational change in the region of the CaM binding domain which allows additional stabilizing interactions with CaM (Putkey and Waxham, 1996). It has been postulated that this autophosphorylation may be involved in prolonging the effects triggered by a transient calcium signal (Miller and Kennedy, 1986) and also may induce changes in the subcellular distribution of the enzyme in *Aplysia* and *Drosophila* cells (Saitoh and Schwartz, 1985; Willmund et al., 1986).

With the exception of *Drosophila*, there are no reports on the characterization of CaM kinase II of other insects. Adult *Drosophila* head contains three

species of CaM kinase II with molecular mass of 54/55, 58 and 60 kDa (Cho et al., 1991; Ohsako et al., 1993). These cross-react with anti-rat CaM kinase antibody. These isoforms are generated from a single gene by alternate splicing (Ohsako et al., 1993, Griffith and Greenspan, 1993). Both amino acid sequence and tissue specificity of the rat kinase are highly conserved in *Drosophila* (Cho et al., 1991).

As a result of extensive studies over the last few years, it was established that CaM kinase II plays a crucial role in physiological and behavioural plasticity in both vertebrates (Malenka et al., 1989, Malinow et al., 1989, Silva et al., 1992a, b; Nayak et al., 1996; Rotenberg et al., 1996; Strack et al., 1997b) and invertebrates (Willmund et al., 1986; Griffith et al., 1993; Wang et al., 1994; Broughton et al., 1996b, Kahn and Matsumato, 1997). CaM kinase II was shown to be necessary for induction of LTP in rat hippocampus (Malenka et al., 1989; Malinow et al., 1989; Strack et al., 1997b). Also mouse strains lacking the a- subunit of this kinase, induced by the " knockout " technique, were found to be deficient in long-term potentiation (LTP) and spatial learning (Silva et al., 1992a, b; Rotenberg et al., 1996, Strack et al., 1997b). Long-term changes in the properties and subcellular distribution of CaM kinase in *Drosophila* heads have been demonstrated following prolonged visual adaptation (Willmund et al., 1986). 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). In contrast to the learning mutants *dunce* and *rutabaga,* which are defective in cAMP cascade, inhibition of CaM kinase II in *ala* transformants caused increased sprouting at larval neuromuscular junctions near the nerve entry point, rather than altering the higher order branch segments. In addition, synaptic facilitation and potentiation were altered in a manner different from that observed in the cAMP mutants (Wang et al., 1994).

One class of independent protein kinases has been found to preferentially phosphorylate acidic proteins, such as casein and phosvitin, and these enzymes are referred to as casein kinases (Hathaway and Traugh, 1982). Two types of casein kinases, known as casein kinase I (CK 1) and casein kinase II (CK II), have been

identified and were found to have widespread distribution in animals (Hathaway and Traugh, 1982). CK II has been extensively studied and it was found to be a cyclic nucleotide and calcium independent protein kinase and is considered to be a tetramer with a a' β_2 or α_2 β_2 form (Tuazon and Traugh, 1991). It is stimulated by polyamines such as spermine, spermidine and inhibited by heparin (Jacob et al., 1983; Hathaway and Traugh, 1984). In insects, CK II activity has been characterized in *Drosophila* (Glover et al., 1983; Birnbaum et al., 1992), and in the house cricket, *Acheta domesticus* (Degrelle et al., 1997). Analysis of CK II genes in *Drosophila* indicates a high degree of evolutionary conservation (Saxena et al., 1987).

Little is known about the functional roles of CK 11 in nervous tissue but few studies have speculated a role in hormonal regulation (Grande et al., 1988; Kandror et al., 1989) and processes of cell proliferation and transformation (Issenger, 1993). It is interesting to note that under *in vitro* conditions, several proteins were phosphorylated by CK II in the brain homogenates of *Acheta domesticus* (Degrelle et al., 1994) and that calmodulin and tubulin have been demonstrated to be substrates of CK II in the prothoracic glands of *Manduca sexta* (Song et al., 1994). More recently, it was also shown that *in vivo* activity of HOX protein *antennapedia* of *Drosophila* was modified because of the phosphorylation by CK II (Jaffe et al., 1997).

Protein tyrosine kinases (PTKs) are relatively recently discovered class of enzymes which specifically catalyze the phosphorylation of proteins at tyrosine residues. PTK activities have been shown to be intrinsic to the oncogene products of certain retroviruses (Hunter and Cooper, 1985) and to the receptors of several mitogenic polypeptide growth factors including epidermal growth factor (EGF), platelet derived growth factor (PDGF), insulin and fibroblast growth factor (Yarden and Ullrich, 1988). In addition, high levels of PTK activities, which are not related to either growth factor receptors or retroviral oncogene products, have also been detected in normal, uninfected cells and tissues (Swarup et al., 1983; Srivastava, 1990).

The expression of high levels of PTK activities in several differentiated and even in non-nucleated cells suggests that these kinases play important roles not only in oncogene and growth factor mediated mitogenesis, but are also involved in the regulation of differentiated and growing cells (Srivastava, 1990). Several receptor tyrosine kinases (RTKs) have been identified in *Drosophila*. The specification of the R7 photoreceptor cell in the developing eye is dependent upon the activation of *sevenless* receptor tyrosine kinase (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, including photo-receptor determination, wing formation, pattern formation at the anterior and posterior termini of the embryo (Doyle and Bishop, 1993; Duffy and Perrimon, 1994; Schweitzer and Shilo, 1997). A recent study suggested that bacterial lipopolysaccharide-stimulated exocytosis of non-self recognition protein from insect haemocytes depend on protein tyrosine phosphorylation (Charalambidis et al., 1995). Insulin receptor-like tyrosine kinase activity has also been reported in the prothoracic glands of *Manduca sexta* (Smith et al., 1997).

Few preliminary reports are available indicating regulation of protein kinase activity by insect hormones. Metamorphic hormone, 20-hydroxyecdysone (20E) has been shown to stimulate the *in vitro* phosphorylation of few fat body proteins of *Mamestra brassicae* (Sass, 1988) and *Sarcophaga peregrina* (Itoh et al., 1985). Injection of 20E into *Acheta domesticus* inhibits CK II activity in the brain (Degrelle et al., 1997). Juvenile hormone I (JH I) was shown to be involved in the activation of a specific Na',K - ATPase via PKC in the follicle cell membranes of *Rhodinus prolixus* (Sevala and Davey, 1989). Yamamoto et al., (1988) have reported the stimulatory role of JH on protein synthesis in male accessory glands of *Drosophila* through the activation of PKC. Eclosion hormone which triggers pupal ecdysis has been shown to stimulate the cGMP-PK activity in the CNS of *Manduca sexta* (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

The analysis of the physiological functions of CaM kinase II has drawn special attention over the last few years. Three considerations justify this special interest in its role in neuronal signal transduction: (i) it is highly concentrated in brain and is localized on both sides of the synapse, where events central to neurotransmission are likely to be directly regulated, (ii) many of its substrates are involved in neuronal signalling - neurotransmitter release, synthesis and LTP, (iii) brief calcium signals activate this kinase and stimulate an autophosphorylation which allows the kinase to maintain its activated state beyond the duration of a particular calcium signal, and this would play an important role in synaptic plasticity. Although extensive investigations on CaM kinase II have been carried out with the vertebrate nervous system, little information is available for the insect nervous system. With the exception of *Drosophila,* there are no reports on the characterization of this kinase in other insects. The less complex insect nervous system may reveal more clearly the many roles of CaM kinase II in cellular functions. As characterization of the protein kinase is the first logical step in investigating its functional role, in the present study, an attempt was made to identify and characterize this multifunctional kinase in the CNS of the silkworm, *Bombyx mori* and to identify its endogenous substrate proteins. Since there are no reports on the localization of CaM kinase II protein in insects including *Drosophila,* the present study also makes an attempt towards this end. Finally, the present investigation also addresses the probable role of major insect hormones on general protein phosphorylation in *Bombyx* nervous system.

CHAPTER 2

MATERIALS AMD METHODS

CHEMICALS

[γ^{32}P]ATP (3000 Ci/mmole) was obtained from Bhabha Atomic Research Centre, Trombay, India EDTA, EGTA, DTT, calmodulin, HEPES buffer, cAMP, cGMP, phosphatdylserine, diacylglycerol and CaM kinase II substrate peptide - syntide 2 were obtained from Sigma Chem. Co. (St. Louis, MO, USA). Okadaic acid was obtained from GIBCO-BRL (Ohio, USA). Alkaline phosphatase-conjugated anti-mouse secondary antibody, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were from Pierce (Rockford, IL, USA). Monoclonal rat anti-CaM kinase II a antibody was purchased from Boehringer Mannheim (Darmstadt, Germany). Monoclonal anti-phosphotyrosine antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). P-81 phosphocellulose sheets were purchased from Whatman (Maidstone, UK). KN-62(1-[*N,O*-bis(5-isoquinoline-sulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpipera-zine) was a gift from Dr. H. Hidaka of Department of Pharmacology, Nagoya University School of Medicine, Japan The sources of all other chemicals was the same as mentioned in part I- Chapter 2.

METHODS

Protein estimation:

Protein was estimated according to the microprotein assay method of Bradford (1976) mentioned in Part I - Chapter 2.

Preparation of tissue sample:

Intact CNS (brain + ventral nerve cord) was rapidly dissected from animals of different developmental stages, frozen in liquid nitrogen and stored at -70° C till use. Frozen tissue was hand homogenized (4 CNS/50 µl) in 50 mM HEPES buffer (pH

7.4) containing 1 mM EDTA and 1 mM DTT using an all glass microhomogenizer (Kontes). Homogenates were centrifuged at 1000 x g for 5 min to remove debris. Fat body and thoracic muscle homogenates were also prepared in similar manner. Freshly prepared homogenates were used for phosphorylation reaction.

***In vitro* phosphorylation of endogenous proteins in *Bombyx* tissue homogenates:**

Incubations were carried out in 1.5 ml Eppendorf tubes in a total volume of 40 µl, containing 20 ug of CNS homogenate protein. The reaction mixture consisted of 50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT, 10 uM 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 µCi of [γ^{32}P]ATP. After 1 min, reaction was terminated by the addition of 20 µl 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 ug protein) was subjected to SDS-PAGE (Laemmli, 1970). The gels were silver stained (Blum et al., 1987) as mentioned in Part 1 - Chapter 2

Phosphorylation reactions were also carried out under similar conditions to study the effect of calmodulin (2 uM), phosphatdylserine (10 uM) + diacylglycerol (100 µM), cAMP (10 µM), cGMP (10 uM), KN-62 (10 uM), okadaic acid (1-500 nM), juvenile hormone I (7 x 10^{-8} M) and 20-hydroxyecdysone (5 x 10^{-6} 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.

Western blotting and immunostaining:

Proteins electrophoresed by SDS-PAGE were electroblotted on to a nitrocellulose membrane as mentioned in Part 1- Chapter 2. After the transfer, membrane was air dried and incubated for 1 h at room temperature with 3% (w/v) bovine serum albumin (BSA) in TBS (10 mM Tris, 150 mM NaCl, pH 7.5) to block non-specific binding sites. The blot was incubated overnight with 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 then 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).

Assay of CaM kinase II in *Bombyx* CNS homogenates:

CaM Kinase II activity was assayed by the phosphorylation of syntide 2 essentially 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/pmol), 30 μM syntide, 2 μM calmodulin, 1 mM $CaCl_2$, 0.1 mM EGTA and suitable amounts of homogenate protein in a total volume of 50 ul. A control reaction was carried out in the presence of 1 mM EGTA. After an incubation at 30° C for 1 min, the reaction was terminated by adding 10 ul of 0.4 M EDTA and a 50 ul aliquot was spotted on 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 (2 min each) in phosphoric acid with gentle agitation. After drying in an oven (100° C, 5 min), the radioactivity was measured by liquid scintillation spectrophotometry using toluene scintillation fluid (0.5 gm POPOP, 5 gm PPO/ litre of toluene). Assay conditions were standardized using CNS homogenates from freshly emerged adult moths.

CaM kinase II immunocytochemistry:

(i) *Tissue preparation:* The CNS of freshly emerged adults were dissected in ice-cold TBS (0.1 M, pH 7.4) and fixed in Bouin's fixative for 8 h. Tissue was washed in TBS, dehydrated through graded series of ethanol, cleared in xylene, and embedded in paraffin, following standard histological technique. Ten micron thick sections were prepared, deparaffinized, rehydrated and were processed for immunocytochemical staining.

(ii) *Immunocytochemical staining:* The sections were first incubated with 1% non-immune goat serum to block nonspecific binding sites. Then they were sequentially incubated with monoclonal anti-CaM kinase II antibody (10 μg/ml), for 24 h at 4° C, alkaline phosphatase-conjugated goat anti-mouse IgG (1:100) for 1 h. All antibodies were diluted in 50 mM TBS (pH 7.5) containing 1% non-immune goat serum. Each incubation with antibody was followed by three 10 min washes in TBS. These sections were finally stained with NBT/BCIP as described by Meltzer et al., (1997). Staining specificity was assessed by replacing the primary antibody with the IgG fraction derived from non-immune mouse serum. Slides were photographed under a Nikon Labophot II microscope.

