CHAPTER 2

MATERIALS AND METHODS
CHEMICALS

[\gamma^{32}\text{P}]\text{ATP (3000 Ci/m mole)}\text{ was obtained from Bhabha Atomic Research Centre, Trombay, India EDTA, EGTA, DTT, calmodulin, HEPES buffer, cAMP, cGMP, phosphatidyserine, 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 μg of CNS homogenate protein. The reaction mixture consisted of 50 mM HEPES (pH 7.4), 10 mM MgCl₂, 1 mM DTT, 10 μM ATP, 1 mM EGTA or 0.1 mM EGTA and 1 mM CaCl₂. Reaction mixture was preincubated for 5 min at 30° C for temperature equilibration. Reaction was initiated by the addition of 4 μCi of [γ³²P]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 μg 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 μM), phosphatidylserine (10 μM) + diacylglycerol (100 μM), cAMP (10 μM), cGMP (10 μM), KN-62 (10 μM), 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 MgCl₂ and 100 mM NaCl, pH 9.5) containing 0.033% nitroblue tetrazolium (NBT) and 0.0165% 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

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 MgCl₂, 0.1 mM [γ³²P]ATP (2000-4000 cpm/pmol), 30 μM syntide, 2 μM calmodulin, 1 mM CaCl₂, 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 $7 \times 10^{-7}$ or $7 \times 10^{-8}$ 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 $[^{32}\text{P}]$ 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.