Introduction and Review of Literature
## Contents for Introduction and Review of Literature

<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insects</td>
<td>3</td>
</tr>
<tr>
<td>Types of insects</td>
<td>3</td>
</tr>
<tr>
<td>Molting and metamorphosis</td>
<td>3-4</td>
</tr>
<tr>
<td>Metamorphosis and its physiological significance</td>
<td>4</td>
</tr>
<tr>
<td>Hormones and metamorphosis</td>
<td>4-5</td>
</tr>
<tr>
<td>Endocrine control of metamorphosis</td>
<td>5-7</td>
</tr>
<tr>
<td>Choice of the last instar for present study</td>
<td>7</td>
</tr>
<tr>
<td>Chemical nature of ecdysteroids</td>
<td>7</td>
</tr>
<tr>
<td>Juvenile hormones (JHs) and its analogues</td>
<td>7-8</td>
</tr>
<tr>
<td>Regulation of haemolymph ecdysteroids and JHs titer</td>
<td>8-9</td>
</tr>
<tr>
<td>Regulation of ecdysteroid biosynthesis</td>
<td>9-10</td>
</tr>
<tr>
<td>Evidences of factor(s) from fat body, haemolymph and other insect tissues for the regulation of ecdysteroidogenesis</td>
<td>10-11</td>
</tr>
<tr>
<td>Regulation of ecdysteroidogenesis by protein phosphorylation and dephosphorylation</td>
<td>11-12</td>
</tr>
<tr>
<td>Regulation of enzymes of ecdysteroidogenic pathways</td>
<td>12</td>
</tr>
<tr>
<td>Haemolymph proteins as a source of carrier for ecdysteroids</td>
<td>12-13</td>
</tr>
<tr>
<td>Mode of action of ecdysteroids</td>
<td>13-14</td>
</tr>
<tr>
<td>Mechanism of steroid hormone action</td>
<td>14-15</td>
</tr>
<tr>
<td>Steroid hormone receptor a member of nuclear receptor super family</td>
<td>15-16</td>
</tr>
<tr>
<td>Ecdysone receptor in insects</td>
<td>16</td>
</tr>
<tr>
<td>Classification of steroid hormone action</td>
<td>16-17</td>
</tr>
<tr>
<td>Examples and characteristics of nongenomic effects of steroid hormones</td>
<td>17-18</td>
</tr>
<tr>
<td>Classification of nongenomic action of steroids</td>
<td>18-19</td>
</tr>
<tr>
<td>Membrane receptors for nongenomic action of steroids</td>
<td>19-20</td>
</tr>
<tr>
<td>Modulation of genomic and nongenomic actions of steroids by a cross-talk</td>
<td>20</td>
</tr>
<tr>
<td>Nongenomic actions of ecdysteroids</td>
<td>20-21</td>
</tr>
<tr>
<td>Regulation of ecdysteroids action</td>
<td>21-22</td>
</tr>
<tr>
<td>Fat body tissue and its significance for the present study</td>
<td>22-23</td>
</tr>
<tr>
<td>Protein phosphorylation: a general account</td>
<td>23-24</td>
</tr>
<tr>
<td>Protein phosphorylation: role of 20E and other insect hormones</td>
<td>24-25</td>
</tr>
<tr>
<td>Calcium dependent protein kinases: role of 20E</td>
<td>25-27</td>
</tr>
<tr>
<td>Tyrosine kinases: role of 20E</td>
<td>27-29</td>
</tr>
<tr>
<td>Hexamerins: synthesis, release and receptor mediated uptake</td>
<td>29-30</td>
</tr>
<tr>
<td>Hexamerin receptor and role of 20E on its activation for hexamerin uptake</td>
<td>30-31</td>
</tr>
<tr>
<td>Acid phosphatases: a general account</td>
<td>31-32</td>
</tr>
<tr>
<td>Autophagy and metamorphosis: regulation by ecdysteroids</td>
<td>33</td>
</tr>
<tr>
<td>Why present study</td>
<td>33-34</td>
</tr>
<tr>
<td>Objectives of the present study</td>
<td>34</td>
</tr>
</tbody>
</table>
Insects-

Insects that encompass more than 70% of entire animal kingdom are the most successful group of organism living on earth. Their existence dates back to nearly 250-500 million years and are adaptable to live in air, water or land. These insects can be divided into three convenient groups i.e., harmless, injurious and beneficial. The injurious insects referred as pests annually destroy between 6-30% of agricultural harvest in developing countries. These losses become even more significant for stored cereal products than pre-harvest losses because post-harvest costs are much higher than the cost of production (USDA-Agricultural Research Service Information Bulletin, 1995). A wide range of lepidopteran pests cause damage and constitute a major factor that reduce the agricultural harvest globally including India. To combat such losses increased emphasis on developing safer, more effective and eco-friendly methods through modern biotechnology advances are required.

Types of insects-

Insects on the basis of their ability to undergo metamorphosis are broadly classified into ametabolous (no metamorphosis), hemimetabolous (incomplete metamorphosis) and holometabolous (complete metamorphosis). The holometabolous group has distinct larval and pupal stages and undergoes some of the most complex transformations seen in animal kingdom (Sehnal et al., 1996; Truman and Riddiford, 1999). The present study deals with this group of insect and most of the studies are carried using rice moth, Corcyra cephalonica.

Molting and metamorphosis-

These are the characteristic features of all insects including holometabola during the postembryonic development i.e., the ontogeny accomplished after hatching. Molting refers to the period that begins with apolysis. Apolysis is the separation of the epidermis from the old cuticle followed by a more or less pronounced morphogenesis and deposition of new cuticle and ends with ecdysis i.e., the shedding of the old cuticle. Hence, molting is the shedding of the hard external covering, which is necessary to accommodate growth and changes in morphology. Metamorphosis is marked by abrupt changes in the form and / or structure during the postembryonic development. The larval forms are the juveniles of holometabola that lack the external rudiments of wings and genitalia but possess imaginal discs (an invaginated group of undifferentiated embryonic stem cells). The larvae are voracious feeders.
and have different habitat and niche from the adult stage. The non-feeding pupal stages are usually hidden or somehow protected stage. The tissue degeneration and rebuilding mainly occurs at the pupal stage, which also possesses the external rudiments of wings and genitalia. The adult stage of holometabolous insect is morphologically very different from the previous stages and they are usually prolific breeders (For review see- Wigglesworth, 1934, 1939, 1954; Safranek and Williams, 1984; Sehnal et al., 1996; Buszczak and Segraves, 2000; Tissot and Stocker, 2000; Truman and Riddiford, 1999, 2002).

**Metamorphosis and its physiological significance**-

Metamorphic developments actually are the manifestation of sequential polymorphism produced by the same genome (Highnam, 1981; Nijhout and Wheeler, 1982). A group of hormones by several cascades of events control these developments and decide whether a cell remains at the present stage or advances to the next one (Wigglesworth, 1954; Nijhout, 1994; Sehnal et al., 1996; Gilbert et al., 1996; Truman and Riddiford, 2002). For example in epidermal cells, the hormones as well as their titers determine the type of cuticle produced successively in the larva, pupa and adult (Riddiford, 1982; Willis, 1996). The larval cuticular proteins are produced under high juvenile hormone (JH) titer, whereas moderate JH titer, facilitates pupal cuticular protein synthesis and in the absence of JH, the imaginal cuticular proteins are produced (Piepho, 1951; Willis et al., 1982). Some authors regard the transition from larval to pupal and then to adult functional state as a developmental process during which time, it produces the imaginal cuticle (Anderson et al., 1995). During postembryonic and adult development, each stage (larva, pupa and adult) is strictly determined and can be neither omitted nor mixed with other stages (Slama, 1975).

**Hormones and metamorphosis**-

As mentioned above, the postembryonic development in insects involves growth, molting and metamorphosis. It is now established that metamorphosis, a seemingly abrupt morphological transition as viewed externally is in reality a smooth continuation of precisely regulated events (Sehnal et al., 1996). These events are controlled by the endocrine cues that are mainly secreted by the brain, corpora cardiaca, corpora allata and prothoracic glands (PGLs). A choreographic precision of titer of mainly the morphogenetic hormones *i.e.*, the juvenile hormones (JHs) and ecdysteroids and their interaction is required for the molting and metamorphic events to occur normally (Gilbert et al., 1996). Since 1930, the role of
hormones in the regulation of insect postembryonic development has received lot of attention resulting in several studies (Wigglesworth, 1934, 1939, 1954; Safranek and Williams, 1984; Sehnal et al., 1996; Henrich et al., 1999; Truman and Riddiford, 1999, 2002; Tissot and Stocker, 2000; Riddiford et al., 2001; Gilbert et al., 2002). However, knowledge regarding the regulation of hormone dependent actions is very limited and the field remains largely unexplored with a demand for further research.

**Endocrine control of metamorphosis**

![Flow chart of 20-hydroxyecdysone biosynthesis in lepidopteran insects (a) and endocrine control of insect metamorphosis (b) (source: http://www.devbio.com/article.php?id=179&search=metamorphosis).](image)

**Fig. A:** Simplified flow chart of 20-hydroxyecdysone biosynthesis in lepidopteran insects (a) and endocrine control of insect metamorphosis (b) (source: http://www.devbio.com/article.php?id=179&search=metamorphosis).

