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Synthesis and metabolism of vertebrate-type steroids by tissues of insects: A critical evaluation

L. Swevers*, J. G. D. Lambert[a] and A. De Loof

Zoological Institute, Catholic University of Leuven, Naamsestraat 59, B-3000 Leuven (Belgium), and [a] Experimental Zoology, State University of Utrecht, Transitorium III, Padualaan 8, NL-3584 Utrecht (The Netherlands)

Summary. This review covers the synthesis and the metabolism of vertebrate-type steroids (progesterone, testosterone, estradiol, corticosteroids) by insect tissues and discusses the significance of the reactions for insect physiology. Biosynthesis of vertebrate-type steroids from cholesterol hitherto has been demonstrated in only two insect species, i.e. the water beetle *Acilius sulcatus* (Coleoptera) and the tobacco hornworm *Manduca sexta* (Lepidoptera). In *Acilius*, steroid synthesis is associated with exosecretion (chemical defense). Nothing, however, is known about a physiological role of the C_{21} steroid conjugate present in ovaries and eggs of *Manduca*.

No synthesis of vertebrate-type steroids was observed in any other insect investigated to date. Most metabolic conversions of steroids by insects concerned oxidoreduction of oxygen groups (hydroxysteroid dehydrogenase activity) and (polar and apolar) conjugate formation. All important enzymatic steps involved in synthesis and catabolism, as known from studies with tissues of vertebrates, were not, or hardly observed.

The conclusion is drawn that typical vertebrate-type (C_{21}, C_{19} and C_{18}) steroids probably do not act as physiologically active substances in insects.

Key words. Vertebrate-type steroids; insects; metabolism; hormones.

Introduction

In recent years, it has become clear that the endocrine system of insects is much more complicated than previously assumed and, in addition, that it shares many common features with that of vertebrates[16,18,106]. Thus, several families of peptides have members in both vertebrates and insects[19,93]. Homology was also found regarding bio-active amines and prostaglandins[5,99]. The moulting hormone of insects, ecdysone, possesses a steroid nucleus and is synthesized from cholesterol, a situation similar to that of the steroid hormones in vertebrates[44]. Molecular analysis of genes involved in pattern formation during *Drosophila* development has led to the isolation of cDNAs which display considerable sequence homology to vertebrate growth factors[76].

A peculiar feature of the emerging picture is the discovery that the classical sex steroid hormones of higher vertebrates (progesterone, testosterone, estradiol) are present in insect tissue extracts[3,17,20,69,78]. The presence of steroids in members of a phylum phylogenetically so far removed from the vertebrates as arthropods is remarkable since it suggests that the 'vertebrate-type' steroid hormone system (including corticosteroids, progestogens, androgens and estrogens) is very old and may be shared by several or even all phyla of the animal kingdom. Such a suggestion was made by Sandor and Mehdi[89] and seemed to be confirmed recently when vertebrate-type steroids were identified in insects using specific and sensitive analytical techniques such as gas chromatography-mass spectrometry (GC-MS) and radioimmunoassay (RIA)[13,14,59,68,72,77,111]. In several species of other invertebrate phyla (coelenterates, molluscs, crustaceans, echinoderms), vertebrate-type steroids were also detected[39,50,74,92].

However, the presence of vertebrate-type steroids in tissue extracts of insects is not necessarily an indication of their physiological relevance. The steroids may even have no function at all, since they may be simply derived from a dietary source, at least in some cases[22]. Alternatively, it is possible that the identified steroids are but intermediates or side-products in an elaborate biosynthetic or catabolic pathway, while the active steroids remain to be identified. Application of the typical steroids of higher vertebrates indeed did not result in significant effects on insect physiology[6]. It is therefore of major importance to investigate the capacity of insects to convert vertebrate-type steroids. If no vertebrate-type steroid biosynthesis exists in insects, an endocrinological role for steroids in this class can be excluded.

The study of the metabolism of vertebrate-type steroids is an obvious and easy approach to the question of the significance of vertebrate-type steroids in insects. Two principal 'waves' of metabolic studies can be found in literature, one at the end of the 1960s and the beginning of the 1970s, which culminated in the review by Lehoux and Sandor[53], and a second one which started more recently when vertebrate-type steroids were identified in insect tissue extracts beyond doubt by GC-MS[15,20,70,101,102,103]. This paper reviews the synthesis and metabolism of vertebrate-type steroids by insect tissues and discusses its significance in insect physiology.

Survey of methods generally used

The metabolism of vertebrate-type steroids was studied in a very straightforward way, i.e. by administration of steroids to insects or insect tissues, followed, after an incubation period, by the identification of the metabolites.

In most studies, radioactive ([^{3}H] or [^{14}C]) labelled steroids were used as precursors[15]. All intermediates in the well-documented biosynthetic pathway in vertebrates are indeed commercially available in radioactive labelled form (cholesterol, pregnenolone, progesterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, dehydroepiandrosterone, androstenedione, testosterone, 5α-dihydrotestosterone, estrone and estradiol). The use of labelled substrates also results in great sensitivity. Conversions of very small amounts of steroids (to less than ng quantities) can easily be measured. These amounts may closely match the physiological concentrations of endogenous substrates; concentrations of vertebrate-type steroid-like immunoreactive substances, as measured by RIA, may reach ng per g tissue values in insects[68]. For conclusive identification[88], steroid metabolites underwent the following procedures: (1) comigration with added steroid carriers or steroid references on successive thin-layer-chromatography (TLC); (2) derivatization, followed by comigration with derivatized steroid standards during chromatography; (3) recrystallizations to constant specific activity with authentic unlabelled steroid.