Back phosphorylation:

Back phosphorylation was carried out as described by Forn and Greengard (1978). Intact CNS from late-last instar larvae was dissected out and incubated in TC-100 insect culture medium for 4 h to deplete the endogenous hormone. Tissue was incubated with 7 x 10^{-7} or 7 x 10^{-8} M JH I in fresh culture medium for 1 and 2 h. Equal volume of the carrier solvent (0.1 % ethanol) was added to the control cultures. At the end of this incubation, CNS was removed from the culture vials, rinsed thoroughly with insect Ringer, homogenized and subjected to *in vitro* phosphorylation.

Liquid scintillation spectrophotometry:

The extent of [^{32}P] incorporation into individual proteins were measured by liquid scintillation spectrophotometry of labeled bands excised from the dried gel in toluene scintillation fluid in a LKB liquid scintillation spectrometer.

Molecular weight determination and densitometric scanning:

Molecular weight determination of the polypeptides separated on SDS-PAGE and densitometric scanning of the immunoblots were done as mentioned in Part I - Chapter 2.

Statistical analysis:

Statistical analysis was performed by one way ANOVA followed by comparisons of the means by student-Newmann-Keuls multiple comparison test using the Sigma Stat software. $p<0.05$ was defined as the criterion for statistical significance. The data were represented as mean ± S.D.

CHAPTER 3

RESULTS

***In vitro* phosphorylation of endogenous proteins of *Bombyx* CNS**

In vitro phosphorylation of CNS proteins from freshly emerged adults in presence of EGTA revealed the phosphorylation of few proteins of which a 48 kDa band was the major protein labeled with [^{32}P] (Fig. 1b, lane 1). Inclusion of 1 mM $CaCl_2$ stimulated the phosphorylation of several proteins, particularly 59/60 kDa (Fig. 1b, lane 2) and this stimulation was further enhanced by the addition of 2 uM calmodulin (Fig. 1b, lane 3). However, PKC activators, phosphatydlserine + diacylglycerol had no effect on the phosphorylation of this protein (Fig. 2, lane 3). This increase in phosphorylation of 58/60 kDa protein was inhibited by 80% when the homogenate was incubated for 5 min with 10 uM KN-62, a specific inhibitor of CaM kinase II (Fig. 3, lane 2) The phosphorylation of the 48 kDa band was unaffected by Ca^{2+}, CaM and other effectors.

Identification of 59 and 60 kDa proteins as isoforms of CaM kinase II

Monoclonal rat CaM kinase II a-antibody was used to identify the CaM kinase II in the CNS homogenates of *Bombyx* and the results are presented in figure 4a. In rat brain homogenate, this antibody showed strong 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 2). In *Bombyx* CNS homogenates, two closely migrating proteins of molecular weights 59 and 60 kDa cross reacted with the antibody (lane 1). Autoradiography of the immunoblot revealed incorporation of [^{32}P] into these two bands (Fig. 4b). These bands were excised separately from the blot and the [^{32}P] incorporated was measured by liquid scintillation spectrophotometry. This confirmed the autophosphorylation of CaM kinase II. Since these two bands migrate closely, the signal on the autoradiograph appears as a single band. No other protein in the CNS homogenate of *Bombyx* cross reacted with the antibody.

Dephosphorylation of autophosphorylated CaM kinase II and the effect of okadaic acid

Studies on the time course of phosphorylation of major phosphoproteins revealed maximal [^{32}P] incorporation by 1 min at 30° C. Of interest was the observation of

rapid dephosphorylation of the autophosphorylated CaM kinase II with increase in reaction time. With 5 min of incubation time the [^{32}P] incorporation decreased by 1/4th of that seen at the end of 1 min (Fig. 5), suggesting the involvement of a protein phosphatase in this process. One nanomolar okadaic acid had no effect on the dephosphorylation of the kinase (Fig. 6, lane 6) while higher concentrations (10 and 200 nM) inhibited the activity of the phosphatase in a dose dependent manner (Fig. 6, lanes 7 and 8).

(a M kinase II is more abundant in the CNS than other tissues

In addition to CNS, *in vitro* phosphorylation studies were also carried out with homogenates of fat body and thoracic muscle and the results are presented in figure 7. Protein kinase activity was found to be high in neural tissue when compared with fat body and muscle. Autophosphorylation of CaM kinase II was maximal in extracts from both brain and ventral nerve cord (Fig. 7b, lanes 1 and 2) and there was no corresponding signal in the samples from fat body and muscle (Fig 7b, lanes 3 and 4). However, longer exposure (6 days) revealed the autophosphorylation of CaM kinase II in fat body as well as muscle (Fig. 7c) and the signal was more intense in fat body than muscle. In addition, a 46 kDa protein was phosphorylated only in brain and phosphorylation of a 30 kDa protein was observed to be more in ventral nerve cord than brain (Fig. 7b). These proteins, probably are endogenous substrates of CaM kinase II.

Catalytic properties of *Bombyx* CaM kinase II

The degree of phosphorylation of syntide-2 was linear up to 15 μg of homogenate protein (Fig. 8). The rate of phosphorylation of syntide-2 by *Bombyx* CaM kinase II showed normal Michaelis-Menten kinetics with respect to the concentration of ATP and syntide-2 (Figs. 9a and b). In the presence of saturating amount of syntide-2, the K_m value for ATP was 21.3 μM. The apparent K_m for syntide-2 was 12.5 μM with a V_{max} of 2.68 nmol/min/mg homogenate protein.

Changes in the activity of CaM kinase II in the CNS of *Bombyx* during larval-pupal-adult transformation

CaM kinase II was assayed in the CNS homogenates from different developmental stages and the results are presented in figure 10. A comparison of the CaM kinase II activity revealed significant differences at different stages of development, with specific activities varying from 1.3 to 2.5 nmoles/min/mg homogenate protein. The enzyme exhibited highest activity in late-pupal stage and lowest in early-last instar larval stage. During the development, two peaks in the activity of the kinase was observed, one in the late-last larval instar and another towards the end of the pupal period.

Developmental changes in the levels of CaM kinase II

Immunoblotting of equal amount of CNS proteins extracted from various developmental stages was performed to find out correlation, if any, between the activity and quantity of the enzyme (Fig. 1 la). This study revealed that changes in the levels of CaM kinase II at various developmental stages was, in general, accompanied by a proportionate increase/decrease in the enzyme activity. Laser scanning densitometry of the immunoblot revealed that the amount of the enzyme protein was maximal in late-pupal and lowest in the early-last larval stage (Fig 11b).

Immunocytochemical localization of CaM kinase II in the CNS **of** *Bombyx*

Light microscopic study demonstrated more or less even distribution of immuno-reactivity in coronar sections of the adult brain. Cell bodies in the pars- intercerebralis and tritocerebrum showed intense staining when compared to the neuropil (Fig. 12b). Giant neurons of metathoracic ganglion in the adult, showed a strong cross reactivity in the cytoplasm and it was totally absent in the nucleus (Fig. 13b).

Juvenile hormone I stimulates the phosphorylation of the 48 kDa protein

As mentioned earlier, *in vitro* phosphorylation of CNS proteins from mid-last or late-last instar larvae in presence of 1 mM EGTA revealed the phosphorylation of a 48

kDa polypeptide (Fig.lb, lane 1). Addition of calcium or calmodulin had no effect on the degree of phosphorylation of this polypeptide (Fig.lb, lanes 2 and 3). In addition, cyclic nucleotides like cAMP and cGMP also had no effect on the phosphorylation of this protein (Fig. 14).

Since phosphorylation of the 48 kDa protein was neither dependent on calcium nor on cyclic nucleotides, it might be mediated by a tyrosine kinase. Immunoblot analysis (Fig. 15b) of the phosphorylated protein with anti-phosphotyrosine monoclonal antibody confirmed the above tenet, as the 48 kDa protein showed a clear cross reactivity with the antibody.

Incubation of the homogenate in presence of 7 x 10^{-8} M JH I, specifically stimulated the phosphorylation of the 48 kDa protein significantly (Fig. 16b, lane 2).

Back phosphorylation studies

Back phosphorylation experiments were carried out to verify whether the 48 kDa protein was also phosphorylated under *in vivo* conditions and whether JH I had a stimulatory effect on this process. The rationale in the experiment is as follows : if this protein is already phosphorylated under *in vivo* conditions in response to JH 1 treatment, then these phosphorylated sites in the protein would be occupied by endogenous unlabeled phosphate and they would be less available /unavailable for accepting labeled phosphate during the *in vitro* reactions. The pattern of phosphorylation obtained with the extracts of CNS treated with 7 x 10^{-8} M JH I for 1 and 2 h (Fig. 17, lanes 3 and 4) shows considerably less incorporation of [^{32}P] into the 48 kDa protein when compared to the solvent (0.1% ethanol) treated control (Fig. 17, lanes 1 and 2). Furthermore, treatment of CNS with higher doses of JH I (7 x 10^{-7} M) for 1 h (Fig. 17, lane 5) under similar conditions resulted in the total loss of [^{32}P] incorporation into the 48 kDa protein. The 48 kDa protein was the only one which consistently showed this change in response to the exposure to the JH 1.

Developmental profile of the 48 kDa protein phosphorylation

Developmental profile (Fig. 18) of the ***in*** *vitro* phosphorylation of the 48 kDa protein in the CNS during the last larval instar revealed that the degree of [^{32}P] incorporation was low in early-last instar (lane 1), high in mid-last instar (lane 2) and late-last instar (lane 3) larval stages and least during the prepupal (lane 4) and early-pupal (lane 5) stages.

Fig. 1 - *In vitro* phosphorylation of *Bombyx* CNS proteins: following incubation of homogenates with [$^{32}\gamma$-P] ATP under phosphorylating conditions (see Part II - materials and methods). (a) - SDS-PAGE, (b) - autoradiograph of the same gel. The incubations were under the following conditions: lane 1 : 1 mM EGTA + 2 μM calmodulin, lane 2 : 1 mM $CaCl_2$, and lane 3 : 1 mM $CaCl_2$ + 2 μMcalmodulin. Note the phosphorylation of the 59/60 kDa proteins (◄) which was significantly stimulated in presence of calcium and calmodulin. Lane M - molecular weight markers. 10 μg protein was loaded in each lane.

Fig. 2 - Autoradiograph showing the effect of PKC activators on the phosphorylation of *Bombyx* CNS proteins: Lane 1 : 1 mM EGTA, lane 2 : 1 mM $CaCl_2$ and lane 3 : 1 mM $CaCl_2$ + 10 mM phosphatydlserine and 100 mM diacylglerol. Note that PKC activators did not have any stimulatory effect on 59/60 kDa proteins (◄).

Fig. 3 - Autoradiograph showing the effect of KN-62 on the phosphorylation of CNS **proteins:** The homogenate was incubated for 5 min with 10 μM KN-62 prior to the phosphorylation reaction. Lane 1 : - KN-62 and lane 2 : + KN-62. Phosphorylation of 59/60 kDa proteins was significantly inhibited (◄). 10 μg protein was loaded in each lane.

Fig. 4 - (a) - Immunoblot analysis of the CaM kinase II in the CNS homogenates of *Bombyx:* Homogenate protein (30 μg) was phosphorylated in presence of Ca^{2+} and calmodulin, separated by SDS-PAGE and blotted to nitrocellulose membrane, followed by immunostaining (lane 1). Rat brain homogenate (20 μg) was applied in parallel as a positive control (lane 2). (b) - autoradiograph of lane 1 of the immunoblot. Note the phosphorylation of 59/60 kDa proteins which cross reacted with rat monoclonal CaM kinase II a antibody (◄).

