Ecdysteroid Biosynthesis and its Inhibition

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Doctor of Philosophy

by

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Chapter 1

General Introduction

1.1. Hormonal control of insect moulting

The process of moulting and metamorphosis in insects involves the interplay of three major categories of hormones : prothoracicotropic hormone (PTTH), juvenile hormones (JH) and ecdysteroids (Fig. 1.1).

1.1.1. Prothoracicotropic hormone (PTTH)

The neuropeptide PTTH, is released during specific intervals (gates) in the daily photoperiodic cycle (Truman, 1972), in response to both environmental (photoperiod and temperature) and developmental stimuli (JH) (Bollenbacher *et al.*, 1987). PTTH is produced by the neurosecretory cells of the mid-dorsal and/or lateral regions of the insect brain, being transferred to the corpora allata for release (Agui *et al.*, 1979; Mizoguchi *et al.*, 1987). Recently the hindgut of certain Lepidopteran insects has also been shown to be a site of PTTH synthesis (Gelman *et al.*, 1993). PTTH acts upon competent prothoracic glands during various developmental stages of the insect eliciting an increase in ecdysteroid synthesis in the gland (Bollenbacher, 1988).

The structure of PTTH has been investigated primarily in two insect species, *Manduca sexta* and *Bombyx mori* , where it has been found to exist in multiple forms, which based on their molecular weight fall into two main categories: big PTTH (22-29K) and small PTTH (4-7K). The two forms of PTTH in *M. sexta* have been shown to have different developmental stage actions, with the small PTTH having greater activity in larval prothoracic glands than pupal glands, in contrast to the big PTTH which elicits equal glandular activity at both developmental stages (Bollenbacher *et al.*, 1984). The big PTTH present in the brain and developing embryos of *B. mori* appears to serve as the true prothoracicotropic hormone in this species, whereas the small PTTH (bombyxin) has no known function in this species, although it has a prothoracicotropic effect in *Samia cynthia* (Ishizaki *et al* ., 1983; Chen *et al* ., 1987; Fugo *et al.*, 1987 Ishizaki., 1989).

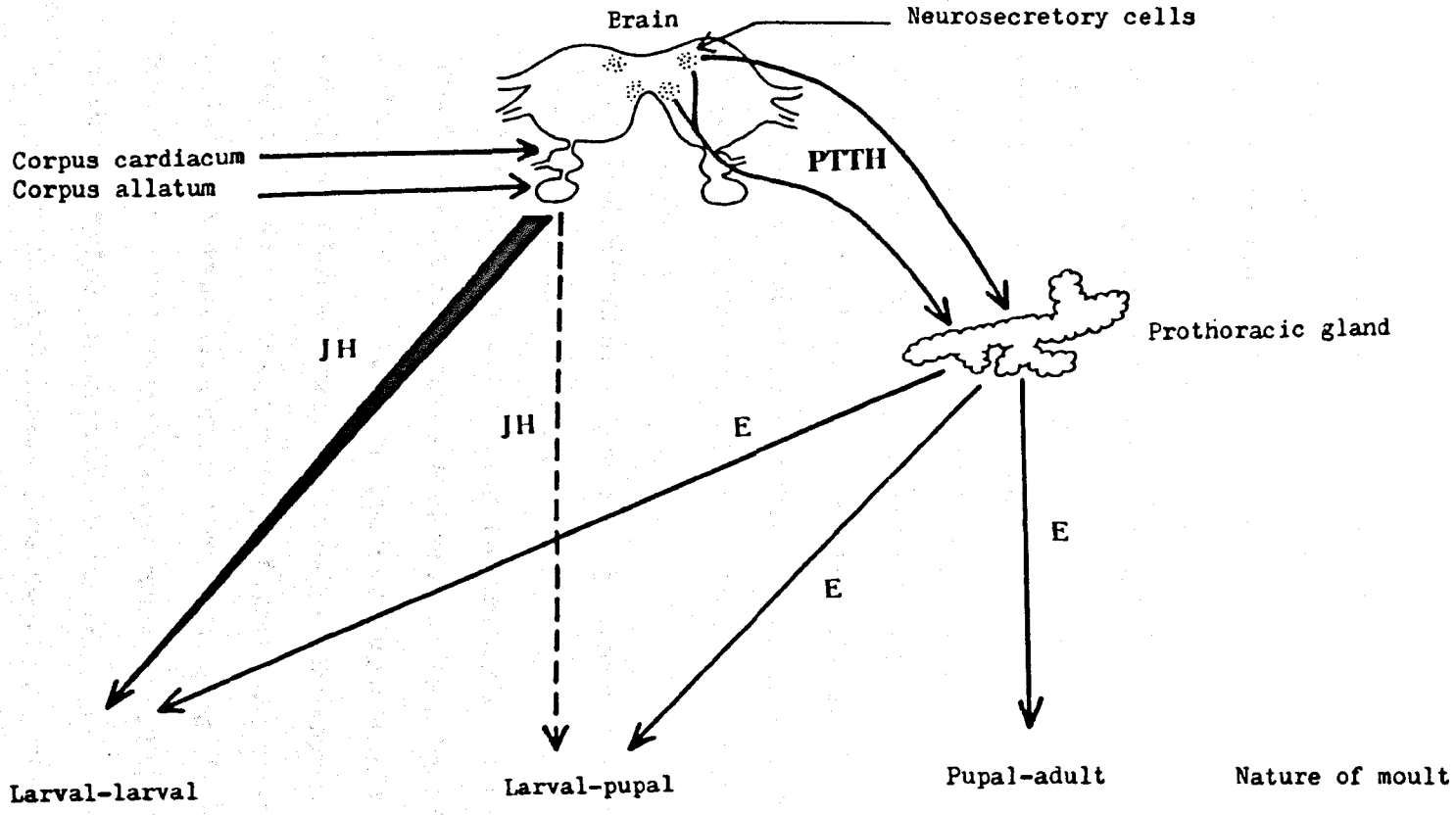


Fig. 1.1. The hormonal control of moulting in insects

Key: PTTH, prothoracicotropic hormone; JH, juvenile hormone; E, ecdysteroids.

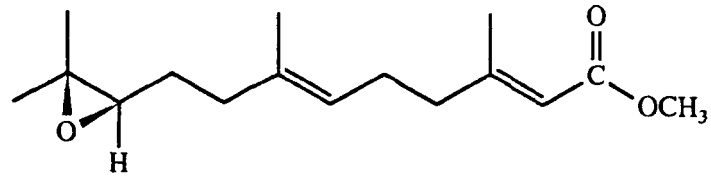
1.1.2. Juvenile hormone (JII)

JH III is the most prevalent form of JH, being present in a number of insect orders, although this is not the case in Lepidopteran insects, were JH exists as a number of homologues (JH III, JH II, JH I, JH0 and 4-MeJH I; Fig. 1.2), although the roles of the different forms of JH are unclear (Schooley *et al.*, 1984). Certain Dipteran insects have been found to possess the bisepoxide form of JH111, as well as its precursor compound methyl farnesoate (Richard *et al.*, 1990). Methyl farnesoate is also found in crustaceans, where it is the major JH (Cusson *et al.*, 1991).

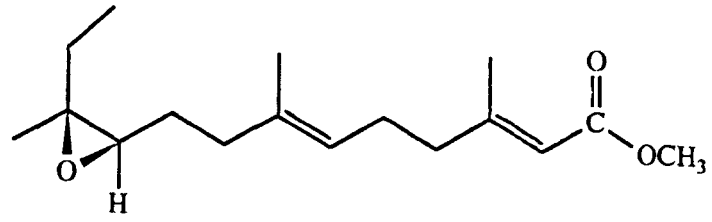
JH is synthesized and secreted by the corpora allata, its titre in the haemolymph at a critical period prior to moulting determining the nature of the moult (ie larval-larval, larval-pupal and pupal-adult) (Granger and Bollenbacher , 1981). High JH titres being responsible for larval-larval moults whereas larval-pupal moults and pupal-adult proceed in the presence of low JH titres and the virtual absence of JH, respectively (Sehnal, 1989).

The role of JH in the moulting process will be referred to in relation to a model proposed for the fifth larval instar of the tobacco hornworm, *M. sexta*, which has received a preponderance of the research interest in this field. The JH and ecdysteroid titres throughout the fifth instar of *M. sexta* are shown in Fig. 1.3.

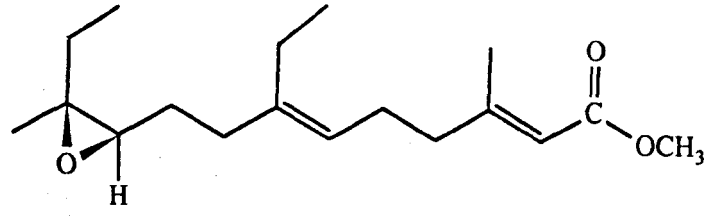
During initial stages of the instar there is a decline in the JH titre of the haemolymph, due to both a reduction in the synthetic activity of the corpora allata, and the appearance at this time in the haemolymph of JH-specific esterases (Jones *et al.*, 1982; Hammock *et al.*, 1984). The decline in the titre of JH results in a gated release of PTTH, allowing the prothoracic glands to become competent to respond to PTTH, thus, eliciting the ecdysteroid pupal commitment peak. The rise in the ecdysteroid titre of the haemolyph forming the pupal commitment peak causes the release of JH acids by the corpora allata, a minority of which are methylated to form their respective JH homologues by the peripheral tissues (Sparagana *et al.*, 1985). The synthetic activity of the corpora



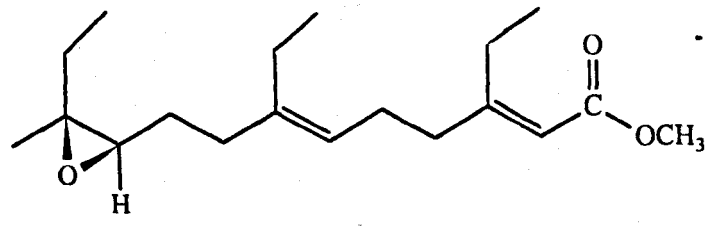
JH III



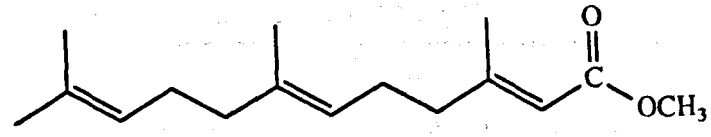
JH II



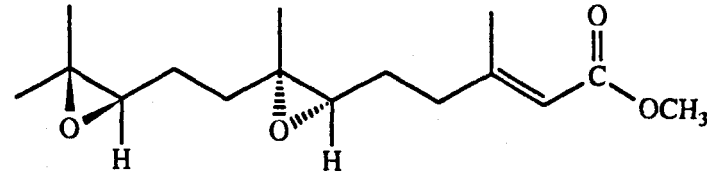
JH I



JH 0



Methyl Farnesoate



JH III Bisepoxide

Fig. 1.2. Examples of common juvenile hormones

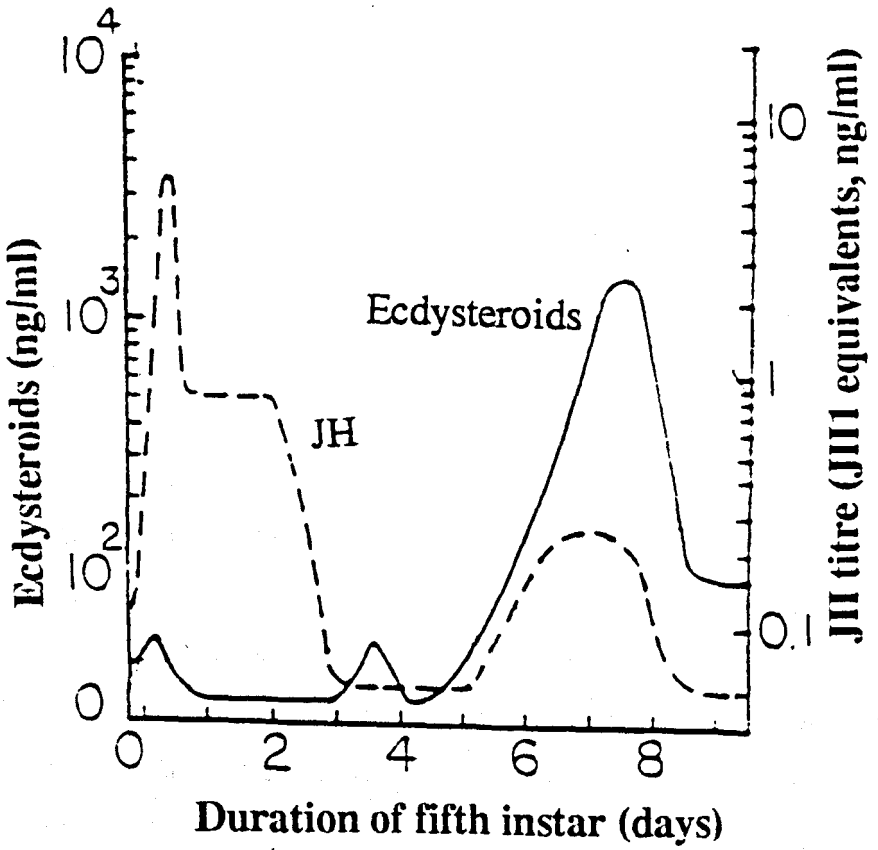


Fig.1.3. The ecdysteroid and juvenile hormone(JH) titre throughout the fifth instar of *M. sexta* (after, Riddiford, 1980)

allata is thought to be controlled by direct nervous and/or neuroendocrine innervation of the gland, with neuropeptide allatotropins and allatostatins mediating this regulation (Granger *et al.*, 1984; Rankin *et al.*, 1986; Granger and Janzene, 1987). The resulting rise in the titre of JH acids and their JH homologues results in a release of PTTH, and an increase in ecdysteroid synthetic activity of the prothoracic glands. The resulting post-commitment ecdysteroid peak inhibits the synthesis of JH by the corpora allata and results in a decline in the titre of JH towards the end of the instar (for review see Riddiford, 1980; Bollenbacher, 1988).

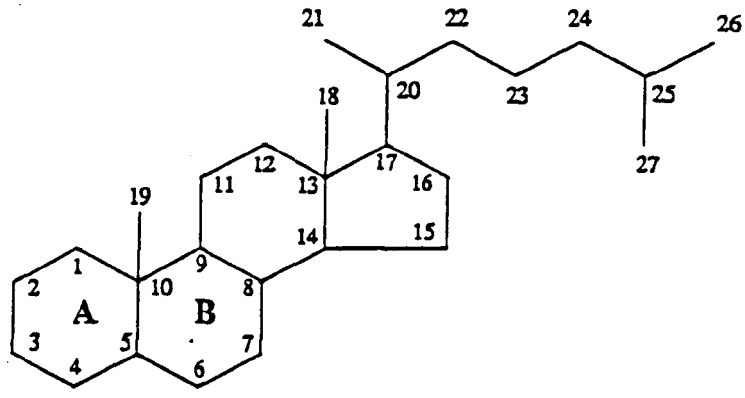
1.1.3. Ecdysteroids

Ecdysone (2β, 3β, 14α , 22R, 25-pentahydroxyl 5β-cholestan-7-en-6-one) was isolated in 1954 from the pupae of *B. mori* by Buntendant and Karlson. Since this time, a number of compounds structurely related to ecdysone have been discovered, being collectively known as ecdysteroids (Goodwin *et al.*, 1978; Rees, 1989a).

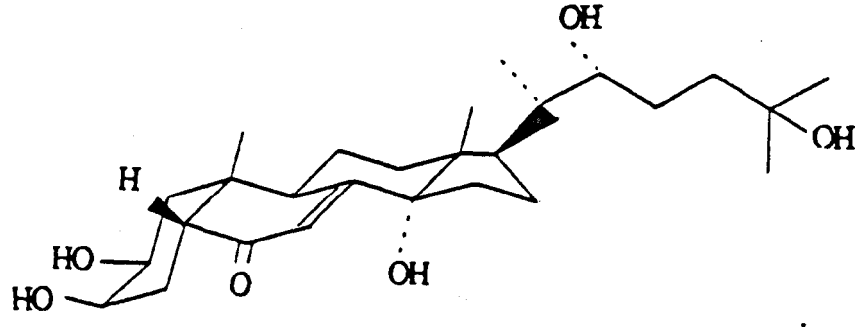
1.2. Ecdysteroid structure

Ecdysteroids are polyhydroxylated steroid hormones, possessing the four ring nucleus of steroids and the full side chain of cholesterol (Fig. 1.4a). Characteristic structural features of ecdysteroids which cause them to differ markedly from vertebrate type steroid hormones are the presence of a keto (C=0) group at C-6, which is in conjugation with a double bond at C-7, and the A/B *cis* ring junction (5β hydrogen). This causes the A ring to deviate away from the plane of the other rings, resulting in the non-planar structure of ecdysteroids as compared to vertebrate steroid hormones, which posses an A/B *trans* ring junction (Fig. 1.4b, c).

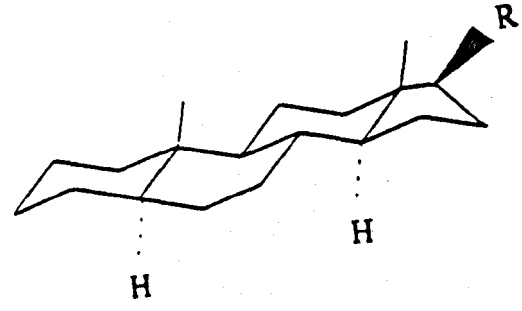
Ecdysteroids exist in a number of different forms. Representative examples of the common ecdysteroid groups are shown in Fig. 1.5. The ecdysteroid molecule itself is subject to a number of enzymic reactions yielding a whole host of ecdysteroid type



1.4a. The numbering system of steroids

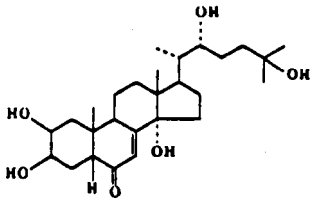


1.4b. The three dimensional structure of ecdysone

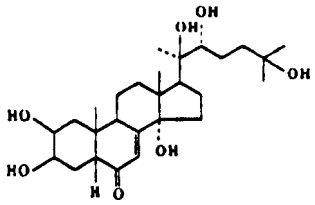


1.4c. The three dimensional structure of vertebrate A/B *trans* steroid

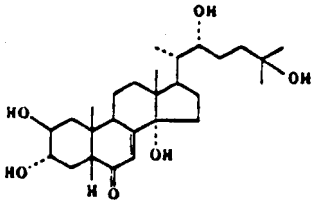
Fig. 1.4. Steroid structures



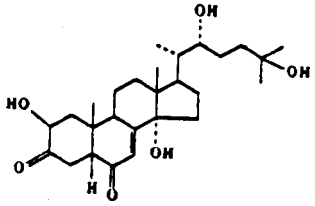
Ecdysone



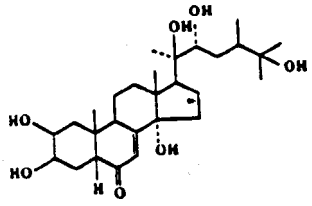
20-Hydroxyecdysone



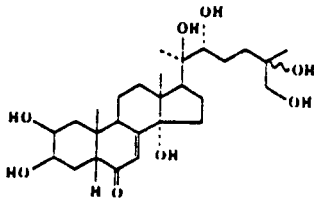
3-Epiecdysone



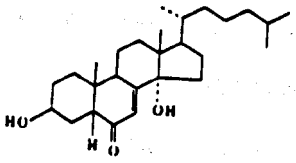
3-Dehydroecdysone



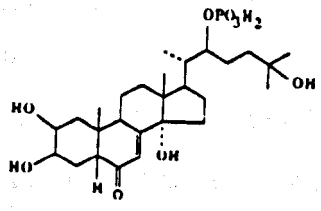
Makisterone A



20,26-Dihydroxyecdysone



2,22,25-Trideoxyecdysone



Ecdysone 22-phosphate

Fig. 1.5. The structure of various ecdysteroid types

molecules (Fig. 1.6). These reactions include hydroxylation, which at the C-20 position produces the active moulting hormone, 20-hydroxyecdysone. However, hydroxylation at the C-26 position and subsequent oxidation produces the inactivation product, ecdyson 26-oic acid. Esterification by phosphorylation, long chain fatty acylation and glycosylation (with the possibility of double esterification occurring at hydroxyl groups on both the side chain and steroid nucleus) gives rise to both double phosphate and phospho-acetate esters. Esterification of the side chain at the C-22 and C-26 positions in the ovarian follicle cells is generally reversible, with the conjugates acting as storage forms of ecdysteroids for early embryogenesis. In contrast, conjugate formation at the C-3 position is irreversible resulting in the formation of an inactivation product. Oxidation of the hydroxyl group at the C-3 position results in the formation of 3-dehydroecdysone, this compound can then be reduced to form either ecdysone or the inactive 3-epiecdysone (for review see Isaac and Slinger, 1989; Lafont and Connat, 1989; Koolman, 1990).

1.3. Occurrence in nature

Ecdysteroids are generally accepted as the moulting hormones of Arthropods, their occurrence being demonstrated in the classes; Insecta, Crustacea and Arachnida (for reviews see Splindler *et al.*, 1980; 1984; Splindler, 1989). Outside the phylum Arthropoda, ecdysteroids have been detected in Plathelminths, Nemathelminths, Mollusca, Annelida and Coelenterata (for review see Frank and Kauser, 1989; Barker *et al.*, 1990). The role of ecdysteroids in these phyla is as yet uncertain. In the case of Nemathelminths it has been demonstrated through exogenous application studies, that ecdysteroids have a role in the moulting process, oocyte development and reproduction in adult females. However, the inability to demonstrate ecdysteroid synthesis in any of these phyla outside the Arthropods puts the origin of their ecdysteroid content into question. Ecdysteroids have been detected in the blood and urine samples of man and

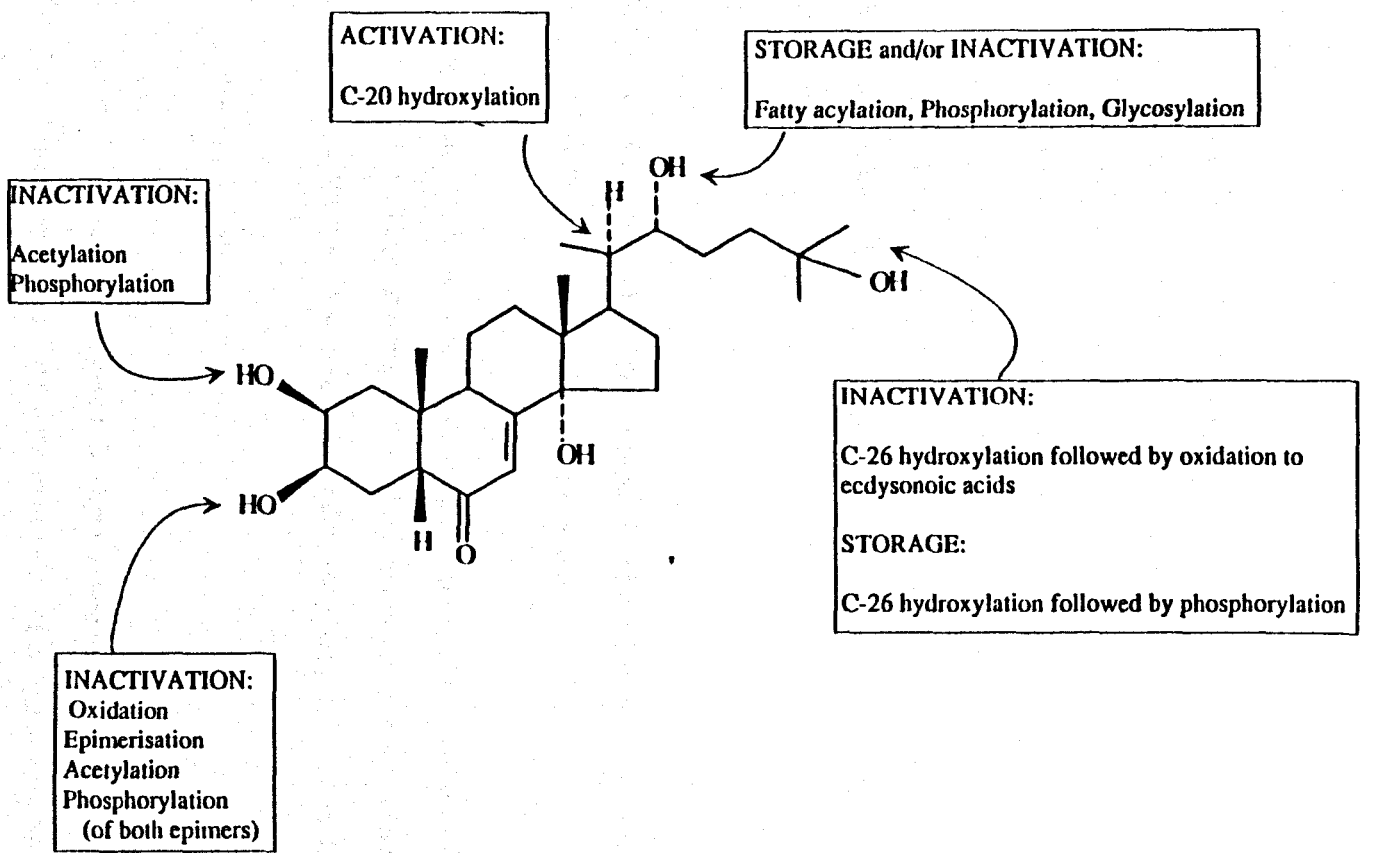


Fig. 1.6. Composite scheme of the modifications to the ecdysone molecule

initially it was believed to be a direct result of nematode infection (Koolman and Moeller, 1986). However, it has now been claimed that the presence of these ecdysteroids is due directly to pathological changes in the gut flora (Gharib *et al.*, 1991). For a comprehensive review of known animal ecdysteroids (zooecdysteroids) see Rees (1989a).

Numerous ecdysteroids (phytoecdysteroids) have been isolated from plants, being most abundant in the embryophytes (ferns, gymnosperms and angiosperms) and to a lesser extent in the lower plants (thallophytes). The ecdysteroid concentration in the plants may be comparatively high, sometimes several orders of magnitude higher than that of Arthropods. Although there appears to be no clear cut physiological function for these phytoecdysteroids in plants, it has been proposed that they may serve to bestow the plant with some degree of resistance to phytophagous insects (for review see Lafont and Horn, 1989).

1.4. Sites of synthesis of ecdysteroids, their forms and functions in the different developmental stages of insects

Ecdysteroids are found in all the developmental stages of insects: in embryos, larvae, pupae and adults. Their occurrence throughout development suggests that they have different stage-specific functions, and that they may be synthesized in different tissues.

1.4.1. Prothoracic glands

Prothoracic glands and ring glands in Diptera (which consist of prothoracic glands fused to other endocrine glands) are the major sites of ecdysteroid synthesis in larval and pupal insects. Their ecdysteroid biosynthetic activity has been confirmed by numerous *in vitro* culture studies (Chino *et al.*, 1974; King *et al* ., 1974 ; Hirn *et al.*, 1979; Redfern , 1983; Kauser *et al.*, 1988) In some insect species, ecdysteroid biosynthesis by

the prothoracic glands of the female insect exceeds that of the male (Roussel, 1992b). Ecdysone, once secreted by the prothoracic glands is converted into the major physiologically acting moulting hormone, 20-hydroxyecdysone by some peripheral tissues, such as the fat body and Malpighian tubules (King, *et al.*, 1974).

In vitro culture studies with prothoracic glands from both larval and pupal stages of *M. sexta* have revealed that 3-dehydroecdysone predominates over ecdysone as products of the glands (Warren *et al.*, 1988a). 3-Dehydroecdysone appears to be a precursor of ecdysone, due to the low ecdysone-3-oxidase activity associated with the glands (Warren *et al.*, 1988b). Once secreted by the prothoracic glands, 3-dehydroecdysone is reduced to ecdysone by a 3β-ketoreductase enzyme present in the haemolymph (Warren *et al.*, 1988b; Sakurai *et al.*, 1989). In species besides Lepidoptera, 3-dehydroecdysone does not appear to be a preponderant product of the prothoracic glands, if it is produced at all (Kiriishi *et al.*, 1990).

In the nymphal and larval stages of certain insect orders including, Hymenoptera and Hemiptera and to a lesser extent, Diptera possess a C_{28} ecdysteroid moulting hormone known as makisterone A. Its appearance in these insects has been associated with their inability to dealkylate plant sterols at the C-24 position (for review see Feldlaufer and Svobada, 1986 ; Feldlaufer, 1989).

During the terminal stages of larval instars, the ecdysteroid titre of the haemolymph increases dramatically to form a distinct peak which is responsible for eliciting moulting processes such as cessation of endocuticle deposition, apolysis, secretion of ecdysial droplets, proliferation of epidermal cells and the secretion of a new cuticle (for review see Sehnal, 1989). In Holometabolous insects, the last larval instar is characterised by a small peak (commitment peak) in the haemolymph ecdysteroid titre during the first half of the instar. This is responsible for changes in larval behaviour, such as wandering and the reprogramming of the larval epidermis from larval synthesis to pupal synthesis. This is then followed by the much larger post-commitment ecdysteroid peak which elicits the larval-pupal moult (for review see Steel and Vafopoulou, 1989).

In accordance with the IUPAC-IUB Recomendations for the Nomenclature of Steroids (IUPAC-IUB, 1989) the carbons of a C-24 ethyl group, C-28 and C-29 now become C-24^1 and C-24^2, respectively. However, throughout this thesis the numbering of steroids according to IUPAC-IUB (1976) recommendations has been referred to.

The control of ecdysteroid titres throughout the moulting process involves a complex interaction of PTTH and JH on the ecdysteroid biosynthetic activity of the prothoracic glands (refer to sections 1.1.1 and 1.1.2). It must be stressed that ecdysteroids are secreted into the haemolymph every day independently of the developmental profile of the insect; this circadian rhythm is directly regulated by the prothoracic glands (Cymboroski *et al.*, 1991; Vafoulout and Steel, 1991). Ecdysteroid titres vary both quantitatively and qualitatively throughout post-embryonic development. Larval-pupal and larval-adult moults are induced by ecdysteroid titres of higher magnitude and/or duration than those for larval-larval moults. In addition, the ratio of ecdysone to 20-hydroxyecdysone in the insect haemolymph is not constant during post-embryonic development but varies drastically, with 20-hydroxyecdysone being the predominant constituent of the ecdysteroid titre moulting peak in larval insects. However, in contrast ecdysone may be of greater concentration than 20-hydroxyecdysone in the haemolymph during the pupal stages of insect development (Smith, 1985).

The decline in the ecdysteroid titre during the terminal stages of the instar, appears to be responsible for triggering a number of events associated with the termination of the moult (Slama, 1980), the events including ecdysis and cuticle melanisation (Curtis *et al.*, 1984). The decline in the ecdysteroid titre of the haemolymph is a result of both a decrease in the synthetic activity of the prothoracic gland and an increase in the inactivation and excretion of circulating ecdysteroids (ecdysone and 20-hydroxyecdysone). Inactivation of ecdysteroids occurs in a number of tissues including the fat body, the gut and the Malpighian tubules, where ecdysteroids are converted to ecdysonoic acids, 3-epiecdysteroids and phospho- and acyl- conjugates, respectively, before being excreted via the gut (Lafont *et al.*, 1983, Modde *et al* ., 1984 ; Milner *et al.*, 1985; Warren and Gilbert, 1986).

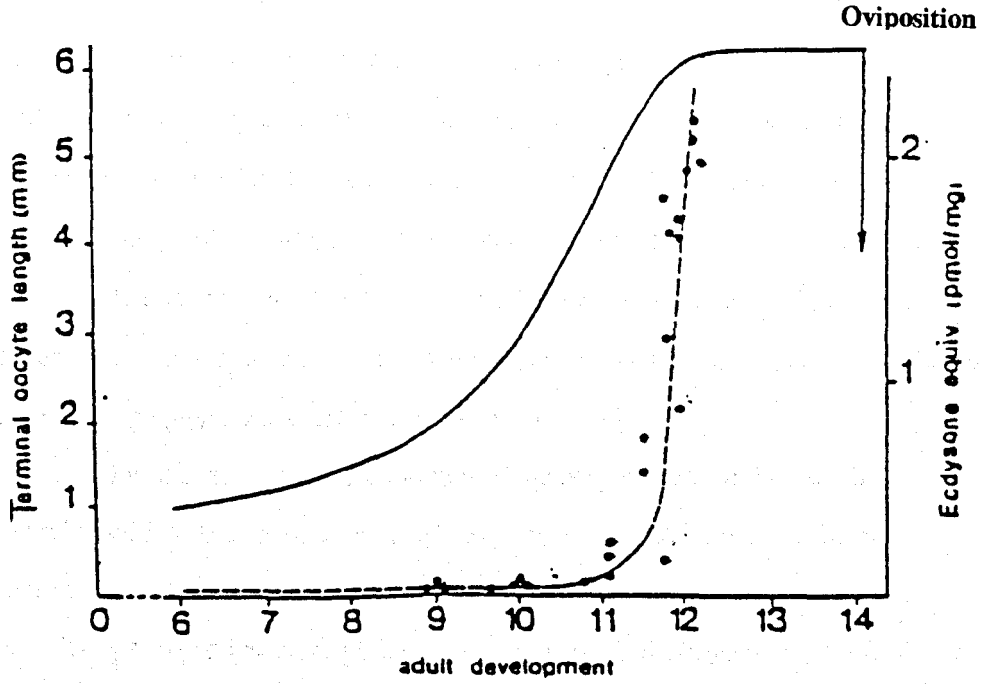
1.4.2. Ovary

The ovaries of a number of reproductively competent female insect species have been shown to have ecdysteroid biosynthetic activity, their synthetic activity being conclusively demonstrated under *in vitro* conditions (Hagedorn *et al.*, 1975; Lagueux *et al.*, 1977; Rubenstein, 1982; Hagedorn *et al.*, 1985; Smith and Bollenbacher, 1985). The ovaries of adult *L. migratoria* synthesize ecdysteroids essentially during the last stages of oocyte maturation, when vitellogenesis is well advanced, just prior to chlorionation (Lagueux *et al.*, 1977).The actual site of ovarian ecdysteroid synthesis is the follicle cells present in the epithelium surrounding the terminal oocyte (Glass *et al.*, 1978; Goltzene *et al.*, 1978). Follicle cells attain ecdysteroid biosynthetic activity at a terminal oocyte length of approximately 4.5mm, reaching maximum activity at 5.7mm, thereafter declining as chlorionation of the terminal oocyte proceeds (Fig. 1.7).

Generally 95-98% of the total ovarian ecdysteroid content synthesized by the follicle cells is transferred to the oocyte, being present in newly laid eggs predominantly as the polar conjugates, ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate (ratio 1:2) bound to vitellin. (Lagueux *et al.*, 1981;84). In another Orthopteran insect species, *Schistocherca gregaria* the bulk of ecdysteroids present in the newly laid egg consist of the ecdysteroid 22-phosphate forms of ecdysone, 2-deoxyecdysone and 20-hydroxyecdysone (Isaac *et al.*, 1983). In addition to polar conjugates a number of apolar conjugates have been identified in the eggs of the insect species *Acheta domesticus* (Whitting and Dinan, 1988; 1989) and *Periplaneta americana* (Slinger *et al.*, 1986).

Once transferred to the developing oocyte, ovarian ecdysteroids have two major functions :

Firstly, in the developing oocytes of *L. migratoria* it has been demonstrated that surges in the concentration of free ecdysone prior to ovulation and at egg laying are responsible for triggering the concurrent meiotic reinitiation which also occurs at these times (for review see Lanot *et al.*, 1989). Ecdysone is able to induce meiotic reinitiation



(Solid line: length of terminal oocytes , expressed in mm)

(Dotted line: pmol equivalents of ecdysone per mg of ovary)

Fig. 1.7. Ecdysteroid production during the first gonotropic cycle of the terminal oocytes of *Locusta migratoria* (after, Lagueux *et al.*, 1977)

by a mechanism involving a reduction in the intracellular concentration of cAMP (Lanot *et al.*, 1990).

Secondly, ecdysteroid conjugates present in newly laid eggs act as inactive storage forms of ecdysteroids, which can be hydrolysed at specific times to yield their respective free ecdysteroids as embryogenesis proceeds (Lagueux *et al.*, 1981; Rees and Isaac, 1984; Whiting *et al.*, 1993). In the developing embryos of *L. migratoria* four peaks of ecdysteroid titre have been correlated with four cycles of cuticulogenesis. The first two peaks occur before the differentiation of the embryonic prothoracic glands, implicating maternal ecdysteroid conjugates as the source of hormone (Lagueux *et al.*, 1979). Maternal ecdysteroids once utilised by the embryo are metabolised into ecdysteroid 26-oic acids and a range of inactive phosphate and acyl esters (Lagueux *et al.*, 1984; Rees and Isaac, 1984; Dimarc *et al.*, 1987). In contrast, maternal ecdysteroid (26-hydroxyecdysone 26-phosphate) is metabolised into an inactive 22-glucoside conjugate in the developing eggs of *M. sexta* (Thompson *et al.*, 1987).

In Dipteran insects, an appreciable amount of the ecdysteroid synthesized by the ovary is released into the haemolymph, where it is believed to have a role in the synthesis and secretion of vitellogenin by the fat body (Hagedorn, 1985). In *Aedes aegypti*, 20-hydroxyecdysone has been found to increase vitellogenin synthesis in fat body under *in vitro* conditions (Fallon *et al*, 1974). Racioppi *et al.* (1986) have suggested that 20-hydroxyecdysone is able to stimulate vitellogenin synthesis in fat body by increasing the level of transcription of the yolk protein genes. JH is also thought to play an essential role in vitellogenesis by enhancing the expression of yolk protein genes, therefore, reducing the concentration of 20-hydroxyecdysone required for stimulation of vitellogenesis *in vitro* (Borovsky *et al.*, 1985). In addition, JH allows the fat body to become competent, enabling it to respond to 20-hydroxyecdysone, possibly by stimulating the production of 20-hydroxyecdysone receptors (Racioppi *et al.*, 1986).

1.4.3. Testes

Ecdysteroids have been detected in the testes of various insects, including *Calliphora vicinia* (Koolman *et al.*, 1979) , *Heliothis virescens* (Loeb *et al.*, 1984) and *Spodoptera littoralis* (Jarvis *et al.*, 1994a). Immunological and *in vitro* studies have suggested the testis sheath or covering as the site of testicular ecdysteroid biosynthesis in *H. virescens* (Loeb *et al.*, 1982; Loeb., 1986). The testes appear to gain ecdysteroid biosynthetic activity after the middle of the last larval instar, producing at least twelve different ecdysteroids under *in vitro* conditions, three of which have been identified as 20-hydroxyecdysone, makisterone A and 20,26-hydroxyecdysone (Loeb *et al.*, 1982; Loeb *et al.*, 1984). In contrast, only six ecdysteroids have been detected in the testes of *Ostrinia nubilalis*, comprising of 20-hydroxyecdysone, ecdysone, 26-hydroxyecdysone and three unknown polar ecdysteroids (Gelman *et al.*, 1988). Metabolism studies with radiolabelled ecdysone have demonstrated that the primary ecdysteroid product of the testes of both *O. nubilalis* (Gelman *et al.*, 1985) and *H. virescens* (Loeb *et al.*, 1989) is 20-hydroxyecdysone.

Exogenous 20-hydroxyecdysone has been shown to elicit a positive feedback effect on the endogenous ecdysteroid production by late larval testes of *H. virescens* under *in vitro* conditions (Loeb, *et al.*, 1986). In addition, a testes ecdysiotropin extracted from the brain of larval and pupal forms of *H. virescens* has the ability to initiate ecdysteroid production in non-synthetic testes, in the presence of exogenous 20-hydroxyecdysone (Loeb *et al.*, 1987). These data possibly reflect the regulatory mechanisms involved in the control of testicular ecdysteroid biosynthetic activity *in vivo* .

Ecdysteroids synthesized by the testes are thought to play an essential role in the reproductive system of the male insect, stimulating both eupyrene and apyrene spermiogenesis in larval insects (Gelman *et al.*, 1986), as well as maintaining spermiogenesis in adult insects (Dumser and Davy, 1974). Testicular ecdysteroids are also implicated in the cessation and stimulation of sperm production during diapause (Friedlander and Benz, 1982 ; Friedlander and Reynolds, 1992). In pupal stages of

insects, testicular ecdysteroids are associated with the development of the male genital tract (Nowock, 1972). However, in adult insects, ecdysteroids are thought to mediate sperm release (Thorson and Riemann, 1982) and may have a role in the sexual behaviour of the insect (Hagedorn, 1981).

1.4.4. Other sites

The ability of larval and pupal insects to undergo the moulting process in the absence of prothoracic glands has been reported in certain insect species, including *P. americana* (Gersh, 1977) and *M. sexta* (Sakurai *et al.*, 1991). Additionally, the isolated abdomens of a number of these species are capable of inducing a moult autonomously (Delbecque *et al.*, 1978 ; DeWild *et al* ., 1980; Sakurai *et al.*, 1991), implying an alternative source of ecdysteroid biosynthesis in addition to the prothoracic glands. Isolated abdomens have been demonstrated to convert radiolabelled cholesterol into ecdysone and 20-hydroxyecdysone in a number of insect species, including *Mamestra brassicae* (Gersh and Strurzebecher, 1971), *B. mori* (Nakanishi *et al.*, 1972) and *Musca domestica* (Studinger and Willig, 1975). In addition, the ability of the thorax as well as the abdomen to produce ecdysteroids under *in vitro* culture has been reported in larval and pupal stages of *A. aegypti* (Jenkin *et al.*, 1992). The possible source of integument ecdysteroid biosynthetic activity has been associated with both the oenocytes and the epidermal tissue (Studinger and Willing, 1975; Cassier *et al.*, 1980), and Romer *et al.* (1974) have reported the ability of oenocytes to convert radiolabelled cholesterol into 20-hydroxyecdysone. Pupal wings, which consist almost entirely of epidermal tissue have been confirmed as an active ecdysteroid source in *Tenebrio molitor* (Delbecque *et al.*, 1986; 1988). Cell lines of epidermal origin have also been widely reported to synthesize and secrete ecdysteroids under culture conditions (Porcheron *et al.*, 1988). The ability of the integument to synthesize ecdysteroids is not restricted to the larval and pupal stages of the insect, as the abdominal integument with the adjacent fat body of

adult female *Gryllus bimaculatus* has been reported to produce ecdysone from both endogenous and radiolabelled substrates in culture (Hoffmann *et al.*, 1992).

The ecdysteroid biosynthetic ability of these alternative sources has not yet been unequivocally proven, although it could explain the source of ecdysteroids in Coleopteran insects whose prothoracic glands degenerate before the pupal-adult moult.

1.5. Ecdysteroid biosynthetic pathway

The incorporation of radiolabelled cholesterol into ecdysone was first demonstrated *in vivo* by Karlson and Hoffmeister in 1963. Since this time a host of *in vitro* and *in vivo* studies have confirmed the distal precursor role of cholesterol in the ecdysteroid biosynthetic pathway (Romer *et al.*, 1974; Sakurai *et al.*, 1977; Warren *et al.*, 1987).

Insects are unable to perform *de novo* synthesis of sterols from small molecules such as acetate, and therefore, require a dietary source of these compounds (Clayton, 1964). Carnivorous insects are able to obtain cholesterol directly from their diet, whereas phytophagous insects must first dealkylate C_{28} and C_{29} phytosterols such as campesterol, stigmasterol and sitosterol at the C-24 position in order to obtain the C_{27} sterol, cholesterol (Fig. 1.8, after Rees, 1989b). The ability to dealkylate phytosterols is not universal amongst all phytophagous insect species, and the appearance of makisterone A in the insect orders, Hemiptera and Hymenoptera is a direct result of the ability of these insects to utilize the C_{28} phytosterol, campesterol as a distal precursor for ecdysteroid biosynthesis (for review see Feldlaufer, 1989).

During ecdysone biosynthesis from cholesterol, extensive modification of the steroid nucleus occurs before side chain hydroxylations (Rees, 1985), Fig. 1.9 shows a proposed biosynthetic scheme for the conversion of the distal precursor, cholesterol into 20-hydroxyecdysone.

7-Dehydrocholesterol has been suggested as an early intermediate in the transformation of cholesterol into ecdysone (Horn *et al.*, 1974) and has been detected endogenously in the prothoracic glands of *M. sexta* (Thompson *et al.*, 1973; Warren *et*

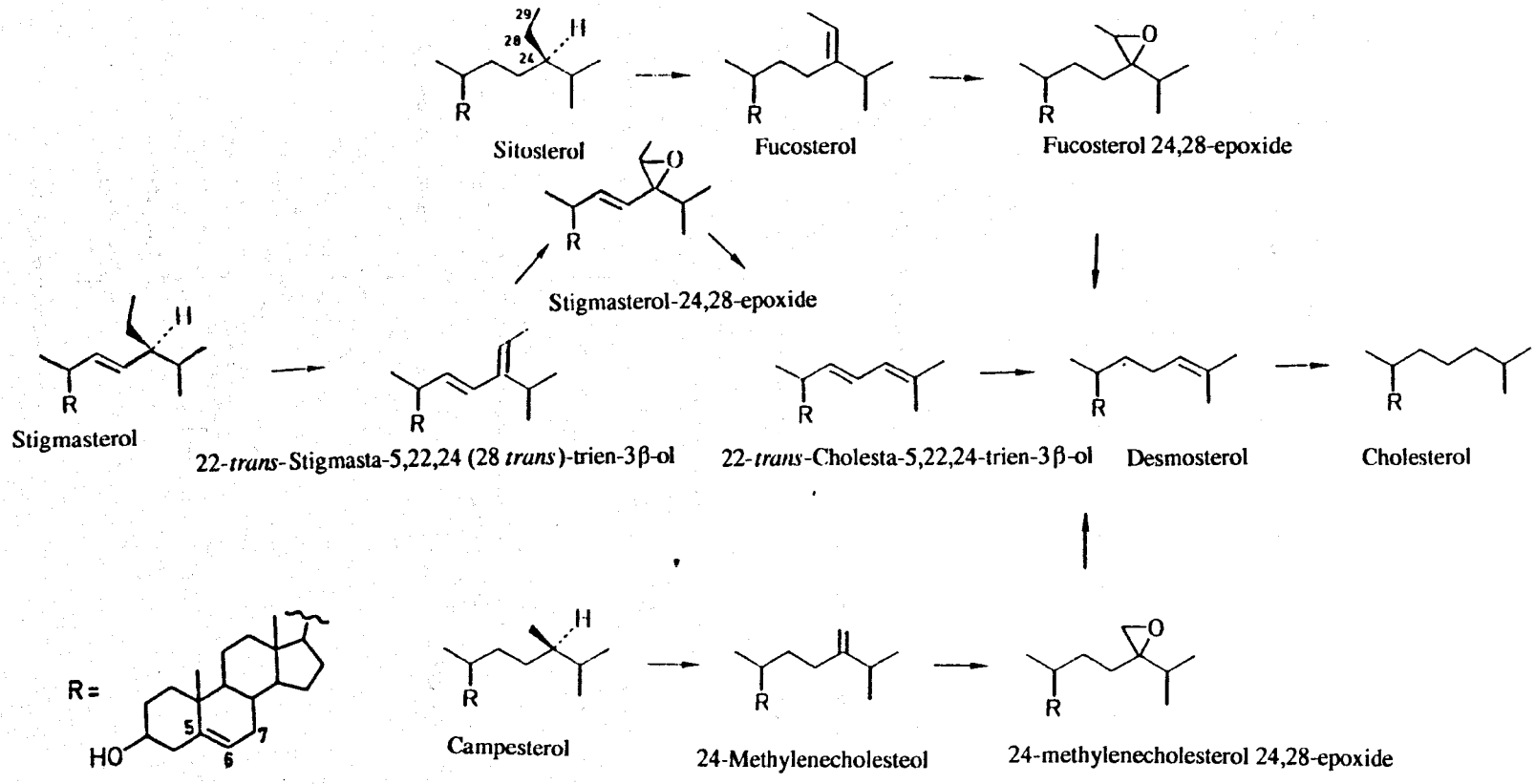


Fig. 1.8. The conversion of sitosterol, stigmasterol and campesterol into cholesterol in insects

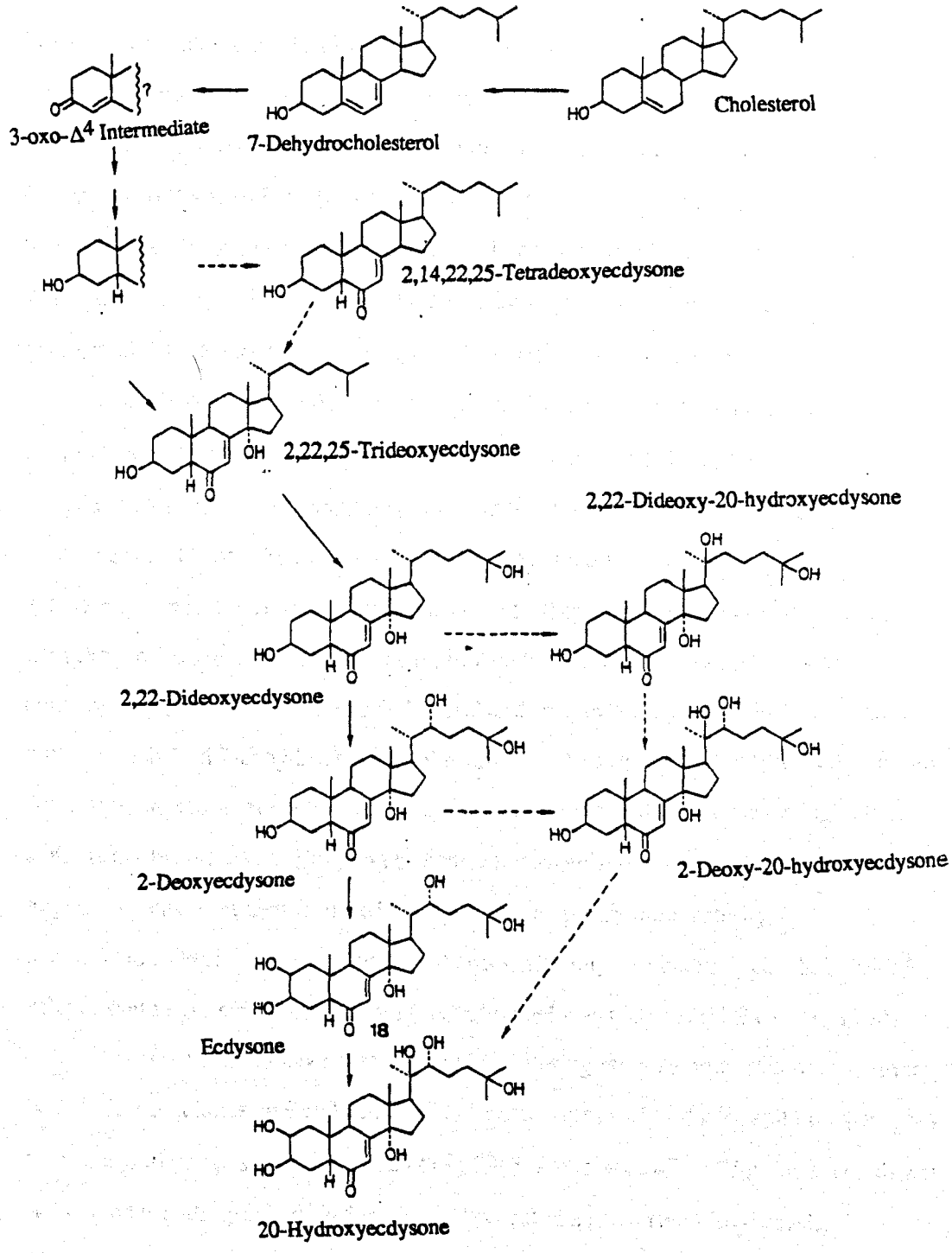


Fig. 1.9. A possible pathway of ecdysteroid biosynthesis (Rees, 1989)

al., 1988a; Grieneisen *et al.*, 1991) and *P. americana* (Thompson *et al.*, 1973). Fluctuations in the concentration of 7-dehydrocholesterol throughout larval development have been shown to be consistent with its involvement in ecdysteroid biosynthesis (Sakurai *et al.*, 1986; Warren *et al.*, 1988a). The *in vivo* incorporation of 7-dehydrocholesterol into ecdysteroids has been demonstrated in the insect species, *Calliphora stygia* (Horn *et al.*, 1974), *S. littoralis* (Milner *et al.*, 1986) and *S. gregaria* (Johnson and Rees, 1977a). However, the interconvertible relationship that exists between 7-dehydrocholesterol and cholesterol has made it difficult to ascertain whether 7-dehydrocholesterol is incorporated directly into ecdysteroids or via reduction to cholesterol. The demonstration that [4-^{14}C, 7-^{3}H]7-dehydrocholesterol was incorporated into ecdysteroids in larval *S. littoralis* with the complete retention of the tritium label was consistent with the proposed direct incorporation of 7-dehydrocholesterol into ecdysteroids, rather than by prior reduction to cholesterol (Milner *et al.*, 1986). The irreversible nature of the incorporation of 7-dehydrocholesterol into ecdysteroids has been further confirmed by *in vitro* studies with the prothoracic glands of *M. sexta* , in which radiolabelled cholesterol was converted directly into 7-dehydrocholesterol and ecdysteroids. In addition, incubation with radiolabelled 7-dehydrocholesterol resulted in the synthesis of ecdysteroids with no detectable formation of cholesterol (Warren *et al.*, 1987; 1988a). The resulting 1000g supernatant from the centrifugation of gently homogenized ovarian follicle cells from *L. migratoria* has been demonstrated to convert high specific radioactivity 7-dehydrocholesterol into a range of ecdysteroids, although the inability of whole follicle cells to synthesize ecdysteroids from this radiolabel was reported (Dolle *et al.*, 1990).

Stereospecific studies have demonstrated that during the formation of the Δ^7 bond in both 7-dehydrocholesterol and ecdysteroids there is removal of the 7β and 8β hydrogens from the cholesterol substrate (Cook *et al.*, 1973; Johnson *et al.*, 1975); this is consistent with an intermediary role of 7-dehydrocholesterol in ecdysteroid biosynthesis. Grieneisen *et al.* (1993) have proposed that a microsomal cytochrome P450-dependent

enzyme is responsible for the conversion of cholesterol to 7-dehydrocholesterol in the prothoracic glands of *M. sexta.*

The subsequent steps of the ecdysteroid biosynthetic pathway are less well known, and require further investigation. The complete removal of the 3α and 4β hydrogens and the retention of the 4α hydrogen from cholesterol during its incorporation into ecdysteroids in *S. gregaria* has been interpreted by the envolvement of a 3-oxo -Δ^4-intermediate in the formation of the A/B cis ring junction in ecdysteroids, (Davies *et al.*, 1981, Rees, 1985).

The successful incorporation of 3β, 14α-dihydroxy cholesta-4,7-dien-6-one (a potential 3β-hydroxy -Δ^4-intermediate) into 20-hydroxyecdysone has been demonstrated in *C. stygia* (Kinnear *et al.*, 1978), although this could not be repeated *in vitro* with the prothoracic glands of *M. sexta* (Gilbert *et al.*, 1980). The failure to incorporate the 3-oxo-Δ^4 compounds, cholest-4-en-3-one and cholesta-4,6-dien-3-one into the ecdysteroid biosynthetic pathway of *S. gregaria* has been attributed to the requirement of other structural features in these compounds, in addition to possible permeability factors (Milner *et al.*, 1986). The isolation of the tentatively identified intermediate (cholest-7-en-3β, 5α, 6β -triol) from the *in vitro* culture of radiolabelled cholesterol with disrupted prothoracic glands of *M. sexta* has suggested the possibility that 7-dehydrocholesterol 5, 6α-epoxide has an intermediary role in the early stages of ecdysteroid biosynthetic pathway (Grieneisen *et al.*, 1991).

Prothoracic glands of *B. mori* when cultured *in vitro*, synthesized 3β-hydroxy-5α cholestan-6-one from radiolabelled cholesterol, which could then be converted into ecdysone (Sakurai *et al.*, 1977). However, this compound could not be incorporated into ecdysteroids either by the prothoracic glands of *M. sexta* cultured *in vitro* (Gilbert *et al.*, 1980) or *in vivo* into adult stages of *S. gregaria* (Milner *et al.*, 1986). Therefore, the intermediacy of this compound in ecdysteroid biosynthesis has yet to be confirmed.

2,14,22,25-Tetradeoxyecdysone (5β-ketol) has been found endogenuosly in the ovaries of *L. migratoria* (Hetru *et al.*, 1978; 1982). However, radiolabelled 5β-ketol could be only poorly incorporated into 20-hydroxyecdysone in pupal *C. stygia in vivo*

(Faux *et al.*, 1979). In addition, prothoracic glands from *M. sexta* (Bollenbacher *et al.*, 1977) and *L. migratoria* (Haag *et al.*, 1987) converted 5β-ketol into 14-deoxyecdysone *in vitro*. This suggested that the 14-hydroxylation step must occur at an early stage in the ecdysteroid biosynthetic pathway, possibly before the completion of nuclear modification in the steroid. In addition, the synthesis of ecdysone lacking the carbonyl group at C-6 from radiolabelled 5β-cholest-7-en-3β, 6α,14α-triol by *L. migratoria* prothoracic glands *in vitro*, suggests that the carbonyl group at C-6 is also introduced at an early stage of ecdysteroid biosynthesis (Schwab and Hetru, 1991).

2,22,25-Trideoxyecdysone (5β-ketodiol) is generally accepted as an intermediate in the ecdysteroid biosynthetic pathway of a number of insect species, being isolated from the ovaries of *L. migrotoria* (Hetru *et al.*, 1978; 1982), in addition to its synthesized from radiolabelled cholesterol by *L. migratoria* ovarioles *in vitro* (Hetru *et al.*, 1982). Radiolabelled 2,22,25-trideoxyecdysone (5β-ketodiol) is converted into ecdysone by *M. sexta* prothoracic glands *in vitro* (Bollenbacher *et al.*, 1977), and into 20-hydroxyecdysone *in vivo* by larval and pupal stages of *Calliphora vicinia* (Meister *et al.*, 1987). Follicle cells of *L. migratoria* when cultured *in vitro* with radiolabelled 5β-ketodiol synthesized a range of ecdysteroids including the polar ecdysteroid conjugates of 2-deoxyecdysone and ecdysone (Kappler *et al.*, 1986a). Prothoracic glands of this species also accept 5β-ketodiol as a precursor for ecdysteroid synthesis (Meister *et al.*, 1985).

Recently, the radiolabelled 3-dehydro derivative of 2,22,25-trideoxyecdysone, known as 3-dehydro-2,22,25-trideoxyecdysone, has been synthesized (Dolle *et al.*, 1991) as a proposed ecdysteroid precursor of 3-dehydroecdysone, which is synthesized by the prothoracic glands of a number of Lepidopteran insects *in vitro* (Warren *et al.*, 1988a; Kiriish *et al.*, 1990). Radiolabelled 3-dehydro-2,22,25-trideoxyecdysone, on incubation with prothoracic glands of *L. migratoria* (Dolle *et al.*, 1991) and *Pieris brassicae* (Blais and Lafont, 1991), was efficiently converted into ecdysteroids, yielding both ecdysone and 3-dehydroecdysone as final products in both cases. In conjunction with this radiolabel study, the prothoracic glands of *P. brassicae* were also demonstrated to

synthesize both 3-dehydroecdysone and ecdysone under *in vitro* conditions, whereas *L. migratoria* prothoracic glands have so far only been reported to synthesize ecdysone *in vitro* (Hirn *et al.*, 1979; Blais and Lafont, 1991). However, 3-dehydroecdysone synthesis by the prothoracic glands of *L. migratoria* is suggested by the presence of 3-oxoecdysteroid 3-reductase activity in the haemolymph of this insect species (Kiriishi *et al.*, 1990; Roussel, 1992a). Further investigation of the ecdysteroids synthesized from radiolabelled 3-dehydro-2,22,25-trideoxyecdysone in the *L. migratoria* prothoracic gland culture has revealed that they consist of both the common 3β-hydroxy and the 3-dehydro derivatives of the ecdysteroid compounds, in equal proportions (Roussel, 1992a).

The sequence of hydroxylations of 2,22,25-trideoxyecdysone during the terminal stages of the ecdysteroid biosynthetic pathway have been well studied in the ovarian follicle cells and prothoracic glands of *L. migratoria. In vitro* culture studies with radiolabelled 2,22,25-trideoxyecdysone have yielded, 2,22-dideoxyecdysone, 2-deoxyecdysone and ecdysone, together with 22,25-dideoxyecdysone and 22-deoxyecdysone in both cases. However, on isolation and reincubation of 22,25-dideoxyecdysone and 22-deoxyecdysone with ovarian follicle cells no conversion into ecdysone or 20-hydroxyecdysone occurred (Kappler *et al.*, 1986a; Meister *et al.*, 1985).

Incubation of radiolabelled 2,22-dideoxyecdysone with *L. migratoria* ovarian follicle cells and prothoracic glands *in vitro* yielded both 2-deoxyecdysone and ecdysone (Haag *et al.*, 1988). In addition, radiolabelled 2-deoxyecdysone has been shown to be converted into ecdysone, 20-hydroxyecdysone and 2-deoxy-20-hydroxyecdysone on incubation with *L. migratoria* follicle cells *in vitro* (Kappler *et al.*, 1986a), being converted into ecdysone only on incubation with prothoracic glands (Kappler *et al.*, 1986b). These foregoing incubation studies together with the isolation of 2,22-dideoxyecdysone, 2-deoxyecdysone and ecdysone from the ovaries of *L. migratoria* (Hetru *et al.*, 1978; 1982) suggest a privileged sequence of hydroxylation of 2,22,25-trideoxyecdysone into ecdysone of C-25, C-22 and C-2 in the prothoracic glands and ovarian follicle cells of *L. migratoria.*

The isolation of 2-deoxy-20-hydroxyecdysone 22-phosphate, 2,22-dideoxy-20-hydroxyecdysone 3-phosphate and 22-deoxy-20-hydroxyecdysone from the ovaries of *B. mori* (Ohnishi *et al.*, 1989; Kamba *et al.*, 1994), together with the isolation of 2-deoxy-20-hydroxyecdysone 22-phosphate from the ovaries of *S. gregaria* (Isaac *et al.*, 1983) suggest that the terminal stages of the biosynthesis of 20-hydroxyecdysone may be branched in these insect species. In addition, the *in vitro* culture studies with radiolabelled 3-dehydro-2,22,25-trideoxyecdysone have suggested a branched pathway, consisting of the 3-β-hydroxy and 3-dehydro derivatives of each ecdysteroid moiety at each level of hydroxylation, for the terminal stages of the ecdysteroid biosynthesis in the prothoracic glands of a number of insect species (see Grieneisen, 1994; Fig. 1.10).

The enzymes involved in the terminal stages of the ecdysteroid biosynthetic pathway have been studied in the prothoracic glands of *L. migratoria,* where the 25-hydroxylase was found to be a microsomal enzyme, which together with the mitochondrial 22-hydroxylase, displayed classical cytochrome P450-dependent monooxygenase characteristics. The mitochondrial 2-hydroxylase enzyme however, displays atypical cytochrome P450-dependent monooxygenase behaviour in its insensitivity to carbon monoxide (Kappler *et al.*, 1988). Additionally, a microsomal 25-hydroxylase and mitochondrial 2- and 22-hydroxylases have been shown to be responsible for the conversion of 3-dehydro-2,22,25-trideoxyecdysone into 3-dehydroecdysone and ecdysone in the prothoracic glands of *M. sexta* (Grieneisen., *et al.*, 1993). It has been suggested by Roussel (1992a) that the hydroxylase enzymes do not discriminate between 3-dehydroecdysteroids and 3β-hydroxyecdysteroids.

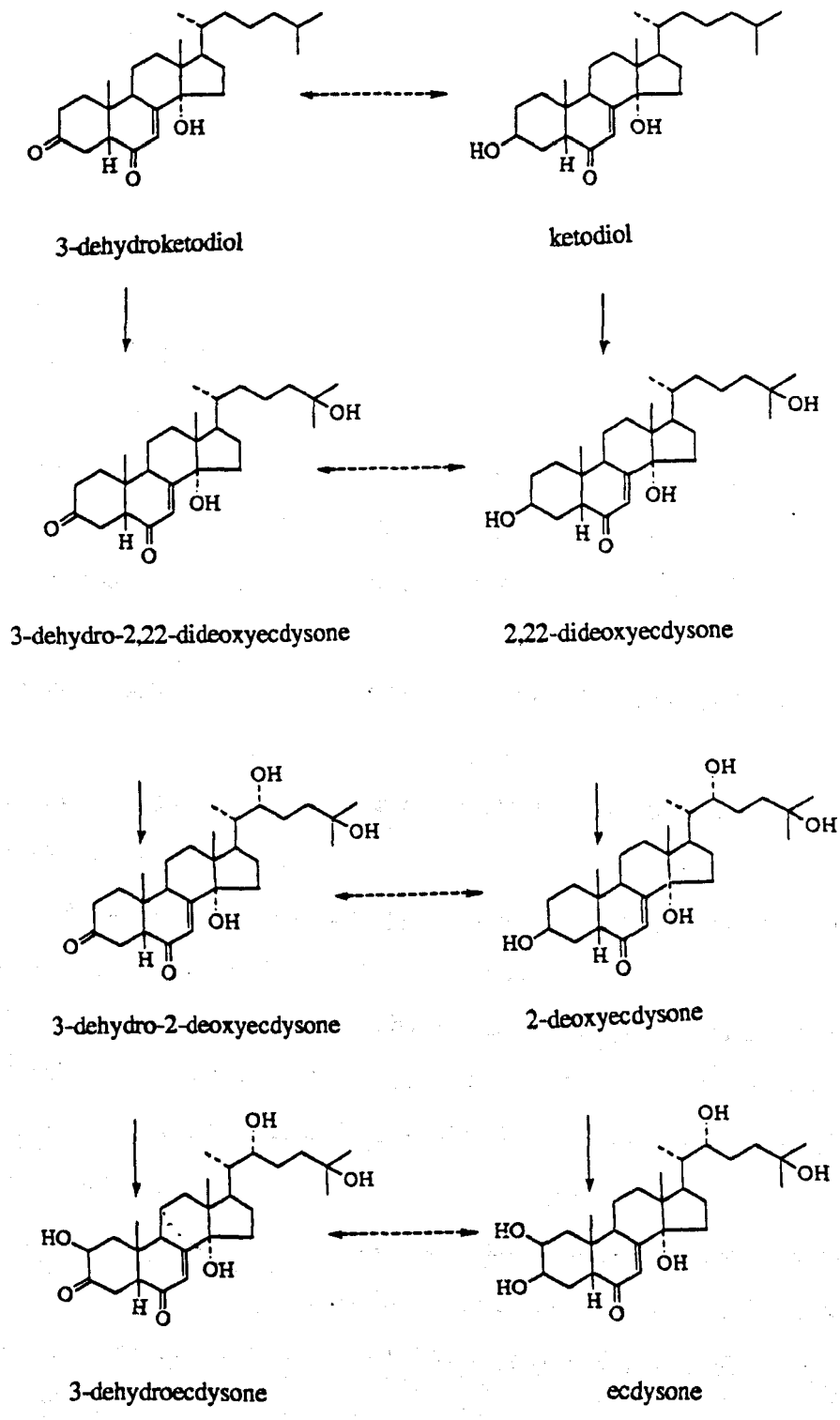


Fig. 1.10. The proposed branched pathway for the final stages of ecdysteroid biosynthesis in the prothoracic glands (after, Grieneisen, 1994)

1.6. Control of ecdysteroid synthesis

Prothoracicotropic hormone (PTTH) is known to play a central role in the control of the ecdysteroid biosynthetic activity of the prothoracic glands (see section 1.1). The mechanism of action of PTTH at the cellular level will now be discussed in relation to previous studies using the prothoracic glands of *M. sexta.* The PTTH- stimulated increase in the ecdysteroid synthetic activity of larval and pupal prothoracic glands can be mimicked *in vitro* by agents that increase the intracellular concentration of cAMP. On incubation with PTTH, the cAMP synthesis in larval and pupal prothoracic glands was found to increase. These results were interpreted in terms of a secondary messenger role for cAMP in PTTH action. However, larval and pupal glands differed markedly in the degree to which PTTH stimulated cAMP accumulation, with a significant accumulation in larval glands only (Smith *et al.*, 1984). The lack of accumulation of cAMP in pupal prothoracic glands has been shown to be due to a marked increase in phosphodiesterase activity in the gland at this time. This enhanced phosphodiesterase activity does not impair the responsiveness of the gland to PTTH (Smith and Pasquarello, 1989). PTTH stimulation of ecdysteroid synthesis in pupal prothoracic glands *in vitro* has been shown to be dependent on the presence of extracellular calcium. In contrast, the basal ecdysteroid synthetic activity of the gland was not found to be calcium-dependent. In addition, the calcium ionophore A23187 has been shown to mediate the steroidogenic effects of PTTH by stimulating the synthesis of cAMP in the prothoracic glands (Smith *et al.*, 1985; Smith and Gilbert, 1986). The PTTH-stimulated ecdysteroid synthesis in prothoracic glands *in vitro* has been shown to be accompanied by the activation of a cAMP-dependent protein kinase, together with the phosphorylation of a single 34 kD membrane associated protein, which is thought to be rate-limiting enzyme in the ecdysteroid biosynthetic pathway (Smith *et al.*, 1987; Smith, 1993). For a proposed model of action of PTTH on ecdystereoid biosynthesis in prothoracic glands, see Smith and Sedlmeier (1990).

