Annu. Rev. Entomol. 2002. 47:883–916

CONTROL AND BIOCHEMICAL NATURE OF THE ECDYSTEROIDOGENIC PATHWAY

Lawrence I. Gilbert, Robert Rybczynski, and James T. Warren

Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-3280; e-mail: lgilbert@unc.edu; rybczy@bio.unc.edu; jwarren@bio.unc.edu

Key Words molting, cyclic AMP, ecdysone, prothoracicotropic hormone, protein kinase, steroid hormone

■ **Abstract** Molting is elicited by a critical titer of ecdysteroids that includes the principal molting hormone, 20-hydroxyecdysone (20E), and ecdysone (E), which is the precursor of 20E but also has morphogenetic roles of its own. The prothoracic glands are the predominate source of ecdysteroids, and the rate of synthesis of these polyhydroxylated sterols is critical for molting and metamorphosis. This review concerns three aspects of ecdysteroidogenesis: (*a*) how the brain neuropeptide prothoracicotropic hormone (PTTH) initiates a transductory cascade in cells of the prothoracic gland, which results in an increased rate of ecdysteroid biosynthesis (upregulation); (*b*) how the concentrations of 20E in the hemolymph feed back on the prothoracic gland to decrease rates of ecdysteroidogenesis (downregulation); and (*c*) how the prothoracic gland cells convert cholesterol to the precursor of E and then 20E, a series of reactions only now being understood because of the use of a combination of classical biochemistry and molecular genetics.

CONTENTS

0066-4170/02/0101-0883$14.00

INTRODUCTION

Although insects are not the only animals that molt[1] (see 2, 143 for a discussion of the monophyletic taxon Ecdysozoa), insects have used that process efficiently over hundreds of millions of years in order to grow at times when predators and other endangering environmental factors (e.g., temperature or photoperiod shifts) were less threatening. Evolutionary biologists suggest molting arose as an adaptation to allow an increase in size of secondary locomotory devices such as cuticular spines (170). Many of the morphological, physiological, biochemical, and molecular events occurring during the molting process are elicited and/or modulated by the principle molting hormone, 20-hydroxyecdysone (20E), although its precursor ecdysone (E) likely has role(s) independent of 20E, as do several peptidergic hormones. Therefore, current thinking accepts the premise that 20E is the major, although hardly the sole, player. As the years pass, there is little doubt that myriad factors will be identified and characterized that are influential in controlling or modulating the virtually countless complex events comprising molting and metamorphosis.

It should, therefore, be obvious that studies of the control of the hemolymph and tissue titers of 20E and E are critical if we are to design physiologically relevant experiments in the hope of understanding the complexities of insect morphogenesis. As has been demonstrated consistently, arthropods cannot synthesize sterols from a simple precursor molecule such as acetate, but they require sterols in their diet [e.g., cholesterol (C), sitosterol] (51). Therefore, the study of the biosynthesis of E or 20E is in essence the analysis of a sequence of molecular alterations of the C molecule resulting in the formation of polyhydroxylated ecdysteroids in the insect's prothoracic glands. It is of interest that molting in nematodes is also compromised in the absence of dietary sterols (26), although

[1]We use the term molting to include all those phenomena that at times are referred to as the molting process, i.e., retraction of the epidermis (apolysis), deposition of new cuticular elements, retrieval of elements from the old cuticle, and the shedding of the almost completely digested old cuticle (ecdysis).

there is, as yet, no concrete evidence that nematodes synthesize ecdysteroids (187). In this chapter, we not only discuss ecdysone biosynthesis, many of the detailed steps having eluded investigators for decades until recently, but further, we present what is now known about how ecdysteroidogenesis in the prothoracic glands is regulated. This comprises a balance between stimulation of ecdysteroidogenesis by the brain neuropeptide prothoracicotropic hormone (PTTH), the details of which we are beginning to understand, and feedback inhibition of biosynthetic events, of which we know little at the biochemical or molecular levels.

A great deal of the data discussed here has been generated using the tobacco hornworm *Manduca sexta* and the fruit fly *Drosophila melanogaster*, the former because of its large size, relatively short life cycle, and paired prothoracic glands, each of which secretes ecdysteroid at a rate basically identical to the other so that in vitro studies have inherent controls, and the latter for its ability to serve as an object of molecular genetic analysis.

Although it is generally conceded that research on insects can benefit humanity by providing a basis for the design of new control agents to combat agricultural pests and vectors of disease, studies on the molecular genetics of *Drosophila* have also revealed telling insights into protein function in human pathologies via the study of *Drosophila* homologs of genetically based human disease (39).

CONTROL OF ECDYSTEROID SYNTHESIS

Sources of Ecdysteroids

Development and growth are dependent on the coordinated and orderly expression of genes, and this is particularly evident in the periodic molts undergone by insects, especially holometabolous insects that undergo metamorphic molts (146). The primary proximate regulators of this episodic gene activity are the ecdysteroid hormones, especially 20E (72). In pre-adult lepidopterans, 20E is a product of the two-step conversion of the prohormone 3-dehydroecdysone (3dE). 3dE is produced by the prothoracic glands (138, 179), unique organs comprising a single steroidogenic cell type in most insects, although in the higher flies, the prothoracic gland is part of a composite organ, the ring gland, which also includes the corpus cardiacum and corpus allatum (31, 145). 3dE is sequentially converted to ecdysone by a hemolymph reductase and then to 20E by an intracellular 20 monooxygenase found in a number of cell types but not in the prothoracic gland itself (55). Ecdysteroids are also produced by epidermal and gonadal cells in some insects, especially during adult life, by which time the prothoracic glands have completely or nearly completely disappeared owing to programmed cell death during pupal-adult development (32, 50). This section concentrates on the regulation and synthesis of ecdysteroids by lepidopteran prothoracic glands under the control of PTTH because the ecdysteroid titer is for the most part a consequence of prothoracic gland regulation.

Prothoracicotropic Hormone and Other Ecdysiotropic Factors Affecting the Prothoracic Gland

Eight decades ago, it was shown that the brain produced a diffusible factor required by a second organ (the prothoracic gland) posterior to the brain in order for normal molting to occur (97). This diffusible factor, now known as PTTH, has been both purified and cloned, first from *Bombyx mori* (89) and subsequently from a variety of other Lepidoptera [*Samia cynthia* (79), *Antheraea pernyi* (142), *Hyalophora cecropia* (F. Sehnal, personal communication), *M. sexta* (H. Kataoka, personal communication)]. PTTH is produced by a pair of large lateral neurons in the brain whose axons course posteriorly and terminate in the corpus allatum, the neurohemal organ for PTTH (1). PTTH release from this neurohemal organ is under complex control that integrates, presumably at the level of the brain, environmental factors such as photoperiod, time of day, and physiological factors such as nutritional state of the animal (18). In the Lepidoptera, PTTH-stimulated surges in ecdysteroid synthesis and the resultant peaks in the hemolymph ecdysteroid titer occur once per instar, with the exception of the last larval instar. In the last larval instar, there are at least two PTTH-dependent ecdysteroid peaks, the first peak being small and unique to this stadium (19). It results in changes in commitment (gene activity) so that at the subsequent molt, under control of the typical larger ecdysteroid peak, metamorphosis occurs (i.e., ecdysis to the pupa) (127).

Analysis of the PTTH protein, cDNA, and genomic sequences revealed that this hormone is synthesized as a prohormone and released as a shorter, glycosylated, homodimeric molecule (25–30 kDa) with a single intermonomer cystine-cystine bond and three intramonomeric cystine-cystine bonds (78, 89). The molecular structure specified by these latter bonds is essential for the bioactivity of the hormone, with dimerization and glycosylation of the hormone being less important for PTTH activity (78). Despite the strong conservation of these cystines and a number of other amino acid residues, pair-wise comparisons of the amino acid sequence of the five lepidopteran PTTHs known to date indicate that these neuropeptides have diverged considerably. Only the *Samia* and *Hyalophora* sequences exhibit strong similarity (94% identity in 125 amino acids); the other paired comparisons yield identities from 47% to 65%. Nevertheless, these lepidopteran PTTHs clearly comprise a family of related proteins when structural features such as the distribution of charged amino acids or hydrophilic regions are considered (72). PTTHs are essentially species specific in action, suggesting that the primary amino acid sequence is critical in eliciting the ecdysteroidogenic response. Outside of the Lepidoptera, the only PTTH to have been characterized in any detail is that of *D. melanogaster*. The semipurified *Drosophila* protein is considerably larger than the moth PTTHs and requires significant glycosylation for activity (93). Searches of the *Drosophila* genome database using any of the moth-PTTH amino acid sequences have not yielded any obvious homologs (R. Rybczynski, unpublished observations). This suggests two possibilities. First, PTTHs have diverged from an ancestral molecule to such a degree that PTTHs from different major taxa may

exhibit minimal amino acid conservation. Second, PTTHs from different major taxa have evolved different PTTHs whose only similarity is the ability to elicit ecdysteroid synthesis by the prothoracic gland. The surprising ability of a lepidopteran PTTH (from *B. mori*) to stimulate ecdysteroidogenesis by a hemipteran prothoracic gland (*Rhodnius prolixus*), albeit requiring relatively high doses, suggests that the first possibility may apply (169). However, the inability of cyclic AMP (cAMP) analogs to stimulate *Drosophila* ring gland ecdysteroid synthesis (71) is in contrast to the well-characterized action of this second messenger in *Manduca* (see below) and suggests that there might be multiple nonhomologous families of PTTHs that activate different intracellular transductory cascades.

Factors other than PTTH may elicit ecdysteroidogenesis by the prothoracic gland, but our knowledge of the nature of such molecules, their biological significance, and their modes of action is rudimentary. The best characterized of these factors is the brain-derived small PTTH (MW < 10,000) of *Manduca*. Small PTTH stimulates ecdysteroid synthesis in larval glands but exhibits poor activity when applied to pupal glands (18). Small PTTH appears to stimulate the same second messenger cascades as big PTTH [Ca^{2+} and cAMP (68); see below], which suggests that it might be an active proteolytic fragment of PTTH generated during PTTH purification. However, pure or recombinant small PTTH is not available in contrast to big PTTH (54), so that all studies on the action of small PTTH have utilized crude extracts. Conclusive evidence that small PTTH is released into the circulation is also lacking. It is clear that purification and sequencing of this molecule are necessary to determine its physiological role, if any. One or more ecdysteroidogenic factors, acting in vitro on the prothoracic gland, have also been partially purified from lepidopteran proctodaea (*M. sexta*, *Lymantria dispar*, and *Ostrinia nubilalis*) (47, 49), but definite physiological roles have yet to be demonstrated for these incompletely characterized insect molecules. Similarly, the dose-dependent stimulatory and inhibitory action of an ovarian-derived dipteran oostatic factor on lepidopteran prothoracic gland PTTH-stimulated ecdysteroid production is intriguing (48), but the significance of these data remains to be determined. A small prothoracicostatic peptide has been isolated from brains of *B. mori* (76). This peptide can block PTTH-stimulated ecdysteroidogenesis in vitro in a dose-dependent manner, but its natural occurrence in the hemolymph has yet to be demonstrated.

The PTTH Transductory Cascade

Early studies of the *Manduca* prothoracic gland revealed that levels of cAMP, an important intracellular second messenger, were elevated at times of increased ecdysteroid synthesis (171). Further studies, using an in vitro system, demonstrated that PTTH stimulated a rapid (<5 min) increase in larval gland cAMP content (154). Pupal glands metabolize cAMP more rapidly owing to an increased expression of cellular phosphodiesterases, requiring the use of phosphodiesterase inhibitors to easily detect the PTTH-stimulated increases in cAMP (152, 157, 158).

The generation of cAMP is not the first intracellular event triggered by PTTH, however. Further experiments in vitro revealed that cAMP generation was dependent on the influx of extracellular Ca^{2+}, which suggests that cAMP synthesis required Ca^{2+}-calmodulin-dependent adenylyl cyclase activity, which is indeed present in lepidopteran prothoracic glands (63, 108, 155). Based on the use of various channel-blocking drugs, PTTH appears to open either an L-type or T-type plasma membrane Ca^{2+} channel (11, 12, 57), but the identification of channel types based on drugs used in pharmacological analyses of vertebrate systems may be problematic and could be dependent on methodological details [i.e., in one study the L-type Ca^{2+} channel inhibitor nitrendipine failed to block PTTH-stimulated ecdysteroidogenesis (11), whereas, in a second study, just the opposite was found (57)]. The mechanism by which PTTH opens prothoracic gland Ca^{2+} channels is also not clear. Based on a variety of pharmacological studies coupled with measurements of prothoracic gland Ca^{2+}, Birkenbeil (12) concluded that G-proteins were not involved in Ca^{2+} channel opening, but these conclusions may not be applicable to all stages of development. For instance, Birkenbeil (12) utilized prothoracic glands from wandering last stage *M. sexta*, a time at which prothoracic gland ecdysteroid synthesis is anomalously high in vitro in the absence of PTTH (60). Thus, the failure of the poorly hydrolyzable GDP analogue, GDPβS, to block PTTH-stimulated Ca^{2+} influx in prothoracic glands from last instar wandering animals and by implication to block PTTH-stimulated ecdysteroid synthesis (12) contrasts with the ability of this compound to inhibit PTTH-stimulated ecdysteroid synthesis earlier in development and with the extracellular Ca^{2+}-dependence of a GTP analogue, GTPγS, to stimulate ecdysteroid synthesis (S. Kellough, R. Rybczynski & L.I. Gilbert; unpublished data). A further complication is evident in the apparent inability of either GTPγS or GDPβS to exert these effects on early pupal prothoracic glands (S. Kellough, R. Rybczynski & L.I. Gilbert; unpublished data). It is clear that our knowledge of the earliest events in PTTH action is incomplete, and these include PTTH-receptor interactions.

All mammalian adenylyl cyclases are activated by $G_s\alpha$, the α subunit of the GTP-bound G-protein (77), and this is likely true of the insect adenylyl cyclases as well. As expected, when intracellular levels of cAMP rise, the cAMP-dependent kinase, protein kinase A (PKA), is activated in prothoracic glands (153). The physiological targets of PTTH-stimulated PKA are not known nor has a requirement for PKA activity in PTTH-stimulated ecdysteroidogenesis been demonstrated unequivocally. Active cell-permeable analogues of cAMP elicit prothoracic gland ecdysteroidogenesis (62, 153, 158), and an inhibitory stereoisomeric analogue of cAMP inhibits PTTH-stimulated ecdysteroidogenesis (158). However, these observations do not eliminate the possibility that cAMP-dependent proteins other than PKA may eventually transduce the PTTH signal into a stimulation of ecdysteroid synthesis. Intriguing in this regard is the report of a mammalian cAMP-dependent guanine nucleotide exchange factor that activates small G-proteins (90) that in turn stimulate further phosphorylation-dependent events, including the activation of the 70-kDa S6 kinase, a known moiety in PTTH action (see below).

