Enzymes of Ecdysteroid Activation and Inactivation in the Cotton Leafworm, *Spodoptera littoralis*

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by

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SUMMARY

The overall purpose of the work has been to characterize aspects of activation and inactivation of ecdysteroids in last (sixth) instar larvae of *Spodoptera littoralis*.

Firstly, it was established that the major secretion from the prothoracic glands was 3-dehydroecdysone (82%), with smaller amounts of ecdysone. This was in agreement with previous reports of the product(s) of Lepidopteran prothoracic glands. The production of 3-dehydroecdysone raises the possibility that the 3-oxo group may be introduced early in the pathway. Therefore, 3-oxo-5β-ketodiol and 5β-ketodiol (2,22,25-trideoxyecdysone) were examined as potential ecdysteroid precursors. Interestingly, the latter was only incorporated into ecdysone (6.0%), but the former was converted into both ecdysone and 3-dehydroecdysone (32.5%). This implied that 3-oxo-5β-ketodiol is a potential intermediate for ecdysteroid biosynthesis in this system.

A 3-dehydroecdysone 3β-reductase enzyme in the haemolymph that reduces 3-dehydroecdysone secreted from the prothoracic glands, was purified almost to homogeneity and had preference *in vitro* for NADPH as cofactor.

Ecdysone inactivation by transformation via 3-dehydroecdysone into 3-epiecdysone and by phosphorylation were investigated in midgut cytosol and the products characterized. Phosphorylation at both C-2 and C-22 positions was observed.

The cofactor preferences of the reactions were assessed, including the requirement for molecular oxygen by ecdysone oxidase. NADPH was the favoured cofactor of the crude midgut 3-dehydroedysone 3α-reductase, whereas the phosphotransferases required the presence of both ATP and $MgCl_2$. The conversion of 3-dehydroecdysone into ecdysone, catalysed by 3-dehydroedysone 3β-reductases in the midgut, was considered to represent an activation of hormonal activity, analogous to the action of the haemolymph enzyme. However, the cofactor preference of the crude midgut enzyme was for NADH in contrast to the haemolymph reductase.

Developmental profiles of enzymic activity throughout the last larval instar were established and the enzymes were studied with respect to optimal pH, reaction time, temperature and protein linearity. Following this, the enzymes were partially purified by DEAE-cellulose chromatography, Mono-Q FPLC and Phenyl Superose hydrophobic interaction FPLC. In the case of the midgut enzymes, one peak of activity was found for the ecdysone oxidase and the ecdysone 2-phosphotransferase throughout the chromatography steps. However, Mono-Q FPLC revealed the occurrence of two 3-dehydroecdysone 3α-reductase peaks, both displaying a slight preference for NADPH and two 3-dehydroecdysone 3β-reductase activities, the first being exclusively NADH-dependent and the second having a greater preference for NADH than NADPH.

ACKNOWLEDGEMENTS

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"All rising to a greater place is by a winding stair"

Sir Francis Bacon 'Essays'

For Mum, Dad and Duncan

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Chapter 1

General Introduction

Chapter 1

1.1 BACKGROUND

Insects represent an extremely diverse class within the Arthropod phylum. They continue to exploit an extensive variety of ecosystems, forming the largest and probably the most successful group in the Animal Kingdom. Their highly resilient capacity and ability to adapt to adverse conditions have presented problems in the field of pest management. Loss of food and fibre crops and the fight against vector-borne diseases (e.g. malaria, yellow fever and many others) is continuous, despite huge international efforts. Chemical pesticides are currently out of favour because of their persistence in the environment and their limitations due to the development of insect resistance. The search for a so-called "green" alternative has led to the increased interest in insect hormones as growth regulating factors, especially in light of their indifferent action towards man and other vertebrates.

Interference in moulting hormone (ecdysteroid) metabolism would disrupt the correct development of the insect: growth is punctuated by a discrete series of immature stages, separated by a shedding of the exoskeleton. Moult inhibitors such as the herbicide dimilin (PH 60-40) have had some success and the degree of resistance has been less than conventional insecticides. The action of PH 60-40 is thought to involve the inhibition of chitin synthesis, either by the prevention of deposition, or its final formation, (Clarke, et al. 1977; Ker, 1977). However, since all Arthropods have a common moulting process, non-target organisms will be affected. Indepth studies of potential sites of inhibition are required as a strong basis for future insect moulting inhibitors.

1.2 INSECT ENDOCRINOLOGY AND DEVELOPMENT

The moulting process is a carefully time-controlled series of events, involving a cascade of three major categories of hormones: prothoracicotropic hormone (PTTH), juvenile hormones (JH) and ecdysteroids, (figure 1.1).

1.2.1 PTTH

The release of PTTH from the cerebral neurosecretory cells occurs in the scotophase and is probably environmentally (photoperiod, temperature) and developmentally (JH) cued. In the tobacco hornworm, *Manduca sexta,* and the silk worm, *Bombyx mori,* PTTH has been found to be a mixture of two active types of neuropeptide. "Big" PTTH (22-29kDa) and "small" PTTH (4-7kDa) exist, however their individual role is not yet clear and most progress has been made in the study of the former (for review, see Smith and Gilbert, 1989 or Bollenbacher et al., 1987).

In *M. sexta,* big PTTH is released via nerve axons from the corpora cardiaca, into the ductless glands of the corpora allata, and finally into the haemolymph. The receptive prothoracic glands are stimulated to produce ecdysteroid(s): PTTH acts directly on the cell membrane by increasing the free Ca^{2+} and thereby activating adenylate cyclase, to liberate cAMP. A cAMP-dependent protein kinase phosphorylates a specific membrane protein of 34kDa, and ultimately stimulates ecdysteroid biosynthesis, (Rountree et al., 1987; Smith and Gilbert, 1989).

It has been established that the circadian rhythmicity [including such events as ecdysis (Truman, 1985), oxygen consumption (Taylor,

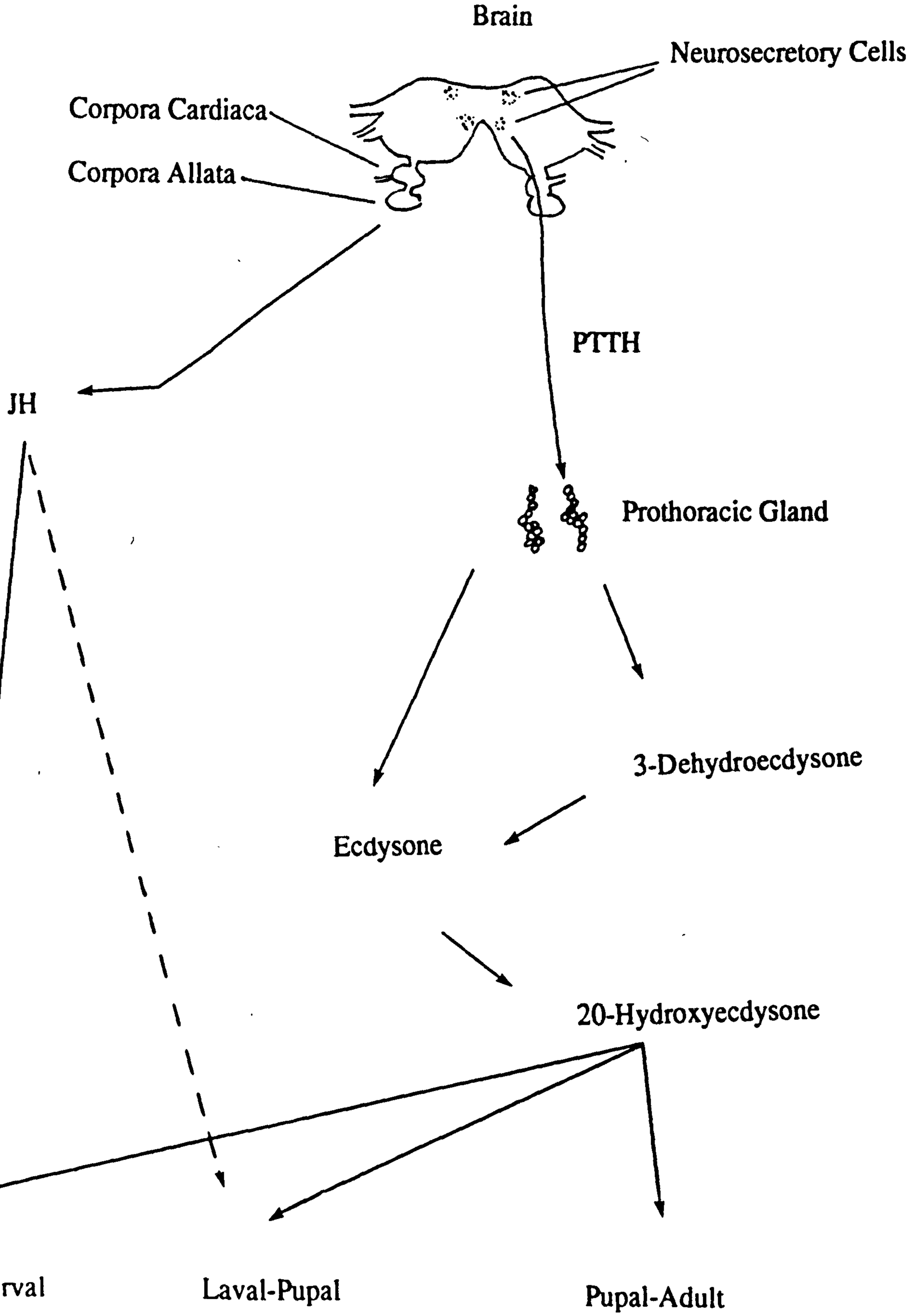


Figure 1.1. The hormonal control of moulting in insects

1977) and so on] is controlled not by the brain, but by the prothoracic glands (Cymborowski et al., 1991). Consequently, this implies a rhythmicity in both endocrinology and development. In *Rhodnius prolixus,* huge daily oscillations in the haemolymph ecdysteroid titre were found to occur, just preceding larval-pupal ecdysis. Moreover, daily fluctuations are irrespective of the overall increasing or decreasing levels. Therefore, ecdysteroids are added to the haemolymph everyday *albeit* in small amounts (Vafopoulou and Steel, 1991).

The nature of the secretory product of the prothoracic glands has promoted renewed interest in this particular topic. A fascinating paper by Warren et al (1988) reported that the major secretion from the prothoracic glands of *Manduca sexta* was 3-dehydroecdysone, not ecdysone as previously believed. Furthermore 3-dehydroecdysone is now regarded as the major secretion of Lepidopteran prothoracic glands (Kiriishi et al, 1990; Sakurai et al, 1989b; Blais and Lafont, 1991). The metabolite from the prothoracic glands can be linked to the levels of a haemolymph protein factor (HPF), considered to be a 3-oxoecdysteroid 3β-reductase. From the insects thusfar studied, Lepidopterans have the greatest enzyme activity.

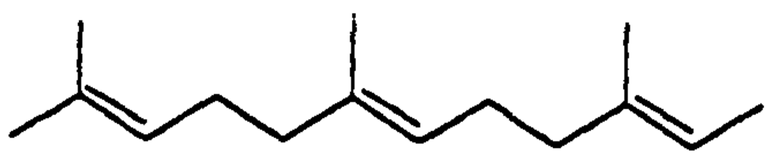
3-Dehydroecdysone is converted into ecdysone in the haemolymph by this enzyme and the latter is hydroxylated at C-20 in certain peripheral tissues and hence, forms the most active hormonal metabolite, 20-hydroxyecdysone. The enzyme has also been discovered in several organs, such as the suboesophageal glands, proctodeae and brain in *Ostrinia nubilalis* (Gelman et al., 1991), and gut of *Manduca sexta* (Nigg et al., 1974; Thompson et al., 1974) and *Spodoptera littoralis* (Milner and Rees, 1985). The exact function of these isozymes is, as yet, unclear.

1.2.2 Juvenile Hormones (JHs)

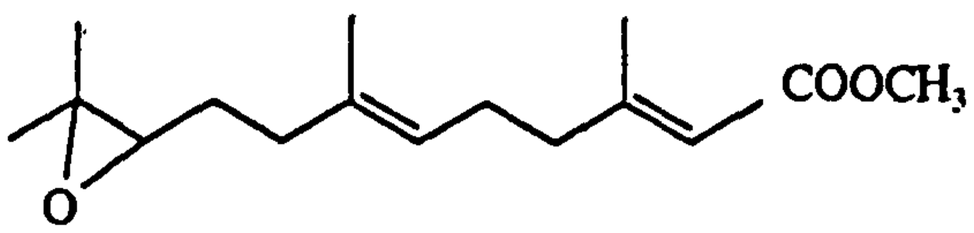
In most species, one JH is responsible for the effects of the corpora allata, that is JH III, but in Lepidoptera, different homologues occur at different stages in development, although the biological role of each is not yet clear (Schooley et al., 1984). Among Lepidopterans, more complex forms of JH include JH 0, JH I, 4-methyl JH I and JHII (figure 1.2). Recently, JH III bisepoxide has been isolated, but is only thought to be produced within the Dipteran order, (Richard et al, 1990). Although regarded as the precursor of JH III, methyl farnesoate is included in the list; it too is believed to have some JH activity in insects (Lanzrein et al., 1984), and it is the main crustacean JH (for review see Cusson et al., 1991).

In terms of its morphogenic role, JH is required to determine the type of moult, high titres of JH leading to larval-larval moults, while larval-pupal and pupal-adult moults occur in the virtual absence of JHs. *Manduca sexta* is often used as a model for insect studies and has the typical ecdysteroid and JH titres shown in figure 1.3. In both the larval and pupal moults, the ecdysteroid titre rises to a distinct peak approximately two days before ecdysis and initiates the moult process. Development from pupa to adult is brought about by a high ecdysteroid titre, which continues throughout the development of the adult, only to decline upon emergence.

The penultimate larval moult is characterized by a high JH titre for the duration of high ecdysteroid levels. In contrast, during the last larval moult (Vth. instar in *Manduca sexta*), the level of JH falls to indistinguishable levels during the instar. This is caused by a) a decrease in the secretory activity of the corpora allata, and b) by an increase in activity of JH esterases in the haemolymph (Jones et al., 1982).