Cyclic AMP has also been suggested to act as a secondary messenger for the 0.5 -1.5kD hindgut ecdysiotropin factor reported by Gelman *et al.* (1993), which has been demonstrated to stimulate ecdysteroid synthesis of *Lymantria dispar* prothoracic glands *in vitro*. The physiological function of such a peptide is at present unknown, however, it might have a role in the initiation of ecdysteroid production reported in isolated abdomens. The alleged secondary messenger responsible for the initiation and augmentation of ecdysteroid synthesis in testes of larval and pupal *L. dispar* by the testis ecdysiotropin has been suggested as diacyl glycerol, in contrast to cAMP. However, cAMP is thought to modulate the effects of the testis ecdysiotropin in inducing ecdysteroid synthesis by cross communication between the cAMP and inositol phosphate systems (Loeb *et al.*, 1993).

Ovarian ecdysteroid synthesis in mature insects is controlled by the Egg Development Neurosecretory Hormone (EDNH), crude extracts of which have been shown to stimulate ecdysone production in the *A. aegypti* (mosquito) ovaries *in vitro* (Hagedorn *et al.*, 1979; Hanaoka *et al.*, 1980). EDNH is synthesized in the corpora cardiaca of the brain complex, and has been shown to be a 18.7kD polypeptide monomer in *A. aegypti* (Borovsky and Thomas, 1985). In contrast to the stimulatory action of EDHN on ecdysteroid synthesis in ovaries, an oostatic factor has been isolated from the ovaries of *A. aegypti* able to prevent vitellogenesis, and therefore, egg development, by blocking the ecdysteroid production by the ovaries (Borovsky , 1985). The action of these factors at the cellular level has yet to be elucidated.

In addition to the extrinsic control mediated on the ecdysteroid biosynthetic pathway by ecdysiotropic factors, intrinsic control of the ecdysteroid biosynthetic pathway has also been proposed. This has been observed in the prothoracic glands of *P. brassicae* (Beydon and Lafont, 1983), *Mamestra configurata* (Bodnaryk, 1986) and *M. sexta* (Sakurai and Williams, 1989) where the terminal products of the larval and pupal ecdysteroid biosynthetic pathway, ecdysone and 20-hydroxyecdysone, have been found to exert a feedback inhibition effect on ecdysteroid biosynthesis of the gland (see Chapter 3).

1.7. Exogenous inhibitors of ecdysteroid synthesis

The ecdysteroid biosynthetic pathway has been shown to be susceptible to inhibition by a number of agents. Several acetyl cholesterol derivatives containing an acetylenic functional group at the C-22 position have been shown to inhibit ecdysteroid synthesis in *L. migratoria* prothoracic glands *in vitro*. One of these derivatives was able to selectively and irreversibly inhibit the 22-hydroxylase (Burger *et al.*, 1987). Increasing the length of the C-22 acetylenic side chain or ensuring it was in the *R*-configuration further enhanced the inhibitory effect of these derivatives on ecdysteroid biosynthesis. (Mauvais *et al.*, 1993a). However, the conversion of the steroid nucleus of these acetyl derivatives to an ecdysone moiety did not further enhance their inhibitory effect (Mauvais *et al.*, 1991; 1993b).

The cytochrome P450-dependent ecdysone 20-monooxygenase has been extensively studied (Johnson and Rees, 1977b; Weirich *et al.*, 1984) and, consequently, has been used as a model system to test the effect of putative inhibitors of insect steroid hydroxylase activity. Recently, using such an *in vitro* system, a number of imidazole derivatives have been shown to inhibit the activity of the microsomal ecdysone 20-monooxygenase present in the fat body of *Neobellieria bullata* (Darvas *et al.*, 1992). A number of plant allelochemicals have also been shown to inhibit the ecdysone 20-monooxygenase of insects, including the ecdysis inhibitor, azadirachtin (Smith and Mitchell, 1988), the chitin synthetase inhibitor, plumbagin and its 2-demethyl derivative, juglone, (Mitchell and Smith, 1988), in addition to several plant flavonoids (Mitchell *et al.*, 1993). The non-steroidal ecdysone agonist, RH 5849, has also been found to inhibit the ecdysone 20-monooxygenase activity of *M. sexta* mid gut *in vitro* in a dose-related and apparently competitive manner (Keogh and Smith, 1991). The observation that RH 5849 completely inhibits the ecdysone 20-monooxygenase but has no effect on any of the terminal hydroxylase enzymes of ecdysone synthesis in *M. sexta* prothoracic

glands *in vitro*, suggests that RH 5849 acts as a specific ecdysone 20-monooxygenase substrate mimic and not a general cytochrome P450 inhibitor (Grieneisen *et al.*, 1993).

1.8. Aims of the project

The aims of this research project were to elucidate further the ecdysteroid biosynthetic pathway of insects and to investigate its inhibition.

Putative inhibitors of ecdysteroid biosynthesis were examined on well-characterised *in vitro* ecdysteroid biosynthetic systems of ovarian follicle cells and prothoracic glands of *L. migratoria* and the mitochondrial ecdysone 20-monooxygenase from the fat body of *S. littoralis*. The effect of these putative inhibitors were assessed on both endogenous ecdysteroid biosynthesis and the metabolism of radiolabelled ecdysteroid precursors in these insect systems.

The potential intermediacy of 3-dehydro-2,22,25-trideoxyecdysone in the ecdysteroid biosynthetic pathway of ovarian follicle cells of *L. migratoria* was investigated by both metabolic studies with the radiolabelled compound and the development of GC-MS techniques to detect its presence endogenously in the cells. 3-Dehydro-2,22,25-trideoxyecdysone has so far only been suggested as a precursor of 3-dehydroecdysone synthesis in larval prothoracic glands (Blais and Lafont, 1991; Dolle *et al.*, 1991; Grieneisen *et al.*, 1993) and the possibility of implicating it in the ecdysteroid biosynthetic pathway of a mature insect system, where no 3-dehydroecdysteroids have so far been detected would suggest a reassessment of the terminal stages of ecdysteroid biosynthesis in the mature insect.

Finally, *Spodoptera frugiperda* (SF21) and *M. brassicae* cell lines were investigated as possible ecdysteroid biosynthetic systems.

Chapter 2

Materials and Methods

2.1. Materials

2.1.1. Solvents

General Solvents

Methanol: redistilled; Department of Chemistry, University of Liverpool.

Ethanol: Redistilled; Department of Chemistry.

Cyclohexane: Sigma Chemical Company, St. Louis, U.S.A.

Chloroform: B.D.H. Ltd., Poole, Dorset, U.K.

Diethyl ether: dried over sodium-lead alloy and redistilled over reduced iron ; B.D.H. Ltd.

Petroleum ether: dried over sodium-lead alloy and redistilled; B.D.H. Ltd.

Tetrahydrofuran: dried over molecular sieve 4A and redistilled; FSA Laboratory Supplies, Loughborough, U.K.

Pyridine: dried over molecular sieve 4A and redistilled; Aldrich Chemical Company Ltd., Dorset, U.K.

Water: Glass redistilled.

HPLC Solvents

Methanol: HPLC Grade; F.S.A. Laboratory Supplies, Loughborough, England.

Acetonitrile: HPLC Grade; F.S.A. Laboratory Supplies.

Dichloroethane: HPLC Grade; F.S.A. Laboratory Supplies.

Hexane: H.P.L.C. Grade; F.S.A. Laboratory Supplies.

Gas Chromatography-Mass Spectrometry (GC-MS) Solvents

Hexane: HPLC Grade redistilled; F.S.A. Laboratory Supplies.

Ethyl Acetate: HPLC Grade; F.S.A. Laboratory Supplies.

2.1.2. Radiochemicals

[1α,2α-$^{3}H_2$]Cholesterol: (45.6Ci/mmol); Amersham International P.L.C., Amersham, England.

[23,24-$^{3}H_2$]Ecdysone: (89Ci/mmol); New England Nuclear (N.E.N.) Boston, U.S.A.

[22,23,24,25-$^{3}H_4$]2,22,25-Trideoxyecdysone: (107Ci/mmol); gift from Dr. C. Hetru and Prof. J.A. Hoffmann, University Louis Pasteur, Strasbourg, France.

[1,2-$^{3}H_2$]3-Dehydro-2,22,25-trideoxyecdysone: (47Ci/mmol); gift from Dr. C. Hetru and Prof. J.A. Hoffmann.

[22,23-$^{3}H_2$]2,22-Dideoxyecdysone: (59.5Ci/mmol); gift from Dr. C.Hetru and Prof. J.A. Hoffmann.

[23,24-$^{3}H_2$]2-Deoxyecdysone: (108Ci/mmol); gift from Dr. C. Hetru and Prof. J.A. Hoffmann.

[22,23-$^{3}H_2$]22-Deoxyecdysone: (59.5Ci/mmol): gift from Dr.T. Jarvis, Department of Biochemistry, University of Liverpool, U.K.

[23,24-$^{3}H_2$]3-Epiecdysone: (89Ci/mmol); gift from Dr.T.Cole, Department of Biochemistry, University of Liverpool, U.K.

[23,24-$^{3}H_2$]3-Dehydroecdysone: (89Ci/mmol); gift from Dr.T.Cole, Department of Biochemistry.

[23,24-$^{3}H_2$]3-Dehydro-2-deoxyecdysone: (108Ci/mmol); prepared as described in Chapter 6.

[23,24-$^{3}H_2$]3-Epi-2-deoxyecdysone: (108Ci/mmol); prepared as described in Chapter 6.

[22,23-$^{3}H_2$]3-Dehydro-2,22-dideoxyecdysone:(59.5Ci/mmol); prepared as described in Chapter 6.

2.1.3. Ecdysteroids

Ecdysone: Simes, Milan, Italy.

20-Hydroxyecdysone: gift from Dr. G.B. Russell, D.S.I.R., Palmerston North, New Zealand.

2-Deoxyecdysone: isolated by Dr. R.E.Isaac, University of Liverpool.

Ecdysone 22-phosphate: isolated from the eggs of *S. gregaria.*

2-Deoxyecdysone 22-phosphate: isolated from the eggs of *S. gregaria.*.

3-Dehydroecdysone: gift from Dr.T.Cole, Department of Biochemistry.

3-Epiecdysone: gift from Dr.T.Cole, Department of Biochemistry.

3-Epi-2-deoxyecdysone: gift from Dr. G.B. Russell, D.S.I.R., Palmerston North, New Zealand.

3-Epi-20-hydroxyecdysone: gift from Dr. L.N. Dinan, Department of Biochemistry

2-Deoxy-20-hydroxyecdysone : isolated by Dr. M. Kabbouh.

2,22,25-Trideoxyecdysone : gift from Dr. Chen Yu-Gun, Shanghai Institute of Organic Chemistry, P.R. China.

22,25-Dideoxyecdysone: Zoecon Corporation, California, U.S.A.

3-Dehydro-2,22,25-trideoxyecdysone : prepared as described in Chapter 7

5α-2-Deoxyecdysone: prepared as described in Chapter 6.

2.1.4. Enzymes

Collagenase: from *Clostridium histolyticum* (570 units/mg); Sigma Chemical Company, St. Louis, U.S.A.

Crude Aryl Sulphatase: from *Helix pomatia* (22.9 units/mg); Sigma. The preparation is known to contain phosphatase activity (Isaac *et al.*, 1982).

2.1.5. Antisera

DHS-1-13.5 was kindly donated by Prof. J.D. O`Connor, University of California, L.A., U.S.A.

H-22 was a gift from Prof. L.I.Gilbert, University of North Carolina, U.S.A.

2.1.6. Sterols

Cholesterol: Sigma Chemical Company, Poole, Dorset, U.K.

Lanosterol: Sigma.

Cholesteryl Palmitate: Sigma.

5α-Cholestan-3β-ol: Sigma.

Stigmasterol: Sigma.

Sitosterol: Sigma.

Campesterol: Sigma.

Cycloartenol and 24-methylenecycloartanol : extracted from hydrolysed Oryzanol (as described in chapter 4), a gift from Dr. T. Akahisi, Nihon University, Tokyo, Japan to Dr. L.J. Goad, Department of Biochemistry, University of Liverpool.

2.1.7. Inhibitors

Ketoconazole: gift from I.C.I Agrochemicals, Bracknell, Berkshire, U.K.

Imazalil: gift from I.C.I. Agrochemicals.

ICI-U-384: gift from I.C.I Agrochemicals.

ICI-U-166: gift from I.C.I. Agrochemicals.

ICI-U-634: gift from I.C.I Agrochemicals.

ICI-L-209: gift from I.C.I. Agrochemicals.

ICI-L-520: gift from I.C.I. Agrochemicals.

ICI-L-635: gift from I.C.I. Agrochemicals.

Buprofezin: gift from I.C.I. Agrochemicals.

Propiconazole: gift from Rhone Poulenc Ltd., Ongar, Essex, U.K.

Azaconazole: gift from Janssen Pharmaceuticals.

KK42: gift from Professor E. Kuwano, the Department of Agricultural Chemistry, Kyushu University, Fukuoka, Japan.

2.1.8. Buffers

M.E.S. Buffer: 0.2M 2-(*N*-morpholino)ethane sulphonic acid (BDH Limited, Poole, Dorset, U.K.), adjusted to pH 5.4 with sodium hydroxide.

Borate Buffer: 0.1M (pH 8.4); 0.1M boric acid, 0.025M sodium tetraborate, 0.075M sodium chloride.

HEPES Buffer: 0.037M HEPES [*N*-(2-hydroxyethyl)piperazine-*N*-(2-ethane sulphonic acid)], 0.1M potassium flouride, 0.3M sucrose(isotonic buffer), 0.05M sucrose (hypotonic buffer), adjusted to pH 7.4 with sodium hydroxide.

Sodium phosphate Buffer: 100mM (pH 7.4); 16.8mM sodium di-hydrogen orthophosphate, 80mM di-sodium hydrogen orthophosphate, 50mM potassium chloride, 2mM 2-mercaptoethanol, 0.25M sucrose (isotonic buffer), 0.05M sucrose (hypotonic buffer).

Tris-HCl Buffer: 10mM (pH7.4).

2.1.9. Culture media

T.C.100 Medium: containing 10% (v/v) foetal calf serum : Gibco Ltd., Paisley, Scotland.

Graces Insect T.C. Medium : Gibco Ltd.

Murashige and Skoog Basal Plant Culture Medium: Murashige and Skoog basal medium (4.0g/l; Sigma Chemical company, Poole, Dorset, U.K.), glucose (30.0g/l), Agar (1%(v/v).

Landureau's Medium (Y4 Medium); prepared using the following for 1litre of medium:

Salts and Sucrose:

Sodium chloride (9.5g),

Potassium chloride (1.0g),

Magnesium sulphate (0.3g),

Magnesium chloride (0.8g),

Manganese sulphate (0.1g),

Glucose (0.5g),

Sucrose (2.0g),

Choline-HCl (0.1g).

Buffer:

Tris-base (2.0g),

Potassium dihydrogen orthophosphate (0.2g),

Disodium hydrogen orthophosphate (1.2g),

Sodium hydrogen carbonate (0.2g).

Vitamins solution:

Folic acid (0.2g),

Biotin (0.1g),

Calcium pantothenate (2.0mg),

Cyanocobalamin (0.4mg),

Inositol (1.0mg),

Pyridoxal-HCl (0.5mg),

Riboflavin (4.0mg),

Thiamine (16.0mg).

Antibiotics Solution:

Streptomycin (63.0mg),

Penicillin-G (125.0mg).

L-Amino Acids Solution:

Glycine (0.5g),

Glutaminic acid (0.5g),

Arginine-HCl (0.2g),

Cysteine-HCl (0.2g),

Histidine (0.2g),

Isoleucine (0.2g),

Leucine (0.4g),

Lysine-HCl (0.3g),

Methionine (0.1g),

Phenylalanine (0.1g),

Proline (0.1g),

Threonine (0.2g),

Tryptophan (0.1g),

Valine (0.1g),

Serine (0.1g),

Asparagine (0.2g),

Glutamine (0.2g).

Precursors of Nucleic Acids:

Adenine (7.0mg),

Hypoxanthine (7.0mg),

Uridine (25.0mg),

Deoxycytidine (11.0mg)

Tyrosine: (200.0mg).

Calcium chloride: (0.3g).

Bovine Serum albumin (BSA): (2.0g).

Foetal bovine serum: (2.0% (v/v).

Phenol Red: (0.5% (v/v).

Trehalose; (3.0g).

pH adjusted to 7.3

2.1.10. Chromatography

Kieselgel (60G and 60H): Merck Darmstadt, Germany, both used to prepare T.L.C. plates.

Kieselgel F_{254} aluminium backed pre-prepared T.L.C. plates : Merck.

C_{18} Sep-Pak cartridges: Millipore (U.K.) Ltd., Watford, Herts, U.K.

Kieselgel 60: Merck, was used for silicic acid column chromotography.

Alumina: Woelm Pharma.F.R.G., used for Alumina column chromotography.

C_{18} Nova-Pak and amino-propyl silica HPLC cartridges (10 x 8mm, i.d. 4μm particle size) : Millipore.

2.1.11. Liquid Scintillation Fluid

Optiphase `Safe` and Optiphase `Hisafe`: FSA Supplies, Loughborough, Leics., U.K.

Flo-Scint III flow-detection scintillation fluid: Canberra-Packard, Pangbourne, Berks., U.K.

2.1.12. Derivatisation Reagents

***O*-Methylhydroxylamine hydrochloride (MOX)** : Fluka Chemicals Ltd., Dorset, England.

***N*, *O*-bis(Trismethylsilyl)triflouroacetamide (BSTA)** : Pierce, Rockford, U.S.A.

***N*-Trimethylsilylimidazole (TMSI)** : Pierce.

2.1.13. Other Chemicals

Sodium borohydride and platinum (1V) oxide: Aldrich Chemical Co.

All other reagents used in this project were of high analytical grade; purchased from Sigma, BDH Ltd and Aldrich Chemical Co.

2.1.14 Insects and Cell Lines

***S. littoralis*: (cotton leaf worm)** obtained from Rhone - Poulenc, Ongar, Essex.

***L. migratoria*: (migratory locust)** obtained from Timstar, Biological Suppliers Ltd., Cheshire, England.

***S. frugiperda*-21 (Sf21) cell line:** kindly provided by Dr. R. Posse, Institute of Virology, Oxford, U.K. Derived from pupal ovary (Vaughn *et al.*, 1977)

***M. brassicae* (Mb1203) cell line:** Kindly provided Dr. R. Posse , Institute of Virology, Oxford, U.K. Derived from the ovary and dorsal vessel of fourth/fifth instar larvae (Miltenburger and David, 1976).

2.2. General experimental methods

2.2.1. Maintenance of insect cultures and cell lines

S. littoralis were maintained at 28°C and a relative humidity of 60-70% under a lighting regime of 8 hour light and 6 hour dark. Larvae were fed on a wheat germ agar-based diet (Hoggard, 1989) ; adults were fed on a 5% (v/v) sucrose solution.

L. migratoria were maintained at 30-35°C during the 12 hour light period, and at 27°C during the 12 hour dark period. The insects were fed on wheat bran and cabbage.

M. brassicae and *S. frugiperda*-21 (SF-21) cell lines were grown in TC100 medium containing 10% (v/v) foetal calf serum supplemented with 0.4% (v/v) fungizone and 1%

(v/v) penicillin-streptomycin at 29°C. Passaging of cells was carried out at weekly intervals.

2.2.2. Preparation of ovarian follicle cells from *L. migratoria*

Ovaries of vitellogenic adult female *L. migratoria* were exposed by a dorsal incision along the length of the abdomen. Terminal oocytes (4.5-5.7mm) with their follicular epithelium were then excised individually from the ovary, combined and incubated with collagenase (1600U in 1ml Landureau`s medium/insect) for 10min at 32°C. The tissue was further dispersed by repeated agitation with a silanised Pasteur pippette and the yolk was removed by low-speed centrifugation (150g for 5min at 4°C). The resulting follicle cell pellet was rinsed twice with Landureau`s medium and resuspended in fresh medium.

2.2.3. Preparation of the mitochondrial fraction from fat body of *S. littoralis*

Fat body was removed from seven *S. littoralis* larvae 88h into the sixth instar, a time which corresponds to high ecdysone 20-hydroxylase activity within the tissue (Milner, 1984), by a dorsal incision made along the body cavity, and stored on ice in isotonic HEPES buffer, pH 7.4. The fat body was then homogenised by 20 passes of a Potter-Elvehjem homogeniser, the homogenate was centrifuged at 600g for 5min at 4°C in a µP Koolspin centrifuge (Burkard Scientific Ltd, Middlesex, U.K.).The supernatant was collected and centrifuged at 10,000 g for 20min at 4°C. The pellet was resuspended in hypotonic HEPES buffer, (pH 7.4; 1.2ml) and homogenised by ten passes in a teflon homogeniser. The homogenate was left for 40min at 0°C before use.

2.2.4. Preparation of the cytosolic fraction from the midgut of *S. littoralis*

Midguts were excised from fifteen *S. littoralis* larvae by a dorsal incision made along the length of the body cavity. The food boluses were removed by gentle squeezing of the contents of the gut, which were then thoroughly homogenised by 24 passes of a Potter-Elvehjem homogeniser in 10mM Tris-HCl buffer, pH 7.4. The homogenate was centrifuged at 10,000g for 20 min at 4°C. The pellet was resuspended in Tris-HCl buffer, rehomogenised and centrifuged as before. The resulting supernatants were combined and centrifuged at 100,000g for 1h at 4°C, and the supernatant (cytosol fraction) was dialysed against 2L of Tris-HCl buffer for 18 h at 4°C before use.

2.2.5. Thin-layer chromatography (TLC)

Free sterols were separated on Kieselgel 60G (0.25mm or 0.5mm) TLC plates, and steryl acetates were separated on Kieselgel 60H (0.25mm) TLC plates impregnated with 5% (w/w) silver nitrate TLC plates. Plates were developed in chloroform, and the bands were visualised under U.V. light after spraying with 1% (w/v) berberine sulphate in methanol/acetone (1:1).

Ecdysteroids (2,22,25-trideoxyecdysone and 3-dehydro-2,22,25-trideoxyecdysone) were separated on Kieselgel F_{254} TLC plates developed using 20% (v/v) methanol in chloroform and the bands were visualised directly under U.V. light.

2.2.6. Silicic acid column chromatography

Columns were prepared using Kieselgel 60 suspended in chloroform (5:1 column height to width , 1g silica/10mg of sample). Samples were adsorbed on to Celite and applied to the top of the columns. The columns were eluted sequentially (10 x weight of silica) with chloroform (elutes apolar materials), 30% (v/v) methanol in chloroform (elutes free ecdysteroids) and methanol (elutes polar ecdysteroids).

2.2.7. C_{18} Sep-Pak fractionation

Procedure 1 :

Cartridges were activated with methanol (5ml) and washed with water (10ml). The sample (no more than 5mg) was applied in 10% (v/v) methanol in water (1ml), and then eluted sequentially with 10% (v/v) methanol in water (3ml; elutes salts and protein), 30% (v/v) methanol in water (4ml ; elutes polar ecdysteroid fraction), 60% (v/v) methanol in water (6ml ; elutes free ecdysteroid fraction) and methanol (6ml ; elutes apolar ecdysteroid fraction).

Procedure 2:

The sample was applied in water (1ml) to an activated cartridge and then eluted sequentially with water (2ml), 25% (v/v) methanol in water (3ml) and methanol (6ml).

In the case of the [^{3}H]3-dehydro-2,22,25-trideoxyecdysone samples, the following different procedure was used.

2.2.8. Hlgh-performance liquid chromatography (HPLC)

Ecdysteroids were analysed by reversed-phased HPLC (RP-HPLC) (systems 1-7) using a C_{18} Z-module Nova-Pak cartridge (10cm x 8mm i.d. ; 10µm pore size) or by adsorption HPLC (systems 8-9) using a µBondapak-NH2 cartridge (10cm x 8mm i.d. ; 10µm pore size), using a Waters Model 440 U.V. detector set at 254nm. Radioactivity was detected by an on-line scintillation monitor, using a Radiomatic A250 scintillation monitor (Canberra Packard), using Flo-scint III scintillation fluid.

<u>Reversed-phase systems</u>

System 1: A linear gradient of 35% (v/v) methanol in water to methanol over 30min at 1ml/min.

System 2: 0.1% (v/v) trifluoroacetic acid in acetonitrile, isocratic at 1ml/min.

System 3: 20% (v/v) acetonitrile in water, isocratic at 1ml/min.

System 4: A linear gradient of 20% (v/v) acetonitrile in water to 30% (v/v) acetonitrile in water over 20min at 1ml/min.

System 5: 40% (v/v) acetonitrile in water, isocratic at 1ml/min.

System 6: 60% (v/v) acetonitrile in water, isocratic 1ml/min.

System 7: A linear gradient of 80% (v/v) acetonitrile in water to acetonitrile over 30min at 1ml/min.

Adsorption systems:

System 8: 8% (v/v) methanol in 1,2-dichloroethane, isocratic at 1ml/min.

System 9: 6% (v/v) methanol in 1,2-dichloroethane, isocratic at 1ml/min.

2.2.9. The retention times of various ecdysteroids on reversed-phase HPLC

ECDYSTEROID	RETENTION TIME (min)
	*R.P.-HPLC SYSTEM 1**
2,22,25-Trideoxyecdysone	34.2
3-Dehydro-2,22,25-trideoxyecdysone	34.2
22,25-Dideoxyecdysone	32.9
2,22-Dideoxyecdysone	28.9
22-Deoxyecdysone	26.5
2-Deoxyecdysone	20.5
2-Deoxy-20-hydroxyecdysone	18.2
Ecdysone	15.6
20-Hydroxyecdysone	12.3

* For details of HPLC system see section 2.2.8.

2.2.10. Derivatisation

Preparation of methoxime-trimethylsilyl (MO-TMSI) derivative of ecdysone for gas chromatography - mass spectrometry (GC-MS)

8% (v/v) O-methoxylamine hydrochloride in dry pyridine (100µl) was added to ecdysone (10µg) and heated for 30min at 80°C in a 2-dram sample vial. Pyridine was removed under N_2, leaving the methoxime-ecdysone derivative.

TMSI (80µl) was added to the sample under a constant stream of N_2. The sample was then heated for 15h at 100°C. The MO-TMS ecdysone derivative was purified by adsorption chromotography using an alumina column (1.5g Woelm Brockmann Grade 1, neutral), prepared in a Pasteur pipette plugged with glass wool. The column was prewashed successively with 30% (v/v) ethylacetate in hexane (2ml) and hexane (2ml). Hexane (20µl) was added to the derivatised sample, which was loaded on to the column, and eluted with 30% (v/v) ethyl acetate in hexane. The eluent was reduced to dryness under N_2 and redisolved in a minimum volume of hexane (5-10µl) prior to GC-MS analysis.

Preparation of sterols for gas chromatography (GC) analysis

Free sterols (10-100µg) were converted into trimethylsilylethers by addition of BSTFA (1 drop) and dry pyridine (1 drop) and heated for 1 hour at 60°C.

2.2.11. Gas-liquid chromatography (GC)

Analysis of sterols was performed on a Hewlett Packard (5890, Series II) gas chromatograph equipped with a flame ionisation detector interfaced with a Spectra Physics SP427 integrator. Samples in cyclohexane were introduced by an on-column

injector onto a fused silica BP5 capillary column (25 x 0.25mm i.d. x 0.25μm film thickness; S.G.E., Milton Keynes, U.K.). The gas chromatograph oven was programmed to rise from 50°C to 120°C at a rate of 50°C/min, being maintained at this temperature for 1min, then programmed to 280°C at a rate of 10°C/min before being held isothermally for 35min.

2.2.12. Gas chromatography-mass spectrometry (GC-MS)

GC-MS of ecdysteroids was carried out on a Pye-Unicam 204 gas chromatograph coupled to a VG Micromass 7070H mass spectrometer. Samples were introduced in a solution of hexane via a S.G.E. OC1 III on-column injector onto a fused-silica BP1 capillary column (25m x 0.22mm i.d. x 0.1μm film thickness ; S.G.E Ltd, U.K.). The gas chromatograph oven temperature was raised ballistically from ambient temperature to 200°C, then programmed to 320°C at a rate of 12°C/min before being held isothermically. The GC-MS interface incorporated a wide bore re-entrant jet allowing the capillary column to feed directly into the ion source. In the case of the MO-TMS ecdysone derivatives, full electron impact (EI) spectra were taken (m/z 90-1000).

2.2.13. Mass spectrometry (MS)

Electron impact mass spectrometry (EIMS) was carried out on a VG Micromass 7070H mass spectrometer at an electron energy of 20eV.

Thermospray ionisation mass spectrometry (TSP/MS) was carried out on a VG Quattro quadrupole mass spectrometer. The source and thermospray probe temperature were maintained at 250°C and 190-210°C, respectively. The repeller voltage was varied between 150 and 270V. Positive ion full scan mass spectra were taken in the range *m/z* 80-550 in a scan time of 2s. Selected ion monitoring was undertaken for a range of ecdysteroids and 3-dehydroecdysteroids.

Electrospray mass spectrometry (ES/MS) was performed on a VG Quattro quadrupole MS in the positive ion ESI mode. The source temperature was maintained at 70°C, with the sampling cone voltage at 50V. The capillary tip voltage was in the range of 3 to 3.5kV, with a counter electrode voltage of 0.6kV, and the ion energy between 0 and 1V. Positive ion full scan mass spectra were taken in the range *m/z* 40-700, in a scan time of 2s.

2.2.14. High performance liquid chromatography-thermospray ionisation mass spectrometry (HPLC-TSP/MS)

HPLC-TSP/MS was performed using a Waters 600-MS solvent delivery system. A CAP (continuously adjustable piston) column (25m x 4.6mm i.d.) containing a reversed-phase Econosphere C_{18} microparticulate packing (5µM; Alltech Assoc. U.K.) eluted with either a linear gradient of 35% (v/v) methanol in water to 90% (v/v) methanol in water containing 0.1M ammonium acetate over 30min. at 1ml/min (system A), or solvent system 7, see section 2.2.8 containing 0.1M ammonium acetate (system B). Detection of eluting ecdysteroids was by a Waters 486 variable wavelength U.V/visible detector set at 254nm positioned between the column and the mass spectrometer. The detector was connected directly to the thermospray interface of the mass spectrometer.

2.2.15. Hydrolysis of ecdysteroid conjugates

Polar ecdysteroid conjugates were dissolved in M.E.S. buffer, pH 5.4 and incubated with so-called crude arylsulfatase at a concentration of 250 I.U./ml for 18h at 37°C. The reaction was terminated by addition of ethanol (2ml), and the precipitated protein was sedimented by centrifugation (600g). The resulting supernatant was blown down under N_2, redissolved in water or 10% (v/v) methanol in water and applied to a C_{18} Sep-Pak.

2.2.16. Protein assay

Protein was estimated by the Bradford assay (Bradford, 1976), using bovine serum albumen (B.S.A) as the protein standard.

2.2.17. Radioimmunoassay (RIA)

DHS1-13.5 antiserum was raised against 20-hydroxyecdysone 2-succinylthyroglobulin amide, and showed greater specifity for the side chain rather than the nucleus of the ecdysteroid molecule (Soumoff *et al.*, 1981). The H-22 antiserum was raised against 20-hydroxyecdysone 22-succinylthyroglobulin, and showed greater specificity for the ecdysone nucleus than the side chain (Horn *et al.*, 1976).

A series of duplicate standard tubes containing increasing amounts ecdysone in the range 0-1000pg were set-up along with three tubes containing no ecdysone and sample tubes containing unknown amounts of ecdysone. Solvent was removed from the tubes in a Gyrovap Centrifugal Evaporator (Uniscience Ltd.). [^{3}H]Ecdysone (100µl in 0.1M borate buffer, pH 8.4; 9000 cpm/ tube) was added to each tube, which were mixed and the diluted antiserum (100µl/tube diluted 1:1500 in 5%(v/v) inactivated rabbit serum in borate buffer) was added. The tubes were gently mixed and incubated for 18h at 4^{o}C. After this time, saturated ammonium sulphate in borate buffer (200µl) was added to each tube, mixed and stood at 4^{o}C for 40min. The tubes were then centrifuged (10,000g for 7min at 4^{o}C), and the supernatant withdrawn and discarded. The pellet was resuspended in 50% (v/v) saturated ammonium sulphate solution in borate buffer and left to stand for 20min at 4^{o}C, and then centrifugation and supernatant withdrawal were repeated. Water (100µl) was then added to each tube (to dissolve the protein pellet) followed by scintillation fluid (1ml; Optiphase `Hisafe` II). After mixing, the tubes were placed in scintillation vials for determination of their radioactivity.

2.2.18. Ecdysone 20-monooxygenase assay

The standard assay for the ecdysone 20-monooxygense activity in the mitochondrial fraction of *S. littoralis* fat body contained the following in hypotonic HEPES buffer, pH 7.4; total volume 300µl : [^{3}H]ecdysone (0.14µCI; 0.23pmole), unlabelled ecdysone (85pmole) , NADPH (6µmole), mitochondrial fraction to be assayed (50µl) and additional buffer (250µl). After incubation at 37°C for 20min, the reaction was terminated by the addition of ethanol (1ml). The samples were then centrifuged (8,800g for 10min at 4°C) and the supernatant removed and reduced to dryness under N_2.

2.2.19. Follicle cell incubation conditions

Ovarian follicle cells from *L. migratoria* (prepared as described in section 2.2.2) were incubated in duplicate (0.5 ovary equivalents/sample vial) in Landureau`s medium (0.5ml) for 8h (or otherwise stated) at 37°C with or without labelled substrate present. Substrates were solubilized in Landureau`s medium by extensive vortexing, unless otherwise stated, before being introduced into the incubation vial. The reaction was terminated by the addition of ethanol (0.5ml).

2.2.20. Silanisation of glassware

All glassware, unless otherwise stated was subject to silanisation before experimental use throughout this research project.

Glassware was rinsed with dimethyldichlorosilane solution (about 2% (v/v) in 1,1,1-trichloroethane ; B.D.H. Ltd.), and baked at 100°C for one hour. The glassware was then rinsed with hexane and baked as before, followed by a final rinse with distilled water and dried prior to use.

Chapter 3

An investigation into possible feedback regulation in the ecdysteroid biosynthetic pathways of *L. migratoria* ovarian follicle cells and prothoracic glands *in vitro*

CHAPTER 3

3.1. Introduction

The prothoracic glands are the source of ecdysteroids in larval and pupal stages of insect development. This has been demonstrated through numerous *in vitro* studies (Chino *et al*., 1974; King *et al*., 1974; Hirn *et al*., 1979). Ecdysone, once released into the haemolymph is converted into the generally more active moulting hormone, 20-hydroxyecdysone by several peripheral tissues including the fat body and Malpighian tubules.

The ovaries of adult insects have been shown to be a site of ecdysteroid synthesis in several insect species (Hagedorn *et al*., 1975; Zhu *et al*., 1983; Smith and Bollenbacher *et al.*, 1985). In the case of adult *L. migratoria,* whose ovaries have been shown to synthesise large quantities of ecdysteroids under *in vitro* culture conditions (Lagueux *et al.*, 1977), the actual site of this ecdysteroid synthesis has been shown to be the ovarian follicle cells located in the epithelium surrounding the terminal oocytes (Goltzene *et al.*, 1978; Glass *et al.*, 1978).

Of the total ecdysteroid content of the ovary, 98% is present in the terminal oocyte (Lagueux *et al*., 1977; Lagueux *et al*., 1979). More than 95% of this ecdysteroid consists of phosphate conjugates of 2-deoxyecdysone and ecdysone in the ratio of 2:1, the remainder being composed mostly of their respective free forms (Lagueux *et al*., 1981). It is surmised that the ecdysteroid conjugates act as storage forms of ecdysteroids in newly laid eggs, being metabolised to free ecdysteroids during embryonic development (Lagueux *et al.*, 1981).

The ecdysteroid 2,22,25-trideoxyecdysone has been found endogenously in the ovaries of *L. migratoria* at a time of ecdysteroid synthesis (Hetru *et al.*, 1978; 1982), and can be synthesized from radiolabelled cholesterol when ovarioles are incubated *in vitro* (Hetru *et al.*, 1982). The intermediacy of this compound in the ovarian ecdysteroid

biosynthetic pathway has been confirmed by the ability of *L. migratoria* ovarian follicle cells to synthesize ecdysone and polar ecdysteroid conjugates from radiolabelled 2,22,25-trideoxyecdysone *in vitro* (Kappler *et al.*, 1986a). In addition *L. migratoria* prothoracic glands have been demonstrated to convert radiolabelled 2,22,25-trideoxyecdysone into ecdysteroids (Meister *et al.*, 1985). However, recently, a 3-dehydro derivative of 2,22,25-trideoxyecdysone, 3-dehydro-2,22,25-trideoxyecdysone has been shown to be efficiently incorporated into ecdysteroids by *L. migratoria* prothoracic glands *in vitro* (Dolle *et al.*, 1991; Roussel, 1992a)

Feedback regulation of ecdysteroid biosynthesis has been studied previously in the pupae of *P. brassicae* where exogenously applied 20-hydroxyecdysone and to a lesser extent, ecdysone were found to inhibit ecdysteroid synthesis in prothoracic glands *in vitro*. 20-Hydroxyecdysone appeared to elicit its effect by direct action on the prothoracic gland (Beydon and Lafont, 1983). Injection of 20-hydroxyecdysone into the pupae of *M. configurata* also caused a similar inhibitory effect on ecdysteroid production by the insect (Bodnaryk, 1986). In *M. sexta* prothoracic glands of high activity (larvae, pupae and developing pupae) were inhibited by ecdysone and 20-hydroxyecdysone. Low activity prothoracic glands (feeding larvae, non-diapausing pupae and diapausing pupae) were activated by both ecdysone and 20-hydroxyecdysone *in vivo* and *in vitro* (Sakurai and Williams, 1989). This suggests that the stage of insect development is critical as to whether positive or negative feedback regulation occurs.

The work described in this chapter had a two-fold aim:

Firstly, to investigate possible feedback regulation in the late stages of the ecdysteroid biosynthetic pathway of *L. migratoria* using an *in vitro* ovarian follicle cell system. Possible feedback regulation in the later stages of the ecdysteroid biosynthetic pathway was suggested by Jarvis (1991), from the effect of the ecdysteroid phosphates, ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate on the incorporation of radiolabelled 2,22,25-trideoxyecdysone into ovarian follicle cells, with the 22-hydroxylase enzyme being indicated as the prime site of this late control in the ecdysteroid biosynthetic

pathway. The current work was an extension of that study of a possible late control point in the ecdysteroid pathway involving the incorporation of radiolabelled ecdysteroids (2,22,25-trideoxyecdysone, 2,22-dideoxyecdysone and 2-deoxyecdysone) into ecdysteroids in the follicle cells *in vitro* in the presence of the ecdysteroid products of the biosynthetic pathway. This work was supplemented by an attempt to incorporate radiolabelled cholesterol into follicle cells *in vitro*, in addition to a study of the effect of the exogenous ecdysteroid phosphates, ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate on the endogenous ecdysteroid synthesis of follicle cells *in vitro*.

Secondly, to develop a viable *in vitro* ecdysteroid biosynthetic system from the prothoracic glands of larval *L. migratoria* in order to study possible feedback regulation in the ecdysteroid biosynthetic pathway of prothoracic glands.

3.2. Experimental and results

3.2.1. The incorporation of [^{3}H]2,22,25-trideoxyecdysone into ovarian follicle cells of *L. migratoria* in the presence of exogenous ecdysone and 20-hydroxyecdysone

In the first part of this chapter the aim is to substantiate evidence for a feedback effect on the later stages of the ecdysteroid biosynthetic pathway by the end products, owing to the low incorporation of [^{3}H]cholesterol into ecdysteroids the intermediate 2,22,25-trideoxyecdysone was chosen as a substrate for this study. Since potentially, ecdysone and 20-hydroxyecdysone, and the 22-phosphate conjugates of ecdysone and 2-deoxyecdysone could be regarded as the end products of the biosynthetic pathway, all of these compounds were examined for a potential feedback effect. The concentration of exogenous ecdysteroids added to the *in vitro* cultures was based on the concentrations of ecdysone and 20-hydroxyecdysone responsible for eliciting a feedback effect on *M. sexta* prothoracic glands *in vitro* (Sakurai and Williams, 1989). Also, the exogenous ecdysteroid concentration present in the culture medium was in the endogenous

ecdysteroid synthetic range of the *L. migratoria* ovarian follicle under the *in vitro* culture conditions (see section 3.2.7).

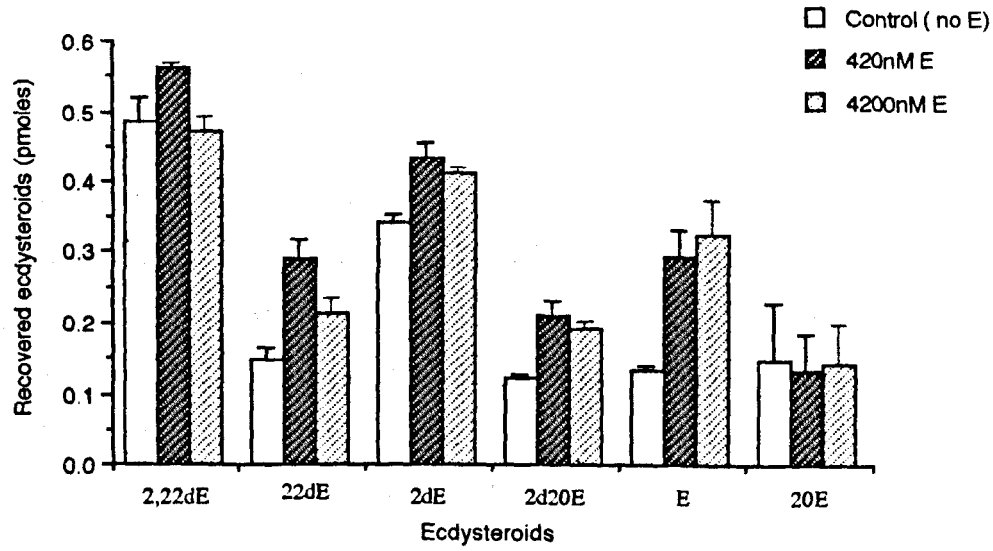
Over an eight hour period the concentration of ecdysteroids present in *L. migratoria* ovarian follicle cells have been shown to increase five fold under *in vitro* culture conditions, with their ecdysteroid secretory activity increasing proportionately for the first 8 hours in culture, before reaching a plateau after approximately 12 hours (Lagueux *et al.*, 1984).

Follicle cells from 5 adult females were prepared as described in section 2.2.2. The resulting follicle pellet was incubated as described in section 2.2.19 with [^{3}H]2,22,25-trideoxyecdysone (0.5μCi; 107Ci/mmol) in the presence of ecdysone and 20-hydroxyecdysone, both at 420nM and 4200nM final concentrations. The reaction was terminated by the addition of ethanol (0.5ml), and the contents of the vials were extracted 3 times in methanol (2ml) in a ground glass hand-held homogeniser. The extracts were hydrolysed by crude aryl sulfatase treatment (125 I.U./0.5ml) and subjected to C_{18} Sep-Pak fractionation (see section 2.2.7, procedure 1). The 60% (v/v) methanol in water fractions and the methanol fractions were analysed by reversed-phase HPLC (RP-HPLC; system 1, see section 2.2.8) with injection of authentic standards; for retention times see section 2.2.9.

Fig 3.1 shows the effect of (1) ecdysone and (2) 20-hydroxyecdysone (each at 420nM and 4200nM, final concentrations) on the metabolism of [^{3}H]2,22,25-trideoxyecdysone in follicle cells. The results are expressed as pmoles of ecdysteroid recovered from RP-HPLC (system 1, see section 2.2.8), and are the mean of duplicate incubations with the range shown.

When referring to concentrations these will be a minimum value because values are worked out assuming no dilution of radioactive substrate with endogenous intermediates. In practice this will not be the case; however this method of expressing

(1) Ecdysone



(2) 20-Hydroxyecdysone

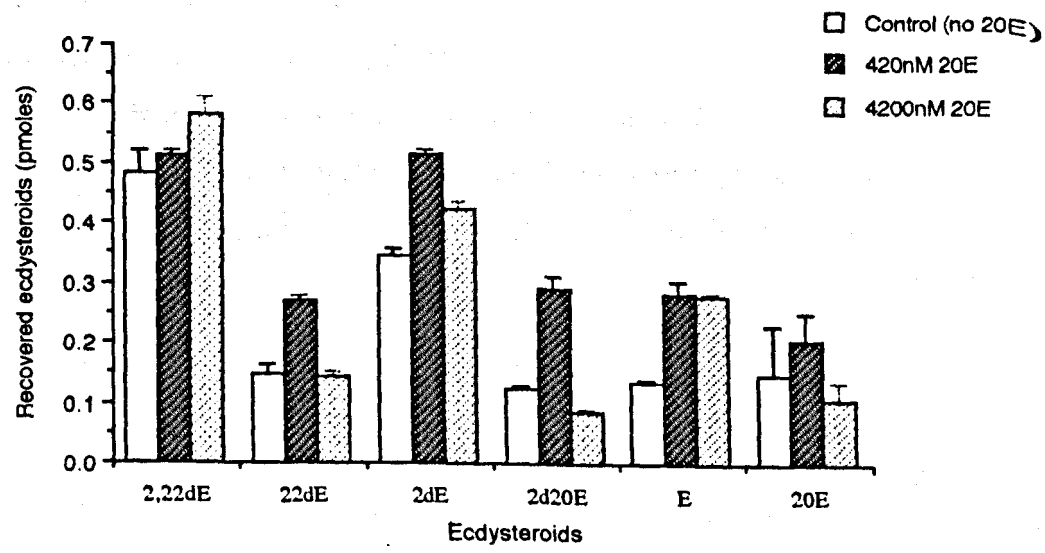


Fig. 3.1. The effect of (1) ecdysone and (2) 20-hydroxyecdysone (each at 420nM and 4200nM, final concentrations) on the metabolism of [^{3}H]2,22,25-trideoxyecdysone in follicle cells, expressed as pmoles of recovered ecdysteroids from RP-HPLC (system 1, see section 2.2.8). Results are the mean values of duplicate incubations with the range shown.

the results gives some indication of the level and of the extent of the transformations. The conclusion would be valid since results of the test incubation are compared to those of the controls.

In the follicle cell incubations containing the terminal hydroxylation products (ecdysone and 20-hydroxyecdysone) the overall rates of conversion were much higher than in the control (no exogenous ecdysteroid). The metabolites were identified as, 2,22-dideoxyecdysone (2,22dE), 22-deoxyecdysone (22dE), 2-deoxyecdysone (2dE), 2-deoxy-20-hydroxyecdysone (2d20E), ecdysone (E) and 20-hydroxyecdysone (20E). by co-chromatography with marker compounds on RP-HPLC (system 1 see section 2.2.8). The addition of exogenous ecdysone (420nM and 4200nM) to the cell preparations caused the amount of all metabolites (apart from 20-hydroxyecdysone and 2,22-dideoxyecdysone in the presence of the higher concentration of ecdysone) to increase significantly compared to the amount present after the incubation in the absence of any terminal hydroxylation products. Similarly the addition of exogenous 20-hydroxyecdysone (420nM and 4200nM) generally caused the amount of metabolites to increase significantly compared to the level present in the absence of terminal end products. However, the amount of 2-deoxy-20-hydroxyecdysone and 20-hydroxyecdysone fell in the presence of the higher concentration of 20-hydroxyecdysone(4200nM). The effect of ecdysone and 20-hydroxyecdysone were not pursued further, since no apparent inhibition of ecdysteroid synthesis was observed.

3.2.2. The incorporation of [^{3}H]2,22,25-trideoxyecdysone into ovarian follicle cells in the presence of exogenous 2-deoxyecdysone 22-phosphate and ecdysone 22-phosphate

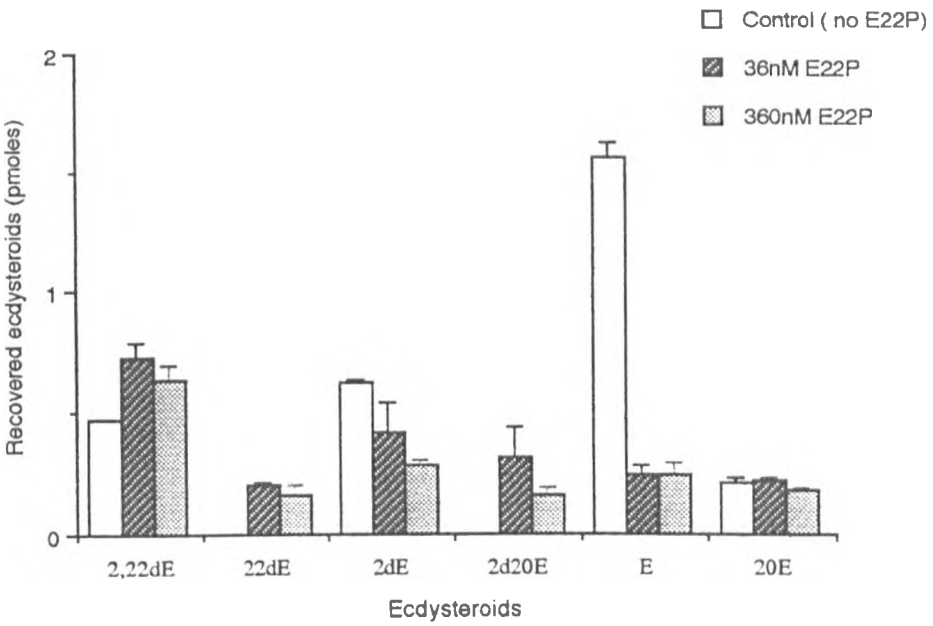
Follicle cells were prepared as described in section 2.2.2. Aliquots of the preparation were incubated as described in section 2.2.19 in the presence of [^{3}H]2,22,25-trideoxyecdysone (0.5μCi) with the addition of ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate, both at 36nM and 360nM final concentrations. The reaction was terminated by the addition of ethanol (0.5ml) and extracted and analysed as described previously in section 3.2.1.

Fig. 3.2 shows the effect of (1) ecdysone 22-phosphate and (2) 2-deoxyecdysone 22-phosphate (each at 36nM and 360nM, final concentrations) on the metabolism of [^{3}H]2,22,25-trideoxyecdysone in follicle cells. The overall rates of metabolism of [^{3}H]2,22,25-trideoxyecdysone were much lower in the presence of ecdysone 22-phosphate (E22P) and 2-deoxyecdysone 22-phosphate (2dE22P), compared to the conversion rate in their absence.

The amount of recovered total ecdysteroids (2,22dE, 22dE, 2d20E, 2dE, E and 20E) decreased from 2.88pmoles in the absence of exogenous phosphates to 2.14pmoles with addition of ecdysone 22-phosphate (36nM), and to 1.68pmoles in the presence of 360nM ecdysone 22-phosphate. The addition of ecdysone 22-phosphate caused the amounts of both 2,22-dideoxyecdysone and 22-deoxyecdysone to rise, although the increases in these two metabolites were not dose related. The amount of 2-deoxyecdysone fell in a dose-dependent manner. The amount of ecdysone fell greatly on addition of ecdysone 22-phosphate (36nM) with little further change on increasing the concentration of ecdysone 22-phosphate (360nM).

On addition of 2-deoxyecdysone 22-phosphate the amount of the total recovered ecdysteroids decreased from 2.88pmoles (in the absence of exogenous phosphate) to 1.94-1.92pmoles (with the addition of 2-deoxyecdysone 22-phosphate 36-360nM). The

(1) Ecdysone 22-phosphate



(2) 2-Deoxyecdysone 22-phosphate

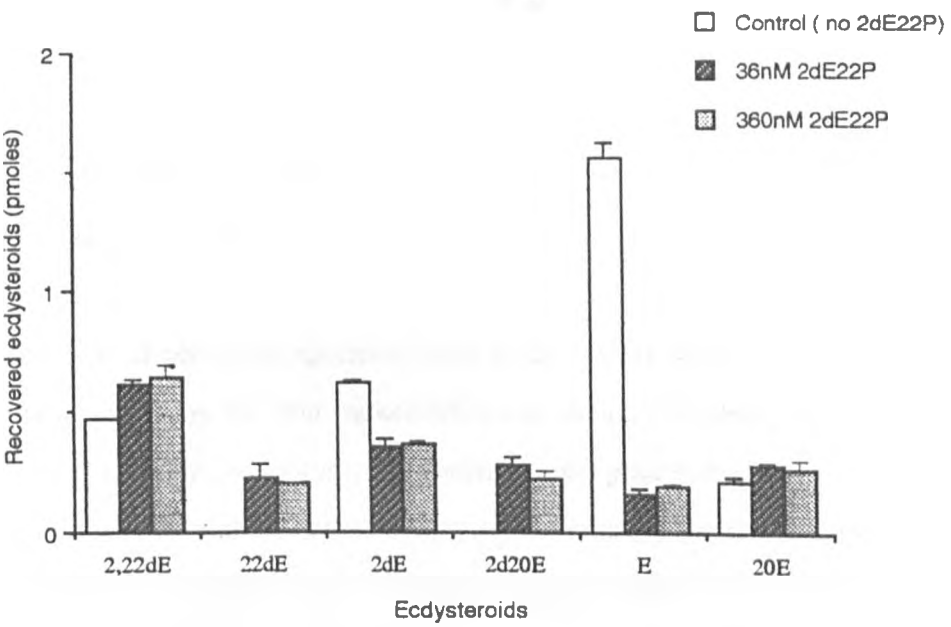


Fig.3.2. The effect of (1) ecdysone 22-phosphate and (2) 2-deoxyecdysone 22-phosphate (each at 36nM and 360nM, final concentrations) on the metabolism of [^{3}H]2,22,25-trideoxyecdysone in follicle cells, expressed as pmoles of recovered ecdysteroids from RP-HPLC (system 1, see section 2.2.8). Results are the mean values of duplicate incubations with the range shown.

amount of 2,22-dideoxyecdysone rose in a dose-dependent manner on addition of the exogenous 2-deoxyecdysone 22-phosphate, the amount of 22-deoxyecdysone also increased, although this did not appear dose related. The amount of 2-deoxyecdysone fell, on addition of exogenous 2-deoxyecdysone 22-phosphate, though this did not appear to be dose related. The amount of ecdysone decreased dramatically on addition of 2-deoxyecdydsone 22-phosphate (36nM), but was not further reduced on increasing the concentration of 2-deoxyecdysone 22-phosphate (360nM). Finally the amount of 20-hydroxyecdysone did not appear to be affected by the addition of exogenous 2-deoxyecdysone 22-phosphate.

The results of the foregoing experiments have been reprocessed to show the effect of the exogenous ecdysteroid phosphates [(1) ecdysone 22-phosphate and (2) 2-deoxyecdysone 22-phosphate] on the individual hydroxylation enzymes involved in the terminal stages of the ecdysteroid biosynthetic pathway (25 , 22 and the 2-hydroxylases) (Fig.3.3). The results are expressed as pmoles of ecdysteroid formed per incubation, and are the mean of duplicate incubations with the range shown.

The products of the hydroxylase enzymes are;

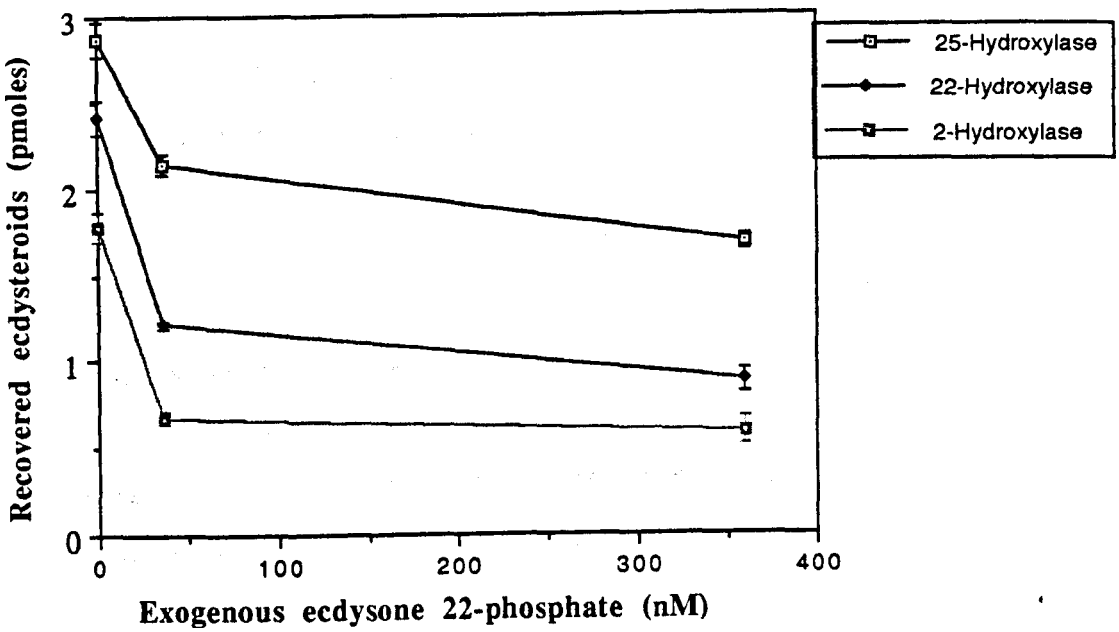
25-hydroxylase: 2,22dE, 22dE, 2dE, 2d20E, E and 20E.

22-hydroxylase: 2dE, 2d20E, E and 20E.

2-hydroxylase: 22dE, E and 20E.

Exogenous ecdysone 22-phosphate apparently reduced the activity of all 3 hydroxylase enzymes, although the most marked effect was on the 22-hydroxylase enzyme. Since the 22-hydroxylase catalyses the enzymatic step prior to the 2-hydroxylase a decrease in the activity of the 22- hydroxylase enzyme would reduce the amount of substrate for the 2-hydroxylase, resulting in an apparent reduction in the observed activity of the 2-hydroxylase. Exogenous 2-deoxyecdysone 22-phosphate also reduced the activity of the three hydroxylase enzymes, the effect being more pronounced on the 22 and the 2-hydroxylases. The lower activity of the 22-hydroxylase was also

(1) Ecdysone 22-phosphate



(2) 2-Deoxyecdysone 22-phosphate

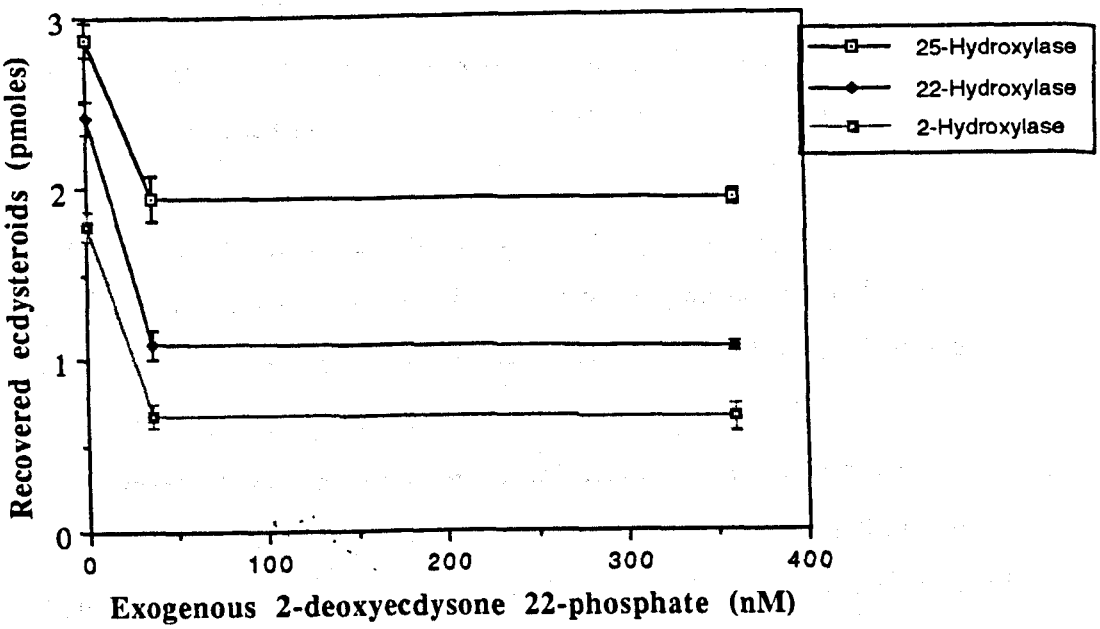


Fig. 3.3. The effect of (1) ecdysone 22-phosphate and (2) 2-deoxyecdysone 22-phosphate on the individual 25, 22 and 2-hydroxylase enzymes involved in the later stages of the ecdysteroid biosynthetic pathway. Results are expressed as pmoles of recovered ecdysteroids from RP-HPLC (system 1, see section 2.2.8), and are the mean values of duplicate incubations with the range shown.

reflected in the activity of the 2-hydroxylase. The addition of the higher concentration of 2-deoxyecdysone failed to cause further inhibition of the hydroxylase enzymes.

3.2.3. Incorporation of [^{3}H]2,22,25-trideoxyecdysone into ovarian follicle cells in the presence of exogenous unlabelled 2,22,25-trideoxyecdysone

Since the [^{3}H]2,22,25-trideoxyecdysone used in the foregoing experiments is of high specific activity, it was necessary to increase the concentration of this labelled substrate by adding increasing concentrations of unlabelled material to examine whether feedback effects of the ecdysteroid phosphates were still observed. However, initially it was necessary to use various concentrations of substrate to determine a suitable one to maintain a reasonable level of transformation of ^{3}H into ecdysteroids

.

Follicle cells were prepared as described in section 2.2.2 and incubated as described in section 2.2.19 in the presence of [^{3}H]2,22,25-trideoxyecdysone (0.5μCi) with addition of unlabelled 2,22,25-trideoxyecdysone (12.7nM, 31.7nM, 63.4nM, 126.8nM, and 253nM, respectively). The incubations were terminated by the addition of ethanol (0.5ml), and were extracted and analysed as described previously (see section 3.2.1).

Fig. 3.4 shows the effect of increasing the concentration of unlabelled 2,22,25-trideoxyecdysone on the conversion of [^{3}H]2,22,25-trideoxyecdysone in follicle cells. The results are expressed as a percentage of the total recovered ecdysteroid from the RP-HPLC (system 1, see section 2.2.8). The addition of unlabelled 2,22,25-trideoxyecdysone up to a concentration of 63.4nM had little effect on the overall incorporation of ^{3}H from 2,22,25-trideoxyecdysone into ecdysteroids in follicle cells. This can be observed directly by comparison with the control sample which contained only undiluted [^{3}H]2,22,25-trideoxyecdysone. However, in the incubation containing diluted substrate (8.1 x dilution), there was a much higher effective conversion into ecdysteroids in terms of nmoles /h.

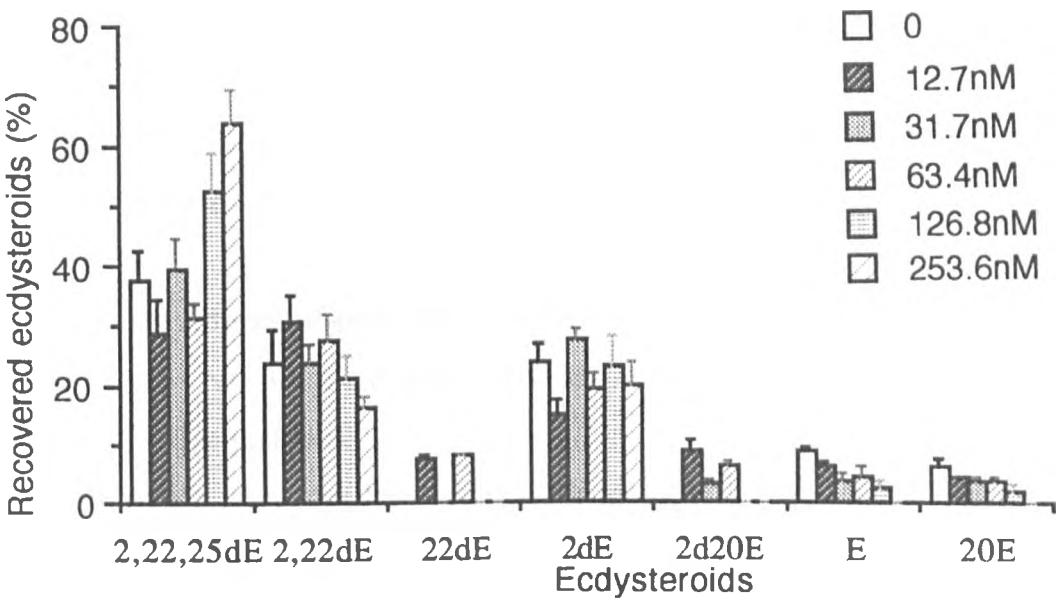


Fig. 3.4. The effect of increasing the concentration of unlabelled 2,22,25-trideoxyecdysone on the metabolism of [^{3}H]2,22,25-trideoxyecdysone in follicle cells, expressed as a percentage of the total recovered ecdysteroid from RP-HPLC (system 1 , see section 2.2.8). Results are the mean values of duplicate incubations with the range shown.

Therefore the concentration of 2,22,25-trideoxyecdysone in the incubation mixture could be increased to 72.3nM (8.92nM and 63.4nM from the labelled and unlabelled substrate respectively) without a noticeable reduction in the incorporation of ^{3}H into the ecdysteroid metabolites. This would allow ample supply of 2,22,25-trideoxyecdysone to sustain the incubation reaction for 8h.