PTTH stimulates a number of rapid protein phosphorylations in the prothoracic gland via PKA, mitogen-activated protein kinases (MAPKs) (132), and/or other uncharacterized kinases, with a protein of ≈34 kDa being perhaps the most prominent and consistent substrate (27, 129, 130, 162). This 34-kDa protein was postulated to be the ribosomal protein S6 based on molecular weight and precedence from vertebrate steroidogenic systems (52). Its identity as S6 was verified by two-dimensional gel analysis of ribosomal proteins and the sensitivity of its phosphorylation to the 70-kDa S6 kinase pathway inhibitor rapamycin (162, 163). Rapamycin blocks not only S6 phosphorylation in the prothoracic gland but also PTTH-dependent ecdysteroid and protein synthesis (163). In vertebrate systems, S6 phosphorylation increases the rate of translation initiation, especially of mRNAs possessing a polypyrimidine tract at their 5′ transcriptional start (37), and it likely plays a similar role in insect cells.

Vertebrate and insect steroidogenic cells are surprisingly homologous in the mechanisms that regulate acute changes in steroidogenesis. In both taxa, sharp increases in steroid hormone synthesis are regulated primarily through the action of peptide hormones produced by neurons or other brain-associated cells. It is of interest that nearly 17% of the cells of the insect central nervous system produce neuropeptides, and although the number of neuropeptides characterized has increased greatly in the past decade, the physiological role of the great majority of them remains unknown (45, 120). PTTH is a notable exception. Peptide hormones normally bind to plasma membrane receptors (presumed in the case of PTTH) that are often associated with G-proteins and with the subsequent generation of cAMP as well as transient increases in intracellular Ca^{2+}. In both insect and vertebrate systems, the final step in the acute upregulation of steroid hormone production appears to be the rapid translation of one or more short-lived proteins critical in removing a rate-limiting bottleneck in the steroid synthesis pathway. In vertebrate steroidogenesis, two proteins, StAR (steroidogenic acute regulatory protein) and DBI (diazepam-binding inhibitor), are believed to facilitate the movement of cholesterol across the mitochondrial membrane to the side chain cleavage P_{450} enzyme (118, 165). It is this rate-limiting step that results in the conversion of cholesterol to pregnenelone, which is alleviated by the rapid translation that in turn is stimulated by a peptide hormone such as adrenocorticotropic hormone (ACTH). A *Manduca* DBI homolog has been cloned, and current evidence is consistent with a role for DBI in basal ecdysteroid synthesis (159, 160), but experiments to explicitly test its role in acute PTTH-stimulated ecdysteroidogenesis have yet to be reported. Analysis of the *Drosophila* genome database indicates that an insect StAR homolog may not exist (R. Rybczynski, unpublished observation). Nevertheless, it is clear that PTTH-stimulated ecdysteroid synthesis requires new translation, as evidenced by the ability of translation inhibitors such as cycloheximide to block such ecdysteroid synthesis (92, 135). Furthermore, past data indicate movement of an ecdysteroid precursor between intracellular compartments (endoplasmic reticulum and mitochondrion) is required, rate-limiting in *Manduca* and *Drosophila* ecdysteroid synthesis, and a carrier protein may be involved (172, 175).

Analysis of protein synthesis in *Manduca* prothoracic glands revealed that PTTH stimulates overall translation at most developmental stages and that some proteins, perhaps as many as ten, are differentially translated against the background of this general increase (56, 133). Two of these proteins have been identified and cloned: a β tubulin (135, 136) and a cognate 70-kDa heat shock protein (hsc70) (134, 137). The slow and prolonged temporal dynamics of hsc70 synthesis suggest that this protein functions in supporting translation, acts as a chaperone for the ecdysone receptor, or participates in other long-term effects of PTTH not directly connected to acute changes in ecdysteroidogenesis. On the other hand, PTTH-stimulated β-tubulin synthesis is rapid and transitory, which suggests a more direct role for this cytoskeletal protein (135). Indeed, treatment of pupal prothoracic glands with microtubule-disrupting drugs inhibits PTTH-stimulated ecdysteroid synthesis (181); however, this effect is not seen in larval glands (R. Rybczynski & L.I. Gilbert, unpublished data). Furthermore, the pupal inhibition is associated with translation inhibition that can be overridden by treatment of glands with a cAMP analogue, indicating that physical coupling between the PTTH receptor and a protein acting early in the transductory cascade is disrupted by such drugs rather than the drugs affecting the ecdysteroid precursor movement (R. Rybczynski & L.I. Gilbert, unpublished data).

The above discussion clearly indicates that although a number of important PTTH-related events are understood, our comprehension of the control of ecdysteroid synthesis by PTTH is still relatively rudimentary. For instance, although PTTH-dependent cAMP generation suggests strongly that the PTTH receptor is a protein that belongs to the G-protein-coupled receptor family, the members of which are plasma membrane proteins with seven transmembrane domains, confusion about the gating of the PTTH-activated Ca^{2+} channels makes this conclusion conjectural.

PTTH probably has a number of other functions in both the prothoracic gland and elsewhere. PTTH stimulates both general and specific protein synthesis in the prothoracic gland, and it is possible to inhibit the former without affecting the PTTH steroidogenic effect (133). This indicates that PTTH probably acts as a trophic factor for the prothoracic gland, which undergoes significant changes in cell size, basal ecdysteroidogenesis, and protein content (66, 109, 135). Significant levels of PTTH are present in adult brains (114), a stage at which the prothoracic glands are no longer present due to their programmed cell death (32), indicating that PTTH likely regulates tissues and processes other than prothoracic gland ecdysteroid synthesis (e.g., ovarian ecdysteroidogenesis).

In higher animals, the intracellular transmission of extracellular signals is often mediated by several sets of MAPK cascades, each consisting of three levels of protein kinases that sequentially activate each other by phosphorylation. The activation of these cascades is initiated by either a small GTP-binding protein or an adaptor protein, which transmits the signal either directly or through a mediator kinase to the MAPK kinase kinase level. The ability of activated MAPKs to translocate into the nucleus and trigger transcription completes a signaling pathway from the

receptor to the nucleus, which mediates the induction of several cellular processes including those related to development. Because of their importance, MAPKs have been investigated for a possible role in the control of *Manduca* prothoracic gland upregulation (132). PTTH activates a MAPK pathway, resulting in a rapid increase in the phosphorylation activity of an ERK (extracellular receptor activated kinase) (132). ERKs can regulate a wide variety of intracellular processes from transcription, by relaying signals from the cell membrane to the nucleus, and translation to microtubule stability (103). However, activation of the ERK pathway in the prothoracic glands by PTTH does not regulate ecdysteroidogenesis (132). The role of ERKs in prothoracic glands is complicated because all gland ecdysteroidogenesis, both basal and PTTH-stimulated, appears to be dependent on the maintenance of a small basal population of active (phosphorylated) ERK molecules (132). Which signal(s) maintains this basal level of ERK phosphorylation is unknown.

PTTH stimulates not only translation but also transcription as noted above (92), but at present, it is unclear if PTTH-stimulated transcription is necessary for the acute ecdysteroidogenic effect of PTTH. Blocking transcription significantly reduces PTTH-stimulated ecdysteroid synthesis (92, 135), but it also rapidly decreases translation to a large extent (135), which raises questions of the specificity of the effect in the prothoracic gland. In vertebrate steroid hormone-producing cells, steroidogenic protein hormones like ACTH regulate the expression of genes that code for enzymes comprising the steroid hormone pathway (82). These longer-term effects, requiring hours to occur, may also be found true for PTTH and the ecdysteroidogenic machinery (e.g., the trophic function of PTTH). The answers to such questions should become apparent once the ecdysteroidogenic enzymes and other proteins (see following section) have been cloned and characterized, and their mRNA and protein levels are determined via molecular, biochemical, and immunological methods. A summary of the presently known and postulated PTTH-regulated intracellular signaling pathways leading to increased ecdysteroid synthesis is presented in Figure 1.

PTTH Release and the Dynamics of Ecdysteroid Synthesis

The periodic increases in ecdysteroid titer critical to insect development reflect to a large degree the episodic activation of the prothoracic gland by PTTH that has been released from the corpus allatum. PTTH release occurs in particular diel time windows, after the organism integrates a variety of factors such as time since last molt, nutritional status, and physical size. The identity of the cells that sense such factors and how the information is transmitted to the brain neurosecretory cells producing PTTH, or to the cell endings in the corpus allatum, are unknown. However, some knowledge of the immediate neural events is available, indicating that PTTH release may be controlled by the muscarinic class of cholinergic neurons (102, 149). Neuroendocrine modulation of PTTH release has also recently been demonstrated in *Periplaneta americana*, acting via the neurohormone melatonin (126). Although, melatonin is a common mediator of day-night physiological

differences in a variety of organisms, neither melatonin nor PTTH temporal cycling was measured in *Periplaneta*. However, melatonin could be involved in controlling the diel cycle of PTTH release as described in *Rhodnius* (168) and presumably occurring in other insects as well.

Several studies have addressed developmental cycles of PTTH release in *Bombyx*, using a prothoracic gland assay system in vitro (148) or anti-PTTH antibodies (112). Although these latter two studies are not in complete agreement as to the timing and number of PTTH hemolymph peaks during the final larval instar, both investigations indicate that major peaks of ecdysteroid secretion are preceded by, and partially cotemporal with, high PTTH titers, and that some larval and pupal-adult periods of increased PTTH titer are not associated with obvious ecdysteroid production. The latter is best illustrated by a late–pupal-adult development PTTH peak (112), at which time ecdysteroid levels are low and the prothoracic glands have presumably undergone programmed cell death. These data support the suggestion that PTTH has functions other than the regulation of prothoracic gland ecdysteroidogenesis.

Other Sources of Ecdysteroids

Although prothoracic glands are the major source of ecdysteroids during insect larval and pupal-adult development, other tissues also contribute ecdysteroids in some insects, especially during pupal-adult development and adult life (34). For example, during pupal-adult development, isolated *Manduca* abdomens devoid of prothoracic glands and lacking gut or testes synthesize ecdysone (139, 177). The sources of ecdysone in such preparations are not yet identified but could be the oenocytes and the epidermis (34). The importance and possible roles of these nonglandular sources in intact animals have been questioned (139), but no real conclusions can be made at this time. Notable, however, is the observation of integumental ecdysteroid synthesis in a noninsect arthropod, the hard tick *Amblyomma hebraeum*, which lacks a prothoracic gland, and the finding that such synthesis can be stimulated by agents increasing intracellular levels of cAMP (105).

A potentially important but as yet unproven area of research concerns the production of ecdysteroid by lepidopteran gonads in vitro. The secretion of ecdysteroid by the larval testis is stimulated by a small testes ecdysiotropin (TE) originating in the brain (104). The transductory cascade has been characterized partially using various pharmacologic agents, is quite complex, and awaits clarification that can only be achieved by actual determination of changes in second messengers. The amount of ecdysteroid synthesized by the testis is only a small fraction of that produced by the prothoracic glands, suggesting a paracrine or autocrine role.

The synthesis of ecdysteroids by ovarian follicle cells is also well established, and these steroids may be important in several aspects of reproduction. Ecdysteroids modulate the production of yolk proteins by the fat body, and maternally derived ecdysteroids in the egg undoubtedly are important during embryonic development. Ovarian ecdysteroids are produced by the nurse and/or the follicle

cells, based on the recent cloning of gene products believed to be involved in ecdysteroid synthesis (21, 40). The data suggest that several extracellular signals, utilizing distinct intracellular signal transduction pathways, are involved in controlling ovarian ecdysteroidogenesis. In the blowfly *Phormia regina*, analysis of the activity of brain extracts in this species revealed both cAMP-sensitive and -insensitive ovarian ecdysteroid synthesis (106). Among the candidate molecules that regulate ovarian ecdysteroid synthesis in a cAMP-independent manner are insulin-like factors. An insulin receptor homolog has been cloned from the ovaries of *Aedes aegypti* (59), and bovine insulin elicits ecdysteroidogenesis by *Aedes* ovaries (128). The natural ligand for the insect insulin receptor remains unknown, but candidates include members of the bombyxin family, with at least 32 genes in *B. mori* (188), and an insulin-related peptide identified in *Locusta* (74). A protein recognized by antisera against the human insulin receptor is present in several *Manduca* tissues, including the prothoracic gland (156), which suggests that this pathway may also modulate ecdysteroid synthesis outside the ovary. A likely target for any insulin-mediated regulation of prothoracic gland ecdysteroid synthesis is the MAPK pathway, which regulates steroid production dynamically in the gland independent of PTTH (132).

DOWNREGULATION OF THE PROTHORACIC GLANDS: ROLE OF EcR

The EcR Complex of the Prothoracic Gland

For the ecdysteroid-responsive sequence of events to occur at multiple times during the life of an insect, there must be a sensing mechanism in the glands with the ability to discern 20E in both qualitative and quantitative terms and then transduce this information to the cell's biosynthetic machinery so that the rate of ecdysteroidogenesis can be adjusted. Furthermore, this sensing mechanism must distinguish between the active ecdysteroids such as 20E and inactive metabolites. To begin to examine this putative mechanism, our laboratory has investigated the heterodimeric ecdysteroid "ecdysone" receptor (EcR) in the prothoracic glands of *M. sexta* by analyzing the receptor constituents and how they may react to an increasing titer of 20E.

Insect molting and metamorphosis are directly or indirectly regulated by the interaction of 20E with the EcR complex that recognizes specific DNA response elements and triggers a cascade of gene activity that directs the molting process (72). Nuclear receptors comprise a superfamily of ligand-activated transcription factors that regulate diverse functions in metazoans (e.g., development, differentiation, metamorphosis, reproduction). As homodimers or heterodimers, they bind to hormone response elements, ultimately leading to changes in gene activity. The EcR heterodimer important to insect molting and a variety of other physiological events is composed of EcR and ultraspiracle (USP) proteins, the latter being a homolog of the mammalian retinoid X receptor (RXR) (186). Although receptors

for mammalian steroid hormones are ligand-activated modulators of transcription that bind to specific hormone response elements, it is still not understood exactly how the receptor system regulates transcriptional efficacy in vivo (8). Both EcR and USP are members of the nuclear hormone receptor family that is characterized by a DNA-binding region and a carboxyl terminal ligand–binding domain (35). Genes for both *EcR* and *USP* were cloned and sequenced from *Drosophila* (73, 95, 117, 147) and subsequently from representatives of a variety of insect orders (24).

The EcR complex from the *M. sexta* prothoracic gland also contains the immunophilin FK-binding protein (FKBP46) (161), although its role in the EcR complex remains conjectural. In the case of mammalian steroid hormone receptors, immunophilins are postulated to act as molecular chaperones (119), to be involved in receptor trafficking from cytoplasm to nucleus (29, 30), and to function as potentiators of receptor activation (98) or modulators of receptor phosphorylation (110). FKBP46 is also associated with a nuclear kinase that phosphorylates the immunophilin in the presence of Mg^{2+} and ATP and forms a complex with nuclear TP2 (i.e., it is a DNA-binding protein) (3). All of the above point to critical roles for FKBPs, and it would be tempting to speculate about their possible roles (e.g., FKBP46 as a corepressor or coactivator acting in conjunction with nuclear hormone receptors in the modulation or reprogramming of insect gene activity). The present findings suggest that the insect EcR complex may be an excellent model system for defining the role of FKBPs associated with nuclear hormone receptors.