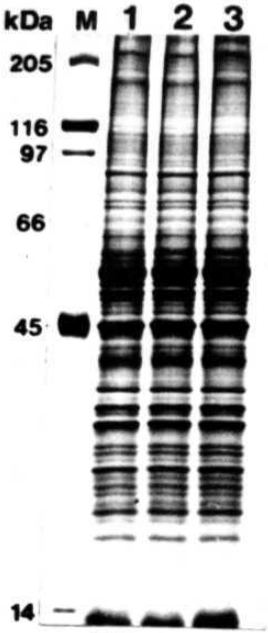

Fig.1a

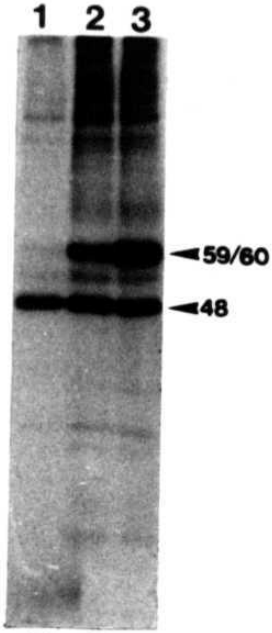

Fig.1b

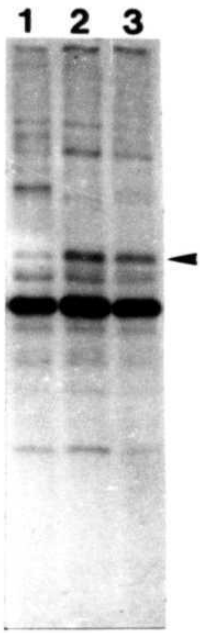

Fig.2

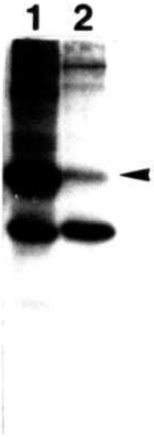

Fig.3

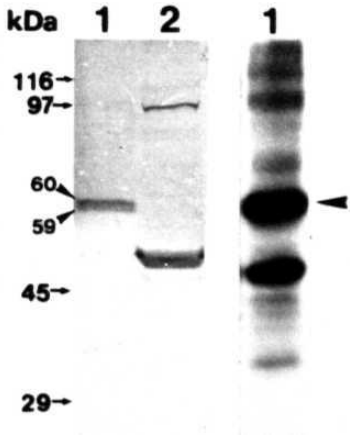

Fig.4a b

Fig. 5 - Graph showing the effect of reaction time on the dephosphorylation of major phosphoproteins of *Bombyx* CNS: Inset - Autoradiograph. Individual bands corresponding to the major phosphorylated polypeptides (46, 48, and 59/60 kDa) were excised from the dried SDS-PAGE and the extent of [^{32}P] incorporation was measured by liquid scintillation spectrophotometry. Values in CPM were plotted against reaction time.

Fig. 6 - Effect of okadaic acid (OA) on the dephosphorylation of autophosphorylated CaM Kinase II: The CNS extracts of adult were phosphorylated in presence of Ca^{2+}, calmodulin and different concentrations of OA for 1 min (lanes 1-4) and 5 min (lanes 5-8). Lanes 1 & 5 : control (- OA), lanes 2 & 6 : 1 nM OA, lanes 3 & 7 : 10 nM OA and lanes 4 & 8 : 200 nM OA (◄ denotes 59/60 kDa CaM kinase II bands). 8 µg protein was loaded in each lane.

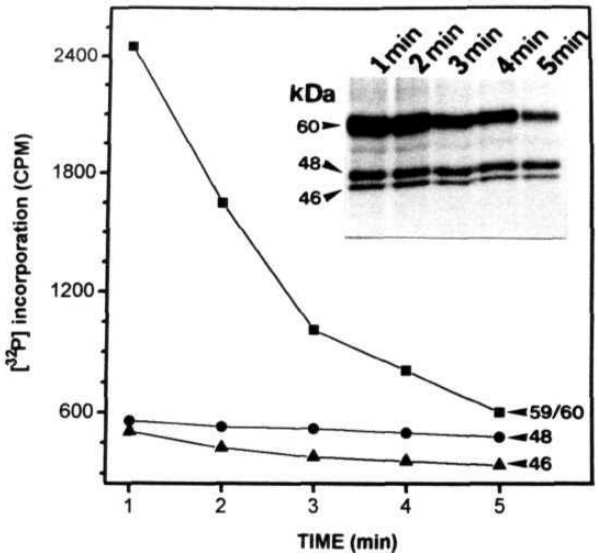
2400
1800
1200
600
[32P] incorporation (CPM)
1
2
3
4
5
TIME (min)
59/60
48
46
kDa
60
48
46
1min
2min
3min
4min
5min

Fig.5

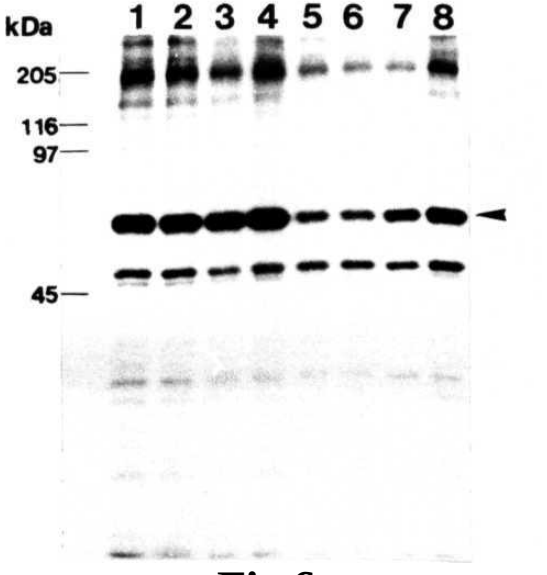
kDa
205
116
97
45
1
2
3
4
5
6
7
8

Fig.6

Fig. 7 - Autophosphorylation of CaM kinase II in various tissues of *Bombyx* - After phosphorylation reaction in presence of Ca^{2+} and calmodulin, the protein extracts were separated by SDS-PAGE (a). (b) - autoradiograph of the same gel exposed for 2 days. Note the presence of phosphoproteins (◄) with molecular weights of 59/60 (CaM kinase II), 46 kDa and 30 kDa. c - autoradiograph of lanes 3 & 4 exposed for 6 days. Lane 1 : brain, lane 2 : ventral nerve cord, lane 3 : fat body and lane 4 : thoracic muscle

Fig. 8 - Graph showing the incorporation of [^{32}P] into syntide-2 as a function of homogenate protein concentration.

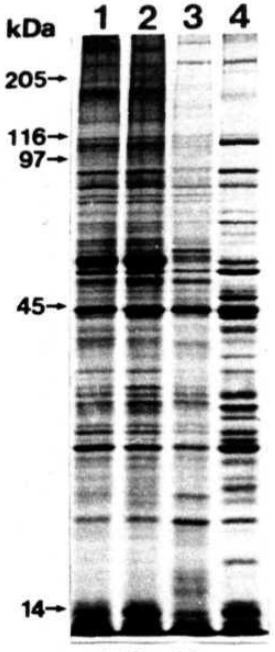

Fig.7a

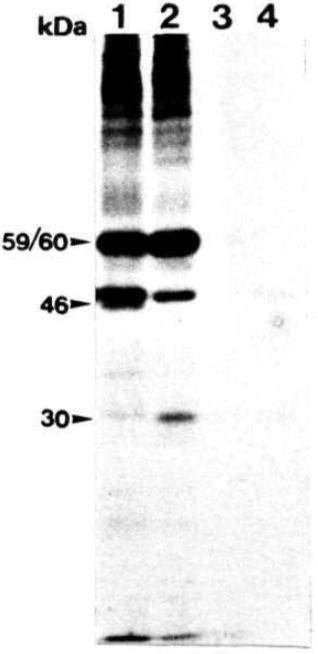

Fig.7b

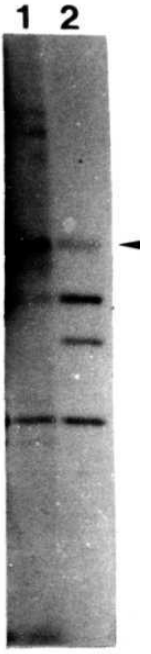

Fig.7c

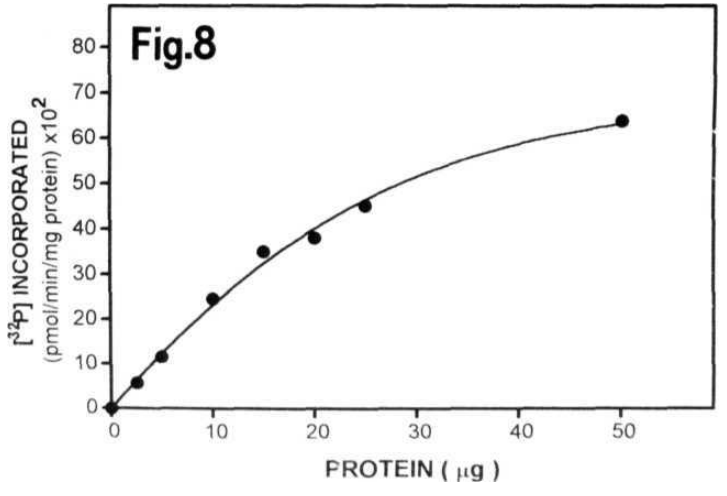

Fig. 9 - Lineweaver-Burk plots of the phosphorylation of syntide 2 by the endogenous CaM kinase II of *Bombyx* CNS - Initial rates were measured under standard conditions for 1 min, using filter paper assay. Syntide 2 concentration (a) ATP concentration (b) were varied as indicated.

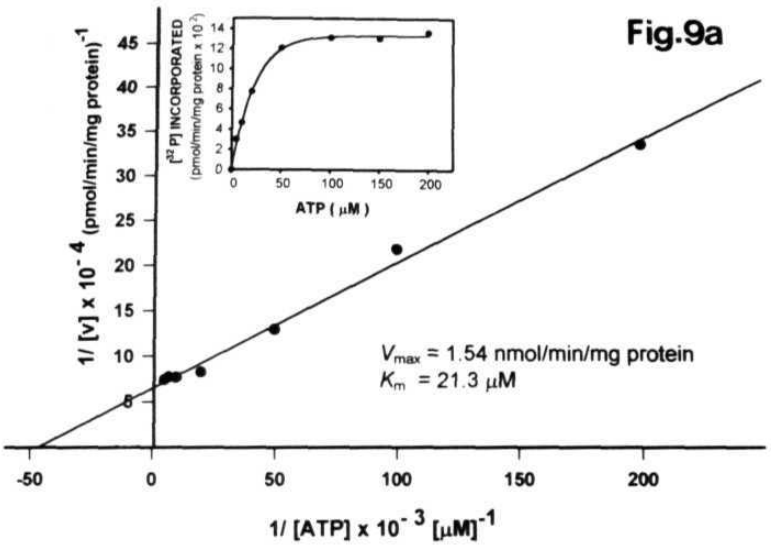
Fig.9a
1/ [v] x 10 - 4 (pmol/min/mg protein) -1
45
40
35
30
25
20
15
10
5
-50
0
50
100
150
200
1/ [ATP] x 10 - 3 [μM] -1
[32 P] INCORPORATED
(pmol/min/mg protein x 10-2)
14
12
10
8
6
4
2
0
0
50
100
150
200
ATP (μM)
Vmax = 1.54 nmol/min/mg protein
Km = 21.3 μM

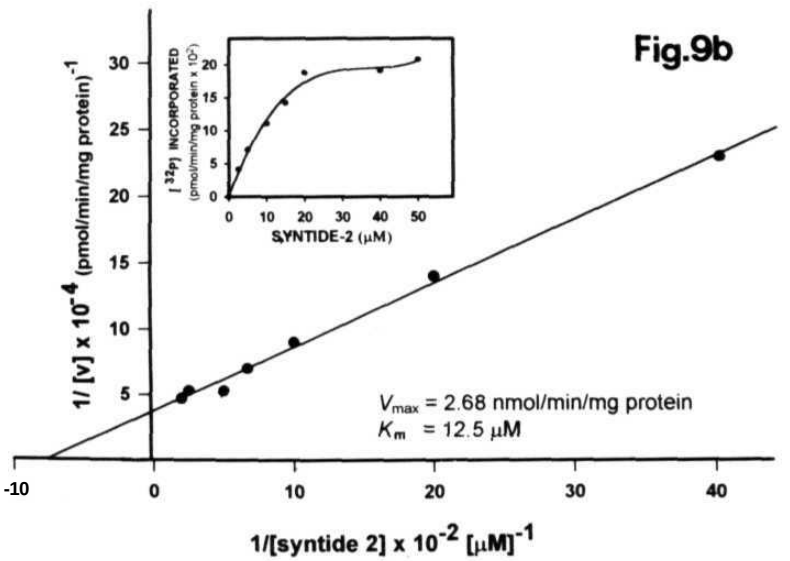
Fig.9b
1/ [v] x 10-4 (pmol/min/mg protein) -1
30
25
20
15
10
5
-10
0
10
20
30
40
1/[syntide 2] x 10-2 [μM] -1
[32P] INCORPORATED
(pmol/min/mg protein x 10-2)
20
15
10
5
0
0
10
20
30
40
50
SYNTIDE-2 (μM)
Vmax = 2.68 nmol/min/mg protein
Km = 12.5 μM

Fig. 10 - Developmental changes in the activity of CNS CaM kinase II of *Bombyx* during larval-pupal-adult transformation - Each value is the mean ± S.D. of four independent determinations. The stages used were ELI, ML1, LL1, PP, EP, MP, LP and Adult. * Significantly different from the previous developmental stage ($p<0.05$) as determined by one-way analysis of variance with Sigma Stat software.

Fig. 11 - (a) **Immunoblotting analysis of the changes in the CaM kinase II levels in the CNS during post-embryonic development of *Bombyx*** - Equal amount of homogenate protein (20 μg) from different developmental stages were loaded in each lane. Lane 1 - ELI, lane 2 - MLI, lane 3 - LL1, lane 4 - PP, lane 5 - EP, lane 6 - MP, lane 7 - LP and lane 8 - Adult.

