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 x 10^{-7} M) or 20E (5 x 10^{-6} M)and [³⁵S]-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):

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Molecular Mass (kDa)</th>
<th>Iso-electric point (pI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48.8</td>
<td>5.5</td>
</tr>
<tr>
<td>2</td>
<td>40.9</td>
<td>5.4</td>
</tr>
<tr>
<td>3</td>
<td>39.0</td>
<td>5.5</td>
</tr>
<tr>
<td>4</td>
<td>36.8</td>
<td>4.9</td>
</tr>
<tr>
<td>5</td>
<td>32.0</td>
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<td>24.9</td>
<td>5.2</td>
</tr>
<tr>
<td>9</td>
<td>24.1</td>
<td>6.2</td>
</tr>
</tbody>
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*In vitro phosphorylation of endogenous proteins of Bombyx CNS*

*In 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 [³²P] (Fig. 9, lane 1). Inclusion of 1 mM CaCl₂ 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. 3 - Changes in the *in vitro* $[^{35}\text{S}]-\text{methionine}$ 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* $[^{35}\text{S}]-\text{methionine}$ 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 (CHASE 46 kDa) and tubulin (CHASE 55 kDa) proteins in the CNS of *Bombyx* 15 $\mu$g protein was loaded in each lane.
Fig. 5 - Developmental profile of actin (≈45kDa) 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.
Fig. 6 - Developmental profile of β-tubulin (\(^{55}\text{kDa}\)) 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 (\(^{-}\)).
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. 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 I 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. 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. 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.