When prothoracic gland homogenates from *M. sexta* larvae were immunoprecipitated by antibodies specific to EcR, USP, and FKBP46, a native EcR complex containing EcR, USP, and FKBP46 was detected in the precipitate by Western blot analysis (161). Most importantly, this immunoprecipitated native EcR complex bound ponasterone A, a phytoecdysteroid commonly used for EcR-binding assays (25), with a K_d of 7.04×10^{-9} M. Using a functional approach with *Drosophila* cells, it was shown that several auxiliary factors are necessary, at least transiently, for the initial association of the EcR/USP heterodimer with the DNA-binding site (6). However, these factors are neither required to maintain DNA binding nor required for the binding of ligand (ecdysteroid) to the EcR/USP complex. These factors include FKBP, HSP 70, HSP 90, HIP, and HOP (53).

Western blot analysis of the immunoprecipitated *Manduca* EcR complex as well as prothoracic gland homogenates revealed that the EcR complex contains three EcR proteins (76, 78, and 80 kDa), two USP proteins (47 and 49 kDa), and the FKBP46 protein. Two more forms of USP (p52 and p54) exist in the gland homogenate but are not part of the EcR complex (161, 164). The 80-kDa EcR isoform was identified as the phosphorylated form of EcR-B1 and the 78-kDa isoform as EcR-A by the use of specific antibodies (164).

The four isoforms (p47, p49, p52, p54) of USP discerned in homogenates of the prothoracic glands during developmental studies display two distinct expression patterns depending on developmental stage (164). The p52 and p54 isoforms that do not heterodimerize with EcR are expressed maximally at days 1–6 of the fifth larval

stadium ($V_{1–6}$) and $P_{0–2}$ (days 0–2 after pupation) when hemolymph ecdysteroid levels are low, whereas p47 and p49 were expressed maximally at $V_{7–9}$ and $P_{3–5}$, when the hemolymph ecdysteroid titers are at their highest level and when the p52 and p54 isoforms were barely detectable. The dramatic change in USP isoform expression indicates that USP may play a critical role in regulating EcR complex function.

The developmental profiles of EcR, USP, and FKBP46 expression reveal three peaks of EcR and USP at V_3, V_7, and P_3 in concert with peaks in the hemolymph ecdysteroid titer (19, 174), while that of FKBP46 remained relatively constant. It should be reiterated that the hemolymph ecdysteroid peak at V_3 elicits cellular reprogramming (127), whereas those at V_7 and P_3 initiate the molt from larva to pupa and pupa to adult, respectively (55). These results indicate that the ecdysteroid titer may regulate EcR and USP expression, perhaps through transcriptional and/or translational regulation (81, 88).

The EcR Complex and the Regulation of Ecdysteroidogenesis

The most reasonable role for the EcR complex in the prothoracic gland is in down-regulation of ecdysteroidogenesis because E and especially 20E inhibit ecdysteroid production by the lepidopteran prothoracic gland (10). Other data also support the supposition that 20E acts on the prothoracic gland as a negative-feedback regulator at particular developmental periods (140). If negative feedback is to occur during the regulation of gland activity, then the functional EcR complex must be present at the time when the hemolymph ecdysteroid titer reaches a critical level. To test this hypothesis, gland homogenates from both V_5 glands that possess the predominant p52 and p54 isoforms and V_7 glands that contain mainly the small isoforms were immunoprecipitated. The results indicated that only the p47 and p49, but not the p52 and p54, dimerized with the EcR in the gland homogenate and that the EcR complex is much more abundant at stage V_7 than at V_5 (164). These results suggest that when p47 and p49 predominate in the presence of a high-ecdysteroid titer, feedback inhibition of ecdysteroidogenesis may occur via a cascade of transcriptional factors. Perhaps the p52 and p54 isoforms of USP partner with other receptor moieties for other purposes.

To provide direct evidence that the increased expression of p47 and p49 at $V_{7–9}$ and $P_{3–4}$ resulted from the rising hemolymph ecdysteroid titers, V_5 prothoracic glands were incubated with 20E. The data showed that increased expression of p47 and p49 was elicited by 20E in vitro in a dose- and time-dependent manner (164). A concentration as low as 1 μM 20E was effective with maximum expression of the small forms of USP occurring at 10 μM 20E, concentrations close to the physiological concentrations in the hemolymph for both V_7 and P_3 animals. The increased expression of p47 and p49 occurred simultaneously with a drastic decrease in the quantity of p52 and p54. E was effective but only at a concentration of 100 μM, a pharmacological dose for $V_{7–9}$ and $P_{3–4}$ animals. These in vitro data demonstrate that 20E is more effective than E in eliciting the expression of the

small forms of USP, as it is in decreasing the synthetic activity of the prothoracic glands (10).

The fact that high levels of p47 and p49 occurred almost simultaneously with peaks in the hemolymph ecdysteroid titer and the finding that 20E regulates the expression of these isoforms suggested that high titers of hemolymph 20E may regulate ecdysteroidogenesis by the prothoracic glands via specific expression of p47 and p49, which then dimerize with EcR to form the active EcR complex. This may then lead to a cascade of translational and/or transcriptional events that culminate in the downregulation of ecdysteroid production. In an attempt to provide more direct evidence for this hypothesis, prothoracic glands from V_5 larvae were incubated in the presence or absence of 20E and then challenged with PTTH. Incubation with 20E for 6 h or longer inhibited PTTH-stimulated ecdysteroidogenesis severely (~75–90%) (164). 20E also inhibited the basal level of ecdysteroid production (i.e., not stimulated with PTTH). These data strongly suggest that PTTH-stimulated ecdysteroidogenesis is feedback-regulated by a high hemolymph titer of 20E, probably via selective expression of specific forms of USP that presumably dimerize with EcR and together with a variety of chaperones form the functional EcR complex (53). The resultant increase in EcR complexes, after binding to 20E, could ultimately lead to gene activity that downregulates the expression of one or more critical components of the ecdysteroidogenic pathway. This is surely an important area for research on the regulation of insect molting that has been all but ignored.

Because two USP transcripts are present in *Manduca* (80), it was of interest to investigate how the four forms of USP in the prothoracic glands relate to these transcripts. The developmental expression pattern of p49 is identical to that of p47, which suggests that perhaps p49 is the phosphorylated form of p47 because all members of the nuclear receptor superfamily are phosphoproteins (119). The same relationship may be true for p54 and p52. To test this hypothesis, partially purified p47, p49, p52, and p54 proteins were treated with λ-protein phosphatase (λ-PPase) and then subjected to Western blot analysis. The data revealed the complete disappearance of the p49 and p54 bands (164), indicating that p49 is the phosphorylated form of p47 and p54 is the phosphorylated form of p52. Thus, there are only two true USP isoforms in the prothoracic glands, each of which can exist in a phosphorylated or unphosphorylated state. To study the effect of phosphorylation on the function of the EcR/USP complex, preliminary binding studies were performed. Dephosphorylation did not disrupt the EcR/USP complex but did alter the ligand- and DNA-binding ability of the complex (Q. Song & L.I. Gilbert, unpublished information), which suggests that phosphorylation and dephosphorylation may play important regulatory roles with the binding activity of the complex.

An increase in the EcR complex at times when the hemolymph ecdysteroid titer is high suggests that a critical titer of active EcR complex is a consequence of the high titer of 20E, that the complex requires the 20E-elicited small forms of USP, and that the complex is instrumental in the downregulation of ecdysteroidogenesis. The subsequent cascade of events leading to decreased ecdysteroidogenesis is the final

act in feedback inhibition, returning the rate of ecdysteroidogenesis to the basal level and thus modulating the ecdysteroid peaks so typical of, and influential in, the physiology of the insect (19). Indeed, several critical physiological phenomena are elicited by a decreasing ecdysteroid titer (55). Research in the future must address the question of exactly how the active EcR receptor interacts with the genome to achieve decreased rates of ecdysteroidogenesis. This may include inhibition of the synthesis of specific enzymes or sterol translocation proteins (see subsequent discussion), or effects on specific transcription factors.

ECDYSTEROID BIOSYNTHESIS

Steroid Biosynthesis in Insects Versus Vertebrates

Although the basic mode of action of insect and higher vertebrate peptide and steroid hormones are quite similar, these animal groups differ most notably in the manner in which they obtain and use cholesterol (C). Vertebrates synthesize C from small carbon units, e.g., acetate, pyruvate, whereas arthropods do not and so must ultimately obtain it from their diet (51). Nevertheless, both vertebrates and invertebrates have developed closely analogous systems that control precisely the level of this critical intermediate in their respective steroidogenic tissues. In mammals, the initial delivery of C to the outer mitochondrial membrane of steroidogenic glands, then subsequently across the aqueous intramitochondrial space to the inner mitochondrial membrane/matrix, is rate limiting for steroid hormone biosynthesis (165). In the mammalian mitochondrial matrix, C undergoes the first committed reaction in steroid hormone biosynthesis, i.e., irreversible side chain cleavage mediated by cytochrome $P450_{SCC}$, a complex multistep monooxygenase (151). In arthropods, following the novel dehydrogenation of dietary C to 7-dehydrocholesterol (7dC) in the ER (Figure 2*a*), it is postulated that this critical pre-ecdysteroid intermediate must also translocate first to, and then within, the mitochondria of the prothoracic glands (172, 175). In the insect prothoracic gland, this process may be rate limiting and under the control of PTTH. Within the mitochondria of these glands, the enzyme system that mediates the oxidation of 7dC to a pre-ecdysteroid molecule may also involve a complex multistep catalysis by one or more insect-specific P450 monooxygenases (Figure 3).

The steroid hormones of insects and vertebrates also differ in the chemical structures and properties of the active hormones. Active ecdysteroids, i.e., 20E, E, and other closely related molecules (Figure 2*b*), are polar steroids, almost sugar-like in their solubility properties and so are soluble in the aqueous cell environment and in the more lipophilic cell membranes, thus distributing relatively easily throughout the open circulatory system of the insect. Mammalian steroid hormones have more variable structures but universally lack the polyhydroxylated side chain characteristic of ecdysteroids and so are quite nonpolar. As a result, these hormones are more restricted in their movement and compartmentalization within the organism, thus affecting their functional capacity.

Side Chain Dealkylation of Plant and Fungal Sterols: The Key Role of Cholesterol

Most insects studied can dealkylate and reduce the common 24(R)-alkyl plant sterols β-sitosterol (Figure 2*a*), campesterol, and stigmasterol to C (166). Those unable to do so must synthesize and use alkylated analogues of 20-hydroxycholesterol (Figure 2*b*), i.e., makisterone A and C, to control aspects of growth and metamorphosis (36, 123). A few species dispense with the requirement for such Δ^5-sterols (including C), circumvent the first enzyme in the pathway (the $C_{7,8}$-dehydrogenase), and utilize food providing Δ^7- or $\Delta^{5,7}$-sterols (i.e., *Drosophila pachea*) (38, 58, 70; see below). Others must ingest C, i.e., carnivorous Diptera, blood-sucking Hemiptera.

Conversion of β-sitosterol to C occurs only in the insect gut, and many of the intermediates involved have been identified and the corresponding enzymatic reactions partially characterized (Figure 2*a*). β-sitosterol is first dehydrogenated to fucosterol, perhaps via a C_{28}-hydroxylated intermediate (167), but the enzyme responsible has not been characterized, although it may be a cytochrome P450-oxygenase. Fucosterol is then epoxidized, perhaps by a P450, and the $C_{24,28}$-epoxy intermediate has been isolated (113). The epoxide is then opened (hydrolyzed), apparently in conjunction with the neighboring C_{25} hydrogen, by an epoxide lyase (121). Further oxidation then results in the eventual elimination of the two-carbon unit and the production of desmosterol, a reaction common to sterol dealkylation pathways (41, 42). Desmosterol, unsaturated at the $C_{24–25}$ position, is then finally reduced to C under the control of a microsomal $\Delta^{24,25}$-reductase (43, 150).

Cholesterol to 7-Dehydrocholesterol: The 7,8-Dehydrogenase

The dehydrogenation of C to 7dC (Figure 2) and the importance of this conjugated $\Delta^{5,7}$-sterol intermediate in ecdysteroidogenesis have been the object of study over several decades. Early feeding studies showed that 7dC could support insect growth in the absence of C or plant sterols (75). Later radiochemical investigations in vivo established that the enzymatic reaction involved the stereospecific removal of both the 7β- and 8β-hydrogens, i.e., from the more sterically hindered β-top face of the planar C molecule (28). In vitro radiochemical and bioanalytical studies using lepidopteran tissue have since shown that the reaction is rapid, basically irreversible, and catalyzed by a microsomal P450 present only in prothoracic glands (61, 111, 178). The substrate specificity of this P450 is not absolute because in many species it also mediates the desaturation of several above-mentioned side chain alkylated plant sterols to their respective 7-dehydro-analogues (141). It also converts 25-hydroxycholesterol, a relatively polar sterol analogue, to 7-dehydro-25-hydroxycholesterol (15, 175), and most surprisingly, in *Manduca* it could utilize B-ring C analogues (i.e., α-5,6-epoxy-and α-5,6-iminocholesterols) as substrates (177). One species of fruit fly, *D. pachea*, has lost its ability to dehydrogenate C to 7dC (180). As a result, it is destined to live its entire life cycle in and around a single desert cactus species that it must ingest to survive because this cactus

synthesizes the Δ^7-sterol that fulfills this insect's bizarre sterol requirement (58). In the laboratory, this species subsists well on an artificial diet containing 7dC (70, 180). A *D. melanogaster* low-ecdysteroid mutant [*woc*; *without children* (185)] also lacks the C 7,8-dehydrogenase and can be partially rescued by feeding 7dC (180; see below).

The 7,8-dehydrogenation of C is not considered to be the rate-limiting step in the acute upregulation of insect ecdysteroidogenesis by PTTH because 7dC is normally a prominent and persistent sterol in the prothoracic gland throughout periods of growth and development and not just at ecdysis (60). Instead, one could consider the 7,8-dehydrogenase and/or the concentration of 7dC in the gland to be rate-enabling for continued active 3dE synthesis over the long term. That is, the endogenous concentrations of 7dC both fall and then rise precipitously, in a manner counter to that of C during the times of peak ecdysteroid biosynthesis in the last larval instar of *Manduca* (61, 178).