(b) histogram showing the quantitative changes in CaM Kinase II levels: obtained by the computed laser scanning densitometry of the immunoblot

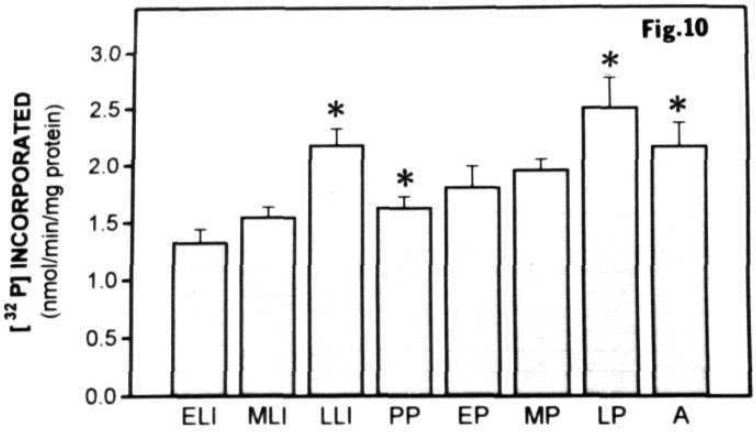
Fig.10
[32P] INCORPORATED
(nmol/min/mg protein)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
ELI
MLI
LLI
PP
EP
MP
LP
A

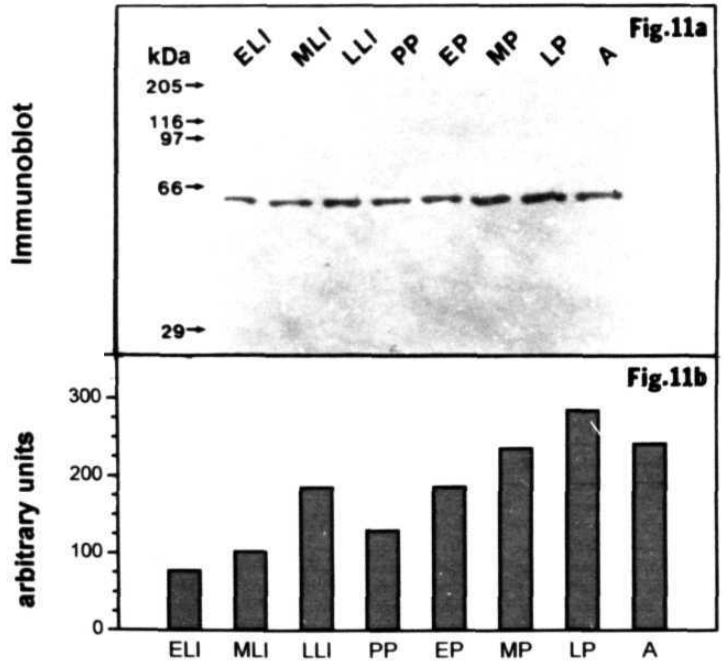
Fig.11a
Immunoblot
kDa
ELI
MLI
LLI
PP
EP
MP
LP
A
205
116
97
66
29
Fig.11b
arbitrary units
300
200
100
0
ELI
MLI
LLI
PP
EP
MP
LP
A

Fig. 12 -Immunocytochemical localization of CaM kinase II - Light micrograph of coronar section through the adult *Bombyx* brain showing the immunoreactivity (b). Note the strong cross reactivity of the cell bodies present in the pars intercerebralis (PI) and tritocerebrum (Tc). Control section (a). Scale bar = 50 µm.

Fig. 13 - Light micrograph showing the localization of CaM kinase II in a giant neuron of the metathoracic ganglion of adult moth (b). Note the strong cross immunoreactivity in cytoplasm (c) which is absent in nucleus (n). control section (a). Scale bar = 10 urn.

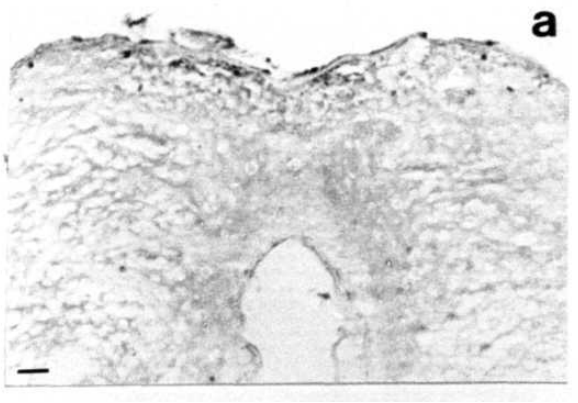

Fig.12

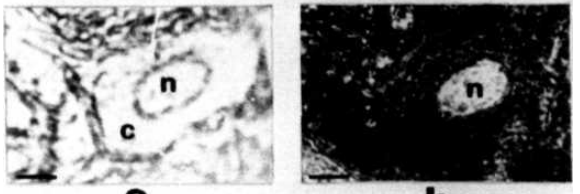

Fig.13

Fig. 14 - Autoradiograph showing the effect of cyclic nucleotides on the phosphorylation of the 48 kDa protein. Lane 1 : + EGT A, lane 2 : +10 mM cAMP and lane 3 : +10 mM cGMP. 10 μg protein was loaded in each lane.

Fig. **15 - Immunoblot of the phosphorylated CNS proteins from late-last instar larvae probed with anti-phosphotyrosine antibody. (a)** - autoradiograph, (b) - immunoblot Note the cross reactivity of the 48 kDa phosphoprotein with the antibody (◄).

Fig. 16 - Effect of JH 1 (7 x 10^{-8} M) on the phosphorylation of the 48 kDa protein (◄). The homogenate was incubated for 5 min with the hormone prior to the phosphorylation reaction. (a) - SDS-PAGE. (b) - autoradiograph of the same gel. Lane M - Molecular weight markers, lane 1 : control (- JH) and lane 2 : experimental (+ JH).

Fig. 17 - Autoradiograph of the gel obtained from back-phosphorylation experiment showing the effect of JH I on phosphorylation of the 48 kDa protein (◄). The intact CNS from late-last instar larva were dissected out and incubated in TC-100 insect culture medium for 4 h to deplete the endogenous JH. Then they were treated with 7 x 10^{-8} M JH 1 for 1 h (lane 3), 2 h (lane 4) and 7 x 10^{-7} M JH I for 1 h (lane 5). Lanes 1 and 2 are respective controls for 1 and 2 h treatment. 10 μg protein was loaded in each lane.

Fig. 18- Autoradiograph showing the developmental profile of the *in vitro* phosphorylation of the 48 kDa protein (◄). The reaction was carried out in the presence of 1mM $CaCl_2$ and 2 μM calmodulin. Lane 1 : ELI, lane 2 : MLI, lane 3 : LLI, lane 4 : PP and lane 5 : EP. 10 μg protein was loaded in each lane.

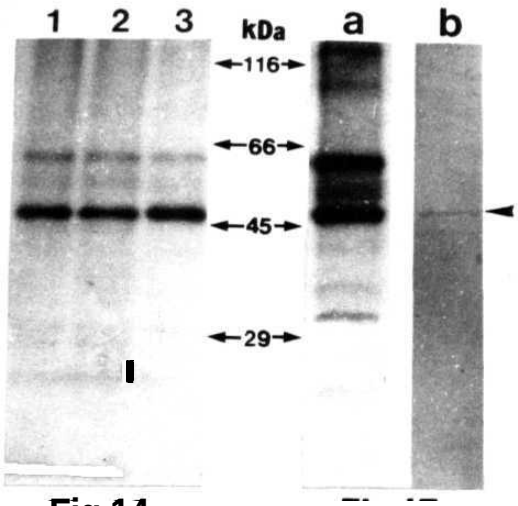

Fig.14

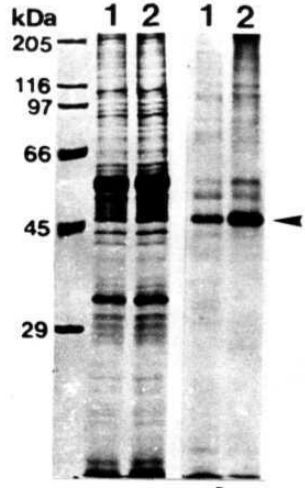

Fig.16a b

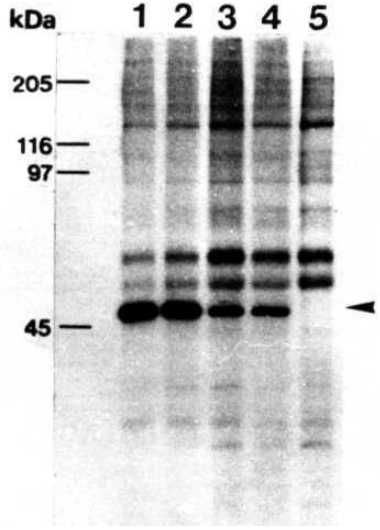

Fig.17

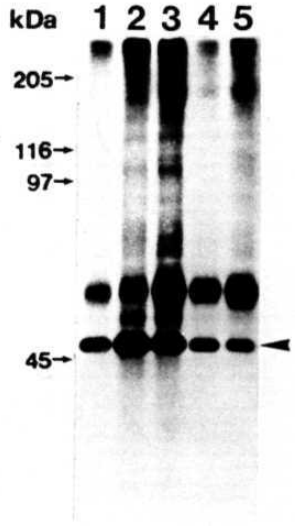

Fig.18

CHAPTER 4

DISCUSSION

Identification and characterization of CaM kinase II of *Bombyx* CNS

In the present study, CaM kinase II has been identified and characterized from *Bombyx* CNS. The kinase consists of two species with molecular weights of 59 and 60 kDa and both exhibited Ca^{2+} and calmodulin-dependent autophosphorylation. *Drosophila* head contains three major species of CaM kinase II with molecular masses of 54/55, 58, and 60 kDa, which cross reacted with anti-rat CaM kinase II antibody (Cho et al., 1991 ; Ohsako et al., 1993). However, in *Bombyx* only two species of CaM kinase were observed, the possibility of other isoforms of same molecular mass, migrating together in SDS-PAGE, cannot be ruled out.

Several potential substrate proteins for CaM kinase II have been identified in vertebrates (Hanson and Schulman, 1992). Among insects, Combest and Gilbert (1986b) reported the phosphorylation of 42 and 25 kDa protein in the brain of tobacco hornworm, *Manduca sexta,* which was stimulated by Ca^{2+} and calmodulin. Recently, the photoreceptor-specific protein, phosrestin I has been identified as a substrate of this kinase in *Drosophila* (Kahn and Matsumoto, 1997). Present study revealed the phosphorylation of 46 kDa protein in brain and 30 kDa protein in brain and ventral nerve cord of *Bombyx.* These proteins might be the endogenous substrates of CaM kinase II

KN-62, a potent inhibitor of CaM kinase II has been extensively used in investigating the physiological role of this enzyme (Tokumitsu et al., 1990; Nayak et al., 1996). It was shown to inhibit the autophosphorylation of this kinase in a dose dependent manner. In the present study, about 80% inhibition in autophosphorylation of the kinase was observed with 10 uM KN-62.

Several lines of evidence suggest that protein phosphatase 1 (PP1), rather than protein phosphatase 2A (PP2A) is more likely to be involved in the dephosphorylation of CaM kinase II in mammals (Shields et al., 1985; Shenolikar and Nairn, 1991; Mulkey et al., 1993; Strack et al., 1997a, b). Protein phosphatase 2C (PP2C) has also been reported to be involved in the dephosphorylation of autophosphorylated CaM

kinase II in rat cerebellar granule cells (Fukanaga et al., 1993). There are no reports on the identification of the phosphatase involved in the dephosphorylation of CaM kinase II in insects. However, PP1 deficient mutant of *Drosophila* showed an impairment in associative learning and visual conditioning (Asztalos et al., 1993). Okadaic acid has been extremely useful in identifying protein phoshatases involved in dephosphoryla-tion of proteins (Bialojan and Takai, 1988; Cohen et al., 1990; Hwang et al., 1996). The activity of PP2A was shown to be completely inhibited with 1 nM okadaic acid ; however, PP1 activity was completely inhibited by 1 μM okadaic acid (Cohen et al., 1990). Present studies with okadaic acid suggested that PP1 might be involved in the dephosphorylation of autophosphorylated CaM kinase II of *Bombyx*.

The high degree of autophosphorylation of CaM kinase II in the neural tissue observed in the present investigation corroborates with earlier reports on *Drosophila* demonstrating high expression of CaM kinase 11 transcripts and its protein product in the head than in the body (Cho et al., 1991; Ohsako et al., 1993). Using *in situ* hybridization, Griffith and Greenspan (1993) reported the presence of CaM kinase II mRNA in both, neuronal and non-neuronal tissues of *Drosophila* Autophosphorylation of this kinase observed in non-neuronal tissues like fat body and muscle, in the present study, supports the view that they 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).