In a few studies, large (unphysiological) amounts of steroids (μg to mg quantities) were added to insects or insect tissues[29,108]. Metabolites were identified by rigorous chemical and physical methods such as GC, MS and nuclear magnetic resonance (NMR).

Both in vitro and in vivo studies were carried out. Insect tissues for in vitro studies were prepared in two forms: as homogenates, in the presence of the appropriate cofactors (NADPH and NAD$^+$), and as minced tissue in culture medium. Most work has concentrated on insect gonads. From the viewpoint of comparative endocrinology, gonads indeed seem the most likely organs to demonstrate biosynthetic reactions. At first sight, insects do not possess an adrenal-like organ, which is the second important site of steroid synthesis in vertebrates. Moreover, reports of the presence of corticosteroids in the haemolymph of insects are less convincing, when compared with findings on sex steroids[53].

In most in vivo studies, vertebrate-type steroids were injected in the haemocoel of the insects. Metabolites were isolated from the whole body[15,23] or from specific organs[70]. In some studies, vertebrate-type steroids were fed to the insect[101,108].

What metabolic conversions can be expected?

The metabolites, which will be identified most easily, are of course the steroids which are also produced by tissues of vertebrates and for which standards are available. Besides such easily identifiable metabolites, it is probable that some other steroids are produced by insect tissues, which do not comigrate with any reference known from studies in vertebrates. The production of unknown steroids from an early percursor, however, is not necessarily an indication for the bioconversion of the precursor to more physiologically relevant steroids. The observed reactions may indeed be simply the results of a detoxification strategy. For instance, the production of polar products may result from the introduction of hydroxyl groups by polysubstrate cytochrome P-450 enzymes involved in the detoxification of xenobiotics[21]. To claim the presence of a vertebrate-type steroid system in insects, one should demonstrate the occurrence of the same set of steroid metabolic enzymes which is remarkably conserved throughout all classes of vertebrates. Eventually, when this set is indeed present, the production of unusual metabolites might be of physiological significance. For easy discussion purposes, the set of enzymes, characteristic for the vertebrate-type steroid system, is divided into four major groups.

Enzymes involved in steroid biosynthesis

The synthesis of androgens and estrogens from cholesterol requires the action of the following principal enzyme systems[46, 61]: (1) cholesterol side-chain cleavage (SCC); (2) 3β-hydroxysteroid dehydrogenase (HSD)/5-ene-4-ene isomerase; (3) 17α-hydroxylase; (4) $C_{17}-C_{20}$ lyase; (5) aromatase. Corticosterone is synthesized from progesterone by the action of 21-hydroxylase and 11β-hydroxylase. (Cortisol and aldosterone are not considered here since they are not common to all classes of vertebrates.)

Some of these enzymes (3β-HSD/isomerase, $C_{17}-C_{20}$ lyase, aromatase) are not only found in the gonads or adrenals, but occur also in many non-steroidogenic tissues, i.e. skin, brain, intestine, kidney and liver[1, 9, 36].

Enzymes involved in steroid activation or inactivation

A distinction can be made between steroids with the 3-keto-4-ene structure (progesterone, corticosterone, testosterone) and aromatic steroids (estrogens).

The reduction of the 4-ene bond to either 5α- or 5β-reduced steroids is the most important reaction to inactivate or (less frequently) activate 3-keto-4-ene steroids. 5α- and 5β-reductase activities result in irreversible reactions and are present in steroidogenic as well as non-steroidogenic tissues of all vertebrates[9, 28, 37, 47, 66].

In estrogen metabolism, hydroxylations are the most important reactions, for instance 16α-hydroxylation[45] and the formation of catecholestrogens (2-hydroxylation[107]).

Hydroxysteroid dehydrogenase enzymes

HSD enzymes are also involved in both the activation and inactivation of steroid hormones. In contrast with the second group of enzymatic conversions, however, the action of HSD enzymes does not result in an irreversible (in)activation. They simply catalyze the oxidoreduction of ketone/hydroxyl functions on different positions of the steroid molecule. These enzymes are named according to the positions of the hydroxyl groups which are formed or oxidized: 3α-HSD, 3β-HSD, 11β-HSD, 17β-HSD, 20α-HSD and 20β-HSD are the most frequently observed activities[36]. These enzymes occur in all tissues of vertebrates but cannot be regarded as key enzymes in steroid synthesis or catabolism. Moreover, it is known that many different dehydrogenases occur in animal tissues. It is, therefore, quite possible that some dehydrogenases specialized for one function also have the capacity to carry out the oxidoreduction of oxygen functions of steroids when these are added to the tissue in relatively high amounts. Thus, the occurrence of HSD activity in a given tissue may be the result of the action of an enzyme normally involved in another metabolic process. To have physiological relevance, the presence of HSD activity in an animal therefore should be 'supported' by the presence of more essential steroid converting enzymes (group 1 and 2).

Enzymes involved in conjugate formation

Steroids with sterically unhindered hydroxyl groups can be coupled to non-steroidal substrates such as glucose, glucuronic acid, sulphate and fatty acids (enzymes: glucosyltransferase, glucuronyltransferase, sulphotransferase and acyltransferase[45, 46, 94]). Steroid conjugate formation usually is involved in inactivation and excretion[36]. In fish, however, steroid glucuronides are physiologically active as sex pheromones[82]. As is the case with HSD activity, the demonstration of steroid conjugate formation alone is not conclusive for the significance of vertebrate-type steroids. Polar conjugates may result from the action of non-specific enzymes involved in detoxification[21] or other metabolic processes[49]. The synthesis of (vertebrate-type) steroid fatty acid esters is also generally considered as a non-physiological reaction, resulting from the addition of relatively high amounts of precursor steroid to the tissues[94].