Translocation of ER-Localized 7-Dehydrocholesterol to, and within, the Mitochondria

Based on enzyme kinetics data from subcellular fractions of homogenized insect prothoracic glands and by analogy with the ACTH-mediated control of C movement within the mammalian adrenal mitochondria (165), it has been hypothesized that the rate-limiting step in PTTH-stimulated ecdysteroidogenesis is the movement of the poorly water soluble 7dC from the ER where it is synthesized to the mitochondria where it is oxidized to ecdysteroid. Neither 7dC nor its polar analogue 7-dehydro-25-hydroxycholesterol was metabolized after first being formed from C or 25-hydroxycholesterol by the microsomal fraction of *Manduca* prothoracic glands (175). Furthermore, in prothoracic gland homogenates, the kinetics of conversion of C (or 25-hydroxycholesterol) via 7dC (or 7-dehydro-25-hydroxycholesterol) to ecdysteroids was much slower than when these preformed dehydrogenated intermediates were supplied directly to the homogenates. That is, $\Delta^{5,7}$-sterol synthesized in the ER was translocated more slowly to the interior of the mitochondria prior to its oxidation to ecdysteroid when compared with the exogenously supplied $\Delta^{5,7}$-sterol. Radiochemical incorporation studies in the low ecdysone *Drosophila* mutant, *ecd1^{ts}* (*ecdysoneless*), indicated that the mutation might be affecting the movement of 7dC within the ring gland rather than the biosynthesis of 7dC (172), as in *D. pachea* and the low-ecdysteroid mutant *woc*, *without children* (180). Similar feeding of 7dC to *ecd1^{ts}* animals had no effect on the phenotype of the mutant (J. Warren & V. Henrich, unpublished information).

The movement of 7dC synthesized in the ER to the outer membrane of the prothoracic gland mitochondria may be analogous to the movement of ER-localized C to the adrenal cortex outer mitochondrial membrane. Such a redistribution of sterol could well be facilitated by carrier protein-based translocation or by processes that allow for the close proximity of these membranes, i.e., involving microtubules and/or intermembrane spanning protein moieties (67, 122, 181). In addition, PTTH-stimulated movement of 7dC within the insect mitochondrion

may be controlled by proteins analogous to STAR (steroidogenic acute regulatory protein) (5, 165) and DBI (diazepam-binding inhibitor) (118) that have been implicated in the rate-limiting movement of C within the adrenal cortex mitochondria.

Oxidation of 7-Dehydrocholesterol: The "Black Box"

In arthropods and plants, the so-called Black Box mitochondrial oxidations of 7dC (Figure 3) that may result in the formation of a long-hypothesized intermediate, the Δ^4-diketol (cholesta-4,7-diene-3,6-dione-14α-ol), would be different from any vertebrate steroidogenic reactions and may, therefore, constitute a desirable target of opportunity for insect control (14, 61). However, no intermediates between 7dC and any molecule bearing the characteristic ecdysteroid structure, i.e., the *cis* 5β(H)-3β-ol- or 3-dehydro-6-one-7-ene-14α-ol functionalities, have been characterized. These clearly complex oxidations and transformations may operate in concert, perhaps mediated by a single P450 (100, 124, 176). In any case, these reactions must occur much faster, and the resulting intermediates must be much more labile than those involved in mammalian C side chain cleavage ($P450_{SCC}$) or 14-demethylation ($P450_{14DM}$). The mechanisms underlying these latter classical multistep oxidations have long been understood since the intermediates were isolated and fully characterized, leading to the development of potent pharmaceuticals that block the action of these enzymes (20).

Following these unique Black Box reactions, the proposed Δ^4-diketol intermediate is converted to the diketol with a *cis*-A,B-ring fusion by a 5β(H)-reductase, at least in crustacean Y-organs (Figures 2 and 3) (14). The analogous structural transformations leading to mammalian steroid hormones have been well characterized. In contrast to the insect system, 5α-reductases mediate the conversion of the 3-ketoΔ^4-functionality to the active 5α-configuration, i.e., to hormones with a *trans*-A,B-ring junction. Truly analogous reduction to the 5β-configuration generally gives rise to inactive steroids in mammals, although bile acids are synthesized in this manner (20).

Once saturated, the A-ring 3-ketone group of these vertebrate steroids can then be reduced reversibly to the 3β-alcohol under the control of 3β-hydroxysteroid dehydrogenases (20). In like fashion, similar enzymatic activities in insects result in the interconversion of various products of terminal ecdysteroid hydroxylation (33), including the ultimately secreted (pro)hormones E, 3dE (94, 178, 179) (Figure 2*b*), and 25-deoxyecdysone (99), the latter being the precursor of the potent invertebrate hormone (and plant sterol) ponasterone A (115). It is unfortunate that classical biochemical paradigms have failed to result in the complete characterization of any of the P450s or other enzymes involved in E biosynthesis. However, recently, the potent combination of *D. melanogaster* molecular genetics and biochemical techniques has resulted in the first complete characterization of two endogenous ecdysteroidogenic cytochrome monooxygenases (see below).

For more than three decades, great effort has been expended to decipher the mechanism(s) underlying the biosynthetic oxidation of 7dC to an intermediate

having the basic required functionality of the ecdysteroid molecule (Figures 2 and 3). In insects, the stereospecific loss of the 3α, 4β, and 6-hydrogens during the metabolism of specifically labeled C analogues, via 7dC, to E was indicated. However, 20–30% of the 3α-hydrogen and 10% of the 6-hydrogen were recovered in the product, i.e., the loss was not all or none. These data led to the early hypothesis of a 3-dehydro-Δ^4-sterol intermediate (124), i.e., one with an oxidized and unsaturated A-ring conjugation. In other studies, the incorporation of various postulated radiolabeled intermediates into ecdysteroids by prothoracic glands was investigated. These experiments led to the generally accepted notion that, in addition to all the biochemical restraints mentioned above, the complex and novel 3-dehydro-6-keto-7-ene-14α-ol functionality characteristic of ecdysteroids must be produced in a concerted fashion (i.e., simultaneously). That is, in insects both in vivo and in vitro, the 14α-hydroxy function could not be introduced after the formation of the 6-keto-7-ene system (17, 65). In addition, ecdysteroid analogues with a 6-hydroxyl group could not be oxidized subsequently to the 6-ketone (144). Furthermore, the 3β-hydroxy analogue of the Δ^4-diketol (Δ^4-ketodiol) is not a good substrate for the newly characterized Δ^4-diketol-5β(H)-reductase (14).

Perhaps a brief discussion of the basic organic chemistry of 7dC and its oxidized derivatives would alleviate some of the confusion resulting from the above-noted radiotracer and precursor incorporation studies. Unlike C, 7dC (pro-vitamin D_3) is rather unstable and is subject to a facile photochemical B-ring fission reaction leading to the nonenzymatic formation of vitamin D_3 (20). In addition, it also undergoes facile photo-induced Diels-Alder addition of atmospheric oxygen across the $\Delta^{5,7}$-conjugated diene system to form the 5α,8α-epidioxide (13). Curiously, under mild nonenzymatic conditions, this highly oxygenated molecule and its 3-dehydro derivative isomerize and rearrange into a wide variety of polyfunctional oxygenated sterols (13, 83). Some of these sterols are identical to hypothesized intermediates in the biosynthesis of E, such as the highly conjugated 3-dehydro-$\Delta^{4,6,8(14)}$-triene (Figure 3) (61) and even the Δ^4-diketol itself (Figure 2). It is unfortunate that the 3-dehydro-5α,8α-epidioxide, although extensively metabolized, was not converted to identifiable ecdysteroids in either insect prothoracic glands or crustacean Y-organs (J. Warren, C. Dauphin-Villemant & R. Lafont, unpublished data). Further, the relatively stable α-5,6-epoxy-7dC molecule (177), long hypothesized as an intermediate, and its more stable $\Delta^{4,6,8(14)}$-triene product (61), were not substrates for ecdysteroid synthesis (84). Thus, there are few remaining possible structures that are stable and thus potentially identifiable molecules for Black Box intermediates.

The early proposals of the intermediacy of 3-dehydro-Δ^4 sterols in ecdysteroid biosynthesis were strengthened greatly when it was discovered that 3dE, and not E, was the major (if not only) ecdysteroid secreted by the prothoracic glands of many insects and the Y-organ of crustaceans. However, the question of when the oxidation of the 3β-hydroxyl group of C occurs remains open (Figure 2*a*). Because it is unstable (4, 101), it is unlikely that 3-dehydro-7dC is formed outside the mitochondria. No trace of its characteristic 3-dehydro-$\Delta^{4,7}$-diene facile isomerization

product could be found following the incubation of *Manduca* prothoracic glands with radiolabeled C or 25-hydroxycholesterol, both of which are converted in situ in high yield to 7dC or 7-dehydro-25-hydroxycholesterol, respectively (175). This distinctive product also could not be detected following the direct incubation of the latter two dehydrogenated compounds with prothoracic glands (J. Warren & L.I. Gilbert, unpublished information).

Nevertheless, for arthropods it is hypothesized that 3-dehydro-7-dehydrocholesterol is a logical initial intermediate that immediately precedes, and is an integral and concerted part of, the overall mitochondrial oxidations leading to the Δ^4-diketol (Figure 3). Its formation would function to activate the entire leading surface of the A, B, C, and D rings of the steroid molecule for further oxidation. This may be best visualized by first considering the structure of the "enol" of 3-dehydro-7dC, the delocalized $\Delta^{3,5,7}$-triene that is considered to coexist to some degree with the 3-dehydro-7dC. This $\Delta^{3,5,7}$-triene constitutes one extreme thermodynamic "boundary condition" for this system. The other extreme is the 3-dehydro-$\Delta^{4,6,8(14)}$-triene, an even more delocalized molecule that is an often-isolated nonenzymatic product of the further oxidation and subsequent rearrangement of the 3-dehydro-7dC (83). This latter isomerization is basically much like what was discussed above [i.e., a rearrangement to achieve a lower-energy, more delocalized (conjugated) system via electron stabilization]. The transition state is a hypothetical composite of these two stable extremes. It is a high-energy, and therefore a reactive, hypothetical intermediate whose actual existence would only be possible because of the planar extensively delocalized electronic character of its conjugated π-orbitals (Figure 3, shown in red). Formed at the active site of the P450, this transient molecule could undergo a variety of rapid and facile oxidation reactions. For example, it could be attacked in a concerted fashion by oxygen or peroxide across carbons 6 and 14, i.e., from below, therefore forming a classically stable, 6-membered structure (another epidioxide) (Figure 3, shown in blue). This high-energy intermediate would then fragment and stabilize as the Δ^4-diketol. Alternatively, rapid sequential hydroxylations by the P450 on this transition state first at C_{14} and then at C_6 followed by additional oxidation, similar to $P450_{SCC}$ or $P450_{14DM}$, could result in the formation of the same product, perhaps even involving high-energy epoxide intermediates (61, 69).

The Δ^4-Diketol-5β(H)-Reductase

The recent discovery of an enzyme in crustaceans that is most active in the Y-organs, which specifically and selectively converts the Δ^4-diketol to the 5β(H)-diketol and subsequently into E (Figure 2*b*), is quite important. Although characterized in a cytosolic fraction but also present in the mitochondrial and microsomal pellets, this enzyme requires NADPH (14). As such, it appears similar to vertebrate 3-oxo-Δ^4-steroid 5β(H)-reductases involved in steroid hormone catabolism and bile acid synthesis (20). Its complete characterization, not only in crabs but also in insects and plants, is critical to our understanding of ecdysteroid biosynthesis.

Ecdysteroid Terminal Hydroxylation

A series of hydroxylation reactions at side chain carbons 25, 22 and A-ring carbon 2 are vital for ecdysteroid biosynthesis and lead directly to E and 3dE. They have been characterized extensively in arthropod ecdysial glands and other tissues at various stages of development (Figure 2*b*). In general, the three responsible monooxygenases (hydroxylases) exhibit unusually varied substrate specificities, in addition to some rather unusual reaction characteristics. The most unexpected observation was the lack of tissue specificity exhibited by these various mitochondrial and microsomal cytochrome P450 enzymes activities (87).

All three hydroxylases require molecular oxygen and NADPH, and their reaction rates are reduced by the classical P450-inhibitors, metyrapone, piperonyl butoxide, and fenarimol. However, unlike the 25- and 22-enzymes, the 2-hydroxylase is not inhibited by carbon monoxide. Although present in all prothoracic glands and Y-organs studied, these enzymes, or similar enzymatic activities, are also found in the fat body, Malpighian tubules, gut, and epidermis of a variety of insect and crustacean species (64, 107).

25-HYDROXYLATION Generally considered to be the first of the three terminal hydroxylases in the sequence of hydroxylation, ecdysterol 25-hydroxylase is a classical microsomal P450 (87). This enzyme is not required for the synthesis of 25-deoxyecdysone, and thus ponasterone A in crustaceans, but exhibits high activity in *Manduca* prothoracic gland homogenates (60, 61). In contrast to the vertebrate P450 that mediates the 25-hydroxylation and activation of vitamin D_3 (44), the insect enzyme is not stereospecific and exhibits varied substrate specificity (16).

22-HYDROXYLATION Thought to be the second enzyme in the sequence of hydroxylations (see below), the 22-hydroxylase is a mitochondrial P450. As a result, in addition to NADPH, Krebs cycle intermediates can also sustain appreciable reaction rates in vitro (also true of the 2-hydroxylase). Both this reaction and the subsequent 2-hydroxylation occur with the retention of configuration at these positions (87). Mechanism-based, suicide inhibitors of this enzyme have been described and this has obvious practical implications (131). Recently, the lack of endogenous ecdysterol 22-hydroxylation activity has been identified as the cause of the *Drosophila* mutation, *disembodied* (see below). This gene (CYP302a1) has been cloned (21) and specifically expressed in an insect cell line, and the resultant protein catalyzes the efficient metabolism of the ketotriol (Figure 2*b*) to 2-deoxyecdysone (J. Warren, G. Marques, A. Petryk, M. O'Connor & L.I. Gilbert, unpublished information).

2-HYDROXYLATION In the prothoracic gland and Y-organs, this mitochondrial enzyme probably functions last in the sequence of terminal hydroxylations because ecdysteroid precursors prematurely hydroxylated at C_2 are not good substrates for subsequent 22-hydroxylase reactions (33). As a result, some intermediates such as

22-deoxyecdysone and 22,25-dideoxyecdysone were not metabolized to E, even under lengthy in vitro incubations of ecdysial glands with the ketodiol (125). However, when these same intermediates were administered in vivo they were generally converted to E (86), although not always (9), i.e., the reaction is especially slow during larval stages. These results suggest that the specificities and therefore the identities of the ecdysial gland hydroxylases may differ from those present at various stages in other insect tissues. As with the 22-hydroxylase, the molecular characterization of another *Drosophila* mutant (*shadow*) has recently resulted in the complete characterization of the 2-hydroxylase as P450 CYP315a1 (see "Halloween" mutants below). This approach should result in a more-complete understanding of the identity and specificity of enzymes mediating ecdysteroidogenesis.

3-β-Hydroxysterol Dehydrogenases

The ecdysteroid 3β-ketoreductase requires NADPH and is present in the hemolymph and in many other insect tissues and converts 3dE to E (23, 94, 138). In contrast, the 3α-ketoreductase is present only in the gut and functions in concert with ecdysone oxidase to inactivate ecdysteroids (22). All intermediates distal to the Δ^4-diketol, and perhaps the Δ^4-diketol itself, are subject to oxidation and reduction of the 3β-alcohol/3-ketone functionality. With the diketol/ketodiol and 3-dehydro-ketotriol/ketotriol pairs, this reaction may be truly reversible and catalyzed by enzymes mechanistically similar to mammalian 3β-hydroxysterol dehydrogenases/reductases (Figure 2*b*). With more hydroxylated products like 2-deoxyecdysone and E, ecdysone oxidase mediates the irreversible oxidation of the 3β-alcohol functionality to the 3-dehydro derivative (96). However, it does not catalyze the dehydrogenation of the 3α-epimeric analogues. Reduction of the 3-keto function to either the 3β (active) or 3α (inactive) stereochemistry is achieved by two different basically irreversible reactions (22, 23).