Molting and metamorphosis are hormonally regulated, mainly by JHs and ecdysteroids. Several workers have proposed a basic model of the endocrine control of postembryonic development (Fukuda, 1944; Nijhout and Williams, 1974; Grieneisen, 1994; Gilbert et al., 1996). According to them, the specific neurosecretory cells in the insect brain synthesize a neuropeptide, prothoracicotropic hormone (PTTH), which is transported to the
Introduction and Review of Literature

corpora cardiaca and corpora allata that also act as neurohemal sites in lepidoptera (Agui et al., 1980; Smith and Gilbert, 1989; Smith and Sedlmeier, 1990). Once released into haemolymph as a result of neural, hormonal, physiological or environmental factors (photoperiod, temperature and humidity), PTTH acts on the prothoracic glands and stimulates ecdysteroid synthesis (Gilbert et al., 1988). Thus 3-dehydroecdysone is released into the haemolymph where it is reduced by a ketoreductase to ecdysone (Warren et al., 1988a, b; Sakurai and Williams, 1989; Sakurai and Gilbert, 1990). The prohormone ecdysone is converted to the principal molting hormone 20-hydroxyecdysone (20E) in the mitochondria and microsomes of peripheral tissues such as fat bodies, Malpighian tubules and epidermal cells (Smith et al., 1983; Smith, 1985; Zhu et al., 1991a; Riddiford et al., 2001). The 20E finally exerts its effect and causes apolysis and secretion of larval, pupal or adult cuticle (Smith and Gilbert, 1989). In addition to sequestering PTTH, the corpora allata synthesize and secrete JHs into haemolymph, the second major effector hormone in insect life. In haemolymph, the hormones are bound to JH-binding proteins, which enhance the solubility of JH, protect it from esterase degradation and facilitate its entry into the target cells (Riddiford, 1996; Willis, 1996; Gilbert et al., 2000).

The relative titer and interplay between JHs (sesquiterpenes) and ecdysteroids (a group of steroid hormones) orchestrates the progression of one developmental stage to the next i.e., egg-larva, larva-larva, larva-pupa and pupa-adult. During postembryonic development, the ecdysteroids initiate the onset and timing of molt (the producer). The titers of JH, determines the result of the molt either by maintaining it in juvenile condition during the larval-larval molt or by allowing it to transform during larva-pupal molt. JH thus regulates the quality of molt (the director) (Sehnal and Meyer, 1968; Sehnal, 1985; Smith, 1985; Gilbert et al., 1988, 1996; Rachinsky et al., 1990; Chang, 1993; Gilbert et al., 2000; Davey, 2000).

In holometabolous insects JH levels are high throughout the larval instars, declines in the last instar but rise again before the pupal molt during the prepupal stage and absent during the pupal stage. So molting in the presence of high JH titer would result in larval-larval molt while in the presence of reduced JH titer would result in larval-pupal molt and in the absence of JH would result in pupal-adult molt. Ecdysteroid levels rise prior to a molt and decline just before actual ecdysis. In the last larval instar, there is a small peak of ecdysteroids that occurs at a time when JH is absent. This peak of ecdysteroids along with the following JH peak,
signals that the next molt will be a pupal molt (For review see- Smith, 1985; Riddiford, 1996; De Kort et al., 1996; Davey, 2000)

**Choice of last instar and prepupal stages for present study**

During the postembryonic development, the cells may change their functions and these changes may or may not be related to cell divisions (Wigglesworth, 1954; Sehnal et al., 1996). The extent of change is usually much greater in the last larval instar and pupa than in preceding instars making these developmental stages an ideal choice for the present study.

**Chemical nature of ecdysteroids**

Ecdysteroids are well defined term for all compounds structurally related to ecdysone. It includes true ecdysteroid and ecdysteroid related compounds. The biologically active ecdysteroids refer to the molting hormone. Thus the hormonally active nonsteroidal compound such as RH5849 and its derivatives (Wing, 1988) are molting hormone agonist but not an ecdysteroid or ecdysone (Karlson, 1995). Chemically ecdysone is the trivial name of a specific compound (22R)-2β,3β,14α,22,25-pentahydroxy-5β-cholest-7-en-6-one, a derivative of cholesterol. 20-Hydroxyecdysone (20E) is the active molting hormone, which is a result of ecdysone 20-monoxygenase catalyzed hydroxylation (Grieneisen, 1994; Rees, 1995). The two molting hormones ecdysone and 20E were originally designated as α and β ecdysone respectively (Horn and Bergamasco, 1985). In arthropods, 20E is one of the most ubiquitously distributed ecdysteroid utilized by the molt cycle and is also associated with various physiological events (Gilbert et al., 2002).

**Juvenile hormones (JHs) and its analogues**

The JHs are a unique group of sesquiterpenoid hormones. These are synthesized and secreted by the corpora allata under the influence of allatotropins and allatostatins, which are released from brain neurosecretory cells (Schooley and Baker, 1985). The existence of JH was first reported in *Rhodnius* by Wigglesworth (1934, 1936). The term “juvenile” hormone was introduced because of its role in the retention of larval characteristics or the restraining of development toward the adult form. To date, six different JHs have been identified from various insect orders. In the lepidoptera, five JHs are produced JH I, JH II, JH III, JH 0 and 4-methyl-JH I (Williams, 1956; 1959; Yin et al., 1994; Gilbert et al., 2000). Methyl farnesoate is the predominant JH like molecule in the crustacean. Different JH homologs have different
levels of biological activity. All the JHs have a methyl ester on one end and an epoxide on the other end. Both of these structural features are required for activity.

![Prothoracicotropic hormone (PTTH)](image1)

![Ecdysone](image2)

**Fig. B.** Major hormones of insects

**Regulation of haemolymph ecdysteroids and JHs titer-**

Extensive studies have been carried out on the regulation of hormone titer as well as their synthesis (Gilbert et al., 1980a, b, 1997, 2002; Gruetzmacher et al., 1984a, b; Song and Gilbert, 1998). These studies orient towards the temporal, quantitative and qualitative regulations of haemolymph titers of ecdysteroids and JHs. During the larval-pupal development of insects, there is a precise temporal fluctuation in the haemolymph titer of the JHs that appears to affect changes in the haemolymph titre of ecdysteroids and vice versa (Nijhout and Williams, 1974; Riddiford and Truman, 1978; Smith, 1985). This sequence of interaction begins early in the last larval instar when the JH titre is at its peak and the ecdysteroid titre at its lowest. In the presence of this high JH and low ecdysteroid titre, the prothoracicotropic, synthesize and release PTTH in the haemolymph. The PTTH then
activates the PGL to synthesize ecdysone resulting in an initial subtle increase in the ecdysteroid titre, which evokes wandering behaviour and pupal commitment. This ecdysteroid surge in turn stimulates the corpora allata via the brain to synthesize JH, leading to a second increase in the JH titre, which is necessary for normal metamorphosis to the pupa (Kiguchi and Riddiford, 1978). This peak of JH titre also stimulates the PGL, indirectly contributing to the second major increase in ecdysteroid titre, which elicits the pupal moult. This model suggests that the titre and action of the ecdysteroids are of critical importance in the governance of the physiological activities associated with growth, metamorphosis and reproduction (Doane, 1973).

Detailed studies reveal that the basal concentration of ecdysteroids in the haemolymph of various lepidopteran insects during postembryonic development ranges from $10^{-8}$ to $10^{-5}$ M, which is species specific as well as stage dependent (Calvez et al., 1976; Dutta-Gupta and Ashok, 1998). A major peak of the ecdysteroid during larval-larval and larval-pupal development is usually present during the later half of each stadium, the duration of which is once again species specific (Smith, 1985; Tissot and Stocker, 2000). During the pupal-adult development, the major peak occurs in the first half or middle of the pupal stadium (Dean et al., 1980). This pupal peak is normally 1.5 to 2.0 times greater in magnitude and duration and is attributed to the accentuated need of these compounds for extensive remodeling and differentiation of different tissues. In addition to the major peaks, there are non-molting peaks during inter-molt period which are of lower magnitude and duration. Some of the inter-molt peaks have been found to correlate temporally with metabolic activities such as synthesis of DNA, RNA, protein and other macromolecules (Truman and Riddiford, 2002). The haemolymph ecdysteroid titre at any given moment is therefore a reflection of several metabolic processes, which include ecdysteroid biosynthesis, secretion, and transport to target tissues, tissue uptake, degradation and excretion. Thus the changing haemolymph titre of ecdysteroids in holometabolous insects is responsible for eliciting the change in commitment and is necessary for metamorphosis as well as for the critical sequence of behavioral, physiological and biochemical events termed molting (Gilbert et al., 1980b; Nijhout, 1994; Riddiford et al., 2001).

**Regulation of ecdysteroid biosynthesis**

The primary and secondary effectors regulate ecdysteroidogenesis. Detailed studies reveal that the cerebral neuropeptide PTTH is the primary effector in the regulation of
ecdysone biosynthesis by the PGLs (Bollenbacher and Bowen, 1983; Bollenbacher and Granger, 1985). Several evidences also suggest that secondary effectors also control the PGL activity. These include environmental signals such as temperature (Meola and Adkisson, 1977), photoperiod (Mizoguchi and Ishizaki, 1982), direct neural input (Richter and Gersch, 1983), humoral factors such as lipoproteins (Chino et al., 1974) and hormones other than PTTH (Safranek et al., 1980; Beylon and Lafont, 1983). These effectors either individually or in combinations precisely regulate the synthesis and release of ecdysone by PGL during postembryonic development. Thus, they control the quantitative and temporal fluctuations in the haemolymph ecdysteroid titre (Bollenbacher et al., 1981; Gilbert et al., 2002).