Unknown enzymes

Some unknown enzymes may be involved in the synthesis of metabolites which are not commonly found in studies with vertebrates (hydroxylations at 'unorthodox' positions, double bond formation,...). These reactions may have physiological relevance only when the enzymatic reactions of both group 1 and 2 are demonstrated.

Metabolism of vertebrate-type steroids by insect tissues

A clear distinction has to be made between two groups of insects. First, some beetles (Coleoptera) possess defensive glands which contain high amounts of steroidal substances. This has been reported in four families of beetles, i.e. Dytiscidae (water beetles), Silphidae (carrion beetles), Chrysomelidae (chrysomelid beetles) and Lampyridae (fireflies). Beetles belonging to the latter two families produce steroidal defensive agents not related to the vertebrate-type steroids: cardenolides (Chrysomelidae[79]) and lucibufagins, a unique group of steroidal pyrones, which are related to the bufadienolid toad poisons (Lampyridae[34]). The steroids of the Dytiscidae, which are stored in the prothoracic defensive glands, on the other hand, include some well-known steroid hormones from vertebrates such as 11-deoxycorticosterone, testosterone and estradiol[90]. For Silphidae, a report has been published on the occurrence of 15β-hydroxyprogesterone and related 5β-pregnane derivatives in the rectal defensive gland of a single species, *Silpha americana*[60].

A second line of research documented that vertebrate-type steroids also occur in tissues and haemolymph of insects which do not possess a defensive gland system[69]. The concentrations that were found (pg/g to ng/g quantities as measured by RIA) were much lower than those found in the beetle defensive secretions (µg to mg/beetle quantities). The vertebrate-type steroids were identified in species of eight different orders, suggesting that the presence of steroids might be common to all insects[3, 13, 59, 69, 73, 98, 109].

The metabolism of vertebrate-type steroids by insects of both groups will be discussed separately. However, one case can be found in literature which is exceptional and hitherto could not be classified clearly in one of both groups. The tobacco hornworm *Manduca sexta* (Lepidoptera) possesses no distinct defensive glands. Nevertheless, high amounts (nearly 100 µg/g) of a vertebrate-type steroid conjugate, i.e. 20β-dihydropregnenolone glucoside, are found in ovaries and eggs of this species[104]. The case of *Manduca sexta* will therefore also be discussed separately.

Synthesis of steroids by water beetles

Up to date, biosynthesis of vertebrate-type steroids was demonstrated in one dytiscid species: *Acilius sulcatus*[10, 90]. Labelled precursor steroids were injected into the body cavity and the presence of metabolites in the prothoracic defensive fluid was investigated. Due to the fact that the experiments were carried out by in vivo injections, the site of production of steroids remains to be established. The possible involvement of associated microorganisms can not be discounted either[90].

In his experiments, Schildknecht[90] used four different precursors. [2-^{14}C]Mevalonate could not be incorporated into the defensive steroids, which is consistent with the widely held dogma that insects are unable to biosynthesize the steroid nucleus[100]. [4-^{14}C]Cholesterol, on the other hand, was converted to 11-deoxycorticosterone and 20α-dihydroprogesterone. Besides the typical steroids of vertebrates, strange derivatives, with an additional double bond at position 6, were also found (4,6-pregnadienes). Also [^{14}C]progesterone and [^{14}C]cholesta-4,6-dien-3-one were precursors of the defensive steroids. The conversion of progesterone to vertebrate-type steroids was much higher than to the unusual 4,6-pregnadienes, while the reverse situation was observed when injecting cholesta-4,6-dien-3-one. Schildknecht concludes that the introduction of the 4,6-diene group occurs preferentially at a very early stage in the biosynthetic pathway, before side-chain cleavage to C_{21} steroids begins[90].

Finally, the stereochemistry of double bond formation at positions 4 and 6 in the 4,6-pregnadienes was studied by Chapman et al.[10]. They concluded that the hydrogen atoms at positions 4β and 7β stereospecifically are removed during the introduction of the double bonds.

Metabolism of steroids by insects not possessing a defensive gland system

An overview of the observed activities is given in the table. In this table, we present both demonstrated and non-demonstrated activities (although the latter were not always explicitly stated in some papers). We will discuss the presence of enzymes according to the 5 major groups presented in the previous section.

(1) *Enzymes involved in steroid biosynthesis*

SCC. In in vitro studies using insect gonads, vertebrate-type steroid synthesis from cholesterol could not be demonstrated[23, 53, 101] (table). It was also not observed after injection of labelled cholesterol in last instar larvae of the fleshfly, *Sarcophaga bullata*[15]. However, the above-mentioned studies of cholesterol metabolism are insufficient to allow a conclusive statement about the absence of the vertebrate-type steroid system in insects. Gonads indeed may not be the (vertebrate-type) steroidogenic organs in insects. When labelled cholesterol is injected in the haemolymph, one can expect that only low amounts will reach a possible steroidogenic organ. These amounts may be too small to allow detection of steroid synthesis. Incubations with labelled cholesterol are also characterized by low conversion rates owing to the very large pool of endogenous unlabelled cholesterol in the cells (up to mg per g dry weight quantities in *Locusta migratoria*[12]). In addition, cholesterol does not function solely as a steroid hormone precursor, but may be converted according to multiple enzymatic pathways[15].