FUTURE DIRECTIONS

With the notable exception of the 3β-ketoreductase (22), the classical techniques of enzyme analysis and purification have failed to conclusively, or completely, characterize any of the other enzymes involved in either ecdysteroidogenesis or the subsequent metabolism of E (Figure 2). Considerable research, however, has resulted in the characterization of the critical ecdysteroid 20-monooxygenase that mediates the conversion of E to 20E (184). Analogous studies of the ecdysteroid 26-hydroxylase (7, 91, 183), together with the 3-dehydroecdysteroid 3α-reductase responsible for molting hormone inactivation, should result in their complete characterization in the near future.

For more than 30 years, the study of various low ecdysteroid *Drosophila* mutants has been helpful in understanding the roles of E and 20E in insect development. The temperature-sensitive mutation *ecd1*^{ts} (*ecdysoneless*) (46, 172) and the *woc* mutation (*without children*) (185) were instrumental in elucidating the early synthesis

of 7dC from C and the subsequent movement of 7dC within the prothoracic gland cells of the ring gland. Unlike *ecd1*, the *woc* mutant lacks the $C_{7,8}$-dehydrogenase, and this enzyme deficiency results in the classical low E phenotype. That is, the ecdysteroid titer could be dramatically increased and mutants could be partially rescued by dietary 7dC (180). However, when the *woc* gene was cloned, it was shown to code for a novel zinc-fingered protein that may function as a transcription factor (185) rather than the expected P450 enzyme. The amino acid sequence of *woc* is upward of 65% similar to genes previously identified in humans that, when variously mutated, have been linked to mental retardation and leukemia. The powerful techniques of gene expression analysis (i.e., differential display), subtractive hybridization, and *Drosophila* gene microarray screening are being pursued with this mutation in order to both isolate the 7,8-dehydrogenase gene and to better understand *woc* control of *Drosophila* development.

Recently, the paradigms of molecular genetics were applied to a series of *Drosophila* embryonic lethal mutations that affect cuticle formation, i.e., the "Halloween" mutants (85, 116, 182). In two mutants, *disembodied* (21) and *shadow* (J. Warren, A. Petryk, L. Gilbert & M. O'Connor, unpublished information), the mutations are associated with low embryonic ecdysteroid levels and a failure to activate downstream ecdysone-responsive genes. Following the identification of the genes, both cytochrome P450s with characteristic N-terminal mitochondrial import sequences, in situ analyses demonstrated mRNA expression only in the embryonic and larval ring gland and in the follicle cells of the adult ovary. The data support the assertion, at least in *Drosophila*, that the 2- and 22-terminal hydroxylation reactions are specific to the ecdysial glands. Similar reactions detected in other tissues must therefore be catalyzed by different enzymes. Transfection of these genes into *Drosophila* S2 cells followed by radiotracer analysis revealed that the *disembodied* gene coded for the terminal 22-hydroxylase and that *shadow* coded for the 2-hydroxylase (J. Warren, G. Marques, A. Petryk, M. O'Connor & L.I. Gilbert, unpublished information). When both genes were transfected into S2 cells simultaneously, the ketotriol was efficiently converted to E (Figure 2*b*). We believe that future analysis of other members of the "Halloween" mutant family will result in the identification and characterization of the other P450 enzymes involved in ecdysteroidogenesis, i.e., those affecting the dealkylation of plant sterols to C and its conversion to E, as well as the subsequent metabolism of E, first to 20E and then to inactive metabolites of the molting hormone. This should provide a solid foundation for an understanding of ecdysteroid titer modulation, a process critical for the design of new agents for insect control and physiologically relevant experiments on ecdysteroid action (173).

ACKNOWLEDGMENTS

We thank Pat Cabarga for helping prepare this contribution and Susan Whitfield for the graphics of Figures 2 and 3. We are also grateful to our past and present colleagues who contributed to the research noted herein from the Gilbert laboratory.

For the PTTH-prothoracic gland activation research, we are grateful to Wendy Smith, Wendell Combest, Noriaki Agui, Walter Bollenbacher, Noelle Granger, Victoria Meller, and Akira Mizoguchi. For the prothoracic gland EcR work, we thank Qisheng Song; and for the ecdysteroid biochemistry research, Mike Grieneisen and Sho Sakurai. The use of low-ecdysteroid mutants was aided critically in the early days by Vince Henrich and Tim Sliter, more recently by Jasmine Wismar and Elisabeth Gateff (*woc*) and Michael O'Connor and his laboratory (Halloween genes). Much of the work from the Gilbert laboratory discussed here was supported by grants from NIH and NSF.

LITERATURE CITED

1. Agui N, Granger NA, Bollenbacher WE, Gilbert LI. 1979. Cellular localization of the insect prothoracicotropic hormone: *in vitro* assay of a single neurosecretory cell. *Proc. Natl. Acad. Sci. USA* 76:5694–98
2. Aguinaldo AMA, Turbeville JM, Linford LS, Rivera MC, Garey JR, et al. 1997. Evidence for a clade of nematodes, arthropods and other moulting animals. *Nature* 387:489–93
3. Alnemri ES, Fernandes-Alnemri T, Nelki DS, Dudley K, DuBois GC, et al. 1994. FKBP46, a novel Sf9 insect cell nuclear immunophilin that forms a protein-kinase complex. *J. Biol. Chem.* 269:30828–38
4. Antonucci R, Bernstein S, Giancola D, Sax K. 1951. $\Delta^{5,7}$ steroids. IX. The preparation of $\Delta^{4,7}$ and $\Delta^{4,7,9}$3-keto-steroid hormones. *J. Org. Chem.* 16:1453–57
5. Arakane F, King SR, Du Y, Kallen CB, Walsh LP, et al. 1997. Phosphorylation of steroidogenic acute regulatory protein (StAR) modulates its steroidogenic activity. *J. Biol Chem.* 272:32656–62
6. Arbeitman MN, Hogness DS. 2000. Molecular chaperones activate the *Drosophila* ecdysone receptor, an RXR heterodimer. *Cell* 101:67–77
7. Bassett MH, McCarthy JL, Waterman MR, Sliter TJ. 1997. Sequence and developmental expression of *Cyp* 18, a member of a new cytochrome P450 family from *Drosophila*. *Mol. Cell. Endocrinol.* 131:39–49
8. Beato M, Sánchez-Pacheco A. 1996. Interaction of steroid hormone receptors with the transcription initiation complex. *Endocrine Rev.* 17:587–609
9. Bergamasco R, Horn DHS. 1980. The biological activities of ecdysteroids and ecdysteroid analogues. In *Progress in Ecdysone Research*, ed. JA Hoffmann, 7:299–324. Amsterdam/New York/Oxford: Elsevier/North Holland Biomed.
10. Beydon P, Lafont R. 1983. Feedback inhibition of ecdysone production by 20-hydroxyecdysone in *Pieris brassicae* pupae. *J. Insect Physiol.* 29:529–33
11. Birkenbeil H. 1998. Intracellular calcium in prothoracic glands of *Manduca sexta*. *J. Insect Physiol.* 44:279–86
12. Birkenbeil H. 2000. Pharmacological study of signal transduction during stimulation of prothoracic glands from *Manduca sexta*. *J. Insect Physiol.* 46:1409–14
13. Bladon P, Sleigh T. 1965. Photo-oxygenation of 3-acetoxyergosta-3,5,7,22-tetraene and related compounds. *J. Chem. Soc.* 6991–7000
14. Blais C, Dauphin-Villemant C, Kovganko N, Girault J-P, Descoins C Jr, et al. 1996. Evidence of the involvement of 3-oxo-Δ^4 intermediates in ecdysteroid biosynthesis. *Biochem. J.* 320:413–19

15. Böcking D, Dauphin-Villemant C, Toullec J-Y, Blais C, Lafont R. 1994. Ecdysteroid formation from 25-hydroxycholesterol by arthropod molting glands in vitro. *C. R. Acad. Sci. Paris* 317:891–98
16. Bollenbacher W, Faux AF, Galbraith MN, Gilbert LI, Horn DHS, et al. 1979. *In vitro* metabolism of possible ecdysone precursors by the prothoracic glands of the tobacco hornworm, *Manduca sexta*. *Steroids* 34:509–26
17. Bollenbacher W, Galbraith N, Horn DHS, Gilbert LI. 1977. *In vitro* metabolism of 3-hydroxy-, and 3,14β-dihydroxy-[3-^{3}H]-5-cholest-7-en-6-one by the prothoracic glands of *Manduca sexta*. *Steroids* 29:47–63
18. Bollenbacher WE, Granger NA. 1985. Endocrinology of the prothoracicotropic hormone. See Ref. 92a, 7:109–51
19. Bollenbacher WE, Smith SA, Goodman W, Gilbert LI. 1981. Ecdysteroid titer during larval-pupal-adult development of the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol.* 44:302–6
20. Brown GD. 1998. The biosynthesis of steroids and triterpenoids. *Nat. Prod. Rep.* 15(6):653–96
21. Chávez VM, Marqués G, Delbecque JP, Kobayashi K, Hollingsworth M, et al. 2000. The *Drosophila disembodied* gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. *Development* 127:4115–26
22. Chen JH, Powls F, Rees HH, Wilkinson MC. 1999. Purification of ecdysone oxidase and 3-dehydroecdysone 3α-reductase from the cotton leafworm, *Spodoptera littoralis*. *Insect Biochem. Mol. Biol.* 29:8899–908
23. Chen JH, Turner PC, Rees HH. 1999. Molecular cloning and characterization of hemolymph 3-dehydroecdysone 3β-reductase from the cotton leafworm, *Spodoptera littoralis*—a new member of the third superfamily of oxidoreductases. *J. Biol. Chem.* 274:10551–56
24. Cherbas P, Cherbas L. 1996. Molecular aspects of ecdysteroid hormone action. See Ref 56a, pp. 175–221
25. Cherbas P, Cherbas L, Lee S-S, Nakanishi K. 1988. 26-[^{125}I]Iodoponasterone A is a potent ecdysone and a sensitive radioligand for ecdysone receptors. *Proc. Natl. Acad. Sci. USA* 85:2096–100
26. Coggins JR, Schaefer FW III, Weinstein PP. 1985. Ultrastructural analysis of pathologic lesions in sterol-deficient *Nippostrongylus brasiliensis* larvae. *J. Invertebr. Pathol.* 45:288–97
27. Combest WL, Gilbert LI. 1992. Polyamines modulate multiple protein phosphorylation pathways in the insect prothoracic gland. *Mol. Cell. Endocrinol.* 83: 11–19
28. Cook IF, Lloyd-Jones JG, Rees HH, Goodwin TW. 1973. The stereochemistry of hydrogen elimination from C-7 during biosynthesis of ecdysones in insects and plants. *Biochem. J.* 136:135–45
29. Czar MJ, Lyons RH, Welsh MJ, Renior J-M, Pratt WB. 1995. Evidence that the FK-506-immunophilin heat shock protein 56 is required for trafficking of the glucocorticoid receptor from the cytoplasm to the nucleus. *Mol. Endocrinol.* 9:1549–60
30. Czar MJ, Owens-Grillo JK, Yem AW, Leach KL, Deibel MR, et al. 1994. The hsp56 immunophilin component of untransformed steroid receptor complexes is localized both to microtubules in the cytoplasm and to the same nonrandom regions within the nucleus as the steroid receptor. *Mol. Endocrinol.* 8:1731–41
31. Dai J-D, Gilbert LI. 1991. Metamorphosis of the corpus allatum and degeneration of the prothoracic glands during the larval-pupal-adult transformation of *Drosophila melanogaster*: a cytophysiological analysis of the ring gland. *Dev. Biol.* 144:309–26
32. Dai J-D, Gilbert LI. 1997. Programmed cell death of the prothoracic glands of *Manduca sexta* during pupal-adult

metamorphosis. *Insect Biochem. Mol. Biol.* 27:69–78
33. Dauphin-Villemant C, Blais C, Lafont R. 1998. Towards the elucidation of the ecdysteroid biosynthetic pathway. *Ann. NY Acad. Sci.* 839:306–10
34. Delbecque J-P, Weidner K, Hoffmann KH. 1990. Alternative sites for ecdysteroid production in insects. *Invertebr. Reprod. Dev.* 18:29–42
35. Evans RM. 1988. The steroid and thyroid hormone superfamily. *Science* 240:889–95
36. Feldlaufer MF, Weirich GF, Lusby WR, Svoboda JA. 1991. Makisterone C: a 29-carbon ecdysteroid from developing embryos of the cotton stainer bug, *Dysdercus fasciatus*. *Arch. Insect Biochem. Physiol.* 18:71–79
37. Ferrari S, Thomas G. 1994. S6 phosphorylation and the p70s6k/p85s6k. *Crit. Rev. Biochem. Mol. Biol.* 29:385–413
38. Fogleman JC, Duperret SM, Kircher HW. 1986. The role of phytosterols in host plant utilization by cactophilic *Drosophila*. *Lipids* 21:92–96
39. Fortini ME, Bonini NM. 2000. Modeling human neurodegenerative diseases in *Drosophila* on a wing and a prayer. *Trends Genet.* 16:161–67
40. Freeman MR, Dobritsa A, Gaines P, Segraves WA, Carlson JR. 1999. The *dare* gene: steroid hormone production, olfactory behavior, and neural degeneration in *Drosophila*. *Development* 126:4591–602
41. Fujimoto Y, Ikuina Y, Kakinuma K. 1989. Mechanism of the conversion of fucosterol epoxide into desmosterol in insects. The stereochemical fate of the diastereotopic C-26 and C-27 methyl groups of the epoxide. *J. Chem. Soc. Chem. Commun.* 464–66
42. Fujimoto Y, Ikuina Y, Nagakari M, Kakinuma K, Ikekawa N. 1990. C-25 prochirality in the fragmentation reaction catalysed by fucosterol epoxide lyase from the silkworm, *Bombyx mori*. *J. Chem. Soc. Perkin Trans.* I:2041–46
43. Fujimoto Y, Nagakari M, Ikuina Y, Kakinuma K. 1991. Stereochemistry of the hydrogen addition to C-25 of desmosterol by sterol-Δ^{24}-reductase of the silkworm, *Bombyx mori*. *J. Chem. Soc. Chem. Commun.* pp. 688–89
44. Fujimoto Y, Ohyama K, Nomure K, Hyodo R, Takahashi K, et al. 2000. Biosynthesis of sterols and ecdysteroids in *Ajuga* hairy roots. *AOCS Press* 35:279–88
45. Gäde G, Hoffmann KH, Spring JH. 1997. Hormonal regulation in insects: facts, gaps, and future directions. *Physiol. Rev.* 77:963–1032
46. Garen A, Kanvar L, Lepesant JA. 1977. Role of ecdysone in *Drosophila* development. *Proc. Natl. Acad. Sci. USA* 74: 5099–103
47. Gelman DB, Beckage NE. 1995. Low molecular weight ecdysiotropins in proctodaea of fifth instars of the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae), and hosts parasitized by the braconid wasp *Cotesia congregata* (Hymenoptera: Braconidae). *Eur. J. Entomol.* 92:123–29
48. Gelman DB, Borovsky D. 2000. *Aedes aegypti* TMOF modulates ecdysteroid production by prothoracic glands of the gypsy moth *Lymantria dispar*. *Arch. Insect Biochem. Physiol.* 45:60–68
49. Gelman DB, Thyagaraja BS, Kelly TJ, Masler EP, Bell RA, et al. 1991. The insect gut: a new source of ecdysiotropic peptides. *Experientia* 47:77–80
50. Gilbert LI. 1962. Maintenance of the prothoracic gland by the juvenile hormone in insects. *Nature* 193:1205–7
51. Gilbert LI. 1967. Lipid metabolism and function in insects. *Adv. Insect Physiol.* 4:169–211
52. Gilbert LI, Combest WL, Smith WA, Meller VH, Rountree DB. 1988. Neuropeptides, second messengers and insect molting. *BioEssays* 8:153–57
53. Gilbert LI, Granger N, Roe M. 2000. The juvenile hormones: historical facts and speculations on future research directions.