**Evidences of factor (or factors) from fat body, haemolymph and other insect tissues for the regulation of ecdysteroidogenesis**-

Among the secondary effectors that regulate the PGLs, JH is of particular interest since it has both stimulatory as well as inhibitory effects on the gland. Several evidences indicate an indirect stimulation of PGL by JH in post-committed last-instar *Manduca sexta* larvae via secretion of a factor from fat body (Williams, 1959; Gilbert and Schneiderman, 1959; Hiruma et al., 1978; Hiruma, 1980; Safranek et al., 1980; Gruetzmacher et al., 1984a, b). This factor was shown to stimulate *in vitro* synthesis of ecdysone by *M. sexta* PGL and its ecdysteroidogenic effect was additive with that of PTTH. Later the factor was identified as a trypsin sensitive heat labile protein with apparent mass of 30 kDa (Watson et al., 1985, 1987). The group hypothesized that chemical nature of the stimulatory protein facilitates the transport of sterol precursor from which ecdysone is synthesized. It was further suggested that the mode of action of this protein is distinctly different from that of PTTH. Their studies also revealed a direct correlation between the activity of the haemolymph factor and titer of JH during development of *M. sexta* (Watson et al., 1988).

The ecdysteroid production may also be suppressed by another mechanism in which the PGLs themselves become refractory to PTTH stimulation during diapause (Agui, 1975; Browning, 1981; Bowen et al., 1984; Ciancio et al., 1986). Meola and Adkisson (1977) observed that release of PTTH occurs at the onset rather than at the termination of diapausing *Heliothis zea* pupae for pharate adult development. Despite the release of this hormone, pupae remain in diapause because an unknown mechanism prevents ecdysone synthesis. Further investigation showed that when PGL from pupa which are maintained in a diapause
sustaining temperature of 19°C, implanted PGLs are able to produce ecdysone only in non-diapausing hosts (Meola and Gray, 1984). However, when haemolymph from non-diapausing host pupae is injected into diapausing insects kept at 19°C, ecdysone synthesis is stimulated. They concluded that the temperature sensitive mechanism controls diapause by regulating the availability of a humoral factor necessary for ecdysone synthesis. It was further reported that the fat body releases a factor into the haemolymph in response to diapause terminating temperature and that the factor is neither a free or conjugated ecdysteroid nor PTTH (Gray et al., 1987). Another interesting mechanism of regulation of ecdysteroid synthesis at the PTTH level was reported in *M. sexta*, where the PGLs synthesized 3-dehydroecdysone, which is rapidly converted to ecdysone through the mediation of a haemolymph enzyme, a 3-β forming 3-keto steroid reductase. It was also reported that the enzyme is trypsin sensitive, heat labile and has a mass between 20 to 30 kDa (Sakurai et al., 1989).

**Regulation of ecdysteroidogenesis by protein phosphorylation and dephosphorylation**

PTTH stimulated ecdysteroid production in PGLs occurs via a cascade of events which is yet to be elucidated completely. Earlier studies on *M. sexta* revealed a correlation between circulating ecdysteroid titers and adenylate cyclase activity in the PGL, suggesting a role for cAMP (Vadeckis et al., 1976; Smith et al., 1985; Smith, 1993). The Ca\(^{2+}\) deprived glands failed to generate cAMP in response to PTTH indicating that cAMP production was downstream of Ca\(^{2+}\)/calmodulin sensitive adenylate cyclase (Meller et al., 1988, 1990). The group also found evidence of G-protein (guanine nucleotide binding protein) involvement in the adenylate cyclase activation. Regardless of the complicated, developmentally dynamic relationships among calcium, calmodulin, G proteins and adenylate cyclase, it is clear that PTTH elicits increased cAMP formation in PGLs. Increase in intracellular cAMP levels can lead to the activation of cAMP dependent protein kinase (PKA) and subsequent phosphorylation (Fig. A). In recent past, Gilbert et al. (1997) reported that during the process of ecdysteroidogenesis, PTTH initiates a cascade of events, that progresses from the influx of Ca\(^{2+}\) and cAMP generation through phosphorylation of the ribosomal protein S6. This is followed by S6 dependent protein synthesis and an increase in the synthesis and export of ecdysone from the PGL. Their studies further suggest that S6 phosphorylation probably controls the steroidogenic effect of PTTH by gating the translation of selected mRNAs whose protein products are required for increased hormone synthesis (Song and Gilbert, 1995, 1997,
1998). They have also shown that the ecdysone produced by the PGL feeds back upon the gland by increasing the expression and phosphorylation of a specific p47 USP isoform, a constituent of the functional ecdysone receptor. Thus, changes in the concentration and composition of the ecdysone receptor complex of the PGL could modulate the gland’s potential for ecdysone synthesis. Recently an enzyme ecdysteroid phosphate-phosphatase has been identified to be responsible for the dephosphorylation of 20-hydroxyecdysone 22-phosphate and ecdysone 22-phosphate for the formation of 20E apart from the de novo synthesis of 20E (Yamada and Sonobe, 2003).

**Regulation of enzymes of biosynthetic pathways of ecdysteroidogenesis-**

In insects, ecdysone 20-monoxygenase catalyzed hydroxylation of ecdysone into the active hormone 20E has been defined as activation (Gilbert et al., 1996). However, during times of decreasing hormone titers, inactivation occurs by several routes including (i) 26-hydroxylation and further oxidation to 26-oic acid, (ii) formation of various conjugates (e.g., phosphates) and (iii) in lepidoptera in particular, ecdysone oxidase catalysed formation of 3-dehydroecdysteroid, which is reduced to 3-epiecdysteroid, followed by phosphotransferase catalyzed formation of phosphate conjugates (Williams et al., 1997). These results indicate that molting hormone stimulates at least one universal route of its own inactivation by inducing 26-hydroxylase activity, thus regulating its activity as well as titer.

**Haemolymph proteins as a source of carrier for ecdysteroids-**

It is generally assumed that after biosynthesis in a specific endocrine tissue, transport of the ecdysteroid hormone or prohormone occurs non-specifically. In vertebrates, almost all the steroid hormones have been known to be regulated by the presence of plasma binding globulins (Rosner et al., 1991). Here the binding or carrier proteins facilitate release of hormone from the endocrine gland, constitute a circular pool of conserved hormone, buffer the effect of a sudden release of active molecules into the bloodstream and protect the hormones from enzymatic degradation that prevents the rapid depletion of hormone titer (Roe and Venkatesh, 1990). In insects, enough information exists on the carrier proteins for JHs. These proteins appear to be present in the haemolymph of a large variety of insect orders (Goodman, 1983). However, only a few reports are available on the existence of possible haemolymph carrier proteins for ecdysteroids. Feyereisen et al., (1977) for the first time, reported the presence of a 280 kDa high affinity ecdysteroid carrier protein in the
Introduction and Review of Literature

haemolymph of *Locusta migratoria*. They further demonstrated that the majority of circulating ecdysteroid bound to this (Feyereisen 1980). Cao et al., (1983) purified a weakly acidic dimeric 270 kDa protein (monomers- 135 kDa) from the haemolymph of adult *L. migratoria* females. Despite these studies, the information regarding the transport of ecdysteroids is not very clear and the area remains largely unexplored.

**Mode of action of ecdysteroids-**

The isolation and purification of ecdysone and 20E by Butenandt and Karlson (1954) revolutionized the field of insect endocrinology. The widespread ramifications of this discovery later led to the present day understanding of eukaryotic gene expression. The pioneering research of Clever and Karlson (1960) and Clever (1964) revealed puffing patterns of the *Chironomous tentans* salivary gland polyprene chromosome by ecdysteroid. This observation of puff regulation was later confirmed in genetic model organism, the fruit fly *Drosophila melanogaster* by several other groups (Becker, 1959; Ashburner et al., 1974; Ashburner and Richards, 1976). Based on these observations as well as through a series of detailed and elegant studies, Ashburner and group (1974, 1976) proposed a model for the regulation of gene expression by 20E. Since then this model became the basis of the knowledge of mechanism of steroid hormone action, which suggest that ecdysteroid could initiate a cascade of gene expression by directly acting on the nucleus. According to this model, the ecdysone upon binding to its specific receptor directly regulates two classes of genes, a small class of early regulatory genes and a large class of late genes. The protein products of the early genes in turn repress their own expression and induce the much larger set of late genes that play a more direct role in controlling the biological response of hormone. Extensive studies based on this model have provided insights into the molecular mechanism of 20E action (Cherbas, 1993; Antonieweski et al., 1993; Henrich and Brown, 1995; Thummel, 1996; Henrich et al., 1999; Riddiford et al., 2001). The focus of these studies have been on two major aspects- (i) studies on the transcription factors induced by 20E and how these factors transduce and amplify the hormonal signal by coordinating the induction of secondary response genes? (ii) Discovery, cloning, characterization and expression of ecdysone receptor proteins (Segraves and Hogness, 1990; Thummel et al., 1990; DiBello et al., 1991; Palli et al., 1992; Koelle et al., 1992; Riddiford et al., 2001; Thummel, 2002). From these studies a clearer understanding of the mechanism by which a
systemic hormonal signal is refined into stage and tissue specific developmental responses has emerged.

**Mechanism of steroid hormone action—**

![Diagram of steroid hormone action](http://www.zoo.utoronto.ca/zoo344s/2003Group2/mechanism_steroid.htm)

**Fig. C: The generalized representation of mechanism of steroid hormone action.** Steroid hormones mostly regulate the biological response in following steps- 1. Carrying of hormones to appropriate target and its dissociation from carrier protein. 2. Transport of hormone into cell cytoplasm or nucleus. 3 & 4. Binding of activated hormone receptor complex to hormone responsive elements as homo or heterodimers. 5. Gene activation and transcription. 6. Release of mRNA from nucleus and its translation into protein. 7. Changes in the cellular activity due to the regulation of newly synthesized protein. In addition to this the steroid hormones may also exert a direct rapid effect possibly through a cell surface receptor mediated by a second messenger system (I) (source-http://www.zoo.utoronto.ca/zoo344s/2003Group2/mechanism_steroid.htm).