3β-HSD/isomerase. 3β-Hydroxy-5-ene steroids (pregnenolone, 17α-hydroxypregnenolone, dehydroepiandrosterone) can be converted to 3-keto-4-ene steroids by insect tissues, but conversion rates were always low (7% of the total activity at most[15, 51, 101]). While 3β-HSD/isomerase is an active enzyme in tissues of vertebrates, this seems not to be the case in insects.

In a remarkable report, Veith et al.[108] demonstrate 'reverse' 3β-HSD/isomerase activity (the conversion of 3-keto-4-ene steroids to 3β-hydroxy-5-ene steroids) when vertebrate-type steroids are fed to the honey bee. Such 'reverse' 3β-HSD/isomerase activity occurs but very rarely in vertebrates[35]. In the same report[108], other peculiar reactions were also found, such as 17α-hydroxylase

Overview of vertebrate steroid conversions by insect tissues (in vivo and in vitro). Indicated are both demonstrated and non-demonstrated activities. Comments on the data presented in this Table are given in the text. Abbreviations: (?) = Identification remains doubtful. (*) = Based on hydrolysis by enzyme preparations (β-glucuronidase and sulphatase) or on saponification of apolar compounds. (**) = The production of 17α-hydroxylated steroids was not involved in androgen synthesis in this study. Instead, it seemed that 17α-hydroxylated steroids were further metabolized according to insignificant pathways. (***) = Precursor steroids were fed to the insect. The involvement of associated micro-organisms in the observed reactions can not be discounted. Moreover, the observed activities seem not to be involved in an active steroid synthesis. (****) = This activity was observed only in Malpighian tubules.

Insect species	Tissue	Precursor	Enzymes present	Enzymes absent	Ref.
Orthoptera					
Gryllus assimilis (common cricket)	Minced tissue in vitro testis, leg muscle	Pregnenolone	3β-HSD/isomerase	17α-hydroxylase	51
	Tissue homogenate testis, leg muscle	Progesterone	20α-HSD 20β-HSD	17α-hydroxylase 5α-reductase 5β-reductase 11β-hydroxylase 21-hydroxylase	54
Gryllus domesticus (house cricket)	Tissue homogenate testis intestine fat body cuticle leg muscle	Cholesterol Androstenedione Testosterone 5α-dihydrotestosterone 5β-dihydrotestosterone 11-ketotestosterone Estradiol	17β-HSD	SCC Aromatase 5α-reductase 5β-reductase 2-hydroxylase 16α-hydroxylase	52 53
Schistocerca gregaria (desert locust)	(1) From whole body after injection in adult females (2) Tissue homogenate: ovary, testis, intestine, muscle, fat body	Cholesterol Progesterone 20α-dihydroprogesterone 20β-dihydroprogesterone 17α-hydroxyprogesterone Dehydroepiandrosterone Androstenedione Testosterone Estrone Estradiol	20α-HSD 20β-HSD 17β-HSD	SCC 3β-HSD/isomerase 17α-hydroxylase $C_{17}-C_{20}$ lyase Aromatase 5α-reductase 5β-reductase 11β-hydroxylase 21-hydroxylase 2-hydroxylase 16α-hydroxylase	23 24
Locusta migratoria (African migratory locust)	(1) Tissue homogenate testis, ovary (2) Minced tissue in vitro integument, fat body, flight muscle, heart, brain, intestine, Malpighian tubules, male accessory glands, spermatheca/ovipositor, testis, ovary (3) from whole body, intestine and faeces after injection or feeding	Cholesterol Pregnenolone Progesterone 17α-hydroxypregnenolone 17α-hydroxyprogesterone Dehydroepiandrosterone Androstenedione Testosterone Estradiol	3β-HSD/isomerase $C_{17}-C_{20}$ lyase 17β-HSD 20α-HSD 20β-HSD Glucuronyltransferase (*) Sulphotransferase (*) Acyltransferase (*) 21-hydroxylase (****)	SCC 17α-hydroxylase Aromatase 5α-reductase 5β-reductase 11β-hydroxylase 2-hydroxylase 16α-hydroxylase	101
Dictyoptera					
Byrsotria fumigata (cockroach)	Minced tissue in vitro testis	Pregnenolone	3β-HSD/isomerase 20β-HSD	17α-hydroxylase	51
		Pregnenolone/progesterone [^{3}H] [^{14}C]	17α-hydroxylase (?) $C_{17}-C_{20}$ lyase (?)	11β-hydroxylase 21-hydroxylase 5α-reductase 5β-reductase	53
Gromphadorina portentosa (hissing cockroach)	Minced tissue in vitro testis	Pregnenolone	3β-HSD/isomerase	17α-hydroxylase	51
	Tissue homogenate testis	Pregnenolone/progesterone [^{3}H] [^{14}C]	17α-hydroxylase (?) $C_{17}-C_{20}$ lyase (?)	11β-hydroxylase 21-hydroxylase	53
	leg muscle	Progesterone	20α-HSD 20β-HSD	17α-hydroxylase 5α-reductase 5β-reductase 11β-hydroxylase 21-hydroxylase	54
Diptera					
Sarcophaga bullata (fleshfly)	From whole body after injection in last instar larvae	Cholesterol Pregnenolone Progesterone 17α-hydroxypregnenolone 17α-hydroxyprogesterone Dehydroepiandrosterone Androstenedione Testosterone	3β-HSD/isomerase 17α-hydroxylase (**) $C_{17}-C_{20}$ lyase 17β-HSD 20α-HSD 20β-HSD Glucuronyltransferase (*) Sulphotransferase (*)	SCC Aromatase 5α-reductase 5β-reductase 11β-hydroxylase 21-hydroxylase	15