3.2.4. Incorporation of [^{3}H]2,22,25-trideoxyecdysone (72.3nM) into ovarian follicle cells in the presence of ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate

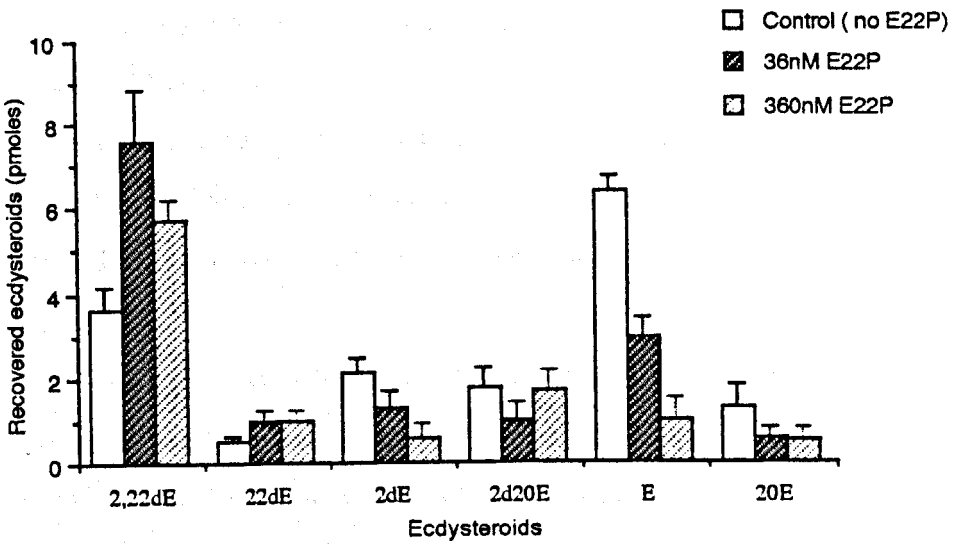
The effect of ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate at final concentrations of both 36nM and 360nM were examined on the metabolism of diluted [^{3}H]2,22,25-trideoxyecdysone.

This experiment was undertaken as described in section 3.2.2 with [^{3}H]2,22,25-trideoxyecdysone at a final concentration of 72.3nM.

Fig. 3.5 shows the effect of (1) ecdysone 22-phosphate and (2) 2-deoxyecdysone 22-phosphate (each at 36nM and 360nM, final concentrations) on the metabolism of [^{3}H]2,22,25-trideoxyecdysone (72.3nM) in follicle cells. The addition of ecdysone 22-phosphate caused the amount of 2,22-dideoxyecdysone and 22-deoxyecdysone to increase, this effect was not dose dependent. The amount of 2-deoxyecdysone and ecdysone both fell in a dose dependent manner on addition of ecdysone 22-phosphate. The amount of 20-hydroxyecdysone was also reduced on addition of ecdysone 22-phosphate, being little affected on addition of 360nM ecdysone 22-phosphate; 2-deoxy-20-hydroxyecdysone was reduced on addition of the lower concentration of ecdysone 22-phosphate, but was not reduced on addition of 360nM ecdysone 22-phosphate.

The addition of 2-deoxyecdysone 22-phosphate (36nM) caused the amount of 2,22-dideoxyecdysone to increase dramatically, although there was little further increase on

(1) Ecdysone 22-phosphate



(2) 2-Deoxyecdysone 22-phosphate

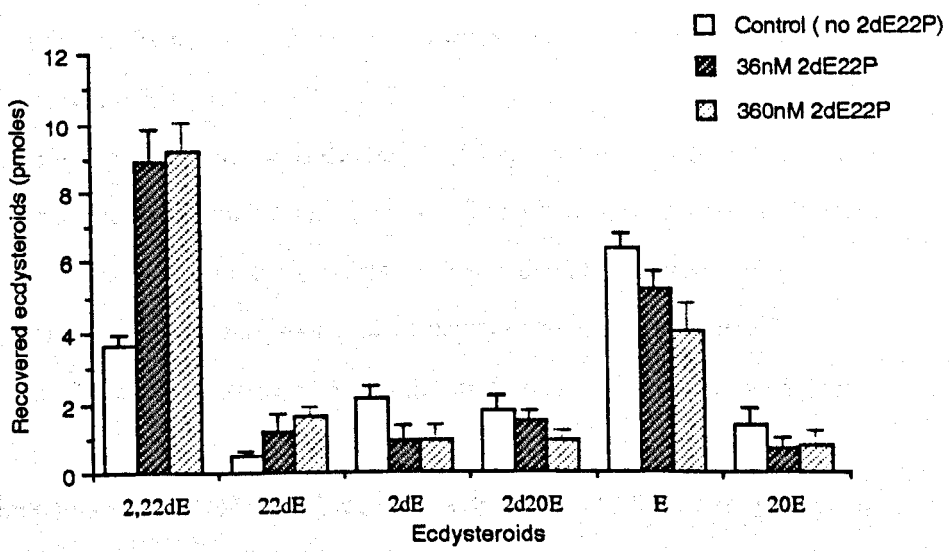


Fig. 3.5. Effect of (1) ecdysone 22-phosphate and (2) 2-deoxyecdysone 22-phosphate (each at 36nM and 360nM, final concentrations) on the metabolism of [^{3}H]2,22,25-trideoxyecdysone (72.3nM) in follicle cells, expressed as pmoles of recovered ecdysteroids from RP-HPLC (system 1 , see section 2.2.8). Results are the mean of duplicate incubations with the range shown.

increasing the concentration of 2-deoxyecdysone 22-phosphate to 360nM. The amount of 22-deoxyecdysone increased on addition of 2-deoxyecdysone 22-phosphate, although again further addition of 2-deoxyecdysone 22-phosphate (360nM) had little effect. The amount of 2-deoxyecdysone, 2-deoxy-20-hydroxyecdysone, ecdysone and 20-hydroxyecdysone fell on addition of 2-deoxyecdysone 22-phosphate, with 2-deoxy-20-hydroxyecdysone and ecdysone falling in a dose dependent manner.

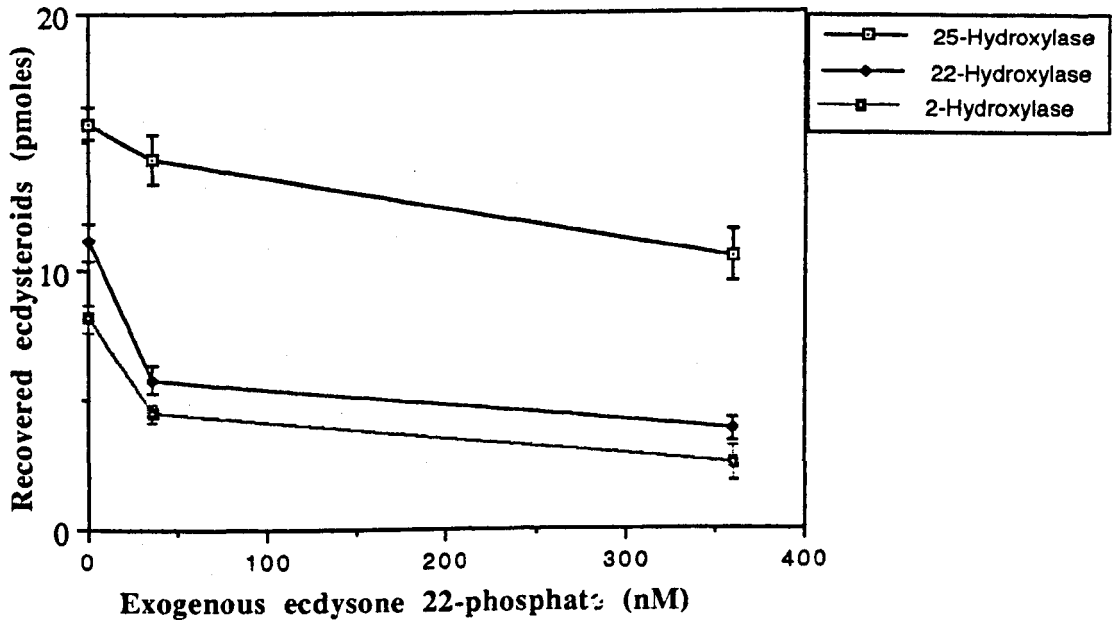
Fig 3.6 shows the effect of the ecdysteroid phosphates [(1) ecdysone 22-phosphate and (2) 2-deoxyecdysone 22-phosphate] on the individual enzymes, 25, 22 and the 2-hydroxylases when [^{3}H]2,22,25-trideoxyecdysone (72.3nM) was used as a substrate. Addition of exogenous ecdysone 22-phosphate caused an apparent reduction in the activity of the three enzymes, 25, 22 and the 2-hydroxylase, although the effect on the 22-hydroxylase was most marked. However, as discussed previously the substrate for the 2-hydroxylase may become limiting following inhibition of the preceding step (22-hydroxylase).The activity of the hydroxylase enzymes decreased as the concentration of ecdysone 22-phosphate increased.

On addition of exogenous 2-deoxyecdysone 22-phosphate the activity of the 22 and the 2-hydroxylase decreased, as the activity of the 25-hydroxylase increased somewhat. This increase in the activity of the 25-hydroxylase was due to an increase in the concentration of 2,22-dideoxyecdysone and 22-deoxyecdysone. Addition of 2-deoxyecdysone 22-phosphate at the higher concentration caused little further effect.

3.2.5. The incorporation of [^{3}H]2,22-dideoxyecdysone and [^{3}H]2-deoxyecdysone into ovarian follicle cells in the presence of the ecdysteroid phosphates, ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate

The apparent inhibition of the 22-hydroxylase enzyme which occurred during the incorporation of [^{3}H]2,22,25-trideoxyecdysone into ecdysteroids in follicle cells in the presence of the ecdysteroid phosphates, ecdysone 22-phosphate and 2-deoxyecdysone

(1) Ecdysone 22-phosphate



(2) 2-Deoxyecdysone 22-phosphate

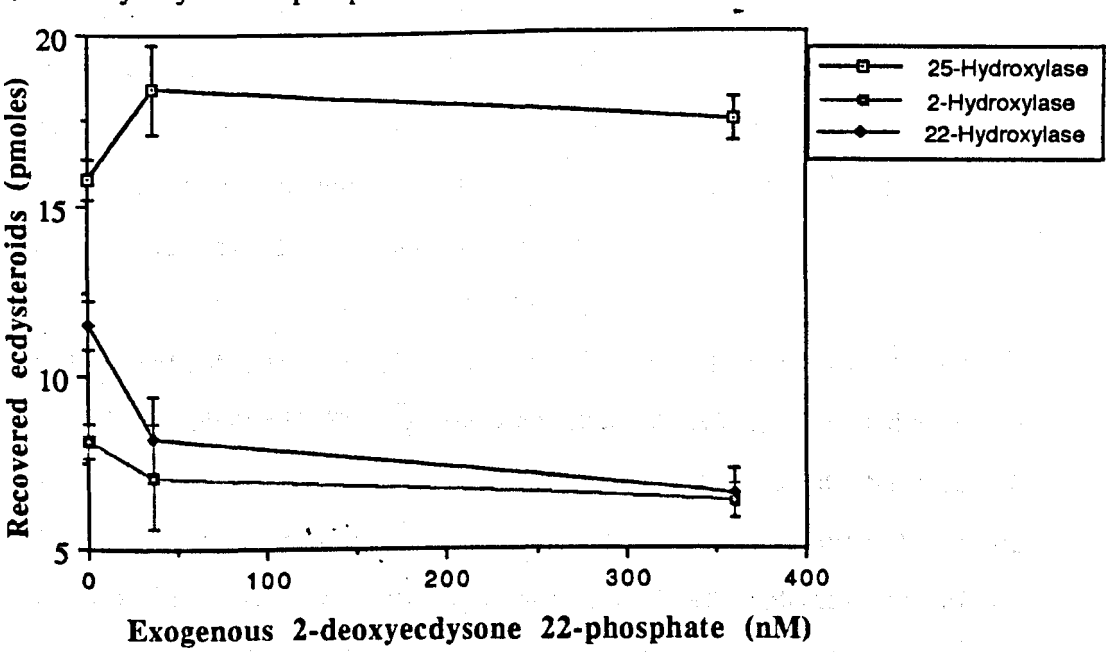


Fig. 3.6 The effect of (1) ecdysone 22-phosphate and (2) 2-deoxyecdysone 22-phosphate on the individual 25, 22 and 2-hydroxylase enzymes involved in the later stages of the ecdysteroid biosynthetic pathway, when [^{3}H]2,22,35-trideoxyecdysone (72.3nM) was used as a substrate. Results are expressed as pmoles of recovered ecdysteroids from RP-HPLC (system 1 , see section 2.2.8), and are the mean values of duplicate incubations with the range shown.

22-phosphate was further examined by using [^{3}H]2,22-dideoxyecdysone as a substrate. This allowed the 25-hydroxylase enzyme stage to be bypassed, and thus enabled a more direct study of the effect of the ecdysteroid phosphates on the 22-hydroxylase enzyme.

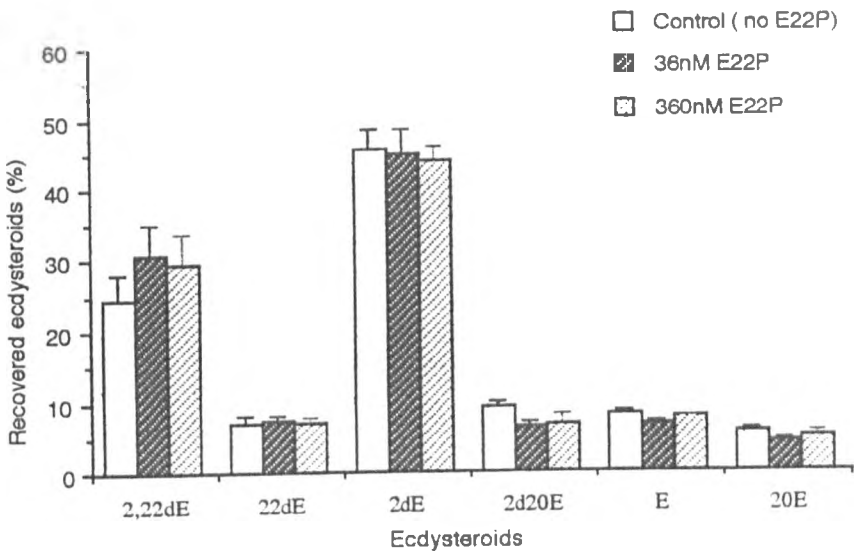
Since the 2-deoxyecdysone substrate for the 2-hydroxylase was likely to be limiting in the foregoing experiments owing to the feedback inhibition of the preceeding 22-hydroxylase, the feedback effect of the ecdysteroid phosphates was examined directly on the 2-hydroxylase, using [^{3}H]2-deoxyecdysone as a substrate.

Follicle cells were prepared as described in section 2.2.2 and incubated as described in section 2.2.19 for 5h in the presence of [^{3}H]2,22-dideoxyecdysone (0.5μCi) and 3h in the presence of [^{3}H]2-deoxyecdysone (0.5μCi), with the addition of the ecdysteroid phosphates, ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate, both at 36nM and 360nM final concentrations. The reaction was terminated by addition of ethanol (0.5ml) and extracted and analysed as described in section 3.2.1.

Fig. 3.7 shows the effect of (1) ecdysone 22-phosphate and (2) 2-deoxyecdysone 22-phosphate (each at 36nM and 360nM , final concentrations) on the metabolism of [^{3}H]2,22-dideoxyecdysone in follicle cells. The results are expressed as a percentage of the total recovered ecdysteroid from RP-HPLC (system 1, see section 2.2.8) and are the mean of duplicate incubations with the range shown.

On addition of either ecdysone 22-phosphate or 2-deoxyecdysone 22-phosphate the amount of unmetabolised 2,22-deoxyecdysone increased slightly, although there was no corresponding decrease in the amount of 2-deoxyecdysone synthesised. The amounts of 22-deoxyecdysone, ecdysone and 20-hydroxyecdysone synthesized were little affected on addition of ecdysone 22-phosphate (36nM or 360nM). Although the amount of 2-deoxy-20-hydroxyecdysone was reduced on addition of ecdysone 22-phosphate, this effect was not dose related.

(1) Ecdysone 22-phosphate



(2) 2-Deoxyecdysone 22-phosphate

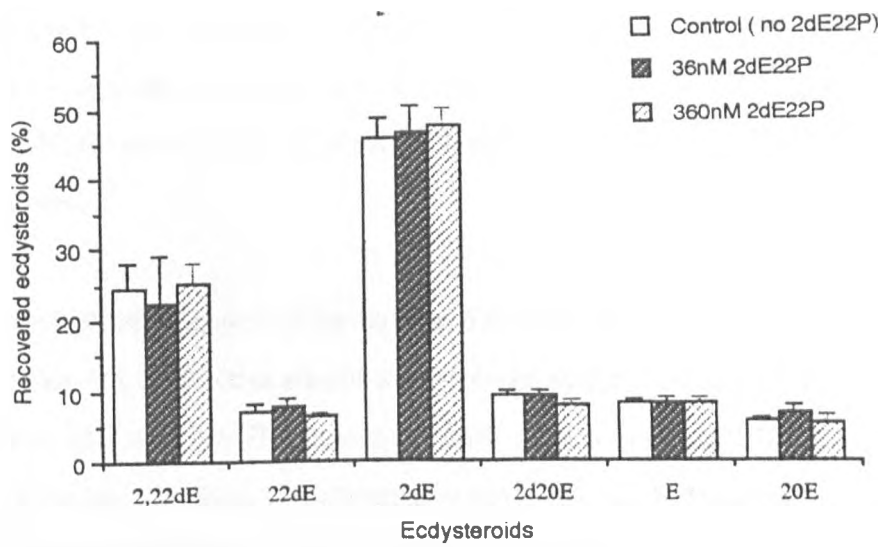


Fig. 3.7. The effect of (1) ecdysone 22-phosphate and (2) 2-deoxyecdysone 22-phosphate (each at 36nM and 360nM, final concentrations) on the metabolism of [^{3}H]2,22-dideoxyecdysone in follicle cells, expressed as a percentage of the total recovered ecdysteroids from RP-HPLC (system 1 , see section 2.2.8). Results are the mean values of duplicate values with the range shown.

The addition of 2-deoxyecdysone 22-phosphate had no overall effect on the incorporation [^{3}H]2,22-dideoxyecdysone into ecdysteroids in follicle cells.

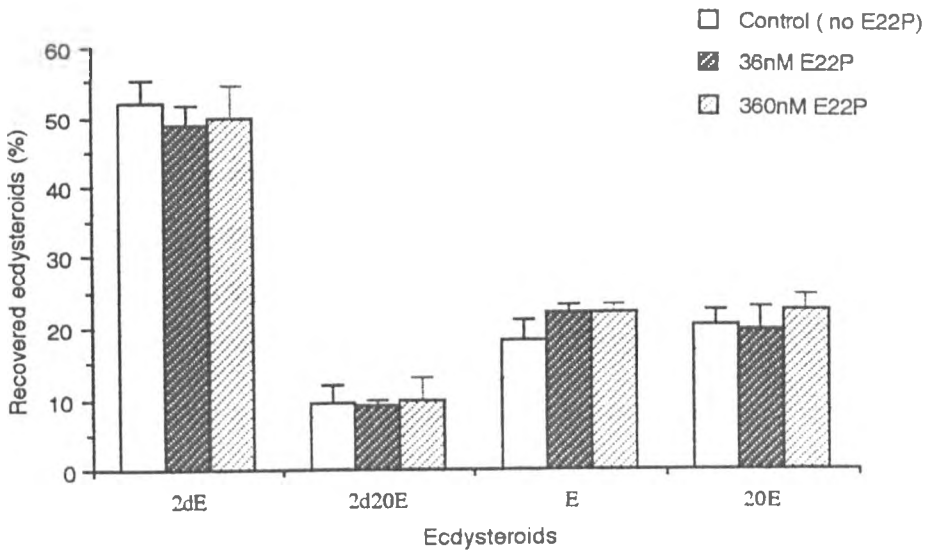
Fig. 3.8 shows the effect of (1) ecdysone 22-phosphate and (2) 2-deoxyecdysone 22-phosphate (each at 36nM and 360nM, final concentrations) on the metabolism of [^{3}H]2-deoxyecdysone in follicle cells. The addition of either ecdysone 22-phosphate or 2-deoxyecdysone 22-phosphate (36nM or 360nM) did not affect the incorporation of [^{3}H]2-deoxyecdysone into ecdysteroids in the follicle cells, with the synthesis of 2-deoxy-20-hydroxyecdysone, ecdysone and 20-hydroxyecdysone being little affected on the addition of either of these ecdysteroid phosphates.

3.2.6. The incorporation of [^{3}H]cholesterol into ovarian follicle cells

Since incorporation of the distal precursor, [^{3}H]cholesterol into ecdysteroids in follicle cells would aid further examination of the feedback effects, incorporation of this substrate was explored. The method of incorporation of [^{3}H]cholesterol into follicle cells was based on methods used by Dolle *et al.* (1990), which enabled the efficient incorporation of [^{3}H]7-dehydrocholesterol into ecdysteroids in *L. migratoria* ovarian follicle cells *in vitro.*

Two aliquots of follicle cells were prepared from 6 adult female *L. migratoria* as described in section 2.2.2. The first aliquot was incubated as described in section 2.2.19 in the presence of TLC-purified [^{3}H]cholesterol (15µCi) emulsified in 0.01% (w/v) Tween 20 in Landureau`s medium. [$^3$H]Cholesterol was emulsified in Tween 20 by evaporation of a solution of hexane (1ml) containing [$^3$H]cholesterol (30µCi) and Tween 20 (0.1mg) under $N_2$ in a silanised 2-dram vial. The resulting residue was resuspended in Landureau`s medium (1ml) by vortex mixing. The second aliquot was then resuspended in isotonic 100mM phosphate buffer (0.4ml), pH 7.3 and gently homogenised in a teflon-glass Potter- Elvehjem homogeniser (4 passes). The

(1) Ecdysone 22-phosphate



(2) 2-Deoxyecdysone 22-phosphate

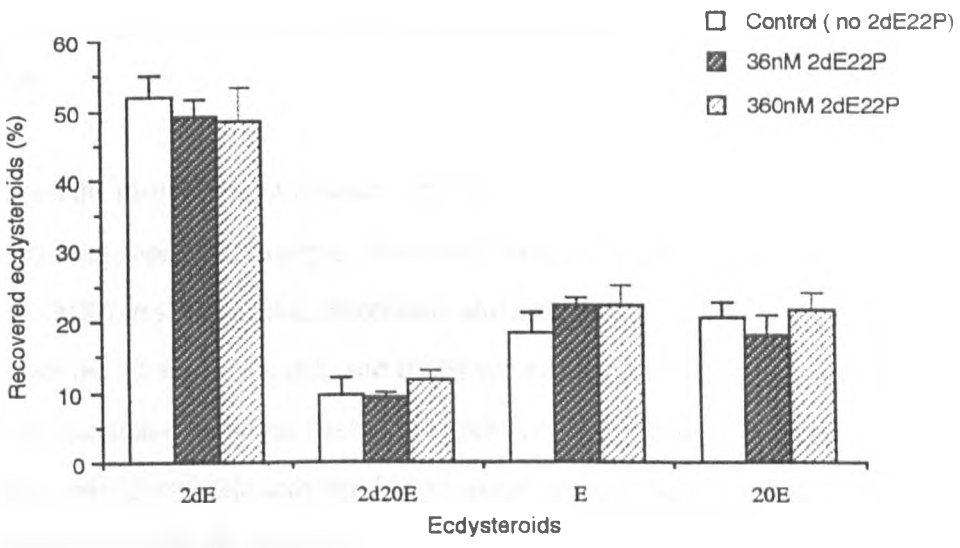


Fig. 3.8. The effect of (1) ecdysone 22-phosphate and (2) 2-deoxyecdysone 22-phosphate (each at 36nM and 360nM, final concentrations) on the metabolism of [^{3}H]2-deoxyecdysone in follicle cells, expressed as a percentage of the total recovered ecdysteroids from RP-HPLC (system 1 , see section 2.2.8). Results are the mean values of duplicate values with the range shown.

homogenate was centrifuged (1000g for 10min at 4°C) to remove cell debris. To the resulting supernatant, [^{3}H]cholesterol (10μCi) emulsified in 0.01% (w/v) Tween 20 in hypotonic 100mM phosphate buffer (0.1ml; containing, 0.2mM NADPH, 2.5mM glucose-6-phosphate, and 0.25U glucose-6-phosphate dehydrogenase) was added. The addition of hypotonic 100mM phosphate buffer will reduce the osmotic pressure of the isotonic buffer, and therefore increase the possibility of lysis in the membranes of the mitochondria and endoplasmic reticulum during the course of the incubation, enhancing the permeability to the substrate.The sample was incubated for 4h at 37°C. Both reactions were terminated by the addition of ethanol (0.5ml); the contents of the vials were extracted once with hexane (2ml) and 3 times with methanol (2ml) using a hand-held ground glass homogeniser. The resulting extracts were subjected to silicic acid column fractionation (for procedure see section 2.2.6). The methanol fractions (polar ecdysteroids) were subjected to crude aryl sulfatase treatment (see section 2.2.15), followed by C_{18} Sep-Pak fractionation procedure 2 (see section 2.2.7). The resulting methanol fraction and the 30% (v/v) methanol in chloroform fractions (free ecdysteroids) from the silicic acid fractionation were analysed by RP-HPLC (system 1, see section 2.2.8).

Table 3.1 Shows the distribution of radioactivity (μCi) from the whole follicle cell and 1000g follicle cell supernatant samples after the elution of the silicic acid column with chloroform, 30% (v/v) methanol in chloroform and methanol.

Analysis of both the whole follicle cell and 1000g supernatant samples (methanol and 30% (v/v) methanol in chloroform fractions) by RP-HPLC revealed no conversion of [^{3}H]cholesterol into identifiable ecdysteroid compounds (results not shown). This experiment was repeated with the same result.

	Radioactivity recovered (μCi)		
	Silicic acid column fractions (% methanol in chloroform)		
	0	*30*	*100*
Follicle cell samples			
whole cells	**12.0**	**0.6**	**0.09**
1000g supernatant	**7.9**	**0.8**	**0.05**

Table 3.1. The distribution of radioactivity expressed in μCi detected in the silicic acid column fractions from the whole follicle cell and the 1000g follicle cell supernatant samples after incubation for 8h and 4h, respectively, at 37°C with [^{3}H]cholesterol.

3.2.7. The effect of the exogenous ecdysteroid phosphates, ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate, on the endogenous ecdysteroid biosynthesis in ovarian follicle cells

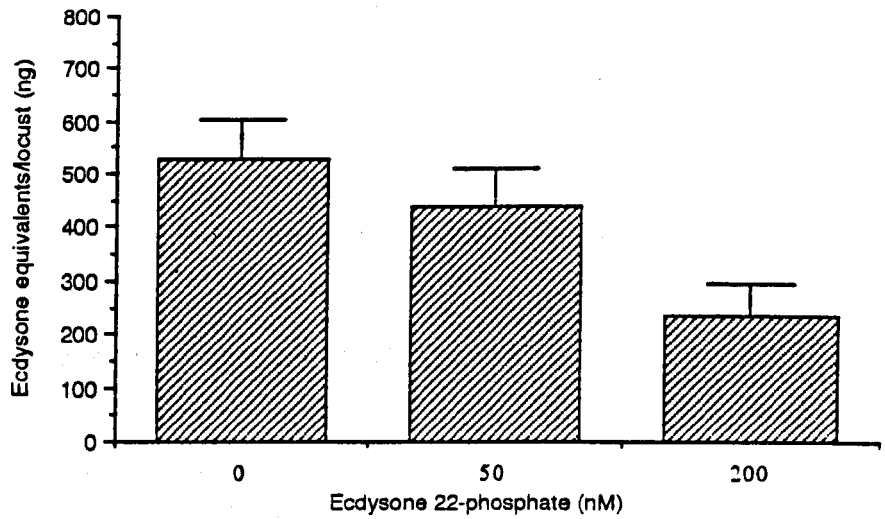
Having demonstrated a possible feedback effect of the ecdysteroid phosphates on a late step in the ecdysteroid biosynthetic pathway (22-hydroxylase), it was necessary to determine whether these phosphates had an effect on the overall amounts of ecdysteroids synthesised. Such an experiment would reflect feedback effects at any stage of the biosynthesis. In fact, a major effect would be expected at an early regulatory step.

Follicle cells were prepared as described in section 2.2.2. Incubations were carried as described in section 2.2.19 in the presence of ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate each at 50nM and 200nM, final concentrations. The ecdysteroid phosphates were quantified by their U.V. absorbance at 242nm. The reactions were terminated by the addition of ethanol (0.5ml). The samples were extracted 3 times in a ground glass hand held homogeniser. The extract was then subjected to enzyme hydrolysis by crude aryl sulfatase (see section 2.2.15), and subjected to C_{18} Sep-Pak fractionation as described in section 3.2.1. The 60% (v/v) methanol in water fractions were analysed by R.I.A. (see section 2.2.17) using DHS-1-13.5 antiserum.

Since the exogenously added ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate would also be enzymatically hydrolysed, the amounts of free ecdysteroids derived from these were subtracted from the total ecdysteroids detected at the end of the incubations.

Fig. 3.9 shows the effect of (1) ecdysone 22-phosphate and (2) 2-deoxyecdysone 22-phosphate (each at 50nM and 200nM, final concentrations) on the endogenous ecdysteroid biosynthesis in ovarian follicle cells, expressed as immunoreactive ng

(1) Ecdysone 22-phosphate



(2) 2-Deoxyecdysone 22-phosphate

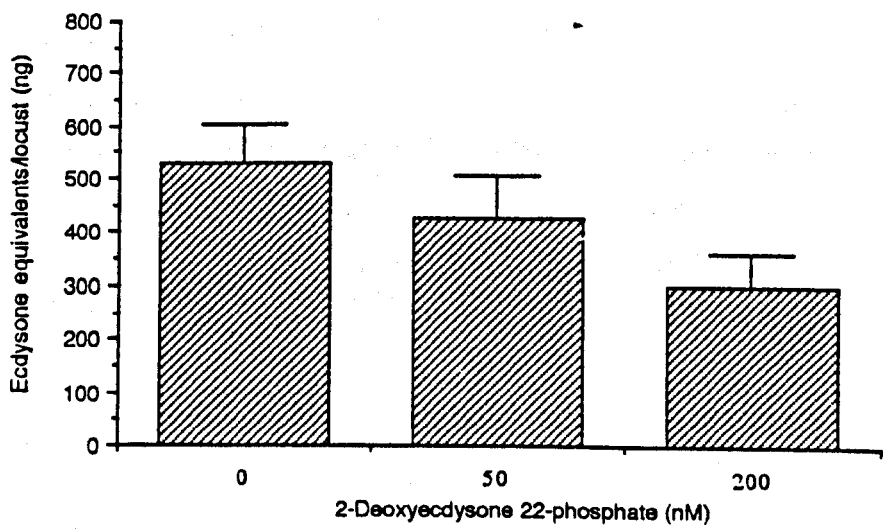


Fig. 3.9. The effect of (1) ecdysone 22-phosphate and (2) 2-deoxyecdysone 22-phosphate (each at 50nM and 200nM, final concentrations) on the endogenous ecdysteroid biosynthesis in follicle cells, expressed as ng immunoreactive ecdysone equivalents/locust. Results are the mean values of triplicate incubations from two experiments ± S.E.M.

At time 0h and 8h control incubations (no ecdysteroid phosphates present) contained mean values of 73.4ng ± 15.5ng and 603ng± 75ng ecdysone equivalents/ locust total ecdysteroid content, respectively.

ecdysone equivalents produced per locust. The results are the mean of triplicate incubations from 2 separate experiments ± S.E.M.

The concentration of endogenous immunoreactive ecdysteroid equivalents in follicle cells rose from 73.4ng ± 15.3ng /locust to 603ng ± 75ng /locust after 8 h incubation at 37°C. The addition of ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate at each concentration reduced the endogenous ecdysteroid synthesis of the follicle cells. The lower concentration of 2-deoxyecdysone 22-phosphate (50nM) caused a slightly greater inhibition (19.8%) than the lower concentration (50nM) of ecdysone 22-phosphate (16.9%), respectively. This situation was reversed in the presence of the higher concentrations (200nM) of ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate causing 56.1% and 43.4 % inhibition, respectively. Since the major ecdysteroids synthesized by follicle cells are the ecdysteroid phosphates, 2-deoxyecdysone 22-phosphate and ecdysone 22-phosphate in the ratio 2: 1 (Lagueux et al., 1981), and as the DHS-1-13.5 antiserum has a cross reactivity to 2-deoxyecdysone of 3.8 relative to ecdysone (Jarvis, 1991), it is therefore possible that the estimated total amount of endogenous ecdysteroid synthesized in the follicle cell samples during the course of the *in vitro* culture may not reflect the true value of endogenous ecdysteroid synthesis. Therefore the effect of the ecdysteroid phosphates on the endogenous ecdysteroid synthesis in follicle cells may not be fully apparent when using this quantitative estimate of ecdysteroid synthesis.

3.2.8. Synthesis of ecdysteroids in prothoracic glands from second day, fifth instar larvae of *L. migratoria*

The ability to set-up a viable *in vitro* ecdysteroid biosynthetic prothoracic gland system would enable an investigation into possible feedback regulation in the ecdysteroid biosynthetic pathway of the gland. Since the ecdysteroid biosynthetic activity of prothoracic glands varies throughout the larval instar, undergoing both high and low levels of activity, prothoracic glands were excised from second day fifth instar *L. migratoria* larvae at a time corresponding to high ecdysteroid biosynthetic activity (Hirn *et al.*, 1979).

Pairs of prothoracic glands were excised from second day fifth larvae; and suspended in Landureau`s medium (0.5ml) in silanised 2-dram vials (2 glands from 1 insect/ incubation vial). To half of the incubation vials ethanol (0.5ml) was immediately added; the remainder were incubated for 4h at 33°C, the reaction being terminated by the addition of ethanol (0.5ml). The contents of the vials were extracted and fractionated as described in section 3.2.1. The 60% (v/v) methanol in water fractions (free ecdysteroids) were analysed by R.I.A. (see section 2.2.17), using DHS-1-13.5 antiserum.

Fig. 3.10 shows the amount of ecdysteroid, before and after incubation of pairs of prothoracic glands (2 glands from 1 insect/ incubation vial) for 4 h at 33°C, expressed as ng ecdysone equivalents. The results are the mean of six individual incubations ± S.E.M.

Negligible ecdysteroid concentration was found in the non incubated pairs of prothoracic glands, and therefore it can be assumed that no storage of ecdysteroid occurs in these glands. On incubation, pairs of prothoracic glands synthesised 2.88ng±0.15ng of ecdysone equivalents.

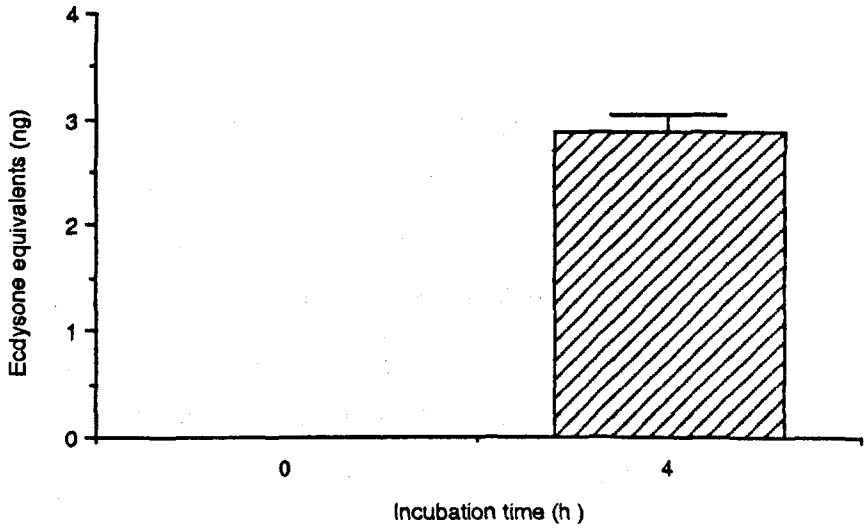


Fig. 3.10. The total amount of ecdysteroid synthesized by one pair of prothoracic glands (one pair/ incubation vial) after a 4h incubation at 33°C. The results are expressed as ng ecdysone equivalents and are mean of six individual incubations ± S.E.M.

3.2.9. The incorporation of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone into pairs of partially homogenised prothoracic glands from second day, fifth instar larvae of *L. migratoria* in the presence of exogenous 20-hydroxyecdysone

In order to investigate possible feedback regulation in the terminal stages of the ecdysteroid biosynthetic pathway of *L. migratoria* prothoracic glands, [^{3}H]3-dehydro-2,22,25-trideoxyecdysone was used as a substrate in the presence of exogenous 20-hydroxyecdysone, which is the terminal product of the ecdysteroid biosynthetic pathway in larval insects. Concentrations of exogenous 20-hydroxyecdysone chosen were based on the concentrations of exogenous ecdysteroids required to elicit inhibition of ecdysteroid synthesis of *M. sexta* prothoracic glands *in vitro* (Sakurai and Williams, 1989), although the concentration of exogenous 20-hydroxyecdysone was reduced to take into account the different rates of ecdysteroid synthesis between *M. sexta* and *L. migratoria* prothoracic glands *in vitro*. [^{3}H]3-Dehydro-2,22,25-trideoxyecdysone has been shown to be incorporated to a much greater extent than [^{3}H]2,22,25-trideoxyecdysone into ecdysteroids in *Pieris brassicae* prothoracic glands *in vitro* (Blais and Lafont, 1991). In addition, [^{3}H]3-dehydro-2,22,25-trideoxyecdysone has also been found to be more efficiently incorporated than [^{3}H]2,22,25-trideoxyecdysone into ecdysteroids in *L. migratoria* prothoracic glands (results not shown), and therefore, appears to be a viable ecdysteroid intermediate in the later stages of the ecdysteroid biosynthetic pathway in prothoracic glands.

Prothoracic gland pairs from individual insects (2 glands from 1 insect/ incubation vial) were suspended in Landureau`s medium (0.4ml) and partially homogenised in a hand held ground glass homogeniser (0.5 turn). Incubations were carried out in Landureau`s medium (0.5ml) in the presence of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone(0.5µCi) with addition of 20-hydroxyecdysone (20nM and 40nM, final concentrations) for 4 h at 33^{o}C. After the reaction had been terminated by the addition of ethanol (0.5ml) the contents of each vial were extracted 3 times with methanol in a

hand-held ground glass homogeniser and the resulting extract subjected to subjected to C_{18} Sep-Pak fractionation (see section 2.2.7, procedure 2) followed by analysis by RP-HPLC (system 1, see section 2.2.8).

Fig. 3.11 shows a typical RP-HPLC (system 1 see section 2.2.8) radiochromatogram of the labelled ecdysteroids extracted from an incubation of partially homogenised prothoracic glands with [^{3}H]3-dehydro-2,22,25-trideoxyecdysone.

On incubation of prothoracic glands with [^{3}H]3-dehydro-2,22,25-trideoxyecdysone, the 3-dehydroecdysteroid and 3β−hydroxyecdysteroid derivatives , 2,22-dideoxyecdysone, 22-deoxyecdysone, 2-deoxyecdysone and ecdysone were synthesised. On RP-HPLC (system 1 see section 2.2.8) the 3β-hydroxy and 3-dehydro derivatives of ecdysteroid compounds are found to co-chromatograph , and so the combined 3β−hydroxy and 3-dehydroecdysteroid derivatives will be referred to as ecdysteroid regions. The effect of 20-hydroxyecdysone on the incorporation of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone will be referred to in relation to these individual regions.

Fig. 3.12 Shows the effect of 20-hydroxyecdysone(at 20nM and 40nM, final concentrations) on the metabolism of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone in partially homogenised prothoracic glands, the radioactivity in each region being expressed as a percentage of the total recovered ecdysteroid from RP-HPLC (system 1, see section 2.2.8). On addition of 20-hydroxyecdysone (20nM or 40nM) there was little effect on the metabolism of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone as compared to the control incubation (no 20-hydroxyecdysone).

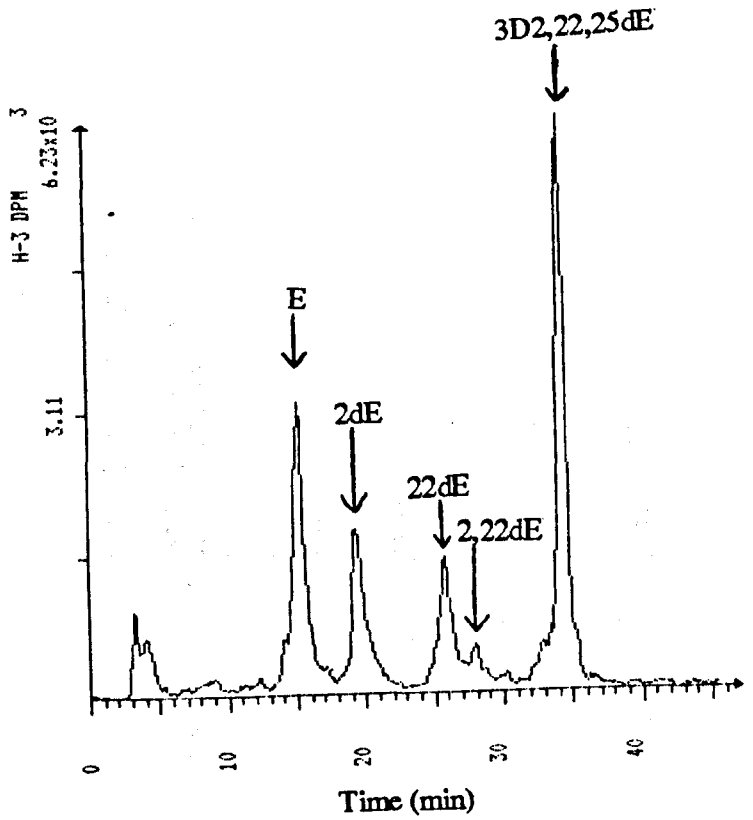


Fig. 3.11. A typical RP-HPLC (system 1, section 2.2.8.) radiochromatogram of the labelled ecdysteroids extracted from an incubation of partially homogenised prothoracic glands with [^{3}H]3-dehydro-2,22,25-trideoxyecdysone. The positions of authentic standards are shown: 3-dehydro-2,22,25-trideoxyecdysone (3D2,22,25dE), 2,22-dideoxyecdysone (2,22dE), 22-deoxyecdysone (22dE), 2-deoxyecdysone (2dE) and ecdysone(E)

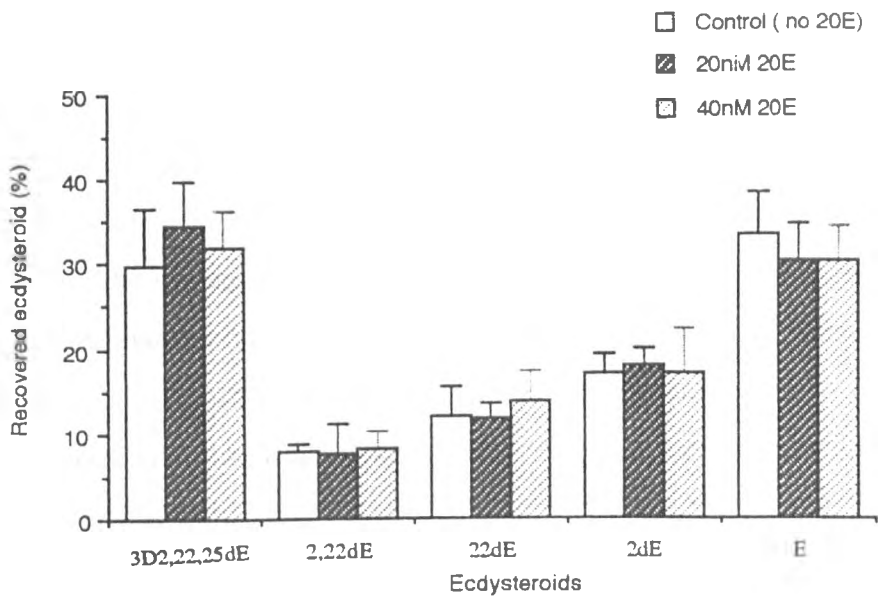


Fig. 3.12. The effect of exogenous 20-hydroxyecdysone (at 20nM and 40nM, final concentrations) on the metabolism of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone in pairs of partially homogenised prothoracic glands, expressed as a percentage of the total recovered ecdysteroids in each region from RP-HPLC (system 1, see section 2.2.8.). The results are the mean values of duplicate incubations from two experiments $\pm$ S.E.M.

3.2.10. The effect of 20-hydroxyecdysone and ecdysone on the endogenous ecdysteroid biosynthesis of individual prothoracic glands from second day, fifth instar larvae of *L. migratoria*

The effect of the terminal products of the ecdysteroid biosynthetic pathway in larval insects, ecdysone and 20-hydroxyecdysone, were investigated on the overall amount of ecdysteroid synthesized by prothoracic glands. Such an investigation enabled possible feedback regulation to be gauged on the whole ecdysteroid biosynthetic pathway. The effect of the exogenous ecdysteroids were measured on individual glands, with their contralateral glands acting as controls. To ensure comparable rates of ecdysteroid synthesis between individual prothoracic glands and their respective contralateral glands an *in vitro* study was undertaken.

The left and right glands from four prothoracic gland pairs were separated and incubated in Landureau`s medium (0.4ml) for 4h at 33°C. The reaction was terminated by the addition of ethanol (0.5ml), and the contents of the vials were analysed by DHS1-13.5 antiserum.

Fig. 3.13 shows the amount of ecdysteroids synthesized by the individual prothoracic glands after incubation for 4h at 33°C. The results are expressed as pg ecdysone equivalents, and are the mean of four incubations ± S.E.M.

On incubation for 4h at 33°C the left and right prothoracic glands produced 1137pg± 119pg and 1182pg± 97pg of ecdysteroids, respectively, the right prothoracic gland producing approximately 4% more than the left gland.

The effect of 20-hydroxyecdysone and ecdysone on the endogenous ecdysteroid biosynthesis of individual prothoracic glands was examined using the contralateral gland as a control. Prothoracic glands were excised from second day fifth instar larvae of *L.*

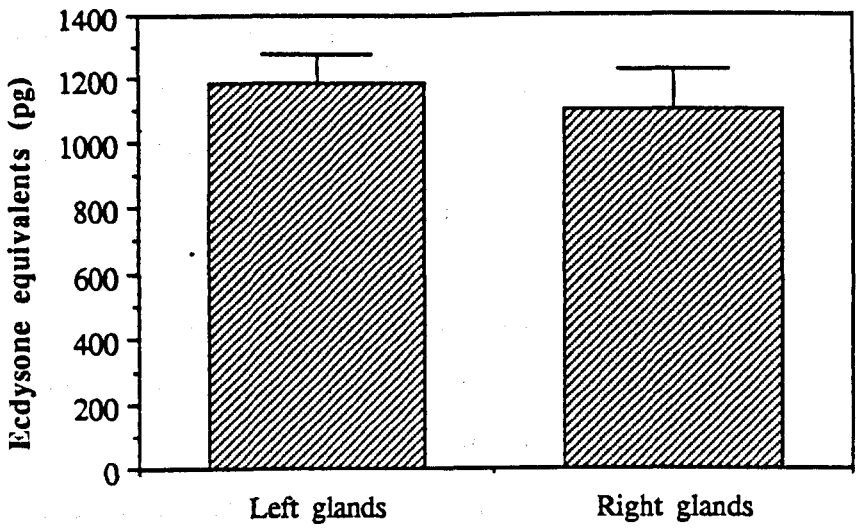


Fig. 3.13. The total amount of ecdysteroid synthesized by individual prothoracic glands and their respective contralateral glands after a 4h incubation at 33°C. Results are expressed as pg equivalents of ecdysone, and are the mean of four individual incubations ± S.E.M.

migratoria. Pairs of glands were separated, so that the right gland was incubated in the presence of exogenous 20-hydroxyecdysone ecdysone each at 4nM, 10nM and 30nM, final concentrations for 4h at 33°C in Landureau`s medium (0.4ml) , whilst the respective left gland was incubated in hormone free Landureau`s medium. The reaction was terminated by the addition of ethanol (0.5ml) and the contents of the vials were analysed by DHS1-1-13.5 antiserum (section 2.2.17).

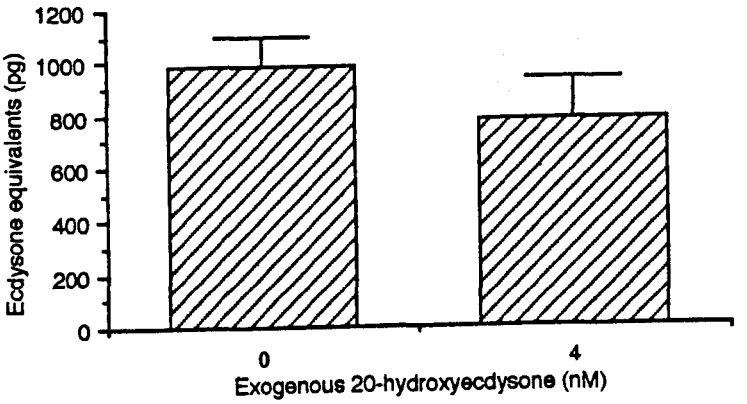
Fig. 3.14 shows the effect of exogenous 20-hydroxyecdsone (4nM, 10nM and 30nM) on the endogenous ecdysteroid biosynthesis of prothoracic glands. Addition of 20-hydroxyecdysone (4nM) caused 21% overall inhibition in the endogenous ecdysteroid biosynthesis, the inhibition increased to 64.5% on further addition of 20-hydroxyecdysone (10nM), reaching a maximum of 80% in the presence of the highest concentration of 20-hydroxyecdysone (30nM).

Fig. 3.15 shows the effect of exogenous ecdysone (4nM, 10nM and 30nM) on the endogenous ecdysteroid biosynthesis of prothoracic glands. Ecdysone (4nM or 10nM) caused no significant inhibition in the endogenous ecdysteroid biosynthesis of the prothoracic glands (1% and 5%, respectively). On addition of the highest concentration of ecdysone (30nM) there was a dramatic reduction of 65% in the endogenous ecdysteroid biosynthesis of the prothoracic glands.

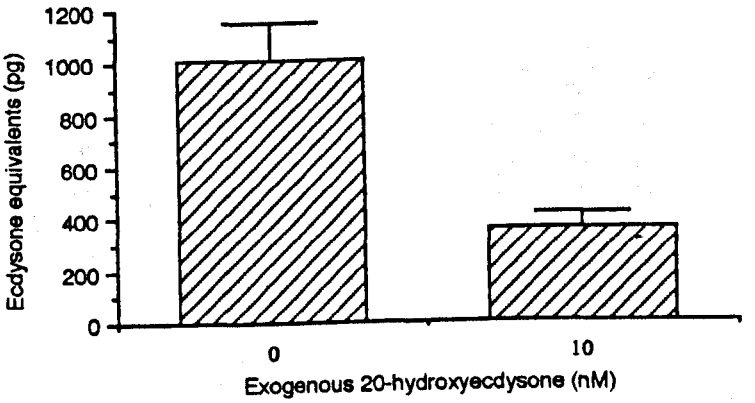
3.3. Discussion

Incorporation of [^{3}H]2,22,25-trideoxyecdysone into ovarian follicle cells of *L. migratoria* allowed an investigation into a possible late control point in the ecdysteroid biosynthetic pathway. The synthesis of ecdysteroids from [^{3}H]2,22,25-trideoxyecdysone was not reduced in follicle cells in the presence of either exogenous ecdysone or 20-hydroxyecdysone at concentrations of 420nM or 4200nM. The ineffectiveness of the

(1) 4nM 20-hydroxyecdysone



(2) 10nM 20-hydroxyecdysone



(3) 30nM 20-hydroxyecdysone

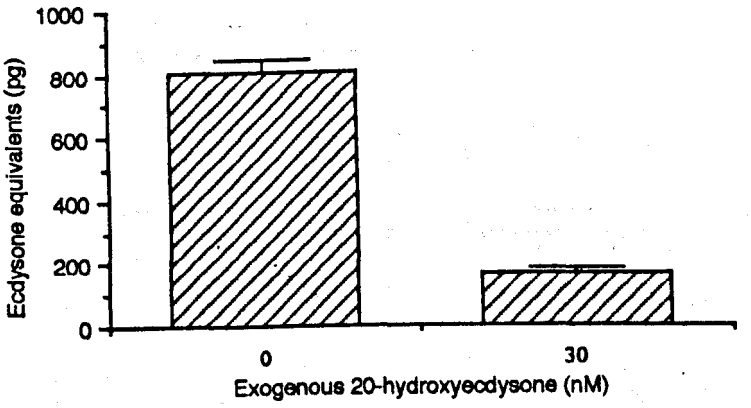
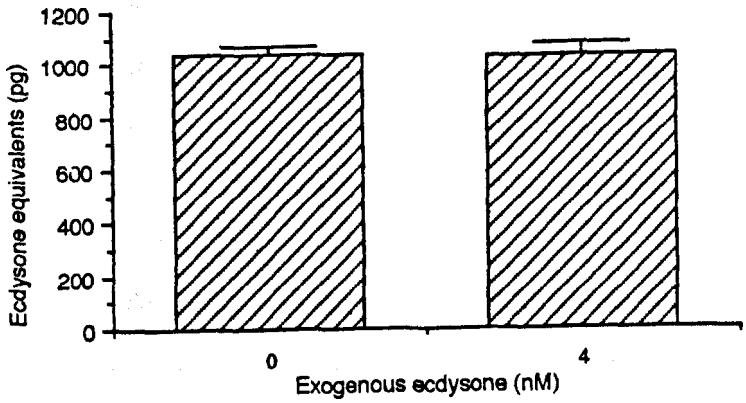
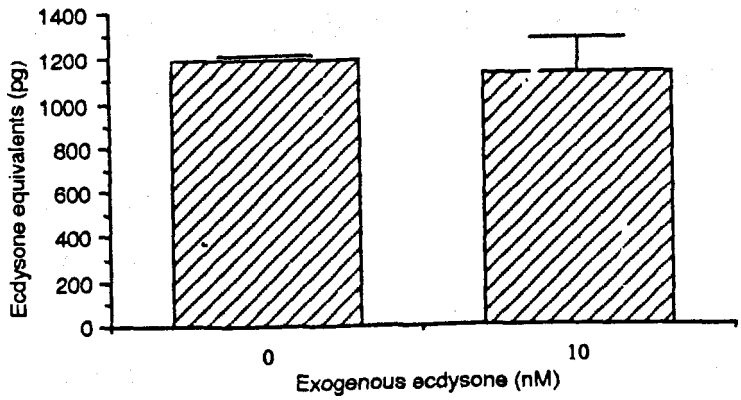


Fig. 3.14. The effect of 20-hydroxyecdysone (4nM, 10nM or 30nM) on the endogenous ecdysteroid biosynthesis of individual prothoracic glands, expressed as pg ecdysone equivalents synthesized. The results are the mean values of 3 triplicate incubations ± S.E.M.

(1) 4nM Ecdysone



(2) 10nM Ecdysone



(3) 30nM Ecdysone

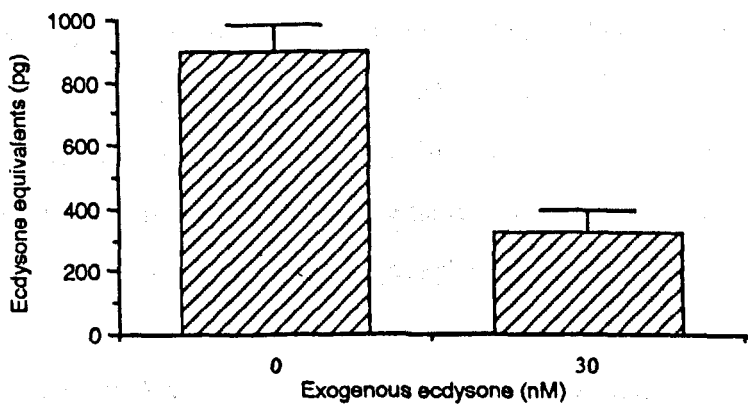


Fig. 3.15. The effect of exogenous ecdysone (4nM, 10nM or 30nM) on the endogenous ecdysteroid biosynthesis of individual prothoracic glands, expressed as pg ecdysone equivalents.synthesized. The results are the mean values of 3 triplicate incubations ± S.E.M.

free ecdysteroids in eliciting feedback control on the later stages of the ecdysteroid biosynthetic pathway was not unexpected, as free ecdysteroids are only minor forms in the developing ovary of the mature insect. In the immature stages of insect development the free ecdysteroids are the major form, and it is during the early development stages that they have been shown to exert feedback regulation on ecdysteroid biosynthesis in the prothoracic glands (Beydon and Lafont, 1983; Bodnaryk, 1986, Sakurai and Williams, 1989). The increase in ecdysteroid synthesis from the [^{3}H]2,22,25-trideoxyecdysone substrate in the presence of exogenous ecdysone and 20-hydroxyecdysone may be due to a stimulation in the activity of the 22-phosphotransferases by these exogenous ecdysteroids, which would therefore serve to remove products from the ecdysteroid biosynthetic pathway.

The ecdysteroid phosphates, ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate appeared to reduce the synthesis of ecdysteroids from [^{3}H]2,22,25-trideoxyecdysone in follicle cells, ecdysone 22-phosphate at a concentration of 360nM being the most effective. On addition of ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate to the follicle cell preparations, the synthesis of 2,22-dideoxyecdysone and 22-deoxyecdysone increased; at the same time the synthesis of the more hydroxylated ecdysteroids, most noticeably 2-deoxyecdysone and ecdysone decreased.

Ecdysone 22-phosphate appears to inhibit ecdysteroid synthesis from [^{3}H]2,22,25-trideoxyecdysone in a dose dependent manner, although this is not the case with 2-deoxyecdysone 22-phosphate, where addition of the higher concentration of 2-deoxyecdysone 22-phosphate has little further effect.

When the concentration of [^{3}H]2,22,25-trideoxyecdysone was raised from 8.92nM to 72.3nM, which is thought to represent a more physiological concentration, the ecdysteroid phosphates, ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate again reduced the synthesis of the more hydroxylated ecdysteroids, 2-deoxyecdysone and ecdysone, with a corresponding increase in the synthesis of 2,22-dideoxyecdysone and 22-deoxyecdysone. Ecdysone 22-phosphate at a concentration of 360nM again appeared to be the most effective inhibitor of ecdysteroid synthesis.

The effect of increasing the concentration of 2,22-dideoxyecdysone and the dead end product , 22-deoxyecdysone (Meister *et al.*, 1985), in conjunction with a decrease in the concentration of 2-deoxyecdysone and ecdysone, suggests an inhibitory effect by the ecdysteroid phosphates, 2-deoxyecdysone 22-phosphate and ecdysone 22-phosphate, on the activity of the 22-hydroxylase enzyme. The ecdysteroid phosphates appear to drastically reduce the activity of the 22 and the 2- hydroxylase enzymes, although their effect on 25-hydroxylase enzyme is less severe. The reduction in the activity of the 22- and the 2-hydroxylase enzymes in relation to the 25-hydroxylase in the presence of a higher concentration of [^{3}H]2,22,25-trideoxyecdysone (72.3nM), may be more representative of a physiological response.

It is likely that the observed decrease in the activity of the 2-hydroxylase was due to a reduction in the available substrate for this enzyme, as a result of decreased 22-hydroxylase activity. The inability of ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate at concentrations of 36nM and 360nM to reduce the incorporation of [^{3}H]2-deoxyecdysone into follicle cells appeared to confirm this hyphothesis; however, the inability of the ecdysteroid phosphates to inhibit the 22-hydroxylase enzyme when [^{3}H]2,22-dideoxyecdysone was used as a substrate appears to contradict the previous results. The possibility arises that endogenously formed substrate of the 22-hydroxylase is required for the ecdysteroid phosphates to elicit their inhibitory effect on the enzyme.

Incorporation of [^{3}H]cholesterol into follicle cells was not observed under *in vitro* conditions, dilution of [^{3}H]cholesterol by the endogenous follicle cell cholesterol pool possibly contributed to this failure to demonstrate incorporation. Therefore, any attempt to investigate possible feedback regulation in the early stages of the ecdysteroid biosynthetic pathway of follicle cells was aborted.

Possible feedback regulation on the ecdysteroid biosynthetic pathway as a whole was investigated by examination of the effect of the ecdysteroid phosphates, ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate, on the endogenous ecdysteroid biosynthesis in follicle cells. In this experiment, the total endogenous ecdysteroid

content of the follicle cells were quantified i.e. the preponderant phosphate conjugates after enzymic hydrolysis, plus the originally free ecdysteroids. The amount of endogenous ecdysteroid synthesised in follicle cells is greatly reduced in the presence of either ecdysone 22-phosphate or 2-deoxyecdysone 22-phosphate (both at a concentration of 50nM and 200nM), the former at a concentration of 200nM being the most effective, although only two concentrations of ecdysteroid phosphates were tested. Ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate appeared to inhibit endogenous ecdysteroid biosynthesis in a dose dependent manner.

The 22-hydroxylase enzyme could possibly represent a control point in the later stages of ecdysteroid biosynthesis, being susceptible to feedback regulation by ecdysone 22-phosphate and 2-deoxyecdysone 22-phosphate. The endogenous ecdysteroid biosynthesis, which can be deemed the synthesis of ecdysteroids from precursor molecules, was also susceptible to feedback regulation by the ecdysteroid phosphates. Presumably, this inhibition is at an early stage. For this possible early control point to be investigated further, incorporation of an early precursor of ecdysteroid biosynthesis must first be achieved. [^{3}H]7-dehydrocholesterol, might be a suitable early precursor, reducing the possibility of dilution by the presumed large pool of endogenous cholesterol, which hampered the investigation of incorporation of [^{3}H]cholesterol into ecdysteroids by follicle cells.

It was shown that pairs of prothoracic glands from second day, fifth instar larvae of *L. migratoria* synthesised ecdysteroids under *in vitro* conditions, the concentration of ecdysteroid product comparing favourably to earlier studies (Hirn *et al.*, 1979).

Possible feedback regulation on the later stages of ecdysteroid biosynthesis in prothoracic glands was investigated by the effect of exogenous 20-hydroxyecdysone (20nM and 40nM) on the incorporation of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone into partially disrupted prothoracic glands from second day, fifth instar larvae of *L. migratoria*. This stage corresponds to a high ecdysteroid production (Hirn *et al.*, 1979) and, therefore, minimal possible activation of the prothoracic glands by the exogenous ecdysteroid. Prothoracic glands of high ecdysteroid synthetic activity have been shown

to be susceptible to feedback inhibition by exogenous ecdysteroids in *M. sexta* (Sakurai and Williams, 1989) whereas glands of low activity display stimulation on addition of exogenous ecdysteroids (Sakurai and Williams, 1989). The glands were disrupted to increase their ability to take up the radiolabel (Grieneisen *et al.*, 1991). 20-Hydroxyecdysone at either concentration examined failed to have significant effect on the synthesis of ecdysteroids from [^{3}H]3-dehydro-2,22,25-trideoxyecdysone, suggesting that there may not be a late control point in the ecdysteroid biosynthetic pathway in the prothoracic glands of *L. migratoria*.

Endogenous ecdysteroid biosynthesis was reduced in individual prothoracic glands in a dose dependent manner in the presence of exogenous 20-hydroxyecdysone (4nM, 10nM and 30nM). Addition of ecdysone (4nM and 10nM) had little effect on the endogenous ecdysteroid biosynthesis of the prothoracic glands. Inhibition of ecdysteroid synthesis was only observed in the presence of the highest concentration of exogenous ecdysone (30nM). Thus 20-hydroxyecdysone appears to be more effective than ecdysone in the inhibition of endogenous ecdysteroid biosynthesis in prothoracic glands. This phenomena has also been shown by *in vivo* studies on pupae of *P. brassicae* (Beydon and Lafont, 1983) and by *in vitro* studies on prothoracic glands from *M. sexta* larvae (Scott 1982, cited in Beydon and Lafont, 1983). 20-Hydroxyecdysone is considered the more physiologically active moulting hormone in larval stages of insect development (Bergamoso and Horn, 1980), and, therefore, its titre would be expected to be subjected to regulation during the various stages of insect development. The *in vitro* study suggests that this regulation is, to some extent, by direct action of 20-hydroxyecdysone on the prothoracic glands.

The acual site in the ecdysteroid biosynthetic pathway of prothoracic glands that is susceptible to feedback regulation is as yet unknown. Grieneisen *et al.* (1991) suggested it was at a stage subsequent to the formation of 7-dehydrocholesterol in the larvae of *M. sexta*. Since no evidence of feedback control was observed when [^{3}H]3-dehydro-2,22,25-trideoxyecdysone was used as a substrate in the prothoracic glands of *L.*

migratoria, it must be surmised that the major control point(s) exists in a region of the ecdysteroid biosynthetic pathway as yet unidentified, but post 7-dehydrocholesterol.

Chapter 4

Cycloartenol in relation to insect development

4.1. Introduction

Artificial manipulations of the sterol content of plants by both fungicidal and genetic means has led to the development of plants with insecticidal properties.

Long term treatment of maize seedlings with low concentrations of the systemic fungicide, fenpropimorph, has led to an almost complete replacement of the Δ^5-sterols (sitosterol, stigmasterol and campesterol) by 9β, 19-cyclopropyl sterols (cycloeucalenol, dihydro-cycloeucalenol and 31-norcyclobranol) and Δ^8-sterols (24-ethyl-5α-cholest-8-en-3β-ol and 24-methyl-5α-cholest-8-en-3β-ol). The 9β, 19-cyclopropyl sterols represented more than 90% of the total sterol content of the plant (Costet *et al.*, 1987; Corio-Costet *et al.*, 1989). This alteration in the sterol content of the maize seedlings could be traced back to a selective inhibition by the fungicide of two enzymes of the biosynthetic pathway of the plant sterols (Rahier *et al.*, 1986). Adult female locusts (*L. migratoria*) showed a dramatic decrease in their cholesterol content when reared on fenpropimorph-treated wheat, with the ecdysteroid concentration of eggs laid by these insects being reduced by up to 80% compared with those of the control insects which were fed on untreated wheat. This severe reduction of the ecdysteroid content was associated with a series of embryonic developmental arrests and/or abnormalities (Costet *et al.*, 1987). Larval *L. migratoria* showed considerable reductions in haemolymph ecdysteroid titre and delayed development when reared on fenpropimorph-treated wheat. In addition, higher mortality rates were observed for larvae which had been fed exclusively on treated wheat since hatching, as compared to the control insects reared on untreated wheat (Charlet *et al.*, 1988). Analysis of the sterol content of adult female *L. migratoria* fed on fenpropimorph-treated wheat has revealed a marked accumulation of 9β,19-cyclopropyl sterols and Δ^8-sterols in the free sterol fraction, and especially in their steryl ester fraction, with a concomitant decrease in the Δ^5, Δ^0 and Δ^7-sterol content of the insect (Corio-Costet *et al.*, 1989). These combined studies suggest that dietary 9β, 19-cyclopropyl sterols and Δ^8-sterols cannot be used by *L. migratoria* in place of Δ^5-sterols for ecdysteroid biosynthesis. This hypothesis is supported by the

ability of fenpropimorph-treated wheat supplemented with either the Δ^5-sterols, cholesterol or sitosterol to restore the ecdysteroid titre in both the adult female and eggs of *L. migratoria* (Costet *et al.*, 1987; Corio-Costet *et al.*, 1989).