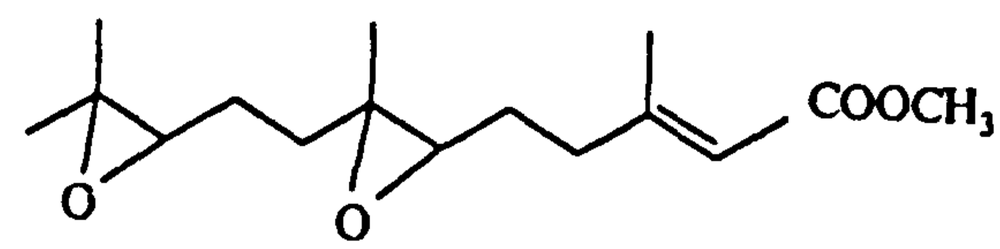


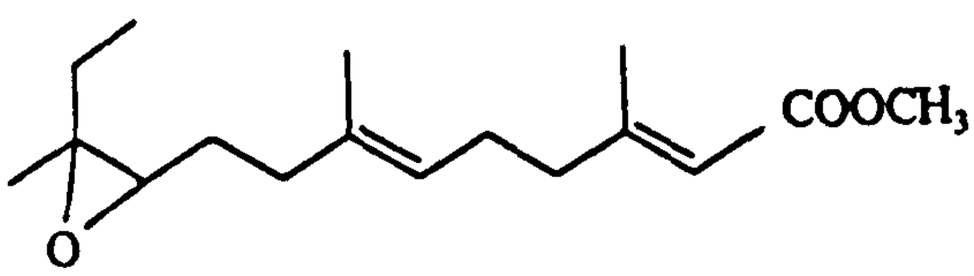
Methyl Farnesoate



JH III

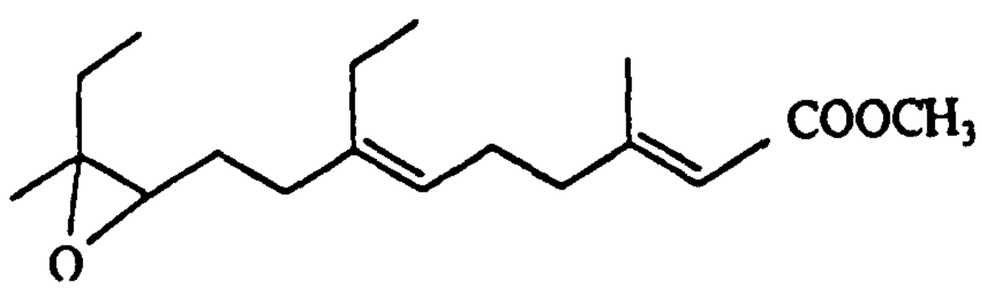
JH III Bisepoxide



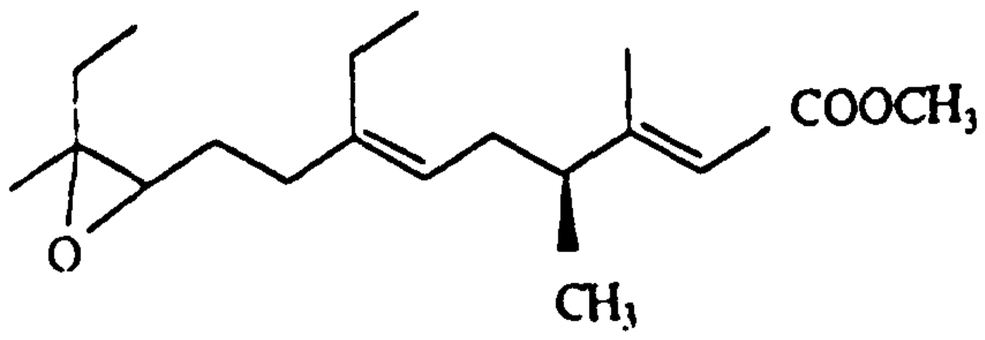


JH II

JH I



4-Methyl JH I



JH 0

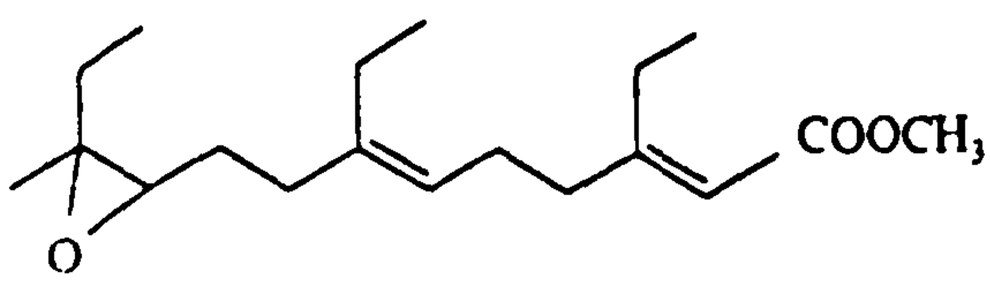


Figure 1.2. Examples of common juvenile hormones

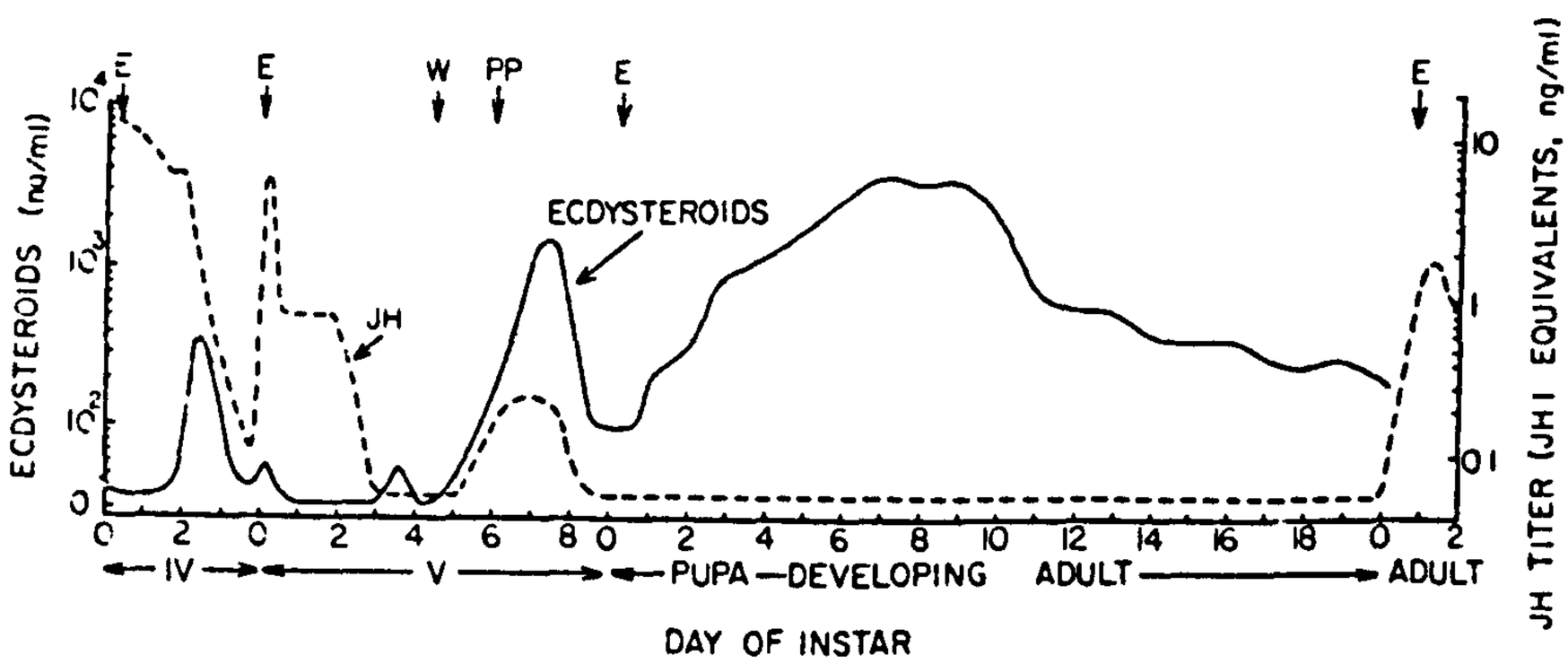


Figure 1.3. The ecdysteroid and juvenile hormone titre of *Manduca sexta* through larval growth and metamorphosis (after Riddiford, 1980)

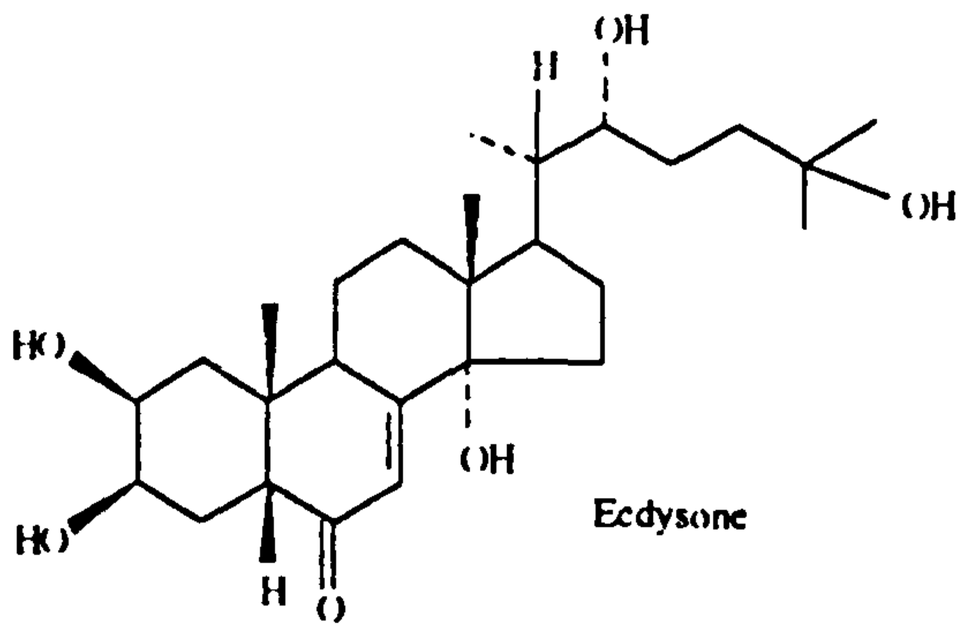
The decline of JH is vital both physiologically and behaviourally. Firstly it results in an increased responsiveness of the prothoracic glands to PTTH, (Sakurai et al, 1989a) and removes the inhibitory effects of JH on ecdysteroid secretion (Fair and Riddiford, 1976). This results in a small rise in the ecdysteroid titre, known as the commitment peak, which prepares the insect for pupal life. Secondly, in the absence of JH, the ecdysteroid induces the larva to stop feeding, leave the plant and begins to burrow into the soil. to construct a pupation cell. The ecdysteroid titre rises once more, to promote the moult. The increasing ecdysteroid level stimulates JH synthesis in the corpora allata, (Watson et al, 1986) and hence, prevents precocious adult development. During the developing adult stage, it is important that the JH titre is negligible, otherwise maturation is impeded. The corpora allata stops JH production and cannot be influenced by the high levels of ecdysteroids (for review see Riddiford, 1980).

1.2.3 Ecdysteroids

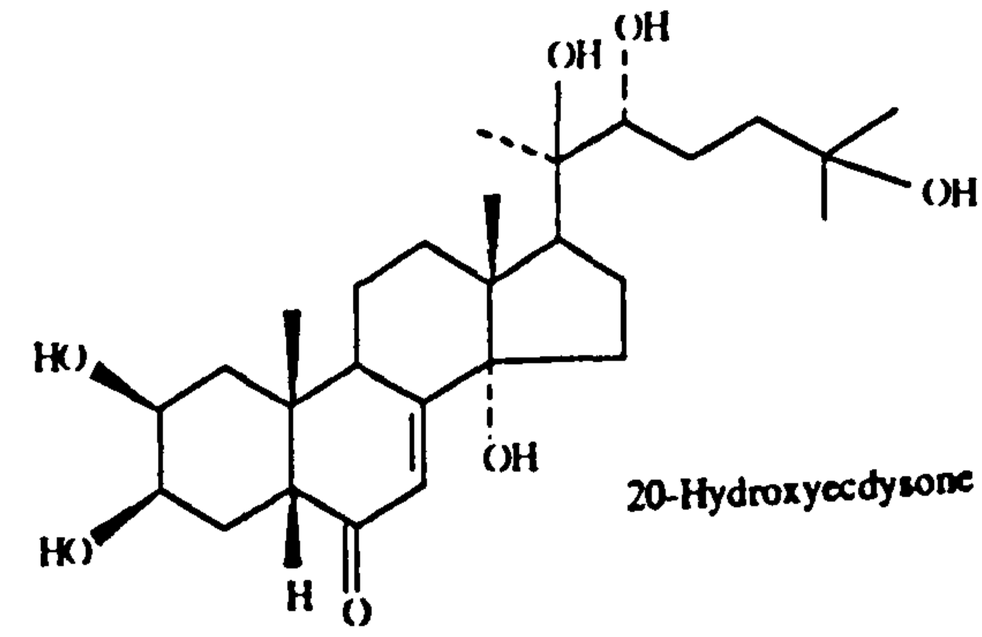
Ecdysone was the first ecdysteroid isolated, from *Bombyx mori* in 1954 by Butendant and Karlson and since this time, a host of structurally related compounds have been discovered. Hence, the generic name, ecdysteroid was coined (Goodwin et al., 1978). Some of the more common examples are given in figure 1.4.

1.3 ECDYSTEROID STRUCTURE

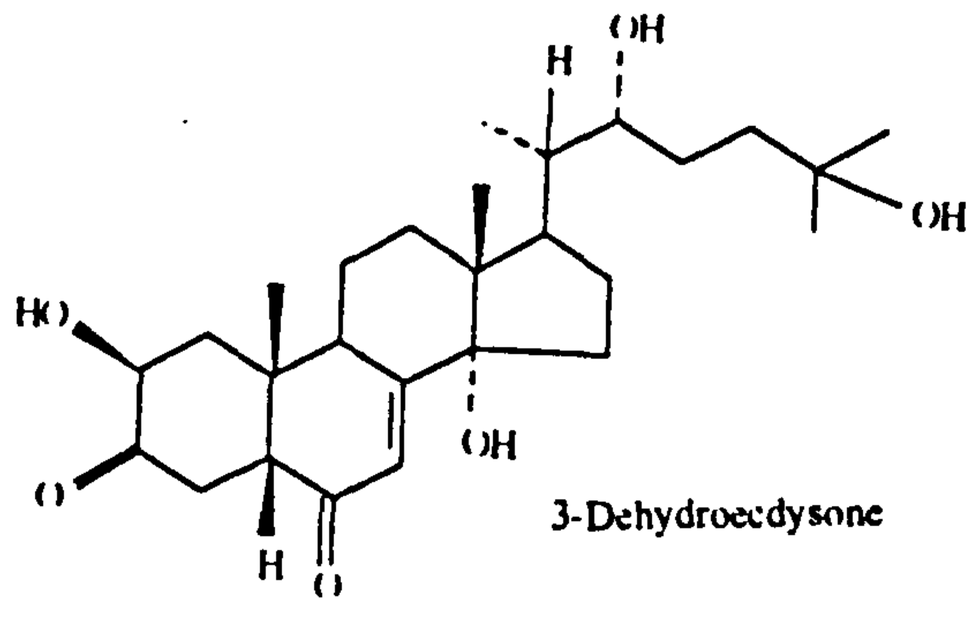
Structurally distinct from vertebrate hormones, ecdysteroids are polyhydroxylated ketosteroids, (figure 1.5a,b,c). They exhibit a distinct 14α-hydroxy-7-en-6-one system, an A/B *cis* junction, (i.e. a 5β