Insect species	Tissue	Precursor	Enzymes present	Enzymes absent	Ref.
	Minced tissue in vitro gonads, 8 non-gonadal tissues	Testosterone Androstenedione	Aromatase (?)	5α-reductase 5β-reductase	20
	From whole body after injection in pharate adults	Androstenedione	17β-HSD Glucuronyltransferase (*) Sulphotransferase (*)	Aromatase 5α-reductase 5β-reductase	102
Lepidoptera					
Bombyx mori (silkworm)	(1) From ovaries and fat body after injection (2) Minced tissues in vitro testis, ovary	Testosterone Estrone Estradiol	17β-HSD α-glucosyltransferase β-glucosyltransferase	Aromatase 5α-reductase 5β-reductase 2-hydroxylase 16α-hydroxylase	70 29
Galleria mellonella (wax moth)	(1) Tissue homogenate whole body (adults) (2) Minced tissue in vitro prothoracic glands (larvae)	Pregnenolone 17α-hydroxyprogesterone Androstenedione	3β-HSD/isomerase $C_{17}-C_{20}$ lyase 17β-HSD 20α-HSD 20β-HSD Glucuronyltransferase (*) Sulphotransferase (*) Acyltransferase (*)	17α-hydroxylase Aromatase 5α-reductase 5β-reductase 11β-hydroxylase 21-hydroxylase	101
Hymenoptera					
Apis mellifera (honey bee)	From whole body after feeding	Progesterone 17α-hydroxyprogesterone	3β-HSD/isomerase 17α-hydroxylase (***) $C_{17}-C_{20}$ lyase 5α-reductase (***) 20β-HSD	Aromatase 5β-reductase 11β-hydroxylase 21-hydroxylase	107
Coleoptera					
Leptinotarsa decemlineata (Colorado potato beetle)	From whole body after injection	17α-hydroxyprogesterone	20α-HSD 20β-HSD Glucuronyltransferase (*) Sulphotransferase (*)	$C_{17}-C_{20}$ lyase 5α-reductase 5β-reductase 11β-hydroxylase 21-hydroxylase	102

and 5α-reductase. However, because the steroids were fed to the insect, it is not demonstrated yet that the insect itself is capable of carrying out these reactions. According to the authors, the action of micro-organisms could be discounted, because essentially the same results were obtained after co-feeding with antibiotics. Unfortunately, however, they have not checked the viability of the micro-organisms in the digestive tract before and after uptake of antibiotics. In our opinion, it seems more plausible that the reactions observed after feeding result from the action of symbiotic micro-organisms, when the reactions in isolated tissues from other insects (see further below; table) are taken into account. It is a well-known fact that micro-organisms are capable of converting steroids in many ways, e.g. hydroxylations at various positions, reduction of ketone groups, reduction of double bonds, isomerizations and side-chain degradation[57]. Finally, in our opinion, an active steroid synthesis is not demonstrated in the experiments of Veith et al.[108]: upon feeding progesterone to honey bees, 17α-hydroxylated steroids, but not androgens, were demonstrated. Synthesis of androgens was observed only after feeding large amounts (500 mg) of the intermediate steroid 17α-hydroxyprogesterone. The conversion of progesterone to androgens does not seem to be an efficient pathway.

17α-hydroxylase. In our experiments with different tissues of *Locusta migratoria* and the wax moth *Galleria mellonella*, special interest was given to the production of 17α-hydroxylated steroids from pregnenolone or progesterone[101]. No conclusive evidence for 17α-hydroxylase activity was obtained in any experiment. Neither was 17α-hydroxylase activity reported in most other metabolic studies using pregnenolone or progesterone as precursor (table). In the review of Lehoux and Sandor[53], preliminary evidence was reported for the formation of androgens from pregnenolone and progesterone (= joint 17α-hydroxylase and $C_{17}-C_{20}$ lyase activities) in incubations of minced testicular tissue of the cockroaches *Byrsotria fumigata* and *Gromphadorina portentosa*. The yield of these metabolites, however, was extremely low, so that unequivocal evidence for their identity could not be obtained. Thus, the presence of 17α-hydroxylase in the testes of cockroaches remains uncertain.

After injection of $[^3H]$progesterone into *Sarcophaga bullata* larvae, De Clerck et al.[15] reported the metabolism into compounds which were identified as 17α-hydroxyprogesterone and testosterone by chromatographic methods. Only low yields (0.2 and 0.9%, respectively) were obtained. A definite identification by recrystallizations was not achieved. Moreover, in similar injection experiments, metabolism of [^{3}H]pregnenolone to 17α-hydroxypregnenolone, progesterone, 17α-hydroxyprogesterone or testosterone was not observed. Fleshfly larvae were capable of converting pregnenolone to 17α-hydroxy-20β-dihydropregnenolone and three unidentified compounds. Although 17α-hydroxylase activity was demonstrated, the metabolite was only further converted according to insignificant pathways.

C_{17}–C_{20} lyase. In some insects, low levels (1–5% of the total activity) of C_{17}–C_{20} lyase were observed (*Sarcophaga bullata, Locusta migratoria* and *Galleria mellonella*: table). In all three species 17α-hydroxyprogesterone appeared to be a much more efficient precursor than 17α-hydroxypregnenolone. In other insects, on the other hand, C_{17}–C_{20} lyase activity seems to be absent (*Schistocerca gregaria, Gryllus assimilis, Leptinotarsa decemlineata*).