Mevalonic acid (MVA) is the specific precursor molecule for a vast array of isoprenoids and phytosterols, which are synthesized and function in plant cells. The synthesis of MVA from 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) is catalysed by HMG-CoA reductase,which has also been ascribed a key regulatory function in phytosterol biosynthesis (Goad, 1983 ; Bach *et al*, 1991). In a transgenic tobacco plant of the strain, *Nicotiana tabaccum* (Amoco Corporation, European patent no. 0 480 730 A2) the copy number of a structural gene encoding a polypeptide having HMG-CoA reductase activity has been increased by tranforming the plant with a truncated HMG-CoA reductase gene (HMGR-Δ227) which has been placed under a cauliflower mosaic virus 35S (CaMV 35S) promoter, the function of which is substantially unaffected by the level of sterol in the transformed plant. The corresponding increase in HMG-CoA reductase enzymic activity of the transgenic plants results in a marked increase in the sterol content of these plants relative to the untransformed (native) tobacco plants, with the level of the 9β, 19-cyclopropyl sterol, cycloartenol, being raised from 3 - 30 percent of the total sterols present in the native plants to 60-70 percent of the total sterols in the transformed plants. First instar tobacco hornworm, *M. sexta* grown on leaves of the transgenic tobacco plant demonstrated retarded development (no progression beyond the second instar) and inhibited growth, compared with insects grown on leaves from the native plants. Chappell *et al.* (1991) have shown that, in contrast to the dramatic increase in the level of cycloartenol in the transgenic tobacco plant, the level of other isoprenoids (sesquiterpenes, phytoalexins and carotenoids) remained unaltered, and together with the observations for fenpropimorph-treated wheat, this result suggests that the inhibitory effect of the transgenic tobacco plant on insect development appears to be related to the high 9β, 19-cyclopropyl sterol content of the plant.

In this chapter, the effect of the 9β, 19-cyclopropyl sterol, cycloartenol will be described on ecdysteroid synthesis in the *in vitro* ovarian follicle and prothoracic gland

systems of *L. migratoria*. In addition, its effect on the growth and development of insects was assessed. MVA was incorporated into both maize seedlings and tomato, *Lycopersicon (α) esculentum* (variety Ailsa craig) callus, in an attempt to mimic the effect of the increased HMG-CoA reductase activity of the transgenic tobacco plant in elevating cycloartenol levels. Callus was used, since it could provide the opportunity to generate leaf tissue from it.

4.2. Experimental and results

4.2.1. Isolation of cycloartenol and 24-methylenecycloartanol from hydrolysed oryzanol extract from rice bran oil

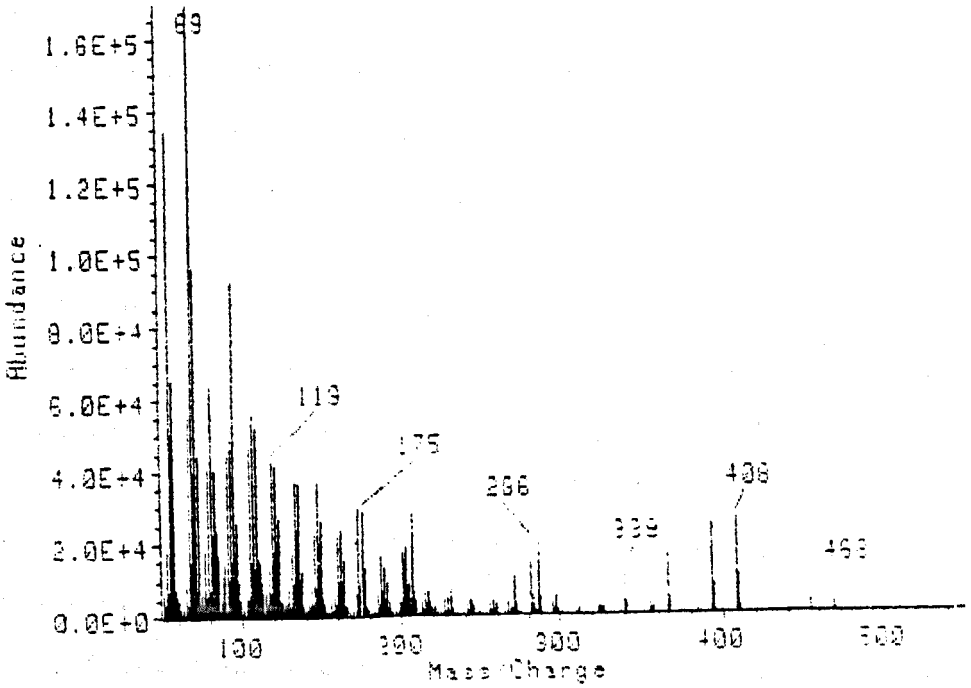
Hydrolysed oryzanol extract obtained from rice bran oil (300mg) was applied to a Woelm acid washed alumina column (30g); the alumina had been previously deactivated to Brockman grade III by accurate addition of 7% (w/w) water (under dry redistilled petrol). The column was sequentially washed with petrol, 2% (v/v) ether in petrol, 4% (v/v) ether in petrol, 6% (v/v) ether in petrol, 9% (v/v) ether in petrol and 20% (v/v) ether in petrol and ether. Aliquots from each fraction were analysed by thin-layer chrmoatography (TLC), using 0.25mm Kieselgel G plates developed in chloroform. The 4% (v/v) ether in petrol fraction was found to contain a single band that co-chromatographed with authentic lanosterol standard on TLC, suggesting the presence of only 4,4-dimethylsterols in this fraction. The 6% (v/v) ether and petrol fraction contained two bands which co-chromatographed with cholesterol and lanosterol standards, respectively, suggesting the presence of both 4,4-dimethylsterols and 4-desmethyl sterols in this fraction. This fraction was rechromatographed on TLC, and the band co-chromatographing with lanosterol standard was collected and combined with the 4% (v/v) ether in petrol fraction. This combined fraction was then evaporated to dryness and subjected to acetylation by dissolving it in pyridine(1.2ml) to which acetic anhydride (2.4ml) was added. The mixture was then allowed to stand over night under

nitrogen at room temp. The mixture was then extracted four times with ether, and the combined extracts washed briefly with 0.5M hydrochloric acid. The solution was then washed with saturated sodium carbonate solution (two times) and water (three times) until neutral. The ether extract was then dried over anhydrous sodium sulphate, filtered, and reduced to dryness. The steryl acetates formed were separated from non-reacted material by column chromatography on acid-washed Brockman grade III alumina, which was eluted with 2% (v/v) ether in petrol (steryl acetates) and 20% (v/v) ether in petrol (non-acetylated sterols). The 2% (v/v) ether in petrol fraction was then subjected to TLC on 0.25mm Kieselgel H plates impregnated with 5% (w/w) silver nitrate which were developed in chloroform. The steryl acetates were resolved into two component bands, which were collected separately and analysed by GC-MS. The identity of the upper band was found to be cycloartenyl acetate because of the presence of ions at *m/z* 468 and 408 in the mass spectrum (Fig. 4.1.1) which correspond to the $[M]^+$ and the $[M-ROH]^+$ ions, respectively. The mass spectrum of the lower band (Fig.4.1.2) corresponded to 24-methylenecycloartanyl acetate, due to the presence of ions at *m/z* 482 and 422 which correspond to the $[M]^+$ and $[M-ROH]^+$ species, respectively (Audier *et al.*, 1966; Williams *et al*; 1967). The two steryl acetates were then subjected to saponification by being dissolved in ethanol (20ml) to which 2ml of 60% (w/v) potassium hydroxide in water was added. The mixture was gently mixed and heated under reflux for 60 min, diluted with water (60ml), and extracted four times with ether (30ml). The ether extract was washed with water (four times) until neutral and evaporated to dryness under vacuum, to yield cycloartenol (16.8mg) and 24-methylenecycloartanol (30.3mg).

4.2.2. Effect of cycloartenol on the ecdysteroid biosynthetic activity of ovarian follicle cells and prothorcic glands of *L. migratoria.*

The inhibited growth and retarded development displayed by first instar *M. sexta* reared on leaves of the transgenic tobacco plant might be suggestive of abnormalities in

(1) Putative cycloartenyl acetate (upper band)



(2) Putative 24-methylenecycloartanyl acetate (lower band)

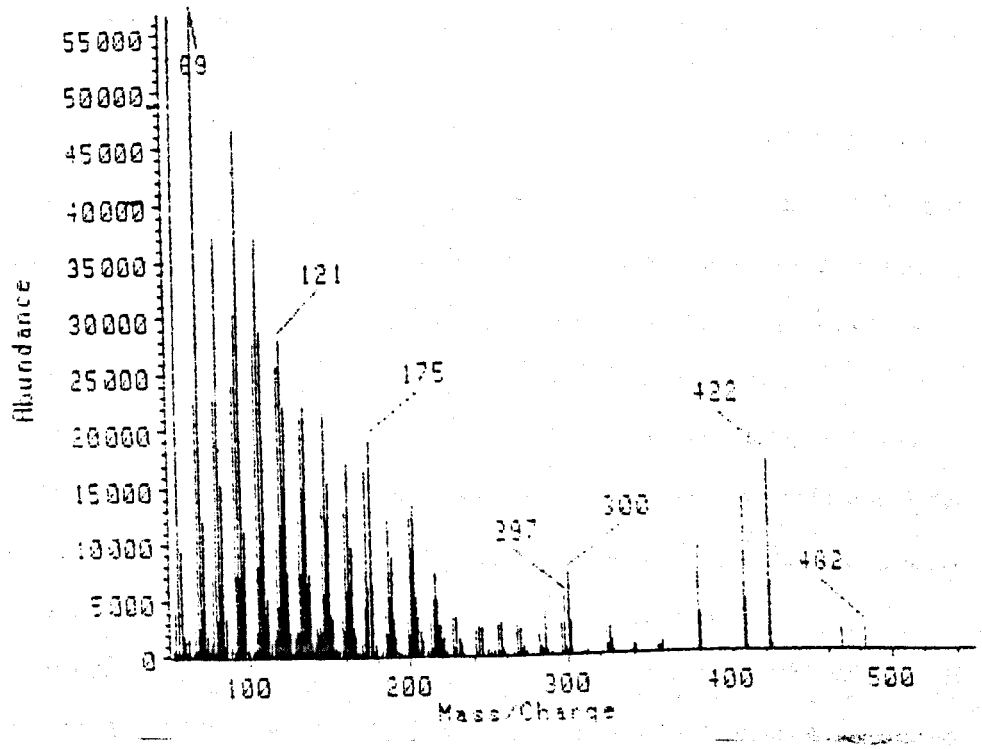


Fig. 4.1. GC-MS (E.I.) of (1) putative cycloartenyl acetate (upper band from $AgNO_3$-silica gel TLC plates) and (2) putative 24-methylenecycloartanyl acetate (lower band) obtained from the hydrolysed oryzanol extract.

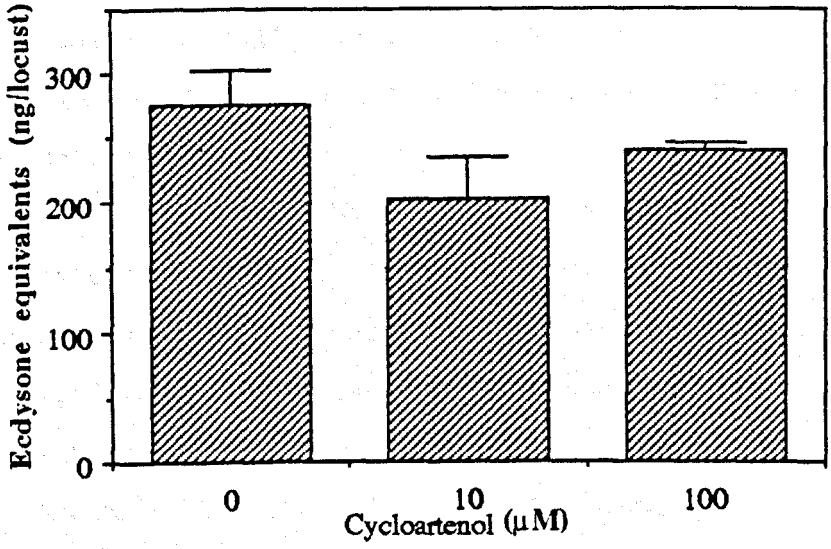
the ecdysteroid metabolism of the insects, as alluded to earlier. The transgenic tobacco plant contains a high cycloartenol content. Thus it is possible that the cycloartenol may be related to the plant`s insecticidal properties. Furthermore, fenpropimorph treated maize accumulating 9β,19-cyclopropyl sterols resulted in low ecdysteroid titres when fed to locusts (Costet *et al.*, 1987; Corio-Costet *et al.*, 1989). Therefore, the effect of cycloartenol was examined directly on ecdysteroid synthetic activity of *L. migratoria* ovarian follicle cells and prothoracic glands *in vitro.*

Follicle cells were prepared as described in section 2.2.2. Incubations were undertaken in triplicate [0.04% (w/v) Tween control sample was in duplicate] as described in section 2.2.19 in the presence of 10μM, 100μM and 1000μM cycloartenol, respectively. The cycloartenol was emulsified in Tween 20 as described in section 3.2.6 [0.01% (w/v)Tween for 10μM and 100μM, 0.04% (w/v) Tween for 1000μM cycloartenol, final concentration]. The reaction was terminated by the addition of ethanol (0.5ml), and samples were extracted three times in methanol (1ml) using a hand-held ground glass homogeniser. The extract was then hydrolysed by *Helix* hydrolase treatment (see section 2.2.15) and subjected to C_{18} Sep-Pak fractionation (see section 2.2.7, procedure 1). The 60% (v/v) methanol in water fractions were then analysed by RIA (see section 2.2.17) using DHS-1-13.5 antiserum.

Fig. 4.2 shows the effect of (1) 10μM and 100μM cycloartenol and (2) 1000μM cycloartenol on the endogenous ecdysteroid biosynthesis in the ovarian follicle cells, . The results are expressed as the mean of triplicate incubations from one experiment ± S.E.M. The addition of 10μM and 100μM cycloartenol reduced the endogenous ecdysteroid synthesis of the follicle cells from 275ng ± 25ng ecdysone equivalents [0.01% (w/v) Tween control] to 202.7ng ± 31.7ng and 239.7ng ± 4.7ng ecdysone equivalents, respectively. The addition of 1000μM cycloartenol reduced the endogenous ecdysteroid synthesis from 235ng ecdysone equivalents [0.04% (w/v) Tween control ; mean of duplicate values] to 133.7ng ± 42ng ecdysone equivalents.

The effect of cycloartenol on the ecdysteroid synthetic activity of prothoracic glands from *L. migratoria* was then investigated. Pairs of prothoracic glands were excised

(1) 10μM and 100μM cycloartenol



(2) 1000μM cycloartenol

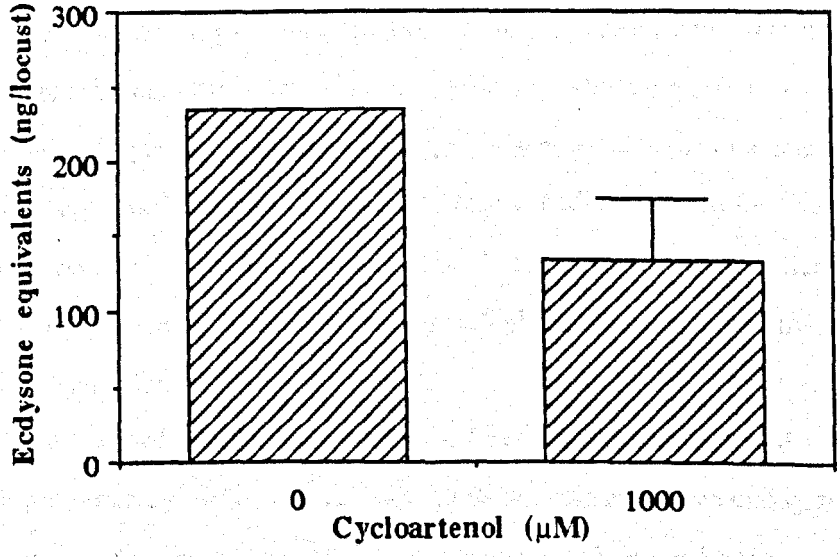


Fig.4.2. Effect of (1) 10μM and 100μM cycloartenol and (2) 1000μM cycloartenol, final concentrations on endogenous ecdysteroid biosynthesis in the ovarian follicle cells of *L. migratoria*, expressed as ng immunoreactive ecdysone equivalents produced per locust. Results are the mean values of triplicate incubations from one experiment ± S.E.M., except in the case of controls for 1000μM cycloartenol (2), where they are the mean values of duplicate incubations

from second day fifth instar *L. migratoria* (two glands from one insect/ incubation vial). Incubations were carried out in triplicate in Landureau`s medium (0.5ml) in the presence of 10μM, 100μM and 1000μM cycloartenol, respectively (0.5ml), emulsified in Tween 20 [0.01% (w/v) final concentration] for 4h at 33°C. The reaction was terminated by addition of ethanol (0.5ml) and the contents of the vials were analysed by RIA (see section 2.2.17), using DHS-1-13.5 antiserum.

Fig. 4.3 shows the effect of cycloartenol (10μM, 100μM and 1000μM, final concentration) on the endogenous ecdysteroid synthesis of pairs of prothoracic glands , expressed as ng ecdysone equivalents. The results are the mean of triplicate incubations from one experiment ± S.E.M. It can be assumed that all the ecdysteroid detected is synthesized during the 4h incubation, since prothoracic glands do not store detectable amounts of ecdysteroids. On addition of 10μM and 100μM cycloartenol, the endogenous ecdysteroid was reduced from 2.7ng ± 0.23ng ecdysone equivalents in the control sample (no cycloartenol) to 1.9ng ± 0.6ng ecdysone equivalents, and to 1.6ng ± 0.7ng ecdysone equivalents, respectively. On addition of 1000μM cycloartenol, the endogenous ecdysteroid synthesis of the prothoracic gland pair was reduced to 0.96ng ± 0.03ng ecdysone equivalents.

The effect of cycloartenol was further examined on the ecdysteroid synthetic activity of prothoracic glands by monitoring its effect on individual prothoracic glands, with the contralateral gland acting as a control. Individual glands were incubated in the presence of 100μM cycloartenol final concentration in Landureau`s medium (0.4ml) for 4h at 33°C, with the respective contralateral gland incubated in cycloartenol-free medium. The reaction was terminated by addition of ethanol (0.5ml) and the contents of the vial were analysed by RIA using DHS1-1-13.5.

Fig. 4.4 shows the effect of 100μM cycloartenol on the endogenous ecdysteroid biosynthesis of prothoracic glands. On addition of 100μM cycloartenol the endogenous ecdysteroid synthesis of the prothoracic glands (1075.5ng ± 113.0ng ecdysone equivalents) was reduced by 34% to 710.0ng ± 93.6ng ecdysone equivalents. This

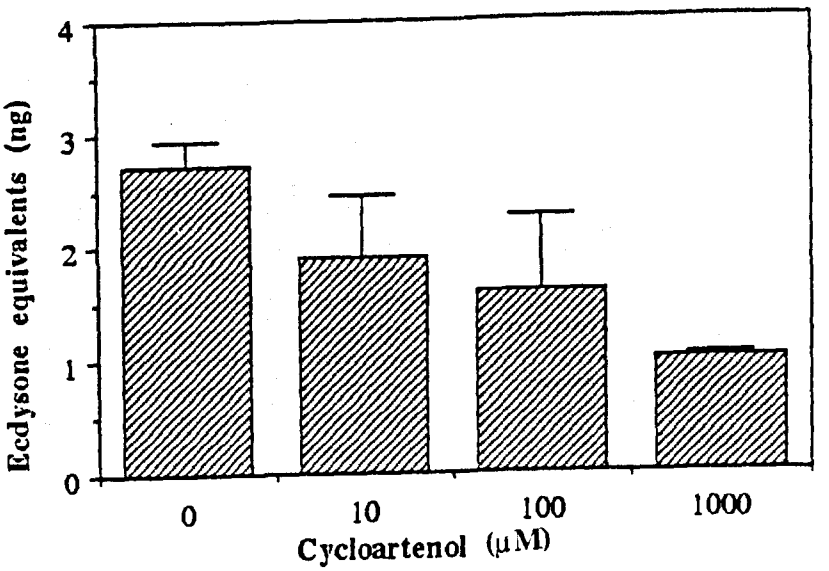


Fig.4.3. Effect of cycloartenol (10μM, 100μM and 1000μM, final concentrations) on the endogenous ecdysteroid synthesis of pairs of prothoracic glands from *L. migratoria* (two glands from one insect/ incubation vial), expressed as ng immunoreactive ecdysone equivalents. Results are the mean values of tripicate incubations from one experiment ± S.E.M.

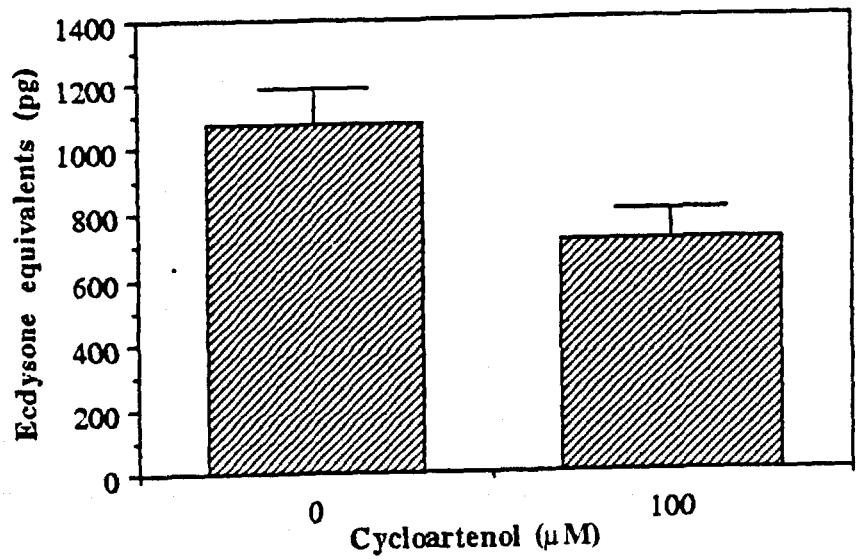


Fig.4.4. Effect of cycloartenol (100μM, final concentration) on the endogenous ecdysteroid synthesis of individual prothoracic glands from *L. migratoria*, expressed as pg immunoreactive ecdysone equivalents. Results are the mean values of four individual incubations from two separate experiments ± S.E.M.

compares with the 40.7% reduction in the previous experiment with 100μM cycloartenol.

4.2.3. Effect of cycloartenol on insect development

In vivo studies were undertaken to investigate the possible correlation between the high cycloartenol content of the leaves of the transgenic tobacco plant and the retarded growth and development expressed by first instar *M. sexta* reared on the these leaves , compared to those insects reared on the leaves of untransformed plants. Cycloartenol will be injected and force-fed into fourth instar *Heliothis virescens*. In addition, first instar *Spodoptera exigua* larvae were reared on an alfalfa bean based diet, in which hydrolysed oryzanol (consisting of approximately 40% (w/w) cycloartenol and 40% (w/w) 24-methylenecycloartanol) were incorporated.

For the injection and force-feeding of fourth instar *H. virescens* larvae, a stock solution of cycloartenol in acetone (20mg/20μl) was prepared. Three insects were injected using a Hamilton microlitre syringe (15mm; 33 gauge) with 0.5μl of the stock solution (50μg cycloartenol) in the region between the dorsal line and lateral line, avoiding the heart and the respiratory apparatus). Three insects were also injected with 0.5μl acetone to act as controls. Insects were then left for three days under culture conditions (temperature of 25^{o}C with a relative humidity of 60%, with a 16h light period and a 8h dark period and with a minimal alfalfa diet of 1g).

In the force-feeding experiment, fourth instar *H. virescens* larvae were secured in foam by use of a miniature vice. With the aid of a binocular microscope the insect was positioned to allow 2-3mm of a needle to enter its mouth ; 0.5μl of stock solution (50μg cycloartenol) was then introduced into the insect from the pre-primed needle. The insect was held in this position for 5 sec to allow the sample to be swallowed before being withdrawn. Insects were force-fed in five groups of two, with control insects being

force-fed 0.5µl of acetone. Insects were then left for 3 days under culture conditions with minimal alfalfa diet (1g).

In the diet incorporation experiment, 30mg of hydrolysed oryzanol was dissolved in 750µl of acetone, 250µl of this solution (10mg) was mixed into the surface of 990mg of alfalfa diet in the insect pots (in triplicate), and the acetone was then left to evaporate. In the control samples, 250µl of acetone only was added to the alfalfa diet (in triplicate) and then allowed to evaporate. Ten first instar *S. exigua* larvae were added to each pot and left under culture conditions for 7 days.

All the fourth instar *H. virescens* larvae injected with 50µg of cycloartenol successsfully passed through the fourth to fifth instar moult, with their morphology and development comparable to the control insects.

In the force-feeding experiment, four out of the five insects successfully passed through their fourth to fifth instar moult (one insect was mortally wounded during force feeding), with their morphology and development comparable to the control insects.

The majority (28/30) of the first instar *S. exigua* larvae fed on alfalfa diet supplemented with hydrolysed oryzanol reached the third instar, with morphological, development and mortality rate comparable to the control insects.

The insect samples and alfalfa diet (990mg) were then extracted in chloroform / methanol (2:1 v/v; three times) using a Polytron homogenizer with the addition of internal standards of 5α-cholestan-3β-ol (20µg; for the 4-desmethylsterols, 4-methyl-sterols and 4,4-dimethylsterols) and of cholesteryl palmitate (20µg; for steryl esters). The extracts were then applied to 0.25mm Kieselgel G TLC plates developed in chloroform. The bands migrating in the region of the lanosterol and cholesterol standards was collected whereas the band co-chromatographing with cholesteryl palmitate were collected and subjected to saponification as described in section 4.2.1. The saponified sterol ester and free sterol fractions were then analysed by GC (see section 2.2.11) as their TMS-ethers with injection of authentic TMS-standards. However, due to the small amount of biological material available, GC analysis of the insect samples did not reveal the presence of sterols, in either the free sterol fractions or

the saponified steryl ester fractions. GC analysis of the alfalfa bean diet (990mg) revealed the presence of stigmasterol (18μg), sitosterol (112μg) and campesterol (21μg) in the free sterol fraction and sitosterol (55μg) in the saponified steryl ester fraction.

4.2.4. Incorporation of MVA into maize seedlings

In an effort to mimic the high level of HMG-CoA reductase activity of the transgenic plant, and increase the level of cycloartenol, an attempt was made to incorporate excess exogenous MVA, the product of this enzyme, into maize seedlings.

As mevalonic acid is supplied as mevalonic acid lactone for stability, the lactone ring was opened before use, by employing the method of Comley and Jaffe (1981) Mevalonic acid lactone (261.0mg) was dissolved in 50mM sodium bicarbonate solution (20ml) and left to stir for 12h at room temperature. After this time the pH of the solution was raised to 7.2 by the addition of 0.1M NaOH, and the volume increased to 2 litres by the addition of distilled water to yield a 2mM MVA solution. Maize seedlings were divided into two groups and either soaked overnight in the 2mM mevalonic acid solution (treated group) or in distilled water adjusted to pH 7.2 with NaOH (control group). The two groups of seeds were placed in separate trays containing Vermiculite and watered daily with either 2mM mevalonic acid (treated seeds) or distilled water at pH 7.2 (control seeds). The seeds were then left to grow at room temperature. After three weeks the seedlings were harvested in groups of thirty and weighed. A group of control seedling (25.7g) and treated seedlings (22.1g) were freeze dried and extracted four times in chloroform/ methanol (2:1 v/v; 15ml) using a Polytron homogeniser with addition of internal standards ; 5α-cholestan-3β-ol (300μg) and cholesteryl palmitate (300μg). The extracts were then applied to 0.5mm Kieselgel TLC plates developed in chloroform, and the combined 4-desmethylsterols, 4α-methylsterols and 4,4-dimethylsterols eluted as well as the steryl esters bands were collected. The steryl ester fractions were saponified as described in section 4.2.1. The saponified steryl ester fractions and the free sterol fractions were then analysed by GC (see section

2.2.11) as their TMS-ethers with injection of authentic TMS-standards. Fig. 4.5 shows the sterol composition of both the free sterol and saponified steryl ester fractions from both the treated and untreated maize seedling samples. On treatment with 2mM mevalonic acid the sterol content of the maize seedlings apparently increased from 457.7µg/ g fresh weight to 481.5µg/ g fresh weight, an overall increase of 5.1%. Cycloartenol was only detected in the saponified ester fraction from the seedlings, being 3.4µg/g fresh weight and 3.1µg/ g fresh weight in the control and treated samples, respectively.

4.2.5. Formation of tomato callus

To enhance the incorporation of MVA into plant cells, callus cultures were prepared from tomato seedlings. Callus culture is a tool adjunct to studies on whole plants, with its decreased level of structural organisation relative to whole plants being conducive to the uptake of metabolites into individual plant cells.

A procedure for the initiation of callus culture from tomato seeds is summarized in Fig. 4.6. Two hundred tomato (*L. esculentum*) seeds of the variety Ailsa Craig were sterilised by soaking for 20min in 10% (v/v) Domestos in distilled water. The seeds were placed in distilled water for 10min and then transferred to fresh distilled water for a further 10min. Washing with distilled water was repeated twice. The sterilised seeds were then placed aseptically into six ounce round powder jars (four /jar) containing agar-solidified Murashigue and Skoog medium (20ml/ jar), pH 5.8 (see section 2.1.8). The jars were then covered with silver foil lids and seeds were then grown for 4 weeks at 25°C with a 12h light : dark period.

The roots and leaves were discarded from the resulting seedlings, and the hypocotyl was divided into 5mm segments (explants). These segments were then placed aseptically into Petri dishes (six/plate) containing agar solidified Murashige and Skoog medium (20ml/plate) and the phytohormones 6-benzylamino purine (BAP; 10^{-5}M) and α-naphthaleneacetic acid (NAA; 10^{-5}M). The Petri dishes were sealed with parafilm and

	CONTROL	TREATED
Free sterol fraction	campesterol (591µg)	campesterol (1182µg)
	stigmasterol (2231µg)	stigmasterol (1496µg)
	sitosterol (3749µg)	sitosterol (2743µg)
	Total = 6571µg (255.7µg/g fresh weight)	**Total** = 5421µg (245.3µg/g fresh weight)
4,4-Dimethylsterol Fraction	undetectable	undetectable
Saponified ester fraction	campesterol (1179µg)	campesterol (1185µg)
	stigmasterol (573µg)	stigmasterol (622µg)
	sitosterol (3440µg)	sitosterol (3413µg)
	Total = 5192µg (202.0µg/g fresh weight)	**Total** = 5220µg (236.2µg/g fresh weight)
	cycloartenol (88µg) (3.4µg/g fresh weight)	cycloartenol (69µg) (3.1µg/g fresh weight)

Fig. 4.5. Composition of the free sterol and ester fractions from thirty treated (0.2mM mevalonic acid) and untreated maize seedlings. The results are expressed in µg. Fresh weight of the treated and untreated maize seedling samples were 22.1g and 25.7g, respectively.

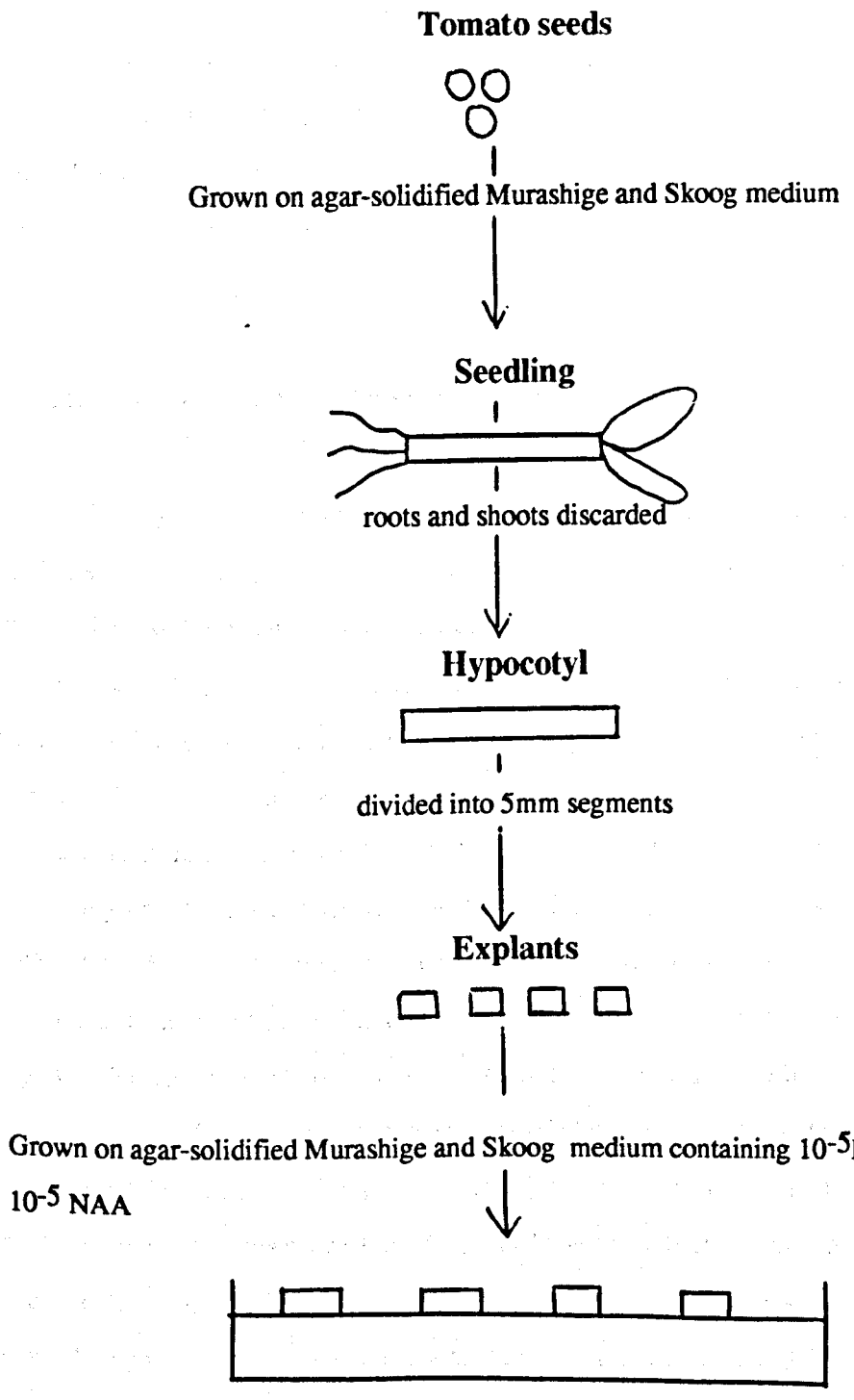


Fig.4.6. Method for the preparation of tomato callus

BAP: 6-benzylamino purine

NAA: α-naphthaleneacetic acid

cultured, under conditions described previously for a period of 4 weeks. The resulting tomato callus was subcultured by dividing individual calli into segments of 3-5mm and tranferring these to fresh plates (6/plate) containing Murashige and Skoog medium (10^{-5}M BAP and 10^{-5}M NAA). The tomato callus was then cultured for 4 weeks. All manipulations of tomato seeds, seedlings and callus were performed in a laminar flow cabinet.

4.2.6. Incorporation of MVA into tomato callus

Sixty Petri dishes, containing Murashige and Skoog medium and 10^{-5}M BAP and 10^{-5} NAA (20ml/plate) in molten agar were set up in a laminar flow cabinet. To the plates, 100µl of filtered-sterilized 50mM sodium bicarbonate solution (10ml) containing varying concentrations of mevalonic acid were added, to yield groups of twelve plates containing final concentrations of 0.5mM, 1.0mM, 2.0mM or 4.0mM MVA. The plates were then left for 2h to allow the agar to solidify.

The subcultured tomato calli (Fig. 4.7) were divided into 4mm segments and placed aseptically onto the plates (6/plate), which were then sealed and cultured for 4 weeks at 25°C. After this time, the individual calli were removed from each plate and weighed to determine the effect of the higher concentration of MVA on their growth (fresh weight; Fig. 4.8). However, due to overall reduction in the accumulation of biomass by the calli in the presence of the different concentrations of MVA, this experiment was repeated with 4mm calli segments being grown individually in universal vials in the presence of the lower concentrations only (0.0mM, 0.01mM, 0.1mM, 0.2mM and 0.5mM MVA) twelve calli samples being subjected to each concentration. Calli were cultured individually to provide sufficient nutrient for longer culture periods. After six weeks in culture when the calli had grown to a sufficient size (Fig. 4.9) the individual calli were weighed to determine the effect of the different concentrations of MVA on their growth (Fig. 4.10). It was found that only in the presence of 0.5mM MVA was there an overall inhibition in the growth of the calli.

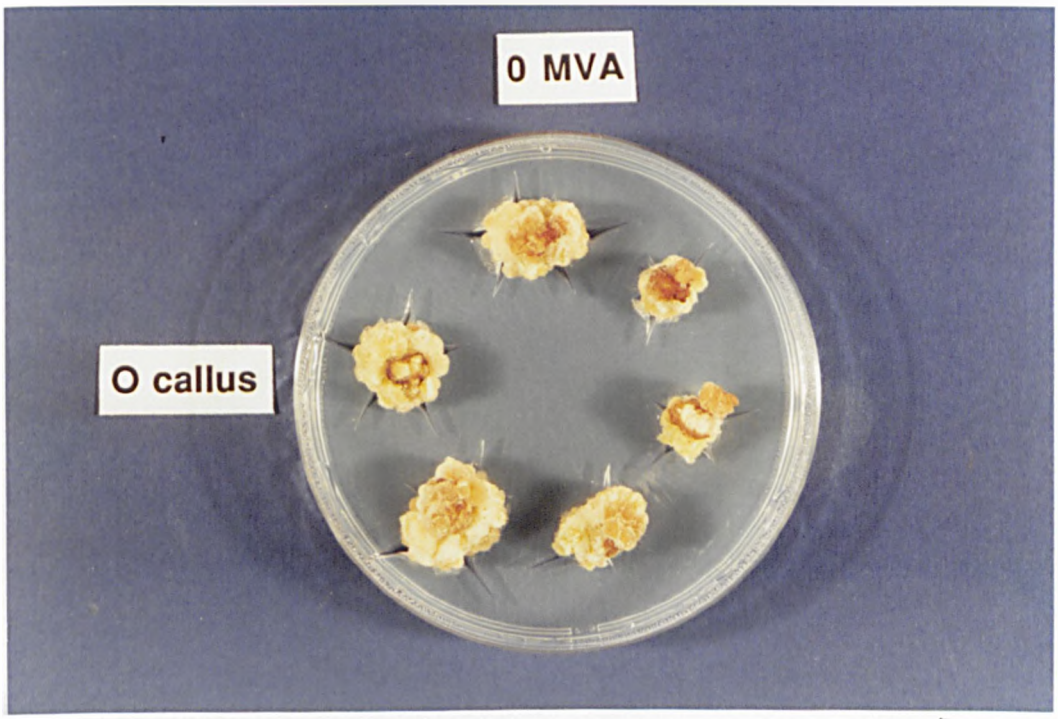


Fig. 4.7. Photograph of subcultured tomato callus sample(six calli/petri dish).

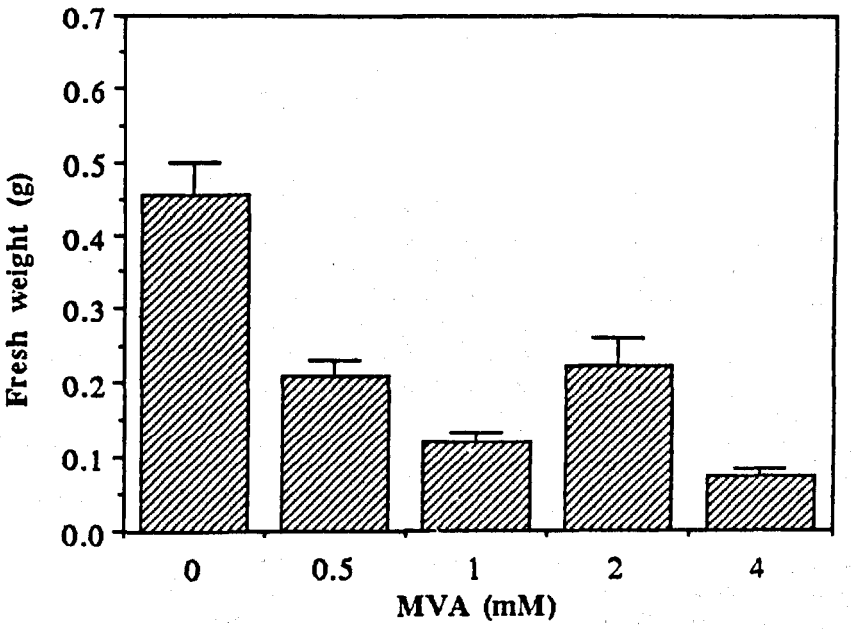


Fig.4.8. Effect of MVA (0.5mM, 1mM, 2mM and 4mM, final concentrations) on the growth of tomato callus. The results are expressed as fresh weights (g) and are the mean of forty individual values ± S.E.M.

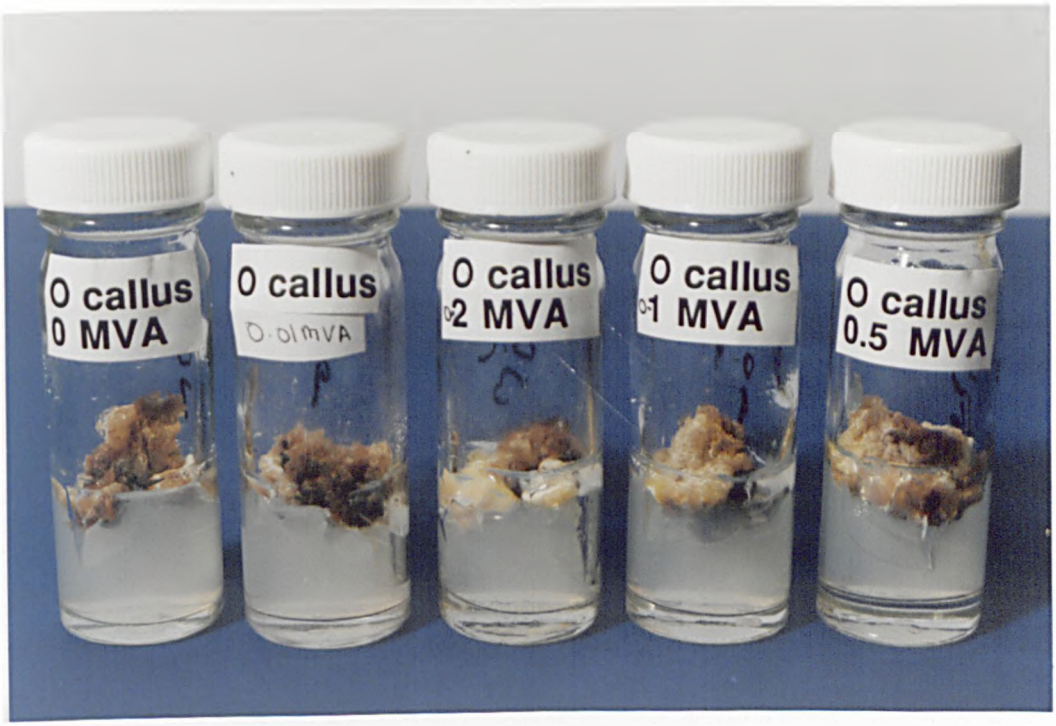


Fig. 4.9. Photograph to show the effect of MVA (0.01mM, 0.1mM, 0.2mM and 0.5mM, final concentrations) on the growth of tomato callus (one callus/ universal vial), relative to the control (no MVA).

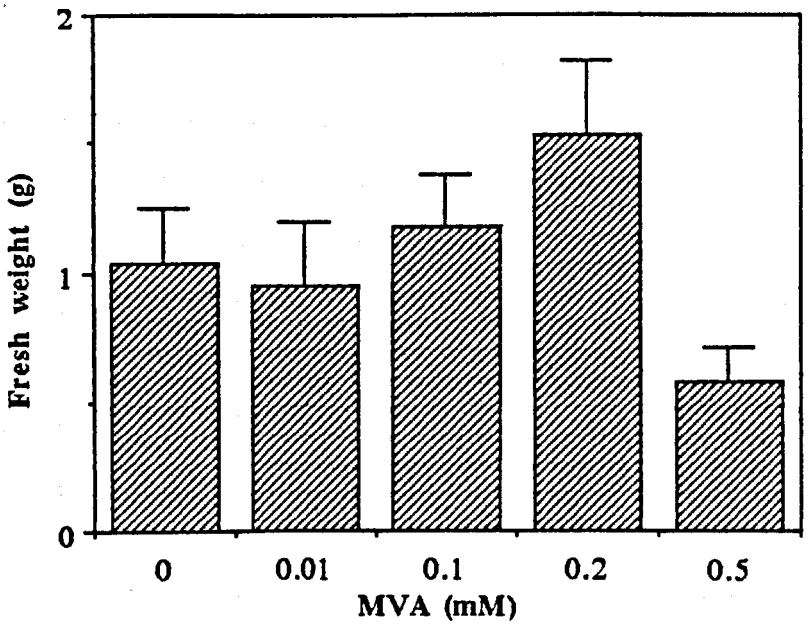


Fig.4.10. Effect of MVA (0.01mM, 0.1mM, 0.2mM and 0.5 mM, final concentrations) on the growth of tomato callus. The results are expressed as fresh weight (g) and are the mean of twelve individual values $\pm$ S.E.M.

Callus samples grown in the presence of 0.0mM, 0.01mM, 0.2mM and 0.5mM MVA were divided into groups of three weighing approximately 3g fresh weight per concentration and freeze dried. These samples were then extracted as outlined below.

Each sample was homogenized in ethanol (10ml) using a Polytron homogenizer with the addition of internal standard 5α-cholestan-3β-ol (100μg). After homogenization, 1g of potassium hydroxide was added to the sample which was saponified by heating under reflux for 1h. The sample was then transferred to a separating funnel and allowed to cool before being diluted with water (40ml) and extracted with ether (30ml). The resulting aqueous layer was re-extracted (4 times) with ether (40ml). The combined ether extracts were washed with water until neutral and reduced to dryness under vacuum. The extracts were then applied to a 0.5mm Kieselgel G TLC plate which was developed in chloroform. The combined 4α-methylsterols, 4-desmethylsterols and 4,4-dimethylsterols were combined and analysed by GC (see section 2.2.11) as their TMS-ethers with injection of authentic TMS-standards. Fig. 4.11 shows a typical GC chromatogram of theTMS-ethers of the total sterols (free and saponified esters) isolated from the tomato callus. Peaks 2,3,4, 5 and 7 were found to co-chromatograph with authentic TMS-standards (Fig. 4.11). It is noteworthy that the cycloartenyl-TMS ether peak was broad in comparison to other peaks, and thus, may have contained another compound. Confirmation of the identity of the compounds was obtained by GC-MS performed on a Hewlett Packard 5970 series Mass Selective Detector under the control of HP ChemStation software. The column employed for the analysis was a 25m x 0.2mm i.d. HP1 (0.33μm film thickness) with the GC oven being programmed from 50°C to 150°C at 25°C min^{-1}, held at this temperature for 1 minute, then programmed to 290°C at 6°C min^{-1}, and held isothermically. Comparison of the mass spectra of peaks 1-6 and 8 in the TIC chromatogram of the total sterol isolated from the tomato callus (Fig. 4.12) with the mass spectra of authentic TMS-standards (Yates, 1991) revealed that the identities of peaks 1,2,3,4,5,6 and 8 were cholesterol, 5α-cholestan-3β-ol (internal standard), campesterol, stigmasterol, sitosterol, isofucosterol and 24-methylenecycloartanol, respectively (see Appendix 1 for mass spectra and

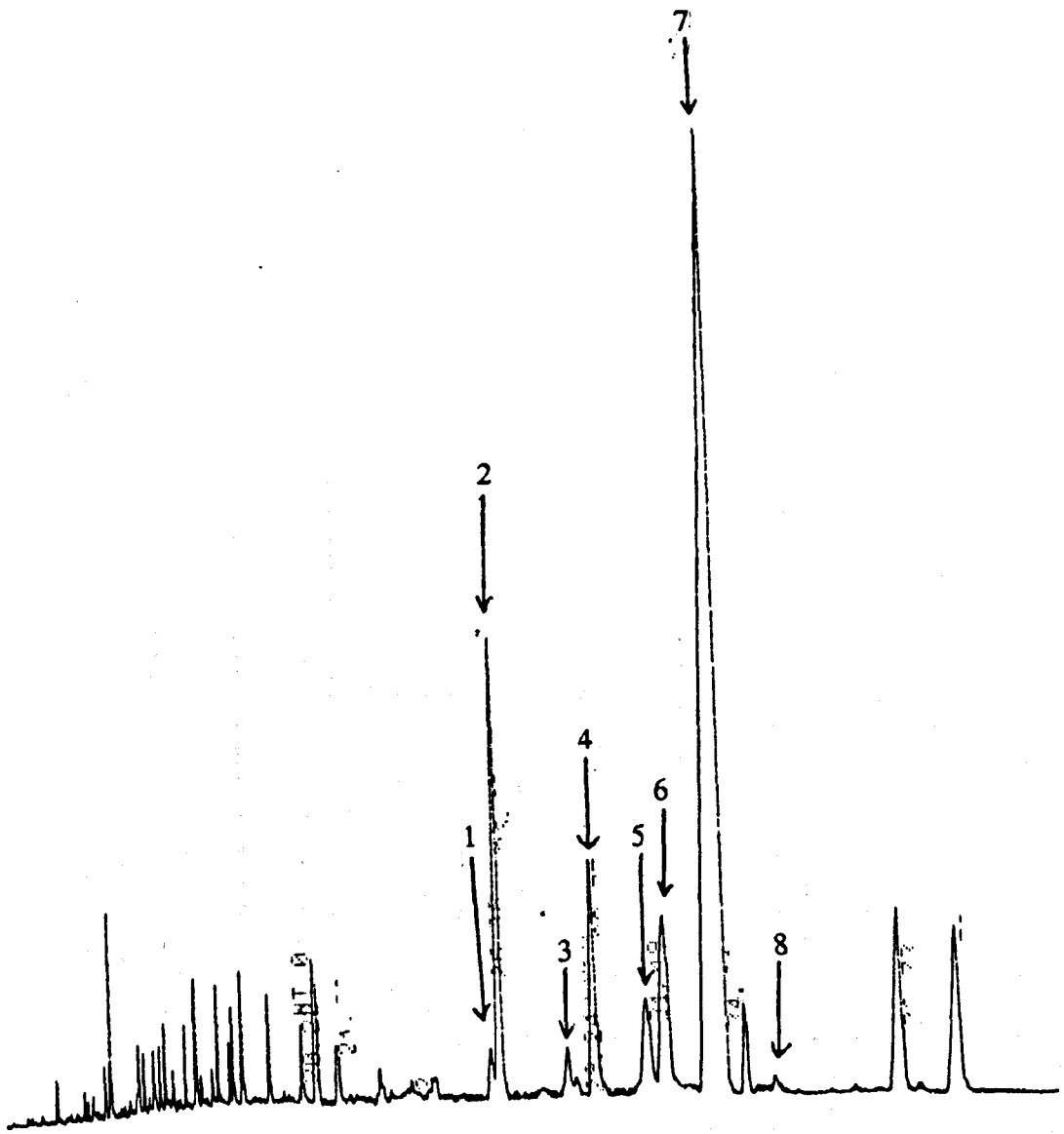


Fig. 4.11. A typical GC chromatogram (control sample) of theTMS-ethers of the total sterols, 4α-methylsterols plus 4,4-dimethylsterols (free and saponified esters, present as their TMS-ethers) isolated from tomato callus. The position of authentic TMS-ether standards are shown: 5α-cholestan-3β-ol (peak 2), campesterol (peak 3), stigmasterol (peak 4), sitosterol (peak 5) and cycloartenol (peak 7).

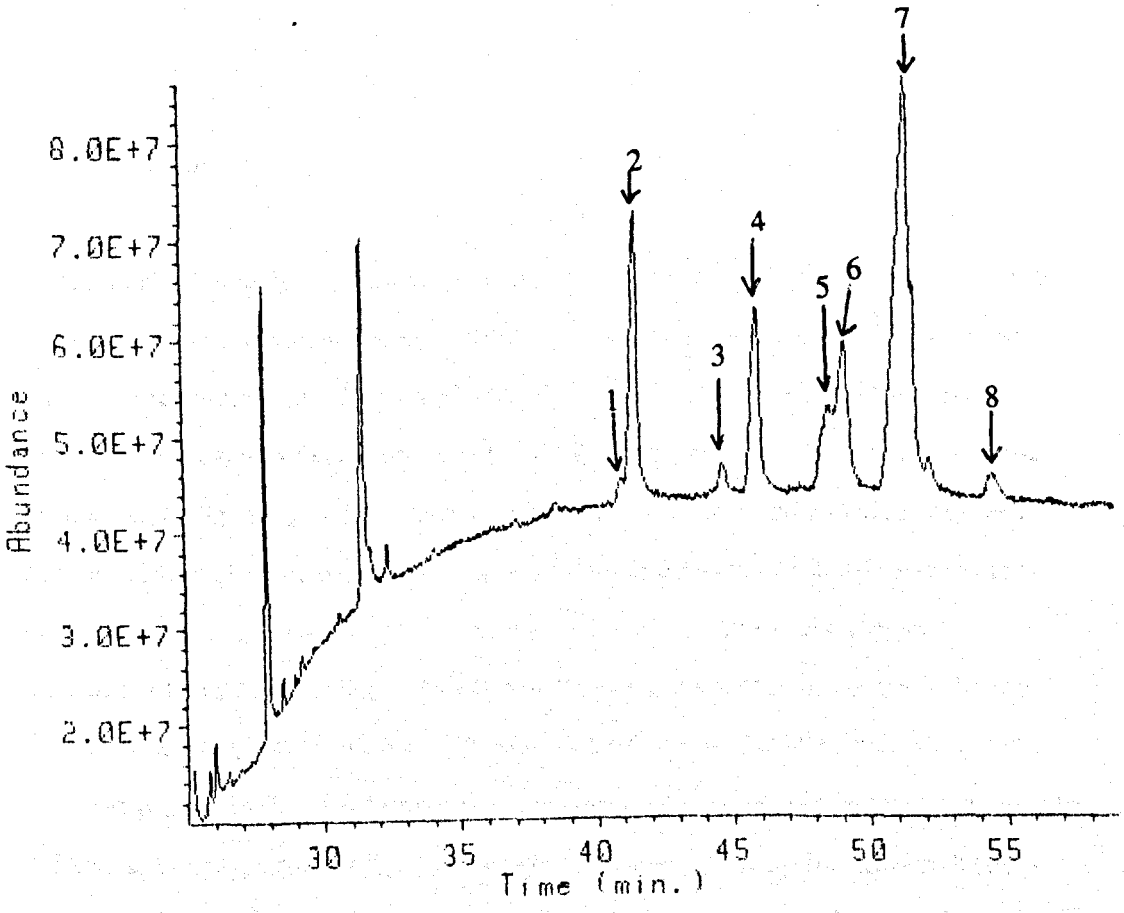


Fig. 4.12. A typical Total ion current chromatogram (TIC; control sample) of the TMS-ethers of the sterols isolated from the tomato callus. Peaks are numbered in accordance with the previous GC chromatogram (Fig. 4.11).

interpretation). As the TIC (Fig. 4.11) showed a shoulder on peak 7, two spectra were taken for this peak. A spectrum was taken at the peak (7a) and a spectrum was taken at the subsequent shoulder (7b). The enhanced spectrum of 7b closely resembled that of cycloartenol, whereas, the identity of spectrum 7a is uncertain (see Appendix 1), but was likely to be a 4α-methylsterol. Clearly, before cycloartenol can be quantified the two compounds co-chromatographing on GC must be separated before analysis by TLC.

4.3. Discussion

On incubation of *L. migratoria* ovarian follicle cells with cycloartenol, an inhibition of the ecdysteroid synthetic activity of the cells was observed in the presence of the highest concentration of this sterol (1000μM; Fig. 4.2). Prothoracic glands also exhibited lower rates of ecdysteroid synthesis in the presence of 100μM and 1000μM cycloartenol (Fig. 4.3 and 4.4). The reason for this decrease in endogenous ecdysteroid synthesis is not clear. However, the reduced ecdysteroid synthetic activity expressed by both ovarian follicle cells and prothoracic glands in the presence of exogenous cycloartenol could be due to a dilution of their endogenous cholesterol pools by this 9β, 19-cyclopropyl sterol. This would result in a reduction in available substrate for enzymes present in the initial stages of the ecdysteroid biosynthetic pathway, and lead to a decrease in ecdysteroid synthesis. The mechanism by which cycloartenol reduces ecdysteroid synthesis in the follicle cells and prothoracic glands could be further elucidated, by an examination of the products of ecdysteroid synthesis by HPLC-RIA and GC-MS.

In the *in vivo* insect studies, no morphological defects, increased mortality or retarded growth and development were observed following injecting or force-feeding cycloartenol into fourth instar *H. virescens* or rearing first instar *S. exigua* larvae on alfalfa diet with incorporated hydrolysed oryzanol extract. As cycloartenol appeared to have no direct inhibitory effect on insect development in these studies, it must be

assumed that the insecticidal properties of the leaves of the transgenic tobacco plant (Amoco Corporation) are not related solely to their high cycloartenol content, but possibly to the elevated ratio of this 4,4-dimethylsterol to the Δ^5-sterols (campesterol, stigmasterol and sitosterol) in their leaves. Although cycloartenol accumulates in the transgenic plants, the insecticidal effect may not necessarily be due to this sterol, but to another isoprenoid product which might be elevated. Insects are unable to synthesize *de novo* the steroid nucleus and depends upon an exogenous source for their sterol economy, in particular the biosynthesis of ecdysteroids (Clayton, 1964). Therefore, if the Δ^5-sterol content of their food source is severely reduced, as it is in fenpropimorph-treated wheat (the Δ^5-sterol content of the plant being almost completely replaced by 9β, 19-cyclopropyl sterols), insects reared on such a food source are unable to utilize 9β, 19-cyclopropyl sterols for ecdysteroid biosynthesis and display severe developmental modifications (Charlet *et al.*, 1988; Corico-Costet *et al.*, 1989). Analysis of the alfalfa diet which *S. exigua* and *H. virescens* were reared on in these studies has revealed a Δ^5 -sterol content of approximately 200µg/ g fresh weight, a level of dietary sterol sufficient to restore any possible disturbance in the ecdysteroid metabolism of fourth instar *H. virescens* larvae force-fed or injected with cycloartenol (50µg). In addition, the ability of insects to discriminate between dietary sterols and preferentially absorb and concentrate the Δ^5-sterols present in the diet (Svoboda and Thompson, 1985; Costet *et al.*, 1987) has possibly contributed to the lack of developmental abnormalities expressed by first instar *S. exigua* reared on an alfalfa diet supplemented with 1% (w/w) oryzanol. However, this would also be expected to apply to the transgenic plant sterols as well.

Due to the difficulty in reproducing artificially the sterol composition of the leaves of the transgenic tobacco plant in insect diets, an attempt was made to mimic the effect of high HMG-CoA reductase activity in this plant by incorporating the product of this enzyme, MVA, into maize seedlings. However, treatment of maize seedlings with MVA resulted in an apparent increase of only a 4.6% in the total sterol content and a decrease in the cycloartenol content compared with control seedlings. To remove the possibility

of permeability and/or transportational factors affecting the incorporation of MVA into plant tissue, callus was prepared from tomato seedlings, enabling a method for the direct uptake of MVA at the level of the plant cell. The total sterols (free and saponified esters) from the tomato callus contained the desmethylsterols, cholesterol, campesterol, sitosterol, stigmasterol and isofucosterol, and the 4,4-dimethylsterols , cycloartenol and 24-methylenecycloartanol. However, because of possible co-elution of another compound with cycloartenol during the GC and GC-MS analysis (Fig. 4.11 and 4.12), the effect MVA on the sterol profile of the callus will require further work involving separation of the components.

Further work to recreate the high cycloartenol environment of the leaves of the transgenic tobacco plant could involve the preparation of agar-based insect diet supplemented with vitamins and amino acids to which the cycloartenol and Δ^5-sterols could be incorporated allowing the sterol composition of the diet to be controlled for *in vivo* insect studies.

Overall, these studies have shown that cycloartenol at a sufficient concentration can reduce ecdysteroid synthetic activity *in vitro*, although at present the mechanism by which it has its effect is unknown. In addition, these studies have suggested that the insecticidal properties of the leaves of transgenic tobacco plant are related to their low Δ^5-sterol content rather than solely to their high cycloartenol content, although the possibility remains that an isoprenoid(s) other than a sesquiterpene, phytoalexin or carotenoid, whose concentrations are not affected by the transformation, is causing this effect (Chappell *et al.*, 1991).

Chapter 5

The effect of cytochrome P450 inhibitors on ecdysteroid synthesis *in vitro*

5.1. Introduction

The enzymes responsible for catalysing the terminal hydroxylation steps in the ecdysteroid biosynthetic pathway of insects are known to be cytochrome P450-dependent monooxygenases requiring both NADPH and molecular oxygen (Kappler *et al.*, 1989). A Cytochrome P450 is a membrane bound enzyme consisting of a single polypeptide chain (40-60 kD) containing an iron protoporphyrin moiety (haem group) non-covalently bound in a hydrophobic pocket of the protein. The haem iron forms four ligand bonds with the nitrogen atoms of the protoporphyrin ring, and a fifth with a cysteine residue from the polypeptide. Substrates can bind to either the protein part of the cytochrome P450 molecule or ligate to the haem iron atom at the sixth coordination position (Gibson and Skett, 1986).

Ecdysone 20-monooxygenase is a well documented cytochrome P450 enzyme, being responsible for the conversion of ecdysone into 20-hydroxyecdysone (Johnson and Rees, 1977b). This enzyme is present in a number of peripheral insect tissues and species, including the fat body, Malpighian tubules, midgut and integument, where it can have either mitochondrial, microsomal or dual sub-cellular location, depending on the tissue (Weirich *et al.*, 1984; Smith, 1985). Ecdysone 20-monooxygenases undergoes distinct changes in activity during post-embryonic development, and it has been postulated that this variation in activity may contribute to the overall regulation in ecdysteroid titre or the titre ratio of ecdysone to 20-hydroxyecdysone (Smith *et al.*, 1983; Smith, 1985).

The 25-, 22- and 2-hydroxylases responsible for the overall conversion of 2,22,25-trideoxyecdysone into ecdysone in the ecdysteroid biosynthetic pathway of insects have been studied in the prothoracic glands of *L. migratoria*, where the mitochondrial 22-hydroxylase and the microsomal 25-hydroxylase were shown to be classical cytochrome P450-dependent monooxygenases. In contrast, the mitochondrial 2-hydroxylase was shown to be an atypical cytochrome P-450 monooxygenase by its relative insensitivity to carbon monoxide (Kappler *et al.*, 1988).

The inhibition of cytochrome P450 enzymes can occur by both reversible and irreversible complex formation with the active site of the protein or the prosthetic haem group. Of the reversible inhibitors, the most effective ones are those which interact strongly with both the lipophilic region of the protein and the iron atom of the prosthetic haem group. These inhibitors include nitrogen-containing aliphatic and aromatic compounds such as imidazole and pyridine derivatives which have found particularly widespread utility as cytochrome P450 inhibitors (Vanden Bossche, 1988).

In insects, the imidazole derivative KK42 (1-benzyl-5-[(E)-2,6-dimethyl-1,5-heptadienyl] imidazole) has been shown to suppress the haemolymph ecdysteroid titre *in vivo* of larval *Bombyx mori*, in addition to inhibiting *in vitro* ecdysteroid synthesis in prothoracic glands from this species and *L. migratoria* (Kadono-Okuda *et al.*, 1987; Roussel *et al.*, 1987). Certain imidazole derivatives including KK42 and the pyridine derivative, metyrapone, have been shown to effectively inhibit under *in vitro* conditions the activity of the microsomal ecdysone 20-monooxygenase from the fat body of fresh fly larvae, *Neobellieria bullata* Parker (Darvas *et al.*, 1992). Metyrapone has also been demonstrated to inhibit *in vitro* the activity of the mitochondrial ecdysone 20-monooxygenases from the fat body of *M. sexta* and Malpighian tubules from *S. gregaria*, in addition to inhibiting the activity of the 25-, 22- and 2-hydroxylases from the prothoracic glands of *L. migratoria* (Greenwood and Rees, 1984; Weirch *et al.*, 1984; Kappler *et al.*, 1988). A number of heterocyclic compounds have been shown to inhibit the *in vitro* ecdysteroid synthesis from radiolabelled 2,22,25-trideoxyecdysone in ovarian follicle cells from *L. migratoria*, with the 22-hydroxylase enzyme being implicated as the most susceptible site of this inhibition (Jarvis *et al.*, 1994b).

In this chapter, the effect of a number of heterocyclic cytochrome P450 inhibitors will be described on activity of the mitochondrial ecdysone 20-monooxygenase from the fat body of sixth instar *S. littoralis*. In addition, by use of radiolabelled 2,22-dideoxyecdysone and 2-deoxyecdysone as substrates in the *in vitro* ovarian follicle cell system of *L. migratoria*, the effect of these putative inhibitors were directly assessed

on the activity of the 22-, 2- and 20-hydroxylase enzymes in the ecdysteroid biosynthetic pathway of this insect species.

5.2. Experimental and results

5.2.1. The effect of a range of cytochrome P450 inhibitors on the activity of the mitochondrial ecdysone 20-monooxygenase from fat body of sixth instar of *S. littoralis*

Initially the effects of various cytochrome P450 inhibitors were investigated on the activity of the mitochondrial ecdysone 20-monooxygenase from fat body from of sixth instar *S. littoralis* larvae. In order to allow comparison between the potency of inhibitors in separate experiments, the imidazole fungicide, ketoconazole, which has been previously shown to inhibit ecdysone 20-monooxygenase (Hoggard, 1989) was included in each experiment. A concentration of 5ppm was determined for this compound, at which approximately 60% inhibition of the ecdysone monooxygenase activity could be demonstrated.

The mitochondrial fraction from the fat body of *S. littoralis* was prepared as described in section 2.2.3 and aliquots of the resulting fraction (0.2 insect equivalents/ sample vial) were then pre-incubated for 10min at 37°C with the individual cytochrome P450 inhibitors which were previously dissolved in hypotonic HEPES buffer, pH 7.4 (100µl/sample vial). After pre-incubation, the samples were assayed for ecdysone 20-monooxygenase activity (see section 2.2.18). Inhibitors were present at final concentrations of 10ppm and 20ppm, respectively, in the incubation vials. All incubations were carried out in duplicate. The products of the assay were then analysed by RP-HPLC (system 2, see section 2.2.8), with co-injection of authentic standards. Ecdysone 20-monooxygenase activity was measured by the conversion of [^{3}H]ecdysone

into [^{3}H]20-hydroxyecdysone as expressed as a percentage of the total ecdysteroid recovered from RP-HPLC.