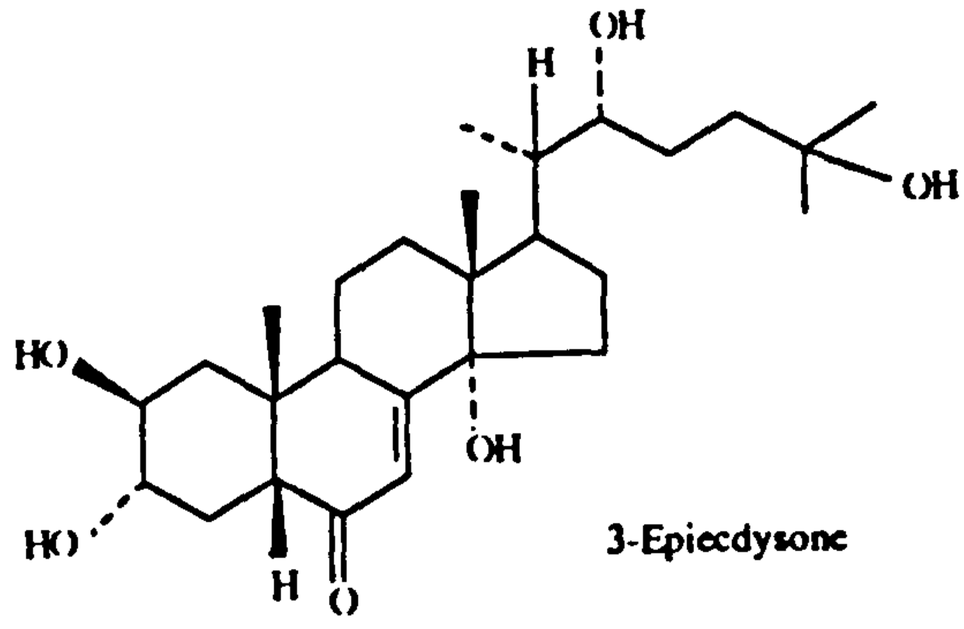
Ecdysone



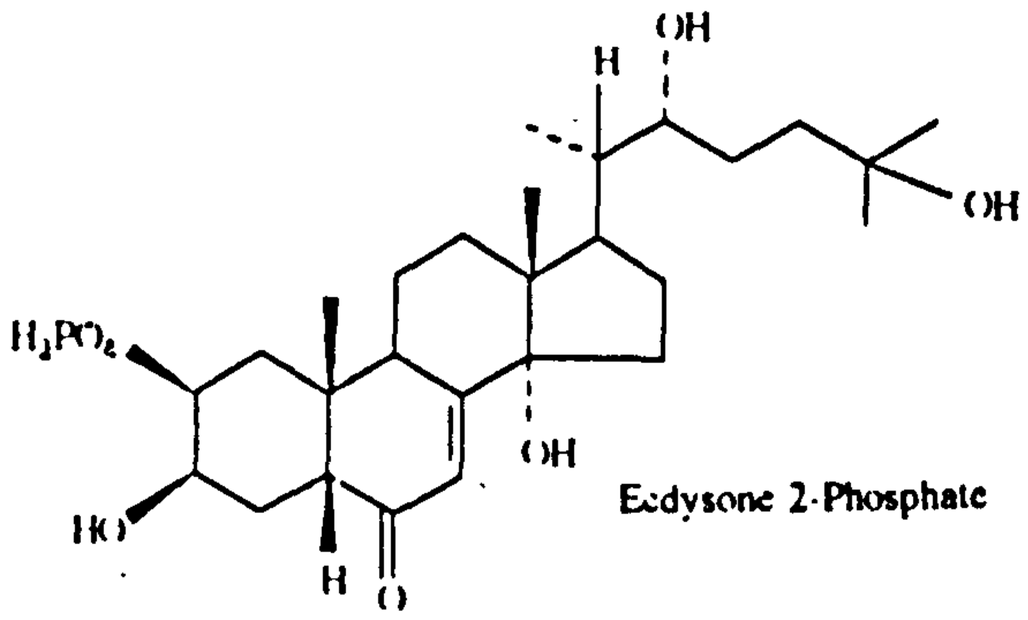
20-Hydroxyecdysone



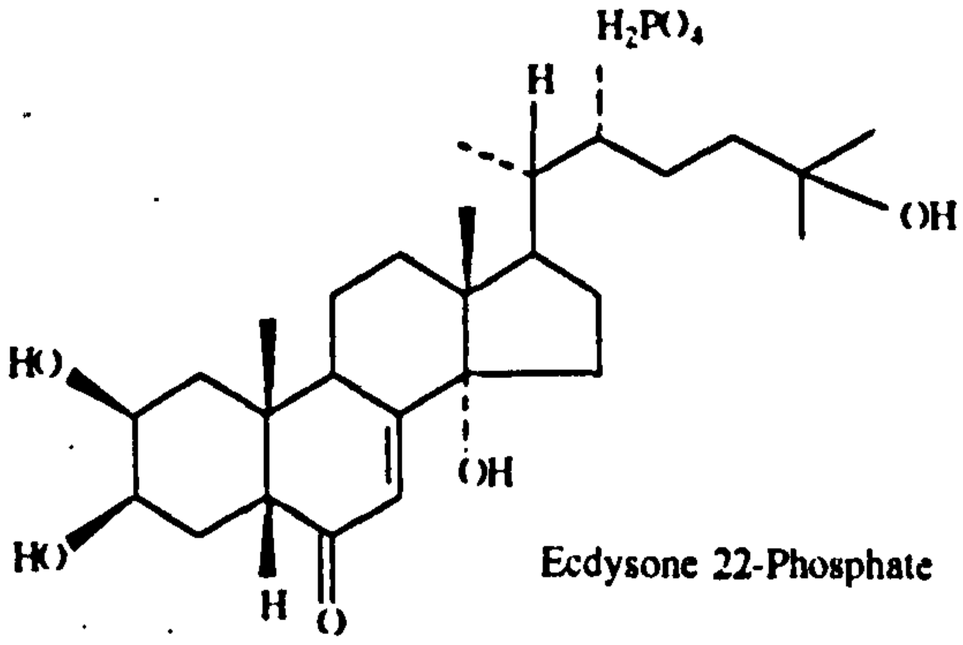
3-Dehydroecdysone



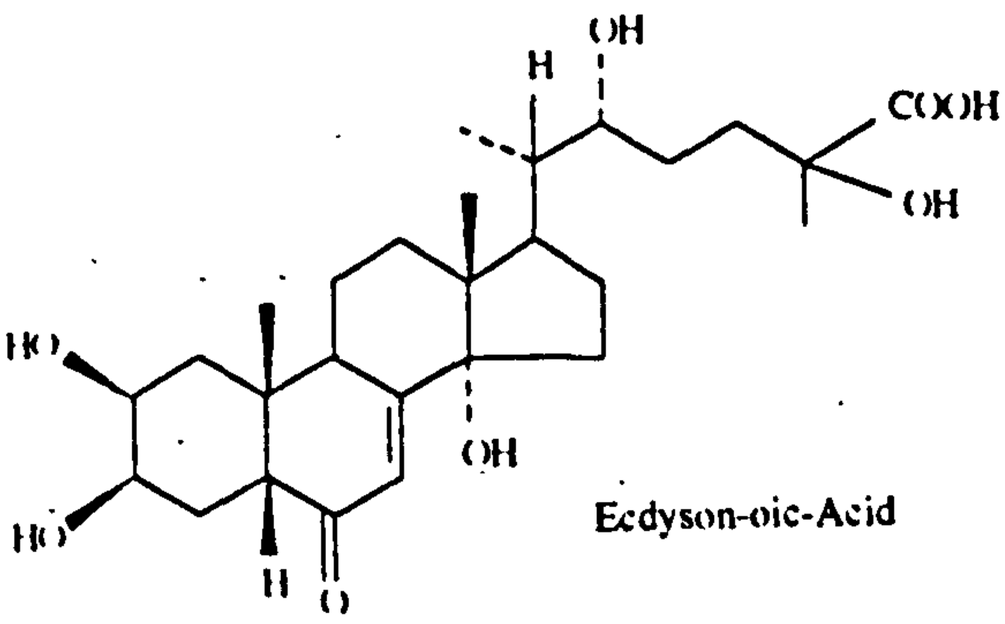
3-Epiecdysone



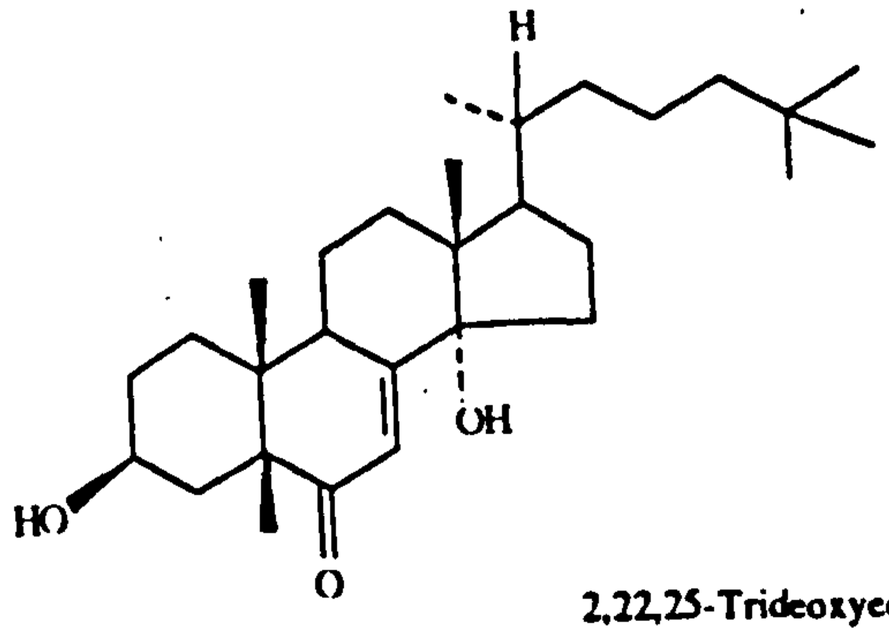
Ecdysone 2-Phosphate



Ecdysone 22-Phosphate

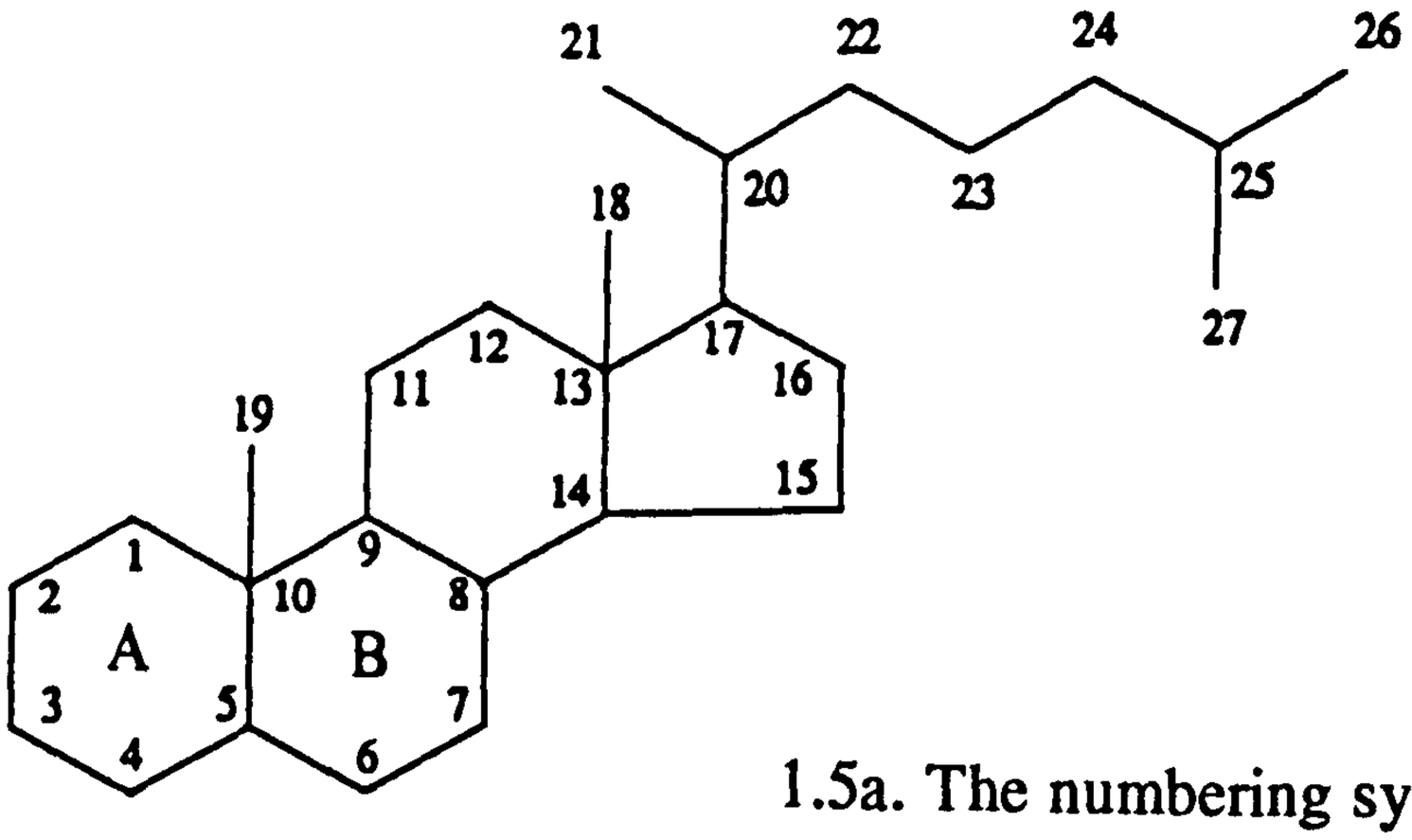


Ecdyson-oic-Acid

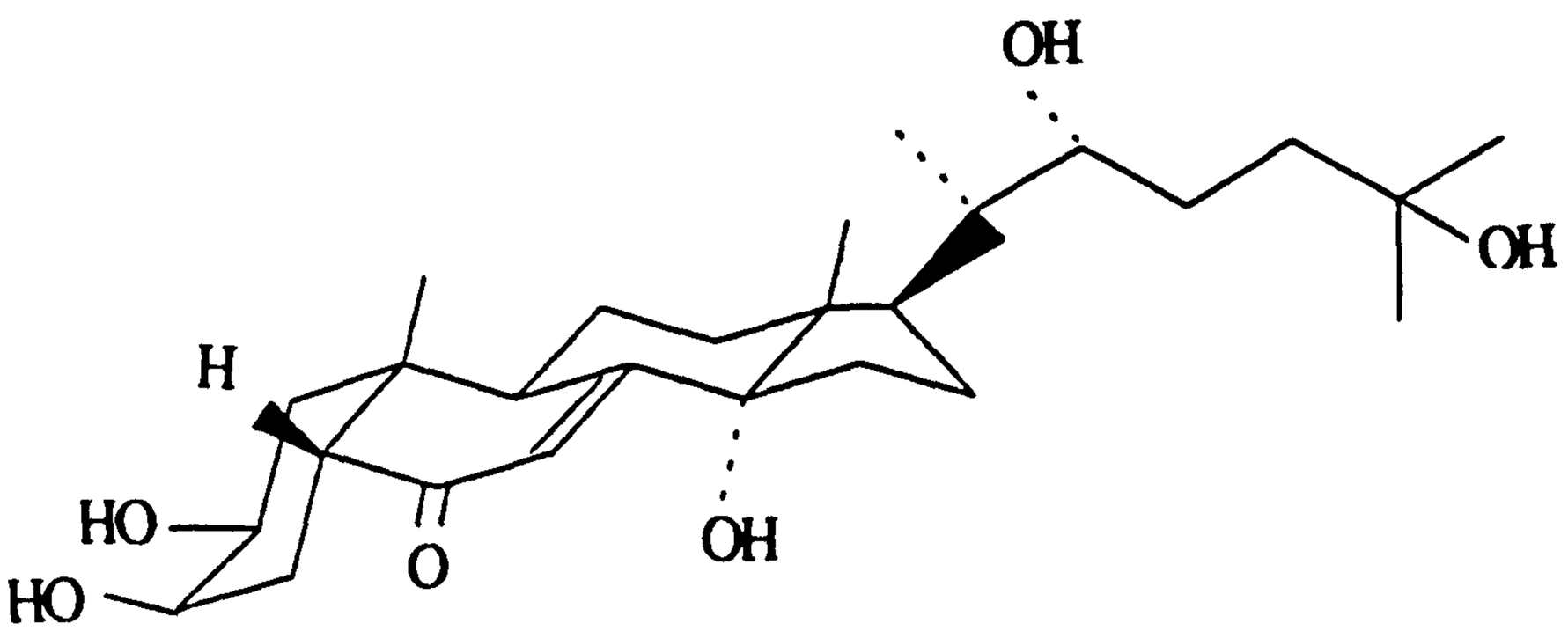


2,22,25-Trideoxyecdysone

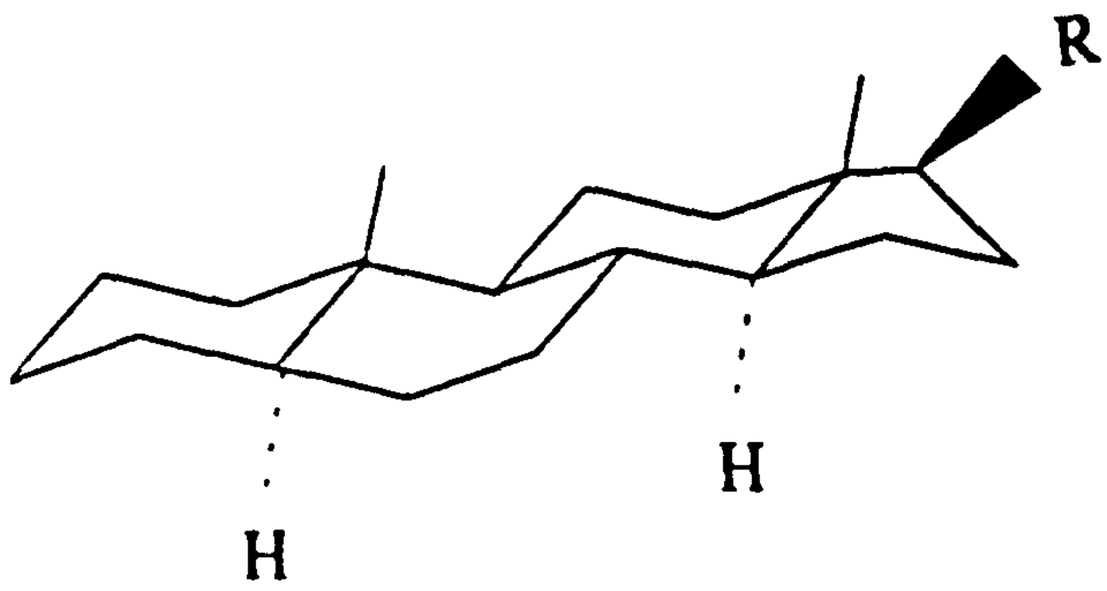
Figure 1.4. Examples of common ecdysteroid structures