Aromatase. A tentative demonstration of aromatase activity in gonadal and non-gonadal tissues of *Sarcophaga crassipalpis* was reported by Denlinger et al.[20]. In these experiments, a conclusive identification was not achieved. Only HPLC using one system was used to identify testosterone metabolites. Moreover, their method used for quantification of aromatase activity in tissues seems erroneous. We demonstrated that the amount of water soluble radioactivity formed in the incubations with [2β-3H]androstenedione probably does not correspond to the loss of water during the aromatization process, but to the formation of water soluble testosterone conjugates[102].

Aromatase activity was not detected in any other study of the metabolism of androgens (table).

11β-Hydroxylase and 21-hydroxylase. When Malpighian tubules of *Locusta* were incubated in the presence of pregnenolone, several polar compounds were produced[101]. Two (minor) compounds were identified as 21-hydroxypregnenolone and 11-deoxycorticosterone, indicating the presence of 21-hydroxylase activity. It was clear, however, that the 21-hydroxylase enzyme of Malpighian tubules was not involved in active corticosteroid synthesis: (1) the metabolism to 21-hydroxylated steroids occurred at a low rate (5% of the total activity); (2) further synthesis of corticosterone or cortisol was not detected, indicating the absence of both 11β-hydroxylase and 17α-hydroxylase enzymes; (3) Malpighian tubules were not capable of synthesizing steroids from cholesterol. In our opinion, the synthesis of 21-hydroxylated steroids from pregnenolone may result from the action of broad specificity cytochrome P-450 detoxification enzymes. High concentrations of cytochrome P-450 are indeed present in the Malpighian tubules of *Locusta*[26].

(2) *Enzymes involved in steroid activation or inactivation*

5α-reductase and 5β-reductase.

Reduction of the double bond at position 4 could not be detected in any in vitro or in vivo study when steroids were injected (table). 5α-Reductase was demonstrated in feeding experiments with the honey bee[108], but in this case the involvement of associated micro-organisms can not be excluded (see above).

Metabolism of estrogens. Only 17β-HSD activity was observed. Unlike vertebrates, insects are not capable of converting estrone or estradiol to estriol (16α-hydroxylase) or catecholestrogens (2-hydroxylase) (table).

(3) *Hydroxysteroid dehydrogenase enzymes*

High activities of HSD enzymes are found in insect tissues (up to more than 90% of the total activity[101]). Androgens and progestogens underwent oxidoreduction of the ketone/hydroxyl function at position 17 (17β-HSD) and at position 20 (20α-HSD and 20β-HSD) respectively (table). In some insects, principally 20α-HSD activity is found (*Gryllus assimilis, Schistocerca gregaria, Locusta migratoria, Galleria mellonella*), while in others 20β-HSD dominates (*Byrsotria fumigata, Gromphadorina portentosa, Sarcophaga bullata, Apis mellifera*). In *Leptinotarsa decemlineata*, different 20-reduced progestogens are produced according to different developmental stages[102].

Kinetics were determined of two insect HSD enzymes: the 17β-HSD of the testis of *Gryllus domesticus* (substrate: testosterone[52]) and the 20α-HSD of *Galleria mellonella* last instar larvae (substrate: 17α-hydroxyprogesterone[103]). Both are soluble enzymes and use preferentially NADP$^+$/NADPH as cofactor. Michaelis-Menten constants (Km), as determined by Lineweaver-Burk plots, were 0.19 µM for the 17β-HSD enzyme and 4.9 µM for the 20α-HSD. These values are comparable to those obtained from studies with mammalian tissues[8, 55, 64, 97].

Attempts have also been made to demonstrate HSD activities histochemically in insect cells. The enzyme 3β-HSD was demonstrated in the neck cells of the testicular tubes of the cockroaches *Byrsotria fumigata* and *Gromphadorina portentosa* by the diaphorase-tetrazolium test[56]. In this report, a weak reaction was also observed in the absence of the substrates. In *Locusta migratoria*, clear 17β-HSD activity was observed in the ovarian follicle cells[67].

(4) *Enzymes involved in conjugate formation*

Water soluble metabolites were produced by tissues of several insects (table). When these metabolites were treated with β-glucuronidase or sulphatase preparations, free steroids could be extracted with an organic solvent, indicating the production of polar conjugates[15, 101–103]. In our metabolic studies with locust gonads[101], evidence was obtained for the production of two types of polar conjugates: (1) conjugates that were hydrolyzed by β-glucuronidase of *Escherichia coli* ('glucuronides'), and (2) conjugates that were hydrolyzed by sulphatase of *Helix pomatia* ('sulphatase'). 'Glucuronides' and 'sulphates' could be demonstrated for all metabolites, possessing an unhindered hydroxyl group. The use of enzymatic preparations, however, is not fully conclusive regarding the nature of the conjugate. Such enzyme preparations are capable of hydrolyzing several different types of conjugates[4]. In one study only, unequivocal identification by physicochemical methods was carried out: polar conjugates of estradiol were identified by NMR as (α- and β-)glucosides[29].

In our incubations of *Locusta* and *Galleria* tissues, the synthesis of apolar conjugates of pregnenolone and testosterone was demonstrated. These apolar conjugates may be fatty acid-esters or acetates. High levels of 'acyltransferase' activity were observed in testes of *Locusta*, six weeks after adult moulting (nearing the end of the life span of the locusts).