Hormones (peptides, amines or steroids) are chemical messengers secreted by certain endocrine tissues into the blood (haemolymph in case of insects) to regulate the activity and function of the other tissues. The mechanism (Fig. C) by which steroid hormones exert their effects is fundamentally different from other types of hormones (Truss and Beato, 1993;
Since the steroids are too hydrophobic to dissolve readily in the blood, they are carried on specific carrier protein from the point of their release to the target tissues. These carrier proteins protect the hormones from enzymatic degradation and extend their half-life. In the target tissues, these hormones pass through the plasma membranes probably by simple diffusion (a process not well characterized) into the cell cytoplasm. The hormone is then mostly transported in to nucleus where it binds to specific receptor. Alternatively receptors may also be present in cytoplasm, where the hormone binds and the complex is transported to nucleus. Once hormone binds to the receptor, the receptor undergoes conformational change and dissociates itself from the heat shock protein and becomes activated. Hence the hormone-receptor complex acts as a ligand activated transcription factor. The activated receptor either dimerises then binds or binds sequentially to its corresponding hormone response elements (HREs) present in the vicinity of target genes to turn on the transcription of particular DNA and then regulates the synthesis of proteins, the gene product, which in turn regulates the cellular and physiological functions (Evans, 1988; Beato, 1989).

**Steroid hormone receptor a member of nuclear receptor super family-**

Fig. D: The generalized representation of the steroid or thyroid hormone superfamily receptor.
The C domain is the highly conserved DNA binding domain (DBD) that contains cysteine rich central region compatible with the formation of two zinc fingers. The D region is responsible for nuclear localization. The E domain contains the ligand binding site and is known as the ligand binding domain (LBD). The relative position of two transactivation domains AF1 and AF2 is also shown (Modified from Bender et al., 1997; Riddiford et al., 2001).
Introduction and Review of Literature

Steroid hormone receptor that acts as transcription factor in vertebrates as well as invertebrates belongs to the nuclear receptor superfamily (Beato et al., 1995; Mangelsdorf, 1995). This superfamily (currently totaling 150 different proteins) consists of receptors for steroids, retinoids, thyroid hormones, fatty acids, prostaglandins and orphan receptors whose ligands are unidentified (White and Parker, 1998). The members of this family are highly related in both (i) primary amino acid sequences and (ii) the organization of functional domain, suggesting that many aspects of their mechanism of action are conserved. In the absence of hormone, the receptor exists as an inactive oligomeric complex with a number of other proteins, including chaperons such as heat shock proteins (Hsp 90 & Hsp 70), cyclophilin 40 and p23 (Smith and Toft, 1993; Pratt and Toft, 1997). The steroid hormone receptors are structurally organized in different domains (Fig. D), which have been confirmed by the results of cDNA cloning experiments (Bender et al., 1997; Riddiford et al., 2001).

Ecdysone receptor in insects-

Like other steroid hormones, the receptor for ecdysone is also a member of the nuclear receptor superfamily (Koelle et al., 1991) that acts as a ligand dependent transcription factor (Mangelsdorf et al., 1995; Freedman, 1997). Unlike the vertebrate steroid receptors which act as homodimers, the functional ecdysone receptor is a heterodimer of EcR with another member of the nuclear receptor superfamily, ultraspiracle (USP) (Yao et al., 1992, 1993). The USP is the insect homolog of the vertebrate retinoid X receptor (RXR) (Oro et al., 1990). The EcR-USP heterodimer binds DNA at ecdysone response elements and acts at molecular and tissue levels (For review see- Riddiford et al., 2001).

Classification of steroid hormone action-

The core paradigm or the central dogma for steroid hormone action has been that steroid hormones bind to their intracellular protein receptors that are ligand activated regulators of the transcription of genes. Hence the steroids trigger a genomic event that leads to transcription and protein synthesis, which in turn is responsible for the long lasting physiological response (Truss and Beato, 1993). Since the mechanism involves transcription and translation, there is a lag time between the binding of hormone to its receptor and to the first observable physiological effect caused by the hormone and are also sensitive to transcriptional or translational inhibitors. (Freedman, 1997). In contrast to this, these hormones may also be responsible for rapid cellular responses independent of gene
transcription and / or translation (Wehling, 1997). For example the steroids may act via cell surface receptor for rapid effects through second messenger system (see Fig. CI). Therefore the steroid hormones action can be classified into two categories-

- **Genomic actions**: well established classical or traditional mode of action
- **Nongenomic actions**: upcoming rapid effects of steroids

**Examples and characteristics of nongenomic effects of steroid hormones**

The first report of rapid steroid effect was published way back in 1942 where intraperitoneal application of progesterone induced a prompt anaesthesia in rats (Selye, 1942). This was followed by several other reports on rapid effects of steroids (Klein and Henk, 1963; Edwardson and Benet, 1974; Pietras and Szego, 1977, 1999). Spach and Streiten (1964) made an excellent observation on *in vitro* effects of physiological concentrations of aldosterone on Na⁺ exchange in dog erythrocytes that lacks nucleus. Their study clearly suggested that steroids can act in a pathway different from the established genomic mode of action. However, it is only recently that there has been an upsurge in the studies of rapid nongenomic actions of virtually all groups of steroids as well as thyroid hormones (Falkenstein *et al.*, 2000a; Davis *et al.*, 2002; Losel and Wehling, 2003). These non-genomic actions represent important new pathways for steroid hormone action on cellular function of varieties of cells and tissues. The important post-transcriptional or other types of rapid steroid effects include the regulations of mRNA stability in the cytoplasm (Cho and Raikhel, 2001; Nomura *et al.*, 2002), changes in membrane electrical activity (Sutter-Dub, 2002), conventional second messenger cascades such as phospholipase C (Civitelli *et al.*, 1990), phosphoinositide turnover (Morley *et al.*, 1992; Morelli *et al.*, 1993), intracellular pH (Jenis *et al.*, 1993; Wehling *et al.*, 1996), free intracellular Ca²⁺ (De Boland and Norman 1990; Wehling *et al.*, 1990), sodium transport (Christ *et al.*, 1995a; Ebata *et al.*, 1999), levels of cAMP (Christ *et al.*, 1999), cGMP (Chen and Chang, 1998), IP₃ + diacylglycerol (Christ *et al.*, 1993) and nitric oxide synthase (Wyckoff *et al.*, 2001). Steroids were also shown to regulate nongenomically the activity of almost all the major classes of protein kinases such as PKC (Sylvia *et al.*, 1993; Christ *et al.*, 1995b), PKA (Harrison *et al.*, 2000), MAP kinases (Endoh *et al.*, 1997) and tyrosine kinases (De Boland and Norman, 1998; Manegold, 1999) etc. All these rapid nongenomic effects of steroids are characterized by at least one of the two basic features-
• **Rapid physiological response:** the first observable cellular effect in response to hormone is seen within seconds to few minutes with few exceptions where it takes little longer time (Losel and Wehling, 2003).

• **No effect of the inhibitors of transcription or translation on cellular response**

**Classification of non-genomic action of steroids**

To address the increasing evidence for rapid effects of steroids and the diversity of mechanisms for rapid steroid signalling, Falkenstein *et al.*, (2000a, b) proposed a Mannheim classification scheme of these rapid non-genomic effects. According to this scheme the rapid non-genomic effects can occur in following ways-

a) **Through a direct effect**- The steroids can directly induce the rapid effect in the absence of receptor. It involves modulation of protein function reflecting changes in membrane physico-chemical properties. The apparent steroid specificity of these effects may thus reflect variable lipophilicity and polarity. *Eg.*, interaction of high concentrations of steroids like progesterone, 17α-hydroxyprogesterone, testosterone and estradiol with membrane vesicles prepared from phosphatidylserine and from lipid extracts of human and hamster spermatozoa (Shivaji and Jagannadham, 1992; Whiting *et al.*, 2000).

b) **Through a rapid non-transcriptional effect of the classical steroid receptor**- *Eg.*, involvement of classical estrogen receptor in rapid stimulation of endothelial nitric oxide synthase activity in response to estrogen, which is insensitive to transcriptional inhibitor actinomycin-D, but is completely inhibited by the antagonist, tamoxifen and ICI 182,780, which bind to classical estrogen receptor (Shaul, 1997).

c) **Through a distinct non-classical receptor that is possibly associated with the plasma membrane (also referred as membrane initiated steroid signalling-MISS)**- This involves majority of rapid steroid effects on cellular signalling and function reported so far. The rapid responses are identified to be transmitted by membrane receptor, unrelated to classical intracellular receptors as it is unaffected by the antagonists of the classical receptors (Watson and Gametchu, 1999; Nadal, 2000; Borski, 2000; Kelly and Levin, 2001; Beyer *et al.*, 2003; Boldyreff and Wehling, 2003). *Eg.*, aldosterone effects on various ion transport mechanism and second
messenger system (Scmidt et al., 2000), rapid stimulation of intestinal Ca\(^{2+}\) transport in perfused chick intestine (transcaltachia) as well as rapid stimulation of PKC and MAP kinase by 1\(\alpha\),25(OH)\(_2\)D\(_3\). (Zanello and Norman, 1997; Sylvia et al., 1998).