1.5a. The numbering system for steroids



1.5b. The three dimensional structure of ecdysone



1.5c. A typical vertebrate A/B *trans* steroid

Figure 1.5. Steroid structures

hydrogen), an α/β saturated oxo group in the B-ring, and retain the side chain of the distal precursor, cholesterol. The A/B *cis* fusion is biologically significant, since *trans* fused rings are hormonally inactive). In stark comparison, the vertebrate hormones are functionally inactive unless the A/B ring is in the *trans* configuration, the saturated oxo group is in the A-ring and side chain cleavage has occurred. Using the IUPAC nomencature therefore, ecdysone becomes 2β-3β-14α,22R,25-pentahydroxy-5β-cholesten-7-en-6-one.

A wide range of ecdysone-related compounds has been isolated, the structures conferring significant changes to the mode of action of the molecule, (figure 1.6). Inactivation (usually for storage or excretion) involves several esterification reactions, including acetylation, phosphorylation, glycosylation, long-chain fatty acylation, double phosphate and phospho-acetate ester formation at various hydroxyl groups, both in the steroid nucleus and on the side chain. Oxidation of the C-3 hydroxyl (to form a keto group) results in 3-dehydroecdysteroids, which classically speaking, have only one tenth hormonal activity of 20-hydroxyecdysone (Richards, 1978; Spindler et al., 1977). It is from the former that irreversible reduction and epimerization takes place to form 3α-hydroxyepimers. These metabolites are phosphorylated and excreted, since they do not serve as active hormones.

Hydroxylation occurring at C-20 produces 20-hydroxyecdysone, thought to be the most active hormone; a similar reaction at C-26 can proceed further to give ecdysteroid-26-oic acids, which are, once again, inactivation products of hormone metabolism.

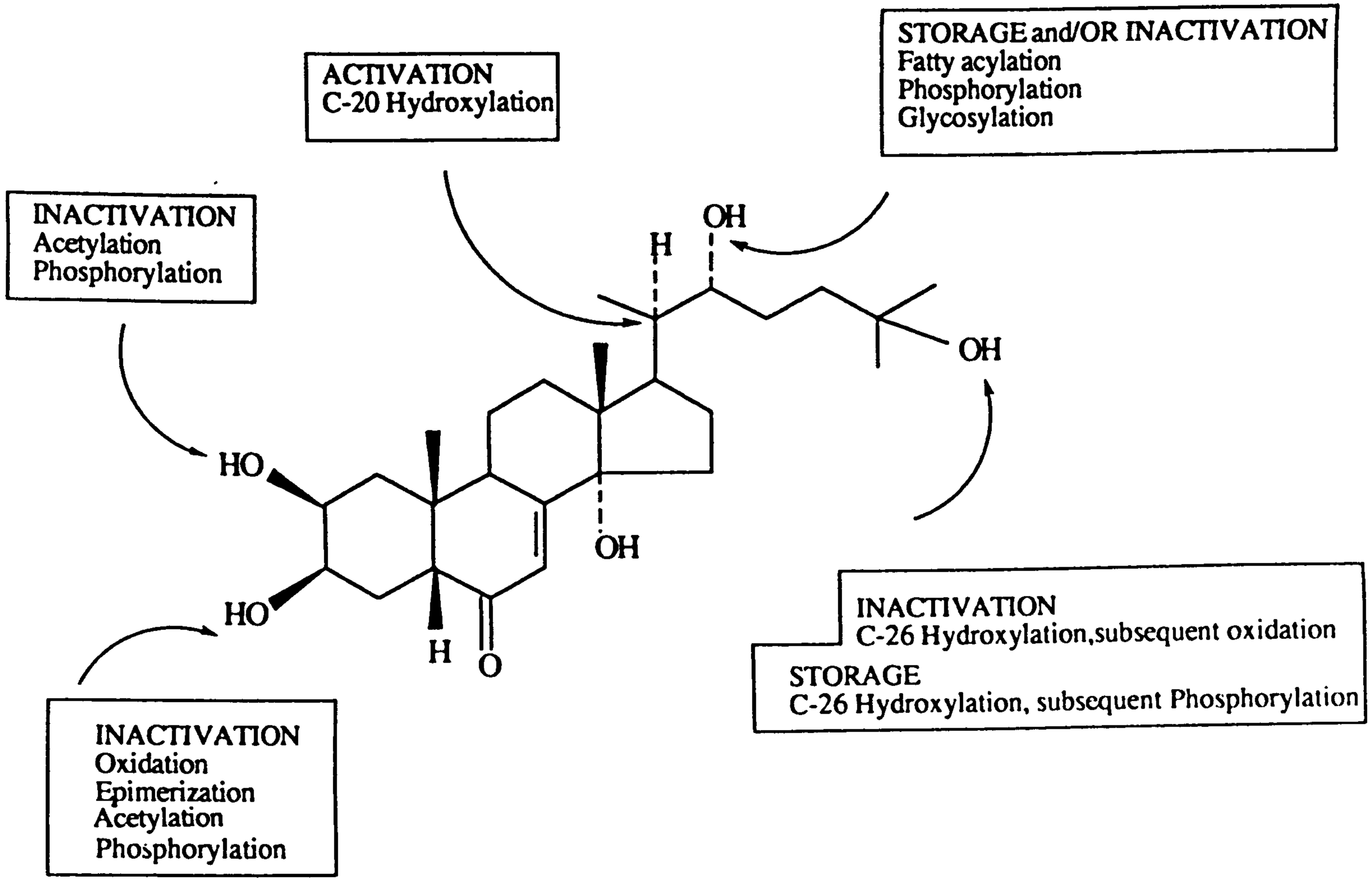


Figure 1.6. Modifications to the ecdysteroid structure, indicating activation and inactivation

1.4 OCCURRENCE

Within the phylum *Arthropoda*, including the classes *Insecta*, and *Crustacea* (for reviews see Horn and Bergamasco, 1985; Spindler et al., 1980; Spindler et al., 1984) and *Arachnida* (Bonaric and De Reggi, 1977) the distribution of ecdysteroids is ubiquitous; moreover, they are also found in smaller quantities in *Echinodermata* (Karlson, 1983), *Mollusca* (Takemoto et al., 1967), *Gastropoda* (Romer, 1979; Whitehead and Sellheyer, 1982), *Annelida* (for a review see Porchet et al., 1984) and in helminths, for example *Nematoda* (Rees and Mendis, 1984; Rees and Mercer, 1986). It is interesting to note that ecdysteroids have been identified in human urine, even in the absence of any parasitic infection, and may provide marker changes in the gut flora, in several pathological conditions, (e.g. cerebral vascular disease, cirrhosis, etc. (Gharib et al., 1991).

Phytoecdysteroids are found in abundance, both in gymnosperms and angiosperms (but with less frequency in lower plants) and hence, provide an excellent source of many ecdysteroid derivatives, since there are many metabolites common to both zoo- and phyto-ecdysteroid classes (for review see Lafont and Horn, 1989). That plants possess these hormones is rather noteworthy, since their exact function is not clear. They are generally believed to play a role in defence against phytophagous insects.

1.4.1 Occurrence in Insects

In the simplest terms, ecdysteroids can be said to a) control moulting, b) be involved in reproduction and c) influence systemic processes such as the control of metabolic rate.

Moulting

Moulting is characteristic of the post-embryonic development of insects (for review see Sehnal, 1989). In the last larval instar, a termination of feeding occurs in preparation for moulting. Gut purge is preceded by a small (commitment) peak of 20-hydroxyecdysone, followed by a larger ecdysteroid peak, promoting ecdysis (Wolfgang and Riddiford, 1986).

The moult begins with the cessation of endocuticle deposition and the secretion of the "moulting fluid" (containing ecdysone and 20-hydroxyecdysone) into the exuvial space. There is extensive apolysis and proliferation of epidermal cells, deposition and sclerotinization of the new cuticle [see Gnatzy and Romer (1984) for a detailed description].

The final steps of the moult process appear to be dependent on the decline of ecdysteroid titre (Sláma, 1980) for example melanization (Curtis et al., 1984). The metamorphosis which the insect undergoes, although largely controlled by JH, may be influenced by the ecdysteroids; in general, larval moults are induced by lower titres of hormone than those of pupae and adults. High doses of ecdysteroids are known to bring about precocious development, beginning with the deposition of the epicuticle and the steps preceding moulting, for instance, epidermal proliferation and differentiation, are often reduced or omitted (Williams 1968). Interestingly the ecdysteroid titre may be lessened by up to 70% and not affect the moult process (Hiruma and Agui, 1985). Surges in ecdysteroid level are also known to affect both cell proliferation and remodelling, for example the movement of the mitochondria in the Malpighian tubules, (Ryerse, 1977).

Reproduction

Although a basal level of gametogonia and spematogenesis may be independent of ecdysteroids, the rise in gametogenesis at metamorphosis is directly due to these hormones (for review Raabe, 1986).

Reproduction in Female Insects

Ovarian follicle cells have been shown to synthesize ecdysteroids (Lagueux et al., 1977, 1984). Ecdysteroids of ovarian origin may be involved in at least three major functions.

i) The regulation of vitellogenesis and oöcyte maturation. Vitellogenesis is the process of yolk synthesis, the yolk proteins of the ovary are referred to as vitellins, but yolk proteins circulating in the haemolymph are referred to as vitellogenins. In many insects, JH regulates vitellogenesis (for review see Bownes, 1989), but in Dipterans, 20-hydroxyecdysone is involved as well (for review see Hagendorn, 1985). In Dipterans, ecdysteroids are released from the ovary into the haemolymph, enter the cells of the fat body and stimulate vitellogenin synthesis. In some insects such as *Drosophila,* yolk protein synthesis is also apparent in the ovary itself.

ii) Results obtained from *Locusta migratoria* indicate that ecdysteroids are also necessary for oöcyte meiotic reinitiation (for review see Lanot et al., 1989). A rising titre of ecdysteroids is observed prior to chorionation and oöcyte enlargement. During oöcyte maturation, there is a meiotic arrest during prophase I, (Kunz, 1967). Ecdysteroid levels remain high and constant until shortly before ovulation, when meiosis is reinitiated and the oöcytes enter metaphase I. A second arrest then occurs, and a second reinitiation upon egg laying is observed, concurrent with a sharp, short-lived peak of ecdysone.

iii) Accumulation of conjugate derivatives, which will be maternally donated to the developing embryo, the most well documented examples being in the *Orthoptera*, (for example locusts). Ecdysteroids are often conjugated with phosphates as a temporary inactive storage form. Identification of the esters found in the eggs have indicated that they are exclusively 22-phosphates of ecdysone, 2-deoxyecdysone, 20-hydroxyecdysone and 2-deoxy-20-hydroxyecdysone (Isaac et al., 1983). Other classes of insects display apolar esters as egg storage forms, such as the C-22 long-chain fatty acyl esters of *Periplaneta americana* (Slinger et al., 1986) and *Acheta Domesticus* (Whiting and Dinan, 1989). In *Manduca sexta*, the major oöcyte ecdysteroid is 26-hydroxyecdysone, which is phosphorylated at the C-26 position (Thompson et al., 1987).

Stored ecdysteroids conjugates of maternal origin are apparently hydrolysed in a controlled manner at specific time points during embryogenesis and provide a possible source of free, active ecdysteroids, at least before the prothoracic glands differentiate (Lagueux et al., 1984; Rees and Isaac, 1984). Elevated levels of free ecdysteroids have been shown to precede the synthesis and deposition of the embryonic cuticle. After utilization by the embryo, ecdysteroids are converted into a range of inactivation products (see Section 1.7).

Reproduction in Male Insects

There is little information on testicular ecdysteroids, however they have been located in such tissue in *Calliphora vicina* (Koolman et al., 1979) and *Heliothis virsescens* (Loeb et al., 1984). Testes of last instar larvae and day 3 pupae of *Heliothis* secrete ecdysteroids into the medium *in vitro*. More specifically, in *Ostrinia nubilalis*, the ecdysteroids have been identified as ecdysone, 20-hydroxyecdysone and highly polar ecdysteroid conjugates (Gelman et al., 1988). Additionally,

the larval testes of *Ostrinia, Heliothis* and *Lymantria dispar* have been reported to convert [^{3}H]ecdysone into 20-hydroxyecdysone (Gelman et al., 1985). It has also been found that the exogenous ecdysteroid is required to initiate or boost the endogenous synthesis *in vitro* (Loeb, et al., 1984, 1986). Although synthesis of ecdysteroid is highly likely in the testes, it is not unequivocally proven.

1.5 MODE OF ACTION

In 1960, Clever and Karlson demonstrated that 20-hydroxyecdysone induced changes in the puffing pattern of the polytene chromosomes of *Chironomus tetans* (puffs are sites of intense mRNA production). Three types of puffs were then described: a) 20-hydroxyecdysone-repressible puffs (seen during intermoult), b) 20-hydroxyecdysone-activated "early" puffs- occurring within minutes of hormone administration. The gene products of these puffs were found to activate c) "late" puffs (Clever, 1964; Ashburner and Richards, 1976).

In *Drosophila,* the hormone-receptor (E-R) induces a locus of the early puffs and inhibits late loci. Products (P) of the early puffs are either proteins or require protein synthesis for their production. They are necessary for negative feedback to switch off the early puffs and, to induce the late ones (Ashburner et al., 1974), (figure 1.7).