Initially, inhibition of the ecdysone 20-monooxygenase was investigated using compounds representative of the various classes of cytochrome P450 inhibitors (azoles, piperazines, pyrimidines and pyridines; Group 1). Fig. 5.1 shows the structure of the Group 1 inhibitors and the effect of the respective compounds on the activity of the mitochondrial ecdysone 20-monooxygenase from fat body of sixth instar *S. littoralis* larvae. The results are the mean of duplicate incubations from one experiment with the range shown.

Ketoconazole at a concentration of 5ppm caused 64% inhibition of the activity of the ecdysone 20-monooxygenase enzyme, and was the most effective inhibitor in Group 1. Metyrapone at concentrations of 10ppm and 20ppm inhibited the activity of the ecdysone 20-monooxygenase by 37.3% and 53.1% , respectively and was the second most potent inhibitor in the group. Triforine and triarimol, each at a concentration of 10ppm had little effect on the ecdysone 20-monooxygenase, causing 4.1% and 6.9% inhibition respectively. At the higher concentration of 20ppm, the inhibitory potency of triarimol increased to 12.3% in contrast to triforine which decreased to 1.5%.

The effect of a number of compounds (Group 2) which have been previously reported to inhibit the synthesis of ecdysteroids from [^{3}H]2,22,25-trideoxyecdysone in the ovarian follicle cells of *L. migratoria,* were investigated on the activity of the ecdysone 20-monooxygenase (Jarvis, 1991). Fig. 5.2 shows the structure of the Group 2 inhibitors and their effect on the activity of the mitochondrial ecdysone 20-monooxygenase. Ketoconazole at a concentration of 5ppm was again the most potent inhibitor, causing a 64.2% inhibition of the activity of the ecdysone 20-monooxygenase. ICI-L-635 caused 55.3% and 60.1% inhibition of the ecdysone 20-monooxygenase at a concentration of 10ppm and 20ppm, respectively, and was the second most potent inhibitor in Group 2. ICI-U-384 and ICI-U-166 caused 35.1% and 8.8% inhibition of the activity of the 20-

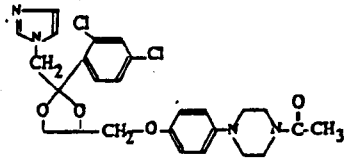
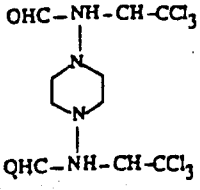
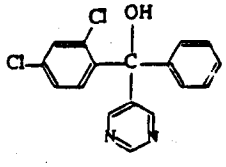
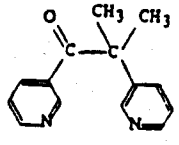
		Percentage inhibition of ecdysone 20-monooxygenase		
	Concentration (ppm)	5	10	20
Ketoconazole (Azole)		64.0±4.4		
Triforine (Piperazine)			4.1±2.2	1.5±1.1
Triarimol (Pyrimidine)			6.9±4.0	12.3±3.0
Metopirone (metyrapone) (Pyridine)			37.3±4.1	53.1±5.2

Fig. 5.1. The structure of the Group 1 inhibitors and their effect (at a final concentration of 10ppm and 20ppm ; ketoconazole 5ppm) on the activity of the mitochondrial ecdysone 20-monooxygenase from sixth instar *S. littoralis*. The results are expressed as a percentage inhibition of the control enzymic activity (no inhibitor present), and are the mean of duplicate incubations from one experiment with the range shown.

In the control incubation 49.3% (mean) of [^{3}H]ecdysone was converted into [^{3}H]20-hydroxyecdysone.

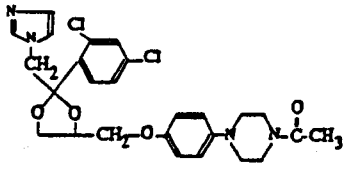
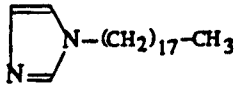
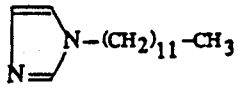
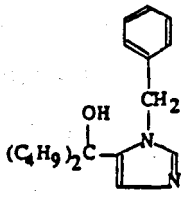
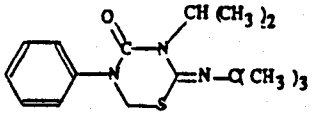
		Percentage inhibition of ecdysone 20-monooxygenase		
	Concentration (ppm)	5	10	20
Ketoconazole (Azole)		64.2±1.1		
ICI-U-384			35.1±4.2	38.1±2.2
ICI-U-166			8.8±2.3	12.2±4.3
ICI-L-635			55.3±5.3	60.1±7.3
ICI-L-161 (Buprofezin)			29.5±4.0	45.1±4.7

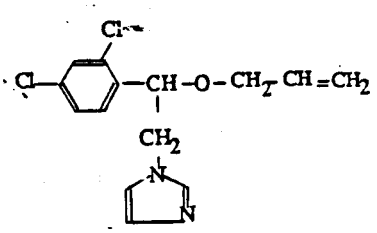
Fig. 5.2. The structure of the Group 2 inhibitors and their effect (at a final concentration of 10ppm and 20ppm ; ketoconazole 5ppm) on the activity of the mitochondrial ecdysone 20-monooxygenase from sixth instar *S. littoralis*. The results are expressed as a percentage inhibition of the control enzymic activity (no inhibitor present), and are the mean of duplicate incubations from one experiment with the range shown.

In the control incubation 42.4% (mean) of [^{3}H]ecdysone was converted into [^{3}H]20-hydroxyecdysone .

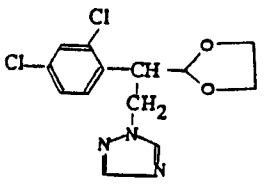
monooxygenase, respectively at a concentration of 10ppm, but at the higher concentration (20ppm) for both of these compounds, little further inhibition was observed. At 10ppm, ICI-L-161 (buprofezin) caused a 29.5% inhibition in the activity of the monooxygenase, increasing to 45.1% at 20ppm.

5.2.2. The effect of a range of cytochrome P450 inhibitors on the activity of the 22-, 2- and 20-hydroxylase enzymes present in the ovarian follicle cells of *L. migratoria*

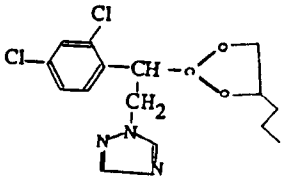
In a previous study, the effect of a number of cytochrome P450 inhibitors was investigated under *in vitro* conditions on the incorporation of [^{3}H]2,22,25-trideoxyecdysone into ovarian follicle cells of *L. migratoria* (Javis *et al.*, 1994b). The use of [^{3}H]2,22,25-trideoxyecdysone as a substrate enabled the effect of the inhibitors to be investigated on the the 25-, 22- and 2-hydroxylase enzymes involved in the terminal stages of the ecdysteroid biosynthetic pathway of the ovarian follicle cells. The results of these experiments suggested that the 22-hydroxylase was the enzyme most susceptible to inhibition of all those involved in the terminal stages of the pathway (Jarvis *et al*, 1994b). By the use of both [^{3}H]2,22-dideoxyecdysone and [^{3}H]2-deoxyecdysone as substrates, the effect of a number of compounds (Fig. 5.3), which have been shown to have inhibitory activity in either the ecdysone 20-monooxygenase system from the fat body of *S. littoralis* (Hoggard, 1989; foregoing section 5.2.1) and in the ovarian follicle cell system of *L. migratoria* (Jarvis, 1991) were investigated on the 22-, 2- and 20-hydroxylase enzymes in the ovarian follicle cells of *L. migratoria*. These inhibitors were representative of various classes of nitrogen heterocyclic compounds, including azoles (propiconazole, azaconazole and imazalil), alkyl imidazoles (ICI-U-384, ICI-U-166 and ICI-U-634), oxadiazoles (ICI-L-520 and ICI-L-209) and aromatic imidazoles (KK42 and ICI-L-635), these inhibitor experiments allowed the potency of these different compounds to be compared to both, the group as a whole as well as their individual heterocyclic classes. Ketoconazole at a concentration of 20μM was present in



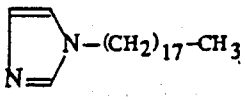
Imazalil



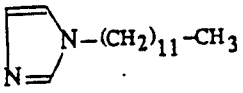
Azaconazole



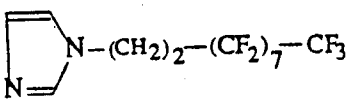
Propiconazole



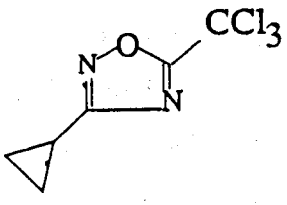
ICI-U-384



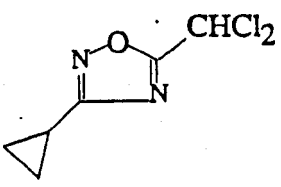
ICI-U-166



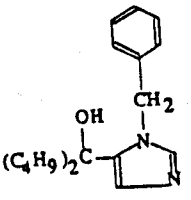
ICI-U-634



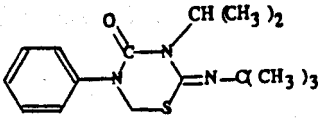
ICI-L-520



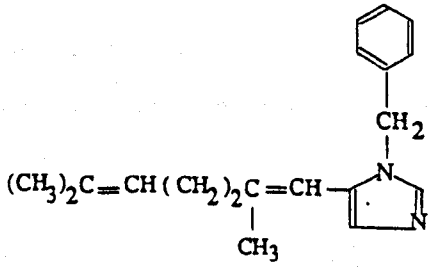
ICI-L-209



ICI-L-635



Buprofezin



KK42

Fig. 5.3. Structure of inhibitors used on the follicle cells of *L. migratoria.*

each experiment to allow the potency of inhibitors to be compared in separate follicle cell preparations.

Ovarian follicle cells were prepared as described in section 2.2.2. The inhibitor compounds were dissolved in methanol and introduced in 2µl from a 10µl Hamilton syringe into Landureau`s medium to make a final volume of 0.5ml in the incubation vial. An aliquot of ovarian follicle cells (0.5 ovary equivalents/ sample vial) was then added, and the vials preincubated for 10min at 37°C. The reaction was then started by addition of either [^{3}H]2,22-dideoxyecdysone (0.5µCi) or [^{3}H]2-deoxyecdysone (0.5µCi), and incubated for 5h or 3h at 37°C, respectively. Inhibitors were present at concentrations of 2µM and 10µM in the final incubation volume of 0.5ml Landureau`s medium, and all incubations were in duplicate. Reactions were terminated by the addition of ethanol (0.5ml) and the contents of the vials were extracted three times in methanol (1ml) in a ground glass hand-held homogeniser. The extracts were then hydrolysed by crude aryl sulfatase (*Helix* hydrolase) treatment (see section, 2.2.15), and then subjected to C_{18} Sep-Pak fractionation (see section 2.2.7, procedure 1). The resulting 60% methanol in water and methanol fractions were analysed by RP-HPLC (system 1, see section 2.2.8) with co-injection of authentic standards (for retention times see section 2.2.9). Blank incubations containing only [^{3}H]2,22-dideoxyecdysone and [^{3}H]2-deoxyecdysone showed there was no conversion of these substrates in the absence of follicle cells.

The activities of the 22-, 2- and 20-hydroxylases were determined from the total amount of ecdysteroid product formed by the individual enzymes, expressed as a percentage of the total ecdysteroid recovered from RP-HPLC (system 1, see section 2.2.8).

22-Hydroxylase: Sum of 2-deoxyecdysone, 2-deoxy-20-hydroxyecdysone, ecdysone and 20-hydroxyecdysone

2-Hydroxylase: Sum of 22-deoxyecdysone (only formed from [^{3}H]2,22-dideoxyecdysone substrate), ecdysone and 20-hydroxyecdysone.

20-Hydroxylase: Sum of 20-hydroxyecdysone and 2-deoxy-20-hydroxyecdysone.

Table 5.1 shows the combined effect of the inhibitors at final concentrations of 2μM and 10μM (separate experiment for each concentration of inhibitor; ketoconazole at 20μM in each experiment) on the 22-, 2- and the 20-hydroxylase enzymes when [^{3}H]2,22-dideoxyecdysone was used as a substrate in the *in vitro* ovarian follicle cell system of *L. migratoria*. The results are expressed as a percentage inhibition of the activity of the enzymes in the control incubation (no inhibitors present), and are the mean of duplicate incubations from one experiment with the range shown.

Of the compounds examined at a concentration of 2μM, the most potent inhibitors of the 22-hydroxylase enzyme were imazalil and propiconazole, causing 19.9%, 12.8 % inhibition of the enzyme, respectively. In contrast, ICI-U-634, ICI-L-520, ICI-L-635 and azaconazole all caused an apparent stimulation of the 22-hydroxylase enzyme at this concentration. On increasing the concentration of the compounds to 10μM , ICI-U-384, ICI-L-209 were found to be the most potent inhibitors of the 22-hydroxylase enzyme, causing, 17.4% and 11.0% inhibition of this enzyme, respectively. Of the inhibitors present at 10μM, apparent stimulation in the activity of 22-hydroxylase (2.8%) was only observed in the presence of KK42. The potency of the control inhibitor, ketoconazole (20μM) on the activity of the 22-hydroxylase enzyme differed markedly between the two experiments (the inhibitors at 2μM and 10μM, respectively) causing 50.9% and 29.3% inhibition, respectively, and therefore suggesting variations in the sensitivity of this enzyme to inhibition between the two experiments. Imazalil, ICI-U-384 and ICI-U-166 were the most potent inhibitors of the 2-hydroxylase enzyme at the 2μM concentration, causing inhibition of 14.5%, 12.7% and 12.7%, respectively. At the higher concentration (10μM) imazalil was again the most potent inhibitor causing 35.0%

Inhibitor	Percentage inhibition of hydroxylases					
	22-Hydroxylase		2-Hydroxylase		20-Hydroxylase	
	20μM	20μM	20μM	20μM	20μM	20μM
Ketoconazole	50.9+7.8	29.3+4.9	65.0+4.2	60.0+4.0	71.0+0.8	83.4+1.7
	2μM	10μM	2μM	10μM	2μM	10μM
Imazalil	19.9±3.7	9.6±2.0	14.5±1.0	35.0±1.8	14.4±2.8	46.4±0.8
ICI-U-384	4.6±1.1	17.4±5.3	12.7±1.4	24.0±6.2	3.8±0.9	18.4±3.3
ICI-U-166	11.5±6.3	6.1±3.6	12.7±2.0	27.9±3.7	25.4±2.0	31.3±2.1
ICI-U-634	+3.9±0.7	3.5±0.9	+3.8±1.9	12.0±2.7	+1.9±2.2	3.6±0.3
ICI-L-209	7.8±1.9	11.0±0.9	3.3±0.9	13.3±0.3	0.2±1.0	13.9±0.2
ICI-L-520	+3.9±1.7	9.3±2.0	7.0±3.8	13.0±3.0	3.5±3.1	12.2±1.3
ICI-L-635	+0.6±0.5	1.8±0.9	+7.0±1.8	8.4±0.6	+3.2±0.5	33.1±0.2
Buprofezin	1.8±3.0	7.2±4.1	4.5±2.7	4.6±2.0	+12.2±1.2	+3.5±2.0
Propiconazole	12.8±9.6	6.2±4.9	6.0±1.0	12.5±4.4	+1.6±0.4	7.6±2.2
Azaconazole	+9.6±1.6	1.7±0.2	+23.0±5.1	7.0±3.8	+4.1±2.2	7.4±1.1
KK42	8.5±0.3	+2.8±1.2	7.6±2.0	8.7±1.5	+6.7±0.9	5.4±1.4

(+ indicates stimulation)

Table 5.1. The effect of inhibitors (at 2μM and 10μM, final concentrations, respectively) on the activity of the 22-, 2- and 20-hydroxylase when [^{3}H]2,22-dideoxyecdysone was used as a substrate in the *in vitro* ovarian follicle cell system of *L. migratoria*. The results are expressed as percentage inhibition of the enzymic activity in the control incubation (no inhibitor present), and are the mean of duplicate incubations from one experiment, with the range shown. A different follicle cell preparation was used for the two inhibitor concentrations.

In the presence of inhibitors at the 2μM concentration, the 22-, 2- and 20-hydroxylases had mean values of, 60.5%, 33.7% and 22.9%, respectively, in the control incubations. For inhibitors at the 10μM concentration, the 22-, 2- and 20-hydroxylases had mean values of, 64.2%, 44.1% and 32.74%, respectively, in the control incubations.

inhibition of this enzyme, with ICI-U-166 and ICI-U-384 expressing subsequent levels of inhibitory activity at 27.9% and 24.0%, respectively. Apparent stimulation of the activity of the 2-hydroxylase was observed in the presence of the inhibitors, ICI-U-634, ICI-L-635 and azaconazole at the 2μM concentration, with this stimulatory activity being abolished at the higher inhibitor concentration (10μM). At the 2μM concentration ICI-U-166 and imazalil were the most potent inhibitors of the 20-hydroxylase enzyme, causing 25.4% and 14.4% inhibition, respectively. The remainder of the inhibitors were responsible for causing stimulation in the activity of the 20-hydroxylase. At the 10μM concentration imazalil, ICI-L-635, ICI-U-166 and ICI-U-384 were the most potent of the inhibitors of the 20-hydroxylase, causing 46.4%, 33.1% and 31.3% inhibition of the enzymic activity, respectively. Buprofezin at the 10μM concentration caused apparent stimulation in enzymic activity.

Table 5.2 shows the effect of the inhibitors at concentrations of 2μM and 10μM on the activity of the 2- and 20-hydroxylase enzymes when [^{3}H]2-deoxyecdysone was used as a substrate in the *in vitro* follicle cell system of *L. migratoria*. The results are expressed as a percentage inhibition of the activity of the enzymes in the control incubation (no inhibitor present).

Imazalil, ICI-U-166 and ICI-L-635 were the most potent inhibitors of the 2-hydroxylase enzyme at the 2μM concentration, causing 26.9%, 25.5% and 22.2% inhibition, respectively of the enzymic activity. At the 2μM concentration apparent stimulation in the enzymic activity of the 2-hydroxylase occurred in the presence of ICI-L-209, ICI-L-520 and azaconazole. At the 10μM concentration imazalil was again the most potent inhibitor of the 2-hydroxylase, causing 26.3% inhibition of the enzymic activity, with subsequent inhibition by KK42 at 25.4% and ICI-U-166 at 24.1% of the 2-hydroxylase.

In the presence of 20μM ketoconazole and 2μM imazalil, no 20-hydroxyecdysone was synthesized from [^{3}H]2-deoxyecdysone, demonstrating that 100% inhibition of this

	Percentage inhibition of Hydroxylases			
	2-Hydroxylase		*20-Hydroxylase*	
Inhibitor				
	20μM	20μM	20μM	20μM
Ketoconazole	64.0±2.0	63.5±1.0	——	77.8±1.0
	2μM	10μM	2μM	10μM
Imazalil	26.9±6.7	26.3±0.6	——	44.4±1.4
ICI-U-384	8.9±3.3	22.5±0.8	78.0±0.3	22.1±2.3
ICI-U-166	25.5±3.8	24.1±0.8	13.8±1.2	41.2±2.0
ICI-U-634	2.5±1.2	0.8±4.3	+28.6±1.5	13.2±1.7
ICI-L-209	+22.2±3.0	1.8±0.5	+6.50±3.0	+4.4±1.1
ICI-L-520	+20.7±3.4	22.3±1.2	+28.7±2.0	12.1±1.1
ICI-L-635	22.2±3.0	17.8±3.8	2.6±1.0	38.0±3.1
Buprofezin	6.7±4.5	21.1±1.6	5.6±1.2	7.6±1.2
Propiconazole	3.5±4.7	16.8±2.5	9.4±1.7	28.5±2.7
Azaconazole	+9.8±1.0	3.2±2.1	+25.1±1.9	9.0±0.7
KK42	15.2±2.2	25.4±3.3	0.9±1.3	30.0±1.1

(+ indicates stimulation)
(—— indicates no products formed by enzymes)

Table 5.2. The effect of the inhibitors (at 2μM and 10μM, final concentrations, respectively) on the activity of the 2- and 20-hydroxylase enzymes when [^{3}H]2-deoxyecdysone was used as substrate in the *in vitro* ovarian follicle cell system of *L. migratoria*. The results are expressed as a percentage inhibition of the enzymic activity in the control incubations (no inhibitors present), and are the mean of duplicate incubations from one experiment with the range shown. A different follicle cell preparation was used for the two inhibitor concentrations.

In the presence of inhibitors at the 2μM concentration, the 2- and 20-hydroxylases had mean values of 29.0% and 24.2%, respectively, in the control incubations. For inhibitors at the 10μM concentration, the 2- and 20-hydroxylases had mean values of 37.3% and 25.2%, respectively, in the control incubations.

enzyme occurred in the presence of these inhibitors in this experiment. ICI-U-384 was the second most potent inhibitor at 2μM, causing 78.0% inhibition in the activity of the 20-hydroxylase. At 10μM imazalil was again the most potent inhibitor of the 20-hydroxylase causing 44.4% inhibition in the activity of the enzyme. ICI-L-209 at the 10μM concentration caused an apparent slight stimulation in the enzymic activity of 4.4%.

5.3. Discussion

By assessing the *in vitro* effect of a number of nitrogen-containing heterocyclic compounds on both the activity of the mitochondrial ecdysone 20-monooxygenase from the fat body of sixth instar *S. littoralis* and ecdysteroid synthesis from [^{3}H]2,22-dideoxyecdysone and [^{3}H]2-deoxyecdysone in the ovarian follicle cells from adult female *L. migratoria* , the effectiveness of these compounds as inhibitors of the cytochrome P450-dependent monooxygenases involved in the terminal stages of ecdysteroid biosynthetic pathway could be ascertained.

On investigating the effect of Group 1 inhibitors on the activity of the mitochondrial ecdysone 20-monooxygenase, the control inhibitor ketoconazole at a concentration of 5ppm was found to be the most effective. Of the remaining Group 1 inhibitors, the pyridine derivative metyrapone, which has been previously shown to inhibit the activity of the ecdysone 20-monooxygenases in a number of insect species had the highest potency, with much lower levels of inhibition being exhibited by the piperazine and pyrimidine derivatives, triforine and triarimol on the enzyme activity. (Greenwood and Rees, 1984;Weirich *et al.*, 1984; Darvas *et al.*, 1992).

In Group 2, the azole ketoconazole was again found to be the most effective inhibitor, with ICI-L-635 being the second most potent inhibitor within this group, with lower levels of inhibition being expressed by buprofezin and ICI-U-384 , respectively. The ability of buprofezin to inhibit ecdysone 20-monooxygenase activity was not

unexpected as studies on the brown rice hopper, *Nilaparata lugens,* have suggested a possible inhibitory action of this compound on the metabolism of ecdysone, possibly at the stage of 20-hydroxyecdysone synthesis (Uchida *et al.*, 1986). As ketoconazole which was used in these experiments as a control inhibitor to allow comparison between different inhibitor groups, had a similar effect on the activity of the ecdysone 20-monooxygenase in each experiment an overall order of inhibitor potency for the heterocyclic compounds can be established: ICI-L-635 > metyrapone > buprofezin > ICI-U-384 > triarimol > ICI-U-166 > triforine.

By the use of [^{3}H]2,22-dideoxyecdysone and [^{3}H]2-deoxyecdysone as a substrate in the *in vitro* ovarian follicle cell system of *L. migratoria* , the effect of a number of cytochrome P450 inhibitors could be studied directly on the activity of 22- and 2- and 20-hydroxylase enzymes involved in the terminal stages of the ecdysteroid biosynthetic pathway. All three of these hydroxylases were found to susceptible to inhibition by these inhibitor compounds, which was not unexpected, as all three of these enzymes are known to be cytochrome P450-dependent monooxygenases (Kappler *et al.*, 1989). The extent of this inhibition varied, however, between the individual enzymes, with the 20-hydroxylase being inhibited to the greatest extent, with subsequent lower levels of inhibition being expressed by the 2-hydroxylase and the 22-hydroxylase enzymes, respectively. Through incubation studies with [^{3}H]2,22,25-trideoxyecdysone in the presence of a number of cytochrome P450 inhibitors Jarvis *et al.* (1994b) have suggested that of the terminal hydroxylases, the 22-hydroxylase was the most susceptible to inhibition. However, in that work the inhibitors were present at a final concentration of 20μM or 100μM, respectively as opposed to 2μM and 10μM concentrations of the inhibitors used in the present experiments, and therefore, this may suggest differential threshold levels of inhibitor sensitivity for the terminal hydroxylases. The possibility arises, however, that the apparent inhibition of 20-hydroxylase may be caused by the lack of available substrate for the enzyme, due to inhibition of the preceding 22- and 2-hydroxylases. Therefore, to study the effect of the cytochrome P450 inhibitors directly on the activity of the 20-hydroxylase the radiolabelled ecdysteroid, [^{3}H]ecdysone

should be used as a substrate for this enzyme. Although variations were found in the overall order of inhibitor potency for these compounds, the azole derivative, imazalil was generally found to be the most potent inhibitor of the 22-, 2- and the 20-hydroxylases. In addition, the alkyl imidazoles, ICI-U-166 or ICI-U-384 were also amongst the most potent inhibitors of these enzymes . At the 2μM concentration a number of the cytochrome P450 inhibitors caused apparent stimulation in the activity of the 22-, 2- and 20-hydroxylase enzymes, which was generally abolished at the higher inhibitor concentration of 10μM. This phenomena has also been reported by Jarvis (1991) who found an overall increase in the synthesis of ecdysteroids from [^{3}H]2,22,25-trideoxyecdysone in the presence of triforine, metyrapone and triarimol at a concentration of 20μM. However, the possible cause of this stimulation in ecdysteroid synthetic activity is unknown.

In contrast to imazalil, the remaining azole derivatives, propiconazole and azaconazole caused much lower levels of inhibition in the activity of the hydroxylase enzymes, with propiconazole being the more potent of the two. As reversible inhibition of cytochrome P450 depends on both the ability to bind with the haem group, as well as the ability to bind to the lipophilic region of the active site of the enzyme, the lower inhibitor potency of propiconazole and azaconazole compared to imazalil must be attributed to differences in both (i) the nitrogen heterocyclic rings contained in these compounds, with imazalil containing an imidazole heterocyclic ring as opposed to the triazole heterocyclic ring present in propiconazole and azaconazole, and (ii) side chain functional groups, with imazalil containing an ether functional group as opposed to the dioxacyclopropane and propyl-dioxacyclopropane fuctional groups present in azaconazole and propiconazole (Ortiz de Montellano and Reich, 1986). Within the alkyl imidazole group of compounds, the much lower inhibitor potency of ICI-U-634 (which is amongst the least potent of the inhibitors) in comparison to ICI-U-166 and ICI-U-384 can be attributed to the variation in the side chain constituents of these derivatives, with both ICI-U-166 and ICI-U-384 possessing hydrocarbon side chains in comparison to ICI-U-634 which contains a fluorinated hydrocarbon substituted side chain.

The oxadiazoles, ICI-L-520 and ICI-L-209 at the 10μM concentration generally caused low to intermediate levels of inhibition in the activity of the hydroxylase enzymes. Overall, from the combined incubation studies with [^{3}H]2,22-dideoxyecdysone and [^{3}H]2-deoxyecdysone, ICI-L-209 appeared to have a higher potency towards the 22-hydroxylase than ICI-L-520, with the 2- and 20-hydroxylases being more supsceptible to inhibition by ICI-L-520 than ICI-L-209. The variation in the inhibitor potencies between these two compounds towards the hydroxylase enzymes can be attributed solely to differences in their side chain constituents, with ICI-L-520 containing an extra chlorine atom (CCl_3) as opposed to the hydrogen atom present in the side chain of ICI-L-209 ($CHCl_2$).

Both the aromatic imidazoles, ICI-L-635 and KK42 had low inhibitor potencies towards the 22-hydroxylase, with KK42 being the more potent of the two inhibitors towards the 2-hydroxylase. However, ICI-L-635 at the 10μM concentration had a high inhibitor potency towards the 20-hydroxylase, being amongst the four most potent inhibitors of this enzyme. The variation in inhibitory potency of ICI-L-635 and KK42 can be attributed to the variation in side chain substituents between the compounds, with ICI-L-635 possessing a branched hydroxylated side chain as opposed to the unsaturated hydrocarbon chain of KK42.

The insect growth regulator, Buprofezin was amongst the least potent of the inhibitors, causing low levels of inhibition in the activities of the three hydroxylase enzymes. It has been suggested by Jarvis *et al.* (1994b) that the insecticidal action of buprofezin was due to inhibition of an enzyme prior to the 22-hydroxylase in the ecdysteroid biosynthetic pathway, and therefore, this would explain the inability of this compound to inhibit to any great extent the activity of the 22-, 2- and 20-hydroxylases in the present study.

In the light of these experimental results it can be seen that the potency of cytochrome P450 inhibitors is not governed solely by the nitrogen heterocyclic class to which they belong, but the structure of the side chain functional groups of the compound must also be taken into account, the latter being responsible for the binding of the inhibitor to the

apoprotein in the vicinity of the substrate binding site (Yoshida, 1988). In addition, the variation in the inhibitor potency of closely related nitrogen heterocyclic compounds to the different hydroxylases may be connected to differences in the secondary structure of these enzymes, which may alter the architecture of the active site, and therefore, inhibitor interaction.

Chapter 6

Evaluation of radiolabelled 3-dehydro-2,22,25-trideoxyecdysone and 2,22,25-trideoxyecdysone as putative ecdysteroid precursors in *L. migratoria* ovarian follicle cells *in vitro*

6.1. Introduction

Recently, it has been shown that the prothoracic glands of a number of Lepidopteran insects synthesize both ecdysone and 3-dehydroecdysone *in vitro* , the ratio of which has been found to vary widely among these species (Warren *et al.*, 1988a; Kelly *et al.*, 1990; Kiriishi *et al.*, 1990). The absence of any edysone oxidase activity associated with the prothoracic glands and the presence of a 3-dehydroecdysteroid 3β−reductase in the insect haemolymph suggested that 3-dehydroecdysone was a precursor rather than a metabolite of ecdysone (Warren *et al.*, 1988b; Gilbert, 1989; Sakurai *et al.*, 1989).

The synthesis of 3-dehydroecdysone by prothoracic glands suggested the possibility that the 3-oxo group of the 3-oxo-Δ^4-intermediate as proposed by Davies *et al.* (1981) was maintained throughout the ecdysteroid biosynthetic pathway. However, the incorporation of putative 3-oxo-Δ^4-intermediates into the ecdysteroid biosynthetic pathway has not yet been possible (Milner *et al.*, 1986). Recently, the radiolabelled 3-dehydro derivative (3-dehydro-2,22,25-trideoxyecdysone) of the well characterised ecdysteroid biosynthetic pathway intermediate, 2,22,25-trideoxyecdysone, has been synthesized (Dolle *et al.*, 1991). This intermediate has been shown to be efficiently incorporated into both ecdysone and 3-dehydroecdysone *in vitro* by the prothoracic glands of *L. migratoria* (Dolle *et al.*, 1991), *P. brassicae* (Blais and Lafont, 1991) and *M. sexta* (Grieneisen *et al.*, 1993).

The ability of prothoracic glands of *M. sexta* and *P. brassicae* to synthesize both 3-dehydroecdysone and ecdysone from [^{3}H]3-dehydro-2,22,25-trideoxyecdysone in a similar proportion to its natural endogenous production, suggests the potential intermediacy of this compound in the ecdysteroid biosynthetic pathway of the glands of these insect species (Blais and Lafont, 1991; Grieneisen *et al.*, 1993).

On further examination of the ecdysteroid products formed from the incubation of radiolabelled 3-dehydro-2,22,25-trideoxyecdysone with *L. migratoria* prothoracic

glands *in vitro* , both 3β-hydroxyecdysteroid and 3-dehydroecdysteroid derivatives were found to be present at each level of hydroxylation, in approximately equal quantities (Roussel, 1992a). The synthesis of both 3-dehydroecdysteroids and 3β-hydroxyecdysteroids from radiolabelled 3-dehydro-2,22,25-trideoxyecdysone has been interpreted in terms of a branched pathway consisting of both 3-dehydroecdysteroid and 3β-hydroxyecdysteroid derivatives at each level of hydroxylation for the terminal stages of ecdysteroid biosynthesis in prothoracic glands (see section 1.5, fig. 1.10). However, the fact that *Locusta* prothoracic glands *in vitro*, produce ecdysone exclusively (Hirn *et al.*, 1979) indicates that the products of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone metabolism by the glands do not reflect the normal situation. This casts doubt on the intermediary role of the latter compound in this tissue.

The enzymes responsible for the hydroxylations at C-25, C-22 and C-2 have been studied previously in the prothoracic glands of *L. migratoria*, where the 2- and 22-hydroxylases were found to have a mitochondrial location. In contrast, the 25-hydroxylase enzyme appeared to be associated with the microsomal fraction (Kappler *et al.*, 1988).

In this chapter, radiolabelled 3-dehydro-2,22,25-trideoxyecdysone was investigated as a potential intermediate in the ecdysteroid biosynthetic pathway of *L. migratoria* ovarian follicle cells *in vitro*. This allowed 3-dehydro-2,22,25-trideoxyecdysone to be investigated as a possible precursor of ecdysteroid synthesis in a mature insect system, where the synthesis of 3-dehydroecdysteroids has not been previously reported, as opposed to previous studies with this putative intermediate in immature insect prothoracic gland systems.

The metabolism of radiolabelled 3-dehydro-2,22,25-trideoxyecdysone was also compared to that of the radiolabelled 2,22,25-trideoxyecdysone, which has been well characterised as an intermediate in the ecdysteroid biosynthetic pathway of ovarian follicle cells of *L. migratoria*. Radiolabelled 2,22,25-trideoxyecdysone has been demonstrated to be readily converted into ecdysteroids in ovarian follicle cells *in vitro*

(Kappler *et al.*, 1986a). In addition, 2,22,25-trideoxyecdysone has been found endogenously in the ovaries of this insect species (Hetru *et al.*, 1978; 1982).

The intracellular distribution of the 25-, 22- and the 2-hydroxylase enzymes was also investigated in the ovarian follicle cells of *L. migratoria*. In addition, the substrate specificity of the 25-hydroxylase was examined for both the [^{3}H]2,22,25-trideoxyecdysone and [^{3}H]3-dehydro-2,22,25-trideoxyecdysone substrates.

6.2. Experimental and results

6.2.1. Incorporation of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone into ecdysteroids in ovarian follicle cells of *L. migratoria*

Initially in this chapter, the possible intermediacy of 3-dehydro-2,22,25-trideoxyecdysone in the ecdysteroid biosynthetic pathway of ovarian follicle cells of *L. migratoria* was investigated. At present 3-dehydroecdysteroids have not been implicated in the ecdysteroid biosynthetic pathway of adult insects. The inability to separate 3β-hydroxyecdysteroids and 3-dehydroecdysteroids on reversed-phase HPLC (RP-HPLC) methanol/ water solvent systems commonly used for ecdysteroid analysis possibly contributes to this phenomenon. The solution to this was approached using a range of RP-HPLC acetonitrile/water solvents systems which have been shown to separate 3β- hydroxy and 3-dehydro derivatives of each ecdysteroid moiety (Roussel, 1992a).

Follicle cells were prepared as described in section 2.2.2, and incubated as described in section 2.2.19 in the presence of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone (0.5μCi; 47Ci/mmol) for either 6.5h or 22h or in the presence of 20μM ketoconazole for 6.5h. The reaction was terminated by the addition of ethanol (0.5ml), and the contents of the vial were extracted 3 times in methanol (2ml) in a hand-held ground glass homogeniser.

The extracts were hydrolysed by *Helix* hydrolase treatment (125 I.U./0.5ml) and subjected to C_{18} Sep-Pak fractionation (see section 2.2.7, procedure 2). The resulting methanol fraction was analysed by RP-HPLC (system 1, 3,4,5,6 and 7, see section 2.2.8) and the components identified by comparison with the retention times of authentic standards (for retention times see section 2.2.19).

The incubation of the follicle cell samples for 22h allowed the terminal hydroxylation products synthesized from [^{3}H]3-dehydro-2,22,25-trideoxyecdysone to accumulate, therefore increasing the amount of ecdysteroid available for study. The cytochrome P450 inhibitor, ketoconazole was employed to inhibit the 2- and 22-hydroxylase enzymes in the terminal stages of the ecdysteroid biosynthetic pathway of the ovarian follicle cells, allowing the accumulation of the initial hydroxylation products (see Chapter 5).

The ecdysteroids extracted from the incubations were first subjected to fractionation on RP-HPLC system 1 (see section 2.2.8); the coeluting 3-dehydro and 3β−hydroxy derivatives of each ecdysteroid compound, known as `regions`, were collected and further analysed on RP-HPLC systems 3,4,5,6 or 7 (see section 2.2.8).

Fig. 6.1 shows the metabolic products formed from [^{3}H]3-dehydro-2,22,25-trideoxyecdysone in ovarian follicle cells from adult female *L. migratoria* after a 6.5h incubation at 37^{o}C. Control incubations with radiolabelled substrates showed no conversion into ecdysteroids in the absence of ovarian follicle cells. After incubation for 6.5h, [^{3}H]3-dehydro-2,22,25-trideoxyecdysone passed through 4 levels of hydroxylation in the ovarian follicle cell to produce 20-hydroxyecdysone. The 3β-hydroxy and 3-dehydro derivatives of 2,22-dideoxyecdysone and 2-deoxyecdysone were also produced in addition to ecdysone and the 3-epiecdysteroids, 3-epi-2-deoxyecdysone and 3-epiecdysone.

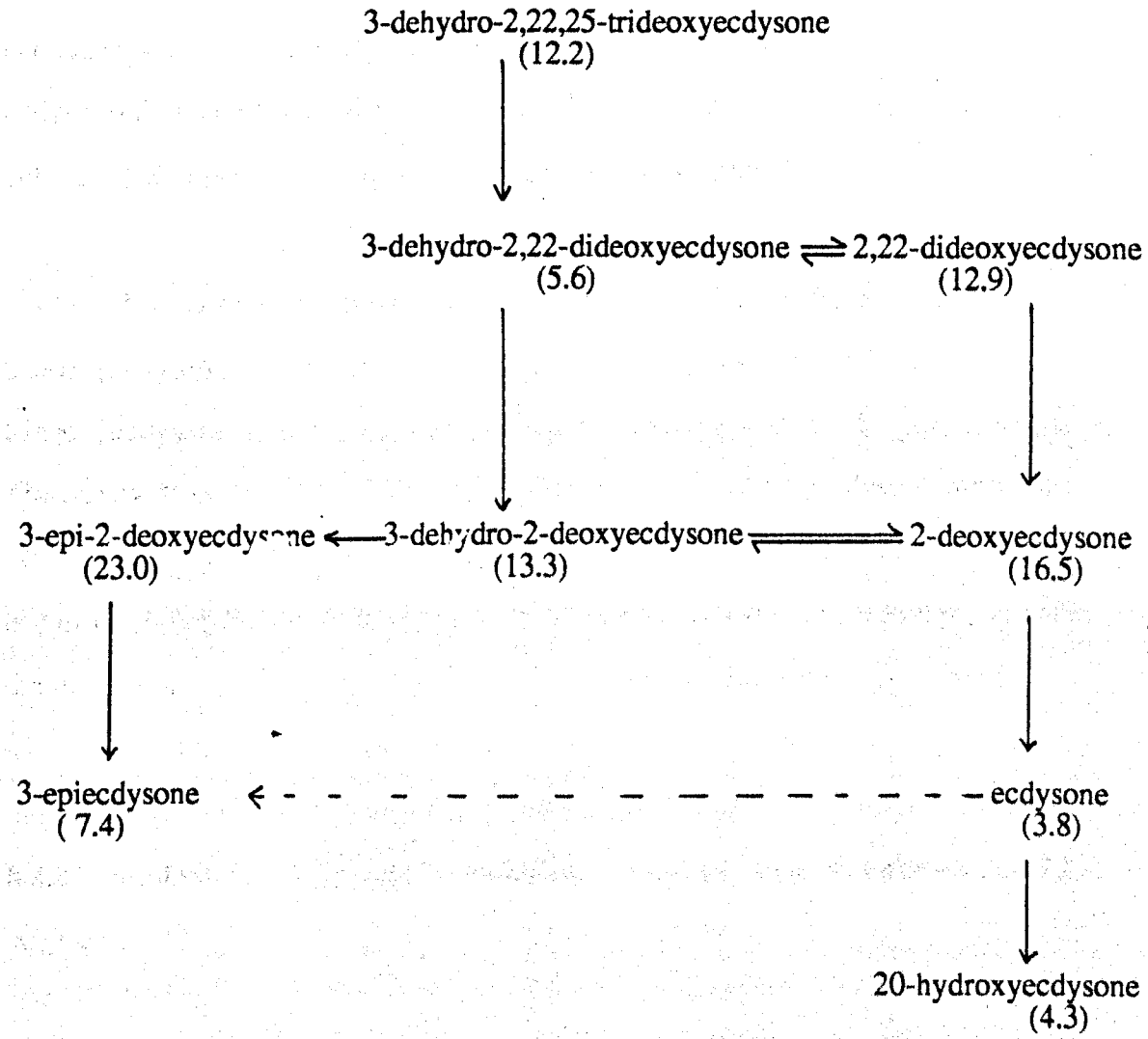


Fig. 6.1. The metabolic products formed from [^{3}H]3-dehydro-2,22,25-trideoxyecdysone after incubation for 6.5h. at 37°C with ovarian follicle cells from *L. migratoria*. The incorporations into various compounds (numbers in parenthesis) are expressed as a percentage of the total recovered ecdysteroids from RP-HPLC (system 1, see section 2.2.8). Full line arrows indicate reactions for which there is evidence. The dotted arrow represents possible reactions.

Fig. 6.2 shows the metabolic products formed from [^{3}H]3-dehydro-2,22,25-trideoxyecdysone in the ovarian follicle cells of *L. migratoria* after a 6.5h incubation at 37^{o}C in the presence of 20μM ketoconazole. [^{3}H]3-Dehydro-2,22,25-trideoxyecdysone underwent 2 levels of hydroxylation to produce 2-deoxyecdysone and 3-epi-2-deoxyecdysone, the latter metabolite accounting for approximately 60% of the total ecdysteroids synthesized. At the first level of hydroxylation, the 3β-hydroxy and the 3-dehydro derivatives of 2,22-dideoxyecdysone could be identified.

Fig. 6.3 shows the metabolic products formed from [^{3}H]3-dehydro-2,22,25-trideoxyecdysone after a 22h incubation at 37^{o}C. [^{3}H]3-Dehydro-2,22,25-trideoxyecdysone underwent 4 levels of hydroxylation to produce 20-hydroxyecdysone. The 3β−hydroxy and the 3-dehydro derivatives of 2,22-dideoxyecdysone were also synthesized in addition to the 3β−hydroxy and 3-epi derivatives of 2-deoxyecdysone and ecdysone. 3-Epi-2-deoxyecdysone was the most predominant ecdysteroid present after the incubation, and accounted for over 50% of the total ecdysteroid synthesized.

6.2.2. Metabolism of [^{3}H]2,22,25-trideoxyecdysone by ovarian follicle cells of *L. migratoria*

The metabolism of [^{3}H]2,22,25-trideoxyecdysone in ovarian follicle cells of *L. migratoria* was re-examined using the recently devised RP-HPLC systems which can resolve both 3β−hydroxyecdysteroids and 3-dehydroecdysteroids (Roussel, 1992a). Classically, 2,22,25-trideoxyecdysone undergoes a conserved sequence of hydroxylations at C-25, C-22 and C-2 to produce ecdysone, and further hydroxylation at C-20 to yield 20-hydroxyecdysone in the ovarian follicle cells of *L. migratoria* (Kappler *et al.*, 1986a). However, the synthesis of 3-dehydroecdysteroids from 2,22,25-trideoxyecdysone in ovarian follicle cells has so far not been reported, since the

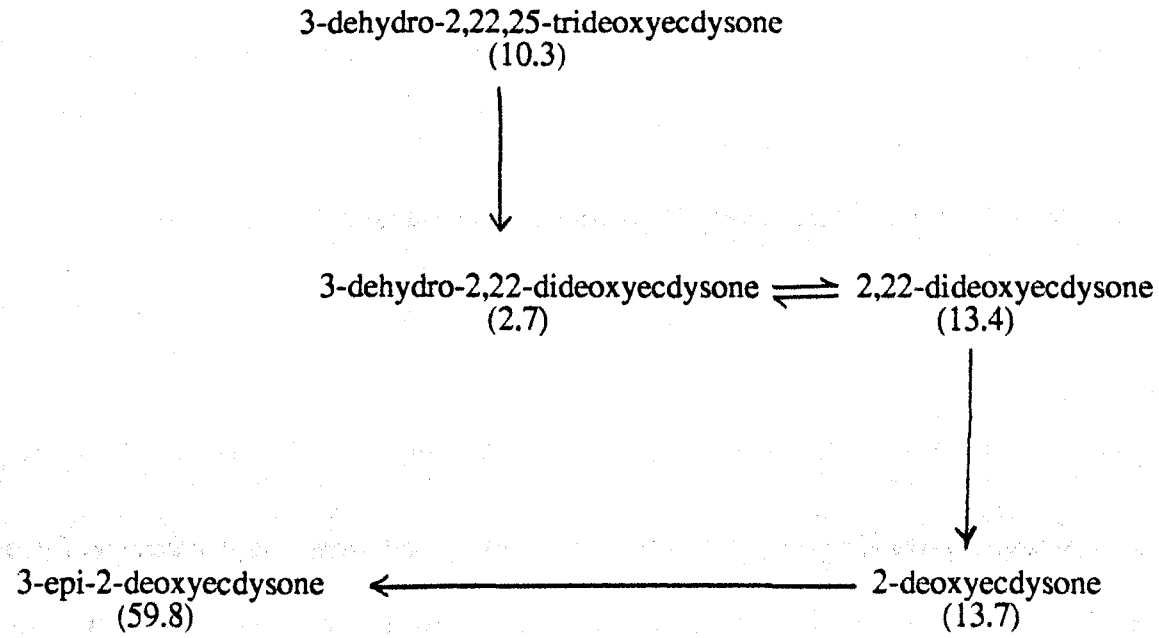


Fig. 6.2. The metabolic products formed from [^{3}H]3-dehydro-2,22,25-trideoxyecdysone after incubation for 6.5h at 37°C with ovarian follicle cells from *L. migratoria* in the presence of 20μM ketoconazole. The incorporations into various compounds (numbers in parenthesis) are expressed as a percentage of the total ecdysteroid recovered from RP-HPLC (systems 1, see section 2.2.8).

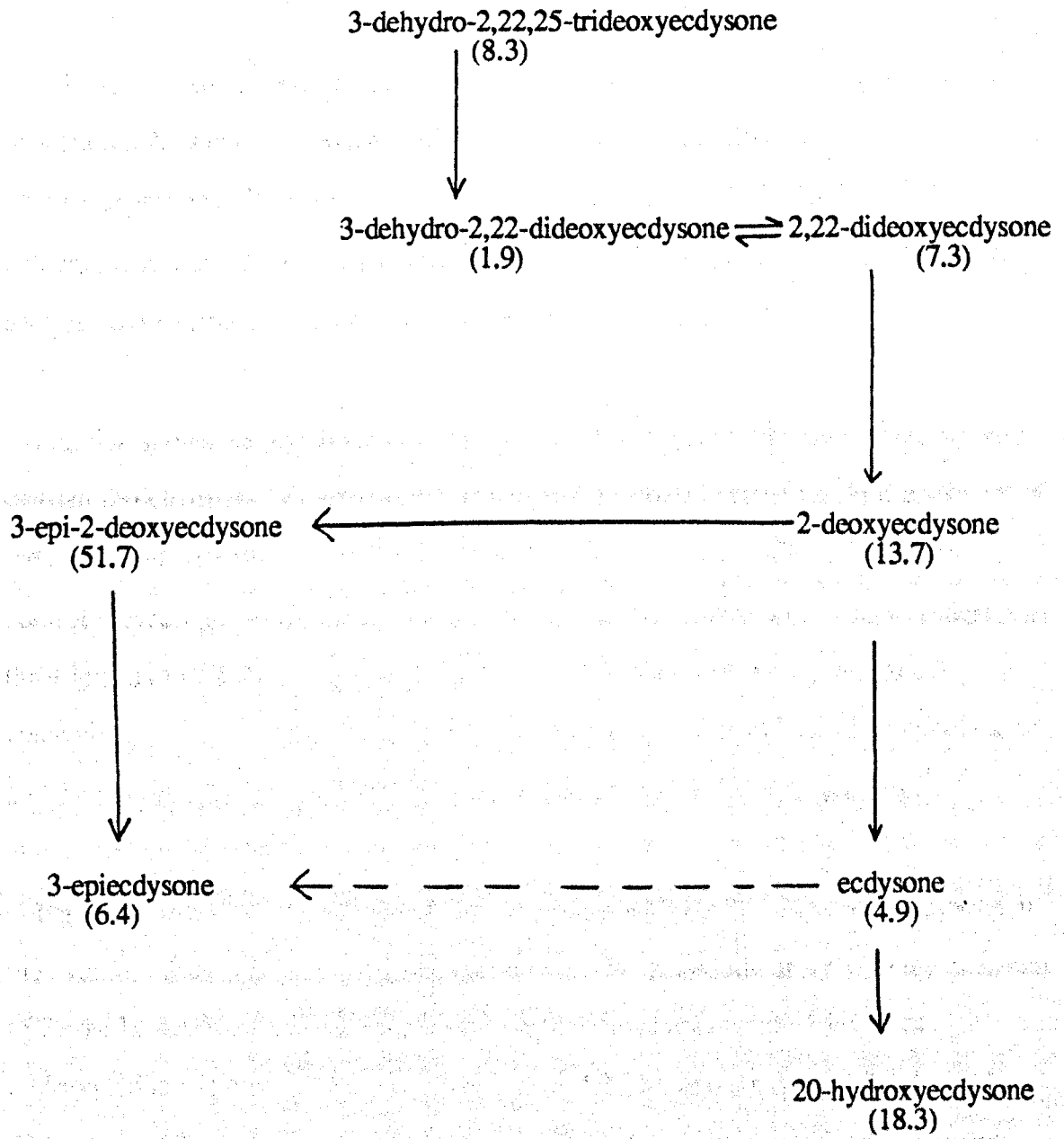


Fig. 6.3. The metabolic products formed from [^{3}H]3-dehydro-2,22,25-trideoxyecdysone after incubation for 22h at 37°C with ovarian follicle cells from *L. migratoria*. The incorporations into various compounds (numbers in parenthesis) are expressed as a percentage of the total ecdysteroid recovered from RP-HPLC (system 1, see section 2.2.8).

chromatographic systems employed would not afford sufficient resolution. It was the aim of this investigation to further address this problem.

Follicle cells were prepared as described in section 2.2.2, and incubated as described in section 2.2.19 in the presence of [^{3}H]2,22,25-trideoxyecdysone (1.15μCi) for 6.5h. In another experiment, incubation was conducted in the presence of 20μM ketoconazole.The reactions were terminated by the addition of ethanol (0.5ml) and the samples were extracted and analysed as described in section 6.2.1.

Fig. 6.4 shows the metabolic products formed from [^{3}H]2,22,25-trideoxyecdysone in ovarian follicle cells of *L. migratoria* after a 6.5h incubation at 37°C. No metabolism of the radiolabelled substrate was observed in the absence of ovarian follicle cells in the control incubations. After a 6.5 h incubation, [^{3}H]2,22,25-trideoxyecdysone underwent three levels of hydroxylation in the ovarian follicle cells to yield ecdysone and 3-epiecdysone. The 3β−hydroxy and 3-dehydro derivatives of both 2,22-dideoxyecdysone and 2-deoxyecdysone were also produced in addition to 3-epi-2-deoxyecdysone.

Fig. 6.5 shows the metabolic products formed from [^{3}H]2,22,25-trideoxyecdysone in the ovarian follicle cells of *L. migratoria* after a 6.5h incubation at 37°C in the presence of 20μM ketoconazole. [^{3}H]2,22,25-Trideoxyecdysone underwent 2 levels of hydroxylation to produce the 3β-hydroxy, the 3-dehydro and the 3-epiecdysteroid derivatives of 2-deoxyecdysone, in addition to 2,22-dideoxyecdysone and 3-dehydro-2,22-dideoxyecdysone. These two latter metabolites represented the majority of the ecdysteroid synthesized from [^{3}H]2,22,25-trideoxyecdysone.

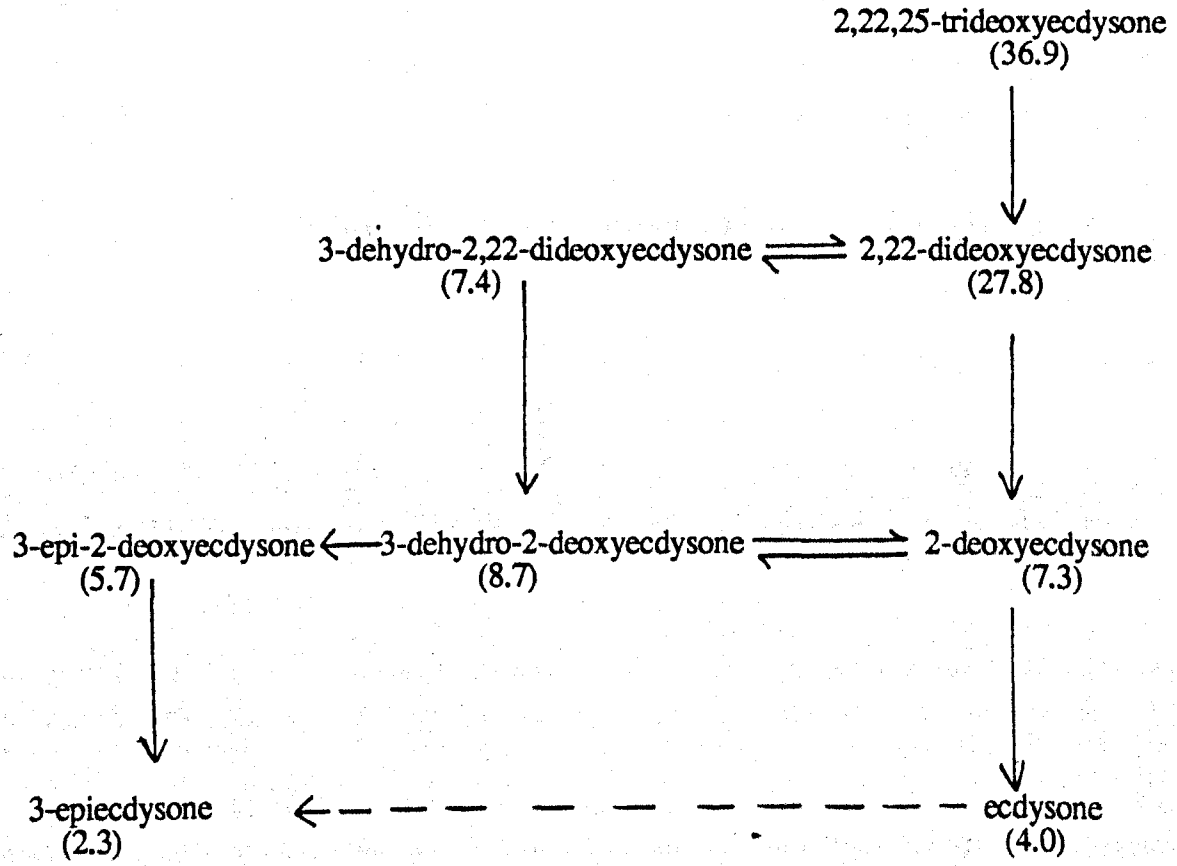


Fig. 6.4. The metabolic products formed from [^{3}H]2,22,25-trideoxyecdysone after incubation for 6.5h at 37^{o}C with ovarian follicle cells from *L. migratoria*. The incorporations into various compounds (numbers in parenthesis) are expressed as a percentage of the total recovered ecdysteroid from RP-HPLC (systems 1, see section 2.2.8).

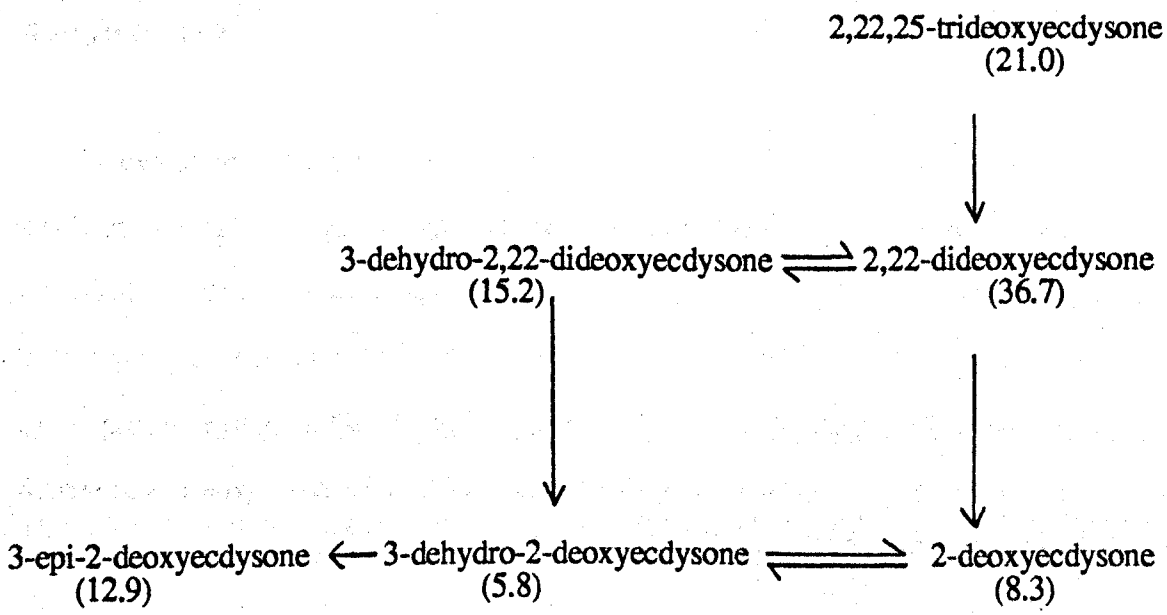


Fig. 6.5. The metabolic products formed from [^{3}H]2,22,25-trideoxyecdysone after incubation for 6.5h at 37^{o}C with ovarian follicle cells from *L. migratoria* in the presence of 20μM ketoconazole. The incorporations into various compounds (numbers in parenthesis) are expressed as a percentage of the total recovered ecdysteroid from RP-HPLC (systems 1, see section 2.2.8).

6.2.3. Preparation of [^{3}H]3-dehydro-2-deoxyecdysone and [^{3}H]3-epi-2-deoxyecdysone

The cytosolic fraction from the midgut of sixth instar larvae of *S. littoralis,* which has been shown by Milner *et al.* (1985) to contain both ecdysone oxidase and a 3-dehydroecdysone 3α-reductase activity will be used to synthesize both [^{3}H]3-dehydro-2-deoxyecdysone and [^{3}H]3-epi-2-deoxyecdysone from [^{3}H]2-deoxyecdysone substrate. In the presence of no cofactors the synthesis of the 3-dehydro derivative of 2-deoxyecdysone by the dialysed cytosolic fraction will be favoured. However, the addition of NADPH will lead to the synthesis of 3-epi-2-deoxyecdysone (Fig. 6.6).

Cytosol from the midgut of sixth instar larvae of *S. littoralis* was prepared as described in section 2.2.4, and incubated with [^{3}H]2-deoxyecdysone (3μCi) for 17h at 37°C in Tris HCl (1ml, pH 7.4) in the absence of cofactors to 3-dehydro-2-deoxyecdysone, or in the presence of NADPH (2.6mM) to form 3-epi-2-deoxyecdysone The reaction was terminated by the addition of ethanol (1ml) to the incubation vials, which were then centrifuged at 1000g for 10min. The resulting pellet was resuspended twice in methanol, being recentrifuged each time. The combined extracts were evaporated to dryness under N_2. The samples were then analysed on RP-HPLC (system 4, see section 2.2.8) and the ecdysteroid products were collected. All samples were co-injected with authentic standards.

To confirm the identity of the putative [^{3}H]3-dehydro-2-deoxyecdysone produced from the incubation of [^{3}H]2-deoxyecdysone with the dialysed midgut cytosol preparation, reduction of this radiolabel with $NaBH_4$ was undertaken. Putative [^{3}H]3-dehydro-2-deoxyecdysone was dissolved in dry 50% (v/v) ethanol in tetrahydrofuran (1ml), followed by treatment with $NaBH_4$ (1mg). The mixture was left at room temperature for 10min and the reaction was terminated by the addition of one drop of

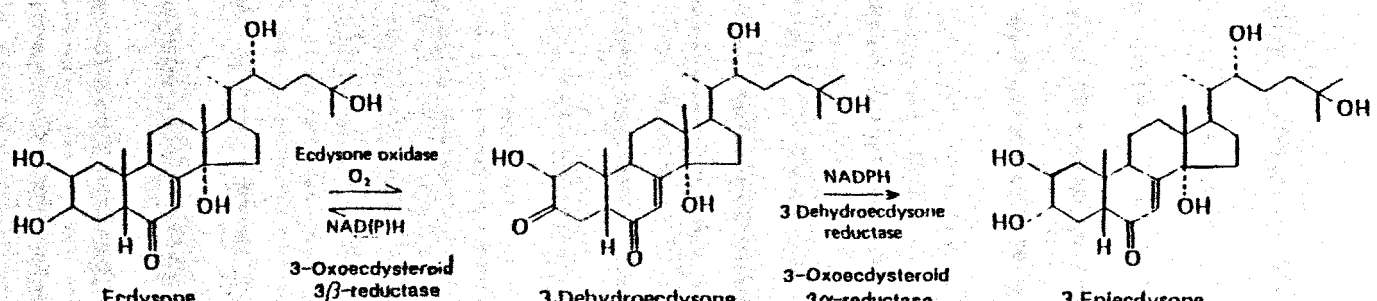


Fig. 6.6. Metabolic scheme for the production of 3-epiecdysone in the midgut of *S. littoralis* (Milner *et al.*, 1985).

glacial acetic acid. The reaction mixture was then evaporated to dryness under N_2, and analysed by RP-HPLC (system 4, see section 2.2.8).

Fig. 6.7 shows the RP-HPLC (system 4, see section 2.2.8) radiochromatogram of the $NaBH_4$ reduction of putative [^{3}H]3-dehydro-2-deoxyecdysone.

On reduction with $NaBH_4$, putative 3-dehydro-2-deoxyecdysone was converted mainly into 3-epi-2-deoxyecdysone, with a much lower amount of 2-deoxyecdysone being produced, both metabolites coeluting with authentic standards on RP-HPLC (system 4, see section 2.2.8). This result agrees with a previous reduction of 3-dehydroecdysone with $NaBH_4$ which yielded both ecdysone and 3-epiecdysone, with the latter ecdysteroid again being the major product (Milner *et al.*, 1985), confirming the identity of the [^{3}H]3-dehydro-2-deoxyecdysone product synthesized from [^{3}H]2-deoxyecdysone in the cytosolic fraction of the midgut from sixth instar larvae of *S. littoralis*. The low specificity ecdysone oxidase activity of the mid gut was also used to prepare [^{3}H]3-dehydro-2,22-dideoxy-3-dehydroecdysone from [^{3}H]2,22-dideoxyecdysone, that was then used as a HPLC marker.

6.2.4. Metabolism of [^{3}H]2-deoxyecdysone, [^{3}H]3-dehydro-2-deoxyecdysone and [^{3}H]3-epi-2-deoxyecdysone in the ovarian follicle cells of *L. migratoria*

On incubation of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone and [^{3}H]2,22,25-trideoxyecdysone with ovarian follicle cells, the 2-deoxyecdysone hydroxylation level has been found to consist of the 3β-hydroxy, 3-dehydro and the 3-epiecdysteroid derivatives of 2-deoxyecdysone. The inter-relationship of these ecdysteroids was examined by metabolic studies using their respective radiolabel forms.

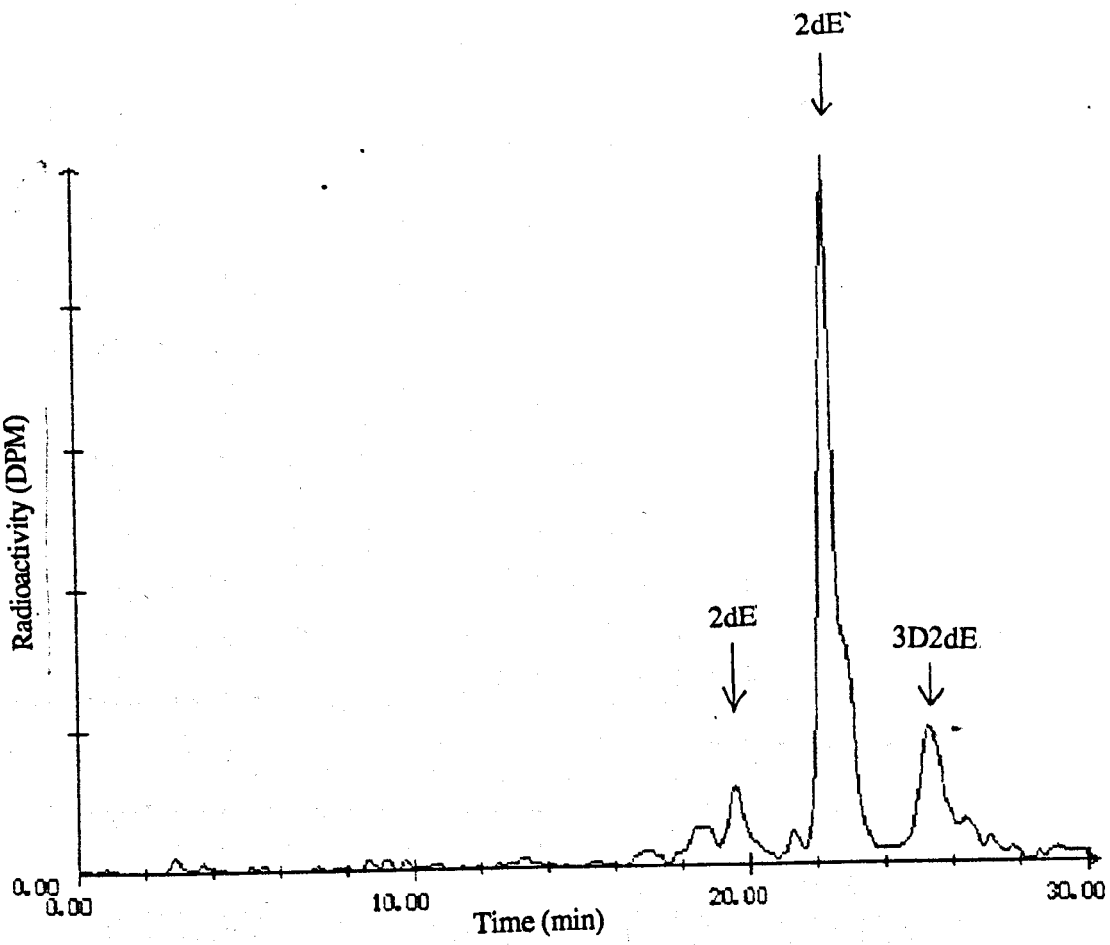


Fig. 6.7. RP-HPLC (system 4, section 2.2.8) radiochromatogram of the $NaBH_4$ reduction products of putative [^{3}H]3-dehydro-2-deoxyecdysone. The positions of authentic 2-deoxyecdysone (2dE) , 3-epi-2-deoxyecdysone (2dE`) and putative 3-dehydro-2-deoxyecdysone (3D2dE) are shown.