**Membrane receptors for nongenomic action of steroids**-

The membrane receptors have been identified for large number of steroids such as estrogen and xenoestrogen (Kelley and Levin, 2001; Nadal et al., 2000), glucocorticoids (Borski, 2000), androgens (Heinlein, 2002), estradiol (Benten et al., 2001), testosterone (Benten et al., 1999), aldosterone (Boldyreff and Wehling, 2003, Losel et al., 2002), Progesterone (Graham and Clarke, 1997) etc. Some authors suggest that these cell surface receptors are G protein coupled receptors (GPCR), which activate second messenger signalling mechanism (Wyckoff et al., 2001). Few evidences suggest that non-genomic effect of androgens and estrogens may occur through cell surface receptor to induce MAP kinase signalling cascade and induce ERK and p38 MAP kinases stemming from G-protein activation and the resulting calcium flux (Benten et al., 2001; Kelley and Levin, 2001; Heinlein, 2002). It is suggested that the membrane steroid receptors for mediation of rapid steroid effects, acts in multiple membrane localization mechanisms and receptor protein can act simultaneously or sequentially in a cell and receptor specific manner (Watson and Gametchu, 2003). Although various steroid hormones have been shown to bind to many biological membranes but the characterization of proteins to which they bind has mostly been limited to the determination of their molecular mass or their tentative identification by antibodies. Meyer et al., (1996), isolated a progesterone binding protein from porcine liver membrane. Further Gerdes et al., (1998) and Bernauer et al., (2001) cloned and analysed two putative progesterone binding membrane proteins from human and porcine liver. However, evidence remains to be seen in terms of cellular effects by such a steroid membrane receptor (Losel and Wehling, 2003).

Recently, Zhu et al., (2003b, c) unequivocally identified and characterized a progesterone membrane receptor in a fish, spotted seatrout responsible for progesterone induced meiotic maturation of oocytes. The group isolated a novel 352 amino acids protein by screening an oocyte expression library using monoclonal antibodies directed against the progestin binding oocyte membrane protein. This seatrout membrane progestin receptor (mPR) showed little sequence homology to GPCRs but contained seven putative hydrophobic transmembrane domains and was therefore considered a novel member of the heptahelical
GPCR receptor family. They further identified a family of mPR proteins from a number of different species including frog, human and mouse, some of which bound to progesterone. This mPR receptor satisfied the seven criteria for its designation as steroid membrane receptor. It had (i) a plausible structure (containing seven domains typical of GPCRs), (ii) tissue specificity (the mRNA detected only in brain and reproductive tissue), (iii) cellular distribution (present only in plasma membrane), (iv) steroid affinity (high affinity, saturable, displaceable, single binding sites for progestins), (v) role in signal transduction (activates MAP kinase and inhibits adenylyl cyclase), (vi) hormonal regulation and (vii) biological relevance. Hence, the identification of mPR gene has further redefined the non-genomic steroid mediated signalling that is often linked to GPCRs (Hammes, 2003). However, several questions such as (i) are these mPR classical GPCRs, (ii) do they bind specifically to a specific steroid or different steroids or even the non-steroids, (iii) what are the factors that direct the steroid binding to an individual target cell and (iv) how the hydrophobic steroids will specifically interact with a receptor that contains no known steroid binding domain, remain unanswered

**Modulation of genomic and nongenomic actions of steroids by a cross-talk-**

It is becoming increasingly clear now that steroid hormones can act at three different cellular levels viz., membrane, cytosol and nucleus with interdependence on each other (Valverde and Parker, 2002; Edwards *et al.*, 2003; Losel and Wehling, 2003). The membrane target includes the non-classical steroid receptor and ligand or voltage activated ion channels. The activation of some of these receptors triggers signalling events, leading to the regulation of various kinases and phosphatases. The major targets are believed to be the classical translocating receptors, which are generally associated with signalling pathways (*eg.*, Src-PI3K. Akt and / or Src-Ras-ERK). The regulation at the membrane or cytosolic targets might ultimately determine the change in gene expression by the interaction of steroids with classical nuclear targets.

**Nongenomic actions of ecdysteroids-**

The classical mode of action of ecdysteroids like all other steroids is the receptor mediated regulation of gene activity influencing transcription and subsequently protein synthesis for the delayed cellular effects. There is also an increasing evidence for rapid non-genomic responses of ecdysteroids although the field is not explored as in the case of steroids
from vertebrate (Tomaschko, 1999). The favored target for the ecdysteroids is the plasma membrane and its associated protein where it interacts with Na\(^+\)-H\(^+\) exchangers and K\(^+\) channels. Thus the ecdysteroids are suggested to regulate nongenomically the ecdysteroid transport, electrolyte transport (Na\(^+\), K\(^+\), H\(^+\), Ca\(^{2+}\), Cl\(^-\)), second messenger (cAMP, Ca\(^{2+}\) level) and protein kinase activity. The uptake of ecdysteroid like other steroids across the membrane into a specific target cell is suggested to occur by diffusion. However, Spindler and his coworkers have suggested a carrier mediated transport for ecdysteroid uptake into crayfish hypodermis (Daig and Spindler 1983a, b; Spindler and Grossman, 1987). In *Sarcophaga peregrina* and *Calliphora vicina* the ecdysteroid mediated activation of hexamerin receptor was found to be independent of transcription and protein synthesis (Ueno et al., 1983; Ueno and Natori, 1984; Chung et al., 1995; Burmester and Scheller, 1997a). The effect of ecdysteroid on selective phosphorylation of protein has also been demonstrated in *S. peregrina* (Itoh et al., 1985, 1986) and *M. brassicae* (Sass, 1988). These are excellent examples of post-translational modification, independent of accompanied protein synthesis.

**Regulation of ecdysteroids action**

A major area with regard to understanding the regulation of 20E action is that of tissue specificity. The diversity in function of the hormone to some extent could be due to the variation of responses among cell types that typifies the action of 20E. The 20E also shows differential effects on same tissue at different developmental stages (De Loof, 1986; Riddiford et al., 2001). Thus studies directed towards the molecular basis of differentiation during the development and the built in regulatory mechanisms at the tissue level largely aid in the understanding of 20E actions. The role of ecdysteroids, particularly 20E in eliciting the molt is no longer in question and has been established as the central dogma of the field. The role of ecdysteroids in postembryonic development of insects is well documented (Sehnal, 1989; Steele and Vafopoulou, 1989; Lanot et al., 1989; Gilbert et al., 1996; Gu and Chow, 1996, 1997). In contrast to vertebrate systems, ecdysteroids perform a wide variety of functions in the entire insect class. Hence, it is often referred that almost the entire insect is target of ecdysteroids (Gilbert et al., 1996). It stimulates the growth and development of imaginal discs, promotes the deposition of cuticle by epidermis, regulates the growth of motor neurons, regulates defensive secretions and controls choriogenesis (Gilbert et al., 1996). The ecdysteroid also initiates the breakdown of larval structures during metamorphosis (Lockshin and Beaulton, 1974; Truman, 1996a, b). In the present work,
studies have been carried out to understand the regulation of this important aspect of metamorphosis by ecdysteroids. The ecdysteroids are regulated at all levels *i.e.*, the biosynthesis, the titer in haemolymph and the action mediated by it (Smith, 1985; Riddiford et al., 2001; Gilbert et al., 2002). As it is clear, there exists a vast amount of information regarding the regulation of ecdysteroids at the synthesis as well as at its titer level. However, knowledge regarding the mechanism of regulation of ecdysteroid dependent actions is not very clear and the field remains largely unexplored.

In majority of holometabolous insects including lepidopterans, the 20E action is regulated to induce transition from the juvenile to adult forms. During this period there is differentiation of undifferentiated stem cells to functional adult structure. Furthermore, the differentiation of various tissues that are required during both the larval and imaginal stages as well as the ones, which are selectively imaginal, depend upon pulses of 20E (Granger and Bollenbacher, 1981; Riddiford, 1985; Sridevi et al., 1988a, b; Gu and Chow, 1993; Wang et al., 1995; Sehnal et al., 1996).

Extensive studies from our laboratory reveal that 20E stimulates synthesis of various proteins, in different tissues during the postembryonic development of lepidopteran insects (Ray et al., 1987a, b; Sridevi et al., 1988a, b, 1989; Ismail and Dutta-Gupta 1990a; Dutta-Gupta et al., 1996; Shanavas et al., 1996). The uptake or sequestration of storage proteins (hexamerins) by the fat body (Ismail and Dutta-Gupta, 1990b; Dutta-Gupta and Ismail, 1990, 1992; Kiran Kumar et al., 1997, 1998) as well as the male accessory reproductive gland (Ismail and Dutta-Gupta, 1990c, 1991; Dutta-Gupta and Ismail, 1992; Ismail et al., 1993) was also shown to be regulated by ecdysteroids. Studies from our laboratory also revealed that the lysosomal activity in the whole body as well as in the fat body exhibits a specific pattern during postembryonic and adult development and that an increase in the lysosomal activity is governed by the elevation of 20E levels (Rao et al., 1984; Ray et al., 1984; Sridevi et al., 1987; Ashok and Dutta-Gupta, 1988; Dutta-Gupta and Sridevi, 1991). Despite these studies, the mechanism of regulation of the majority of ecdysteroid dependent actions are not clear and hence demands for further research.