The locations of a substantial number of puffs have now been mapped (for review see Belyaeva et al., 1989). Early puffs induced by 20-hydroxyecdysone are not inhibited by cycloheximide, and are therefore considered the primary response to hormone. The gene or genes contained within a puff seem to be important in the sequential repression or activation of a number of other genes. It should be added

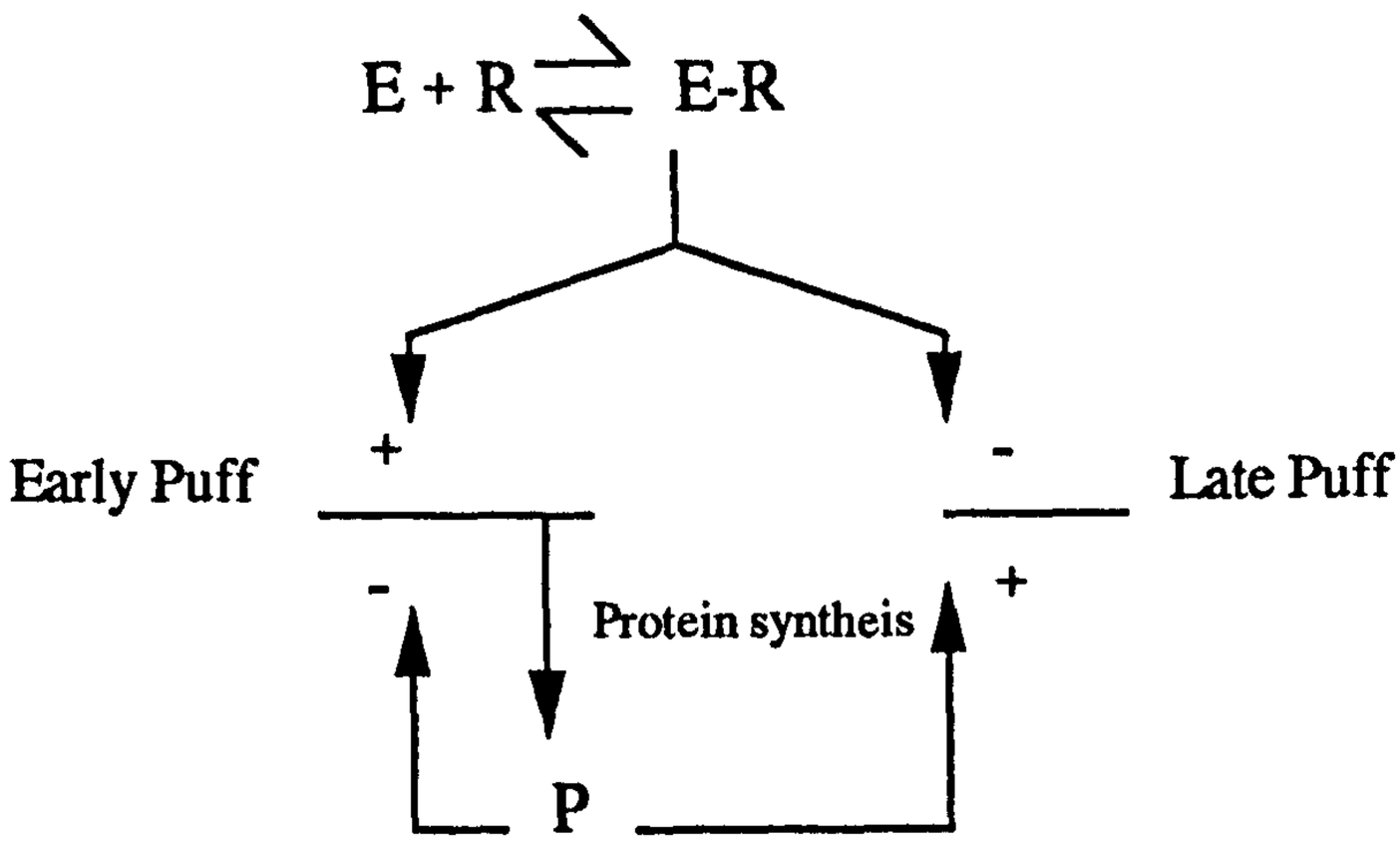


Figure 1.7. The puffing scheme of Ashburner et al., 1974

E= Ecdysteroid
R= Ecdysteroid receptor
P= Products

that for many of these 20-hydroxyecdysone-induced genes, there is no precise description of a physiological role.

Several 20-hydroxyecdysone-responsive genes have now been cloned and investigated (for review see Lepesant and Richards, 1989), including *hsp* genes, salivary glue genes, genes responsible for "early" and "late" puffs in the salivary glands, yolk protein genes, actin and β-tubulin genes and the *Drosophila* fat body genes P1 and LSP2.

In common with other steroid and thyroid hormones, ecdysteroids bind very strongly to a highly specific nuclear receptor (EcR), known to enhance transcription (Evans, 1988; Beato, 1989). The binding of the EcR to the ecdysone response element (EcdRE) is upstream of the ecdysone-responsive gene (Beato, 1989; Martinez and Wahli, 1990). The sequences of the known EcRE suggest that it consists of an imperfect palindromic sequence: 5'-TGA(AC)CY-3' (Riddihough and Pelham, 1987; Ozyhar et al., 1991; Cherbas et al., 1991), suggesting a strong and broad consensus between the response elements of (20-hydroxy)ecdysone, oestrogen, thyroid hormones and retinoic acid. This implied that the EcR and other steroid receptors would also exhibit conservation, which was borne out by Koelle et al., who first cloned the EcR gene from *Drosophila* (1991). Cloning of the EcR gene has now been achieved in *Chironomus tetans* (Imhof et al., 1993) and *Manduca sexta* (Segraves and Woldin, 1993).

The expression of the cloned gene would appear to be developmentally regulated. Late embryo and pre-pupal stages, of *Chironomus* were found to have a high abundance of transcripts (Imhof, et al., 1993; Dorsch-Häsler et al., 1990). Interestingly, the ecdysone titre is also reported to peak during late embryo and prepupal life (Laufer et al., 1986).

In *Drosophila*, at least three proteins are derived from the EcR gene, labelled A, B1 and B2 (Segraves and Hogness, 1990; Hogness, et al., 1992). They differ in their N-termini, but have common DNA- and hormone-binding sequences (Hogness et al., 1992). It is envisaged that the three proteins will bind to the EcREs, but may have different effects on transcription.

The heat shock protein, *hsp 27*, is an example of a typical ecdysone-responsive gene. The EcR binds to a 124bp region, containing the palindromic hexamer -575 to -455bp upstream of the gene start site (Morganelli et al., 1985; Riddihough and Pelham, 1987). A 23bp sequence in the *hsp 27* promoter region is sufficient to convey 20-hydroxyecdysone inducibility on a reporter gene (Ozyhar et al., 1991).

Obviously this is an exciting facet of ecdysteroid action, with many questions as yet unanswered. The regulatory hierarchy of genes controlling the development of the insect is extremely complex. There appear to be profound differences in EcR mRNA levels in the metamorphic response. Almost all tissues are ecdysteroid targets, at some stage of development, but, for example, during the larval-larval moult, known targets are only a small percentage of the total tissues. It remains to be seen what levels of mRNA are evident during pupariation and embryogenesis, and how the production of the EcR itself is regulated.

1.6 BIOSYNTHESIS OF ECDYSTEROIDS

The exact scheme of biosynthesis has not yet been fully established (Warren and Hetru, 1990). Since ecdysteroid concentrations in insects are so low, it has been quite a task to "trap" the intermediates, which, to compound matters, do not accumulate appreciably. Our knowledge is

such that only the first and last steps are fully established, (Rees, 1985). Insects require dietary sterols, to synthesize other steroids and for membrane production, as they cannot perform *de novo* synthesis, (Clayton, 1964). The diet provides a C27 sterol such as cholesterol (1) in carnivores and a C28 or C29 sterol in phytophagous insects, which are dealkylated at C-24. During ecdysteroid biosynthesis, the C27 precursor (cholesterol) undergoes alterations to the steroid nucleus, and is then modified on the side chain (figure 1.8; *cf* vertebrate steroid hormone synthesis, whereby the side-chain cleavage is the initial step).

7-Dehydrocholesterol (2) has been shown to be derived from cholesterol, in the prothoracic glands of several insects (for reviews see Rees, 1985 or 1989). The fact that the 7β- and 8β-hydrogens are removed for the formation of the Δ^7 bond in both 7-dehydrocholesterol and ecdysteroids, is consistent with a role for 7-dehydrocholesterol as an intermediate, (Cook et al., 1973; Johnson et al., 1975).

The following steps of the pathway are less well understood, the 3α- and 4β-hydrogens of cholesterol are eliminated, resulting in a 3-oxo-Δ^4 steroid (3) containing the A/B ring *cis* junction. In the classical pathway, this is reduced at C-3 to form a hydroxy group, there is addition of a 5β−hydrogen at C-5 and reduction of the Δ^4 double bond (5). 3β-Hydroxy-5α-cholest-6-one (16, figure 1.9) has been reported in *Bombyx mori* prothoracic glands *in vitro* when incubated with cholesterol (Sakurai et al., 1977). However, this could not be converted in the larval or adult female of *Schistocerca gregaria in vivo* (Milner et al., 1986) or in *Manduca sexta* prothoracic glands *in vitro* (Gilbert et al., 1980).

5β-Ketol (2,14,22,25-tetradeoxyecdysone) (5) is poorly incorporated into 20-hydroxyecdysteroids in pupariating *Calliphora stygia* (Faux et al., 1979), *Manduca* prothoracic glands (Bollenbacher et

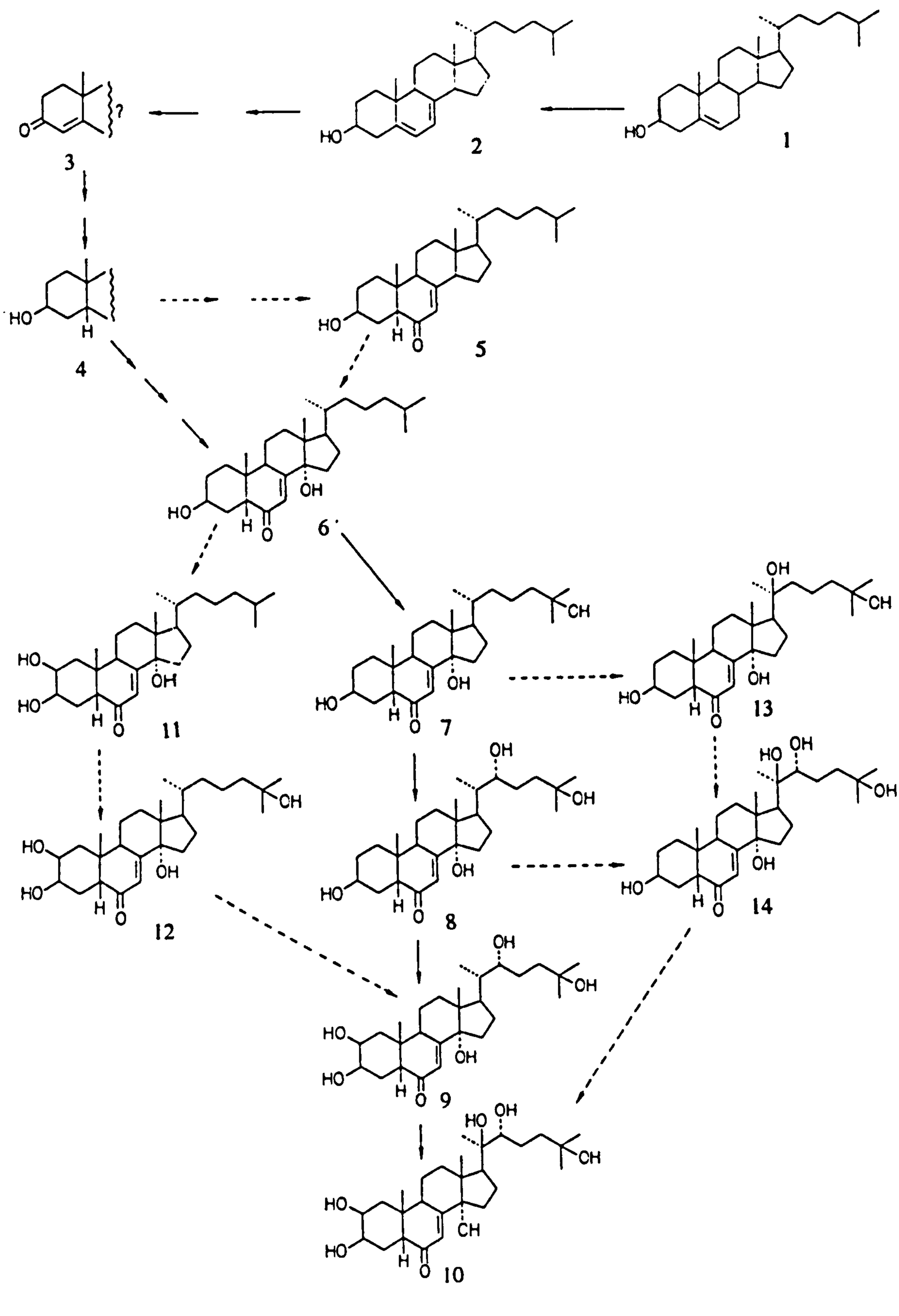
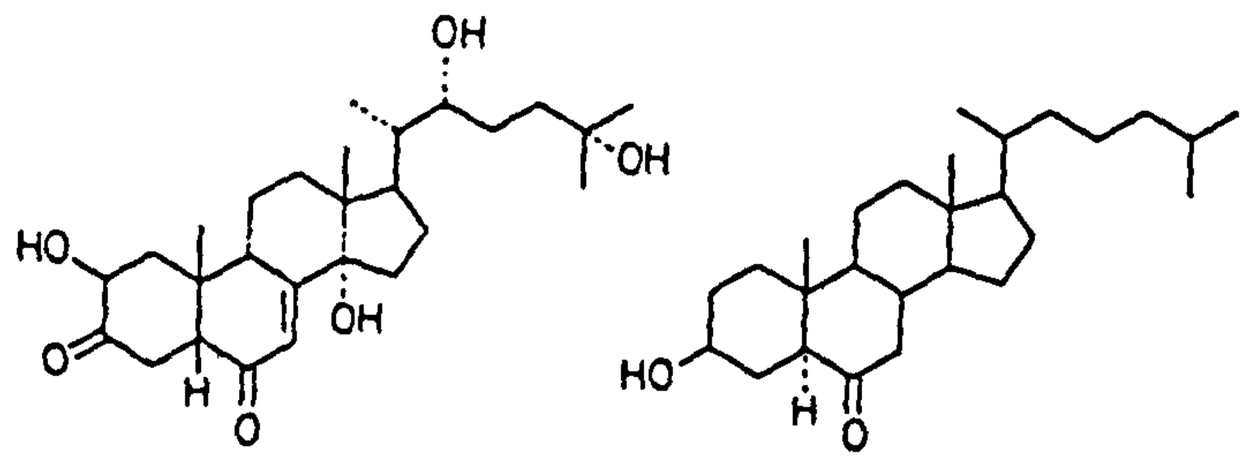


Figure 1.8. The classical scheme of ecdysteroid biosynthesis (after Rees, 1989)



15 16

Figure 1.9. 3-Dehydroecdysone and 3β-Hydroxy-5α-cholest-6-one

al., 1977) or *Locusta* prothoracic glands (Haag et al., 1987); 5β-ketol (5) is primarily converted into 14-deoxyecdysone. This strongly suggests that 14-hydroxylation must occur early on during the nuclear changes. Therefore a substrate other than 5β-ketol has been postulated.

That 2,22,25-trideoxyecdysone [5β-ketodiol, (6)] is involved as an ecdysteroid precursor was first demonstrated by its incorporation into ecdysone in *Manduca* prothoracic glands, (Bollenbacher, et al., 1977). More recently, Kappler et al., (1986a) have shown the transformation into ecdysteroids in the follicle cells of *Locusta*; 5β-ketodiol has also been found to be converted into ecdysteroids in other insect systems (Rees, 1985). Subsequent to this, hydroxylations at C-25, 22 and 2 take place.