Studies on the catalytic properties of *Bombyx* CaM kinase II revealed that K_m values for ATP and syntide-2 were close to the values reported for rat and *Drosophila* (Hashimoto and Soderling, 1987; Ohsako et al., 1993). Developmental changes in CaM kinase II has been well documented in vertebrates. Changes were observed in the enzyme activity, subunit ratios, mRNA levels and subcellular localization of this enzyme during neuronal development (Rostas, 1991; Sugiura and Yamauchi, 1994). A 10 fold increase in CaM kinase II mRNA between day 1 and 21 and further 2 to 5 fold increase by day 90 was observed in rat forebrain (Hanley et al., 1987). An increase in a subunit level peaks near the end of the most active period of synaptogenesis (Kelly et al., 1987) and may be coincident with synaptic maturation

(Rostas, 1991). An interesting observation in the present study was the presence of two peaks in the activity of the enzyme - first peak at late-larval stage and the next peak at late-pupal stage. It is interesting to note that parallel changes have been reported in the ecdysteroid titre in the haemolymph of *Bombyx mori* during postembryonic development (Calvez et al., 1976). Immunoblot analysis of the kinase from different developmental stages indicated that variations in the enzyme activity might be due to changes in the content of enzyme protein. These results suggested a developmental regulation in the expression of CaM kinase II in the CNS of *Bombyx* during metamorphosis. Induction of cellular differentiation in neuroblastoma/glioma cell cultures leads to a significant increase in the activity as well as content of this enzyme, suggesting a clear role for CaM kinase II in the process of development and differentiation of nerve cells (Vallano and Beaman-Hall, 1989).

Immunocytochemical localization of CaM kinase II in rat brain revealed differences in its regional distribution with the highest concentration in the hippocampus (Ouimet et al., 1984 ; Fukanaga et al., 1988). There are no reports on the distribution of CaM kinase II protein in insect brain and the results of present study revealed strong staining of cell bodies when compared to the neuropil. In the neurons of the metathoracic ganglion of the adult, immunoreactivity was very strong in the neuronal cytosol and absent from the nucleus.

CaM kinase II has been postulated to be involved in the induction and maintenance of long-term potentiation (LTP) which is thought to be a form of neural plasticity and a basic process in memory and learning. It has been reported that injection of specific inhibitors of CaM kinase II block induction of LTP in rat hippocampus (Malenka et al., 1989; Malinow et al., 1989; Nayak et al., 1996). In *Drosophila*, visual adaptation with blue light induced change in light/dark choice behaviour whose plasticity was postulated to be a simple form of learning. In this process and in the long term modulation of synaptic transmission, involvement of CaM kinase II has been proposed (Willmund et al., 1986). Transformed strains of *Drosophila* expressing a transgene inhibitor of CaM kinase II were shown to be deficient in an associative conditioning behavioral paradigm (Griffith et al., 1993). A

genetic "knock out' of mouse a-CaM kinase II was reported to be deficient in LTP and spatial learning (Silva et al., 1992a, b; Rotenberg et al., 1996). More recently, it has been shown that CaM kinase II was translocated from cell body to postsynaptic densities (PSD's) in rat hippocampal slices following treatments that induce CaM kinase II autophosphorylation and LTP (Strack et al., 1997b).

At least four CaM kinase II genes have been identified in rat whereas only one gene has been identified in *Drosophila* (Yamanaka et al., 1987; Doyle et al., 1990; Cho et al., 1991; Ohsako et al., 1993) The CaM kinase II gene generates at least 8 transcripts in *Drosophila* (Griffith and Greenspan, 1993), suggesting that need for functional complexity in the gene products might have been compensated by alternate splicing of a single transcript In general, the properties observed for *Bombyx* CaM kinase II in the present study appeared to be similar to those of rat and *Drosophila* CaM kinase II, supporting the concept that CaM kinase II has been highly conserved during evolution.

Juvenile hormone-stimulated phosphorylation of the 48 kDa protein

Although biological actions of JHs are well known, the exact mechanism of their action is not clearly understood (Riddiford, 1996). According to one of the proposed models, JH binds to cytoplasmic/nuclear receptors in target tissues and activates transcription (Riddiford, 1985; Jones and Sharp, 1997). While other studies suggested that JH acts at membrane level and the signal transduction might be mediated by a cascade involving protein kinase C (Yamamoto et al., 1988; Sevala and Davey, 1989). Several JH binding proteins have been reported in various insect tissues (Goodman and Chang, 1985; Lerro and Prestwich, 1990; Shemshedini et al., 1990). In the present study, JH I was used since this has been reported to be the major form of JH in the haemolymph of *Bombyx* larvae (Plantevin et al., 1987).

Presently, a 48 kDa protein was identified in the CNS of *Bombyx* larvae, phosphorylation of which was significantly stimulated by JH I. Further, phosphorylation of this protein was not dependent on Ca^{+} and cyclic nucleotides.

Results of back phosphorylation experiment clearly indicated that JH 1 stimulated the phosphorylation of this protein in isolated intact cultured CNS. JH I titer in the haemolymph during final (fifth) instar larval development of *Bombyx* exhibits a U-shaped curve, with minimal levels at mid-last and late-last instar stage and high levels during early-last larval, prepupal and early-pupal stages (Plantevin et al., 1987). Developmental profile observed in the present study revealed a low degree of [^{32}P] incorporation into the 48 kDa protein in early-last larval, prepupal and early-pupal stages. This suggested that the protein might be existing in a highly phosphorylated state (in response to the high levels of circulating JH I in the haemolymph), hence the number of sites available for phosphate [^{32}P] incorporation during *in vitro* reaction are few. Taking these facts together, it is suggested that JH I either binds or interacts with a tyrosine kinase, thereby increasing the activity of the enzyme, which in turn, stimulates phosphorylation of the 48 kDa protein.

The first evidence for the existence of protein tyrosine kinases (PTKs) was provided by Eckhart et al., (1979). Since then, PTK activities have been shown to be intrinsic to the oncogene products of certain retroviruses (Hunter and Cooper, 1985) and the receptors of several mitogenic peptide growth factors including EGF, PDGF, etc. (Yarden and Ullrich, 1988). In addition, high levels of PTK activities, which are not related to either growth factor receptors or retroviral oncogene products have also been detected in normal, uninfected cells and tissues (Srivastava, 1990) . The 48 kDa phosphoprotein *of Bombyx* cross reacted with anti-phosphotyrosine monoclonal antibody and its phosphorylation was stimulated in the presence of JH I. As parallel changes were noticed in the JH titre (Plantevin et al., 1987) and the phosphorylation of the 48 kDa protein during larval development, it is also suggested that this protein may have a role in the postembryonic development *of Bombyx mori*.

SUMMARY AND CONCLUSIONS

1. *Bombyx* CaM kinase II consists of two isoforms with molecular weights of 59 and 60 kDa and exhibited Ca^{2+} and CaM-dependent autophosphorylation.

2. Protein phosphatase I (PP1) is involved in the dephosphorylation of CaM kinase II.

3. CaM kinase II activity and levels are higher in nervous tissue than other tissues.

4. Immunocytochemical studies: CaM kinase is cytosolic; there exists major variations in its distribution in the adult *Bombyx* brain.

5. CaM kinase II activity and its levels are developmentally regulated.

6. In general, properties of *Bombyx* CaM kinase 11 (regulation by auto-phosphorylation, cross reactivity with rat kinase antibody, cellular localization) appear to be similar to the rat and *Drosophila* CaM kinase II, supporting the concept that this kinase has been highly conserved during evolution.

8. Juvenile hormone stimulates a tyrosine kinase involved in the phospho-rylation of the 48 kDa protein under *in vitro* and *in vivo* conditions.

9. Correlation observed between the phosphorylation status of the 48 kDa protein and juvenile hormone titre in the haemolymph suggests a role in development.

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Identification and Characterization of a Novel Polypeptide in the CNS of *Bombyx mori* During Pupal - Adult Transformation

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Abstract: Detailed one and two dimensional electrophoresis (PAGE) studies carried out on the CNS proteins ot *Bombyx* revealed the appearance of a new nervous system specific acidic polypeptide with a molecular mass of 235 kDa in the pharate adults This polypeptide is totally absent in the late larval, early pupal and mid pupal stages Synthesis of this polypeptide begins in pharate adults and its concentration increases gradually during the adult development and it reaches a very high concentration in 48 h old moths.

Keywords: Metamorphosis; cell death, nervous system protein; *Bombyx mori*

INTRODUCTION

Naturally occurring cell death or programmed cell death is an important feature of developing and metamorphosing nervous system of insects which regulates the size of neuronal population (Truman, 1984; Booker and Truman, 1987; Fahrbach and Truman, 1987). Cell death is required to eliminate the cells which are no longer required as well as for the generation of new cells having specific characteristics. The steroid hormone 20-hydroxyecdysone (20-HE) plays an important role in controlling post-metamorphic neuronal death in *Manducasexta* (Truman and Schwartz, 1984). Furthermore, *in vivo* and *in vitro* studies using RNA and protein synthesis inhibitors reveal that *dc nova* synthesis is required for the post-embryonic neuronal death (Fahrbach and Truman. 1987). The same has been found to be true for the programmed cell death in the inter segmental muscles in *Manduca* (Schwartz *et ai,* 1990).

There is little doubt that much progress has been made towards understanding the process of programmed cell death in insects and significantly towards the actual role of metamorphic hormone (20-HE) in this process. Several approaches are currently being pursued. These include attempts to find mRNAs and/or proteins whose increased expression / synthesis is associated with cell death. Recently, a 40 kDa protein has been identified and implicated with neuronal death in *Manduca* (Montemayor *et al.*.

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1990). In the present study an attempt has been made to analyse the pattern of CNS proteins in *Bombyx mori* using one and two dimensional polyacrylamide gel electrophoresis (PAGE) and silver staining during pupal-adult transformation. At this time, the CNS contains a mixture of neurons that will survive during adult life, neurons that are already degenerating, and neurons that are committed to die but have not yet begun the process of degeneration (Truman, 1983; Truman and Schwartz, 1984). Thus, any changes in protein pattern detected at this time could reflect changes in protein expression in any of these three cell populations.

MATERIALS AND METHODS

Insects:

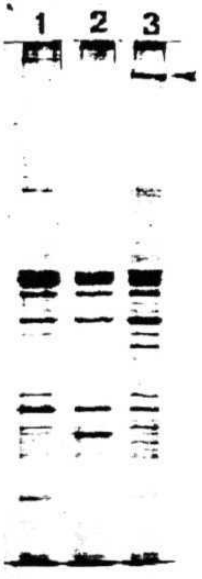
Third instar larvae of *Bombyx mori* (pure Mysore strain) were obtained from local breeding centre and reared in an insect culture room at 26 ± 1°C temperature, 70 ± 5% relative humidity and 14 h: 10 h light-dark period, on fresh mulberry leaves. For the present study, late-last instar larva, pre-pupa, mid-pupa, pharate adult (late-pupa) and adult stages were used.

Preparation of tissue sample:

The intact nervous system and other tissues - muscle, alimentary canal and salivary glands were rapidly dissected out and homogenized in chilled homogenization buffer (10mM Tris; 0.1% Triton X-100; pH 7.1) using a glass microhomogenizer (Kontes). The homogenate was centrifuged at 1000 x g for 2-3 min in a microfuge at 4°C. The supernatant was collected and used for protein estimation and one dimensional electrophoresis. Before electrophoresis, sample was mixed with an equal amount of 2x 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. For two-dimensional electrophoresis, the tissue was homogenized in IEF sample butter containing 9.5 M urea, 2% LKB carrier ampholytes (comprised of 1.6% 5/7 pH and 0.4% 3.5/10 pH), 2% NP-40 and 5% 2-mercaptoethanol.