Follicle cells were prepared as described in section 2.2.2, and incubated as described in section 2.2.19 for 6.5h with [^{3}H]2-deoxyecdysone (0.5μCi), [^{3}H]3-dehydro-2-deoxyecdysone (0.5μCi) or [^{3}H]3-epi-2-deoxyecdysone (0.5μCi). The reaction was terminated by the addition of ethanol (0.5ml) and extracted and analysed as described in section 6.2.1.

Fig. 6.8 shows the metabolic products formed from [^{3}H]2-deoxyecdysone after a 6.5h incubation at 37°C. [^{3}H]2-Deoxyecdysone underwent two levels of hydroxylation to produce both 20-hydroxyecdysone and 3-epi-20-hydroxyecdysone. The 3β−hydroxy and the 3-epiecdysteroid derivatives of ecdysone were also synthesized, in addition to 3-dehydro-2-deoxyecdysone and 3-epi-2-deoxyecdysone.

Fig. 6.9 shows the metabolic products formed from [^{3}H]3-dehydro-2-deoxyecdysone after a 6.5h incubation at 37°C. [^{3}H]3-Dehydro-2-deoxyecdysone underwent two levels of hydroxylation after a 6.5h incubation to produce both 20-hydroxyecdysone and 3-epi-20-hydroxyecdysone. In addition, the 3β−hydroxy and the 3-epiecdysteroid derivatives of 2-deoxyecdysone and ecdysone were also synthesized.

Fig. 6.10 shows the metabolic products formed from [^{3}H]3-epi-2-deoxyecdysone after a 6.5h incubation at 37°C. [^{3}H]3-Epi-2-deoxyecdysone was only poorly incorporated into 3-epiecdysone after a 6.5h incubation with ovarian follicle cells, with approximately 93% of the ecdysteroid being unmetabolised.

6.2.5. Preparation of authenic 5α-2-deoxyecdysone

The presence of 5α−2-deoxyecdysone in both its free and conjugated forms has been previously demonstrated in the ovaries and eggs of *S. littoralis* (Hoggard, 1989). Since 5α−ecdysteroids are not thought to occur naturally *in vivo*, the possibility was considered that these compounds may have arisen by keto-enol tautomerisation during

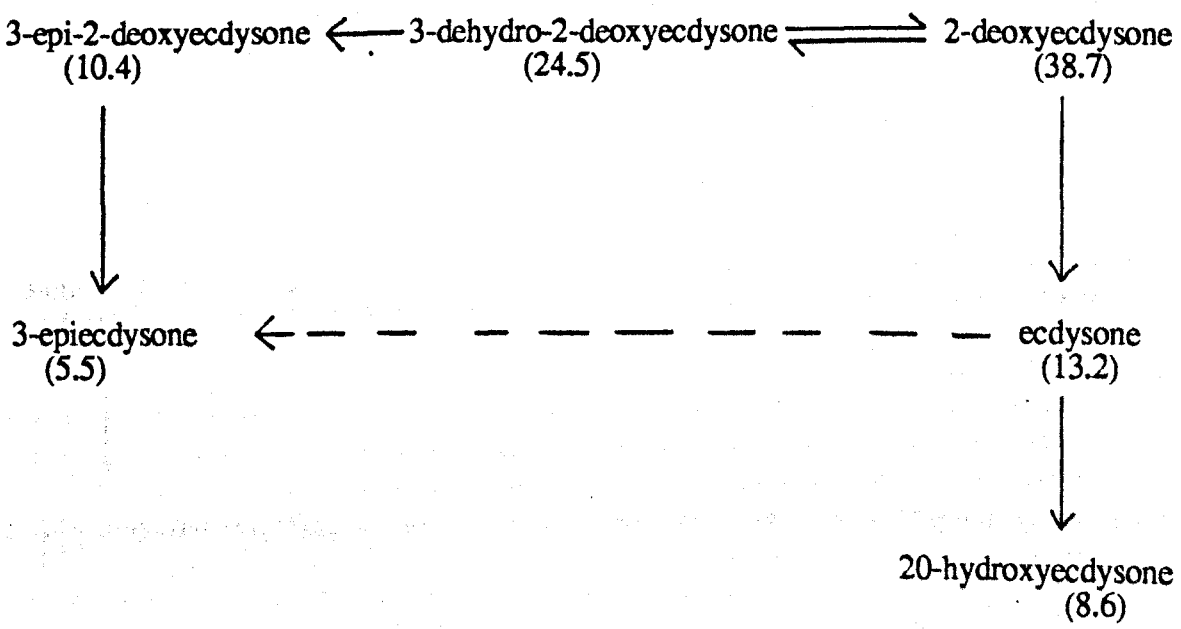


Fig. 6.8. The metabolic products formed from [^{3}H]2-deoxyecdysone after incubation for 6.5h at 37^{o}C with ovarian follicle cells from *L. migratoria*. The incorporations into various compounds (numbers in parenthesis) are expressed as a percentage of the total recovered ecdysteroids from RP-HPLC (systems 3,4, see section 2.2.8).

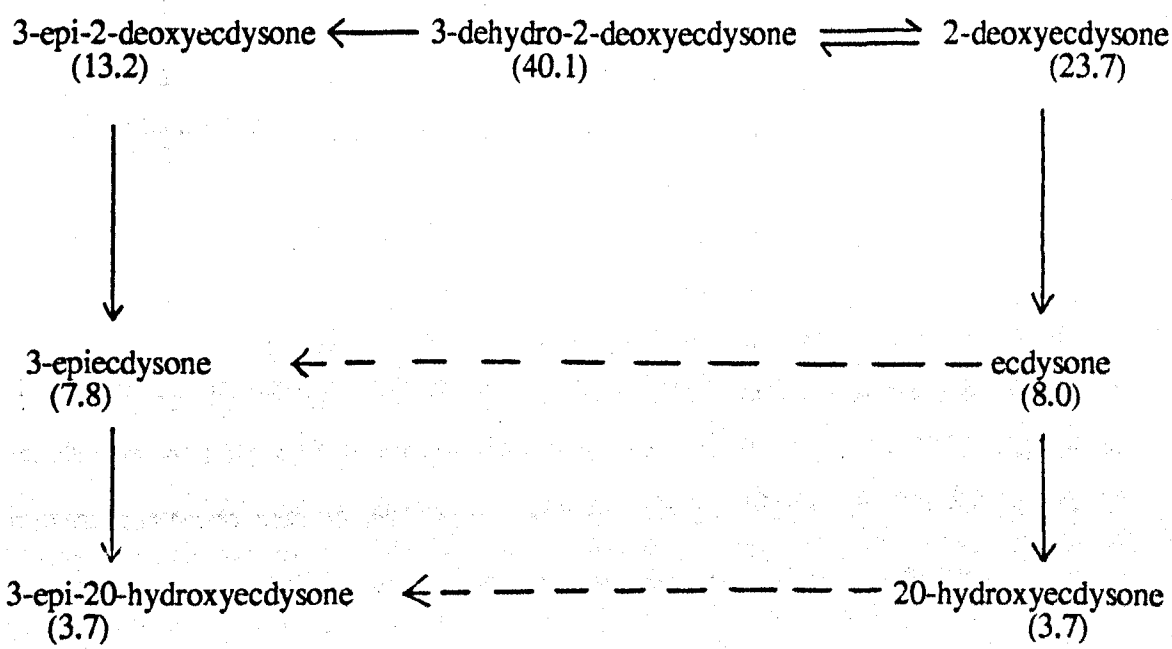


Fig. 6.9. The metabolic products formed from [^{3}H]3-dehydro-2-deoxyecdysone after an incubation for 6.5h at 37°C with ovarian follicle cells of *L. migratoria*. The incorporations into various compounds (numbers in parenthesis) are expressed as a percentage of the total recovered ecdysteroid from RP-HPLC (systems 3,4, see section 2.2.8).

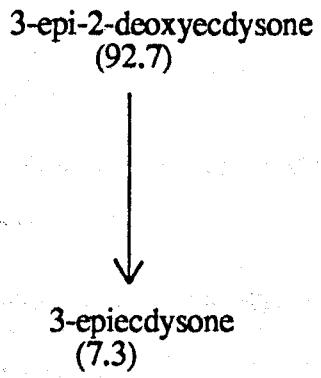


Fig. 6.10. The metabolic products formed from [^{3}H]3-epi-2-deoxyecdysone after incubation for 6.5h at 37^{o}C with ovarian follicle cells of *L. migratoria*. The incorporations into various compounds (numbers in parenthesis) are expressed as a percentage of the total recovered ecdysteroid from RP-HPLC (systems 3,4, see section 2.2.8).

the extraction procedure (Hoggard, 1989). The identity of the 3-epiecdysteroids formed during the previous incubation studies in this work were confirmed by co-elution with authentic standards on RP-HPLC (system 3 and 4, see section 2.2.8). However, the possibility arises that these metabolites could in fact represent the 5α- derivatives of the respective 5β-ecdysteroids, which could conceivably co-elute with authentic 3-epiecdysteroid standards on the RP-HPLC solvent systems employed.

By preparing 5α- 2-deoxyecdysone from 5β-2-deoxyecdysone by the method of Mori *et al*. (1968), the chromatographic properties of 5α and 3-epi derivatives of 2-deoxyecdysone on RP-HPLC system 4 (see section 2.2.8) were investigated.

5β-2-deoxyecdysone (50μg) was dissolved in 1% (w/v) potassium carbonate in 90 % aqueous methanol (500μl). The solution was then heated for 6h at 50°C, before being evaporated under N_2 and subjected to C_{18} Sep-Pak fractionation (see section 2.2.7; procedure 2). The resulting methanol fraction was analysed by RP-HPLC (system 4, see section 2.2.8) with co-injection of authentic standards.

On RP-HPLC system 4 (see section 2.2.8) 5α-2deoxyecdysone (17.9min), 2-deoxyecdysone (19.8min) and 3-epi-2-deoxyecdysone (23.1min) were clearly separated. Therefore, the possibity of co-elution of these ecdysteroids on RP-HPLC sysyem 4 could discounted.

6.2.6. The subcellular distribution of the ecdysteroid 25-, 22- and 2-hydroxylase enzymes in the ovarian follicle cells of *L. migratoria*

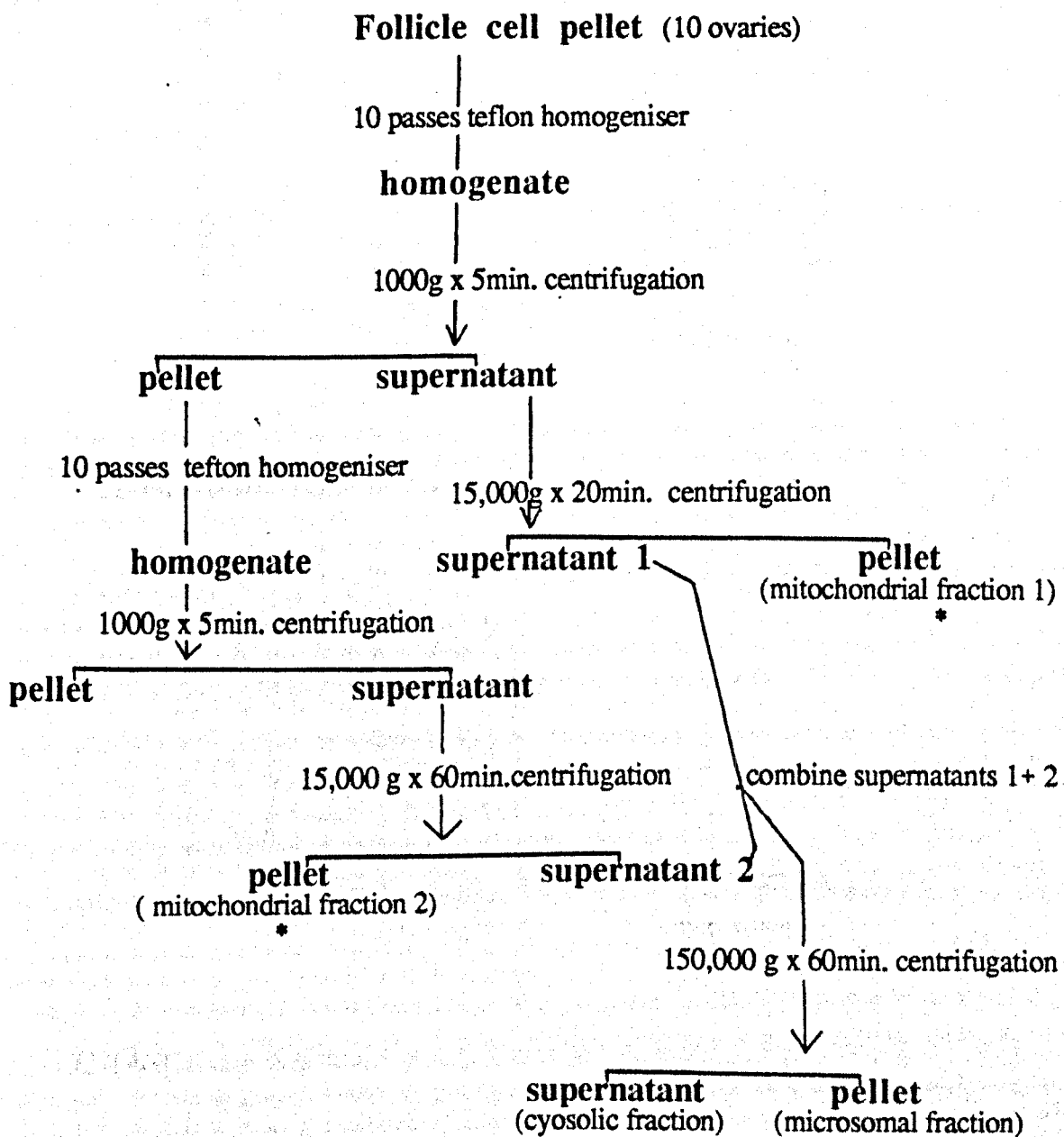
The subcellular distribution of the ecdysteroid 25-, 22- and the 2-hydroxylase enzymes has been previously determined in the prothoracic glands of the immature stages *L. migratoria* (Kappler *et al.*, 1988). In this study, the subcellular location of these enzymes were investigated in the ovarian follicle cells of adult female *L. migratoria*. This involved an examination of the metabolism of the radiolabelled substrates, [^{3}H]2,22,25-trideoxyecdysone, [^{3}H]2,22-dideoxyecdysone and [^{3}H]2-deoxyecdysone

by mitochondrial, microsomal and cytosolic fractions. This approach is based on the findings that a privileged hydroxylation sequence occurs in follicle cells of *L. migratoria* (Kappler *et al.*, 1986a).

Follicle cells were prepared from 10 vitellogenic females as described in section 2.2.2. The resulting follicle cell pellet was suspended in isotonic 100mM phosphate buffer pH 7.2 (1ml), containing 0.1% (w/v) bovine serum albumin and subjected to subcellular fractionation according to the scheme in Fig. 6.11. The resulting mitochondrial fractions were suspended in isotonic 100mM phosphate buffer (500µl). Aliquots of the mitochondrial and microsomal fractions (50µl each) and of the cytosolic fraction (200µl) were incubated for 3h at 37°C in a final volume of 0.5ml hypotonic phosphate buffer, pH 7.2 with [^{3}H]2,22,25-trideoxyecdysone (0.5µCi), [^{3}H]2,22-dideoxyecdysone (0.5µCi) or 2-deoxyecdysone (0.5µCi) in the presence of NADPH (3mM). The reactions were terminated by the addition of ethanol (0.5ml), and the samples were extracted and analysed as described in section 3.1.

Fig. 6.12 shows the activity of the mitochondrial and microsomal fractions towards the radiolabelled substrates, [^{3}H]2,22,25-trideoxyecdysone, [^{3}H]2,22-dideoxyecdysone and [^{3}H]2-deoxyecdysone. The results are expressed as a percentage of the radiolabelled substrate converted into ecdysteroids (see Table 6.1) by the individual subcellular fractions.

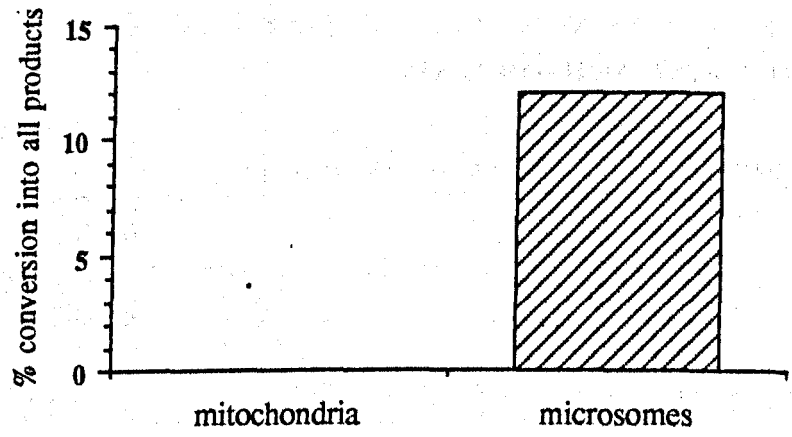
No metabolism of the radiolabelled substrates was observed in the presence of the cytosolic fraction. Conversion of [^{3}H]2,22,25-trideoxyecdysone into ecdysteroids occurred only in the microsomal fraction, with 2,22-dideoxyecdysone being the only ecdysteroid product. The majority of the of the metabolism of the [^{3}H]2,22-dideoxyecdysone and [^{3}H]2-deoxyecdysone substrates occurred in the mitochondrial fraction, with the former compound being converted into 20-hydroxyecdysone, ecdysone, 2-deoxyecdysone and 2-deoxy-20-hydroxyecdysone, and the latter compound being converted into 20-hydroxyecdysone and ecdysone. Much lower activity was



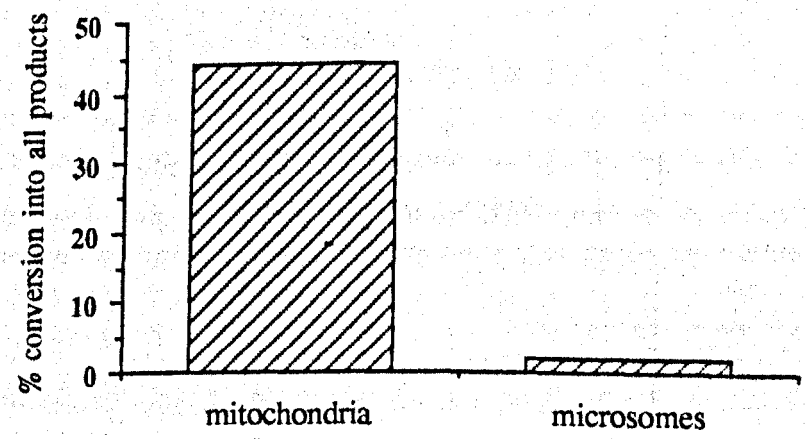
* Mitochondrial fractions 1 and 2 were combined.

Fig. 6.11. Procedure for the subcellular fractionation of ovarian follicle cells from *L. migratoria*

(1) [^{3}H]2,22,25-Trideoxyecdysone



(2) [^{3}H]2,22-Dideoxyecdysone



(3) [^{3}H]2-Deoxyecdysone

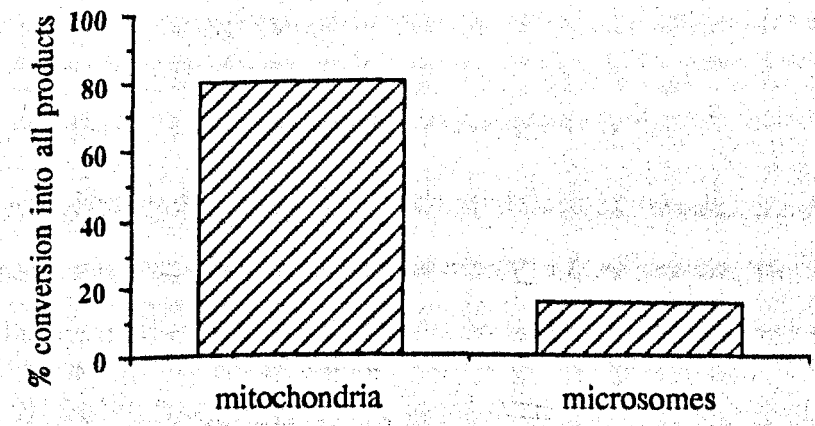


Fig. 6.12. The activity of the mitochondrial and microsomal fractions for the radiolabelled substrates, (1) [^{3}H]2,22,25-trideoxyecdysone, (2) [^{3}H]2,22-dideoxyecdysone and (3)[^{3}H]2-deoxyecdysone. The results are expressed as a percentage of the substrate metabolised into ecdysteroids (see Table 6.1.) by the subcellular fraction.

	Subcellular fractions	
	Mitochondrial	Microsomal
Radiolabelled substrates		
2-Deoxyecdysone	20-hydroxyecdysone (43.6) ecdysone (37.1)	ecdysone (16.3)
2,22-Dideoxyecdysone	20-hydroxyecdysone (16.3) 2-deoxy-2-hydroxyecdysone (6.8) ecdysone (8.5) 2-deoxyecdysone (12.3)	2-deoxyecdysone (2.0)
2,22,25-Trideoxyecdysone	0	2,22-dideoxyecdysone (12.0)

Table 6.1. Ecdysteroid products formed from [^{3}H]2-deoxyecdysone, [^{3}H]2,22-dideoxyecdysone and [^{3}H]2,22,25-trideoxyecdysone in the mitochondrial and microsomal fractions of the ovarian follicle cells of *L. migratoria*. The incorporations into labelled ecdysteroids (numbers in parenthesis) are expressed as a total of the recovered ecdysteroids from RP-HPLC (system 1, see section 2.2.8).

associated with the microsomal fraction, with [^{3}H]2,22-dideoxyecdysone and [^{3}H]2-deoxyecdysone being converted into 2-deoxyecdysone and ecdysone, respectively.

6.2.7. A study of the 25-hydroxylation of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone and [^{3}H]2,22,25-trideoxyecdysone in the ovarian follicle cells of *L. migratoria*

The incorporation of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone and [^{3}H]2,22,25-trideoxyecdysone into ecdysteroids was examined in subcellular fractions of ovarian follicle cells. This investigation allowed a study of the activity of the 25-hydroxylase enzyme towards each of these radiolabelled substrates. This permitted the reported low substrate specificity of the hydroxylase enzymes present in the terminal stages of the ecdysteroid biosynthetic pathway to be specifically examined at the 25-hydroxylation stage of the pathway (Schwab and Hetru, 1991; Roussel, 1992a). In addition, the subcellular location of the 25-hydroxylase was examined using both the 3-dehydro and 3β-hydroxy derivatives of 2,22,25-trideoxyecdysone as substrates. The mitochondrial and microsomal marker enzymes, succinate-cytochrome c reductase and NADPH-cytochrome c reductase, respectively, were used in conjunction with these subcellular studies to enable the purity of the subcellular fractions to be assessed.

Follicle cells were prepared from 30 vitellogenic females as described in section 2.2.2. The resulting follicle cell pellet was resuspended in isotonic 100mM phosphate buffer pH 7.2 (1ml), containing 0.1% (w/v) bovine serum albumen, and subjected to homogenisation in a loose fitting teflon-glass Potter Elvehjem homogeniser (8 passes) in ice. The homogenate was centrifuged at 1000g for 10min at 4^{o}C, the debris pellet (P1) was collected and resuspended in buffer (1ml). The resulting supernatant was centrifuged at 10,000g for 20min at 4^{o}C. The resulting mitochondrial pellet (P10) was resuspended in buffer (1ml) and the supernatant was recentrifuged at 100,000g for 1h at

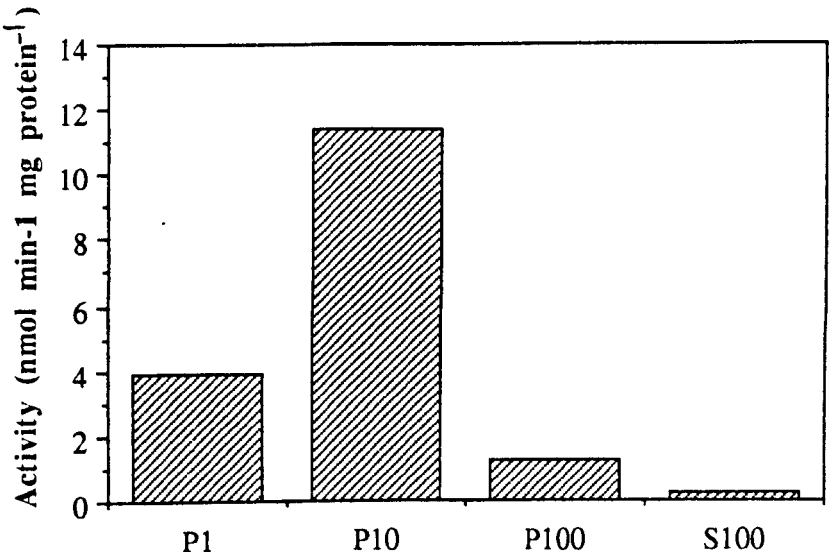
4°C. The supernatant (S100) was collected and the microsomal pellet (P100) was resuspended in buffer (1ml). The subcellular fractions (100µl) were incubated with both [^{3}H]3-dehydro-2,22,25-trideoxyecdysone (0.5µCi) and [^{3}H]2,22,25-trideoxyecdysone (1.15µCi) in hypotonic 100mM phosphate buffer pH 7.2 (0.4ml) for 3h at 37°C in the presence of NADPH (3mM). The reaction was terminated by addition of ethanol (0.5ml), and the samples were extracted and analysed as described in section 6.2.1.

NADPH-cytochrome c reductase activity and succinate-cytochrome c reductase activity in the individual subcellular fractions were determined spectrophotometrically at room temperature, by following the reduction of cytochrome c at 550 nm. (Sottocasa *et al.*, 1967). The assay mixture contained in 1ml of hypotonic 100mM phosphate buffer, pH 7.2 : NADPH (0.1mM) or succinate (2.5mM), KCN (1mM) to prevent reoxidation of cytochrome c and the subcellular fraction (50µl or 100µl). Protein concentration was determined according to the Bradford assay (Bradford, 1976), and was found to be 8.8mg ml^{-1} for the 1,000g pellet (P1), 5.4mg ml^{-1} for the 10,000 pellet (P10), 4.9mg ml^{-1} for the 100,000g pellet (P100) and 6.6mg ml^{-1} for the 100,000g supernatant (S100).

Fig. 6.13 shows the activity of the mitochondrial and microsomal marker enzymes, succinate-cytochrome c reductase and NADPH-cytochrome c reductase , respectively, in the subcellular fractions. The activity of the enzymes are expressed in nmol min^{-1} mg protein^{-1}.

The 1,000g pellet (P1; debris fraction) was contaminated by both the mitochondrial and microsomal fraction, as can be seen from the activity of the marker enzymes in this fraction, presumably reflecting the presence of appreciable numbers of whole cells. Succinate-cytochrome c reductase showed the highest activity in the 10,000g pellet (P10), which corresponds to the mitochondrial fraction, but this fraction also showed contaminant microsomal activity. In addition, the 100,000g microsomal pellet (P100)

(1) Succinate-cytochrome c reductase



(2) NADPH-cytochrome c reductase

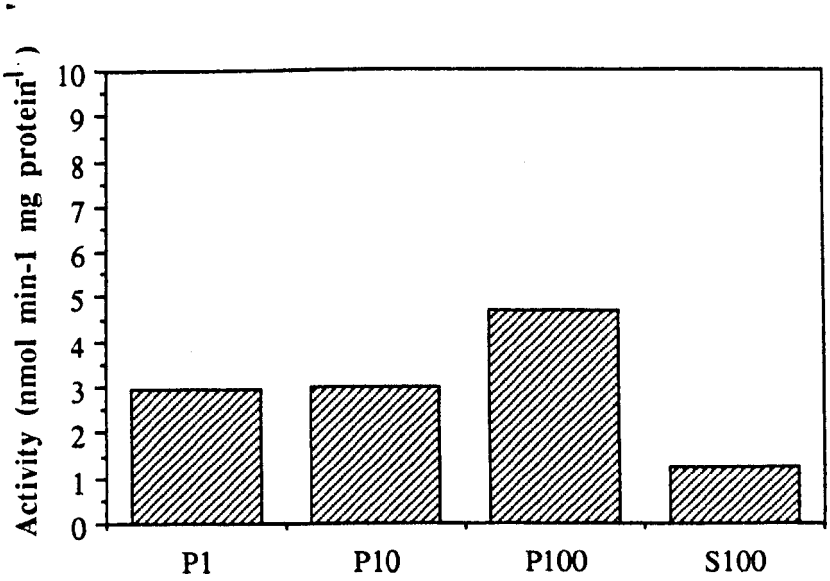


Fig. 6.13. Activity of the mitochondrial and microsomal marker enzymes, (1) succinate-cytochrome c reductase and (2) NADPH-cytochrome c reductase, respectively, in the 1,000g pellet (P1), 10,000g pellet (P10), 100,000g pellet (P100) and the 100,000g supernatant (S100) prepared by centrifugation of the follicle cell homogenate from 30 adult female *L. migratoria*. The activity of the enzymes is expressed as nmol min^{-1} mg $protein^{-1}$.

showed the highest NADPH-cytochrome c reductase activity, with low succinate-cytochrome c reductase activity, reflecting little mitochondrial contamination. The 100,000g supernatant (S100) which corresponds to the cytosolic fraction showed the lowest marker enzyme activity, with some contamination by the microsomal fraction

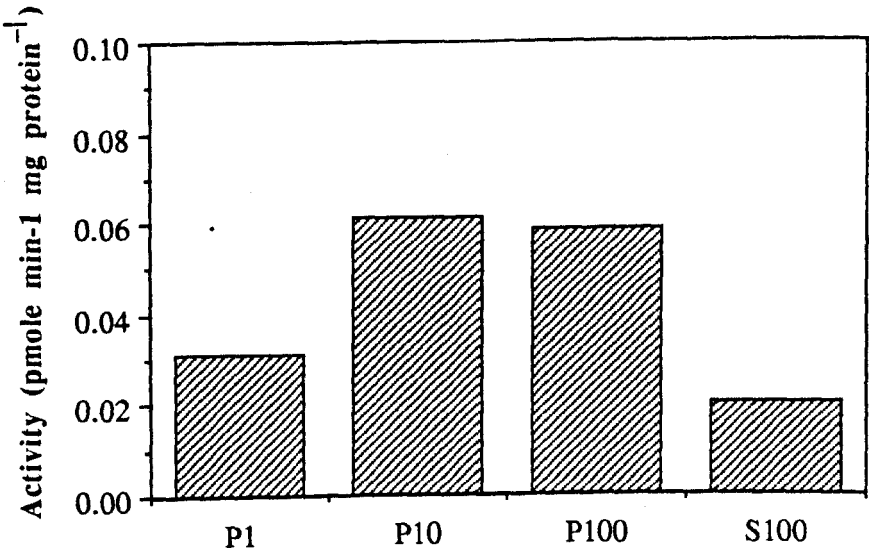
Fig. 6.14 Shows the activity of the 25-hydroxylase using both the (1) [^{3}H]3-dehydro-2,22,25-trideoxyecdysone and (2) [^{3}H]2,22,25-trideoxyecdysone as substrates in the 1,000g pellet (P1), 10,000g pellet (P10), 100,000g pellet (P100) and 100,000g supernatant (S100). The enzymic activity is a measure of the conversion of the radiolabelled substrates into all of their 25-hydroxylated ecdysteroid products (see Table 6.2) and is expressed in pmole min^{-1} mg $protein^{-1}$.

The P10 (mitochondrial fraction) and the P100 (microsomal fraction) showed the highest 25-hydroxylase activity using the [^{3}H]3-dehydro-2,22,25-trideoxyecdysone substrate, with both fractions having approximately equal enzymic activity. 25-Hydroxylase activity in the 100,000g supernatant (cytosolic fraction) and 1,000g pellet could possibly be attributed to the mitochondrial and microsomal contamination in these fractions (see Fig. 6.13). The 25-hydroxylase activity for the [^{3}H]2,22,25-trideoxyecdysone substrate was again highest in both the P10 and P100 fractions with approximately equal activity in each. The specific activity of the 25-hydroxylase enzyme differed for both the radiolabelled substrates, being of higher value for [^{3}H]3-dehydro-2,22,25-trideoxyecdysone than [^{3}H]2,22,25-trideoxyecdysone in all the subcellular fractions of the follicle cell.

6.3. Discussion

[^{3}H]3-Dehydro-2,22,25-trideoxyecdysone was efficiently incorporated into ecdysteroids in the ovarian follicle cells of *L. migratoria*, yielding 3-dehydro, common 3β-hydroxy and 3-epi (3α−hydroxy) derivatives of a number of ecdysteroid moieties.

(1) [^{3}H]3-dehydro-2,22,25-trideoxyecdysone



(2) [^{3}H]2,22,25-trideoxyecdysone

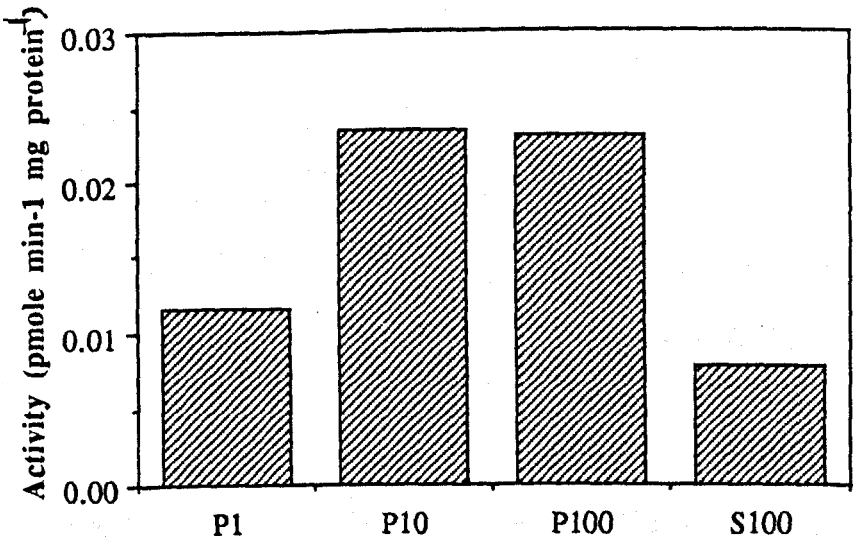


Fig. 6.14. Activity of the 25-hydroxylase enzyme, using (1)[^{3}H]3-dehydro-2,22,25-trideoxyecdysone and (2)[^{3}H]2,22,25-trideoxyecdysone substrates, in the 1,000g pellet (P1), 10,000g pellet (P10), 100,000g pellet (P100) and the 100,000g supernatant (S100) of a homogenate of ovarian follicle cells from *L. migratoria* . The enzymic activity is a measure of the conversion of the radiolabelled substrates into their 25-hydroxylated ecdysteroid products (see Table 6.2) and is expressed in pmole min^{-1} mg protein $^{-1}$.

	Radiolabelled substrates	
	[^{3}H]3D2,22,25dE	[^{3}H]2,22,25dE
Subcellular fractions		
P1	3-dehydro-2,22-dideoxyecdysone (10.2) 2,22-dideoxyecdysone (48.1)	3-dehydro-2,22-dideoxyecdysone (3.3) 2,22-dideoxyecdysone (19.3)
P10	3-dehydro-2,22-dideoxyecdysone (3.8) 2,22-dideoxyecdysone (38.2) 22-dideoxyecdysone (3.5) 3-epi-2-deoxyecdysone (9.0) 3-dehydro-2-deoxyecdysone (1.9) 2-deoxyecdysone (3.3)	3-dehydro-2,22-dideoxyecdysone (6.2) 2,22-dideoxyecdysone (16.1) 22-dideoxyecdysone (2.2) 3-epi-2-deoxyecdysone (1.0) 3-dehydro-2-deoxyecdysone (2.2) 2-deoxyecdysone (1.0)
P100	3-dehydro-2,22-dideoxyecdysone (8.6) 2,22-dideoxyecdysone (51.0) 22-deoxyecdysone (1.3)	3-dehydro-2,22-dideoxyecdysone (2.8) 2,22-dideoxyecdysone (22.0)
S100	3-dehydro-2,22-dideoxyecdysone (4.3) 2,22-dideoxyecdysone (35.7)	3-dehydro-2,22-dideoxyecdysone (1.8) 2,22-dideoxyecdysone (9.6)

Table 6.2. Metabolic products formed from [^{3}H]3-dehydro-2,22,25-trideoxyecdysone (3D2,22,25dE) and [^{3}H]2,22,25-trideoxyecdysone (2,22,25dE) in the 1,000g pellet (P1), 10,000g pellet (P10), 100,000g pellet (P100) and 100,000g supernatant (S100) of a homogenate of ovarian follicle cells of *L. migratoria*. The incorporations into labelled compounds (numbers in parenthesis) are expressed as a total of the recovered ecdysteroids from RP-HPLC (system 1, see section 2.2.8).

However, no 3β-hydroxy derivative of 2,22,25-trideoxy-3-dehydroecdysone was formed. The synthesis of both 3-epiecdysteroids and 3β-hydroxyecdysteroids at the higher levels of hydroxylation suggest that reduction of the 3-oxo group of the radiolabel can yield both the 3α- and 3β- configuration, and implicates the presence of both 3 α- and 3β-forming 3-dehydoecdysteroid reductases in the ovarian follicle cells. The presence of these enzymes has so far not been reported in the adult stages of insect development. However, both 3β- and 3α-reductases occur in the midgut of a number of Lepidopteran insects at both the larval and pupal stages of development, where they are involved in the reduction of 3-dehydroecdysteroids into either 3β-hydroxyecdysteroids or the inactivation products, 3-epiecdysteroids (Milner *et al.*, 1985; Weirich *et al.*, 1989; 1993). 3-Dehydroecdysteroid 3β-reductase enzyme activity has also been widely reported to be present in the haemolymph of a number of larval Lepidoptera, where it is responsible for reduction of 3-dehydroecdysone into ecdysone (Warren *et al.*, 1988a; 1988b ; Sakurai *et al.*, 1989; Kelly *et al.*, 1990; Kiriishi *et al.*, 1990). In addition, a number of organs, including the testes, Malpighian tubules , salivary glands and fat body of fifth instar *Ostrinia nubilis* have also been shown to exhibit 3β-reductase activity (Gelman *et al.*, 1991).

The synthesis of both 3-dehydroecdysteroids and 3β-hydroxyecdysteroids from [^{3}H]3-dehydro-2,22,25-trideoxyecdysone by *L. migratoria* prothoracic glands *in vitro* has recently been reported by Roussel (1992a). However, the origin of this glandular 3β-reductase activity was not determined, and the possibility arises that it was due to haemolymph contamination.

The ability of ovarian follicle cells to synthesize 3-epi-2-deoxyecdysone and 3-epiecdysone from [^{3}H]3-dehydro-2,22,25-trideoxyecdysone is rather anomalous because 3-epi-ecdysteroids have not been detected as `normal` products of follicle cells. Initially it was believed that these ecdysteroid forms were in fact 5α- derivatives formed by keto-enol tautomerism of their respective 5β- isomers. However, this was disproved, at least at the 2-deoxyecdysone level of hydroxylation, by the ability of RP-HPLC system 4 (see section 2.2.8) to clearly separate 5α-2-deoxyecdysone and 3-epi-2-

deoxyecdysone. 3-Epiecdysteroids are considered to be much less hormonally active than their corresponding 3β-hydroxyecdysteroid derivatives, being commonly found in the gut of Lepidopteran insects and the developing eggs of *S. gregaria* and *L. migratoria* , where they are thought to represent the inactivated products of metabolism (Lagueux *et al.*, 1984; Rees and Isaac, 1984; Milner *et al.*, 1985). The inability to detect 3-epi-2-deoxyecdysone in a hydrolysed extract from the day 0 eggs of the Orthopteran insect species, *S. gregaria* (results not shown) was in agreement with the results of Isaac *et al.* (1981) and confirmed that the presence of this compound in the late stage eggs of *S. gregaria* was a direct result of hormone inaction during embryogenesis and not due to previous ovarian ecdysteroid synthesis. Therefore, it must be stressed that the ability of ovarian follicle cells to synthesize 3-epiecdysteroids from the radiolabel may not be an accurate reflection of the endogenous ecdysteroid biosyntheis occurring within the follicle cell. Dinan (1980) observed only low 3-dehydroecdysteroid 3α-reductase activity associated with the fat body of larval *Schistocerca,* and therefore, if the same situation applies for adult *Locusta,* then fat body contamination of the follicle cells would be insufficient to account for the formation of 3-epiecdysteroids from the radiolabelled substrate.

[^{3}H]2,22,25-Trideoxyecdysone has been previously shown to be converted into a number of ecdysteroids by *L. migratoria* ovarian follicle cells *in vitro.* These ecdysteroids included 2,22-dideoxyecdysone, 2-deoxyecdysone, ecdysone and 20-hydroxyecdysone (Kappler *et al.*, 1986a). On re-examination of the ecdysteroids synthesized from the radiolabel by the ovarian follicle cells using the RP-HPLC systems devised by Roussel (1992a), that can separate the 3-dehydro and 3β−hydroxy derivatives of an individual ecdysteroid moiety, 3-dehydro and 3-epi derivatives of a number of ecdysteroids were identified, in addition to the 3β−hydroxyecdysteroids. No 3-dehydroecdysteroid derivative of 2,22,25-trideoxyecdysone could be detected. The synthesis of both 3-dehydro-2,22-dideoxyecdysone and 3-dehydro-2-deoxyecdysone suggests the presence of ecdysteroid 3-oxidase enzymes within the follicle cells. These enzymes can convert the 3β−hydroxy group of the ecdysteroid moiety into the

corresponding 3-oxo group. Ecdysteroid 3-oxidase activity has not been previously detected in mature stages of insect development, but has been extensively studied in the midgut of immature stages of Lepidopteran insects. In the midgut this enzyme, in conjunction with a 3α-reductase, is involved in the 3-epimerisation of 3β-hydroxy ecdysteroids, being responsible for the synthesis of the 3-dehydroecdysteroid intermediate (Milner *et al.*, 1985; Weirich *et al.*, 1993). Ecdysteroid 3-oxidase activity has also been detected in the pupal wings, tegument and fat body of *P. brassicae*, which therefore suggests the possible presence of 3-dehydroecdysteroids within these insect tissues (Blais and Lafont, 1984).

At the 2-deoxyecdysone level of hydroxylation for both the [^{3}H]3-dehydro-2,22,25-trideoxyecdysone and [^{3}H]2,22,25-trideoxyecdysone substrates, the 3-epi, 3-dehydro and the 3β-hydroxy derivatives of 2-deoxyecdysone could be identified. *In vitro* studies with the respective radiolabelled forms of these ecdysteroids in ovarian follicle cells suggested that both the 3β-hydroxy and 3-dehydro derivatives of 2-deoxyecdysone were interconvertible. However, 3-epi-2-deoxyecdysone could not be converted into either 3-dehydro-2-deoxyecdysone or 2-deoxyecdysone confirming the irreversible nature of 3-epimerisation reaction as demonstrated by Milner *et al.* (1985) in the midgut of *S. littoralis*.

The combined incubation results from both the [^{3}H]2,22,25-trideoxy-3-dehydroecdysone and [^{3}H]2,22,25-trideoxyecdysone substrates suggest a tentative pathway for the terminal stages of ecdysteroid biosynthesis in ovarian follicle cells consisting of three parallel pathways of 3-epiecdysteroid, 3-dehydroecdysteroid and 3β-hydroxyecdysteroid derivatives (See Fig. 6.15).

On incubation with either [^{3}H]3-dehydro-2,22,25-trideoxyecdysone or [^{3}H]2,22,25-trideoxyecdysone no synthesis of 3-dehydroecdysone or 3-dehydro-20-hydroxyecdysone could be detected in the ovarian follicle cell cultures. However, the 3-dehydro derivatives of 2,22-dideoxyecdysone and 2-deoxyecdysone were present. This suggests that the reported inability of Kiriishi *et al.* (1990) to detect endogenous 3-dehydroecdysone production in the mosquito *A. aegypti*, and the cockroach, *Leucophea*

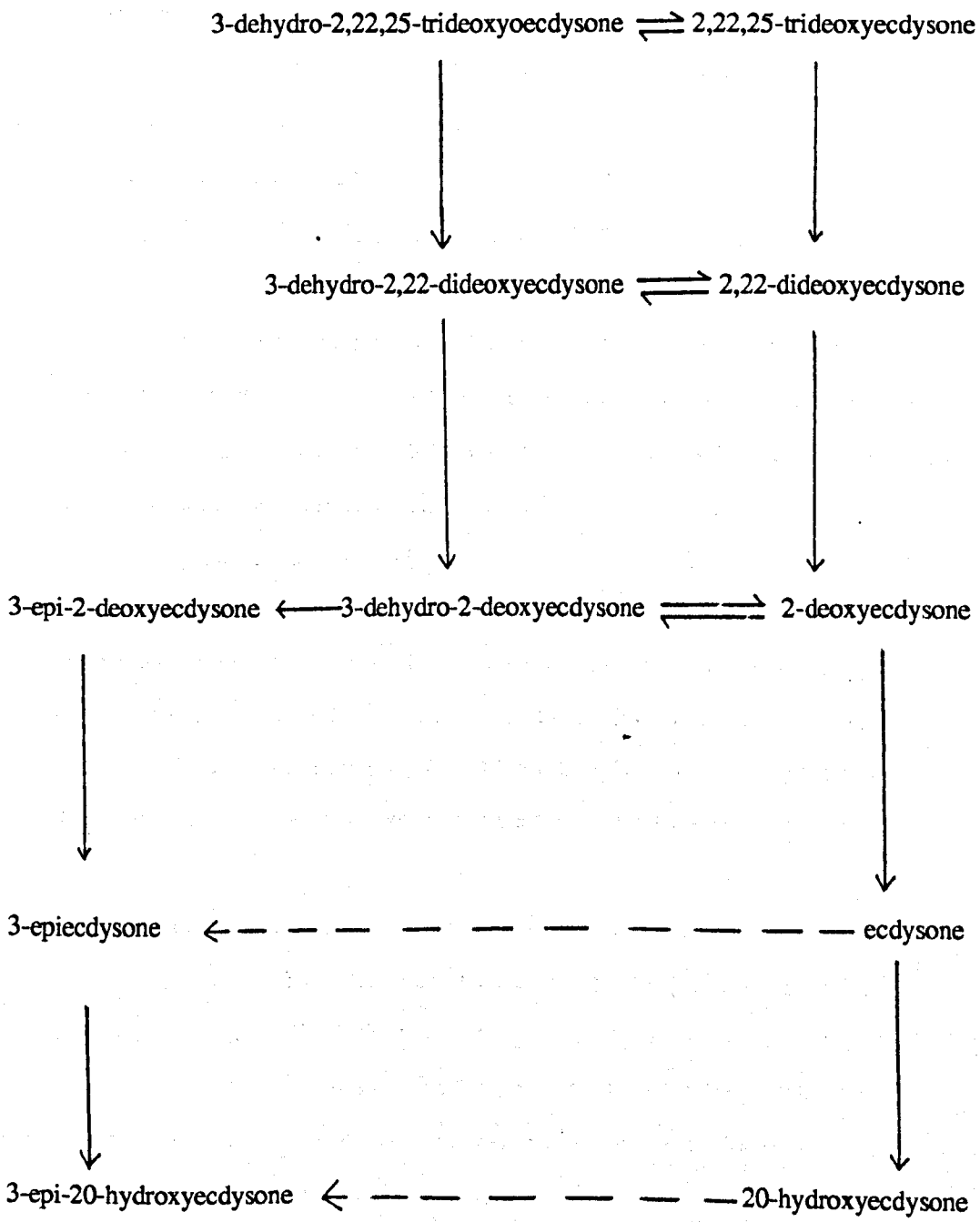


Fig. 6.15. Composite scheme of metabolic reactions observed during metabolism of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone, [^{3}H]2,22,25-trideoxyecdysone, [^{3}H]3-dehydro-2-deoxyecdysone, [^{3}H]3-epi-2-deoxyecdysone and [^{3}H]2-deoxyecdysone in *L. migratoria* ovarian follicle cells *in vitro*.

Full arrows indicate reactions for which there is evidence. Dotted arrows represent possible reactions.

maderae, ovaries *in vitro* does not necessarily preclude the synthesis of other 3-dehydroecdysteroids by these mature insect systems. The high activity of the putative 3α- and 3β-reductases for the 3-dehydroecdysone and 3-dehydro-20-hydroxyecdysone intermediates possibly contributes to the lack of accumulation of these 3-dehydroecdysteroid derivatives in the ovarian follicle cells.

The metabolism of the radiolabelled substrates, [^{3}H]2,22,25-trideoxyecdysone, [^{3}H]2,22-dideoxyecdysone and [^{3}H]2-deoxyecdysone in the subcellular fractions of ovarian follicle cells suggests a possible microsomal location for the 25-hydroxylase, in contrast to the 2- and 22-hydroxylases which appear to be mitochondrial enzymes. The suggested subcellular distribution of these enzymes within the follicle cell is consistent with previous studies on the prothoracic glands of *L. migratoria* and recent studies on the prothoracic glands of *M. sexta*, which have also shown the 25-hydroxylase to be a microsomal enzyme and both the 2- and 22-hydroxylases to be present in the mitochondria (Kappler *et al.*, 1988; Grieneisen *et al.*, 1993). However, since mitochondrial and microsomal marker enzymes were not used in this experiment the origin of the small amount of metabolism of both the [^{3}H]2-deoxyecdysone and [^{3}H]2,22-dideoxyecdysone substrates within the microsomal fraction could not be determined.

Both the mitochondrial (P10) and microsomal fractions (P100) of the ovarian follicle cells showed the highest 25-hydroxylase activity using both [^{3}H]3-dehydro-2,22,25-trideoxyecdysone and [^{3}H]2,22,25-trideoxyecdysone substrates, with approximately equal 25-hydroxylase activity in each fraction. These results can be explained by the postulated occurrence of 25-hydroxylase enzyme within a subfraction of the endoplasmic reticulum, which would aggregate readily with the mitochondrial fraction (Kappler *et al.*, 1988). This is further suggested by the high microsomal marker enzyme activity in the mitochondrial fraction of the follicle cells. The apparently equal subcellular distribution of 25-hydroxylase activity using both the [^{3}H]3-dehydro-2,22,25-trideoxyecdysone and [^{3}H]2,22,25-trideoxyecdysone substrates in the ovarian follicle cell suggests that the C-25 hydroxylation of these radiolabelled substrates is

undertaken by a common enzyme. Schwab and Hetru (1991) have suggested that the hydroxylase enzymes present in the terminal stages of the ecdysteroid biosynthetic pathway do not have strict substrate specificities. This could explain the ability of the 3-epiecdysteroids and 3-dehydroecdysteroids to be accepted as substrates by the enzymes present in the terminal stages of the ecdysteroid biosynthetic pathway of the ovarian follicle cells. This could also, at least partly, account for the higher 25-hydroxylase activity using the [^{3}H]3-dehydro-2,22,25-trideoxyecdysone substrate in contrast to the [^{3}H]2,22,25-trideoxyecdysone substrate in the subcellular fractions of the ovarian follicle cells. It is also possible that differences in permeability to the substrates could arise. The hypotonic conditions of the incubation mixture should have caused sufficient disruption of both the mitochondrial and endoplasmic reticulum membranes, to remove gross permeability differences between the substrates that might affect their substrate availability to the enzyme

The efficient metabolism of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone in the ovarian follicle cells suggests the possible intermediacy of this compound in the ecdysteroid biosynthetic pathway of the cells (Fig. 6.15). However, it will be necessary to detect the endogenous presence of this compound within the follicle cells before the speculated intermediacy of 3-dehydro-2,22,25-trideoxyecdysone can be confirmed. The development of GC-MS methods to aid this detection will be discussed in Chapter 7.

Chapter 7

Development of mass spectrometric methods to detect the presence of endogenous 3-dehydro-2,22,25-trideoxyecdysone and 2,22,25-trideoxyecdysone in ovarian follicle cells of *L. migratoria*

7.1. Introduction

3-Dehydroecdysteroids were initially believed to represent the reversible oxidation products of 3β-hydroxyecdysteroids, being identified as metabolites of radiolabelled ecdysone *in vivo* in a few insect species (Koolman, 1982; Beydon *et al.*, 1987). Their involvement in the 3-epimerisation of 3β-hydroxyecdysteroids into their inactive 3-epiecdysteroid isomers was subsequently demonstrated by *in vitro* studies with enzyme preparations from the midgut of Lepidopteran insects (Blais and Lafont, 1984; Milner *et al.*, 1985). The isolation of the 3-dehydro, 3β-hydroxy and 3-epi derivatives of 2-deoxyecdysone from the late stage eggs of *L. migratoria* is also in accordance with an intermediary role for the 3-dehydroecdysteroid moiety in the 3-epimerisation reaction (Tsoupras *et al.*, 1983).

Recently, the prothoracic glands from a number of Lepidopteran insects have been shown to synthesize and release 3-dehydroecdysone as the major ecdysteroid product of the gland in addition to ecdysone (Warren *et al.*, 1988a; Kiriishi *et al.*, 1990). The inability to detect ecdysone oxidase activity associated with the gland suggested that 3-dehydroecdysone was a precursor rather than a metabolite of ecdysone, being subsequently reduced to ecdysone by the 3-dehydroecdysteroid 3β-reductase enzyme present in the haemolymph (Warren *et al.*, 1988b, Sakurai *et al.*, 1989; Kiriishi *et al.*, 1990). The possibilty that 3-dehydroecdysteroids, in addition to representing precursors and/or metabolites of 3β-hydroxyecdysteroids may have some physiologically active hormonal function in insects was demonstrated by the ability of 3-dehydro-20-hydroxyecdysteroids to initiate ecdysone specific puffs in salivary gland giant chromosomes of *Drosophila*. In addition, this compound has also been shown to be more active than 20-hydroxyecdysone in initiating transcription of the P1 gene in the fat body of larval *Drosophila* (Splindler *et al.*, 1977; Richards, 1978; Somme-Martin *et al.*, 1990).

The inability to detect 3-dehydroecdysteroids *in vivo* in many tissues has been attributed to the reported 3-dehydroecdysteroid 3β-reductase activity associated with a

number of insect tissues including the testes, Malpighian tubules, fat body and salivary glands, in addition to its well documented presence in the haemolymph (Gilbert, 1989; Kiriishi *et al.*, 1990; Gelman *et al.*, 1991). However, the intermediacy of the proposed 3-dehydroecdysone precursor, 3-dehydro-2,22,25-trideoxyecdysone, in the ecdysteroid biosynthetic pathway of prothoracic glands from a number of insect species has been suggested from *in vitro* studies with its radiolabelled form (Blais and Lafont, 1991; Roussel, 1992a; Cole, 1993; Grieneisen *et al.*, 1993). In addition, the ability of ovarian follicle cells from adult female *L. migratoria* to efficiently metabolise radiolabelled 3-dehydro-2,22,25-trideoxyecdysone into a range of ecdysteroids can be interpreted in terms of a possible intermediary role for this compound in the ecdysteroid biosynthetic pathway of the follicle cells (see Chapter 6). However, the ability to detect this steroid endogenously in either the prothoracic glands or the ovarian follicle cells would be first required before the possible intermediacy of 3-dehydro-2,22,25-trideoxyecdysone could be further considered. The low 3-dehydroecdysteroid 3β-reductase activity associated with intact prothoracic glands makes this tissue an ideal candidate for the possible endogenous isolation of this putative 3-dehydroecdysteroid intermediate (Warren *et al.*, 1988b; Gelman *et al.*, 1991; Cole, 1993). However, since prothoracic glands do not accumulate ecdysteroids to any great extent, the possibility of detecting such intermediates would be expected to be very low (King *et al.*, 1974; Hirn *et al.*, 1979). In contrast, the high ecdysteroid biosynthetic activity of ovarian follicle cells of *L. migratoria*, in addition to the subsequent accumulation of these ecdysteroid products within the terminal oocytes, have made this tissue ideal for the isolation of ecdysteroid biosynthetic pathway intermediates. Previous gas chromatography-mass spectrometric (GC-MS) analysis of whole ovary extracts have revealed the presence of the endogenous ecdysteroids, 2-deoxyecdysone, 2,22-dideoxyecdysone, 2,22,25-trideoxyecdysone and 2,14,22,25-tetradeoxyecdysone (Hetru *et al.*, 1978; 1982). However, the inability to detect 3-dehydroecdysteroids in this investigation would not be unexpected, as derivatisation of the ovary extract by silyl ether formation alone would not be sufficient to stabilize these diketonic ecdysteroids for GC-MS analysis, and therefore, the

endogenous presence of 3-dehydroecdysteroids within the ovary of *L. migratoria* cannot be discounted (Hirota *et al.*, 1988).

The work described in this chapter was concerned initially with the development of mass spectrometric methods to detect 3-dehydroecdysteroids enabling them to be distinguished from their respective 3β-hydroxyecdysteroid derivatives. These mass spectrometric methods were then applied to the detection of the endogenous putative ecdysteroid intermediate, 3-dehydro-2,22,25-trideoxyecdysone and its 3β-hydroxyecdysteroid derivative, 2,22,25-trideoxyecdysone in the ovarian follicle cells of *L. migratoria.*

7.2. Experimental and results

7.2.1. Preparation of 3-dehydro-2,22,25-trideoxyecdysone

2,22,25-Trideoxyecdysone was oxidized into its 3-dehydroecdysteroid derivative, 3-dehydro-2,22,25-trideoxyecdysone according to the method of Splindler *et al* (1977).

Platinum (IV) oxide catalyst (25mg) was added to glacial acetic acid (4ml), hydrogen gas was then introduced and the mixture was stirred until the brown platinum (IV) oxide was converted into black platinum (II) oxide. The catalyst was then exhaustively washed with distilled water until all traces of glacial acetic acid were removed. 2,22,25-Trideoxyecdysone (1.4mg), dissolved, with difficulty, by vortexing in 5ml of distilled water, was then added to the catalyst and the solution was stirred at room temperature with a gentle stream of oxygen being bubbled through the reaction mixture. The progress of the reaction was monitored periodically by thin-layer chromatography (TLC), developed with chloroform and a maximum yield of 3-dehydro-2,22,25-trideoxyecdysone was observed after 54 h. The reaction was stopped by the addition of methanol (20ml) and the mixture was then subjected to centrifugation in a bench top centrifuge (1,000g, 10min). The supernatant was removed, the pellet re-extracted twice with 50% (v/v) methanol in dichloromethane (20ml) and the combined supernatants

were blown down under nitrogen. 3-Dehydro-2,22,25-trideoxyecdysone was then separated from the crude reaction products by C_{18} Sep-Pak fractionation (see section 2.2.7, procedure 2). The 2,22,25-trideoxyecdysone and 3-dehydro-2,22,25-trideoxyecdysone present in the methanol fraction were then separated by RP-HPLC (system 7, see section 2.2.8, see Fig. 7.1) before being collected.

The structure of 3-dehydro-2,22,25-trideoxyecdysone was confirmed by electrospray and thermospray mass spectrometry (see section 7.2.3).

7.2.2. GC-MS analysis of methoxime-trimethylsilylated (MO-TMS) ecdysone and trimethylsilylated (TMS) ecdysone.

3-Dehydroecdysteroids and 3β-hydroxyecdysteroids must first be derivatized before they can be analysed by GC-MS. Conver.tionally, 3β-hydroxyecdysteroids are converted into trimethylsilyl ethers (TMS) by treatment with N-trimethylsilylimidazole (TMSi). This reaction converts the hydroxyl groups of the molecule into trimethylsilyl groups (0-Si-$(CH_3)_3$), making the ecdysteroid molecule more volatile and thermally stable for GC-MS analysis. TMSi treatment alone would not be sufficient to fully derivatise 3-dehydroecdysteroids which posses an additional ketone group (C=0) at C-3 in contrast to 3β−hydroxyecdysteroids. Therefore, by analogy to the methods used to derivatise corticosteroids, which are also polyhydroxylated diketonic steroids, 3-dehydroecdysteroids will be first converted into methoxime (MO) derivatives by treatment with methoxime hydrochoride (MOX). This reaction will stabilize the carbonyl groups of the molecule preventing possible enolization occurring which may give rise to silyl enol ethers during the forcing conditions of the subsequent silylation reaction to generate the final MO-TMS derivatisation product (Horning *et al.*, 1968; Aringer *et al.*, 1971).

The suitability of the MOX-TMSi derivatisation procedure for the GC-MS analytical study of ecdysteroids was investigated by comparing the mass spectral behaviour of the MO-TMS-ecdysone derivative with that of its respective well characterised TMS-

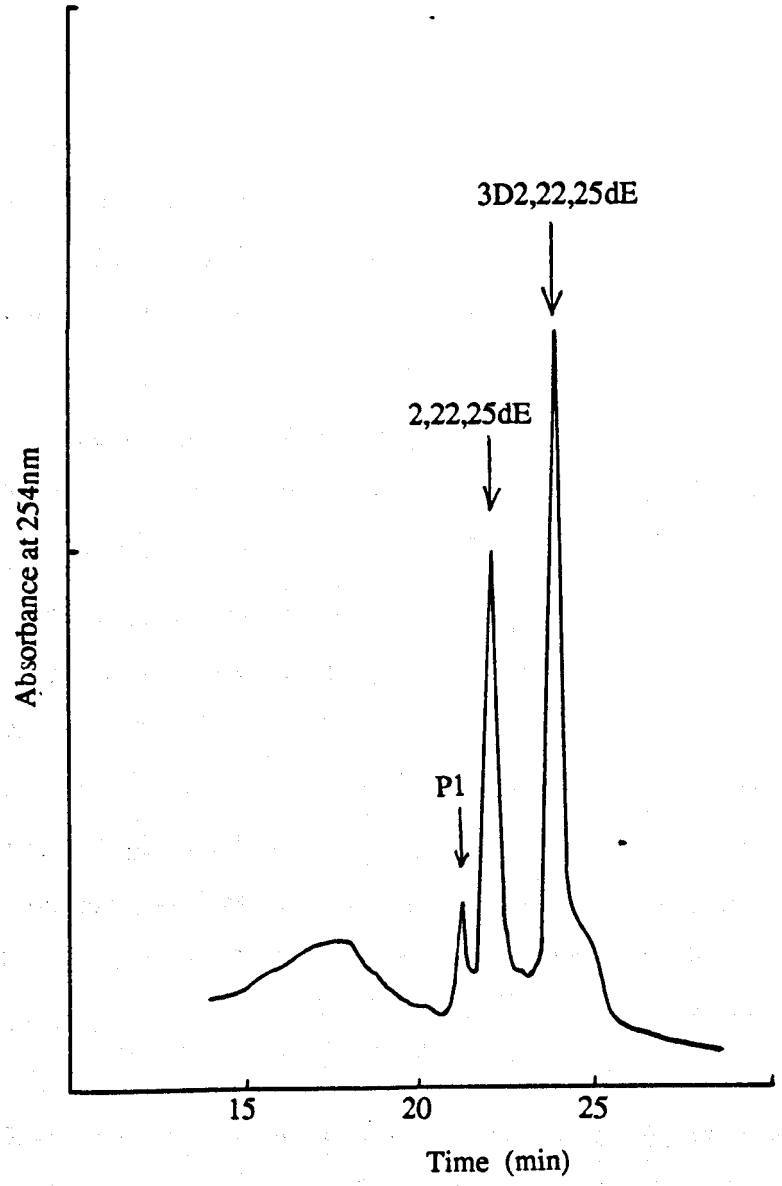


Fig. 7.1. RP-HPLC (system 7, see section 2.2.8) chromatogram of the reaction products from the preparation of 3-dehydro-2,22,25-trideoxyecdysone from 2,22,25-trideoxyecdysone. P1 was an impurity present in the initial 2,22,25-trideoxyecdysone sample. The positions of authentic 2,22,25-trideoxyecdysone (2,22,25dE) and putative 3-dehydro-2,22,25-trideoxyecdysone (3D2,22,25dE) are shown.

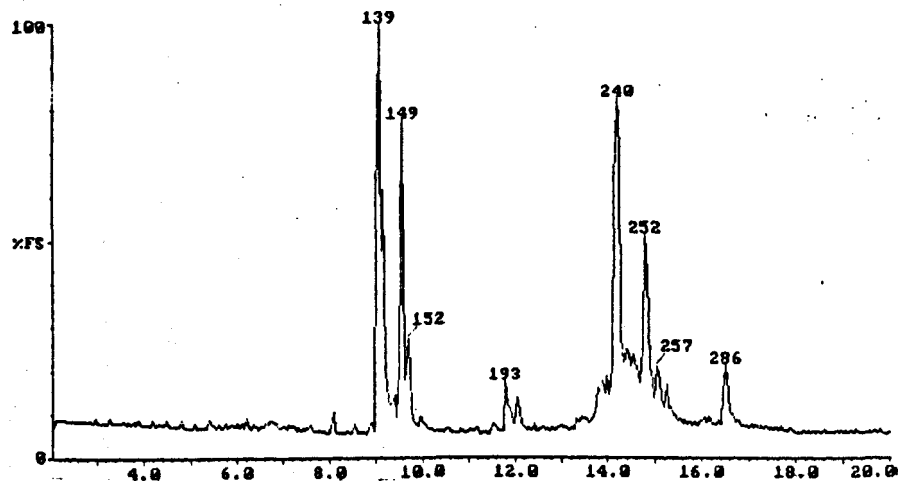
derivative (Lafont *et al.*, 1980). Ecdysteroids were derivatised as described in section 2.2.10.

Fig. 7.2 shows the total ion current (TIC) chromatograms for both trimethylsilylated (TMS) ecdysone and methoxime-trimethylsilyated (MO-TMS) ecdysone. The peak at 14.1 min (scan 240) in the TIC chromatogram of TMS-ecdysone showed ions expected of the fully silylated ecdysone derivative (see below, Fig. 7.3). The two components eluting at 13.4 min (scan 227) and 14.0 min (scan 238), respectively, in the TIC chromatogram of MO-TMS-ecdysone were thought to represent the *syn* - and *anti*-methoxime isomers of the derivative due to the similarity of their retention times to the fully silylated ecdysone moiety (Morgan and Woodbridge , 1971; Ikewa *et al.*, 1972). The mass spectra corresponding to both of these components were analysed and compared to that of TMS-ecdysone, to enable the identification of characteristic *m/z* ions in the former two spectra.

Fig. 7.3 shows the mass spectrum of TMS-ecdysone with the characteristic high mass ion at *m/z* 567, which is indicative of a fully derivatised ecdysone molecule. In addition, the low mass ions present at *m/z* 261 and 171 are thought to represent the fully silylated side chain of the ecdysteroid molecule which has been cleaved between the C-20 and C-22 positions and its partially silylated counterpart, respectively (Lafont *et al.*, 1980).