Since 2,22-dideoxyecdysone (7), 2-deoxyecdysone (8) and ecdysone (9) have all been isolated from the ovaries of *Locusta,* this may suggest the order of the hydroxylations, (Hetru et al., 1978). *In vitro* incubation of follicle cells (able to produce 20-hydroxyecdysteroids), with 2,22,25-trideoxyecdysone (6) leads to the formation of 2,22-dideoxyecdysone (7) and 2,22-dideoxy-20-hydroxyecdysone (13). 2,25- (11) and 22-deoxyecdysone (12) derivatives were also isolated, but were could not be converted into ecdysone or 20-hydroxyecdysone (10), on reincubation, (Kappler, et al., 1986a).

When the prothoracic glands from *Locusta* are incubated with 22,22,25-trideoxyecdysone (6), the same metabolites are isolated, (Meister et al., 1985), leading to the supposition that the major hydroxylation sequence is at C-25, then C-22 and finally C-2. It would however, be quite likely that more than one pathway operates.

The enzymes involved in the biosynthesis of ecdysteroids are not as well studied as for the vertebrate steroid hormones, due in part to the

incomplete elucidation of the biosynthetic pathway. The first step, the conversion to 7-dehydrocholesterol (Grieneisen et al, 1993), as well as the terminal hydroxylations are mediated by cytochrome P-450 enzymes located in mitochondria, [C-2 hydroxylase, (Kappler et al., 1986b; Kabbouh et al., 1987) and C-22 hydroxylase, (Kappler et al., 1989)] and microsomes/ mitochondria, [C-25-hydroxylase, (Kappler, et al., 1989)].

Ecdysone is hydroxylated at C-20, to produce the major active hormone, 20-hydroxyecdysone, (10) in peripheral tissues such as the mitochondria and microsomes of the midgut, and in the microsomes of fat body and Malpighian tubules (Weirich et al, 1984). The ecdysone 20-monooxygenase (EC 1.14.99.22) is cytochrome P-450-dependent and possibly the most well studied enzyme of ecdysone metabolism, (for reviews see Weirich et al., 1984; Smith, 1985).

The reported secretion of 3-dehydroecdysone (15, figure 1.9) from the prothoracic glands of Lepidopteran species (Warren, et al., 1988) puts an interesting perspective on the elucidation of the biosynthetic pathway, since 3-dehydroecdysteroids may have a part to play as precursors to ecdysone.

1.7 ECDYSTEROIDS: ACTIVATION AND INACTIVATION

As alluded to previously, activation of ecdysteroids may be summarized by the following:

a) conversion of the secreted 3-dehydroecdysone into ecdysone (Lepidoptera);

b) conversion of ecdysone into 20-hydroxyecdysone;

c) hydrolysis of stored conjugated ecdysteroids.

At appropriate times after fulfilling their functions, ecdysteroids undergo a variety of inactivation processes. Although ecdysone and 20-

hydroxyecdysone can be excreted unchanged, via the Malpighian tubules and the gut, ecdysteroids are also inactivated to a variety of conjugates, as well as irreversibly into 3α-epimers and ecdysteroid 26-oic acids. The 3α-epimers will be considered more fully in Chapter 5, but ecdysteroid acids and conjugates will be discussed here. Ecdyson-26-oic acid is formed from 26-hydroxyecdysone in tissues such as the fat body. In addition to inactivating ecdysone, the acid formation may also facilitate excretion, (Warren and Gilbert, 1986). Polar and apolar conjugates, for example, phosphates, fatty acyl groups, glucose, double phosphates and aceto-phosphates, are generally considered to be inactivation products. After their formation, the ecdysteroid is prevented from reabsorption by the haemolymph and hence, is excreted, (Warren and Gilbert, 1986).

In closed systems such as pupae and developing embryos, excretion is not possible and irreversible inactivation of ecdysteroids is vital. In locusts, embryos inactivate ecdysteroids by forming a wide range of products, including 3-epi-2-deoxyecdysone 3-phosphate, 3-acetylecdysone 2- or 22-phosphate and (20-hydroxy)ecdyson-26-oic acid, (for comprehensive review see Rees and Isaac, 1984). Similarly, in *Manduca* pupae dispose of ecdysteroids by conversion into predominantly C-26 hydroxy derivatives of ecdysone and 20-hydroxyecdysone, including their conjugates, plus 3-epi(20-hydroxy)ecdysone (Warren and Gilbert, 1986).

The best documented enzymes of inactivation are those of the midgut cytosol (for review see Weirich, 1989). The scheme elucidated is shown in figure 1.10; for simplicity only the conversions for ecdysone are shown, although the same reactions apply to 20-hydroxyecdysone. 3-Epimerization occurs via 3-dehydro-derivatives in several insect species:

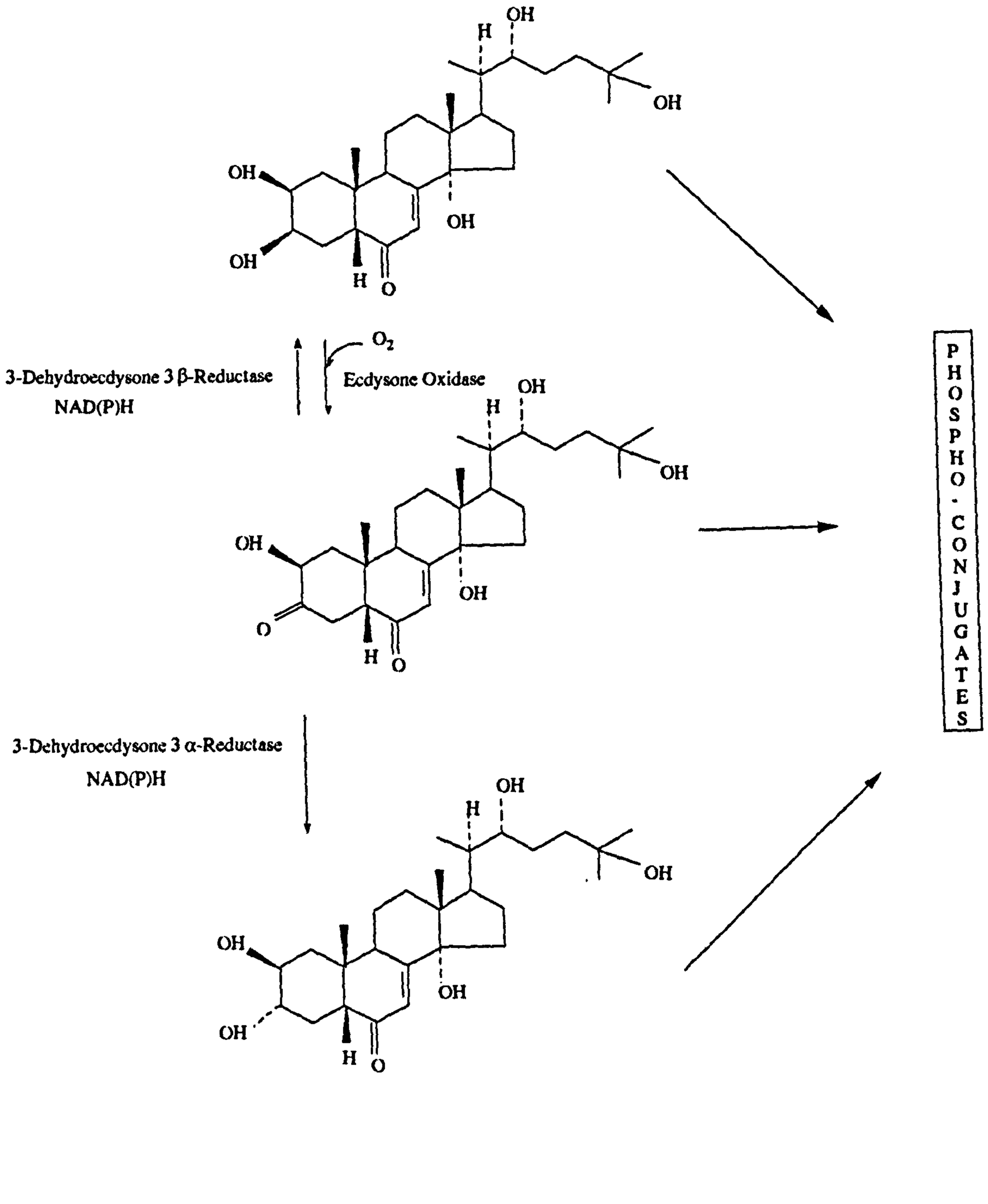


Figure 1.10. Ecdysteroid inactivation in the midgut

Spodoptera littoralis (Milner and Rees, 1985), *Pieris brassicae* (Blais and Lafont, 1984) and *Manduca sexta* (Mayer et al., 1979).

1.7.1 Ecdysone Oxidase

This enzyme is widely distributed in the class *Insecta*, (Koolman, 1978) and can be isolated from post-microsomal supernatants (cytosol) of insect midgut. It catalyses the conversion of (20-hydroxy)ecdysone into 3-dehydro(20-hydroxy)ecdysone. The correct name, ecdysone:O2-oxidoreductase (EC 1.1.3.16) is suggestive of a redox reaction; two hydrogen atoms are transferred from the ecdysteroid at C-3 to molecular oxygen, yielding hydrogen peroxide and 3-dehydro-derivatives. Most of this type of two-electron transferring enzymes are flavoproteins, (e.g. bacterial cholesterol oxidase, Smith and Brooks, 1976). However this oxidase differs very significantly, since cosubstrates FAD, FMN, NAD^+ and $NADP^+$ do not serve to alter the activity. Hitherto, the nature of the prosthetic group of the enzyme is unknown.

1.7.2 3-Dehydroecdysteroid 3α-and 3β-Reductases

When assays for ecdysone oxidase are carried out using a post-microsomal midgut preparation, (i.e. non-dialysed and not having undergone gel filtration), 3-dehydroecdysone is incredibly difficult to detect, since it is rapidly converted into 3-epiecdysone by an active 3-dehydroecdysone 3α-reductase, (EC 1.1.1) (Nigg, et al., 1974). However, by eliminating the appropriate cofactors, the reaction sequence may be limited to the production of 3-dehydroecdysone. NADH and NADPH are commonly removed by extensive dialysis

(Milner and Rees, 1985) or by gel exclusion chromatography, (Weirich et al., 1989) .

In addition, a second enzyme, 3-dehydroecdysone 3β-reductase (EC 1.1.1) may reduce the 3-dehydroecdysone using NAD(P)H to produce the parent ecdysone. Both the 3-dehydroecdysone 3α- and 3β-reductases have activity with either NADH or NADPH as cofactor. *Pieris brassicae* enzymes have only been tested with NADPH and were found to produce ecdysone, with very little of the epimer (Blais and Lafont, 1984). In *Manduca sexta*, the 3β-hydroxy-forming enzyme also has preference for NADPH and the 3α-hydroxy-forming one NADH, (Weirich et al., 1989).

The reverse is true for crude midgut cytosol from *Spodoptera littoralis*, (Milner and Rees, 1985) indicating that the cofactor requirements are species specific.

1.7.3 ATP:Ecdysteroid Phosphotransferases

A host of ecdysteroid conjugates has been isolated and characterized, (Lafont and Koolman, 1984; Rees and Isaac, 1984; Thompson et al., 1985; Thompson et al., 1987). Ironically very few conjugate-forming enzymes have been characterized in detail, (Kabbouh and Rees, 1991a,b; Weirich et al., 1986). In the midgut inactivation sequence, it would seem that generally phosphates are the predominant polar metabolites, (Weirich, et al., 1986; Yang and Wilkinson, 1973), four conjugates of ecdysone and two of 3-epiecdysone were resolved (Weirich, et al., 1986), presumably phosphorylation having taken place on the hydroxyl groups located at C-2, C-3 and C-22. As in the *Schistocerca gregaria* follicle cell preparations, (Kabbouh and Rees, 1991a), phosphotransferases in the midgut of *Manduca sexta* (EC 2.7.1)

would appear to be dependent on ATP and Mg^{2+} ions (Weirich, et al., 1986).

1.8 AIMS OF THE PROJECT

The overall purpose of the work has been to characterize aspects of the activation and inactivation of ecdysteroids in last (sixth) instar larvae of the cotton leafworm, *Spodoptera littoralis.* A complete understanding of the role of 3-dehydroecdysone is pivotal to a fuller appreciation of the metabolic route taken by the ecdysteroid, from the initial secretion to the final inactivation of the molecule. As mentioned previously, a haemolymph 3-dehydroecdysteroid 3β-reductase in *Manduca sexta* (Sakurai et al., 1989b), has been correlated to the secretion of 3-dehydroecdysone from the prothoracic glands (Warren et al., 1988; Kiriishi, et al., 1990). Several questions remain unanswered concerning the enzyme's characterization and purification, these will be addressed in detail.

In view of the fact that 3-dehydroecdysteroids may be precursors on the biosynthetic pathway to active moulting hormone, and additionally inactivation products, it is fascinating to relate the haemolymph 3-dehydroecdysone 3β-reductase enzyme with the inactivation enzymes of the midgut, in particular to compare the two 3-dehydroecdysteroid 3β-reductase activities and to deduce any integrated functions.

In essence therefore, the work will concern the purification and characterization of the enzymes, with attention being given to the quantitative changes in the profiles of ecdysteroid activation/inactivation activities during the instar.

Chapter 2

Materials and Methods

2.1 MATERIALS

Solvents

a) General Purpose Solvents

The following solvents were obtained from FSA Laboratory Supplies, Loughborough, U.K:

Chloroform

1,2-Dichloromethane

Hexane

Tetrahydrofuran

Water was glass distilled

Ethanol and Methanol were purchased from the Department of Chemistry, University of Liverpool, and redistilled before use. In certain applications, dry solvents were required and prepared as follows:

Preparation of Dry, Redistilled Ethanol

Absolute ethanol (7ml) was warmed to 50°C with clean, dry magnesium turnings (0.5g) and iodine (0.05g), until the brown colouration due to iodine disappeared. The solution was then refluxed for 1-2h, such that the magnesium was converted to magnesium ethylate. A further 80ml of absolute ethanol were added and the refluxing continued for 30min. After allowing the apparatus to cool, the ethanol was distilled and collected in a vessel containg molecular sieve 4A. The ethanol was stored over anhydrous calcium chloride, in a dessicator.

Preparation of Dry, Redistilled Acetone and Tetrahydrofuran

The solvent (50ml) was dried over molecular sieve 4A and placed in a round-bottomed flask with a small amount of lithium aluminium hydride (0.1g). The solvent was distilled and used immediately.

b) HPLC-Grade Solvents

All purchased from FSA Supplies:

Acetonitrile

1,2-Dichloroethane

Methanol

Ecdysteroids

Ecdysone was obtained from Simes, Milan, Italy.

20-Hydroxyecdysone was a kind gift from Dr. G.B. Russell, DSIR, Palmerston North, New Zealand.

[23,24-$^{3}H_2$]Ecdysone (≈89.0Ci/mmol) was from New England Nuclear, Boston, Mass., U.S.A.

2,22,25-Trideoxyecdysone (5β-Ketodiol) was a generous gift from Dr. Chen Yu-Gun, Shanghai Institute of Organic Chemistry, PR China.

[22,23,24,25-$^{3}H_4$]2,22,25-Trideoxyecdysone (107Ci/mmol) was a kind gift of Dr. C. Hetru and Prof. J.A. Hoffmann, CNRS, Strasbourg, France.

3-Oxo-2,22,25-trideoxyecdysone (5β-diketol; 3-oxo-5β-ketodiol) was a gift from Mr.I.P. Hargreaves, Department of Biochemistry, University of Liverpool, U.K.

[1,2-$^{3}H_2$]3-Oxo-2,22,25-trideoxyecdysone (5β-diketol; 3-oxo-5β-ketodiol) [47Ci/mmol] was a gift from Dr. C. Hetru and Prof. J.A. Hoffmann, CNRS, Strasbourg, France.

Ecdysone 2,3-acetonide was a gift from Dr. R.M. Magee, Department of Biochemistry, University of Liverpool, U.K.