**Fat body tissue and its significance for the present study-**

The fat body in insects is an important metabolic centre and biochemically most active organ in insects with multiple functions such as metabolism of proteins, carbohydrates
and lipids particularly blood sugar and haemolymph proteins such as vitellogenins and hexamerins. It is also involved in detoxification, synthesis and hormone metabolism (Keeley 1985) and is a possible source of humoral factors (Meola and Gray, 1984, Gray et al., 1987). In view of the complex function performed by fat body, it is often compared to vertebrate liver and hence is a suitable tissue for the studies of the stage and tissue specific expression of genes, post-transcriptional regulation of RNA and post-translational control of proteins (Hansen et al., 2002). The fat body undergoes growth and development along with the other tissues and its function changes in accordance with the developmental stage of the insect (Vanishree et al., 1999). The fat body tissue is structurally organized to provide maximal exposure to the haemolymph and due to its changing metabolic role and integral position in maintaining metabolic homeostasis, it serves as an ideal model for endocrine regulated studies (Kunkel, 1981). Most of the studies with protein expression and sequestration by fat body have been carried out with the entire tissue (Locke and Collins, 1965; 1968). However, there are evidences in both diptera and lepidoptera that suggest regional differences in activity and function of fat body tissue. In case of Helicoverpa zea, the storage proteins are synthesized by peripheral fat body fraction but are sequestered and stored only by perivisceral fat body (Wang and Haunerland, 1994a). In the silkworm Bombyx mori, it has been shown that the dorsal and ventral perivisceral fat body contains the most competent cells for sequestering haemolymph protein as compared to the peripheral and hindgut associated fat body tissue (Vanishree et al., 1999). Another important aspect of studies using insect fat body, as model tissue is that it functions normally under cultured condition. Nakanishi and Garen (1983), reported that ecdysteroid exerts its effect on the cultured fat body system and the expression pattern of LSP-2, P1 and G12 was similar both under in vivo and cultured condition.

Protein phosphorylation: a general account-

Protein phosphorylation is the major regulatory mechanism by which synthetic activities of various tissue or cell types are controlled by external physiological stimuli. It is one of the major post-translational events whose importance is established in hormone action (Cohen, 1982; Boyer et al., 1983; Cochrane and Deeley, 1984). The protein phosphorylation system consists of three primary components, a protein kinase, a protein phosphatase and a substrate protein. When a protein kinase transfers the terminal (γ) phosphate from ATP to the hydroxyl group of a serine or threonine or tyrosine residue of the substrate protein, the
substrate protein is phosphorylated. These phosphate moieties can be removed from the phosphorylated protein by phosphatases. This kind of reversible protein phosphorylation is a post-translational modification well known to play a role in variety of cellular functions (Graves and Krebs, 1999; Davies et al., 2000). It is a highly regulated process by which information can be shuttled from the cell surface to nucleus (Denu et al., 1996). On the basis of the second messenger system required to activate specific kinase, it is classified as cAMP dependent protein kinases (PKA), cGMP dependent protein kinases, multiple types of calcium dependent protein kinase (CaM kinases) and a calcium/phospholipids dependent protein kinase (PKC). In recent years, it has become increasingly evident that many of the cellular actions of calcium in mediating the signalling are by its binding to specific kinases resulting in their activation, which thus phosphorylate a specific protein (Nestler and Greengard, 1984; Pinna and Ruzzene, 1996). Another class of kinase is tyrosine kinase which specifically catalyses the phosphorylation at tyrosine residues. These tyrosine kinases are classified into receptor and non-receptor tyrosine kinases (Hunter and Cooper, 1985).

Although extensive studies on protein phosphorylation systems have been carried in vertebrates, very little information is available on insects. However, in the last few decades, several studies have been initiated on this aspect and kinases such as CaM kinase II (Shanavas et al., 1998), cyclic nucleotide dependent protein kinases like PKA (Chalaye et al., 1988; Muller and Spatz, 1989; Jiang and Struhl, 1995; Lepage et al., 1995; Smith et al., 1996; Muller, 1997a) and cGMP-PK (Foster et al., 1996; Muller, 1997b) have been well characterized from various tissues of different insect species.

**Protein phosphorylation: role of 20E and other insect hormones**

Numerous report convincingly suggest that phosphorylation of specific substrates by a variety of protein kinases appears to be a general mechanism by which many hormones, neurotransmitters and other extracellular signals produce their physiological responses in specific target cells (Greengard, 1978; Graves and Krebs, 1999; Davies et al., 2000; Cohen, 2002). However, only a few reports are available regarding the regulation of protein kinase activity by insect hormones. These studies suggest that 20E not only exerts its action through the modulation of transcription and translation but also exerts its effect at the post-translational level eg., by protein phosphorylation. It has been shown to stimulate *in vitro* phosphorylation of few fat body proteins in *M. brassicae* (Sass, 1988). Itoh et al., (1985, 1986), suggested that the 20E regulated phosphorylation of a 30 kDa protein was responsible
for the conversion of the fat body from a synthetic to storage organ in *S. peregrina*. Casein kinase II activity in the brain of *Acheta domesticus* was shown to be inhibited by the injection of 20E (Degrelle *et al.*, 1997). Earlier studies from our laboratory have revealed autophosphorylation of CaM kinase II in the CNS of *B. mori* (Shanavas *et al.*, 1998) and the phosphorylation of storage protein binding protein (SPBP) in the fat body membranes of *C. cephalonica* (KiranKumar, 1998). These two proteins are developmentally regulated (Shanavas *et al.*, 1998; KiranKumar, 1998) and their phosphorylation in fat body tissue was found to be 20E dependent (Vasanthi, 1999). Hence, the two proteins provided an excellent system for the present study to check if the phosphorylation of these proteins is regulated by ecdysteroid at nongenomic level and evaluate the role or function of haemolymph proteins or humoral factors in these proteins.

In addition to 20E, other insect hormones are also shown to be involved in the signal transduction mechanism. Juvenile hormone (JH) was shown to be involved in the activation of a specific Na\(^{+}\)-K\(^{+}\) ATPase via PKC in the follicle cell membrane of *Rhodnius prolixus* (Sevala and Davey, 1989). It was also shown to stimulate protein synthesis in male accessory glands of *D. melanogaster* through the activation of PKC (Yamamoto *et al.*, 1988). PTTH has been shown to stimulate cAMP-PK activity in the prothoracic glands of *M. sexta* (Smith *et al.*, 1996). The cGMP dependent protein kinase activity in the CNS of *M. sexta* was stimulated by eclosion hormone (Morton and Truman, 1986, 1988).

**Calcium dependent protein kinases: role of 20E-**

Mainly two classes of calcium dependent protein kinases are known and they are calcium/calmodulin dependent protein kinases (CaM kinases) and calcium/phospholipid (phosphatidylserine) dependent protein kinases (PKC).

The CaM kinases act via the interaction of calcium with calmodulin (CaM). Calmodulin is a ubiquitous low molecular weight protein, which undergoes structural changes upon binding with Ca\(^{2+}\) and is thus activated. The Ca\(^{2+}\)/CaM complex then binds and alters the function of other cellular proteins (Cheung, 1980; Carafoli, 1987; Heizmann and Hunziker, 1990). Several studies over the last two decades established that some of the second messenger actions of calcium in a variety of tissues is due to the activation of CaM kinases and protein phosphorylation is one of the important routes by which Ca\(^{2+}\)/CaM signal transduction regulates cellular function (Hanson and Schulman, 1992a, b). Multiple types of
CaM kinases have been demonstrated and characterized in the mammalian neural and non-neural tissues. They include CaM kinase I (Nairn and Greengard, 1987), II (Kennedy and Greengard, 1981), III (Nairn et al., 1985), IV (Kato et al., 1992), myosin light chain kinase (Hagiwara et al., 1989) and phosphorylase kinase (Cohen et al., 1978). CaM kinase II is the most abundant type and has been well characterized in the neural tissue. It was first identified in rat brain as a Ca\(^{2+}\) dependent protein kinase that catalyses the phosphorylation of site 2 and 3 of synapsin 1 (Kennedy and Greengard, 1981). Rat brain CaM kinase II comprises of several related isozymes (Hanson and Schulman, 1992a, b) that 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). A distinct property of CaM kinase II is the autophosphorylation of a threonine residue near its calmodulin binding domain which converts the enzyme to Ca\(^{2+}\) independent form (Miller and Kennedy, 1986; Wang et al., 1994). It was postulated that this autophosphorylation induce changes in subcellular distribution of enzyme in *Aplysia* and *D. melanogaster* (Saitoh and Schwartz, 1985; Willmund et al., 1986) and may also be involved in prolonging the effects triggered by a transient calcium signal (Miller and Kennedy, 1986).