Fig. 7.4 shows the mass spectra for the components eluting at (1) 13.4 min (scan 227) and (2) 14.0 min (scan 238) in the TIC chromatogram for the MO-TMS-ecdysone derivative. The presence of the high abundance low mass ion at *m/z* 171 in both of these spectra suggests that side chain cleavage has also occurred for this derivative. In addition, the ion present at *m/z* 502 in spectrum (1) is representative of the remaining nuclear fragment after the forementioned side chain cleavage and subsequent loss of water. However, the identities of the high mass ions at *m/z* 473 and 401 in spectra (1) and (2), respectively, could not be further elucidated. The possibility exists that they may correspond to nuclear fragments from the ecdysone derivative, but they may also

(1) TMS-Ecdysone



(2) MO-TMS-Ecdysone

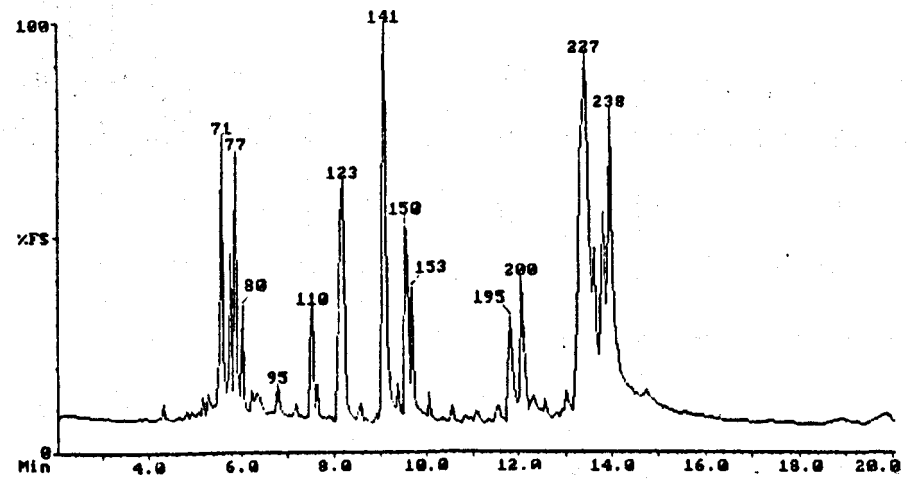


Fig. 7.2. Total ion current (TIC) chromatograms of (1) trimethylsilylated (TMS) ecdysone and (2) methoxime-trimethylsilylated (MO-TMS) ecdysone

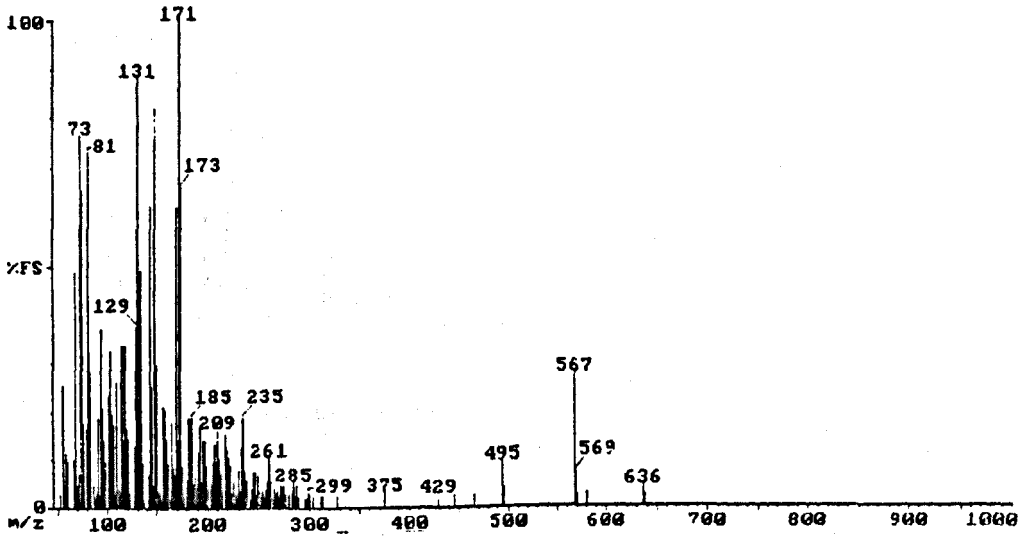
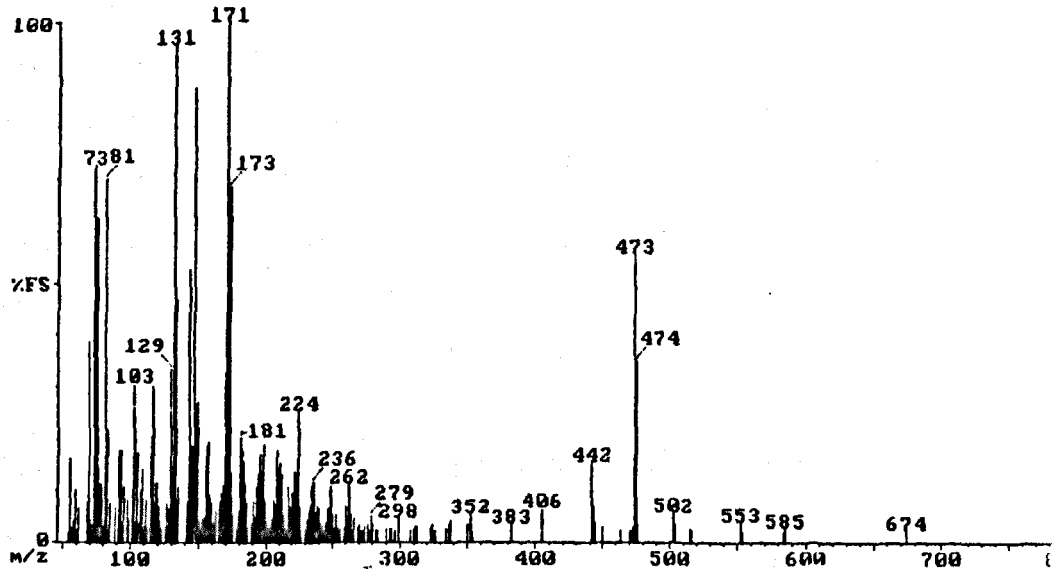


Fig. 7.3. GC-MS (E.I.) of component eluting in 14.1 minutes (scan 240) in the TIC chromatogram of TMS-ecdysone.

(1) 13.4 Minutes (scan 227)



(2) 14.0 Minutes (scan 238)

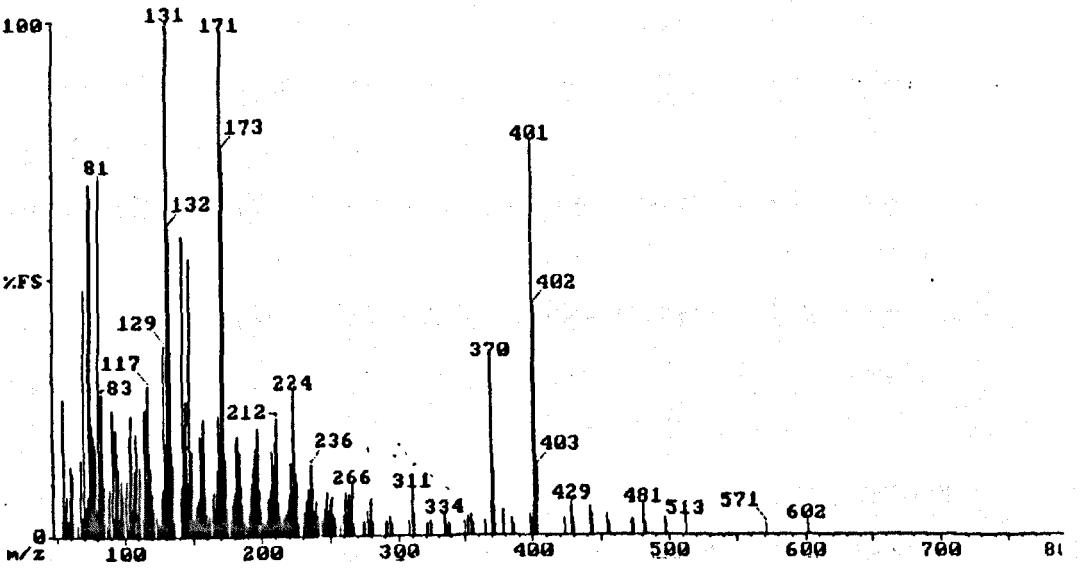


Fig. 7.4 GC-MS (EI) of the components eluting at (1) 13. 4 minutes (scan 227) and (2) 14.0 minutes (scan 238) in the TIC chromatogram of MO-TMS-ecdysone

arise from byproducts of the methoxime reaction which have not been removed during the purification procedure. Therefore, further purification of this derivative using the method of Axelson and Sjovall (1974), which has been applied to the clean up of such MO-TMS-derivatives, would be required before the latter possibility could be dismissed. Due to the initial difficulty in interpreting the mass spectra of the MO-TMS-ecdysone derivatives, coupled with the prolonged derivatization procedure, this method of anaysis was abandoned in favour of both thermospray and electrospray-mass spectrometry, which have the advantage over conventional GC-MS (EI) in that no derivatization of the ecdysteroid is required.

7.2.3. Electrospray and Thermospray mass spectrometric analysis of 3-dehydroecdysteroids and 3β-hydroxyecdysteroids

In vitro metabolism studies with [^{3}H]3-dehydro-2,22,25-trideoxyecdysone in the follicle cells of *L. migratoria* have suggested a possible branched pathway for the terminal stages of ecdysteroid synthesis in these cells, consisting of 3-dehydro and 3β-hydroxyecdysteroid derivatives (Fig. 7.5). Development of mass spectrometry methods will allow examination of the endogenous ecdysteroid content of the follicle cells, enabling the detection of one or both of the 3-dehydro and 3β-hydroxy derivatives of 2,22,25-trideoxyecdysone (ketodiol). In the process it might be possible to examine the pathway between 3-dehydro-2,22,25-trideoxyecdysone (3-dehydroketodiol) and 20-hydroxyecdysone.

Both electrospray (ESP) and thermospray (TSP) mass spectrometry (MS) function via an ion evaporation mechanism, leading to the production of molecular ions or pseudo-molecular ions as opposed to the extensive fragment ions formed during electron impact (EI) MS. As 3-dehydro and 3β-hydroxy derivatives of the same ecdysteroid differ by a molecular weight of only 2 mass units, the ability to reduce fragmentation by

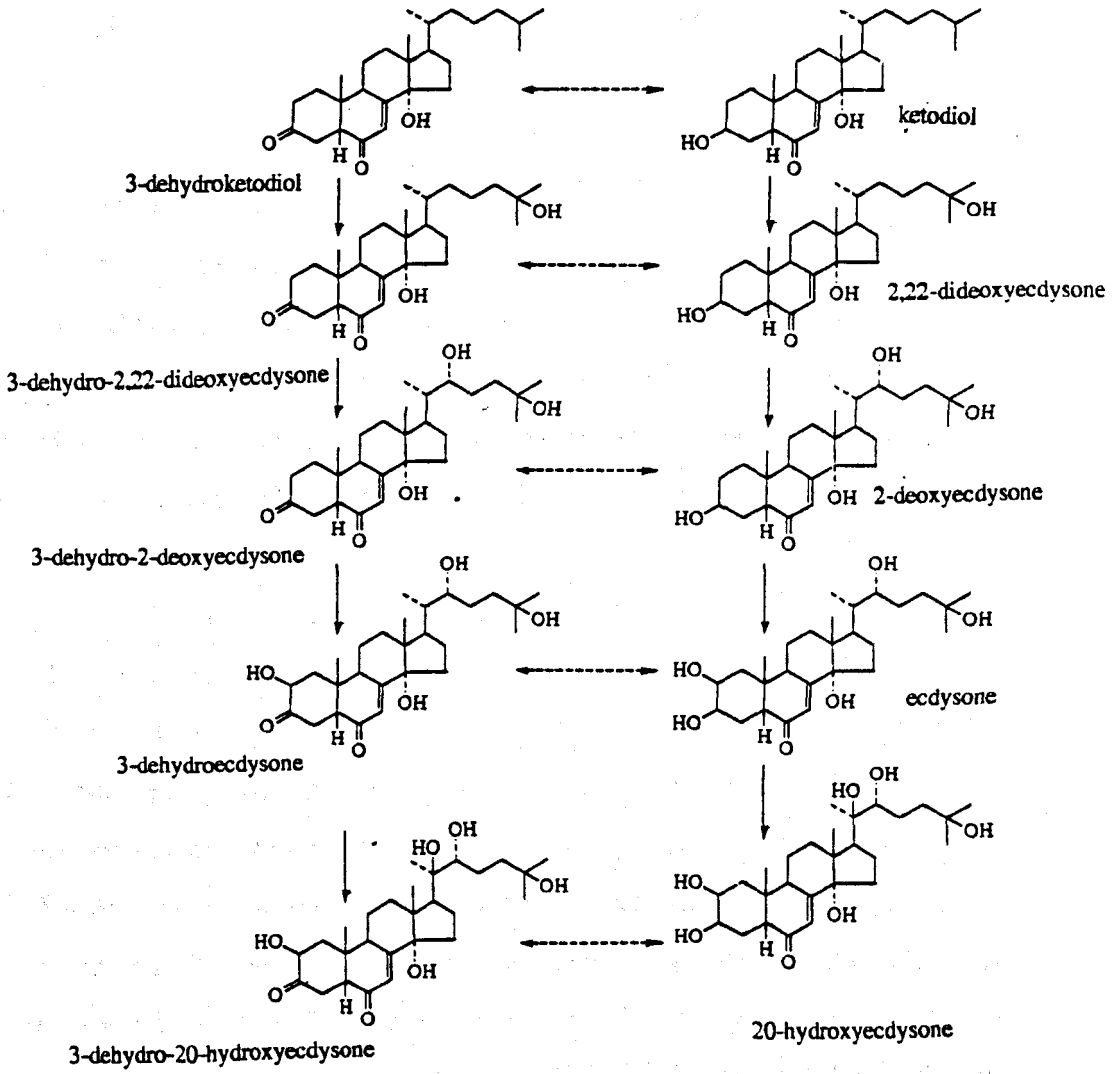


Fig. 7.5. Putative pathway for the terminal stages of ecdysteroid biosynthesis in the ovarian follicle cells of *L. migratoria*.

these gentler mass spectrometry methods would be expected to enhance mass spectrometrical distinction of these ecdysteroids.

ES/MS and TSP/MS were undertaken to analyse the 3-dehydro and 3β-hydroxy derivatives of ecdysone and 2,22,25-trideoxyecdysone in an effort to determine characteristic *m/z* ions by which these ecdysteroids may be distinguished.

Fig.7.6 shows the positive ion electrospray mass spectra obtained from 5μg each of (1) ecdysone and (2) 3-dehydroecdysone. In spectrum (1) the high mass ions at *m/z* 465 and 487 correspond to the pseudomolecular ion $[M+H]^+$, and the sodium adduct ion of ecdysone $[M+23]^+$. In addition, the ions at *m/z* 447 and 429 correspond to $[M-H_2O]^+$ and $[M-2H_2O]^+$, respectively. The ion at *m/z* 365 represents the protonated nuclear fragment remaining from the side chain cleavage between C-20 and C-22. The lower mass ions at *m/z* 142 and 159 are thought to correspond to side chain fragments.

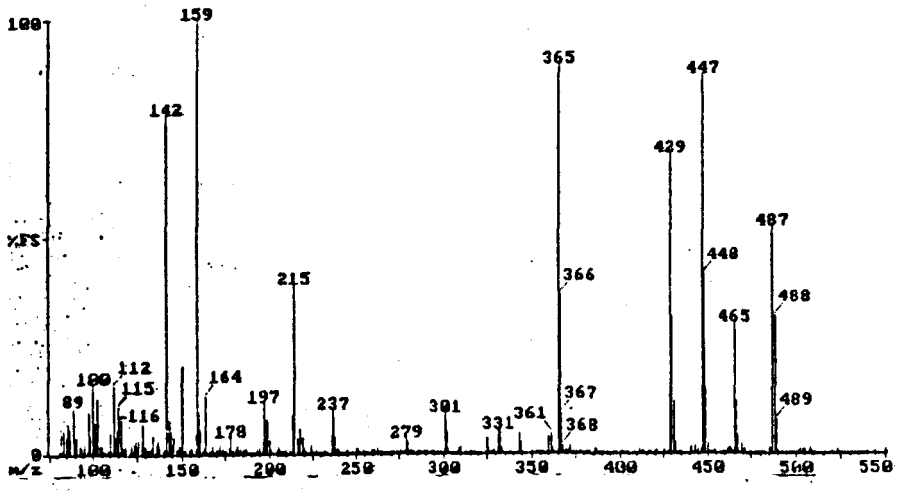
In spectrum (2) for 3-dehydroecdysone the high mass ion at *m/z* 462 represents the molecular ion, $[M]^+$, with the ion at *m/z* 485 corresponding to the $[M+23]^+$ species.

Fig. 7.7 shows the positive electrospray mass spectra obtained from 5μg each of (1) 2,22,25-trideoxyecdysone and (2) 3-dehydro-2,22,25-trideoxyecdysone. The preponderant high mass ion at *m/z* 439 in spectrum (1) represents the sodium adduct of 2,22,25-trideoxyecdysone $[M+23]^+$. In spectrum (2) for 3-dehydro-2,22,25-trideoxyecdysone the high mass ions at *m/z* 437 and 415 represent the $[M+23]^+$ and $[M+H]^+$ species, respectively for this compound.

In thermospray ionisation mass spectrometry, samples were introduced in a solution of hexane containing the volatile electrolyte, 0.1M ammonium acetate, which was added to the solution to provide a source of molecular ions.

The positive ion thermospray mass spectra for (1) 2,22,25-trideoxyecdysone (M.W. 414) and (2) 3-dehydro-2,22,25-trideoxyecdysone (M.W. 416) are shown in Fig. 7.8. The major intensity high mass ions which appears in spectra (1) and (2) at *m/z* 434 and 432, represent the pseudomolecular ion $[M+NH_4]^+$ for 2,22,25-trideoxyecdysone and 3-dehydro-2,22,25-trideoxyecdysone, respectively. In addition to these high intensity

(1) Ecdysone



(2) 3-Dehydroecdysone

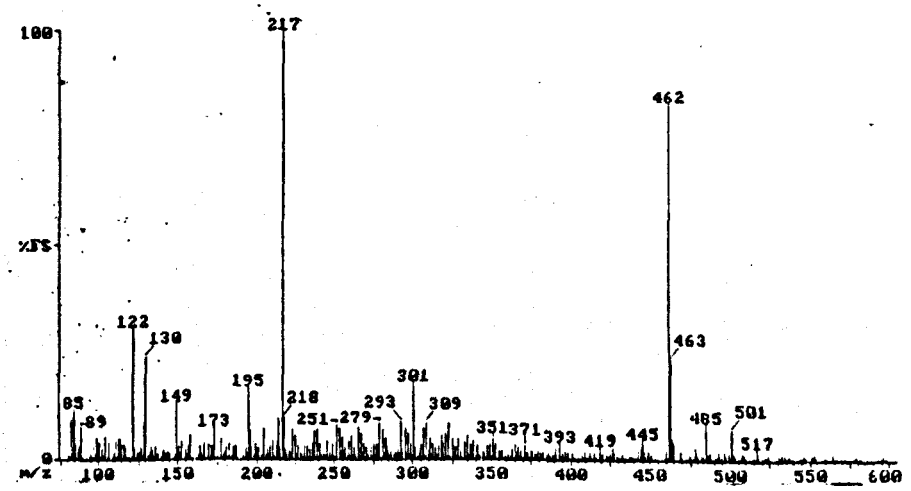
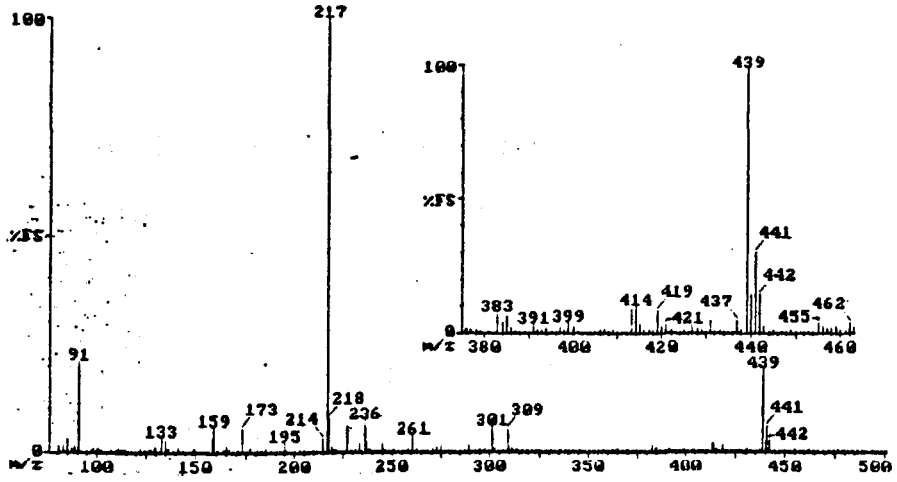


Fig. 7.6. Positive-ion electrospray mass spectra obtained from 5μg each of (1) ecdysone, (2) 3-dehydroecdysone

(1) 2,22,25-Trideoxyecdysone



(2) 3-Dehydro-2,22,25-trideoxyecdysone

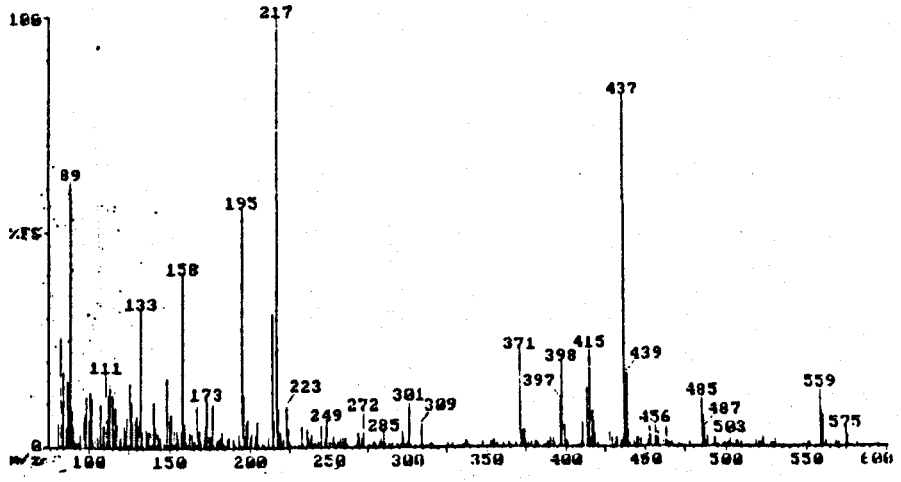
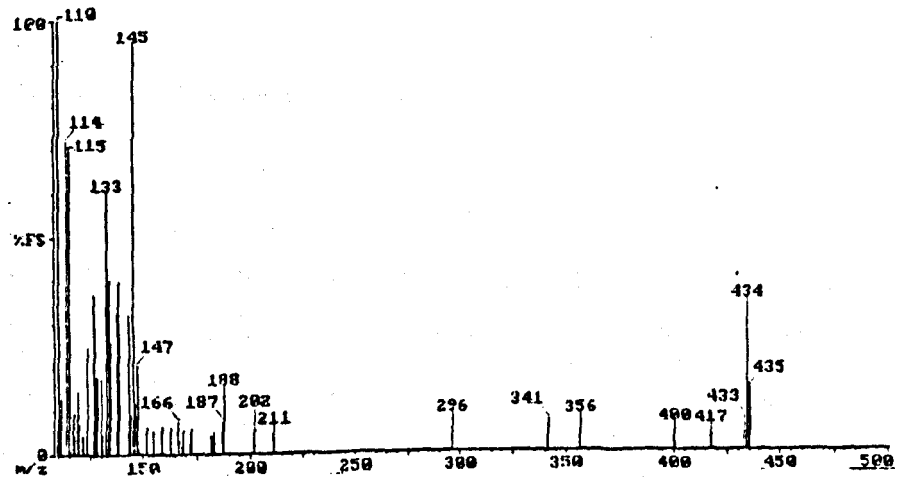


Fig. 7.7. Positive-ion electrospray mass spectra obtained from 5μg each of (1) 2,22,25-trideoxyecdysone and (2) 3-dehydro-2,22,25-trideoxyecdysone

(1) 2,22,25-Trideoxyecdysone



(2) 3-Dehydro-2,22,25-trideoxyecdysone

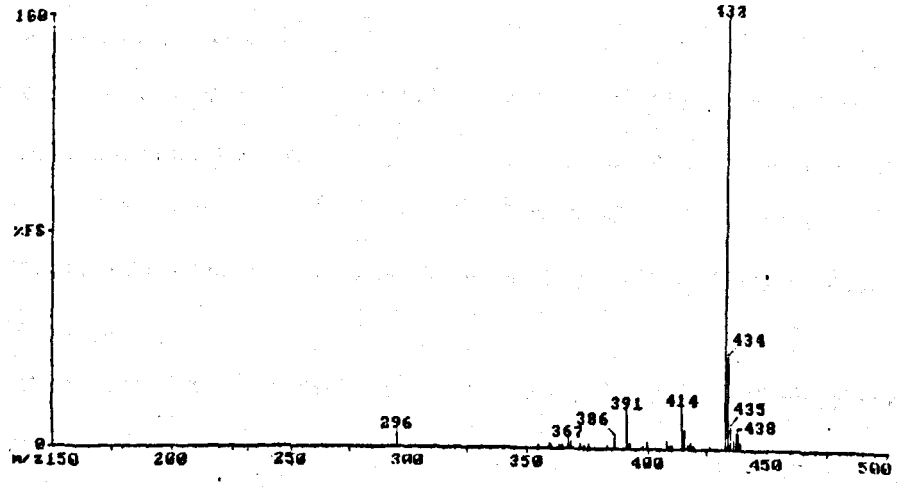


Fig. 7.8. Positive-ion thermospray mass spectra obtained from 5μg each of (1) 2,22,25-trideoxyecdysone and (2) 3-dehydro-2,22,25-trideoxyecdysone

$[M+NH_4]^+$ ion species ones of much lower intensity corresponding to the $[M+H]^+$ species for 2,22,25-trideoxyecdysone at *m/z* 417 and the $[M]^+$ species for 3-dehydro-2,22,25-trideoxyecdysone at *m/z* 414 were also present.

7.2.4. HPLC-TSP/MS analysis of ovarian follicle cell extracts from *L. migratoria*

The presence of endogenous 3-dehydro-2,22,25-trideoxyecdysone and 2,22,25-trideoxyecdysone was investigated in the ovarian follicle cells of *L. migratoria* using HPLC-TSP/MS. To increase the sensitivity of this method, selected ion monitoring (SIM) was employed to detect these compounds. The ions at *m/z* 434 and 432 were selected since these represented the intense high-mass ions corresponding to the $[M+NH_4]^+$species for 2,22,25-trideoxyecdysone and 3-dehydro-2,22,25-trideoxyecdysone, respectively. In order to establish the sensitivity of this technique for the analysis of the ovarian follicle cell sample, a range of methanol solutions containing 100ng, 50ng and 10ng each of 2,22,25-trideoxyecdysone and 3-dehydro-2,22,25-trideoxyecdysone were prepared and subjected to HPLC-TSP/MS analysis. Using these solutions, a detection limit down to 10ng for each of these components was clearly demonstrated as shown in Fig. 7.9 which shows the *m/z* 434 and 432 mass chromatograms for 10ng each of 2,22,25-trideoxyecdysone and 3-dehydro-2,22,25-trideoxyecdysone.

Purification methods were developed to effect a thorough clean up of the follicle cell extract, retaining all the compounds (see Fig. 7.5) between 3-dehydro-2,22,25-trideoxyecdysone and 20-hydroxyecdysone.

Ovarian follicle cells were prepared from approximately 400 ovaries as described in section 2.2.2, which were then subjected to the extraction procedure as shown in Fig. 7.10 which also shows the elution position of 2,22,25-trideoxyecdysone, 3-dehydro-2,22,25-trideoxyecdysone and ecdysone from previous studies with their respective

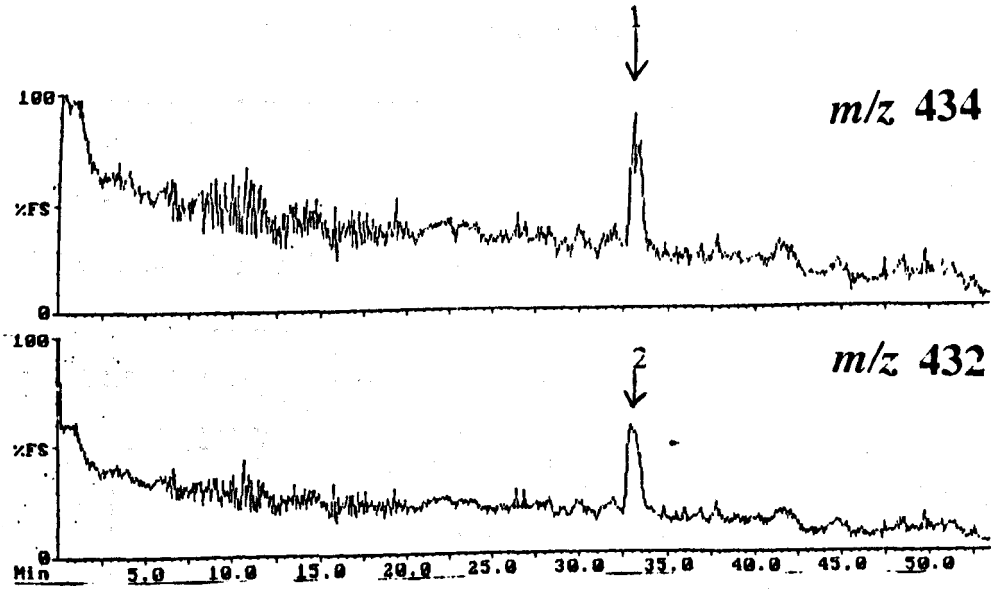


Fig. 7.9. Mass chromatograms for *m/z* 434 and 432 obtained from HPLC-TSP/MS (SIM) using solvent system A (see section 2.2.14) for 10ng each of 2,22,25-trideoxyecdysone(1) and 3-dehydro-2,22,25-trideoxyecdysone (2), respectively.

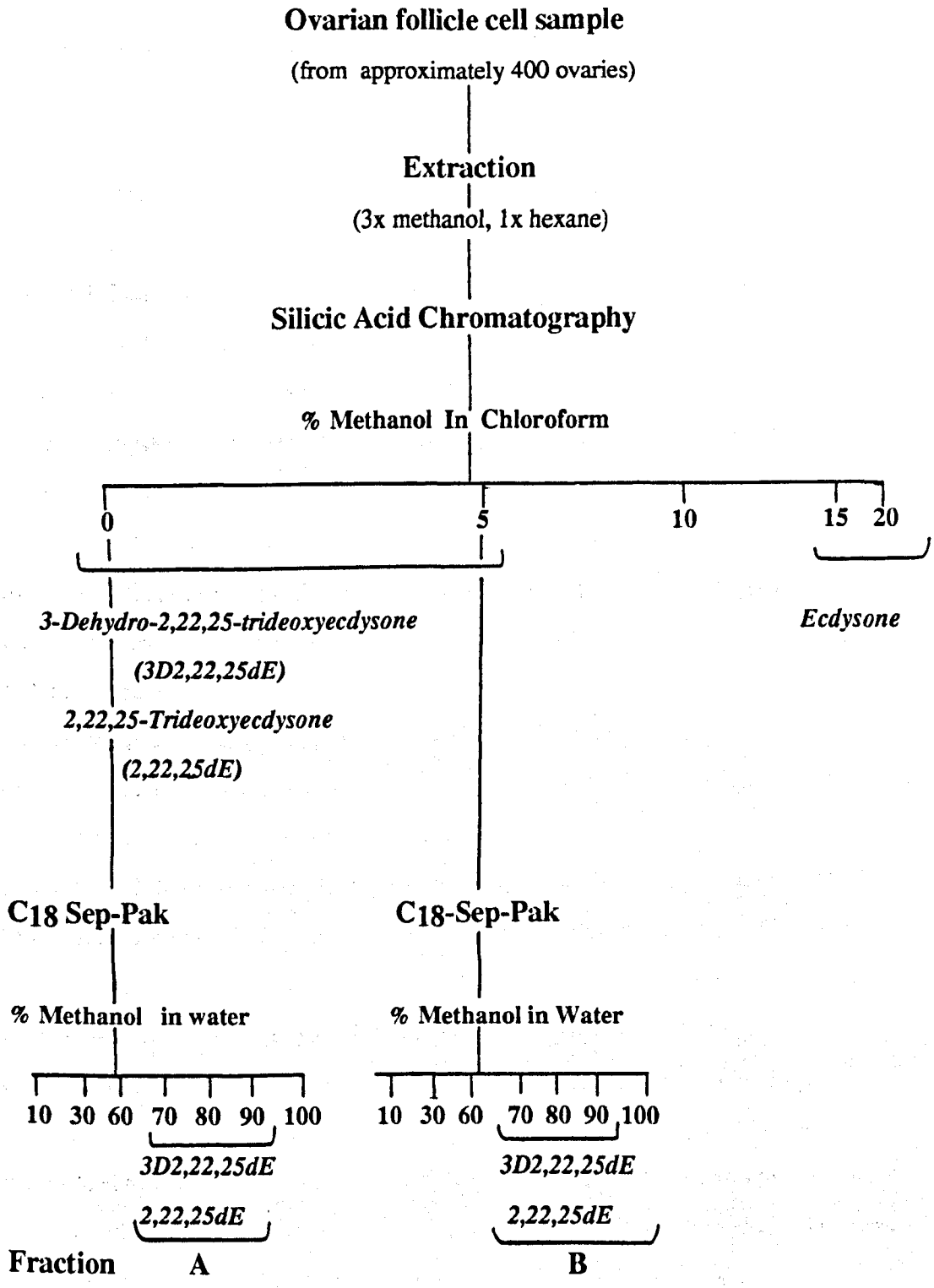


Fig. 7.10. Extraction scheme for the ovarian follicle cell sample from *L. migratoria*.

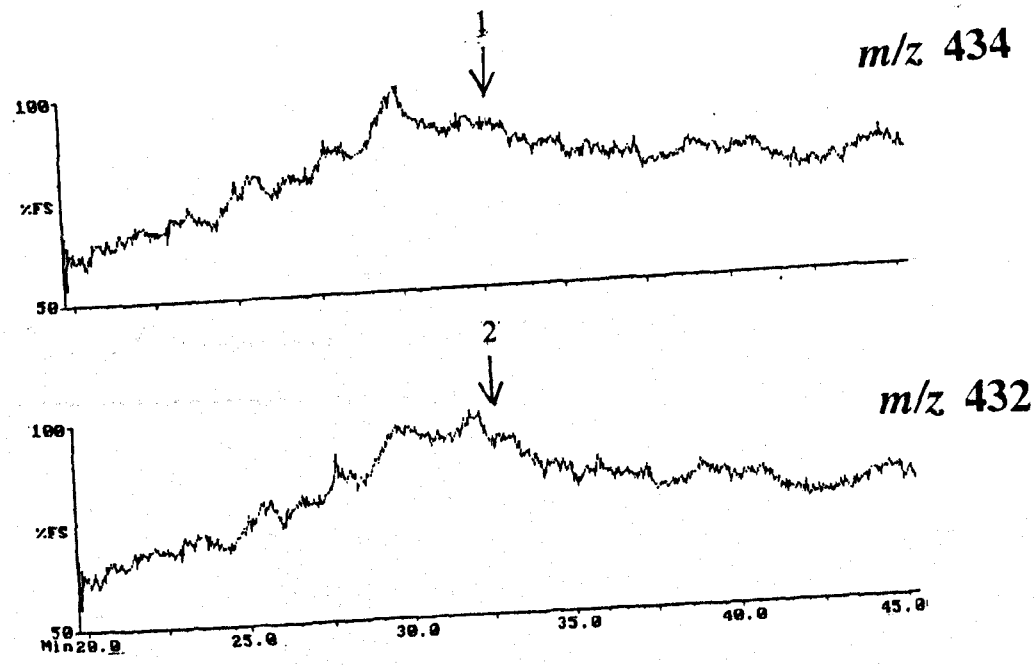
radiolabelled forms. Both fractions A and B were then analysed by HPLC-TSP/MS, using solvent system A (see section 2.2.14).

Fig. 7.11 shows the *m/z* 434 and 432 mass chromatograms obtained from HPLC-TSP/MS analysis of fraction A and B from the ovarian follicle cells. In both the *m/z* 434 and 432 chromatograms for fraction A, no peaks could be observed with retention times (RT) that corresponded to authentic 2,22,25-trideoxyecdysone and 3-dehydro-2,22,25-trideoxyecdysone, which like other 3-dehydro and 3β-hydroxy derivatives of the same ecdysteroid compound will co-elute on a methanol /water RP-HPLC system (see Chapter 6).

The *m/z* 434 and 432 mass chromatograms for fraction B were found to contain components that co-eluted with authentic 2,22,25-trideoxyecdysone and 3-dehydro-2,22,25-trideoxyecdysone, respectively. However, due to the complicated nature and high background in both of these chromatograms, further resolution was required to separate the possible 2,22,25-trideoxyecdysone and 3-dehydro-2,22,25-trideoxyecdysone compounds in sample B from other components displaying similar retention times in solvent system A (see section 2.2.14). By the use of solvent system B (see section, 2.2.14) which could be demonstrated to separate both 3-dehydro-2,22,25-trideoxyecdysone and 2,22,25-trideoxyecdysone (Fig. 7.12), the ovarian follicle cell sample was re-examined by HPLC-TSP/MS. However, no components were found to co-elute with authentic 3-dehydro-2,22,25-trideoxyecdysone or 2,22,25-trideoxyecdysone in the *m/z* 434 and 432 chromatograms, as shown in Fig. 7.13.

2,22,25-Trideoxyecdysone and 3-dehydro-2,22,25-trideoxyecdysone (20ng each) were added to sample B so that the actual detection limit for these compounds in this ovarian follicle extract could be determined. Fig. 7.14 shows the *m/z* 434 and 432 mass chromatograms for such a sample. In the *m/z* 434 chromatogram peak 1 corresponding to authentic 2,22,25-trideoxyecdysone, could be clearly observed. However, in contrast, peak 2 corresponding to authentic 3-dehydro-2,22,25-

Fraction A



Fraction B

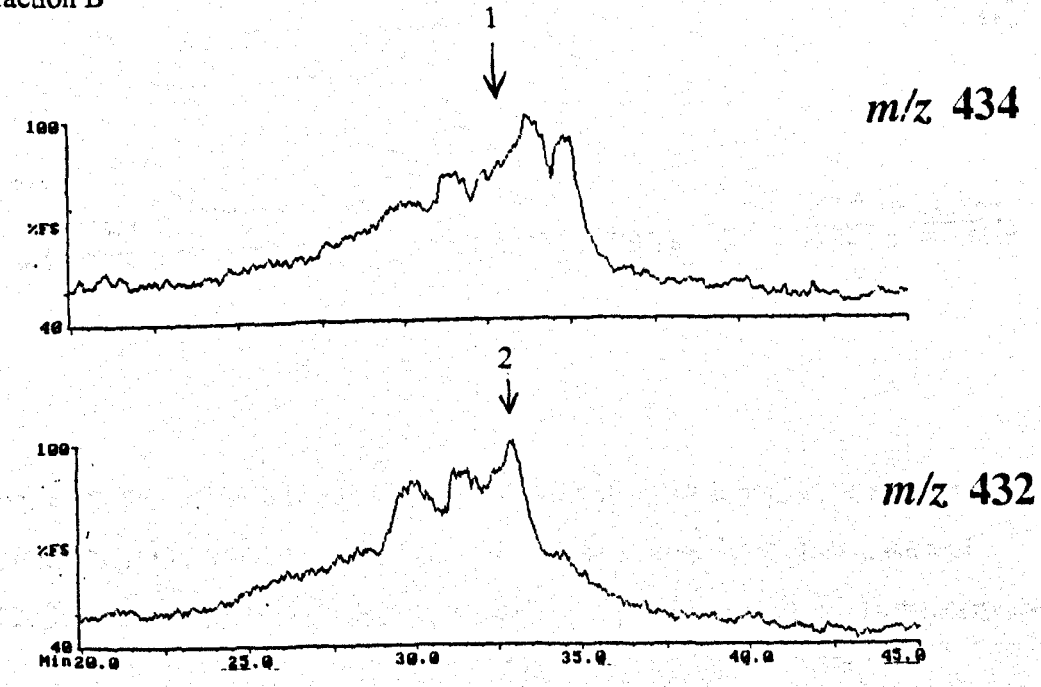


Fig. 7.11. Mass chromatograms for *m/z* 434 and 432 obtained from HPLC-TSP/MS (SIM) using solvent system A (see section 2.2.14) of fractions A and B derived from the ovarian follicle cell extract of *L. migratoria*. The positions of authentic 2,22,25-trideoxyecdysone (1) and 3-dehydro-2,22,25-trideoxyecdysone (2) are shown.

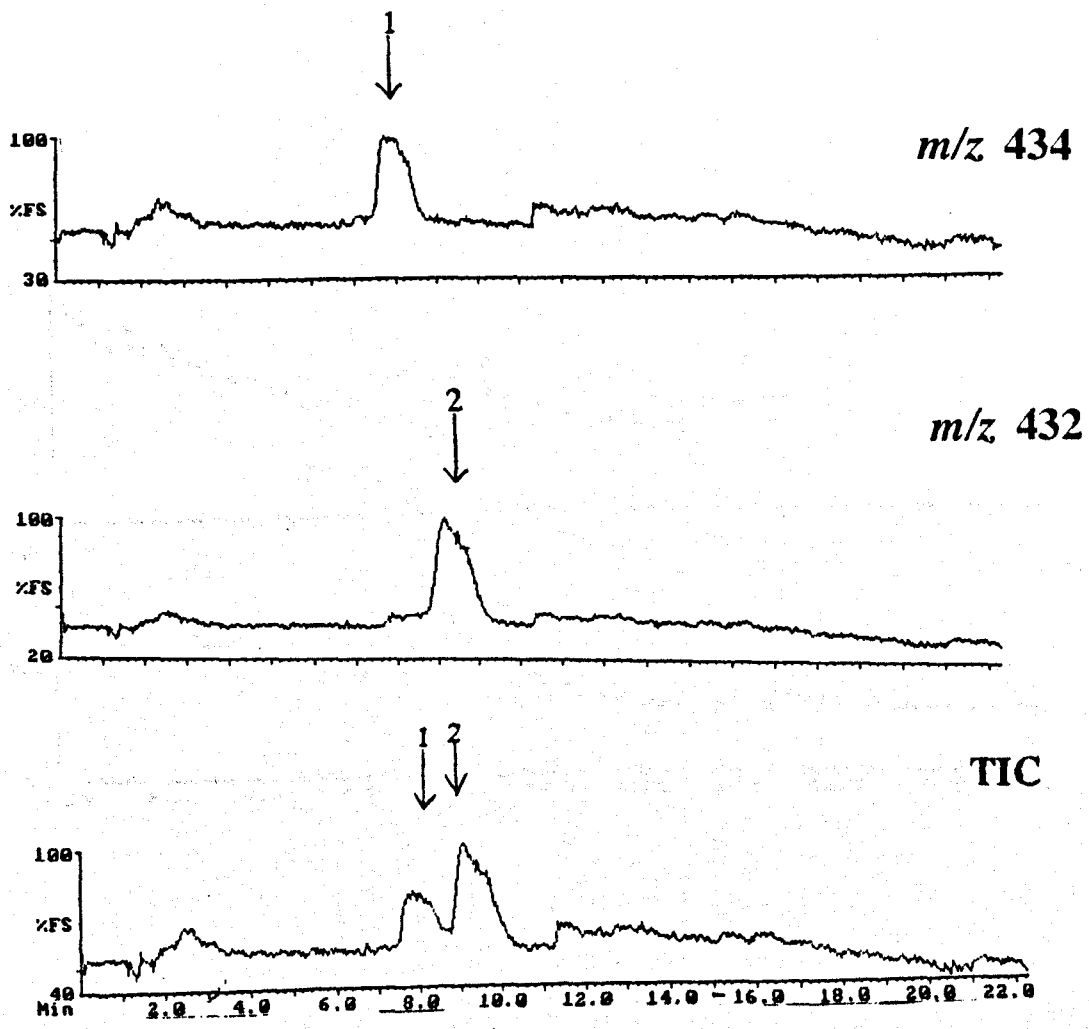


Fig. 7.12. TIC profile and *m/z* 434 and 432 mass chromatograms obtained from HPLC-TSP/MS (SIM) using solvent system B (see section 2.2.14) of 20ng each of 2,22,25-trideoxyecdysone (1) and 3-dehydro-2,22,25-trideoxyecdysone (2), respectively.

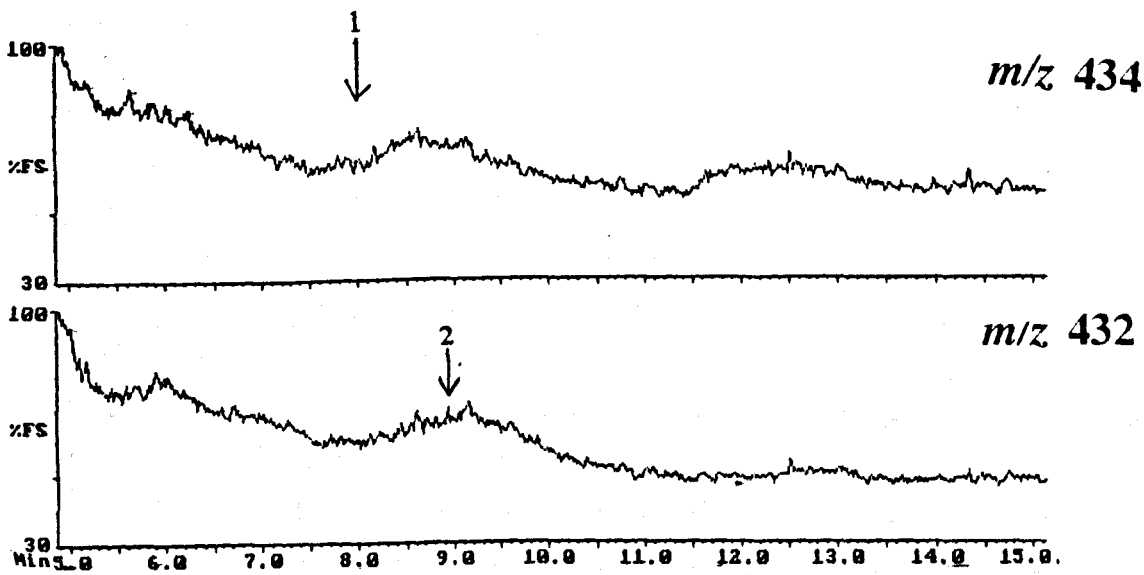


Fig. 7.13. Mass chromatograms for *m/z* 434 and 432 obtained from HPLC-TSP/MS (SIM) using solvent system B (see section 2.2.14) for fraction B from the ovarian follicle cell extract of *L. migratoria*. The positions of authentic 2,22,25-trideoxyecdysone (1) and 3-dehydro-2,22,25-trideoxyecdysone (2) are shown.

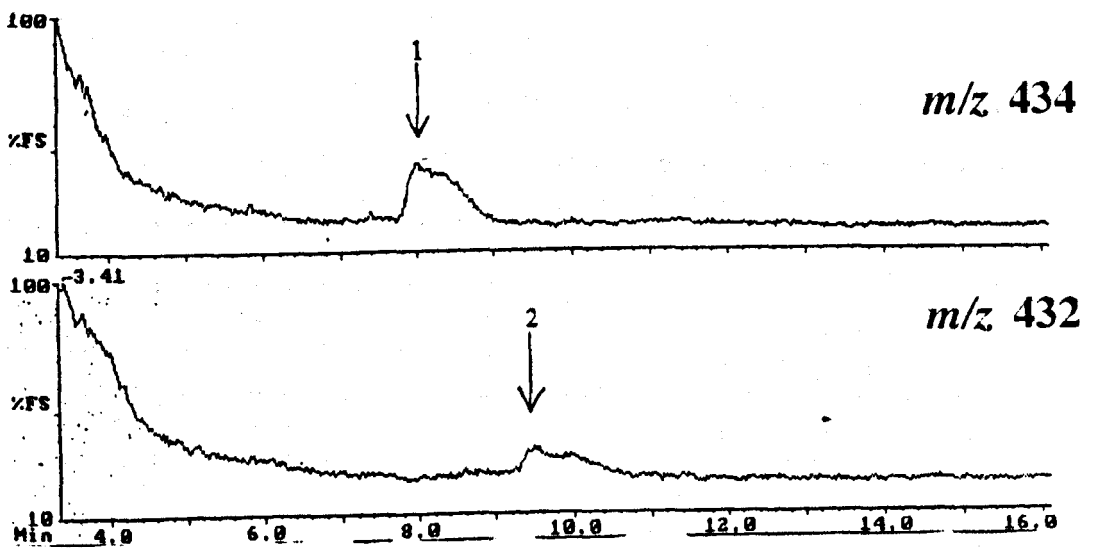


Fig. 7.14. Mass chromatograms for *m/z* 434 and 432 obtained from HPLC-TSP/MS (SIM) using solvent system B (see section 2.2.14) for fraction B from the ovarian follicle cell extract of *L. migratoria* to which 20ng each of 2,22,25-trideoxyecdysone (1) and 3-dehydro-2,22,25-trideoxyecdysone (2) have been added.

trideoxyecdysone in the *m/z* 432 chromatogram was much smaller and harder to discern from the base line.

7.3. Discussion

The high abundance low mass ion at *m/z* 171 which corresponded to the partially silylated side chain fragment of ecdysone, resulting from cleavage between the C-20 and C-22 positions was the only major ion species that could be identifed in the mass spectra of either the *syn* - or *anti*- methoxime isomers of the MO-TMS-ecdysone derivative. Morgan and Woodbridge (1974) have also reported the presence of this low mass ion in a previous mass spectral study of the MO-TMS-ecdysone derivative, where it again represented the most abundant ion species. The formation of methoxime isomers coupled to the difficulty in identifying high mass ions corresponding to fragments from the ecdysone nucleus in either of their respective mass spectra, suggests that this method of derivatisation would not be suitable for detecting 3β-hydroxy and 3-dehydro derivatives of the same ecdysteroid moiety by GC-MS analysis.

The mass spectra resulting from ES/MS analysis of the 3-dehydro and 3β-hydroxy derivatives of ecdysone and 2,22,25-trideoxyecdysone contained a high abundance of a number of high mass ions corresponding to the $[M]^+$, $[M+H]^+$ and $[M+23]^+$ ion species. In addition, TSP/MS analysis of 2,22,25-trideoxyecdysone and 3-dehydro-2,22,25-trideoxyecdysone revealed the presence of high mass ions corresponding to the ion species $[M]^+$, $[M+ H]^+$ and $[M+NH_4]^+$ in their mass spectra, the latter ion representing the preponderance of high mass ions formed. As high mass ions corresponding to whole steroid nuclear fragments are obtained from these ES and TSP mass spectrometry methods, 3-dehydro and 3β-hydroxy derivatives of the same ecdysteroid compound can be clearly distinguished by these methods of mass spectral analysis.

In order to detect the presence of endogenous 2,22,25-trideoxyecdysone and 3-dehydro-2,22,25-trideoxyecdysone in the follicle cells of *L. migratoria,* HPLC-TSP/MS in the selective ion monitoring mode was employed. This allowed the presence of the intense high mass ions corresponding to the $[M+NH_4]^+$ species for both 3-dehydro-2,22,25-trideoxyecdysone and 2,22,25-trideoxyecdysone at *m/z* 432 and 434, respectively, to be monitored in the follicle cell extract. However, the inability to detect these ions in fractions A and B and the 10% (v/v) methanol in chloroform fraction from the silicic acid column (Fig. 7.10; results not shown) from the biological extract, suggested the possible absence of both 3-dehydro-2,22,25-trideoxyecdysone and 2,22,25-trideoxyecdysone within the follicle cell sample. As a detection limit of 10ng for each of these moieties had been established from HPLC-TSP/MS (SIM) analysis of standard solutions, the possibility exists that the follicle cell sample obtained from approximately 400 ovaries contained 3-dehydro-2,22,25-trideoxyecdysone and 2,22,25-trideoxyecdysone, but each at a level below 10ng. However, the difficulty in detecting 20ng of 3-dehydro-2,22,25-trideoxyecdysone standard in the biological sample by HPLC-TSP/MS (SIM) suggests that the detection limit for this compound was above 10ng in the follicle cell extract. The inability to detect 2,22,25-trideoxyecdysone, which has been previously detected in the ovaries of *L. migratoria* by Hetru *et al.* (1978;1982), in the biological extract can be interpreted in terms of a lack of accumulation of ecdysteroid biosynthetic intermediates within the follicle cells. Therefore, the endogenous presence of 3-dehydro-2,22,25-trideoxyecdysone within follicle cells cannot at present be discounted.

In order to enhance the ability to detect endogenous 2,22,25-trideoxyecdysone and the putative intermediate 3-dehydro-2,22,25-trideoxyecdysone within the follicle cells of *L. migratoria,* larger samples of biological material should be employed, therefore providing a larger amount of such ecdysteroids for analysis. It would also be worth extracting whole ovaries as opposed to merely follicle cell preparations. In addition, the possibility of inhibiting ecdysteroid biosynthesis *in vivo* in *L. migratoria* by inhibitors such as ketoconazole, which would allow the accumulation of ecdysteroid pathway

intermediates such as 2,22,25-trideoxyecdysone (see Chapter 5), could also be considered as a means of increasing the amount of these ecdysteroid moieties present within the biological extract.

Chapter 8

Ecdysteroid synthetic activity of insect cell lines

8.1. Introduction

A number of insect lines have been recently demonstrated to produce ecdysteroids.These include the 1AL-TNDi Lepidopteran cell line derived from the imaginal discs of the Cabbage hopper, *Trichoplusia ni,* which has been shown by Lynn *et al.* (1987) to synthesize and accumulate 20-hydroxyecdysone. In addition, the UMBGE-4 cell line derived from the embryo of the German cockroach, *Blattela germanica* and the epithelial cell line derived from the embryo of *Chironomus tentans* have both been demonstrated to produce ecdysone under culture conditions (Ward *et al.*, 1987; Splindler, 1991). However, it is worth noting that the ability to synthesize ecdysteroids was not possessed by all insect cell lines investigated (Ward *et al.*, 1987). A further insight into ecdysteroid synthesis by insect cell lines has been gained by the use of radiolabelled ecdysteroid precursors. The UMBGE-4 cell line has been shown to metabolise radiolabelled 2,22,25-trideoxyecdysone and 2,22-dideoxyecdysone into a number of polar ecdysteroid conjugates. Radiolabelled 2-deoxyecdysone was converted into ecdysone, confirming the presence of 2-hydroxylase activity within the cells. In addition, radiolabelled ecdysone was converted into 20-hydroxyecdysone, 26-hydroxyecdysone and its respective polar and apolar conjugates, both of the latter also being formed from radiolabelled 20-hydroxyecdysone (Ward *et al.*, 1990). In contrast, the *Drosophila melanogaster* tumorous blood cell line could not be demonstrated to either convert radiolabelled ecdysone into 20-hydroxyecdysone, or further metabolise radiolabelled 20-hydroxyecdysone (Dinan, 1985). When the metabolism of radiolabelled ecdysone and 20-hydroxyecdysone was studied in the IAL-PID2 cell line derived from the imaginal wing discs of *Plodia interpunctella*, the latter ecdysteroid was slightly converted into mostly 20,26-dihydroxyecdysone, whereas ecdysone was rapidly converted into 20-hydroxyecdysone (Somme-Martin *et al.*, 1992).

The majority of the insect cell lines which have been shown to be capable of ecdysteroid synthesis are of epidermal origin (including the embryonic UMBGE-4 cells,

with their ability to synthesize chitin suggesting an epidermal origin), a tissue which has been demonstrated to have ecdysteroid synthetic activity in a number of insect species (Delachambre *et al.*, 1984; Delbecque *et al.*, 1986; Delbecque, 1990). Insect cell lines derived from other tissues capable of ecdysteroid synthetic activity may also be expected to retain the ecdysteroid biosynthetic activity of their parent tissue.

In this chapter the ecdysteroid biosynthetic activity of the SF21 cell line derived from the ovary of *S. frugiperda* (Vaughn *et al.*, 1977) and the Mb 1203 cell line derived from both the ovary and dorsal vessel of *M. brassicae* (Miltenburger and David, 1976) were investigated by both metabolic studies with [^{3}H]cholesterol and [^{3}H]3-dehydro-2,22,25-trideoxyecdysone, and measurement of their endogenous ecdysteroid biosynthesis by radioimmunoassay (RIA).

8.2. Experimental and results

8.2.1. The metabolism of [^{3}H]cholesterol and [^{3}H]3-dehydro-2,22,25-trideoxyecdysone by the SF21 and Mb1203 cell lines

By investigating the metabolism of the radiolabelled forms of the distal precursors of ecdysteroid synthesis, cholesterol and the putative ecdysteroid pathway intermediate, 3-dehydro-2,22,25-trideoxyecdysone by the SF21 and Mb1203 insect cells, the ecdysteroid synthetic activities of these cell lines were evaluated.

Cell numbers were determined on a precalibrated model ZBi Coulter counter (Coulter Electronics Ltd., Luton, Bedfordshire, England) to allow a final cell density of 10^6/ml of medium (TC100 medium containing 10% (v/v) foetal calf serum)/ well of a 24-well cell culture plate (Costar). [^{3}H]Cholesterol (40µCi) and [^{3}H]3-dehydro-2,22,25-trideoxyecdysone (40µCi) were evaporated under nitrogen (N_2) in separate silanized 0.5 dram vials and redissolved in DMSO (20µl). The radiolabelled substrates (10µCi) were then introduced in DMSO (5µl) to the culture plate wells containing the Mb1203

and the Sf21 cells (one well per cell line), in addition to wells containing medium only. The culture plates were then incubated for 24h at 29°C. After this time the medium and cells were withdrawn and subjected to low speed centrifugation (600g for 5min). The resulting cell pellet and medium were extracted separately. Ice cold ethanol (2ml) was added to the medium, the precipitated protein was then sedimented by low speed centrifugation as before, and the resulting supernatant was removed and evaporated to dryness under N_2. The cell pellet was extracted 3 times in methanol (2ml) and once in hexane (2ml) in a hand-held ground glass homogeniser. The resulting cell pellet and media extracts were then analysed by RP-HPLC (system 1, see section 2.2.8) with co-chromatography of authentic ecdysteroid standards. No metabolism of [^{3}H]cholesterol or [^{3}H]3-dehydro-2,22,25-trideoxyecdysone was observed in the absence of the insect cells.

Table 8.1 shows the total amount of recovered radioactivity (μCi) from the media and cell pellet extracts of the Mb1203 and the Sf21 cell line cultures after incubation with [^{3}H]cholesterol and [^{3}H]3-dehydro-2,22,25-trideoxyecdysone.

RP-HPLC (system 1, see section 2.2.8) analysis of the media and cell pellet extracts from incubation of [^{3}H]cholesterol with the Sf21 and Mb1203 insect cell lines showed no conversion of this radiolabel into ecdysteroids (results not shown), with all the recovered radioactivity from these extracts being in the form of [^{3}H]cholesterol.

Fig. 8.1 shows the RP-HPLC (system 1, see section 2.2.8) radiochromatograms for extracts obtained from the Sf21 cell cultures after a 24h incubation at 29°C with [^{3}H]3-dehydro-2,22,25-trideoxyecdysone

The medium from the Sf21 cell line incubation (Fig.8.1a) contained metabolites which co-eluted with both 22-deoxyecdysone and 2,22-dideoxyecdysone on RP-HPLC (system 1, see section 2.2.8), and represented 8.9% and 17.4% of the total recovered radioactivity from the RP-HPLC system, respectively. In addition to the unmetabolised 2,22,25-trideoxyecdysone (4% of total recovered radioactivity) a fourth unidentified metabolite was also observed, eluting over a 9 min period in the polar region of the radiochromatogram, from 11.0min to 20min (54.7% of the total recovered radioactivity).

(a) [^{3}H]Cholesterol incubation

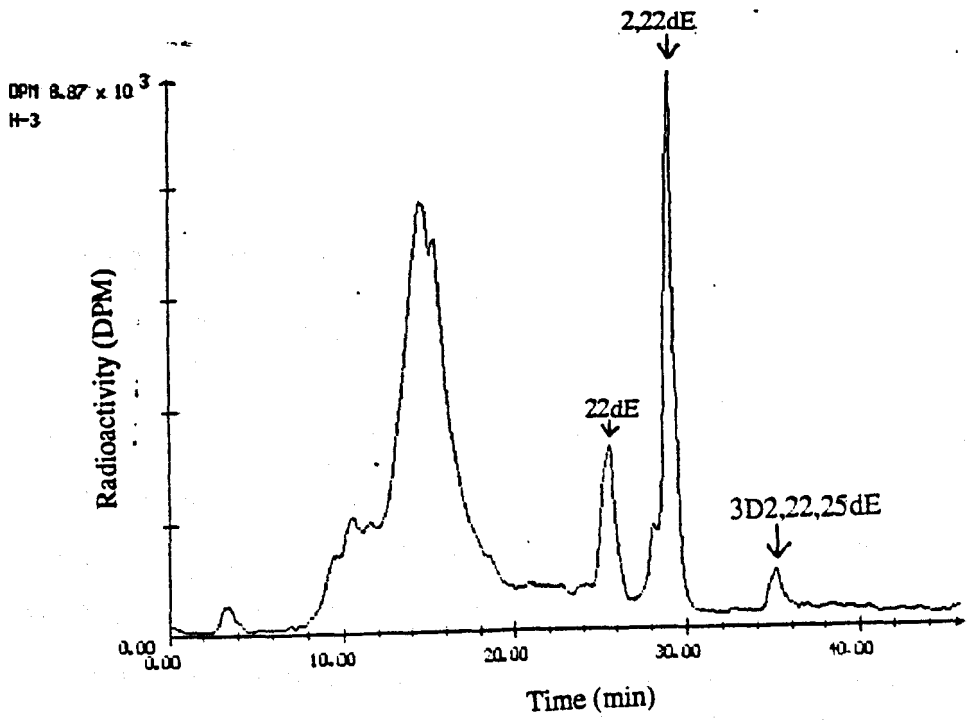
	Radioactivity recovered (μCi)	
Sample Extracts	*Medium*	*Cell pellet*
Insect cell lines		
Mb1203	**6.2**	**0.9**
Sf21	**7.8**	**0.8**

(b) [^{3}H]3-Dhydro-2,22,25-trideoxyecdysone incubation

	Radioactivity recovered (μCi)	
Sample Extracts	*Medium*	*Cell pellet*
Insect cell lines		
Mb1203	**7.2**	**1.4**
Sf21	**6.3**	**1.6**

Table 8.1. Recovered radioactivity (μCi) in the medium and cell pellet extracts obtained from the Mb1203 and Sf21 cell line cultures after incubation with (a) [^{3}H]cholesterol and (b) [^{3}H]3-dehydro-2,22,25-trideoxyecdysone.

(a) Medium extract from Sf21 cell culture



(b) Cell extract from Sf21 cell culture

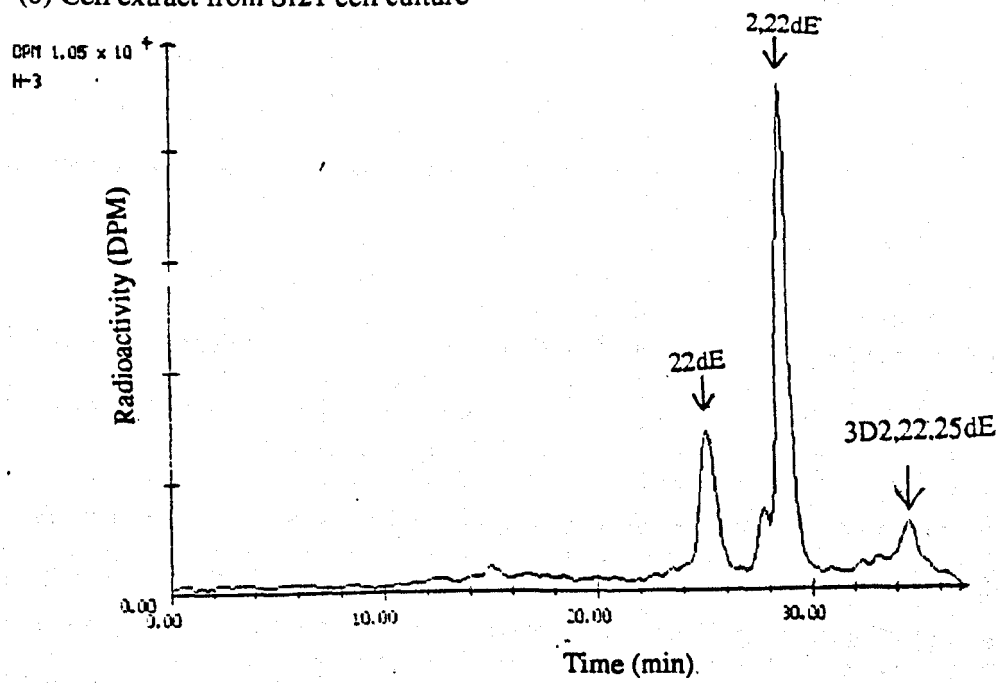


Fig. 8.1. RP-HPLC (system 1, section 2.2.8.) radiochromatograms of the (a) medium and (b) cell pellet extracts obtained from Sf21 cell cultures after a 24h. incubation at 29°C with [^{3}H]3-dehydro-2,22,25-trideoxy-3-dehydroecdysone. The positions of authentic standards are shown: 3-dehydro-2,22,25-trideoxyecdysone (3D2,22,25dE), 2,22-dideoxyecdysone (2,22dE) and 22-deoxyecdysone (22dE).

The cell pellet from the Sf21 cell line incubation (Fig. 8.1b) contained metabolites which co-eluted with 22-deoxyecdysone (28.3% of total recovered radioactivity) and 2,22-dideoxyecdysone (52.7% of total) on RP-HPLC (system 2), in addition to the unmetabolised 3-dehydro-2,22,25-trideoxyecdysone (19.0% of total).

Fig. 8.2 shows the RP-HPLC radiochromatogram obtained from extracts of the Mb 1203 cell culture after incubation with [^{3}H]3-dehydro-2,22,25-trideoxyecdysone.

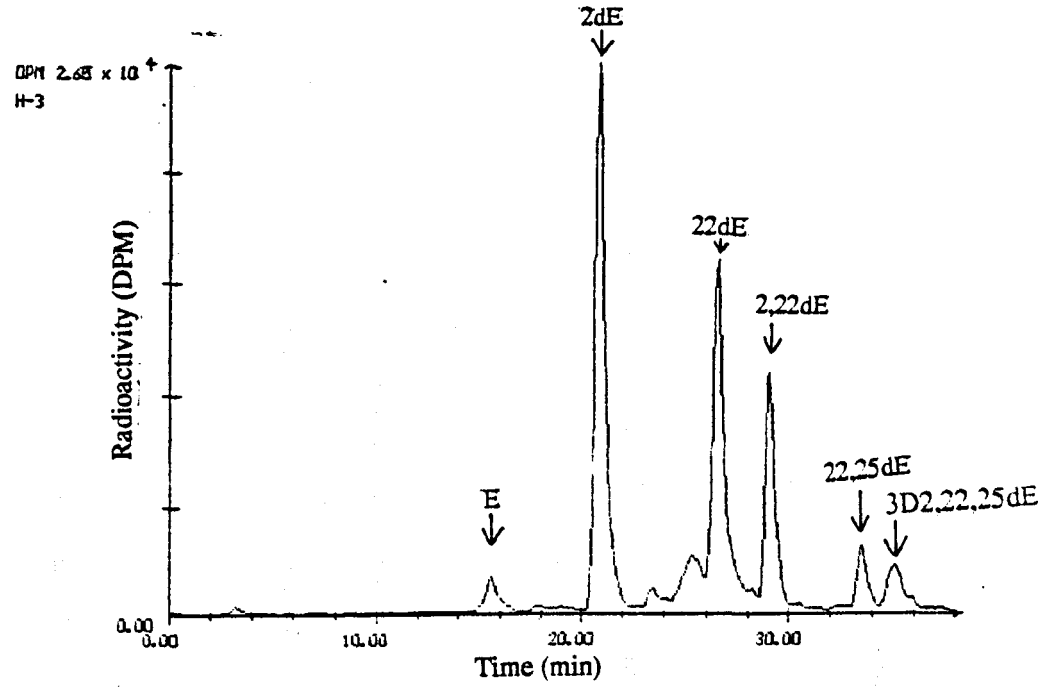
The medium from the Mb1203 cell culture (Fig. 8.3a) contained metabolites which co-eluted with ecdysone (6.4% of the total recovered ecdysteroid from RP-HPLC system 1, see section 2.2.8), in addition to 2-deoxyecdysone (46.2%), 22-deoxyecdysone (31.3%), 2,22-dideoxyecdysone (19.7%), 22,25-dideoxyecdysone (8.6%) and 3-dehydro-2,22,25-trideoxyecdysone (7.2%) on RP-HPLC (system1). Co-chromatography of these metabolites with authentic ecdysteroid standards was also observed on adsorption HPLC (systems 8 and 9, see section 2.2.8).