Electrophoresis:

One dimensional SDS-PAGE was carried out according to the procedure of Laemmli (1970). A 1cm 3.3% stacking gel (pH 6.8) was followed by a 15 cm separating gel (pH 8.8). Tris-glycine buffer (0.025 M) with 0.1% SDS (pH 8.3) was used as the electrode buffer. Proteins were visualized by silver staining (Blum *et al.*, 1987). Two dimensional gel electrophoresis was performed as described by O'Farrell (1975). Ampholyte polyacrylamide tubes were prefocused for 1 h at 200 V to set-up the pH gradient. The gels were run for a total of 10,000 V h. The anolyte used was 0.01 M H_3PO_4, the catholyte used was 0.02 M NaOH. The gels were subsequently transferred to 5 ml equilibration buffer containing 10% glycerol, 5% 2-mercaptoethanol. 2.3% SDS and 0.0625 M Tris-HCl (pH 6.8) and stored at -20°C. The second dimension separation was carried out using 3.3% stacking gel and 10% separating gel. High molecular weight (Sigma) standards were run at the acidic end of several gels. The gels were run at 25mA/gel until the bromophenol blue dye reach the bottom of the gel. The gels were fixed and silver stained. To determine the pH gradient of IEF gels, parallel gels were cut into pieces of 0.5 cm length and incubated for 2 h in 0.5 ml degassed



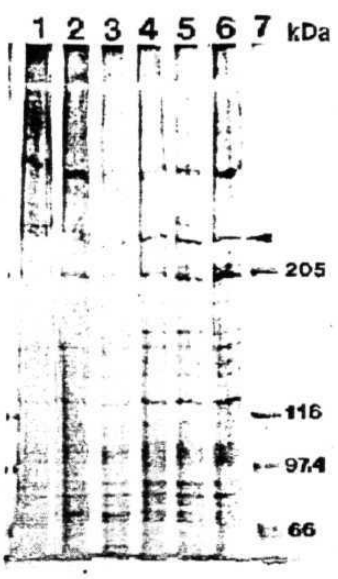


Fig 1 Fig. 2

Fig 1 shows (he polypeplide pattern from the CNS of late-last instar larvae (lane 1). mid-pupa (lane 2) and adult (lane 3) The protein was separated on a 10% SDS-PAGE. and in each lane 10 μg protein was loaded Note the presence of a 235 kDa (◀)polypeptide in the CNS of adult moths Fig 2 shows the polypeptide pattern in the CNS during various stages of development 10μg protein sample from each stage was separated on a 5% SDS-PAGE. The sample loaded in lane 1 is from pre pupa. lane 2 from early pupa. lane 3 from pharate adult (late-pupa). lane 4 from 12 h old adult. lane 5 from 24 h old adult. and lane 6 from 48 h old adult Note that the concentration of the 235 kDa polypeplide (◀(increases in the CNS during the pupal and adult development. Lane 7 shows high molecular weight markers (Sigma)

distilled water. The pH was measured electrometrically. Densitometric scanning of one dimension gels were done with a Bio-Med soft laser scanning densitometer. For all studies equal quantity of protein samples were loaded and comparisons were made. Protein was estimated according to the method of Bradford (1976) in the case of samples for one dimensional electrophoresis and according to the method of Ramagli and Rodriguez (1985) for two dimensional electrophoresis, which allows the quantitation of the proteins in the presence of urea and carrier ampholytes.

RESULTS

Detailed electrophoretic (SDS-PAGE) studies carried out on the CNS proteins of *Bombyx* during pupal-adult transformation revealed the appearance of a new nervous system specific polypeptide with an apparent molecular weight of 235 kDa in pharate

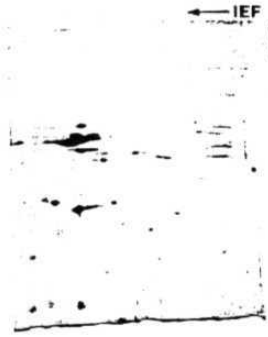


Fig. 3

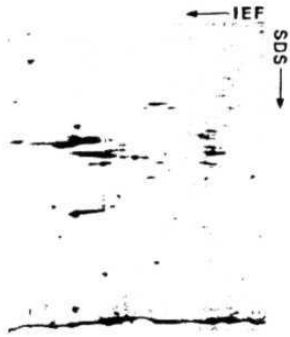


Fig. 4

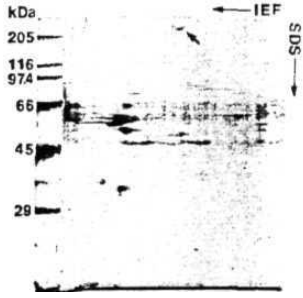


Fig 5

Figs 3, 4 and 5 - Photographs showing the fractionation of CNS proteins from late-last instar larva (Fig 3), early-pupa (Fig 4) and adult (Fig 5) on two dimensional polyacrylamide gel The proteins were separated between 20 to 260 kDa on vertical axis and 4.7 to 7.2 p/ on horizontal axis Note the presence of a new protein (⇐) which appears in the CNS of adult insect

adults. This polypeptide was totally absent in the late-last larval. prepupal and early and mid-pupal stages (Fig. 1, lanes 1 & 2; Fig. 2, lanes 1 & 2). Laser scanning densitometry of dried gels indicated that this polypeptide was present in low concentration in pharate adult (Fig. 2, lane 3) and its content gradually increases during adult development and reaches highest in 48 h old moths. The concentration of this protein remains more or less the same up to 4-5 days (which is the total life span of the adult moth). All the experiments were repeated thrice with tissue samples from three different batches. Subsequent analysis of CNS proteins by two dimensional electrophoresis clearly showed that this 235 kDa polypeptide was expressed only during the pharate

adult and adult development (Fig. 5) and it is totally absent during the larval (Fig. 3) and early pupal (Fig 4) development The p/ value of the polypeptide ranges between 6-6.2. Furthermore, this polypeptide is found to be absent in other tissues like muscles, salivary glands and alimentary canal (data not presented) of larval, pupal and pharate adult stages

DISCUSSION

Programmed cell death has been observed during the development of virtually all metazoan organisms. Extensive studies in *C. elegans* suggest that highly regulated genetic programme is responsible for programmed cell death and the proteins encoded by ced-3 and ced-4 genes act within the dying cells themselves and/or interact with other intracellular molecules to produce cell death (Ellis *et ai,* 1991). While another gene ced-9 product, acts antagonistically to ced-3 and ced-4 products to suppress the cell death (Fanidi and Evan, 1994). DNA fragmentation has been demonstrated in some models of excitotoxin and neurotoxin induced cell death (Dispasquale *et ai,* 1991; Kure *et ai,* 1991) as well as programmed neuronal death (Clarke and Hornung, 1989; Johnson *ex ai,* 1989). Furthermore there increasing evidence for the possible role of nucleases in DNA fragmentation during neuronal death (Ashwell *et ai,* 1994).

In this study we have identified a novel 235 kDa acidic polypeptide in the CNS of *Bombyx mori*, which shows a precise developmental regulation. The synthesis of the polypeptide begins at pharate adult stage and it may play a role in programmed cell death. Montemayor *et ai,* (1990) have reported the appearance of a 40 kDa acidic protein in *Manduca* during neuronal death. However, the apparent molecular weight of the newly synthesized polypeptide in *Bombyx* seems to be nearly six times greater than that reported for *Manduca.* Programmed cell death in the intersegmental muscles of *Manduca* is shown to be suppressed by inhibitors of macromolecular synthesis indicating that it requires new RNA and protein synthesis (Lockshin, 1969; Schwartz *et ai,* 1990).

In conclusion, in the present study we have demonstrated the expression of a developmentally regulated new tissue specific polypeptide which may play a role in CNS remodelling during metamorphosis of *Bombyx mori.* Further studies on identification of protein and its cellular distribution should provide an insight into whether its expression is actually related to the commencement of neuronal death.

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October 31, 1997

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Paper No. B97-432
"Identification, characterization, immunocytochemical localization and developmental changes in the activity of calcium/calmodulin-dependent protein kinase II in the central nervous system of *Bombyx mori* during post-embryonic development"

Dear Dr. Murthy:

I am pleased to inform you that your above manuscript is accepted for publication in J. Neurochem. I will send the revised manuscript to Chief Editor, which will be forwarded to Raven Press.

Yours sincerely,

Eishichi Miyamoto

Eishichi Miyamoto, M.D.

EM:ro

Identification, Characterization, Immunocytochemical Localization and Developmental Changes in the Activity of Calcium/calmodulin-Dependent Protein Kinase II in the Central Nervous System of *Bombyx mori* during Postembryonic development

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Hyderabad 500 046, INDIA

Abstract: In the present investigation *in vitro* phosphorylation of CNS proteins of the silkworm, *Bombyx mori* during the postembryonic development have been studied. SDS-PAGE and autoradiography of phosphorylated proteins, revealed the presence of major phosphoproteins of 59/60 kDa Based on molecular mass, calcium/calmodulin-dependent autophosphorylation, substrate specificity, KN-62 inhibition, apparent Km for ATP and syntide-2, these proteins were identified as Calcium/calmodulin-dependent protein kinase II (CaM kinase II). Anti-rat CaM kinase II monoclonal antibody showed immunoreactivity with *Bombyx* CaM kinase II isoforms. This kinase showed a high degree of autophosphorylation in neural tissue. During postembryonic development of *Bombyx mori*, two distinct peaks of enzyme activity could be noticed - one at the late larval and another at late pupal stage, which were associated with an increase in amount of the enzyme. These results suggested that the expression of CaM kinase II in the CNS of *Bombyx mori* was developmentally regulated.

Running title: *Bombyx* CaM kinase II

INTRODUCTION

Protein phosphorylation is now recognized to be a major regulatory mechanism by which neural activities are controlled by external physiological stimuli (Cohen, 1982; Browning et al., 1985). Considerable evidence now indicates that most of the effects of cAMP, cGMP as well as many of the effects of calcium involve activation of specific protein kinases. There are two types of cAMP-dependent protein kinase and cGMP-dependent kinase in brain, while there are a number of different calcium-dependent protein kinases. Multiple Ca^{2+}/calmodulin-dependent protein kinases and Ca^{2+}/phospholipid-dependent protein kinases (PKCs) have been identified in brain (Rosen and Krebs, 1981; Nestler and Greengard, 1984; Nairn et al., 1985; Hanson and Schulman, 1992; Hidaka and Ishikawa, 1992).

Abbreviations used: CaM kinase, calcium/calmodulin-dependent protein kinase II; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, Dithiothreitol; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl- phosphate; BSA, bovine serum albumin; TBS, tris-buffered saline.

Analysis of the physiological functions of CaM kinase II has drawn considerable attention over the last few years. This kinase is a multifunctional mediator of the activity dependent calcium release in excitable cells, which has been associated with physiological and behavioural plasticity in both vertebrates (Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992a, b; Rotenberg et al., 1996; Strack et al., 1997a) and invertebrates (Wilmund et al., 1986; Griffith et al., 1993; Wang et al., 1994).

Rat brain CaM kinase II comprises of several related isozymes (for review see Hanson and Schulman, 1992). The rat isozymes consists of a catalytic domain, an autoregulatory domain containing a calmodulin-binding site and C-terminal "association domain" that mediates holoenzyme formation (Bennet and Kennedy, 1987; Tobimatsu and Fujisawa, 1989). They are encoded by separate genes and differ mainly in a region between the regulatory and association domains (Hanson and Schulman, 1992). A distinct property of CaM kinase II is that autophosphorylation of its threonine residue near calmodulin binding domain converts it to Ca^{2+} independent state (Miller and Kennedy, 1986) Further, it was shown that calmodulin-dependent autophosphorylation of CaM kinase II induces a conformational change in the region of the calmodulin binding domain which allows additional stabilizing interactions with calmodulin (Putkey and Waxham, 1996). It has been postulated that this autoregulation may be involved in prolonging the effects triggered by transient calcium signal (Miller and Kennedy, 1986) and also may induce its translocation in *Aplysia* and *Drosophila* cells (Saitoh and Schwartz, 1985; Wilmund et al., 1986).

The adult *Drosophila* head contains three isoforms of CaM kinase II with molecular mass of 54, 58 and 60 kDa (Cho et al., 1991; Ohsako et al., 1993). These cross-react with anti-rat CaM kinase antibody. These isoforms are generated from a single gene by alternate splicing (Ohsako et al., 1993; Griffith and Greenspan, 1993). Both amino acid sequence and tissue specificity of the rat kinase are highly conserved in *Drosophila* (Cho et al., 1991).

With the exception of *Drosophila,* there are no reports on the characterization of CaM kinase II of other insects. Here we report our studies on the CaM kinase II of *Bombyx mori,* that has many of the characteristics of the rat and *Drosophila* CaM kinase II including high level of activity in the neural tissue and regulation by autophosphorylation. In addition we also demonstrate changes in the activity and content of this enzyme in the CNS during larval-pupal-adult transformation.

MATERIALS AND METHODS

Animals: Third instar larvae of *Bombyx mori* (pure Mysore strain) were obtained from local breeding centre and reared in an insect culture room at 26 ± 1 °C, 70 ± 5% relative humidity and 14 h : 10 h light-dark period on fresh mulberry leaves. Staging of insects was done based on their age after the 4th ecdysis. One to 2 days old last instar larvae were designated as early-last instar (ELI), 5 to 6 days old as mid-last instar (MLI) and 9-10 days old as late-last instar (LL1). Larvae collected after spinning were designated as prepupa (PP). One to 2 days old pupa as early-pupa (EP), 4-5 days old as mid-pupa (MP) and 9-10 days old as late-pupa (LP). Freshly emerged moths (<12 h old) were used as adult (A).

Materials: [γ^{32}P]ATP was obtained from BARC, Trombay, India. Syntide-2 (CaM kinase II substrate peptide), calmodulin, EGTA, DTT, diacylglycerol, phosphatydylserine and high molecular weight protein markers were obtained from Sigma Chem. Co. (St. Louis, MO).

Monoclonal rat anti-CaM kinase II a antibody was purchased from Boehringer Mannheim (Darmstadt, Germany) Okadaic acid was obtained from GIBCO-BRL (USA). Alkaline phosphatase-conjugated secondary antibody, nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were from Pierce (Rockford, IL). KN-62 was a gift from Dr. H. Hidaka, Nagoya University School of Medicine, Japan. Nitrocellulose sheets were from Millipore, USA. P81 -phosphocellulose papers were from Whatman (Maidstone, UK). All other reagents were of highest grade and were obtained from local sources.