There are only a few reports on the characterization of CaM kinase II in insects (Cho et al., 1991; Ohsako et al., 1993; Shanavas et al., 1998). Adult *D. melanogaster* head contains three species of CaM kinase II with molecular masses of 54/55, 58 and 60 kDa (Cho et al., 1991, Ohsako et al., 1993). The amino acid sequence and tissue specificity of the rat kinase are highly conserved in *D. melanogaster*. Extensive studies from our laboratory suggested that the *B. mori* CNS consists of two species of CaM kinase II with molecular mass of 59/60 kDa, which cross-react with anti-rat CaM kinase II\(\alpha\) monoclonal antibody and show a high degree of autophosphorylation in neural tissue (Shanavas, 1997; Shanavas et al., 1998). Further, two peaks of enzyme activity occurred in the CNS of *B. mori* during the postembryonic development, the first peak at late-larval stage and the next peak at late-pupal stage (Shanavas et al., 1998) that coincided with the reported parallel changes in the ecdysteroid titer in the haemolymph of *B. mori* (Calvez et al., 1976). Transformed strains of *D. melanogaster*, expressing a transgene inhibitor of CaM kinase II have been shown to be deficient in an associative conditioning behavioral paradigm (Griffith et al., 1993; Wang et al., 1994). It was also shown to be involved in the phosphorylation of *D. melanogaster* visual phosrestin, thereby regulating photoreceptor light adaptation (Kahn and Matsumoto, 1997; Kahn et al., 1998).
Another class of calcium dependent protein kinase is the calcium/phospholipid (phosphatidylserine) dependent protein kinases, also known as protein kinase C (PKC). It was originally identified as a serine/threonine kinase that was maximally active in the presence of diacylglycerol (DAG) and calcium. There are at least ten proteins of PKC family that play a critical role in regulating the cellular functions and are involved in the modulation of signal transduction (Nishizuka, 1988, 1995; Newton, 1995). A variety of hormone, growth factors and neurotransmitters are known to regulate inositol phospholipid breakdown to generate DAG and thereby mediate their actions by activating PKC (Kikkawa et al., 1982; Mosior and Epand, 1993). Evidences exist for the activation of JH dependent Na\(^+\)-K\(^+\) ATPase via PKC (Sevala and Davey, 1989). However the information with respect to 20E is not yet clear. In invertebrates, PKC regulated mechanism is suggested to play an important role in the process of neuronal plasticity (Altfelder et al., 1991; Choi et al., 1991; Emptage, 1993; Olds and Alkon, 1993). Recently, interest has shifted to the events downstream of PKC activation cascade, namely to the identification of physiological substrates of PKC to understand the relationship between PKC activation and cellular responses. In an invertebrate, *Hermissenda crassicornis*, a 20 kDa substrate was found to exhibit GTPase activity which reduced K\(^+\) currents. Furthermore, a change in its rate of phosphorylation was seen with learning (Nelson et al., 1990). Muller (1997c) reported a 86 kDa, filament interacting protein in honeybee *Apis mellifera* as a substrate for PKC. Since the interaction of the protein with the cytoskeleton is regulated by Ca\(^{2+}\)/calmodulin and phosphorylation, this PKC substrate was suggested to be a potential site of convergency in regulation of cytoskeleton-membrane rearrangement like the members of the vertebrate myristoylated alanine rich C kinase substrate (MARCKS) family. These studies however do not throw any light on possible effects on PKC or its substrates.

**Tyrosine kinases: role of 20E**

The tyrosine kinases are another very important class of protein kinases, which specifically catalyses the phosphorylation of proteins at tyrosine residues and regulate the cellular functions. These kinases are identified to be of two types, receptor tyrosine kinases (RTKs) and the non-receptor tyrosine kinases. The RTKs contain four domains- (i) an extracellular ligand binding domain, (ii) an intracellular tyrosine kinase domain, (iii) an intracellular regulatory domain and (iv) a transmembrane domain. The amino acid sequences of the tyrosine kinase domain of RTK are highly conserved with those of PKA within the ATP binding and substrate binding regions. The RTKs are classified into at least 14 different
families based upon the structural features in their extracellular portions. Many receptors that have intrinsic tyrosine kinase activity as well as the tyrosine kinases that are associated with the cell surface receptors, contain tyrosine residues, which upon phosphorylation interact with other proteins of the signalling cascade. These other proteins contain amino acid sequence that is homologous to a domain, first identified in the c-Src proto-oncogene and was termed SH2 domain (Src homology domain 2). Another conserved protein-protein interaction domain identified is related to a third domain in c-Src and termed as SH3 domain. The interactions of SH2 domain containing protein with RTKs or receptor associated tyrosine kinase leads to tyrosine phosphorylation of the SH2 containing proteins that have enzymatic activity and caused an alteration in the activity. The non-receptor tyrosine kinases are responsible for phosphorylating variety of intracellular proteins, following activation of cellular growth and proliferation signals. These kinases distinctly belong to two different families- (a) related to Src protein and (b) Janus kinases (Jak). In both families the non-receptor tyrosine kinase couples to cellular receptor that lack enzymatic activity themselves (For review see- Hunter and Cooper, 1985; Koch et al., 1991; Pazin and Williams, 1992; Fantl et al., 1993; Smithgall, 1995; Gerber, 2002).

In insects, several RTKs have been identified in D. melanogaster (Yamamoto, 1994; Freeman, 1996; Raabe et al., 1996). The D. melanogaster homologue of the mammalian EGF receptor has been identified as an RTK and shown to be involved in many stages of development (Doyle and Bishop, 1993; Duffy and Perrimon, 1994; Schweitzer and Shilo, 1997). Insulin receptor like tyrosine kinase activity has been reported in PGLs of M. sexta (Smith et al., 1997). The tyrosine kinase activity was found in the cotton leaf worm Spodoptera littoralis (Pearce et al., 1994). Phosphotyrosine containing proteins have been detected in different tissues of the mediterrenian fruit fly Ceratitis capitata and their role during pupation is discussed (Katsoris et al., 1991). Although the role of insect hormone on tyrosine kinase signalling is not very clear. Some authors have suggested that these kinases stimulate ecdysteroid production in the mosquito Aedes aegypti (Riehle, 1999). In a recent study, the PTTH stimulated ecdysone secretion was shown to be dependent upon the tyrosine phosphorylation in the prothoracic glands of M. sexta (Smith et al., 2003). Inhibition of tyrosine kinases has been suggested to impair axon extension in the nervous system of grasshopper embryo (Menon and Zinn, 1998). Studies from our laboratory suggest that phosphorylation of a 48 kDa protein in neural tissue of B. mori (Shanavas, 1997) and a 120 kDa storage protein binding protein (SPBP) in the fat body of C. cephalonica (KiranKumar,
are probably mediated by tyrosine kinases. In the present study an attempt has been made to understand the physiological significance of the phosphorylation of 48 and 120 kDa proteins as the 120 kDa SPBP is involved in the uptake of hexamerins by the non-feeding prepupal and pupal fat body in *C. cephalonica*. This uptake is an essential process required for the proper growth and development of insects during pupal-adult transformation.

**Hexamerins: synthesis, release and receptor mediated uptake**

As insects do not feed during prepupal and pupal stages, they depend on macromolecular supply (proteins, lipids and carbohydrates) that has previously been accumulated during the larval period and use them as building blocks for the development of imaginal tissues. In all holometabolous insects investigated so far, amino acids and energy are supplied by proteins that have been selectively taken up by the fat body from the haemolymph (Haunerland, 1996; Burmester and Scheller, 1999). Most of the sequestered proteins belong to the family of hexamerins, haemocyanin-related proteins, named according to their composition of six identical or closely related subunits (Telfer and Kunkel, 1991).

Hexamerins (often called larval serum proteins, storage proteins or arylphorins due to high content of aromatic amino acids) are high molecular weight multimers, usually hexamers. Each monomer is composed of subunits in the mass range of 70-90 kDa. This characteristic feature seems to be well retained in the several orders of holometabolous insects as well as in some orders of hemimetabolous insects (Levenbook, 1985; Rahbe *et al.*, 1990; Rehn and Rolim, 1990; Telfer and Kunkel, 1991; Martinez and Wheeler, 1993; Tojo

![Representation of hexamerins synthesis by the actively feeding larval fat body cells, its release into haemolymph and its uptake by the pupal fat body cells (shown in same cell) through a 20E dependent receptor mediated endocytosis. Modified from Haunerland (1996) and Burmester and Scheller (1999).](image)
and Yoshiga, 1994; Haunerland, 1996). In *C. cephalonica*, the native protein is a hexamer (500 kDa) of three subunits with masses 86 kDa (Hex 1), 84 kDa (Hex 2) and 82 kDa (Hex 3) (Ismail, 1991; KiranKumar *et al.*, 1997; Nagamanju *et al.*, 2003). The hexamers are synthesized predominantly by the fat body during the actively feeding larval period and are released into the haemolymph. During the last larval instar of lepidopteran insects, these proteins nearly account for 70-80% of the total soluble protein by weight (Kanost *et al.*, 1990; Telfer and Kunkel, 1991). Shortly before pupation, they are sequestered by the fat body through a receptor mediated endocytosis process and accumulate as dense protein granules to serve as a reserve pool of amino acids required for the remodeling of tissues and for deposition of cuticle during pupal-adult transformation and reproduction (Levenbook, 1985; Bean and Silhacek, 1989; Chrysanthis *et al.*, 1994; Haunerland, 1996; Burmester and Scheller, 1999; Lay *et al.*, 2004).

Endocytosis, mediated by cell-surface receptors, is an essential process in all eukaryotes and required for the uptake of various proteins (including hexamers), hormones, nutrients and vitamins as well as for the recycling of membranes (Pierce and Robinson, 1990; Watts and Marsh, 1992; Schmid, 1995). The uptake of hexamerin from insect haemolymph by the fat body cells is a unique feature of the class “*insecta*” involving a receptor which does not belong to the low density lipoprotein (LDL) superfamily or to any other receptor known to date (Burmester and Scheller, 1997a, b, 1999).