3-Dehydroecdysone and 3-epiecdysone were chemically synthesized as described in section 2.2.13

Antisera

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

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

Enzymes

Crude aryl sulphatase (*ex. Helix pomatia*) was bought from Sigma Chemical Co., Poole, Dorset, U.K.

Peroxidase and glucose oxidase were from Boehringer-Mannheim GmbH, Mannheim, Germany.

Cofactors

NADH, NADPH and ATP were purchased from Boehringer-Mannheim GmbH.

Buffers

Buffer A	10mM Tris-HCl, pH7.4.
Buffer B	10mM Tris-HCl, pH 7.4, containing 0.2%(v/v) mercaptoethanol.
Buffer C	100mM sodium phosphate, pH7.5, containing 1.36M ammonium sulphate and 0.2%(v/v) mercaptoethanol.

Chromatography

Kieselgel GF254 0.063-0.2mm particle size, from Merk, Darmstadt, Germany, was used to prepare TLC plates.

Kieselgel F254 aluminium-backed pre-prepared TLC plates were obtained from Merk.

C18 SEP-PAK cartridges were from Millipore (U.K.) Ltd., Watford, Herts., U.K.

C18 Nova-Pak and amino-propyl silica HPLC columns (10cm x i.d. 8mm, 4µm paricle size) were also obtained from Millipore (U.K.).

DE-52 (DEAE-Cellulose) microgranular was obtained from Whatman, Maidstone, Kent, U.K.

Mono-Q HR5/5 (5cm x i.d.5mm) and phenyl superose HR5/5 (5cm x i.d.5mm) columns were bought from Pharmacia, Uppsala, Sweden.

Liquid Scintillation Fluid

OptiPhase "Safe" and OptiPhase "Hisafe"were produced by FSA Supplies, Loughborough, Leics., U.K. and obtained through LKB Instruments Ltd.

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

Other Chemicals

ABTS (2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid) was obtained from Boehringer-Mannheim GmbH, Mannheim, Germany.

Sodium borohydride and platinum (IV) oxide were from Aldrich Chemical Co., Gillingham, Dorset, U.K.

Glacial acetic acid was purchased from FSA Supplies.

Phenylthiourea and silver nitrate were obtained from Sigma Chemical Co.

All other chemicals were of the highest analytical grade available and puchased from Sigma Chemical Co. or BDH Ltd.

Insects

Spodoptera littoralis were initially obtained from Rhône-Poulenc, Ongar, Essex, U.K.

2.2 GENERAL METHODS

2.2.1 Culture of *Spodoptera littoralis*

Spodoptera littoralis (cotton leafworm) was reared under licence from the Ministry of Agriculture, Fisheries and Food. They were maintained at 28°C and a relative humidity of 60-70% under an 16:8 hour light:dark regime. An artificial agar-based diet was fed to the larvae and a 5%(w/v) sucrose solution to the adult moths, (McKinley, 1970 as modified in Hoggard, 1989).

The last instar larvae were amenable to accurate synchronization: late 5th instar larvae were selected prior to the dark period. At the end of the dark phase (scotophase), all insects which did not have fused head and mouthparts capsule were placed back into the general culture. The newly moulted sixth instar larvae were designated t=3h (±3h), assuming an average moult midway through the dark period.

2.2.2 Dissection

Insects were anaesthetized by immersion into cold water. Midguts were exised in ice-cold Ringer solution [130mM NaCl, 4.6mM KCl, 1.9mM $CaCl_2$, (Bodenstein, 1946)]. Food boluses were removed by

gently squeezing out the contents of the gut. Haemolymph was collected in a capillary tube by making a small incision behind a dorsal pro-leg and transferred to an Eppendorf tube containing a few crystals of phenythiourea.

Prothoracic glands were located by light microscopy, entwined around the trachaea entering the spiracles of the prothorax; glands were carefully extirpated and thoroughly rinsed in Grace's Insect Medium (Gibco Ltd., New York, U.S.A.).

2.2.3 Preparation of Cytosolic Post-Microsomal Supernatant

Approximately 20 *S. littoralis* larvae were routinely dissected in ice-cold Insect Ringer solution and the midguts excised. They were then thoroughly homogenised by 24 passes of a Potter-Elvehjem homogeniser, in 10mM Tris-HCl buffer, pH 7.4, (buffer A), at 4°C. The homogenate was centrifuged at 15,000g for 5min in a Burkard Refrigerated Centrifuge (Koolspin) at 4°C, the pellet was resuspended in the Tris-HCl buffer, rehomogenised and centrifuged again as before. The resulting supernatants were combined and centrifuged at 100,000g for 1h in a Sorvall OTD 50 ultracentrifuge. The cytosol was dialysed against 100 volumes of buffer A for approximately 18h.

Haemolymph was collected and haemocytes and debris were sedimented at 15,000g for 5min. A supernatant dialysate was used for all 3-dehydroecdysteroid 3β-reductase assays.

2.2.4 Standard Enzyme Assay Procedures

The standard enzyme assays were developed on the basis of work carried out in Chapters 4 and 6. The respective substrates and cofactors

used and the optimum reaction conditions are detailed in Table 1. The final incubation concentrations of substrates and cofactors are indicated.

Enzyme preparation (100µl) was added to the required cofactor, dissolved in 100µl 0.2M sodium phosphate buffer, at the appropriate pH. The [23,24$^3H_2$]ecdysteroid substrate (8.4µM) containing 200,000dpm was dissolved in 0.2M sodium phosphate buffer (50µl), and after a 5-10min equilibration period, was added to the incubation mixture (total incubation volume, 350µl).

Reactions were terminated by addition of chilled ethanol (400µl). The mixture was then centrifuged for 10min at 9,000g at 4°C, the supernatant collected, the pellet re-extracted with methanol and the combined alcoholic extracts evaporated to dryness under vacuum. The products of incubation were analyzed by HPLC with monitoring of radioactivity using an on-line detector. Results were expressed in nmol/h/mg protein.

In some experiments (e.g. for the verification of the enzymic product(s) in Chapters 3 and 5 and in the determination of optimal conditions for the ecdysone oxidase and reductases, Chapter 5) activity was assayed using unlabelled ecdysteroid. The reaction products were analyzed following HPLC by UV detection at 254nm.

After partial purification of the enzymes on DEAE-cellulose, the K_m values were determined (See Chapters 4 and 6). The K_m values were in the range 20-230µM, suggesting that the substrate concentrations should be increased.

Table 1 The enzyme assay requirements

Enzyme	Substrate	Cofactor in assay	max. protein[a] (mg/ml)	approx. pH	Temperature °C	max. linear reaction time (min)
Ecdysone oxidase	Ecdysone	O_2	1.7	6.5	40	90
3α-Reductase[b] (NADH)	3-Dehydroecdysone	0.5mM NADH	1.2	7.1	37	20
3α-Reductase[b] (NADPH)	3-Dehydroecdysone	0.5mM NADPH	0.1	7.5	35	20
3β-Reductase[c] (NADH)	3-Dehydroecdysone	0.5mM NADH	1.0	6.5	35	20
3β-Reductase[c] (NADPH)	3-Dehydroecdysone	0.5mM NADPH	0.5	7.9	37	<5
Ecdysone 22-phosphotransferase[d]	Ecdysone	10mM $MgCl_2$ 2mM ATP	0.7	7.8	33	10
Haemolymph 3β-Reductase[c] (NADPH)	3-dehydroecdysone	0.5mM NADPH	1.0	7.5	37	20

[a] maximum concentration of protein in a dialysed cytosolic preparation of enzyme yielding linear rates of reaction

[b] full name: 3-dehydroecdysone 3 α-reductase

[c] full name: 3-dehydroecdysone 3 β-reductase

[d] full name: ATP:ecdysone 22-phosphotransferase

[e] full name: ATP:ecdysone 2-phosphotransferase

2.2.5 Purification of the Enzymes

DEAE-Cellulose Chromatography

The protein was applied to a 30 x 2.5cm column containing DE-52, previously equilibrated with 10mM Tris-HCl buffer, pH 7.4, containing approximately 0.2% mercaptoethanol (buffer B). Protein was eluted using a linear salt gradient generated from 220ml buffer B and 220ml 0.5M NaCl in buffer B, at approximately 40ml/h and 8ml fractions were collected.

Fast Protein Liquid Chromatography (FPLC)

a) Ion- Exchange Chromatography

Appropriate fractions from DEAE-cellulose chromatography were dialysed against buffer A and filtered through a 0.45µm disposable syringe filter (Acrodisc, Gelman Sciences). The protein was then applied to an HR 5/5 (5cm x 5mm i.d.) Mono-Q anion-exchange column (comprised of quaternary amino ethyl substituent groups), pre-equilibrated in buffer B. The enzymes were eluted using a linear gradient of 0.5mM NaCl in buffer B, typically at a flow rate of 1ml/min with collection of 0.5ml fractions.

b) Hydrophobic Interaction Chromatography (HIC)

Appropriate fractions from the previous chromatographic separation were combined, dialysed in buffer B, and ammonium sulphate added to a final concentration of 1.36M. After filtration of the protein through a syringe filter, it was applied to a Phenyl Superose HR 5/5 column (5cm x 5mm i.d.), pre-equlibrated in 100mM sodium phosphate buffer pH 7.5, containing 1.36M ammonium sulphate (buffer C). Elution was by a linear 17ml gradient of buffer C against 10mM

sodium phosphate buffer pH7.5, at approximately 0.5ml/min, with collection of 0.5ml fractions.

2.2.6 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed according to the method of Laemmli, (1970) and Laemmli and Favre (1973), using an LKB 2001 vertical electrophoresis system. The resolving gel was 12.5% acrylamide and the stacking gel 5%. Polypeptide molecular weights were estimated by comparison to the mobilities of standard molecular weight marker proteins, on a calibration curve. Proteins were visualised by either Coomassie Blue, or by silver staining (below).

2.2.7 Silver Staining

Gels were thoroughly washed in ethanol: acetic acid: water mixtures [5:1:4 (2 x 2h); 25:10:65 (2 x 1h); 10: 0.5: 89.5 (2 x 1h)] and then stained according to the method of (Sammons et al., 1981), using silver nitrate (1.9g/l). The excess silver was removed by a short rinse in deionized water (10s), and incorporated silver was reduced by a 10min wash in sodium borohydride (87.5mg/l) and formalin (7.5ml/l of ≈ 40%(v/v) formaldehyde) in 0.75M sodium hydroxide solution. The colours were enhanced by two 1h rinses in sodium carbonate solution (7.5g/l). Gels could be stored for several days in the final solution.

2.2.8 Protein Estimation

Protein was estimated by the folin-phenol method using BSA as the protein standard. (Lowry et al., 1951).

2.2.9 Chromatography

C18 Reversed-Phase SEP-PAK

The cartridges were activated with 5ml methanol, followed by 10ml deionized water. The sample (≤ 5mg steroid) was applied in 10% (v/v) aqueous methanol (2ml) and eluted using 2ml 10%(v/v) methanol/water (to elute salts and proteins), 4ml 30%(v/v) methanol/water (to elute polar ecdysteroids, including 26-oic acids and any unhydrolysed conjugates), 6ml 60%(v/v) methanol/water (to elute free ecdysteroids), 4ml 100%(v/v) methanol (to elute apolar ecdysteroids).

Thin-Layer Chromatography (TLC)

Ecdysteroids were separated on 0.25mm (analytical) or 0.5mm (preparative) plates and developed using 20% (v/v) methanol in chloroform. Bands were visualized by their absorbance under a uv lamp (254nm) and eluted in 50% (v/v) methanol / 1,2-dichloromethane.

High Performance Liquid Chromatography (HPLC)

Samples containing free ecdysteroids were analyzed using a C18 Nova-Pak column (10cm x 5mm, 10µm particle size, Waters Associates, Northwich, Ches., U.K.), linked to Waters model 440 UV detector, set at 254nm. Radioactivity was analyzed by an on-line Flo-One\ Beta radiomatic detector, using Flo-Scint III scintillation fluid. An isocratic

mobile phase of 0.1% (v/v) trifluoroacetic acid in water: acetonitrile (78:22 v/v) was employed at 1.0ml/min (System 1). Adsorption HPLC was carried out using an aminopropyl silica Nova-Pak column (Millipore U.K.) eluted with 8% (v/v) methanol in 1,2-dichloroethane at 1.0ml/min (System 2).

Polar ecdysteroids (conjugates) were identified by two reversed phase systems: i)using a linear 30min gradient of 20-70% (v/v) methanol in 20mM citrate buffer, pH 6.5, at 1.0ml/min (System 3) and ii) a linear 60min gradient of 8-40% (v/v) acetonitrile in 20mM Tris-perchlorate buffer, pH 7.5 at a rate of 1.0ml/min (System 4).

2.2.10 Hydrolysis of Conjugates

The methanolic extracts were dried under nitrogen and fractionated on a Sep-Pak cartridge. The 30% (v/v) methanol/water fraction was evaporated to dryness and dissolved in 1ml 0.1M MES buffer (4-morpholine ethanesulphonic acid), pH 5.5. 250Units of a crude arylsulphatase (*ex Helix pomatia*) preparation were added and the mixture incubated at 37°C; the reaction was stopped after 17h by addition of 4ml methanol and the precipitated protein sedimented by centrifugation. The ecdysteroids were extracted three times with 4ml methanol and the extracts combined and evaporated to dryness. The hydrolysed ecdysteroids were applied to a C_{18} Sep-Pak cartridge in 10% (v/v) methanol/water (2ml), any unhydrolyzed polar ecdysteroids eluted with 30%(v/v) methanol/water fraction and the free ecdysteroids released from conjugates eluted in the 60%(v/v) methanolic fraction.

2.2.11 Negative Ion Fast-Atom Bombardment Mass-Spectrometry (FAB-MS)

Spectra were obtained on a VG 7070H mass spectrometer, using a primary atom beam of xenon and glycerol as the probe solvent. (For conditions see Isaac et al., 1983.)

2.2.12 Nuclear Magnetic Resonance Spectroscopy (NMR)

^{1}H-n.m.r. spectra were obtained at 400.13MHz on a Bruker WH400 spectrometer. Samples were dissolved in [$^{2}H_5$]pyridine and spectra were referenced to trimethylsilane.

2.2.13 Preparation of Ecdysteroids

Ecdysteroids were prepared according to Dinan and Rees (1978).

3-Dehydroecdysone

Platinum (IV) oxide (95mg) was added to 4ml glacial acetic acid in a non-silylanized 4dram vial. Hydrogen was introduced to the mixture, for approximately 1min, the vial was capped and the mixture stirred. As the brown platinum (IV) oxide was reduced, it turned to black platinum (II) oxide and sank to the bottom of the vial, such that the acetic acid could be decanted off. The catalyst was then exhaustively washed with deionized water to remove any traces of acid. 75mg ecdysone were dissolved in 40ml warmed deionized water and added to the catalyst. A gentle stream of oxygen was bubbled continuously through the mixture, which was carefully warmed. The reaction was monitored using TLC on microscope slides, until the optimum amount of was product formed, when 100ml methanol was added. The best yields typically occurred after 6-8hours, with heating, and >48hours

without. The identity of the product was confirmed by FAB-MS (Appendix A, spectrum 2) and NMR (Appendix B, spectrum 2).

3-Epiecdysone

3-Dehydroecdysone (30mg) was dissolved in 2ml dry, redistilled ethanol / dry, redistilled tetrahydrofuran [1:1 (v/v)]. Sodium borohydride (10mg) was carefully added and the mixture allowed to react for 10min at room temperature. The reaction was terminated by addition of one drop of glacial acetic acid. The identity of 3-epiecdysone was verified by FAB-MS (Appendix A, spectrum 3) and NMR (Appendix B, spectrum 3).