Insect Biochem. Mol. Biol. 30:617–44

54. Gilbert LI, Rybczynski R, Song Q, Mizoguchi A, Morreale R, et al. 2000. Dynamic regulation of prothoracic gland ecdysteroidogenesis: *Manduca sexta* recombinant prothoracicotropic hormone and brain extracts have identical effects. *Insect. Biochem. Mol. Biol.* 30:1079–89
55. Gilbert LI, Rybczynski R, Tobe S. 1996. Regulation of endocrine function leading to insect metamorphosis. See Ref. 56a, pp. 59–107
56. Gilbert LI, Song Q, Rybczynski R. 1997. Control of ecdysteroidogenesis: activation and inhibition of prothoracic gland activity. *Invertebr. Neurobiol.* 3:205–16

56a. Gilbert LI, Tata JR, Atkinson BG, eds. 1996. *Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells*. San Diego: Academic. 3rd ed.

57. Girgenrath S, Smith WA. 1996. Investigation of presumptive mobilization pathways for calcium in the steroidogenic action of big prothoracicotropic hormone. *Insect Biochem. Mol. Biol.* 26:455–63
58. Goodnight KC, Kircher HW. 1971. Metabolism of lathosterol by *Drosophila pachea*. *Lipids* 6:166–69
59. Graf R, Neuenschwander S, Brown MR, Ackermann U. 1997. Insulin-mediated secretion of ecdysteroids from mosquito ovaries and molecular cloning of the insulin receptor homologue from ovaries of bloodfed *Aedes aegypti*. *Insect Mol. Biol.* 6:151–63
60. Grieneisen ML, Warren JT, Gilbert LI. 1993. Early steps in ecdysteroid biosynthesis: evidence for the involvement of cytochrome P-450 enzymes. *Insect Biochem. Mol. Biol.* 23:13–23
61. Grieneisen ML, Warren JT, Sakurai S, Gilbert LI. 1991. A putative route to ecdysteroids: metabolism of cholesterol *in vitro* by mildly disrupted prothoracic glands of *Manduca sexta*. *Insect Biochem.* 21:41–51
62. Gu S-H, Chow Y-S, Lin F-J, Wu J-L, Ho R-J. 1996. A deficiency in prothoracicotropic hormone transduction pathway during the early last larval instar of *Bombyx mori*. *Mol. Cell. Endocrinol.* 120:99–105
63. Gu S-H, Chow Y-S, O'Reilly DR. 1998. Role of calcium in the stimulation of ecdysteroidogenesis by recombinant prothoracicotropic hormone in the prothoracic glands of the silkworm, *Bombyx mori*. *Insect Biochem. Mol. Biol.* 28:861–67
64. Haag T, Hetru C, Kappler C, Moustier AM, Hoffmann JA, Luu B. 1988. Study on the biosynthesis of ecdysone, Part IV: synthesis of high specific activity ($^3H_2$-22,23)-2,22-dideoxyecdysone: tissue distribution of the C-22 hydroxylase in *Locusta migratoria*. *Tetrahedron* 44: 1397–407
65. Haag T, Meister M-F, Hetru C, Kappler C, Nakantani Y, et al. 1987. Synthesis of a labelled putative precursor of ecdysone-II [$^3H_4$]3β-hydroxy-5β-cholest-7-ene-6-one: critical re-evaluation of its role in *Locusta migratoria*. *Insect Biochem.* 17:290–301
66. Hanton WK, Watson RD, Bollenbacher WE. 1993. Ultrastructure of prothoracic glands during larval-pupal development of the tobacco hornworm, *Manduca sexta*: a reappraisal. *J. Morphol.* 216:95–112
67. Hapala I, Kavecansky J, Butko P, Scallen TJ, Joiner CH, Schroeder F. 1994. Regulation of membrane cholesterol domains by sterol carrier protein-2. *Biochemistry* 33:7682–90
68. Hayes GC, Muehleisen DP, Bollenbacher WE, Watson RD. 1995. Stimulation of ecdysteroidogenesis by small prothoracicotropic hormone: role of calcium. *Mol. Cell. Endocrinol.* 115:105–12
69. Hedtmann U, Hobert K, Milkova T, Welzel P. 1988. Synthesis of potential ecdysteroid precursors from $\Delta^{7,22}$ sterols. *Tetrahedron* 44:1941–52
70. Heed W, Kircher H. 1965. Unique sterol

in the ecology and nutrition of *Drosophila pachea*. *Science* 149:758–61

71. Henrich VC. 1995. Comparison of ecdysteroid production in *Drosophila* and *Manduca*: pharmacology and cross-species neural reactivity. *Arch. Insect Biochem. Physiol.* 30:239–54
72. Henrich VC, Rybczynski R, Gilbert LI. 1999. Peptide hormones, steroid hormones and puffs: mechanisms and models in insect development. In *Vitamins and Hormones*, ed. G Litwack, 55:73–125. San Diego: Academic
73. Henrich VC, Sliter TJ, Lubahn DB, MacIntyre A, Gilbert LI. 1990. A steroid/thyroid hormone receptor superfamily member in *Drosophila melanogaster* that shares extensive sequence similarity with a mammalian homologue. *Nucleic Acids Res.* 18:4143–48
74. Hetru C, Li KW, Bulet P, Lagueux M, Hoffman JA. 1991. Isolation and structural characterization of an insulin-related molecule, a predominant neuropeptide from *Locust migratoria*. *Eur. J. Biochem.* 201:495–99
75. Horn DHS, Bergamasco R. 1985. Chemistry of ecdysteroids. See Ref. 92a, 7:185–248
76. Hua Y-J, Tanaka Y, Nakamura K, Sakakibara M, Nagata S, Kataoka H. 1999. Identification of a prothoracicostatic peptide in the larval brain of the silkworm, *Bombyx mori*. *J. Biol. Chem.* 274:31169–73
77. Hurley JH. 1999. Structure, mechanism, and regulation of mammalian adenylyl cyclase. *J. Biol. Chem.* 274:7599–602
78. Ishibashi J, Kataoka H, Isogai A, Kawakami A, Saegusa H, et al. 1994. Assignment of disulfide bond location in prothoracicotropic hormone of the silkworm, *Bombyx mori*: a homodimeric protein. *Biochemistry* 33:5912–19
79. Ishizaki H, Suzuki A. 1994. The brain secretory peptides that control moulting and metamorphosis of the silkmoth *Bombyx mori*. *Int. J. Dev.* 38:301–10
80. Jindra M, Huang J-Y, Malone F, Asahina M, Riddiford LM. 1997. Identification and mRNA developmental profiles of two ultraspiracle isoforms in the epidermis and wings of *Manduca sexta*. *Insect Mol. Biol.* 6:41–53
81. Jindra M, Malone F, Hiruma K, Riddiford LM. 1996. Developmental profiles and ecdysteroid regulation of the mRNAs for two ecdysone receptor isoforms in the epidermis and wings of the tobacco hornworm, *Manduca sexta*. *Dev. Biol.* 180:258–72
82. John ME, John MC, Boggaram V, Simpson ER, Waterman MR. 1986. Transcriptional regulation of steroid hydroxylase genes by corticotropin. *Proc. Natl. Acad. Sci. USA* 83:4715–19
83. Johns WF. 1971. Synthesis and reactions of 5α,8-epidioxyandrost-6-enes. *J. Org. Chem.* 36:2391–97
84. Joly RA, Svahn CM, Bennet RD, Heftmann E. 1969. Investigation of intermediate steps in the biosynthesis of ecdysterone from cholesterol in *Podocarpus elata*. *Phytochemistry* 8:1917–20
85. Jürgens G, Wieschaus E, Nüsslein-Volhard C, Kluding H. 1984. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. II. Zygotic loci on the third chromosome. *Roux's Arch. Dev. Biol.* 193:283–95
86. Kaplanis JN, Robbins WE, Thompson MJ, Baumhover AH. 1969. Ecdysone analog: conversion to alpha ecdysone and 20-hydroxyecdysone by an insect. *Science* 166:1540–41
87. Kappler C, Hetru C, Durst F, Hoffmann JA. 1989. Enzymes involved in ecdysone biosynthesis. In *Ecdysone: From Chemistry to Mode of Action*, ed. J Koolman, pp. 161–66. Stuttgart: Georg Thieme-Verlag
88. Karim FD, Thummel CS. 1992. Temporal coordination of regulatory gene expression by steroid hormone ecdysone. *EMBO J.* 11:4083–93
89. Kawakami A, Kataoka H, Oka T, Mizoguchi A, Kimura-Kawakami M, et al.

1990. Molecular cloning of the *Bombyx mori* prothoracicotropic hormone. *Science* 247:1333–35

90. Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, et al. 1998. A family of cAMP-binding proteins that directly activate Rap1. *Science* 282:2275–79
91. Kayser H, Winkler T, Spindler-Barth M. 1997. 26-Hydroxylation of ecdysteroids is catalyzed by a typical cytochrome *P-450*-dependent oxidase and related to ecdysteroid resistance in an insect cell line. *Eur. J. Biochem.* 248:707–16
92. Keightley DA, Lou KJ, Smith WA. 1990. Involvement of translation and transcription in insect steroidogenesis. *Mol. Cell. Endocrinol.* 74:229–37

92a. Kerkut GA, Gilbert LI, eds. 1985. *Comprehensive Insect Physiology, Biochemistry and Pharmacology.* Oxford: Pergamon. Vol. 7

93. Kim A-J, Cha G-H, Kim K, Gilbert LI, Lee CC. 1997. Purification and characterization of the prothoracicotropic hormone of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 94:1130–35
94. Kiriishi S, Rountree DB, Sakurai S, Gilbert LI. 1990. Prothoracic gland synthesis of 3-dehydroecdysone and its hemolymph 3β-reductase mediated conversion to ecdysone in representative insects. *Experientia* 46:716–21
95. Koelle MR, Talbot WS, Segraves WA, Bender MT, Cherbas P, et al. 1991. The *Drosophila EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* 67:59–77
96. Koolman J. 1978. Ecdysone oxidase in insects. *Hoppe-Seyler's Z. Physiol. Chem.* 359:1315–21
97. Kopeć S. 1922. Studies on the necessity of the brain for the inception of insect metamorphosis. *Biol. Bull.* 42:323–42
98. Kralli A, Yamamoto KR. 1996. An FK-506-sensitive transporter selectively decreases intracellular levels and potency of steroid hormones. *J. Biol. Chem.* 271:17152–56
99. Lachaise F, Meister MG, Hetru C, Lafont R. 1986. Studies on the biosynthesis of ecdysone by the Y-organs of *Carcinus maenas*. *Mol. Cell. Endocrinol.* 45:253–61
100. Lafont R. 2000. Understanding insect endocrine systems: molecular approaches. *Entomol. Exp. Appl.* 97:123–36
101. Lakeman J, Speckamp WW, Huisman HO. 1967. Addition to steroid polyenes IV. *Tetrahedron Lett.* 38:3699–703
102. Lester DS, Gilbert LI. 1985. Choline acetyltransferase activity in the larval brain of *Manduca sexta*. *Insect Biochem.* 15:685–94
103. Lewis TS, Shapiro PS, Ahn NG. 1998. Signal transduction through MAP kinase cascades. *Adv. Cancer Res.* 74:49–139
104. Loeb MJ, Kochansky J, Wagner RM, Bell RA. 1994. Transduction of the signal initiated by the neuropeptide, testis ecdysiotropin, in testes of the gypsy moth, *Lymantria dispar*. *J. Insect Physiol.* 40:939–46
105. Lomas LO, Turner PC, Rees HH. 1997. A novel neuropeptide-endocrine interaction controlling ecdysteroid production in ixodid ticks. *Proc. R. Soc. London Ser. B* 264:589–96
106. Manière G, Vanhems E, Delbecque J-P. 2000. Cyclic AMP-dependent and independent stimulations of ovarian steroidogenesis by brain factors in the blowfly, *Phormia regina*. *Mol. Cell. Endocrinol.* 168:31–40
107. Meister MF, Dimarq JL, Kappler C, Hetru C, Lagueux M, et al. 1985. Conversion of a radiolabelled ecdysone precursor, 2,22,25-trideoxyecdysone, by embryonic and larval tissues of *Locusta migratoria*. *Mol. Cell Endocrinol.* 41:27–44
108. Meller VH, Combest WL, Smith WA, Gilbert LI. 1988. A calmodulin-sensitive adenylate cyclase in the prothoracic glands of the tobacco hornworm, *Manduca sexta*. *Mol. Cell. Endocrinol.* 59:67–76