(5) *Unknown enzymes*

Most reports in literature concern the demonstration in insect tissues of enzyme systems which are also present in vertebrates. Few studies have paid attention to the possible synthesis of unknown steroids[15, 101].

Generally, only low amounts of such products could be detected (at most 10% of the total activity). The great majority of metabolites (usually reduced steroids and conjugates) could be identified in most studies, whatever approach was used (in vitro incubation, injection or feeding). In our incubation studies with locust tissues, two exceptions were found: (1) the production of high amounts of a very polar compound from progesterone by immature ovaries, and (2) the synthesis of several unknown polar compounds by Malpighian tubules (precursors: pregnenolone and androstenedione).

Synthesis of 20β-dihydropregnenolone glucoside by Manduca sexta

When [^{14}C]cholesterol was injected into female pupae of *Manduca sexta*, 5-[^{14}C]pregnen-3β,20β-diol glucoside was isolated from ovaries and eggs[105]. Apart from the water beetle *Acilius sulcatus*[90], this is the only report in which synthesis of C_{21} steroids from cholesterol is described. It automatically leads to the question: what is the significance of the synthesis of this compound? Two suggestions were made for answering this question[105].

(1) The physiological function of the compound is exogenous and involved in defense against predators, a situation comparable to that of the water beetles. The amounts of the steroid conjugate in the eggs seem indeed to be sufficiently high (nearly 100 µg/g) for such a role: it has been demonstrated that µg quantities of vertebrate-type steroids are effective as feeding deterrents[60, 62].

(2) 20β-Dihydropregnenolone glucoside is stored in the eggs and serves as a precursor of other steroid hormones during embryogenesis. It is generally assumed that ecdysteroid conjugates serve as hormone precursors during embryonic development of insects[40]. To establish such function, one would have to study the metabolic fate during egg development.

Another intriguing question is whether SCC of cholesterol by *Manduca* is carried out according to the same mechanism as observed in steroidogenic tissues of vertebrates. For instance, would pregnenolone be the primary SCC product? Finally we demonstrated the synthesis of 20β-dihydropregnenolone (both as free steroid and as conjugate) by pupae of *Manduca*, but we did not detect any trace of pregnenolone (Swevers, unpublished results).

Conclusion

A synthesis of vertebrate-type steroids from cholesterol hitherto could be demonstrated in only two insect species. The huge amounts of steroids in the prothoracic glands of *Acilius sulcatus* are involved in defense against vertebrate predators[91]. No evidence was obtained for additional physiological roles of vertebrate-type steroids in the water beetle.

In *Manduca sexta*, on the other hand, nothing is known about a physiological role of the C21 steroid conjugate present in ovaries and eggs[105]. The considerable amounts of steroid conjugate, however, present the idea that they have a function in chemical defense. Moreover, when the metabolism of vertebrate-type steroids by tissues of other Lepidoptera and other insects is taken into account (table), it seems not likely that the synthesis of 20β-dihydropregnenolone is involved in the production of hormones. If the latter would be the case, why then, one could argue, is the vertebrate-type steroid hormone system present in *Manduca* but not in other Lepidoptera (*Bombyx* and *Galleria*; table)? On the other hand, it seems perfectly possible that *Manduca sexta* has managed to carry out SCC of cholesterol as a secondary adaptation to produce effective chemical deterrents.

Besides *Acilius* and *Manduca*, a synthesis of vertebrate-type steroids was not observed in any other insect hitherto investigated (table). Based on the data of the metabolic studies, it is clear that insects lack the capacity to convert vertebrate-type steroids in significant ways. Most metabolic conversions concerned oxidoreduction of oxygen groups (HSD activity) and (polar and apolar) conjugate formation. Several enzymatic steps involved in synthesis and (irreversible) catabolism were not, or hardly, observed (3β-HSD/isomerase, 17α-hydroxylase, C_{17}–C_{20} lyase, aromatase, 11β-hydroxylase, 21-hydroxylase, 5α-reductase, 5β-reductase).

The results from the metabolic studies agree with the lack of convincing reports in which effects of vertebrate-type steroids on the physiology of insects are described (with one exception, see below). For a review of possible effects of vertebrate-type steroids on insect physiology, we refer to Brueggemeier et al.[6]. In summary:

(1) Some reported effects are in conflict with effects found by other investigators or in other species[31, 43, 63].

(2) Some effects could be elicited by several different steroids or even by non-steroidal substances[7, 32]. This indicates a non-specific response of insect tissues to vertebrate-type steroids.

(3) All observed effects were elicited by much higher doses than the actual concentrations in insect tissues[71, 77].

(4) Many reports were involving rather small sample sizes, indicating that more research is needed to confirm earlier results[6].

(5) In several cases, no effects at all were observed[20, 72, 101].

There exists only one example in the literature of an insect that relies on vertebrate-type steroids to coordinate physiological processes. Vertebrate-type hormones (i.a. steroids) regulate the reproductive cycle of the European rabbit flea, *Spillopsyllus cuniculi*[84-86]. This case, however, is exceptional, since it has probably evolved as an adaptation to the parasitic lifestyle of the insect. There is no evidence that the vertebrate-type hormones also act as hormones in the rabbit flea. It seems more likely that the steroids merely act as signals to activate the flea's own endocrine system[87].

An important question that remains concerns, of course, the origin of vertebrate-type steroids in tissue extracts of common insects[69]. In our opinion, three possibilities remain.