The cell pellet from the Mb1203 cell line incubation contained metabolites which co-eluted with 2-deoxyecdysone (8.4% of total ecdysteroid recovered), 22-deoxyecdysone (21.2%), 2,22-dideoxyecdysone (35.8%), 22,25-dideoxyecdysone (17.7%) and 3-dehydro-2,22,25-trideoxyecdysone(16.9) on RP-HPLC (system 1). Again co-chromatography of these metabolites with authentic ecdysteroid standards was observed on adsorption HPLC.

8.2.2. Further RP-HPLC analysis of the ecdysteroid metabolites synthesized from [^{3}H]3-dehydro-2,22,25-trideoxyecdysone by the Sf21 and Mb1203 cell lines

Previous metabolic studies of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone with the prothoracic glands of *L. migratoria*, have revealed the ability of these tissues to convert this radiolabel into a number of 3β-hydroxyecdysteroids and 3-dehydroecdysteroids (see Chapter 6). The 3β-hydroxy and 3-dehydro derivatives of the same ecdysteroid compound have been found to co-elute on both the RP-HPLC methanol/water solvent

(a) Medium extract from Mb1203 cell culture



(b) Cell extract from Mb1203 cell culture

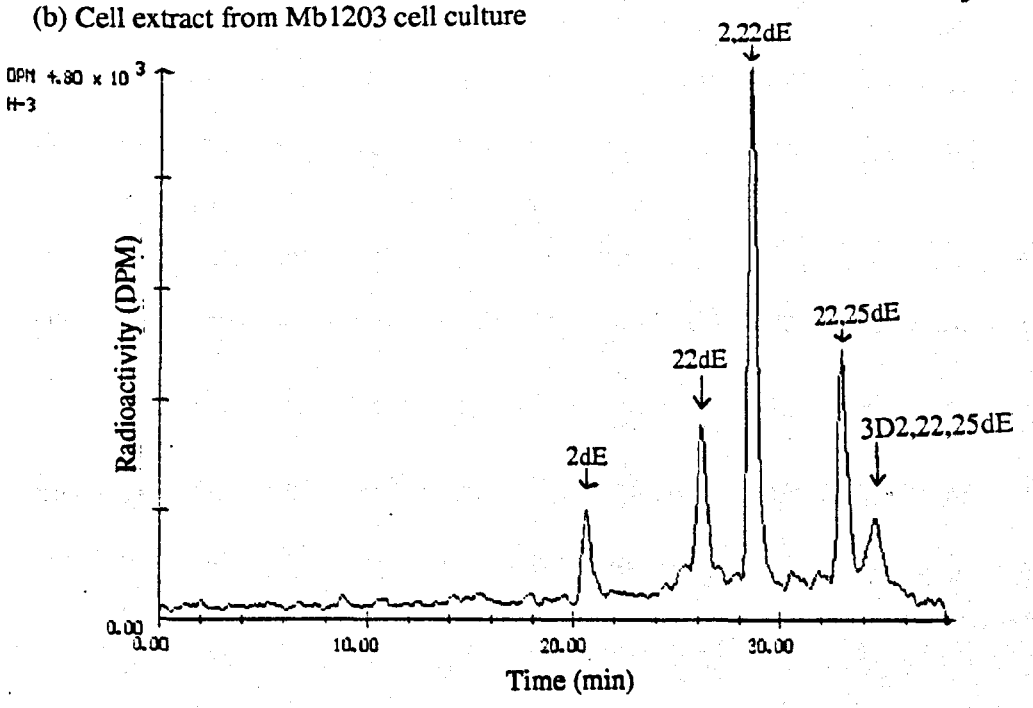


Fig. 8.2. RP-HPLC (system 1, section 2.2.8) radiochromatograms of the (a) medium and (b) cell pellet extracts obtained from the Mb1203 cell cultures after a 24h incubation at 29°C with [^{3}H]3-dehydro-2,22,25-trideoxyecdysone. The positions of authentic standards are shown: 3-dehydro-2,22,25-trideoxyecdysone (3D2,22,25dE), 2,22-dideoxyecdysone (2,22dE), 22-deoxyecdysone (22dE), 2-deoxyecdysone (2dE) and ecdysone (E).

system 1 (see section 2.2.8) and the adsorption HPLC systems (8 and 9) employed in the analysis of the ecdysteroid metabolites formed from the radiolabel by the Sf21 and Mb1203 cell lines. Therefore, by the use of the RP-HPLC acetonitrile/water solvent systems devised by Roussel, (1992a) which have been demonstrated to resolve the 3β-hydroxy and 3-dehydro derivatives of the same ecdysteroid moiety, the ecdysteroid metabolites synthesized from [^{3}H]3-dehydro-2,22,25-trideoxyecdysone by the Sf21 and Mb1203 cells will be further analysed.

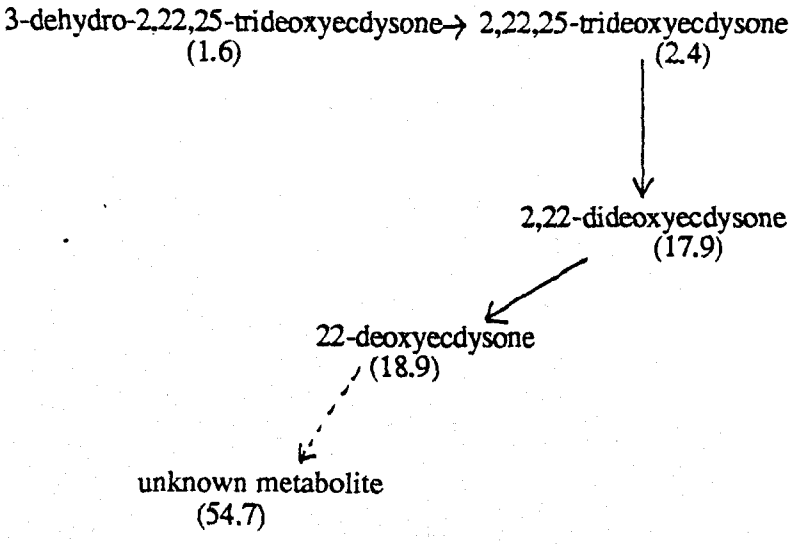
The ecdysteroid moieties present in both the media and cell pellet extracts were separated and collected from RP-HPLC (system 1; see section 2.2.8) and analysed on RP-HPLC systems 3,4,5,6 and 7 together with co-injection of authentic standards. Fig. 8.3 shows the metabolism products of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone in both the medium and cell pellets from the Sf21 cell cultures after incubation for 24h at 29^{o}C. As can be seen in Fig. 8.3a, the medium from the Sf21 cell culture contained both 3-dehydro-2,22,25-trideoxyecdysone (1.6% of total recovered radioactivity from RP-HPLC system 1) and 2,22,25-trideoxyecdysone (2.4%), in addition to the 3β-hydroxy derivatives of 2,22-dideoxyecdysone (17.4%) and 22-deoxyecdysone (8.9%) only. As the unidentified metabolite (54.7%) was found to elute in the polar region of the radiochromatogram (see Fig. 8.1) which corresponds to 20-hydroxyecdysone, ecdysone, 2-deoxy-20-hydroxyecdysone and 2-deoxyecdysone, this metabolite was further analysed on RP-HPLC systems 3 and 4 (which are used for the analysis of these ecdysteroids). However, co-elution of this material with authentic standards was not observed.

The cell pellet from the Sf21 cell culture contained both 3-dehydro-2,22,25-trideoxyecdysone (4.4%) and 2,22,25-trideoxyecdysone (14.6%), in addition to 2,22-dideoxyecdysone (52.7%) and 22-deoxyecdysone(28.3%).

Fig. 8.4 shows the metabolic products of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone in the medium and cell pellet from the Mb1203 cell culture.

The medium from the Mb1203 cell culture (Fig. 8.4a) contained both 3-dehydro-2,22,25-trideoxyecdysone (4.0% of the total recovered ecdysteroids from RP-HPLC

(a) Medium



(b) Cell pellet

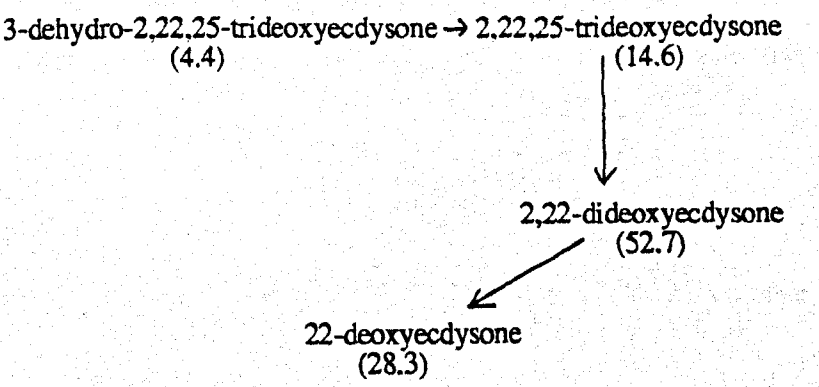
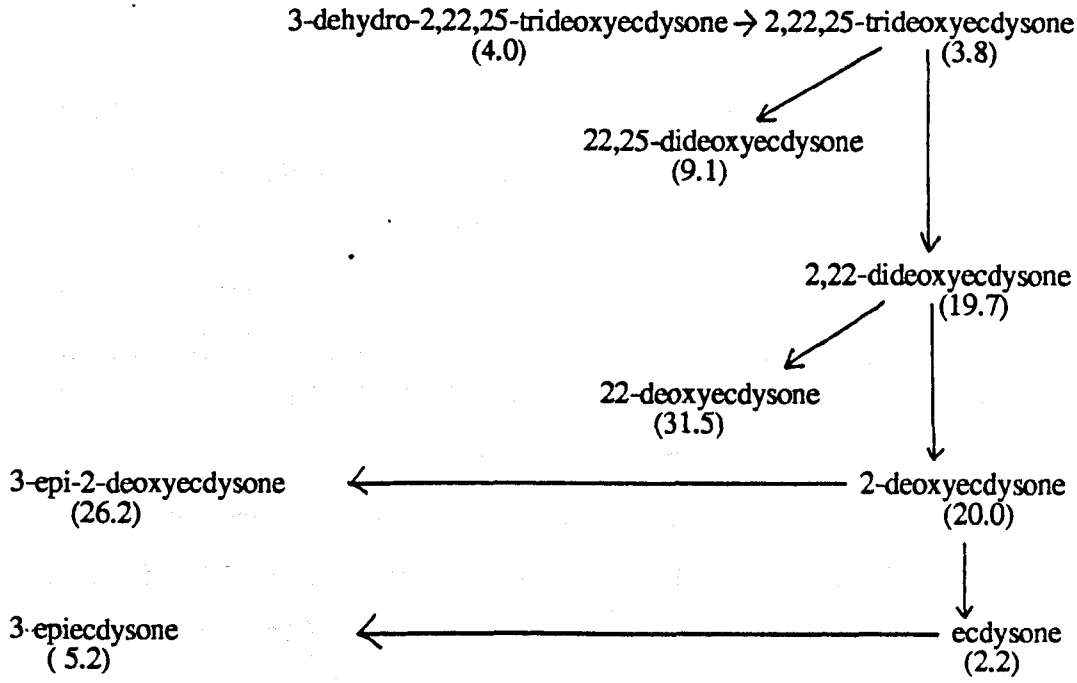


Fig. 8.3. The metabolic products of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone in both the (a) medium and (b) cell pellet extracts from the Sf21 cell culture. The incorporation into each compound (parenthesis) is expressed as a percentage of the total recovered radioactivity from RP-HPLC (system 1, see section 2.2.8).

(a) Medium



(b) Cell -pellet

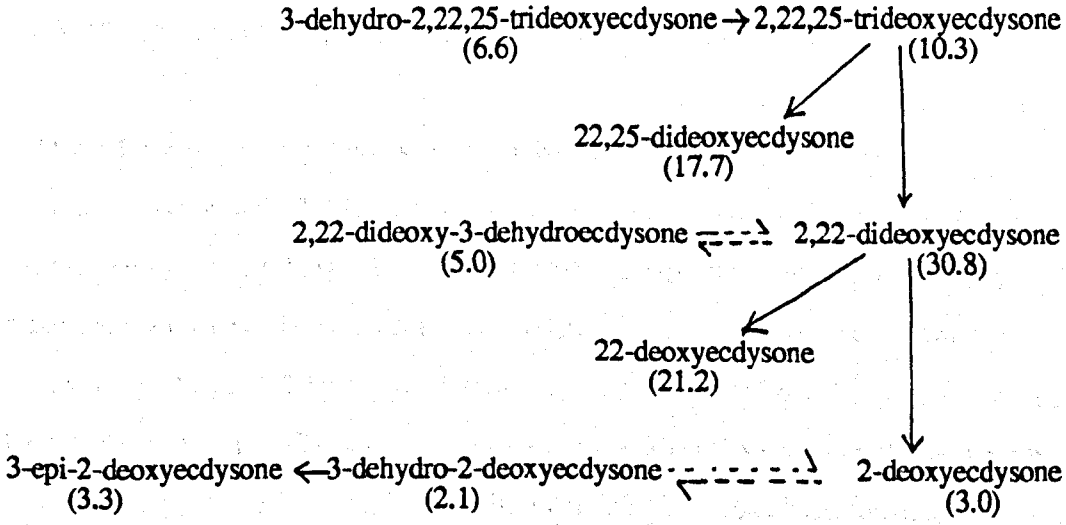


Fig. 8.4. The metabolic products of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone in both the (a) medium and (b) cell pellet extracts from the the Mb1203 cell culture. The incorporation into each compound (parenthesis) is expressed as a percentage of the total recovered ecdysteroids from RP-HPLC (system 1, see section 2.2.8).

system 1) and its 3β-hydroxy derivative, 2,22,25-trideoxyecdysone (3.8%). However, as no 22,25-dideoxy-3-dehydroecdysone standard was available, further analysis of the 22,25-dideoxyecdysone region (9.1%) was not undertaken. At the 2,22-dideoxyecdysone and the 22-deoxyecdysone level of hydroxylation, only the 3β-hydroxy derivatives of these compounds could be identified, representing 19.7% and 31.5%, respectively of the total recovered ecdysteroid. In contrast the 3β-hydroxy and the 3-epiecdysteroid derivatives of 2-deoxyecdysone and ecdysone could be identified, representing 20.0%, 26.2%, 2.2% and 5.2% respectively, of the total recovered ecdysteroid.

The cell pellet from the MB1203 cell culture (Fig. 8.4b) contained the 3β-hydroxy and the 3-dehydroecdysteroid derivatives of both 2,22,25-trideoxyecdysone and 2,22-dideoxyecdysone, representing 6.6%, 10.3%, 5.0% and 30.8% of the total recovered ecdysteroid from RP-HPLC system 1. In addition to 22-deoxyecdysone (21.2%), the 3-dehydro, 3β-hydroxy and 3-dehydroecdysteroid derivatives of 2-deoxyecdysone could also be identified, representing 2.1%, 3.0% and 3.3%, respectively, of the total recovered ecdysteroid. However, again further analysis of the 22,25-dideoxyecdysone level of hydroxylation could not be performed.

8.2.3. Metabolism of [^{3}H]2-deoxyecdysone by the Mb1203 cell line.

Overall, the major ecdysteroids products from [^{3}H]3-dehydro-2,22,25-trideoxyecdysone in the Mb1203 cells were 2-deoxyecdysteroids (2-deoxyecdysone and 3-epi-2-deoxyecdysone). Therefore, the possibility exists that these compounds may represent the major end products of the ecdysteroid biosynthetic pathway in this cell line, with synthesis of 2-hydroxyecdysteroids being at a much lower level. Examining the metabolism of [^{3}H]2-deoxyecdysone by the Mb1203 cells will allow a closer study of the final stages of ecdysteroid synthesis, in addition to further assessment of the ecdysone synthetic activity of the cell line.

Incubation of the Mb1203 cells with [^{3}H]2-deoxyecdysone (4μCi) and extraction of the medium and cell pellet were as described in section 8.2.1.The resulting medium and cell pellet extracts were analysed by RP-HPLC (systems 1, 3 and 4; see section 2.2.8), together with authentic ecdysteroid standards. A total of 2.9μCi and 0.03μCi of radioactivity were recovered from the medium and cell pellet extracts, respectively.

RP-HPLC (system 1, see section 2.2.8) analysis of the medium from the Mb1203 cell line incubation (Fig. 8.5), revealed the presence of two peaks, which co-chromatographed with ecdysone and 2-deoxyecdysone, respectively. The former ecdysteroid accounted for 67.1% of the total recovered ecdysteroid, in comparison to the unmetabolised 2-deoxyecdysone substrate which accounted for 37.1% of the total. RP-HPLC analysis of the medium extract on systems 3 and 4 revealed only the presence of the 3β-hydroxyecdysteroid derivatives of ecdysone and 2-deoxyecdysone. On RP-HPLC (system 1 and 3) analysis of the Mb1203 cell pellet, unmetabolised 2-deoxyecdysone was the only ecdysteroid moiety present (results not shown).

8.2.4. Measurement of the endogenous ecdysteroid synthesis by both the Sf21 and Mb1203 cell lines

As seen in section 8.2.1 both the Sf21 and Mb1203 cell lines have been demonstrated to metabolise the putative radiolabelled ecdysteroid precursor, [^{3}H]2,22,25-trideoxy-3-dehydroecdysone into a number of ecdysteroids metabolites. However, the ability of these insect cells to synthesize ecdysteroids endogenously has yet to be confirmed. Therefore, by the use of radioimmunoassay (RIA), using the DHS-1-13.5 antiserum, the ecdysteroid synthetic activity of these two insect cell lines was investigated.

The Sf21 and Mb1203 cells were seeded at a density of 10^6/ml in 1ml of TC100 medium containing 10% (v/v) foetal calf serum into two 9cm Petri dishes (2 for each cell line). The volume was then made up to 5ml, and the Petri dishes incubated along with two others containing medium only for one week at 29^{o}C. After this time, the cells

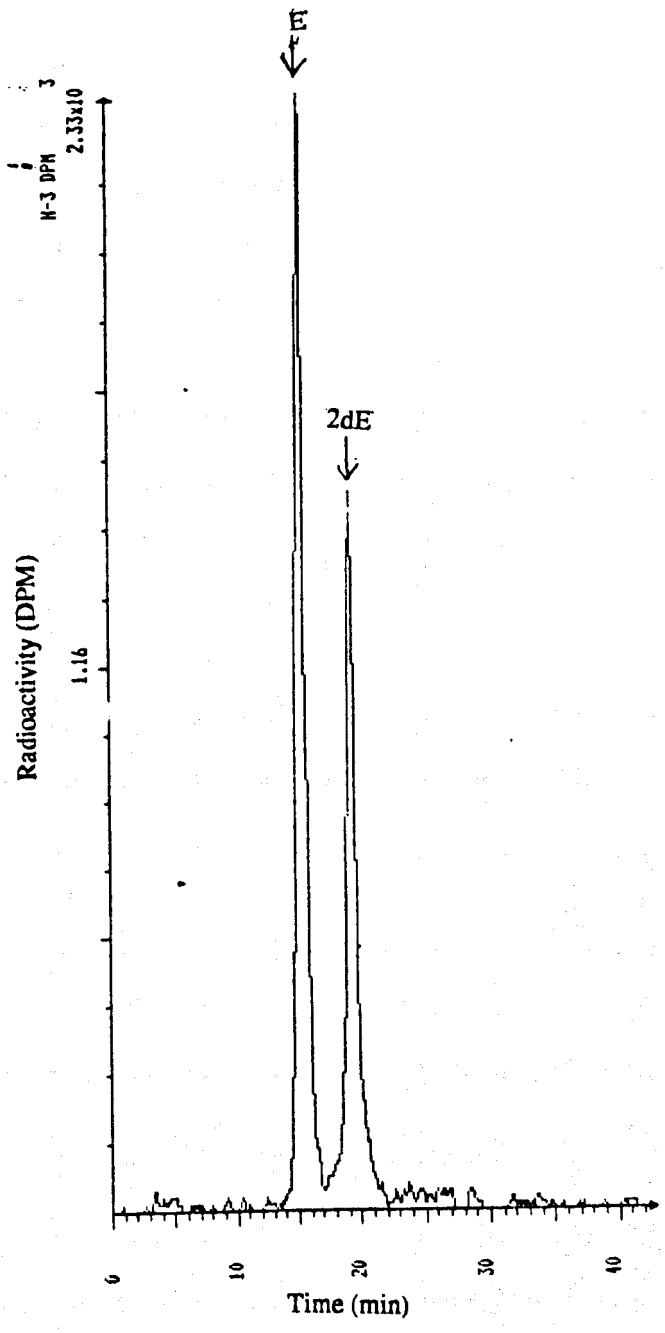


Fig. 8.5. RP-HPLC (system 1, section 2.2.8.) radiochromatogram of the medium extract from the Mb1203 cell culture after a 24h incubation at 29°C with [^{3}H]2 deoxyecdysone. The positions of authentic standards are shown: 2-deoxyecdysone (2dE) and ecdysone (E).

and medium from each plate were removed and subjected to low spin centrifugation (600g for 5min), the cell pellets were discarded and to the resulting supernatant (medium) ice-cold ethanol (5ml) was then added. The samples were again subjected to low spin centrifugation to sediment the precipitated protein. The supernatant was then evaporated to dryness under N_2 before being subjected to silicic acid column fractionation (for procedure see section 2.2.6), eluting with chloroform, 30% (v/v) methanol in chloroform, and methanol. The methanol fraction (polar ecdysteroids) was subjected to *Helix* hydrolase treatment (for method see section 2.2.15). Following enzymic treatment, this fraction, in addition to the chloroform and 30% (v/v) methanol in chloroform fraction (free ecdysteroids), was subjected to C_{18} Sep-Pak fractionation (procedure 2, see section 2.2.7). The methanol fractions were analysed by RIA using the DHS-1-13.5 antiserum (see section 2.2.17).

In the control media samples that had not been exposed to the the Mb1203 and the Sf21 insect cells, no ecdysteroid immunoreactivity was detectable in the post-silicic acid column solvent fractions.

Table 8.2 shows the distribution of detectable ecdysone immunoreactivity present in the chloroform fractions from media extracts. The results are expressed as ng ecdysone equivalents.

As can be seen from Table 8.2 no ecdysone immunoreactivity was detected in the chloroform fraction from either of the media extracts from the cell line cultures. However, 10ng and 5.5ng ecdysone equivalents were detected in the free ecdysteroid fraction (30% (v/v) methanol in chloroform) from both the media extracts from Mb1203 and Sf21 cell cultures, respectively. In addition, ecdysone immunoreactivity was also detectable in the hydrolysed polar ecdysteroid fraction (methanol), with 4.6ng and 8.3ng ecdysone equivalents being detected in the the media extracts from the Mb1203 and Sf21 cell line cultures, respectively.

Insect cell lines	Ecdysone equivalents (ng)		
	Silicic acid column fractions (*% methanol in chloroform)*		
	0	*30*	*100 (after enzymic hydrolysis)*
Mb1203	0	10.0	4.6
Sf21	0	5.5	8.3

Table 8.2. Distribution of ecdysone immunoreactivity detected in the silicic acid column fractions from the media extracts from the Sf21 and Mb1203 cell cultures after incubation for 1 week at 29°C. The results are expressed as ng ecdysone equivalents. The same volumes of initial media contained no detectable ecdysteroid immunoreactivity.

8.3. Discussion

The results from the incubation studies of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone with the Mb1203 and Sf21 cells clearly show that both these cell lines have the ability to convert this radiolabel efficiently into a number of ecdysteroid compounds. Although the synthesis of 22-deoxyecdysone and 2,22-dideoxyecdysone were common to both cell lines, being present in both the media and cell pellet extracts from the cell cultures, the final products of synthesis from [^{3}H]3-dehydro-2,22,25-trideoxyecdysone were found to differ greatly. In the case of the Mb1203 cell line, the final products of synthesis were identified as 2-deoxyecdysone, 3-epi-2-deoxyecdysone, ecdysone and 3-epiecdysone, with the ecdysteroids present at the 2-deoxyecdysone level of hydroxylation representing the preponderance of ecdysteroid synthesis from the radiolabel (46.2%). In contrast, the metabolism of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone by the Sf21 cell line resulted in the production of an unidentifiable metabolite, which eluted in the polar region of the radiochromatogram (the 20-hydroxyecdysone, ecdysone and 2-deoxy-20-hydroxyecdysone region; Fig. 8.1), and therefore, the possibility exists that it may represent a modified form of one of these ecdysteroid compounds. However, the width of the eluting peak corresponding to this material, suggests that it may consist of more than one metabolite, which are not resolved under the HPLC conditions employed. Therefore, further HPLC (RP- and adsorption-phase) analysis and characterisation would be required before the identity of this/these metabolite(s) could be further elucidated. In previous incubation studies with the ovary tissue from *S. littoralis*, a member of the same genus as *S. frugiperda*, [^{3}H]2,22,25-trideoxyecdysone (3β-hydroxy derivative of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone substrate) was converted into a number of ecdysteroids including, ecdysone, 2-deoxyecdysone and 2,22-dideoxyecdysone in addition to a number of polar ecdysteroid moieties (Jarvis, 1991). The differences in metabolism of the radiolabelled substrates displayed by the Sf21 cells (which are derived from the ovary of *S.*

frugiperda) and the ovary tissue of *S. littoralis* may reflect differences in the ecdysteroid synthetic activity between the cell line and its tissue of origin.

Although there is evidence that [^{3}H]cholesterol is taken up by the insect cells (Table 8.1), with 0.9μCi and 0.8μCi being associated with the Mb1203 and Sf21 cell pellets, respectively, the ability of these insect cells to incorporate this radiolabel into ecdysteroids could not be demonstrated. Ward *et al.* (1990) has also reported the inability of the UMBGE-4 cell line derived from *B. germanica* embryos to incorporate [^{3}H]cholsterol into ecdysteroids, with the cholesterol pool size of the medium (from foetal calf serum) probably contributing to the lack of incorporation of the radiolabel.

The low rate of synthesis of ecdysteroids present at the ecdysone level of hydroxylation from [^{3}H]3-dehydro-2,22,25-trideoxyecdysone (7.4% of total ecdysteroid synthesized) in comparison to the 2-deoxyecdysone level of hydroxylation (2-deoxyecdysone and 3-epi-2-deoxyecdysone ; 46.2%), suggested that the latter metabolites represented the major products of the ecdysteroid synthetic pathway of the Mb1203 cell line. However, the ecdysone synthetic activity of the Mb1203 cell line was confirmed by the ability of the respective cells to show considerable incorporation of [^{3}H]2-deoxyecdysone into ecdysone (62.9%). A low ecdysone synthetic activity has been demonstrated for the UMBGE-4 cockroach cell, with no detectable incorporation of either [^{3}H]2,22,25-trideoxyecdysone or [^{3}H]2,22-dideoxyecdysone into ecdysone being demonstrated. However, in the presence of [^{3}H]2-deoxyecdysone a low rate of ecdysone synthesis could be shown (Ward *et al.*, 1990).

The ability of the Sf21 cells to convert [^{3}H]3-dehydro-2,22,25-trideoxyecdysone into 2,22-dideoxyecdysone and 22-deoxyecdysone suggests possible 25- and 2-hydroxylase activity associated with this cell line. In addition, the initial reduction of 3-dehydro-2,22,25-trideoxyecdysone into 2,22,25-trideoxyecdysone suggests the presence of 3-dehydroecdysteroid 3β−reductase activity within these insect cells. Mb1203 cells could convert [^{3}H]3-dehydro-2,22,25-trideoxyecdysone into 22,25-dideoxyecdysone, 2,22-dideoxyecdysone, 22-deoxyecdysone, 2-deoxyecdysone and ecdysone, therefore suggesting the presence of 25-, 22- and 2-hydroxylase activity within the cells. The

synthesis of mainly 3-epiecdysteroids and 3β-hydroxyecdysteroids from [^{3}H]3-dehydro-2,22,25-trideoxyecdysone by the Mb1203 cells, suggests that these insect cells have the ability to reduce the carbonyl (keto) group at the C-3 position in the radiolabel in both the α- and β- configuration, and therefore, implies the presence of both 3-dehydroecdysteroid 3β- and 3α-reductases within these cells. These enzymes are commonly found in the midgut of a number of Lepidopteran insects (Blais and Lafont, 1984; Milner *et al.*, 1985; Gelman *et al.*, 1991), and metabolic studies with [^{3}H]3-dehydro-2,22,25-trideoxyecdysone using the ovarian follicle cells of *L. migratoria* have also suggested the presence of these enzymes within the follicle cells (see Chapter 6). However, the ability of the Mb1203 and Sf21 cell lines to convert putative labelled intermediates into ecdysteroids does not necessarily demonstrate *de novo* ecdysteroid synthesis, as some ecdysteroid hydroxylases have been shown to occur in non-ecdysteroid synthetic tissue such as fat body and Malpighian tubules (Meister *et al.*, 1987). Therefore, it is necessary to demonstrate formation of such ecdysteroid intermediates from cholesterol before the ecdysteroid synthetic activity of the insect cell lines can be confirmed.

Analysis of the endogenous ecdysteroid synthetic products formed by the Sf21 and Mb1203 cell lines revealed the presence of ecdysone immunoreactivity in both the free ecdysteroid fraction [30% (v/v) methanol in chloroform silicic acid column fraction] and the fraction containing ecdysteroids released from polar conjugation (methanol) for each cell type. However, the ratio of ecdysteroids that were originally free to those that were in polar conjugation differed between the two cell lines, being higher for the Mb1203 cells. Examination of the endogenous ecdysteroid content of the ovaries from a number of Lepidopteran insects has revealed the presence of both free and polar ecdysteroid conjugates within these tissues, with the ratio of free ecdysteroid to polar ecdysteroid conjugate varying amongst the different insect species (Watanabe and Ohnishi, 1984; Hoggard, 1989; Jarvis, 1991). Therefore, the ability of the Sf21 and Mb1203 to synthesize both free and polar ecdysteroid conjugates may be a reflection of the ovarian origin of both these cell lines. However, before further conclusions can be drawn, a

further qualitative study would be required, using HPLC-RIA to determine the actual endogenous ecdysteroid profile for each of the insect cell lines.

In conclusion, it can be seen that both the Mb1203 and Sf21 cells possess some ecdysteroid hydroxylase activites, being able to metabolise [^{3}H]3-dehydro-2,22,25-trideoxyecdysone into a range of ecdysteroid derivatives, but in the case of the Sf21 cells, some of the more polar products have not yet been identified. In addition, endogenous ecdysteroid synthetic activity has been demonstrated in both cell lines. Therefore, these initial investigations, along with those of Lynn *et al.* (1987), Ward *et al.* (1990) and Splindler (1991), provide further evidence for the possible use of continuous insect cell lines as tools in the study of the biosynthesis and release of ecdysteroids at the cellular level.

Chapter 9

General Discussion

General discussion

The initial aim of this project was to investigate aspects of control and inhibition of ecdysteroid biosynthesis. Primary regulation of the ecdysteroid biosynthetic pathway would be expected to be exerted by the neuropeptides, PTTH and EDNH in larval and mature females, respectively (see section 1.6). Studies on *L. migratoria* ovarian follicle cells and prothoracic glands have suggested that in addition to this external control, an internal regulatory mechanism is operating within the pathway (Chapter 6). Incorporation of [^{3}H]2,22,25-trideoxyecdysone into follicle cells in the presence of the ecdysteroid phosphates has implicated the 22-hydroxylase as a site of feedback control in the terminal stages of the ecdysteroid biosynthetic pathway. As the major site of control would be expected in the early stages of the pathway, the presence of a late control point is unusual, although not unprecendented, as previous studies on *M. sexta* have also suggested that the 22-hydroxylase is the rate-limiting enzyme in the terminal stages of the pathway (Thompson *et al.*, 1981). In contrast to the follicle cells, no late control was detected in the ecdysteroid biosynthetic pathway of prothoracic glands of fifth instar *L. migratoria*. This result is in agreement with the suggested lack of developmental regulation of the terminal hydroxylases during the fifth instar of *M. sexta* (Grieneisen, 1994). Studies on endogenous ecdysteroid biosynthesis have indicated the presence of an early control point (pre 3-dehydro-2,22,25-trideoxyecdysone stage) in the ecdysteroid biosynthetic pathway of prothoracic glands, and possibly the follicle cells. However, due to the inability to incorporate [^{3}H]cholesterol into follicle cells (Chapter 6) or prothoracic glands (results not shown) the sites of this early control have yet to be elucidated, and present a goal for future work.

The study of inhibition of *in vitro* ecdysteroid synthesis has enabled the selection of a number of compounds with possible insecticide potential (Chapter 4 and 5). The plant 4,4-dimethylsterol, cycloartenol has been shown to moderately inhibit ecdysteroid synthesis in *L. migratoria* prothoracic glands and follicle cells (Chapter 4). However, the mechanism by which this compound elicits its effect has yet to be demonstrated. The

inability of cycloartenol to cause any effect on insect development *in vivo* has suggested that the reported insecticidal property of the leaves of the transgenic tobacco plant (Amoco Corporation European Patent no. 0 480 730 A2) is due to the inability of insects reared on these leaves to obtain sufficient Δ^5-sterols for ecdysteroid synthesis. The possibility also exists that this effect could be due to an isoprenoid compound which has either been formed, or whose concentration has increased, during the transformation of the plant. In addition, if the MVA shunt is in operation in the leaves of this plant, a certain amount of the proposed high MVA content of the leaves (due to the increased activity of HMG-CoA reductase) will be recycled into acetyl-CoA (Nes and Bach, 1985; Bach, 1987). As this compound is a precursor of flavonoids, compounds which have been demonstrated by Mitchell *et al.* (1993) at high concentrations to have ecdysone 20-monooxygenase inhibitory activity, the possible accumulation of these compounds in the leaves may therefore be responsible for the insecticidal properties of the transgenic tobacco plant.

A number of nitrogen heterocyclic compounds were demonstrated to inhibit the ecdysone 20-monooxygenase system of *S. littoralis* and ecdysteroid synthesis in follicle cells of *L. migratoria*. In order to allow comparisons and detect variation between different batches of insect material, a common inhibitor, ketoconazole, was included in each experiment. Of the enzymes examined in the terminal stages of the ecdysteroid biosynthetic pathway of follicle cells the 20-hydroxylase appeared to be the most susceptible to inhibition. This result conflicts with a previous study by Jarvis *et al.* (1994b), who suggested that the 22-hydroxylase was the most susceptible to inhibition of the enzymes present in the terminal stages of the pathway. However, these results may reflect the different inhibitor concentrations used in these two studies (Chapter 5). The fact that the 22-, 2- and the 20-hydroxylases are linked sequentially may have an effect on the interpretation of the inhibitor studies. This is because inhibition of an enzyme present in the early part of the sequence will limit available substrate for the later enzymes, giving rise to an apparent inhibition. Therefore, further inhibitor studies on the individual enzymes would be required before firm conclusions can be drawn.

The SF21 insect cell lines (Vaugh *et al.*, 1977) and the Mb1203 insect cell lines (Miltenburger and David, 1976) , may also be considered as a source of experimental material for future inhibitor work, as radiolabelling studies have indicated the presence of a number of ecdysteroid hydroxylases in these cells (Chapter 8). Use of the cell lines will also alleviate the tedious dissections associated with *in vitro* tissue culture methods.

The second major area of investigation was to examine and further elucidate the terminal stages of the ecdysteroid biosynthetic pathway. [^{3}H]3-dehydro-2,22,25-trideoxyecdysone was synthesized by Dolle *et al.* (1991) to act as putative precursor for 3-dehydroecdysone, which has been shown to be secreted by the prothoracic glands of a number of Lepidopteran insects (Warren *et al.*, 1988a; Kelly *et al.*, 1990; Kiriishi *et al.*, 1990). Work in this thesis has demonstrated that [^{3}H]3-dehydro-2,22,25-trideoxyecdysone can be accepted as a substrate for ecdysteroid synthesis in *L. migratoria* follicle cells, and the Sf21 and Mb1203 insect cells, which are of ovarian origin.

The ability of follicle cells to utilize [^{3}H]3-dehydro-2,22,25-trideoxyecdysone for the synthesis of their normal ecdysteroid products (20-hydroxyecdysone and ecdysone released after hydrolysis of polar conjugates; Kappler *et al.*, 1986a), has suggested an intermediary role for the steroid in the ecdysteroid biosynthetic pathway. However, before the intermediacy of 3-dehydro-2,22,25-trideoxyecdysone can be established a number of criteria have to be fulfilled (Rees, 1985). The compound must be :1) Isolated from the tissue and its structure established, 2) Formed biologically from the distal precursor, 3) Converted biologically into the final products, 4) Formed biologically from the postulated intermediate, 5) converted biologically into the next postulated compound in the scheme, and finally 6) each individual step in the pathway should be demonstrated directly.

In order to fulfil the first criterion, mass spectrometric methods have been developed to allow the detection of 3-dehydro-2,22,25-trideoxyecdysone in the follicle cells (Chapter 7). However, an initial attempt to detect this steroid endogenously has proved

unsuccessful. Since the amounts of ecdysteroids in insects are comparatively low, intermediates would not be expected to accumulate to any extent. Therefore, the possibility of detecting other 3-dehydroecdysteroids which may be present in the terminal stages of the pathway should be considered (possibly 3-dehydro-2-deoxyecdysone, which has been shown to be a product of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone *in vitro* ; Chapter 6). As a possible end product of the pathway, this steroid would be expected to be accumulated to a greater extent than the proposed intermediate, increasing its detectable mass.

Although, the conversion of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone into a number of 3β-hydroxyecdystereoids has been previously demonstrated in the prothoracic glands of *L. migratoria* (Roussel, 1992a) before it was observed in follicle cells, the ability of an ecdysiosynthetic tissue to convert [^{3}H]2,22,25-trideoxyecdysone into 3-dehydroecdysteroids has not been previously reported. The combined incubation studies with [^{3}H]3-dehydro-2,22,25-trideoxyecdysone and [^{3}H]2,22,25-trideoxyecdysone in the follicle cells have suggested a tentative pathway for the terminal stages of ecdysteroid synthesis in the cells, consisting of three parallel pathways of 3-epiecdysteroids, 3-dehydroecdysteroids and 3β-hydroxyecdysteroids (see Fig. 6.15). Grieneisen *et al.* (1993) have also suggested that a branched pathway exists for the terminal stages of ecdysteroid synthesis in Lepidopteran prothoracic glands. As 3-epiecdysteroids are not normal endogenous products of the follicle cells, their formation from precursors could be due to the metabolism of the exogenous substrates not reflecting the endogenous situation. It is also conceivable that the follicle cells were slightly contaminated with fat body, and that this was responsible for the 3α-reductase activity; however, at least larval fat body contains low 3α-reductase activity (Dinan, 1980). In addition 3-epiecdysteroid formation was consistently found with different batches of follicle cells.

The ability of follicle cells to synthesize 3-epi, 3-dehydro and 3β-hydroxyecdysteroids from the radiolabelled substrates ([^{3}H]3-dehydro-2,22,25-trideoxyecdysone and [^{3}H]2,22,25-trideoxyecdysone) suggests the presence of cellular

3-dehydroecdysteroid 3β- and 3α-reductase activity, in addition to ecdysteroid 3-oxidase activity. Gelman *et al.* (1991) has also shown 3-dehydroecdysteroid 3β-reductase activity can also to be associated with a number of insect tissues including the testes, Malpighian tubules and fat body. In addition, Blais and Lafont, (1984) have indicated the presence ecdysteroid 3-oxidase activity in a number of insect tissues. However, 3-dehydroecdysteroid 3α-reductase activity has so far only been detected in the gut of Lepidopteran insects (Milner *et al.*, 1985; Weirich *et al.*, 1993), and therefore, its possible presence in the follicle cells is questionable.

Initial studies on the subcellular metabolism of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone and [^{3}H]2,22,25-trideoxyecdysone in the follicle cells have indicated that, in agreement with work of Roussel (1992a) on prothoracic glands, the terminal hydroxylases, at least at the the C-25 hydroxylation stage, do not appear to discriminate between the oxidation states of the ecdysteroids. This result may suggest that the ability of follicle cells to metabolize [^{3}H]3-dehydro-2,22,25-trideoxyecdysone is a direct consequence of the low substrate specificity of hydroxylases. However, this does not explain the higher 25-hydroxylase activity using [^{3}H]3-dehydro-2,22,25-trideoxyecdysone in contrast to the accepted ecdysteroid biosynthetic pathway intermediate [^{3}H]2,22,25-trideoxyecdysone.

The efficient metabolism of [^{3}H]3-dehydro-2,22,25-trideoxyecdysone in the follicle cells further suggests the feasability of involvement of a 3-oxo-Δ^4-intermediate in the ecdysteroid biosynthetic pathway of the cells (Davies *et al.*, 1981). In addition, incubation studies in locusts with the radiolabelled compound also suggest that the 3-dehydro group of the 3-oxo-Δ^4-intermediate is retained until the 2-deoxyecdysone level of hydroxylation in follicle cells , in contrast to prothoracic glands, where it appears to be maintained throughout the biosynthetic pathway to yield 3-dehydroecdysone (see Section 1.5). However, before these hypotheses can be confirmed, the early part of the pathway must be first elucidated.

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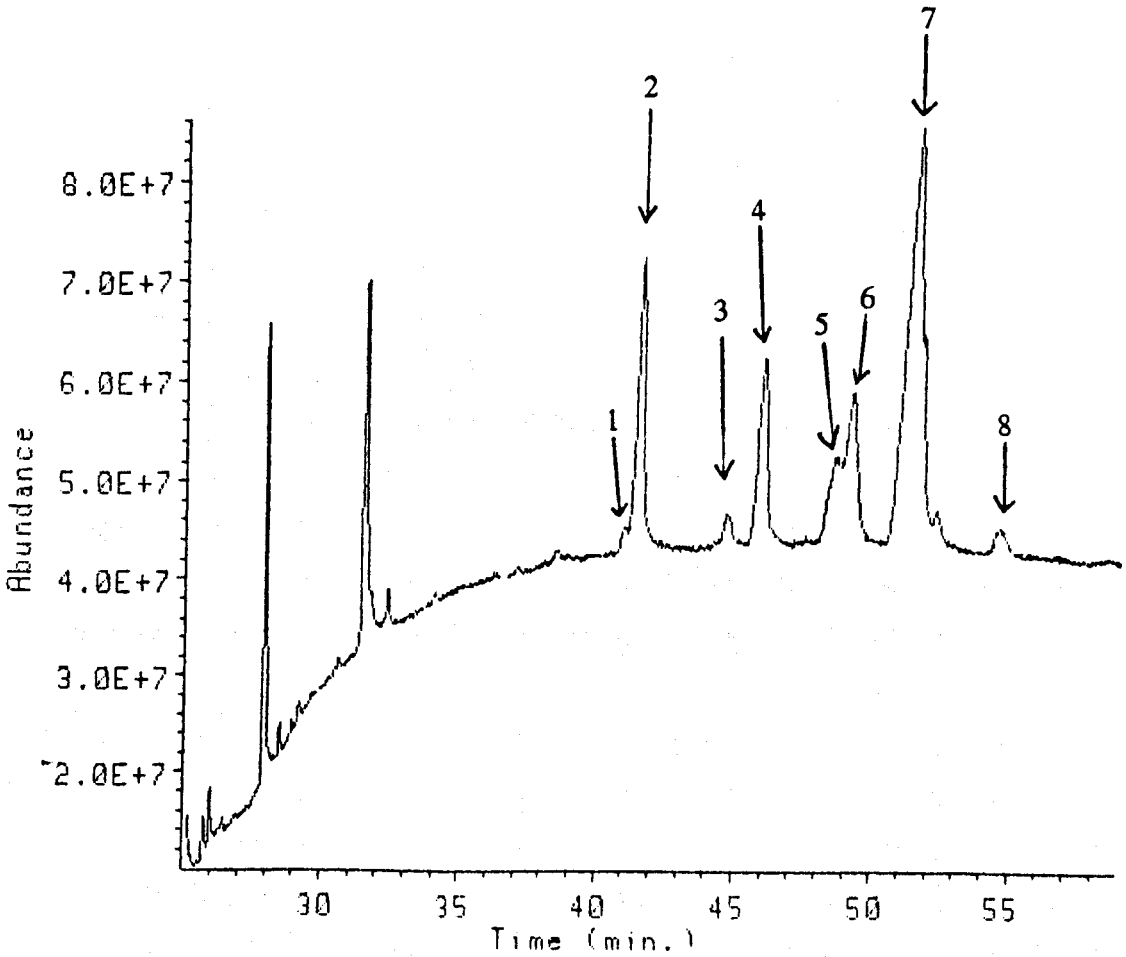
Appendix

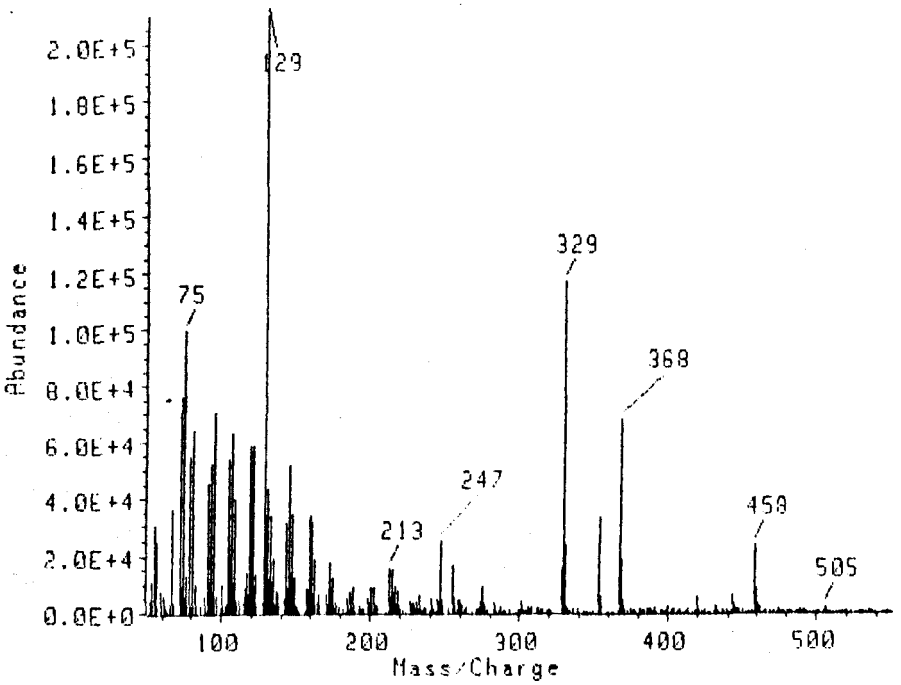
Identification of sterols in tomato callus: mass spectra

Total ion chromatogram from GC-MS of combined sterols, 4α-methylsterols and 4,4-dimethylsterols from tomato callus



Prinicipal ions in the mass spectra were assigned by comparison with those in spectra of authentic compounds (Goad, 1991).

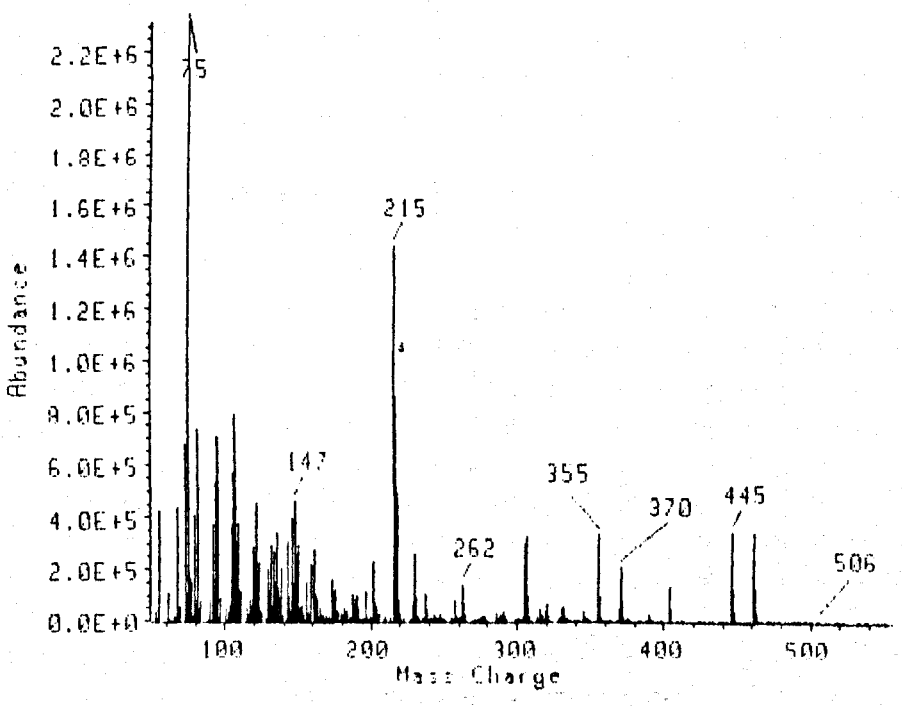
Mass spectra



Peak 1 (Cholesterol)

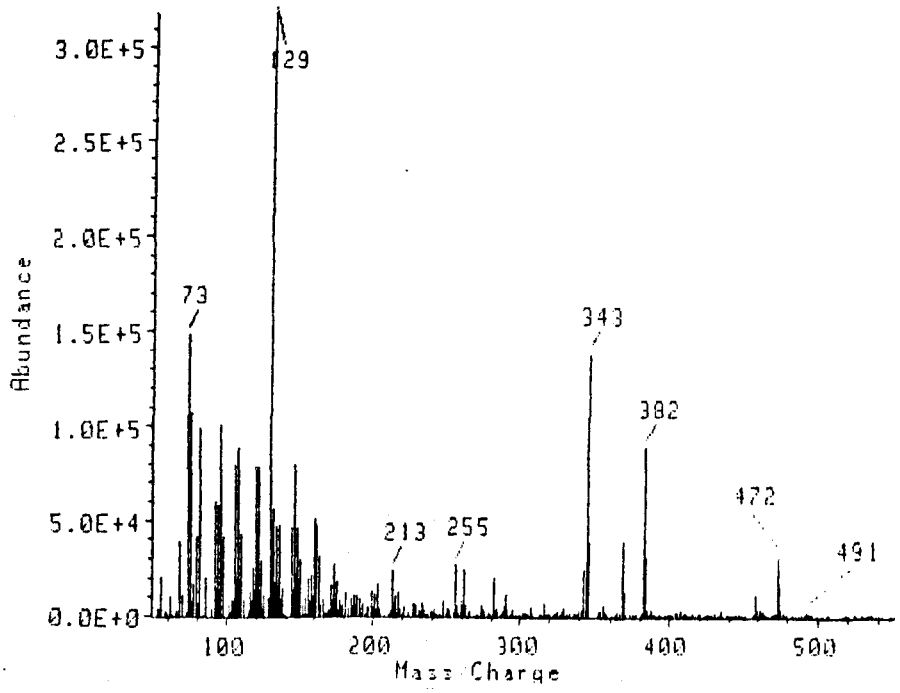
m/z (rel. int.): 458 $[M]^+$ (11), 368 $[M\text{-}TMSOH]^+$ (32), 353 $[M\text{-}TMSOH\ -CH_3]^+$ (11), 329 $[M\text{-}TMSO\text{-}C_3H_4]^+$ (55), 247 (11), 213 $[M\text{-}SC\text{-}TMSOH\text{-}42]^+$ (6), 129 $[TMSO+C_3H_4]^+$ (100).

SC (side chain)



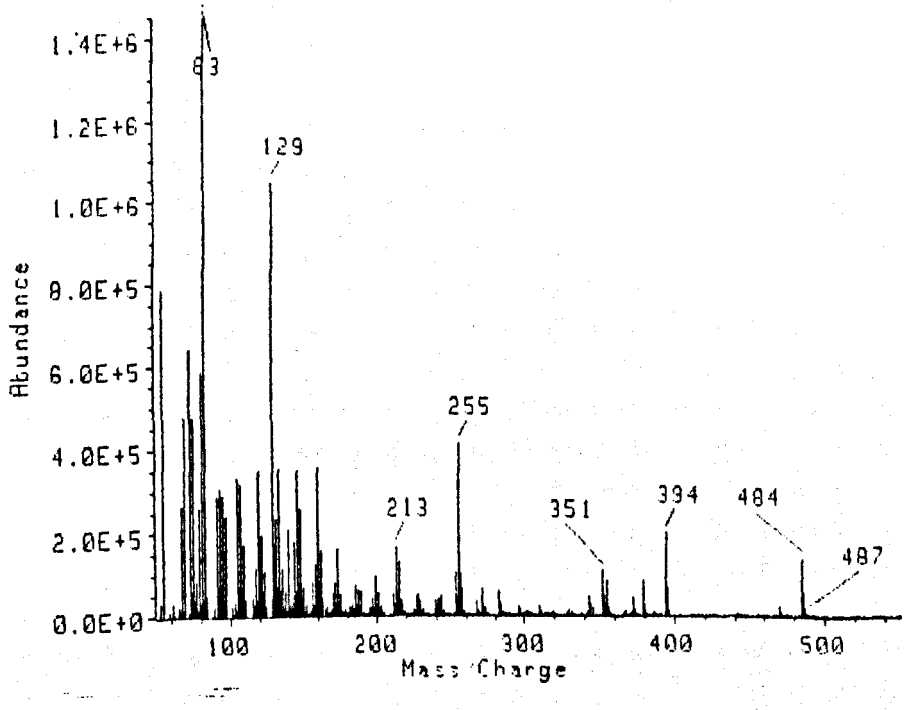
Peak 2 (5α-Cholestan-3β-ol)

m/z (rel. int.): 460 $[M]^+$ (10), 445 $[M\text{-}CH_3]^+$ (10), 370 $[M\text{-}TMSOH]^+$ (8), 355 $[M\text{-}CH_3\text{-}TMSOH]^+$ (15), 303 (13), 262 (5), 215 $[M\text{-}SC\text{-}42\text{-}TMSOH]^+$ (62), 147 (79), 75 (100).



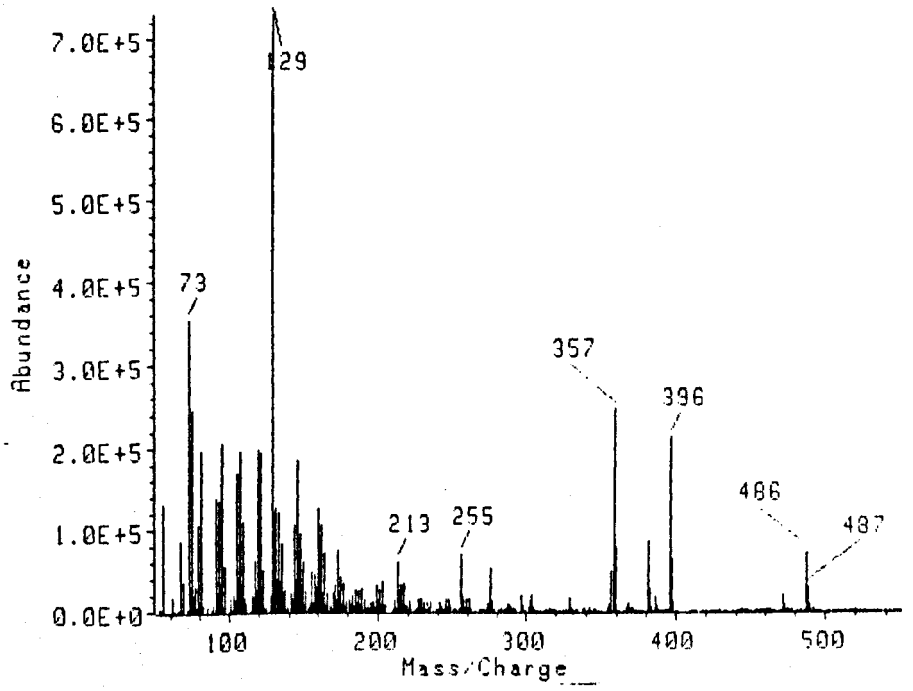
Peak 3 (Campesterol)

m/z (rel. int.): 472 $[M]^+$ (19), 382 $[M\text{-}TMSOH]^+$ (29), 343 $[M\text{-}TMSO\text{-}C_3H_4]^+$ (45), 255 (8), 213 $[M\text{-}SC\text{-}42\text{-}TMSOH]^+$ (7), 129 $[TMSO+C_3H_4]^+$ (100).



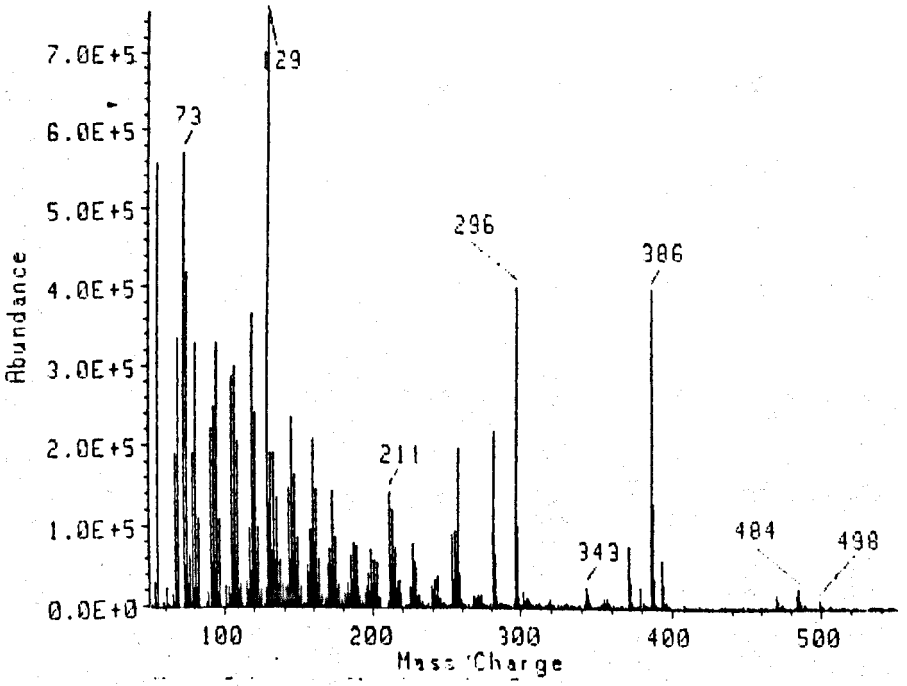
Peak 4 (Stigmasterol)

m/z (rel. int.): 484 $[M]^+$ (9), 394 $[M\text{-}TMSOH]^+$ (14), 351 (7), 255 (27), 213 $[M\text{-}SC\text{-}42\text{-}TMSOH]^+$ (11), 129 $[TMSO+C_3H_4]^+$ (72), 83 (100).



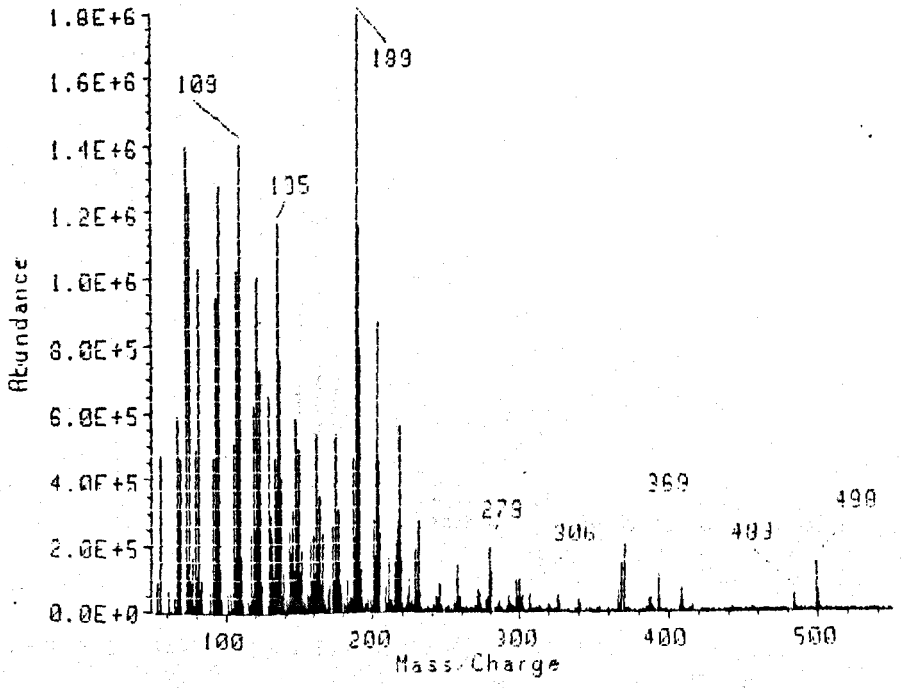
Peak 5 (Sitosterol)

m/z (rel. int.): 486 $[M]^+$ (9), 396 $[M\text{-}TMSOH]^+$ (29), 357 $[M\text{-}TMSO\text{-}C_3H_4]^+$ (33), 255 (9), 213 $[M\text{-}SC\text{-}42\text{-}TMSOH]^+$ (8), 211 (18), 129 $[TMSO+C_3H_4]^+$ (100).



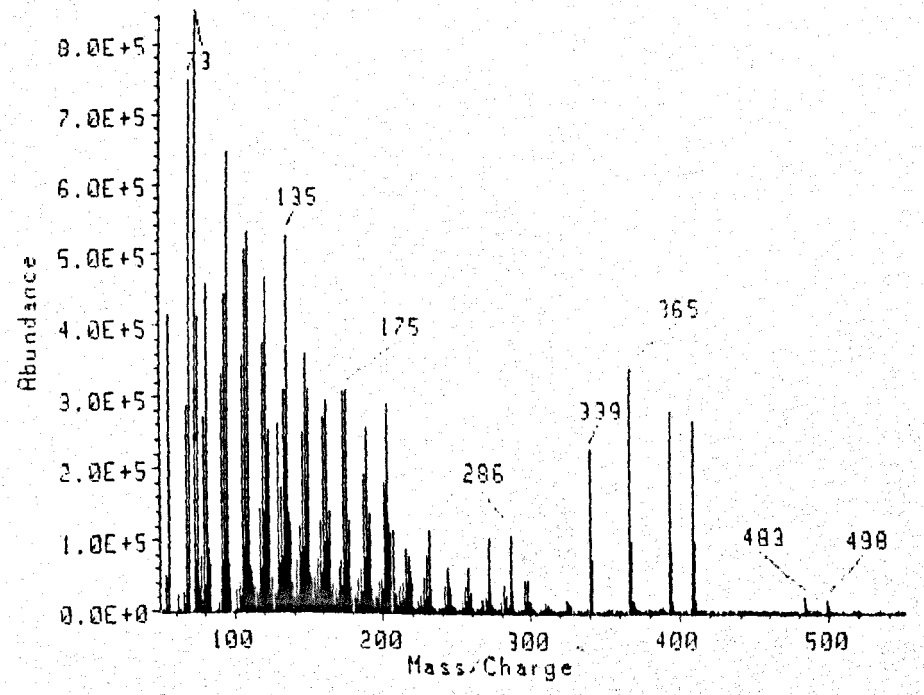
Peak 6 (Isofucosterol)

m/z (rel. int.): 484 $[M]^+$ (2), 386 (52), 343 (3), 296 (53), 213 $[M\text{-}SC\text{-}42\text{-}TMSOH]^+$ (27), 211 (18), 129 $[TMSO+C_3H_4]^+$ (100).



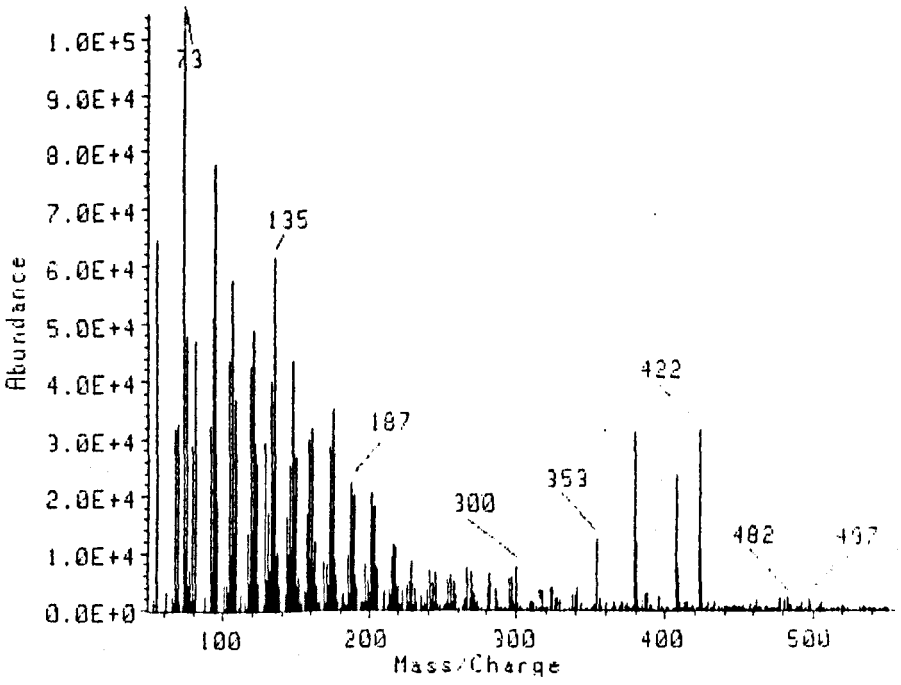
Peak 7a (Unknown)

m/z (rel. int.): 498 (7), 483 (2), 369 (11), 325 (3), 279 (11), 189 (100).



Peak 7b (Cycloartenol)

m/z (rel. int.): 498 $[M]^+$ (2), 483 $[M\text{-}CH_3]^+$ (2), 408 [M-TMSOH]$^+$ (31), 393 [M-TMSOH-CH_3]$^+$ (32), 365 (41), 339 (28), 286 [characteristic of 9β,19-cyclopropane after loss of A ring] (12), 175 (36), 135 (55), 73 (100).



Peak 8 (24-Methylenecycloartanol)

m/z (rel. int.): 497 $[M\text{-}CH_3]^+$ (2), 482 (2), 422 $[M\text{-}TMSOH]^+$ (29), 407 $[M\text{-}TMSOH\text{-}CH_3]^+$ (17), 397 (29), 353 (11), 73 (100).