109. Meller VH, Sakurai S, Gilbert LI. 1990. Developmental regulation of calmodulin-dependent adenylate cyclase in an insect endocrine gland. *Cell. Reg.* 1:771–80
110. Milad M, Sullivan W, Diehl E, Altmann M, Nordeen S, et al. 1995. Interaction of the progesterone receptor with binding proteins for FK506 and cyclosporin A. *Mol. Endocrinol.* 9:838–47
111. Milner NP, Nali M, Gibson JM, Rees HH. 1986. Early stages of ecdysteroid biosynthesis: the role of 7-dehydrocholesterol. *Insect Biochem* 16:17–23
112. Mizoguchi A, Ohashi Y, Hosoda K, Ishibashi J, Kataoka H. 2001. Developmental profile of the changes in the prothoracicotropic hormone titer in hemolymph of the silkworm *Bombyx mori*: correlation with ecdysteroid secretion. *Insect Biochem. Mol. Biol.* 31:349–58
113. Morisaki M, Ohtaka H, Okubayashi M, Ikekawa N, Horie Y, et al. 1972. Fucosterol-24,28-epoxide, as a probable intermediate in the conversion of β-sitosterol to cholesterol in the silkworm. *J. Chem. Soc. Commun.* 1275–76
114. Nagasawa H, Isogai A, Suzuki A, Tamura S, Ishizaki H. 1979. Purification and properties of the prothoracicotropic hormone of the silkworm, *Bombyx mori*. *Dev. Growth Diff.* 21:29–38
115. Nakanishi K, Koreeda M, Sasaki S, Chang ML, Hsu HY. 1966. Insect hormones. The structure of ponasterone A, an insect moulting hormone from the leaves of *Podocarpus nakaii* Hay. *J. Chem. Soc. Chem. Commun.* pp. 915–17
116. Nüsslein-Volhard C, Wieschaus E, Kluding H. 1984. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. I. Zygotic loci on the second chromosome. *Roux's Arch. Dev. Biol.* 183:267–82
117. Oro AE, McKeown M, Evans RM. 1990. Relationship between the product of the *Drosophila Ultraspiracle* locus and the vertebrate retinoid X receptor. *Nature* 347:298–301
118. Papadopoulos V, Amri H, Boujrad N, Cascio C, Culty M, et al. 1997. Peripheral benzodiazepine receptor in cholesterol transport and steroidogenesis. *Steroids* 62:21–28
119. Pratt WB, Toft DO. 1997. Steroid receptor interaction with heat shock protein and immunophilin chaperones. *Endocrinol. Rev.* 18:306–60
120. Predel R, Eckert M. 2000. Neurosecretion: peptidergic systems in insects. *Naturwissenschaften* 87:343–50
121. Prestwich GD, Angelastro M, De Palma A, Perino MA. 1985. Fucosterol epoxide lyase of insects: synthesis of labeled substrates and development of a partition assay. *Anal. Biochem.* 151:315–26
122. Puglielli L, Rigotti A, Greco AV, Santos MJ, Nervi F. 1995. Sterol carrier protein-2 is involved in cholesterol transfer from the endoplasmic reticulum to the plasma membrane in human fibroblasts. *J. Biol. Chem.* 270:18723–26
123. Redfern CPF. 1984. Evidence for the presence of makisterone A in *Drosophila* larvae and the secretion of 20-deoxymakisterone A by the ring glands. *Proc. Natl. Acad. Sci. USA* 81:5643–47
124. Rees HH. 1985. Biosynthesis of ecdysone. See Ref. 92a, 7:249–93
125. Rees HH. 1995. Ecdysteroid biosynthesis and inactivation in relation to function. *Eur. J. Entomol.* 92:9–39
126. Richter K, Peschke E, Peschke D. 2000. A neuroendocrine releasing effect of melatonin in the brain of an insect, *Periplaneta americana* (L.). *J. Pineal Res.* 28: 129–35
127. Riddiford LM. 1996. Molecular aspects of juvenile hormone action in insect metamorphosis. See Ref. 56a, pp. 223–51
128. Riehle MA, Brown MR. 1999. Insulin stimulates ecdysteroid production through a conserved signaling cascade in the mosquito *Aedes aegypti*. *Insect Biochem. Mol. Biol.* 29:855–60
129. Rountree DB, Combest WL, Gilbert LI. 1987. Protein phosphorylation in the

prothoracic glands as a cellular model for juvenile hormone-prothoracicotropic hormone interactions. *Insect Biochem.* 17: 943–48

130. Rountree DB, Combest WL, Gilbert LI. 1992. Prothoracicotropic hormone regulates phosphorylation of a specific protein in the prothoracic glands of the tobacco hornworm, *Manduca sexta*. *Insect Biochem* 22:353–62
131. Roussel J-P. 1994. Synthetic molecules designed as potential inhibitors in ecdysone biosynthesis. *Entomol. Exp. Appl.* 71:193–99
132. Rybczynski R, Bell SC, Gilbert LI. 2001. Regulation of insect steroid hormone synthesis by a MAP kinase. *Mol. Cell. Endocrinol.* In press
133. Rybczynski R, Gilbert LI. 1994. Changes in general and specific protein synthesis that accompany ecdysteroid synthesis in stimulated prothoracic glands of *Manduca sexta*. *Insect Biochem. Mol. Biol.* 24: 175–89
134. Rybczynski R, Gilbert LI. 1995. Prothoracicotropic hormone-regulated expression of a hsp70 cognate protein in the insect prothoracic gland. *Mol. Cell. Endocrinol.* 115:73–85
135. Rybczynski R, Gilbert LI. 1995. Prothoracicotropic hormone elicits a rapid, developmentally specific synthesis of β tubulin in an insect endocrine gland. *Dev. Biol.* 169:15–28
136. Rybczynski R, Gilbert LI. 1998. Cloning of a $\beta 1$ tubulin from an insect endocrine gland: developmental and hormone-induced changes in mRNA expression. *Mol. Cell. Endocrinol.* 141:141–51
137. Rybczynski R, Gilbert LI. 2000. cDNA cloning and expression of a hormone-regulated heat shock protein (hsp70) from the prothoracic gland of *Manduca sexta*. *Insect Biochem. Mol. Biol.* 30:579–89
138. Sakurai S, Warren JT, Gilbert LI. 1989. Mediation of ecdysone synthesis in *Manduca sexta* by a hemolymph enzyme. *Arch. Insect Biochem. Physiol.* 10:179–97
139. Sakurai S, Warren JT, Gilbert LI. 1991. Ecdysteroid synthesis and molting by the tobacco hornworm, *Manduca sexta*, in the absence of prothoracic glands. *Arch. Insect Biochem. Physiol.* 18:13–36
140. Sakurai S, Williams CM. 1989. Short-loop negative and positive feedback on ecdysone secretion by prothoracic gland in the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol.* 75:204–16
141. Sakurai S, Yonemura N, Fujimoto Y, Hata F, Ikekawa N. 1986. 7-Dehydrosterols in prothoracic glands of the silkworm, *Bombyx mori*. *Experientia* 42: 1034–36
142. Sauman I, Reppert SM. 1996. Molecular characterization of prothoracicotropic hormone (PTTH) from the giant silkmoth *Antheraea pernyi*: developmental appearance of PTTH-expressing cells and relationship to circadian clock cells in central brain. *Dev. Biol.* 178:418–29
143. Schmidt-Rhaesa A, Bartolomaeus T, Lemburg C, Ehlers U, Garey JR. 1998. The position of the Arthropoda in the phylogenetic system. *J. Morphol.* 238:263–85
144. Schwab C, Hetru C. 1991. Synthesis and conversion study of a radiolabeled putative ecdysone precursor, 5β-cholest-7-ene-3β, 6α, 14α-triol in *Locusta migratoria* prothoracic glands. *Steroids* 56: 316–19
145. Sedlak BJ. 1985. Structure of endocrine glands. See Ref. 92a, 7:109–51
146. Sehnal F, Svacha P, Zrzavy J. 1996. Evolution of insect metamorphosis. See Ref. 56a, pp. 59–107
147. Shea MJ, King DL, Conboy MJ, Mariani BD, Kafatos FC. 1990. Proteins that bind to *Drosophila* chorion *cis*-regulatory elements: a new C2H2 zinc finger protein and a C2C2 steroid receptor-like component. *Genes Dev.* 4:1128–40
148. Shirai Y, Aizono Y, Iwasaki T, Yanagida A, Mori H, et al. 1993. Prothoracicotropic hormone is released five times in the 5th-larval instar of the silkworm, *Bombyx mori*. *J. Insect Physiol.* 39:83–88

149. Shirai Y, Iwasaki T, Matsubara F, Aizono Y. 1994. The carbachol-induced release of prothoracicotropic hormone from brain-corpus cardiacum-corpus allatum complex of the silkworm, *Bombyx mori*. *J. Insect Physiol.* 40:469–73
150. Short JD, Guo D-A, Svoboda JA, Nes D. 1996. Mechanistic and metabolic studies of sterol 24,25-double bond reduction in *Manduca sexta*. *Arch. Insect Biochem. Physiol.* 31:1–22
151. Simpson ER, Boyd GS. 1996. The cholesterol side-chain cleavage system of the adrenal cortex: a mixed function oxidase. *Biochem. Biophys. Res. Commun.* 24:10–17
152. Smith WA, Combest WL, Gilbert LI. 1986. Involvement of cyclic AMP-dependent protein kinase in prothoracicotropic hormone-stimulated ecdysone synthesis. *Mol. Cell. Endocrinol.* 47:25–33
153. Smith WA, Gilbert LI. 1986. Cellular regulation of ecdysone synthesis by the prothoracic glands of *Manduca sexta*. *Insect Biochem.* 16:143–47
154. Smith WA, Gilbert LI, Bollenbacher WE. 1984. The role of cyclic AMP in the regulation of ecdysone synthesis. *Mol. Cell. Endocrinol.* 37:285–94
155. Smith WA, Gilbert LI, Bollenbacher WE. 1985. Calcium-cyclic AMP interactions in prothoracicotropic hormone stimulation of ecdysone synthesis. *Mol. Cell. Endocrinol.* 39:71–78
156. Smith WA, Koundinya M, McAllister T, Brown A. 1997. Insulin receptor-like tyrosine kinase in the tobacco hornworm, *Manduca sexta*. *Arch. Insect Biochem. Physiol.* 30:579–89
157. Smith WA, Pasquarello TJ. 1989. Developmental changes in phosphodiesterase activity and hormonal response in the prothoracic glands of *Manduca sexta*. *Mol. Cell. Endocrinol.* 63:239–46
158. Smith WA, Varghese AH, Healy MS, Lou KJ. 1996. Cyclic AMP is a requisite messenger in the action of big PTTH in the prothoracic glands of pupal *Manduca sexta*. *Insect Biochem. Mol. Biol.* 26:161–70
159. Snyder MJ, Feyereisen R. 1993. A diazepam binding inhibitor (DBI) homolog from the tobacco hornworm, *Manduca sexta*. *Mol. Cell. Endocrinol.* 94:R1–4
160. Snyder MJ, Van Antwerpen R. 1998. Evidence for a diazepam-binding inhibitor (DBI) benzodiazepine receptor-like mechanism in ecdysteroidogenesis by the insect prothoracic gland. *Cell Tissue Res.* 294:161–68
161. Song Q, Alnemri ES, Litwack G, Gilbert LI. 1997. An immunophilin is a component of the insect ecdysone receptor (EcR) complex. *Insect Biochem. Mol. Biol.* 27:973–82
162. Song Q, Gilbert LI. 1994. S6 phosphorylation results from prothoracicotropic hormone stimulation of insect prothoracic glands: a role for S6 kinase. *Dev. Genet.* 15:332–38
163. Song Q, Gilbert LI. 1995. Multiple phosphorylation of ribosomal protein S6 and specific protein synthesis are required for prothoracicotropic hormone-stimulated ecdysteroid biosynthesis in the prothoracic glands of *Manduca sexta*. *Insect Biochem. Mol. Biol.* 25:591–602
164. Song Q, Gilbert LI. 1998. Alterations in Ultraspiracle (USP) content and phosphorylation state accompany feedback regulation of ecdysone synthesis in the insect prothoracic gland. *Insect Biochem. Mol. Biol.* 28:849–60
165. Stocco DM, Clark BJ. 1997. Regulation of the acute production of steroids in steroidogenic cells. *Endocrinol. Rev.* 17:221–44
166. Svoboda JA. 1999. Variability of metabolism and function of sterols in insects. *Crit. Rev. Biochem. Mol. Biol.* 34:49–57
167. Thompson MJ, Kaplanis JN, Robbins WE, Svoboda JA. 1973. Metabolism of steroids in insects. *Adv. Lipid Res.* 11: 219–65
168. Vafopoulou X, Steel CGH. 1996. The

insect neuropeptide prothoracicotropic hormone is released with a daily rhythm: re-evaluation of its role in development. *Proc. Natl. Acad. Sci. USA* 93:3368–72

169. Vafopoulou X, Steel CGH. 1997. Ecdysteroidogenic action of *Bombyx* prothoracicotropic hormone and bombyxin on the prothoracic glands of *Rhodnius prolixus in vitro*. *J. Insect Physiol.* 43:651–56
170. Valentine JW, Collins AG. 2000. The significance of moulting in Ecdysozoan evolution. *Evol. Dev.* 2:152–56
171. Vedeckis WV, Bollenbacher WE, Gilbert LI. 1976. Insect prothoracic glands: a role for cyclic AMP in the stimulation of ecdysone secretion. *Mol. Cell. Endocrinol.* 5:81–88
172. Warren JT, Bachman JS, Dai J-D, Gilbert LI. 1996. Differential incorporation of cholesterol and cholesterol derivatives by the larval ring glands and adult ovaries of *Drosophila melanogaster*: a putative explanation for the *l*(*3*)*ecd*^{1} mutation. *Insect Biochem. Mol. Biol.* 26:931–43
173. Warren JT, Dai JD, Gilbert LI. 1999. Can the insect nervous system synthesize ecdysteroids? *Insect Biochem. Mol. Biol.* 29:571–79
174. Warren JT, Gilbert LI. 1986. Ecdysone metabolism and distribution during the pupal-adult development of *Manduca sexta*. *Insect Biochem.* 16:65–82
175. Warren JT, Gilbert LI. 1996. Metabolism in vitro of cholesterol and 25-hydroxycholesterol by the larval prothoracic glands of *Manduca sexta*. *Insect Biochem. Mol. Biol.* 26:917–29
176. Warren JT, Hetru C. 1990. Ecdysone biosynthesis: pathways, enzymes and the early steps problem. *Invertebr. Reprod. Dev.* 18:91–99
177. Warren JT, Rybczynski R, Gilbert LI. 1995. Stereospecific, mechanism-based, suicide inhibition of a cytochrome P450 involved in ecdysteroid biosynthesis in the prothoracic glands of *Manduca sexta*. *Insect Biochem. Mol. Biol.* 25:679–95
178. Warren J, Sakurai S, Rountree DB, Gilbert LI. 1988. Synthesis and secretion in vitro of ecdysteroids by the prothoracic glands of *Manduca sexta*. *J. Insect Physiol.* 34:571–76
179. Warren JT, Sakurai S, Rountree DB, Lee S-S, Nakanishi K, Gilbert LI. 1988. Regulation of the ecdysteroid titer of *Manduca sexta*: reappraisal of the role of the prothoracic glands. *Proc. Natl. Acad. Sci. USA* 85:958–62
180. Warren JT, Wismar J, Subrahmanyam B, Gilbert LI. 2001. *Woc* (*without children*) gene control of ecdysone biosynthesis in *Drosophila melanogaster*. *Mol. Cell. Endocrinol.* 181:1–14
181. Watson RD, Ackerman-Morris S, Smith WA, Watson CJ, Bollenbacher WE. 1996. Involvement of microtubules in prothoracicotropic hormone-stimulated ecdysteroidogenesis by insect (*Manduca sexta*) prothoracic glands. *J. Exp. Zool.* 276:63–66
182. Wieschaus E, Nüsslein-Volhard C, Jürgens G. 1984. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. III. Zygotic loci on the X-chromosome and fourth chromosome. *Roux's Arch. Dev. Biol.* 193:296–307
183. Williams DR, Fisher MJ, Rees HH. 2000. Characterization of ecdysteroid 26-hydroxylase: an enzyme involved in molting hormone inactivation. *Arch. Biochem. Biophys.* 376:389–98
184. Winter H, Bilbe G, Richener H, Sehringer B, Kayser H. 1999. Cloning of a cDNA encoding a novel cytochrome P450 from the insect *Locusta migratoria*: CYP6H1, a putative ecdysone 20-hydroxylase. *Biochem. Biophys. Res. Commun.* 259:305–10
185. Wismar J, Habtemichael N, Warren JT, Dai JD, Gilbert LI, et al. 2000. The mutation *without children* (*woc*^{rgl}) causes ecdysteroid deficiency in the third instar of *Drosophila melanogaster*. *Dev. Biol.* 226:1–17
186. Yao T-P, Forman BM, Jiang Z, Cherbas