Preparation of tissue sample: Intact CNS (brain + ventral nerve cord) was rapidly dissected from larvae of different developmental stages, frozen in liquid nitrogen and stored at -70° C. Frozen tissue was homogenized (4 CNS/50 ul) by hand in 50 mM HEPES buffer, pH 7.4, containing 1 mM EDTA and 1 mM DTT using an all glass microhomogenizer (Kontes). Homogenates were centrifuged at 1000 *g* for 5 min to remove debris. Freshly prepared homogenates were used for phosphorylation reaction. Protein content was estimated according to the method of Bradford (1976).

Endogenous phosphorylation in *Bombyx* tissue homogenates: Incubations were carried out in 1.5 ml Eppendorf tubes in a total volume of 40 ul, containing 20 µg of CNS homogenate protein. Incubation mixture consisted of 50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 2 uM calmodulin, 10 uM ATP, 1 mM EGTA or 0.1 mM EGTA and 1 mM $CaCl_2$. This was preincubated for 5 min at 30° C and the reaction was initiated by the addition of 4 µCi of [γ^{32}P]ATP (~ 3000 Ci/mmol). After 1 min, reaction was terminated by the addition of 20 ul of 3x 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 g for 3 min, 30 ul of the supernatant (10 ug protein) was subjected to SDS-PAGE.

In vitro phosphorylation reactions were also carried out under appropriate conditions to study the effect of phosphatydylserine + diacylglycerol, KN-62 and okadaic acid.

Assay of CaM kinase II: CaM Kinase II activity was assayed by the phosphorylation of 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/pmol), 30 uM syntide-2, 2 uM calmodulin, 1 mM $CaCl_2$, 0.1 mM EGTA and suitable amounts of homogenate protein in a total volume of 50 ul. 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 ul of 0.4 M EDTA and a 50 ul aliquot was spotted on phosphocellulose paper squares. Radioactivity was determined as described by Roskosky (1983). Assay conditions were standardized using CNS homogenates from freshly emerged adults.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography: [^{32}P] labeled phosphoproteins in CNS homogenates were separated on 1 mm slab gels of 10% acrylamide using the system of Laemmli (1970). Equal amount of protein was loaded in all the wells. Gels were stained by the silver method of Blum et al., (1987). The stained gels were photographed, dried under vacuum between cellophane sheets using a Hoeffer Gel drier and exposed for 1-3 days to Kodak X-Omat AR film at -70° C.

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 (BioRad) according to the procedure of Towbin et al., (1979) After the transfer, membrane was air dried and was then incubated for 1 h at room temperature with 3% (w/v) bovine serum albumin (BSA) in TBS (10 mM Tris, 150 mM NaCl, pH 7.5) to block non-specific binding

sites. The blot was incubated overnight with monoclonal CaM kinase II antibody (10 μg/ml) 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. The blot was once again 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% NBT and 0.0165% BCIP.

Tissue preparation and immunocytochemical staining: The CNS of freshly emerged adults was dissected in ice-cold 0.1 M TBS and fixed in Bouin's fixative for 8 h. Tissue was washed in TBS, dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Ten micron thick sections were cut, rehydrated and were processed for immunocytochemical staining.

The sections were first incubated with 1% non-immune goat serum to block nonspecific binding sites. Then they were sequentially incubated with monoclonal anti-CaM kinase II antibody (10 μg/ml) for 24 h at 4° C; alkaline phosphatase-conjugated goat anti-mouse IgG (1:100) for 1 h. All antibodies were diluted in 50 mM TBS (pH 7.5) containing 1% non-immune goat serum. Each incubation with antibody was followed by three 10 min washes in TBS. The sections were finally stained in NBT/BCIP as described by Meltzer et al., (1997). Staining specificity was assessed by replacing the primary antibody with the IgG fraction derived from non-immune mouse serum Slides were visualized and photographed with a Nikon Labophot II microscope.

Molecular weight determination and densitometric scanning: Molecular weights of electrophoretically separated polypeptides were determined by co-electrophoresing high molecular weight marker proteins. They included myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and α-lactalbumin (14 kDa). Quantitation of autoradiograms and immunoblots was done using a computing laser scanning densitometer (Molecular Devices).

Statistical analysis: Statistical analysis was performed by one way ANOVA with Sigma Stat software. The data are represented as means ± S.D. Values are considered statistically significant only when $p < 0.05$.

RESULTS

In vitro phosphorylation of endogenous proteins of *Bombyx* CNS

In vitro phosphorylation of CNS proteins in presence of EGTA revealed the phosphorylation of few proteins, of which a 48 kDa band was the major protein labeled with [^{32}P] (Fig. 1b, lane 1). Inclusion of 1 mM $CaCl_2$ stimulated the phosphorylation of several proteins, particularly 59/60 kDa (Fig. 1b, lane 2) and this stimulation was further enhanced by the addition of 2 μM calmodulin (Fig 1b, lane 3). However, PKC activators, phosphatydlserine + diacylglycerol had no effect on the phosphorylation of this protein (data not presented). This increase in phosphorylation of 59/60 kDa protein was inhibited by 80% when the homogenate was incubated for 5 min with 10 μM KN-62, a specific inhibitor of CaM kinase II (Fig. 1c, lane 2).

Identification of 59 and 60 kDa proteins as isoforms of CaM kinase II

Rat CaM kinase II a antibody was used to identify the CaM kinase II in the CNS homogenates *of Bombyx* and the results are presented in figure 2. Rat brain homogenate was used as control. The 50 kDa a subunit of rat CaM kinase II showed strong crossreactivity to the antibody (lane 2). Two closely migrating proteins of molecular weights 59 and 60 kDa from *Bombyx* CNS

homogenate cross reacted with the antibody (lane 1). Autoradiography of the immunoblot revealed [^{32}P] incorporation into these two bands. These bands were excised from the blot separately and the [^{32}P] incorporation was measured by liquid scintillation spectrophotometry. This confirmed the autophosphorylation of CaM kinase II. Since these two bands migrate closely, the signal on the autoradiograph appears as a single band. No other protein in the *Bombyx* CNS homogenate cross reacted with the antibody.

Dephosphorylation of autophosphorylated CaM kinase II and the effect of okadaic acid

Time course studies on the phosphorylation of major phosphoproteins revealed that maximal [^{32}P] incorporation was achieved by 1 min at 30° C. Of interest was the observation of rapid dephosphorylation of the autophosphorylated CaM kinase II with increase in reaction time. With 5 min of incubation time the [^{32}P] incorporation decreased by l/5th of that at the end of 1 min (Fig. 3), suggesting the involvement of a protein phosphatase in this process. Okadaic acid (1 nM) had no effect on the dephosphorylation of the kinase (Fig. 3, lane 6) while higher concentrations (10 and 500 nM) inhibited the activity of the phosphatase in a dose dependent manner (Fig. 3, lanes 7 and 8).

Catalytic properties of *Bombyx* CaM kinase II

The rate of phosphorylation of the peptide substrate syntide-2 by CaM kinase II showed normal Michaelis-Menten kinetics with respect to the concentration of ATP and syntide-2 (Figs. 4a and b). In the presence of saturating amount of syntide-2, the K_m value for ATP was 21 μM. The synthetic peptide proved to be a very effective substrate for *Bombyx* CaM kinase II with an apparent K_m of 12.5 μM and a V_{max} of 2.68 nmol/min/mg homogenate protein.

Changes in the activity of CaM kinase II in the CNS **of *Bombyx* during larval-pupal-adult transformation**

CaM kinase II was assayed in the CNS homogenates from different developmental stages and the results are presented in figure 5. A comparison of the CaM kinase II activity revealed a continuos rise in the activity from early-last instar larvae to adult stage. However, at two stages of development, late-last instar and late-pupa, there was a sudden spurt in the activity when compared to the immediate previous stage (i.e., 47% difference between late-last instar and mid-last instar; 29% difference between late-pupa and mid-pupa). Immediately after this surge, there was a decrease in the activity (-25% between late-last instar and prepupa; -14% between late-pupa and adult).

Developmental changes in the levels of CaM kinase II

Determination of CaM kinase II activity was followed by the immunoblotting of equal amount of CNS protein extracts of various developmental stages to find out the correlation, if any, between the activity and quantity of the enzyme (Figs. 6a and b). This study revealed that changes in the levels of CaM kinase II at various developmental stages may be due to changes in the enzyme content. Amount of the enzyme protein was found to be maximal in late-pupal and lowest in the early-last larval stage.

Immunocytochemical localization of CaM kinase II in the CNS of *Bombyx*

Light microscopic study demonstrated more or less evenly distributed immunoreactivity in coronal sections of the adult brain. Cell bodies in the pars- intercerebralis and tritocerebrum showed intense staining when compared with the neuropil (Fig. 7b). In the giant neurons of the metathoracic ganglion of the adult, the cross reactivity was strong in the cytoplasm and absent in the nucleus (Fig. 8b).

DISCUSSION

In the present study CaM kinase II has been identified and characterized from *Bombyx* CNS. The kinase consists of two species with molecular weights of 59 and 60 kDa and both exhibited Ca^{2+} and calmodulin-dependent autophosphorylation. *Drosophila* head contains three major species of CaM kinase II with molecular masses of 54/55, 58, and 60 kDa, which cross reacted with anti-rat CaM kinase II antibody (Cho et al., 1991 ; Ohsako et al., 1993). However, in *Bombyx* we observed only two species although possibility of other isoforms of same molecular mass, migrating together in SDS-PAGE cannot be ruled out.

KN-62, a potent inhibitor of CaM kinase II has been extensively used in investigating the physiological role of this enzyme (Tokumitsu et al., 1990). It has been shown to inhibit the autophosphorylation of the kinase in a dose dependent manner. In the present study we have observed about 80% inhibition in autophosphorylation of the kinase with 10 uM KN-62.

Several lines of evidence suggest that protein phosphatase 1 (PP1) is more likely to be involved in the dephosphorylation of CaM kinase II in mammals than protein phosphatase 2A (PP2A) (Shenolikar and Nairn, 1991; Strack et al., 1997b). Protein phosphatase 2C has also been reported to be involved in the dephosphorylation of autophosphorylated CaM kinase II in rat cerebellar granule cells (Fukanaga et al., 1993). There are no reports on the identification of phosphatase involved in the dephosphorylation of CaM kinase II in insects. However, PP1 deficient mutant of *Drosophila* show impaired associative learning and visual conditioning (Asztalos et al., 1993). Okadaic acid has been extremely useful in identifying protein phoshatases involved in dephosphorylation of proteins (Biolojan and Takai, 1988). The activity of PP2A has been shown to be completely inhibited by 1 nM okadaic acid ; however, PP1 activity is inhibited only at 1 uM okadaic acid and is resistant to 1 nM okadaic acid (Cohen et al., 1990). Our studies using okadaic acid suggested PP1 as the phosphatase involved in the dephosphorylation of autophosphorylated CaM kinase II of *Bombyx*.

The high degree of autophosphorylation of CaM kinase II in the neural tissue observed in the present investigation corroborates with earlier reports on *Drosophila* demonstrating high expression of CaM kinase II transcripts and its protein product in the head than in the body (Cho et al., 1991; Ohsako et al., 1993). Several studies have identified potential substrate proteins of CaM kinase II in vertebrates (Hanson and Schulman, 1992). Among insects, Combest and Gilbert (1986) have reported the phosphorylation of 42 and 25 kDa protein in the brain of tobacco hornworm, *Manduca sexta*, which was stimulated by Ca^{2+} and calmodulin. Recently, the photoreceptor-specific protein, Phosrestin 1 has been identified as a substrate of this kinase in *Drosophila* (Kahn and Matsumoto, 1997).

Studies on the catalytic properties of *Bombyx* CaM kinase II revealed that K_m values for ATP and syntide-2 are close to the values reported for rat and *Drosophila* (Hashimoto and Soderling, 1987; Ohsako et al., 1993). Developmental changes in CaM kinase II is well documented in vertebrates with noted changes in enzyme activity, subunit ratios, mRNA levels and subcellular localization during neuronal development (Rostas, 1991; Sugiura and Yamauchi, 1994). Rat forebrain shows a 10 fold increase in CaM kinase II mRNA between day 1 and 21 and further 2 to 5 fold increase by day 90 (Hanley et al., 1987). The increase in a subunit levels peaks near the end of the most active period of synaptogenesis (Kelly et al., 1987) and may be coincident with synaptic maturation (Rostas, 1991). An interesting observation in the present study was the presence of two peaks in the activity of the enzyme - first one at the late-larval stage and the next peak at the late-pupal stage. It is interesting to note that parallel changes were reported in the ecdysteroid titre in the haemolymph of *B. mori* during postembryonic development (Calvez et al., 1976). Further studies are required to

understand the functional significance of these changes in the activity. Immunoblot analysis of the kinase from different developmental stages indicates that variations in the enzyme activity may be accounted to some extent by changes in the enzyme content. These results suggest a developmental regulation in the expression of CaM kinase II in the CNS of *Bombyx* during metamorphosis. Induction of cellular differentiation in neuroblastoma/glioma cell cultures leads to a significant increase in enzyme activity as well as enzyme content, suggests a clear role for CaM kinase II in the process of development and differentiation of nerve cells (Vallano and Hall, 1989).