**Hexamerin receptor and role of 20E on its activation for hexamerin uptake**

The transport of hexamers across the fat body cell membrane by the non-feeding prepupal and pupal stages requires the existence of a specific receptor. These receptors have been recognized in dipteran as well as lepidopteran insects but the sequences of the receptors are known only from the dipteran clan, notably from flesh fly *Sarcophaga peregrina* (Chung *et al.*, 1995), blow fly *Calliphora vicina* (Burmester and Scheller, 1995) and fruit fly *D. melanogaster* (Burmester *et al.*, 1999). These receptors show a significant similarity to their ligands, the hexamers suggesting that the receptors evolved from their own ligands even before the divergence of winged insects (Burmester and Scheller, 1996; Burmester, 2002). In *S. peregrina*, a 120 kDa receptor was identified. Under the influence of 20E, the receptor acquires the ability to sequester hexamerin (Ueno and Natori, 1984; Chung *et al.*, 1995). Studies in *C. vicina* revealed that the hexamerin receptor is synthesized as a precursor (130 kDa), which is subject of a three-fold post-translational cleavage to give rise the active
receptor (Burmester and Scheller, 1997a). The onset of hexamerin uptake coincides with the third cleavage, which is initiated by ecdysteroids (Burmester and Scheller, 1997, 1999). Both in *S. peregrina* and *C. vicina*, the ecdysteroid mediated activation of the hexamerin receptor was found to be independent of transcription and protein synthesis suggesting that the receptor activation by hormone occurs at a post-translational level (Ueno and Natori, 1984; Burmester and Scheller, 1997). It has also been shown that a rise in the ecdysteroid titer at the end of larval life triggers the incorporation of hexamerins in the fat body of the fruit fly, *D. melanogaster* through fat body protein 1 (*Fbp-1*) receptor (Burmester *et al*., 1999). Hansen *et al*., (2002) reported the presence of an anterior fat body protein in *C. vicina*, which interacts with the hexamerin receptor and regulates hexamerin uptake by the fat body cells in the posterior part of the organ. The group further identified the binding domains of the receptor by yeast-two-hybrid system (Hansen *et al*., 2002, 2003). These domains do not show any similarity to any functional protein domains known to date. However, the puzzling feature of the hexamerin receptor *i.e.*, the absence of a typical membrane-spanning domain, to explain how the receptor mediates endocytosis remains unclear.

In the bollworm, *Helicoverpa zea*, a single 80 kDa receptor protein was reported to mediate the uptake of VHDL and storage proteins (Wang and Haunerland, 1993; 1994a, b). Our laboratory has identified hexamerins in *C. cephalonica* and *Chilo partellus* and focused on the uptake of these proteins by the fat body and male accessory reproductive glands (MARG) (Ismail and Dutta-Gupta, 1990b, c, 1991; KiranKumar *et al*., 1997). Using ligand binding studies, we have earlier demonstrated the presence of 120 kDa hexamerin receptor in the fat body membrane of *C. cephalonica* (KiranKumar *et al*., 1997). The receptor was found to be present in the last larval instar and at maximal concentration in the prepupal stage. The sequestration of hexamerin in *C. cephalonica*, like in other lepidopteran insects, was not observed during the larval stage (Ismail and Dutta-Gupta, 1990b, c). However, 20E treatment induced a precocious uptake of hexamerins in the late-last instar (LLI) larval fat body (Ismail and Dutta-Gupta, 1990b). These studies suggest that ecdysteroid hormone activates the hexamerin receptor prior to hexamerin uptake.

**Acid phosphatases: a general account**

The acid phosphatases (ACPs) are a group of enzymes capable of hydrolysing esters of orthophosphoric acid in an acid medium. They are widely distributed and represent a heterologous group of enzymes with multiple isoforms and different isozymes (Egawa *et al*.,
1995). The physiological functions of ACPs are to provide inorganic phosphate as a building block in making new cells. They are broadly classified into two types (a) the lysosomal ACPs (EC 3.1.3.2) and (b) the phosphatidic acid phosphatases or PAP (3.1.3.4). The PAPs are further classified into type 1 (PAP1) and type 2 (PAP2) PAPs. The PAP1 (38-43 kDa) is the cytosolic or membrane bound ACP and is involved in the supply of the diacylglycerols in the classical pathway of glycerolipid biosynthesis by dephosphorylating phosphatidic acid phosphate (Martin et al., 1987; Moolenaar et al., 1992). The PAP2 (35-50 kDa) is membrane bound and is involved in signal transduction that is mediated by phospholipase D (Exton, 1990; Kai et al., 1996). In this case, the phosphatidic acid cleaved from the major membrane phospholipid, phosphatidylcholine is converted by PAP to diacylglycerol, which serves as a lipid second messenger by activating protein kinase C (Nishizuka, 1984a, b; Kai et al., 1996).

In the holometabolous insects the larval structures degenerate at the beginning of metamorphosis (Schin and Clever, 1968; Radford and Misch, 1971; Lockshin and Beaulton, 1974). Lysosomal enzymes are known to play important role in histolysis of larval organs, cellular destruction, tissue remodelling and reorganisation. The metabolic fuels for these are provided primarily by the fat body. Acid phosphatase is one of the most commonly used marker enzyme to study the lysosomal activity in insects (Verkuil, 1979, 1980). The enzyme has been identified in every organism studied to date and they exist in multiple forms and different isozymes (Konichev, 1982; Kutuzova, 1991). In D. melanogaster, ACP has often been used as a tool for survey of genetic polymorphism, using a major acid phosphatase gene Acph-1 (MacIntyre, 1966; Chung et al., 1996). A high increase in the activity of the lysosomal marker enzyme ACP is observed at the beginning of the wandering prepupal stages of dipteran as well as lepidopteran insects, preceding the actual metamorphosis (Verkuil et al., 1979; Ashok an Dutta-Gupta, 1988; Fialho et al., 2002). Transplantation experiments with Calliphora erythrocephala and thorax-ligation as well as the exogenous ecdysteroid injection studies suggest that the induction of lysosomal activity is under hormonal control possibly by ecdysteroids (Verkuil et al., 1979; Verkuil, 1980; Ashok and Dutta-Gupta, 1988). In the larval fat body of Calpodes ethlius a relationship could be derived between the autophagic events observed in the ultra-structural study (Locke and Collins, 1968) and variations in ACP activity in homogenates (Collins, 1975). These studies clearly suggest that the rise in ACP activity in the fat body of the insect larvae may be related to increasing autophagic activity leading to the elimination of certain cytoplasmic organelles like endoplasmic reticulum and mitochondria.
**Autophagy and metamorphosis: regulation by ecdysteroids**

It is well established that metamorphosis in insects is the transition from the larval to adult stage and the events are controlled by ecdysteroids (Riddiford *et al.*, 2001; Truman and Riddiford, 2002; Gilbert *et al.*, 2002). It involves the breakdown of larval structures and the formation of new tissues that occurs either by apoptosis of individual cells or autophagy of group of cells (Lockshin and Bealuton, 1974; Thummel, 2001; Trumann and Riddiford, 2002). As a part of cell remodeling during metamorphosis, acidic autophagic vacuoles accumulate in the fat body cell and activity of several lysosomal enzymes such as ACPs increases and cause the lysis of larval tissues (Verkuil, 1980; Sass and Kovacs, 1980; Thummel, 2001; Lee and Baehriecke, 2001). The fat body that fills a large fraction of the insect body and whose function has been considered equivalent to the role of the vertebrate liver in the intermediary metabolism (Kunkel, 1981; Keeley, 1985; Vanishree *et al.*, 1999; Hansen *et al.*, 2002) shows high activity of lysosomal enzymes. The stimulation of the lysosomal activity by ecdysteroids is well demonstrated in several insects including *C. cephalonica* (Verkuil *et al.*, 1979; Verkuil, 1980; Ashok and Dutta-Gupta, 1988; Sass *et al.*, 1989; Kutuzova *et al.*, 1991). Studies also provide evidence that the induction in lysosomal activity by ecdysteroids is governed at a non-genomic level (Verkuil, 1979). However, no evidence has been provided to support the hypothesis. In the present study an attempt has been made to understand the activation of ACP by 20E in the fat body cells of the rice moth, *C. cephalonica*.

**Why present study**

The present interest to characterize haemolymph protein (HP) in order to understand the regulation of ecdysteroid action in insects rose from the repeated interesting observation that injection of exogenous 20E stimulated the lysosomal activity in the fat body of the thorax-ligated larvae of *C. cephalonica* (Ashok and Dutta-Gupta, 1988). However, addition of 20E to larval fat body cultures failed to stimulate the enzyme activity. Studies conducted earlier in *M. sexta* also revealed that the ACP activity was not induced in response to exogenous 20E alone but the reason was elusive (Caglayan, 1990). However, Ashok and Dutta-Gupta (1991) for the first time reported a significant stimulation in the fat body ACP activity, when haemolymph from larval stage of *C. cephalonica* was added to the fat body cultures in the presence of 20E. This suggested that the hormone might require the presence
of some additional factors under *in vitro* condition, which are present in the *in vivo* system to mediate the action, on lysosomal activity. Therefore an attempt was made in the present study to analyze the possible role of haemolymph factor(s) if any, in mediating the 20E action on selected marker proteins. Thus, a protein in the haemolymph named HP19 *i.e.*, haemolymph protein of mass 19 kDa was identified as a possible molecule in regulating few of the 20E mediated actions in some of the lepidopteran insect species.

**Objectives of the present study**-

Postembryonic development in insects involves growth, molting and metamorphosis. These events are controlled mainly by two morphogenetic hormones namely ecdysteroids and juvenile hormones. There is vast amount of information about the regulation of these hormones at synthesis as well as at its titer level. The information also exists about the interaction of these hormones among themselves to regulate metamorphosis. However, knowledge regarding the regulation of hormone dependent actions is limited and the field remains largely unexplored. In the present study, an attempt has been made to understand this important aspect of insect molting hormone, ecdysteroids. The study discusses the appearance of a stage and tissue specifically regulated protein HP19. This protein is identified in the present study and is found to be responsible for the regulation of few of the 20-hydroxyecdysone (20E) dependent actions.

**The specific objectives of the dissertation work are given below:**

1. Confirmation of the presence of factor(s) in *C. cephalonica* larval haemolymph required for 20E stimulation of acid phosphatase.

2. Understand the nature of the haemolymph factor, its isolation, characterization and developmental regulation.


4. Role of HP19 in postembryonic development.

5. Effect on 20E regulated actions.

6. Mechanism of 20E regulated actions that are mediated by HP19.