(1) Vertebrate-type steroids are synthesized according to alternative pathways, different from the classical cholesterol and pregnenolone dependent scheme. To date, no evidence has been obtained for this hypothesis. It would seem strange, indeed, if insects were producing the same hormones but according to totally different biosynthetic pathways. Ecdysone, the insect steroid moulting hormone, is also quite distinct from the 'classical' vertebrate-type steroid hormones, suggesting that major differences exist between insects and vertebrates concerning synthesis and metabolism of steroids. Nevertheless, the ecdysteroid system of insects and the vertebrate-type steroid system probably have a common origin[44]. In our opinion, the further exploration of the ecdysone biosynthetic pathway may prove a more successful approach in the future. By using an early precursor of ecdysteroid synthesis (for instance, 7-dehydrocholesterol[110]), incubations of different insect tissues could lead to the identification of tissue- and stage-specific metabolites. Possibly, a hormonal function for some of these metabolites may then be found. Such experiments may result in the elucidation of (a) bifurcation(s) in the biosynthetic pathway of ecdysteroids, similar to the bifurcation in steroidogenesis of vertebrates (corticosteroids versus sex steroids).

(2) The steroids are simply derived from the dietary source of the insects. While this seems evident for insects feeding on vertebrate food sources (for instance, the fleshfly feeds on bovine liver[42]), it also cannot be excluded for phytophagous insects: vertebrate-type steroids appear to be present in plants as well[2, 30, 33, 38, 81]. However, it seems that steroids (ecdysteroids but possibly also vertebrate-type steroids) are not readily taken up from the gut of insects[27]. Therefore, the dynamics of uptake of vertebrate-type steroids from the food source and the accumulation in insect tissues has to be carefully examined.

(3) The concentrations of vertebrate-type steroids in insect tissues are very low. Steroid concentrations and their fluctuations during insect development were measured by the sensitive and practical technique of RIA[14, 68, 77, 104, 111]. We have to review these results critically, when the findings of the metabolic studies are taken into account. With RIA, there is indeed always the possibility of non-specific detection due to cross-reacting substances[11]. On the other hand, the more specific (but less sensitive) technique of GC-MS was never used to measure steroid concentrations. Although it can not be denied that small amounts of vertebrate-type steroids were identified by GC-MS in insect tissues, the fluctuations of steroid titres during development, as measured by RIA, may be largely the result of variations of the concentrations of molecules, other than the supposed ones. In our opinion, it could very well be that the actual concentrations of vertebrate-type steroids are much lower than previously stated and, therefore, may be too low to have a physiological significance. Such findings would much better fit with the results of the metabolic studies, which suggest the absence of a vertebrate-type steroid hormone system in insects.

Although results illustrating homologies between the insect and vertebrate peptidergic hormone system are now accumulating rapidly[19], harvest of similar data concerning the steroid hormone systems hitherto is relatively poor. Nevertheless, there are indications that several hormones with the action of steroid hormones remain to be discovered in insects. Recently, DNA sequences encoding steroid hormone receptor-like proteins were isolated from *Drosophila melanogaster*[58, 65, 75, 83, 96]. It is suggested that the gene-regulating properties of such DNA-binding proteins are induced or altered after binding of ligands or hormones[95]. One of the ligands was indeed identified as ecdysone[48], while juvenile hormone (JH) also seems a likely candidate. The identification of other ligands may lead to the elucidation of novel hormone systems (steroids, terpenoids?) in insects[25]. Besides ecdysone and JH, the chemical nature of other possible 'steroid-like acting' hormones in insects remains a matter of speculation, despite all efforts undertaken to date.

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* Present address: c/o Prof. K. Iatrou, University of Calgary, Department of Medical Biochemistry, 3330 Hospital Drive N.W., Calgary, Alberta T2N 4N1, Canada.

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Research Articles

A lens-like specialization for photic input in the pineal window of an Indian catfish, *Heteropneustes fossilis*

S. Srivastava and C. B. L. Srivastava[1]

Zoology Department, University of Allahabad, Allahabad (India)

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Summary. An unusual lens-like structure is reported in the pineal window of the Indian nocturnal catfish *Heteropneustes fossilis*. This is the first report of its kind for the pineal window of fishes. This structure, coupled with a pineal fossa and a pineal window, forms a specialization that apparently serves to concentrate the photic input to the intracranially situated pineal organ. This structure may play a significant role in the photoneuroendocrine function of the photosensitive pineal under conditions of low light intensity, controlling the fish's circadian rhythmic activities.

Key words. Pineal window; lens-like tissue; pineal photosensitivity; catfishes.

Vertebrate photoreceptive systems operating through extraretinal[2] (extraocular[3]) photosensory inputs include the superficial dermal receptors, the epithalamic pineal sense organs, and the deep diencephalic photoreceptors. While the eyes serve primarily for visual function, pineal sense organs control photoneuroendocrine events[4], for which they are also endowed with photoreceptor cells and photopigments resembling those found in the retina. The vertebrate photoreceptive systems control circadian and reproductive rhythmic activities. As far as the pineal is concerned, light has an indirect effect, producing inhibition of melatonin synthesis. In tetrapods, light can reach the pineal (situated superficially, though intracranially), by penetrating through the skin and the skull, though its intensity may be attenuated while passing through these tissues. This is also the case in most fishes which lack a pineal foramen. As the amount of light reaching the pineal is dependent, on the one hand, on the nature of the tissues overlying it[5] and, on the other hand, on the light conditions of the species' habitat[6], fishes