Immunocytochemical localization of CaM kinase II in rat brain revealed differences in its regional distribution with the highest concentration in the hippocampus (Ouimet et al., 1984 ; Fukanaga et al., 1988). There are no reports on the distribution of CaM kinase II protein in insect brain and the results of present study revealed strong staining of cell bodies when compared to the neuropil. In the neurons of the metathoracic ganglion of the adult, immunoreactivity was very strong in the neuronal cytosol and absent from the nucleus.

At least four CaM kinase II genes have been identified in rat whereas only one gene has been identified in *Drosophila* (Cho et al., 1991; Ohsako et al., 1993) The CaM kinase II gene generates at least 8 transcripts in *Drosophila* (Griffith and Greenspan, 1993), suggesting that requirement for functional complexity in the gene products is compensated by alternate splicing. In general the properties observed for *Bombyx* CaM kinase II in this report appear to be similar to the rat and *Drosophila* CaM kinase II, supporting the concept that CaM kinase II has been highly conserved during evolution.

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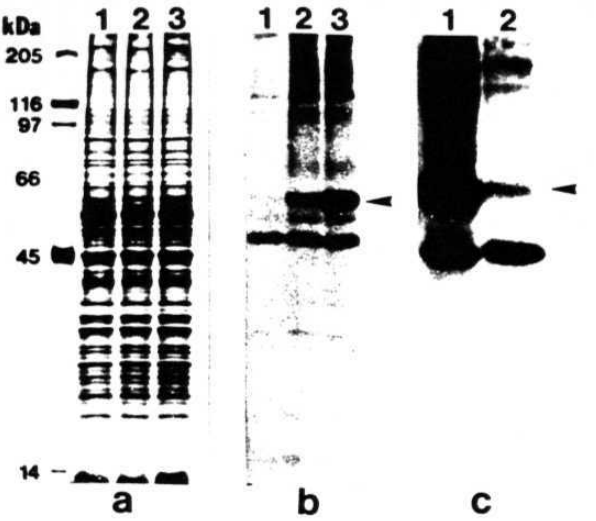

Fig. 1

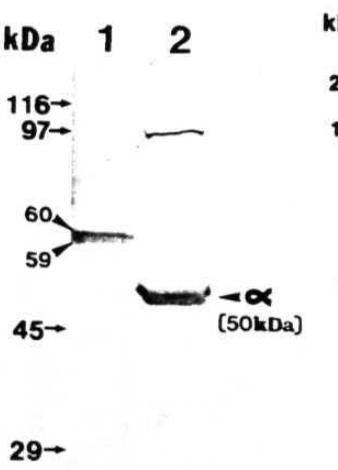

Fig. 2

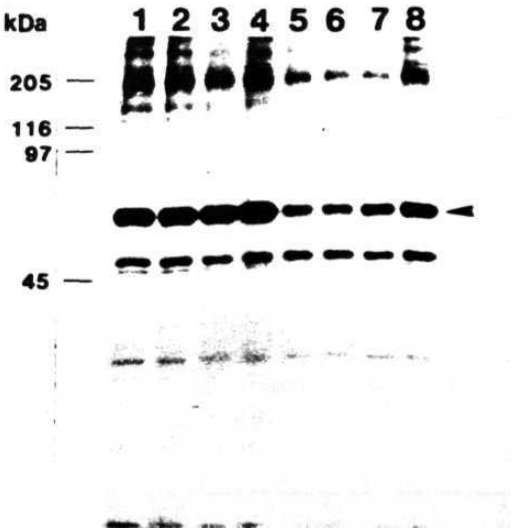

Fig. 3

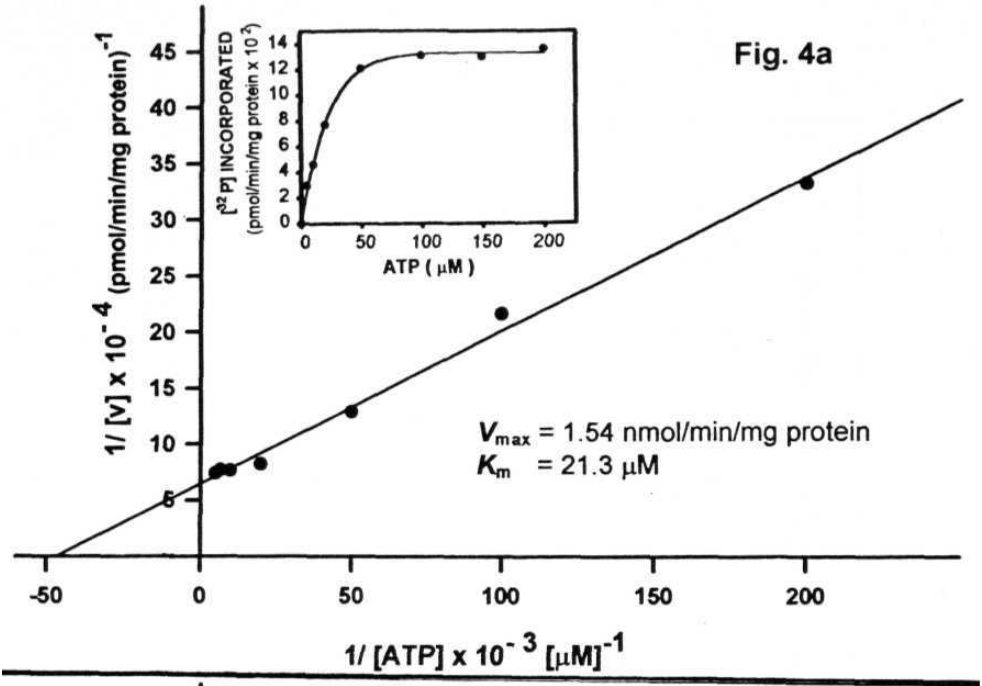
Fig. 4a
1/ [V] x 10 - 4 (pmol/min/mg protein)-1
45
40
35
30
25
20
15
10
5
-50
0
50
100
150
200
1/ [ATP] x 10 - 3 [μM]-1
[32P] INCORPORATED (pmol/min/mg protein x 10-2)
14
12
10
8
6
4
2
0
0
50
100
150
200
ATP (μM)
Vmax = 1.54 nmol/min/mg protein
Km = 21.3 μM

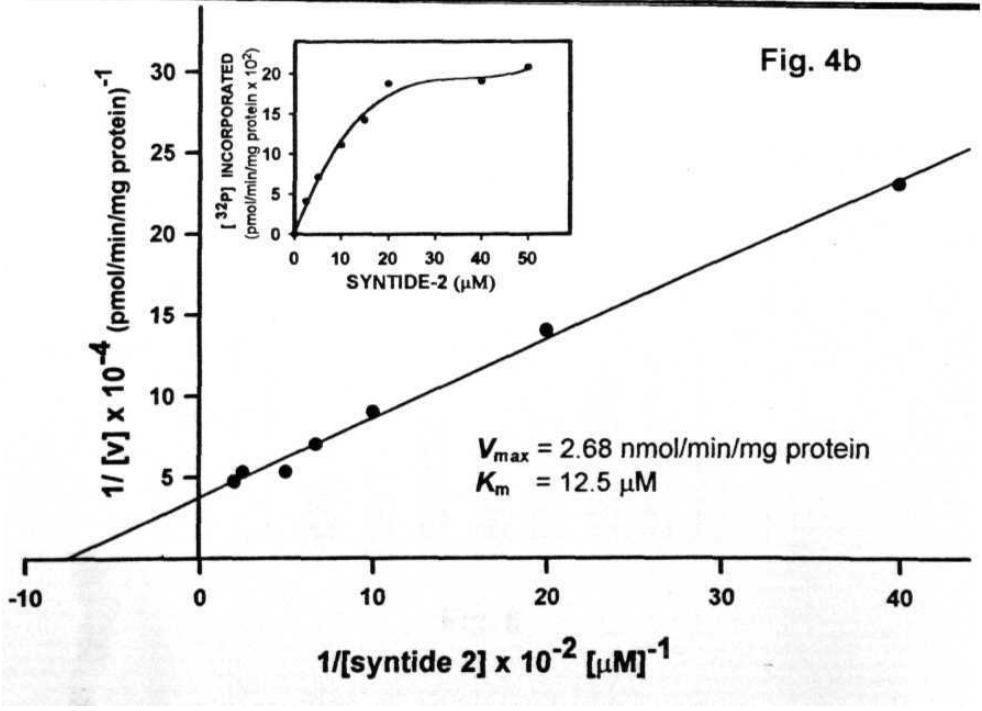
Fig. 4b
1/ [V] x 10-4 (pmol/min/mg protein)-1
30
25
20
15
10
5
-10
0
10
20
30
40
1/[syntide 2] x 10-2 [μM]-1
[32P] INCORPORATED (pmol/min/mg protein x 10-2)
20
15
10
5
0
0
10
20
30
40
50
SYNTIDE-2 (μM)
Vmax = 2.68 nmol/min/mg protein
Km = 12.5 μM

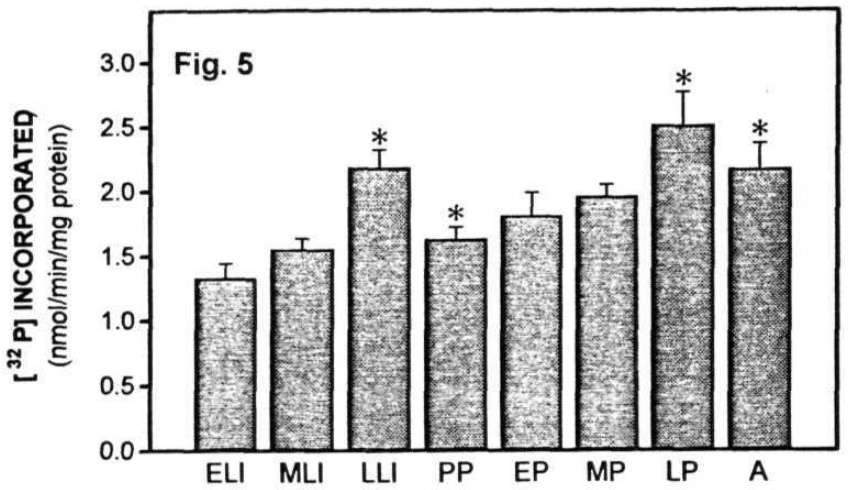
Fig. 5
[32 P] INCORPORATED
(nmol/min/mg protein)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
*
ELI
MLI
LLI
PP
EP
MP
LP
A

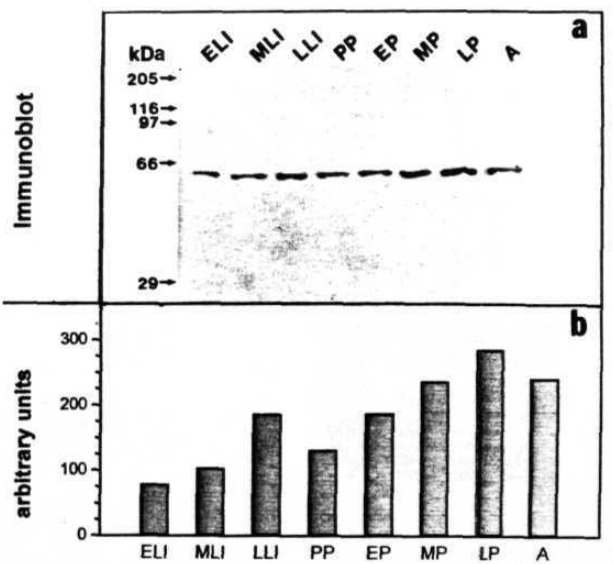
a
kDa
205
116
97
66
29
ELI
MLI
LLI
PP
EP
MP
LP
A
Immunoblot
b
arbitrary units
300
200
100
0
ELI
MLI
LLI
PP
EP
MP
LP
A

Fig. 6

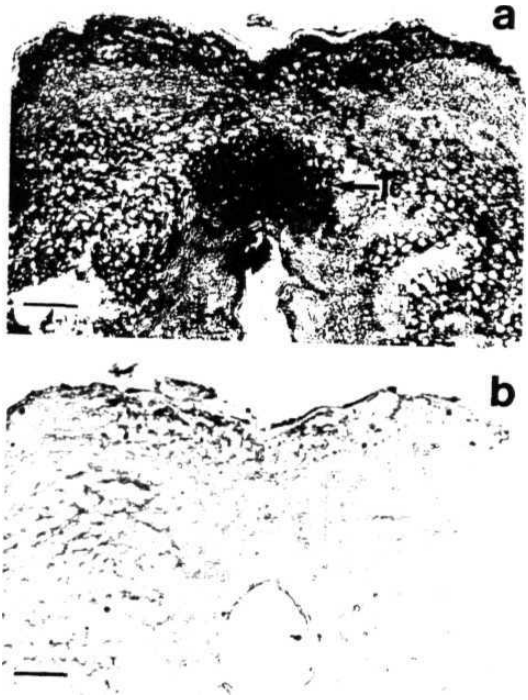

Fig. 7

Fig. 8