L, Chen J-D, et al. 1993. Functional ecdysone receptor is the product of EcR and *ultraspiracle* genes. *Nature* 366:476–79

187. Yochem J, Tuck S, Greenwald I, Han M. 1999. A gp330/megalin-related protein is required in the major epidermis of *Caenorhabditis elegans* for completion of molting. *Development* 126:597–606
188. Yoshida I, Moto K, Sakurai S, Iwami M. 1998. A novel member of the bombyxin gene family: structure and expression of bombyxin G1 gene, an insulin-related peptide gene of the silkmoth *Bombyx mori*. *Dev. Genes Evol.* 208:407–10

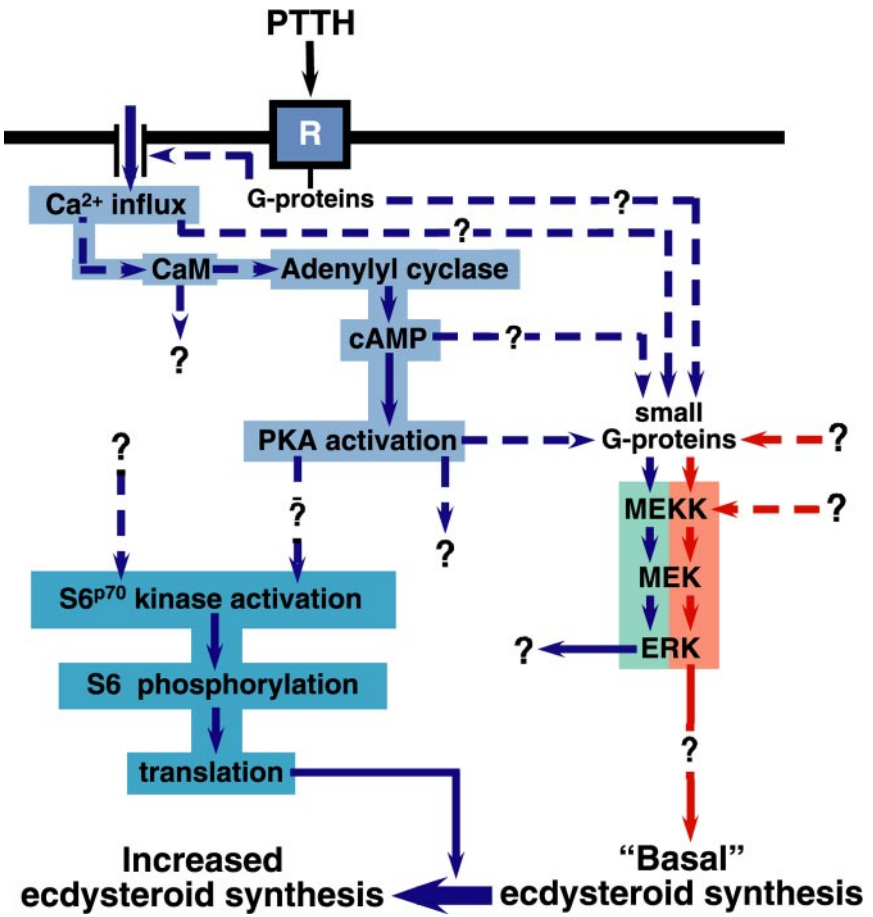
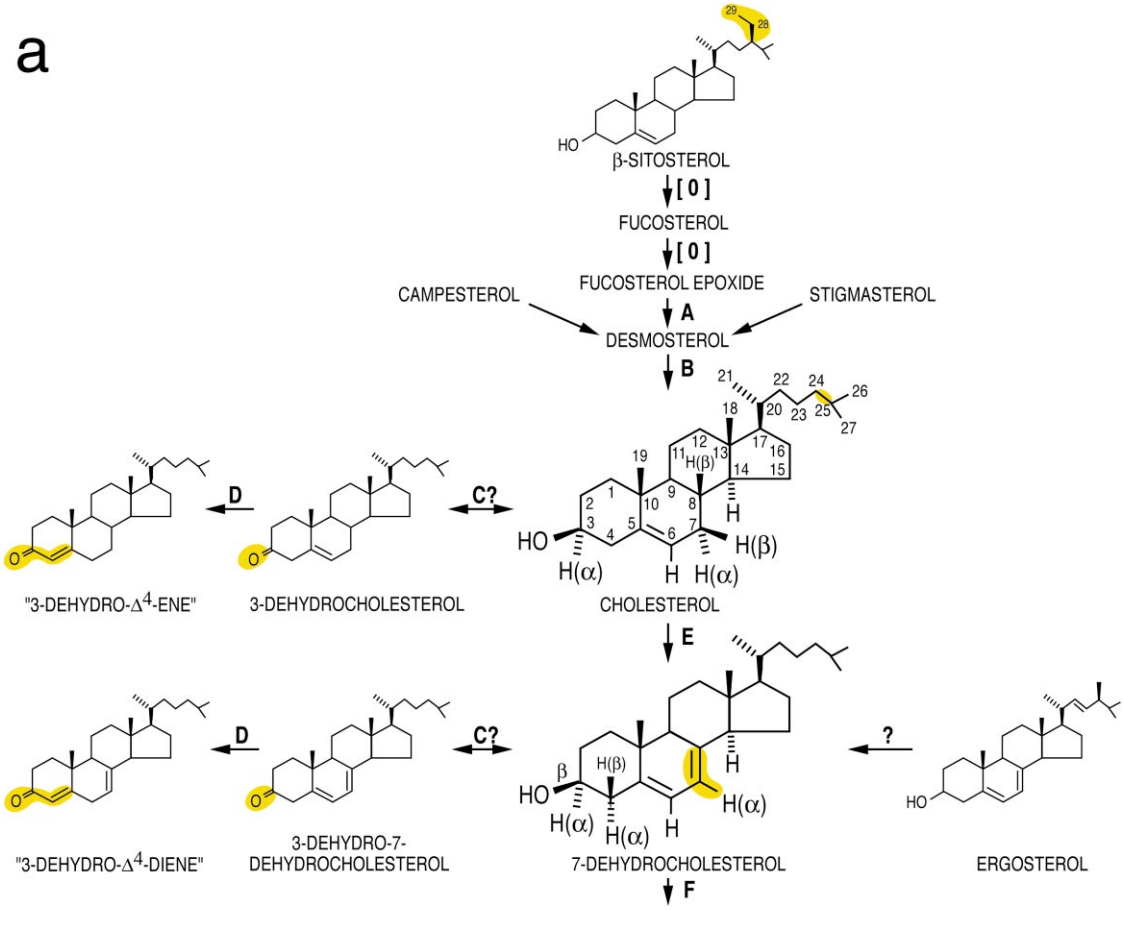


Figure 1 A model for PTTH signal transduction in prothoracic glands. Proteins and cellular processes highlighted by *blue* illustrate well-confirmed PTTH-dependent intracellular events. *Red* highlighting indicates the contribution of factors other than PTTH in the dynamic regulation of ecdysteroid synthesis. *Solid arrows* indicate characterized events, and *dashed arrows* indicate hypothetical relationships between signaling molecules. A *question mark* indicates the probable contribution of one or more unidentified molecules or events to a given pathway. R, PTTH receptor; CaM, calmodulin; PKA, protein kinase A; S6, ribosomal protein S6; $S6^{p70}$ kinase, 70-kDa S6 kinase; MEKK, MAP/ERK kinase kinase; MEK, MAP/ERK kinase; ERK, extracellular signal-regulated kinase.

a



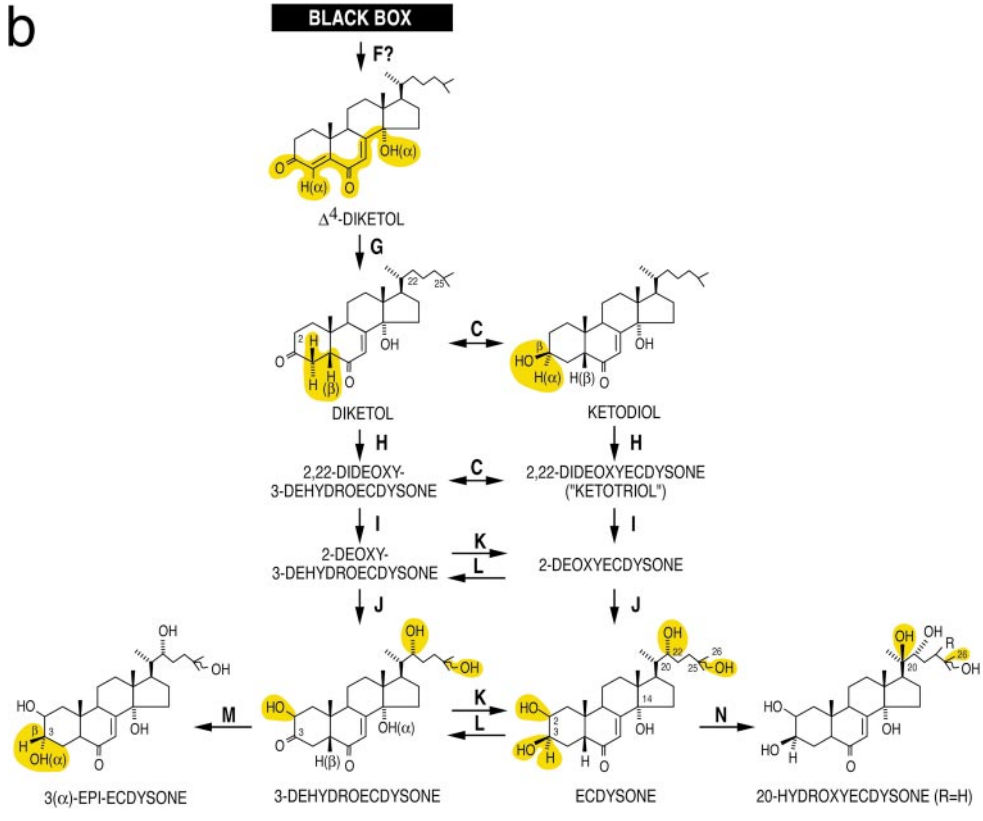


Figure 2 Scheme of ecdysone biosynthesis. *Yellow* highlights the molecular modifications referred to in the text. A *question mark* indicates reactions or processes that have not been completely characterized. (*A*) epoxide lyase; (*B*) desmosterol 24,25-reductase; (*C*) 3β-hydroxysterol dehydrogenase; (*D*) chemical isomerization; (*E*) cholesterol 7,8-dehydrogenase; (*F*) translocation; (*G*) Δ^4-diketol-5β(H)-reductase; (*H*) 25-hydroxylase; (*I*) 22-hydroxylase; (*J*) 2-hydroxylase; (*K*) 3β-ketoreductase; (*L*) ecdysone oxidase; (*M*) 3α-ketoreductase; (*N*) ecdysone-20-monooxygenase; (*O*) oxidation.

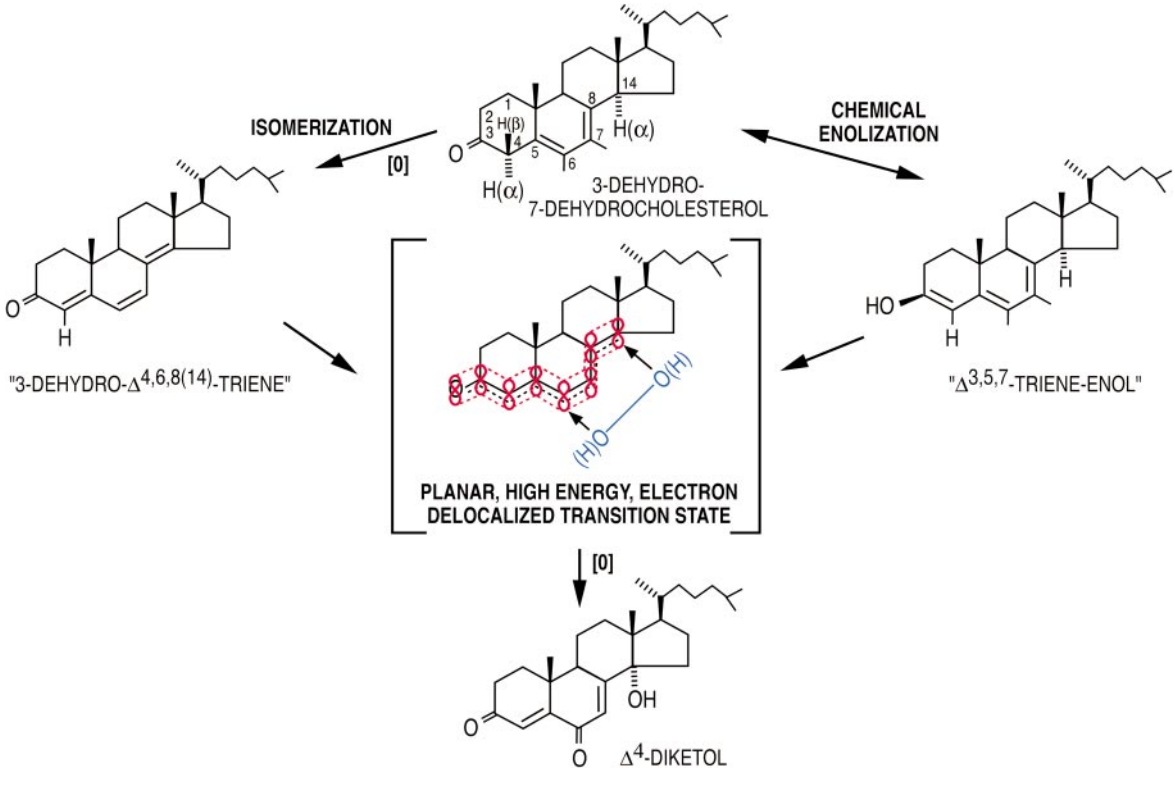


Figure 3 The "Black Box": hypothetical formation and oxidation of a transition state intermediate. Electron delocalization is shown in *red*, attacking oxygen species in *blue*.

Annual Review of Entomology
Volume 47, 2002

CONTENTS