Ecdysteroid Acetonides

The ecdysteroid (50µg) was dissolved in 100µl dry acetone, together with approximately 1mg phosphomolybdic acid as catalyst. The mixture was stirred for 30min at ambient temperature, the reaction terminated by addition of 2ml deionized water and a few crystals of potassium hydrogen carbonate. After evaporating to dryness, the ecdysteroids were fractionated by C_{18} Sep-Pak chromatography. The acetonides of free ecdysteroids eluted in the 60% (v/v) methanol/water fraction, whereas acetonides of polar conjugates eluted in the 30% (v/v) methanol/water fraction.

2.2.14 High Performance Liquid Chromatography-Radioimmunoassay (HPLC-RIA)

a) HPLC

Biological ecdysteroid samples were separated on a Nova-Pak C_{18} Radial-Pak cartridge (8mm x 10cm) developed isocratically with

acetonitrile/ 0.1% TFA in water (22/78, v/v) [system 1]. 1.0ml fractions of eluate were collected for radioimmunoassay.

b) RIA

A series of tubes containing known amounts of ecdysone in methanol (duplicates of 10, 20, 30, 50, 70, 100, 150, 200, 300, 500 and 1000pg) and three containg no ecdysone were set up. Solvent was removed from these tubes and those containg HPLC eluate in a Gyrovap centrifugal evapoator (Uniscience Ltd.).

0.1M Borate buffer was prepared by dissolving boric acid (6.18g), sodium tetraborate (9.54g) and sodium chloride (4.38g) in distilled water, adjusting the pH to 8.4 and making up to 1litre. Sufficient [23,24-$^{3}H_2$]ecdysone was dissolved in borate buffer to give a final amount of radioactivity of approximately 9000cpm/ 100µl. This solution was added to each tube (100µl) and vortex mixed.

DHS-1-13.5 or H-22 antiserum was diluted 1:1500 with 5%(v/v) inactivated rabbit serum and 100µl added to each tube, which was very briefly vortex mixed and incubated at 4°C for 18hours without shaking.

Saturated ammonium sulphate in borate buffer (200µl) was added and the tubes vortex mixed and chilled (4°C, 20min). The tubes were then centrifuged (4°C, approximately 10,000g, 7min.) and the supernatants withdrawn and discarded. The protein pellet was resuspended in 500µl saturated ammonium sulphate/ borate buffer (1:1, v/v), vortex mixed and centrifuged as before. The supernatants were withdrawn and the pellet dissolved in 100µl distilled water. 1.0ml OptiPhase "HiSafe" II was added and after vortex mixing each tube was transferred to a sealed scintillation vial for determination of radioactivity.

Chapter 3

Studies on the prothoracic glands of *Spodoptera littoralis*: the later stages of ecdysteroid synthesis and the nature of the secretory product

3.1 INTRODUCTION

The prothoracic glands are the primary, and in immature stages, possibly the sole source of ecdysteroids in insects. Several factors are known to influence the rate of ecdysteroid secretion, most notably prothoracicotropic hormone, (PTTH). This peptide acts as a positive control on ecdysteroid biosynthesis, by way of a cyclic nucleotide-protein kinase cascade, leading to synthesis and secretion, (Smith, 1985; Rountree et al., 1987). More recently, an ecdysiotropin from the hind-gut has been characterized in *Ostrinia nubilalis* and although the physiological function is unclear, this peptide has been shown to act directly on the prothoracic glands, thereby promoting ecdysteroid synthesis (Gelman et al., 1991). Environmental, nervous and other hormonal factors may also regulate and/or promote ecdysteroid synthesis. Photoperiod (Mizoguchi and Ishizaki, 1982), temperature (Meola and Adkisson 1977), neural activity (Richter and Gersch 1983) and JH (Watson et al., 1987, 1988) have all been listed as secondary effectors, driving PTTH secretion.

In 1988, Warren et al. described how 3-dehydroecdysone, and not ecdysone, was the major secretion from the prothoracic glands, as classically believed. Although all insect orders have not been examined, it would seem that the production of 3-dehydroecdysone takes place largely in Lepidopteran species, the ratio of this product to ecdysone varying widely, depending on species (Warren et al., 1988; Kiriishi et al., 1990). 3-Dehydroecdysone production is not exclusive to Lepidoptera; the prothoracic glands of *Periplaneta americana* secrete a 1:1 ratio of 3-dehydroecdysone : ecdysone (Kiriishi et al., 1990), however, a great many other orders (e.g. Diptera, Orthoptera, Coleoptera) secrete predominantly ecdysone. It is interesting that in

several crustacean species the ecdysiosynthetic Y-organs also produce a markedly greater proportion of 3-dehydroecdysone than that of ecdysone, (Spaziani et al., 1989; Sonobe et al., 1991; Böcking et al., 1992).

That 3-dehydroecdysone is primarily secreted and not ecdysone, has been the motivation for a reconsideration of the mechanism of the biosynthetic pathway. In the past two decades, it has been generally accepted that 2,22,25-trideoxyecdysone ("5β-ketodiol") is an intermediate of ecdysone biosynthesis (for reviews see Rees, 1985, 1989).

In *Manduca sexta* prothoracic glands maintained *in vitro*, [^{3}H]2,22,25-trideoxyecdysone is converted into ecdysone and a product which is probably 22-deoxyecdysone (Bollenbacher et al., 1977). Additionally, the prothoracic glands of *Locusta migratoria* can convert [^{3}H]2,22,25-trideoxyecdysone into 2,22-dideoxyecdysone, 22,25-dideoxyecdysone, 22-deoxyecdysone and 2-deoxyecdysone (Meister et al., 1985). Upon re-incubation, 22,25-dideoxyecdysone and 22-deoxyecdysone are poorly incorporated into ecdysone, but 2,22-dideoxyecdysone and 2-deoxyecdysone can be used as substrates for ecdysone synthesis (Kappler, et al., 1986a). [^{3}H]2-deoxyecdysone is also efficiently transformed into ecdysone *in vitro* by the prothoracic glands of *S. littoralis* (Jarvis, 1991) and the embryos and early larvae of *Locusta* (Meister et al., 1985). This suggests that the preferred route taken, at least in prothoracic glands, is that of hydroxylation at C-25, C-22 and finally C-2. Moreover, 2,22-dideoxyecdysone, 2-deoxyecdysone and ecdysone have all been isolated endogenously from ovaries of *Locusta migratoria* (Hetru, et al., 1978, 1982). However, as the pgs do not appreciably accumulate ecdysteroids, no precursors have been

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precursors have been isolated endogenously. Therefore, the order of hydroxylation is less clear than in ovaries.

Prompted by the fact that 3-dehydroecdysone may be the final end-product, [^{3}H]-3-oxo-5β-ketodiol has been used as a putative intermediate; recent studies have shown it to be incorporated into ecdysteroids and 3-dehydroecdysteroids by isolated prothoracic glands, (Blais and Lafont, 1991; Roussel, 1992). However, precisely when in the pathway the reduction of the C-3 oxo group occurs during biosynthesis of ecdysone, is unclear.

Finally, in insects, a factor located in the haemolymph has been found to convert 3-dehydroecdysone to ecdysone (Sakurai et al., 1989b; Kiriishi et al, 1990; Watson et al., 1985). Although first believed to be an ecdysteroid carrier protein, stimulating ecdysteroid synthesis in prothoracic glands, it is now known to be a 3-dehydroecdysteroid 3β-hydroxy forming reductase, requiring NAD(P)H. Since different insects have been found to possess variations in the level of reductase activity, (Kiriishi et al, 1990), it was important to initiate a study of this enzyme in *Spodoptera littoralis* by verifying the transformation of 3-dehydroecdysone into ecdysone.

The major objectives of the work in this chapter have been to determine the quantitative and qualitative nature of the secretion(s) from the prothoracic glands of *S. littoralis*. The secretion of 3-dehydroecdysone has posed many questions: not only is the exact stage of formation, in the biosynthetic pathway, of the 3-oxo group uncertain, but also whether this grouping has been maintained from the early putative precursor, the so-called "3-oxo-Δ^4 steroid" (See Figure 1.8). Both 5β-ketodiol and 3-oxo-5β-ketodiol have been examined as potential precursors to 3-dehydroecdysone / ecdysone in larval *Spodoptera* prothoracic glands.

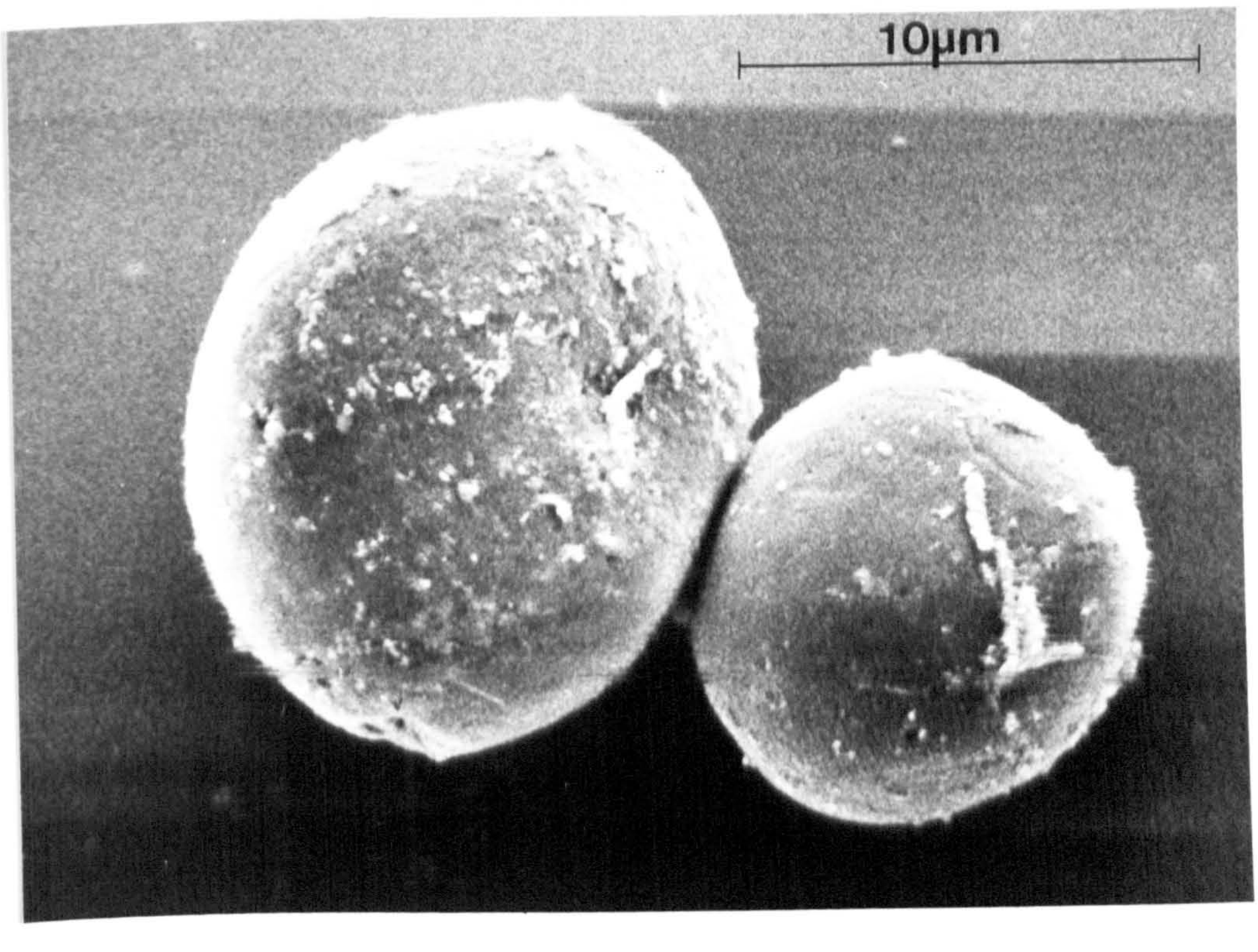


Figure 3.1 Scanning elecron micrograph of two prothoracic gland cells.

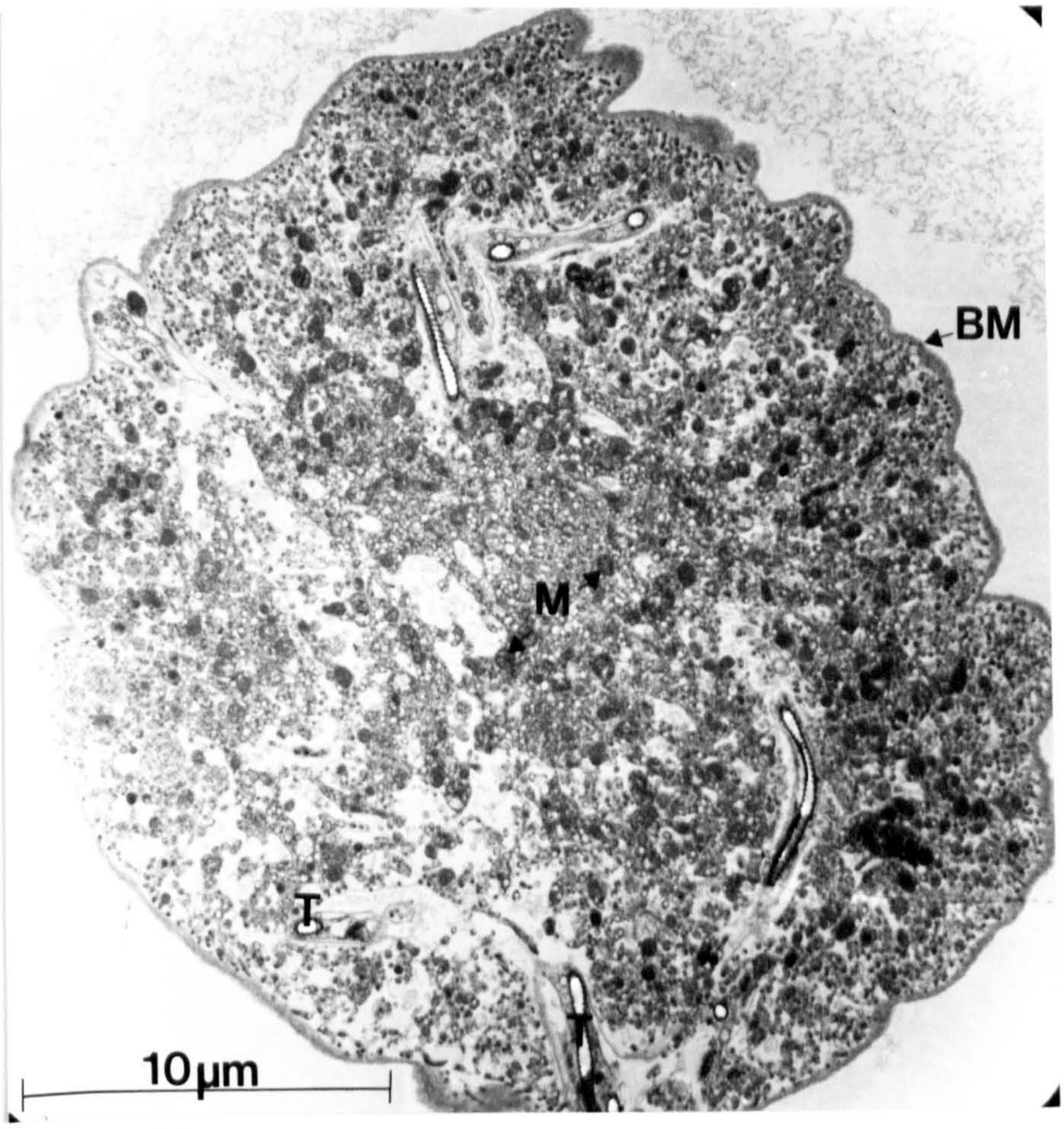


Figure 3.2 Transmission elecron micrograph of a prothoracic gland cell, indicating the prominent features: basement membrane (BM), mitochondria (M) and tracheoles (T). Magnification x 4,000.

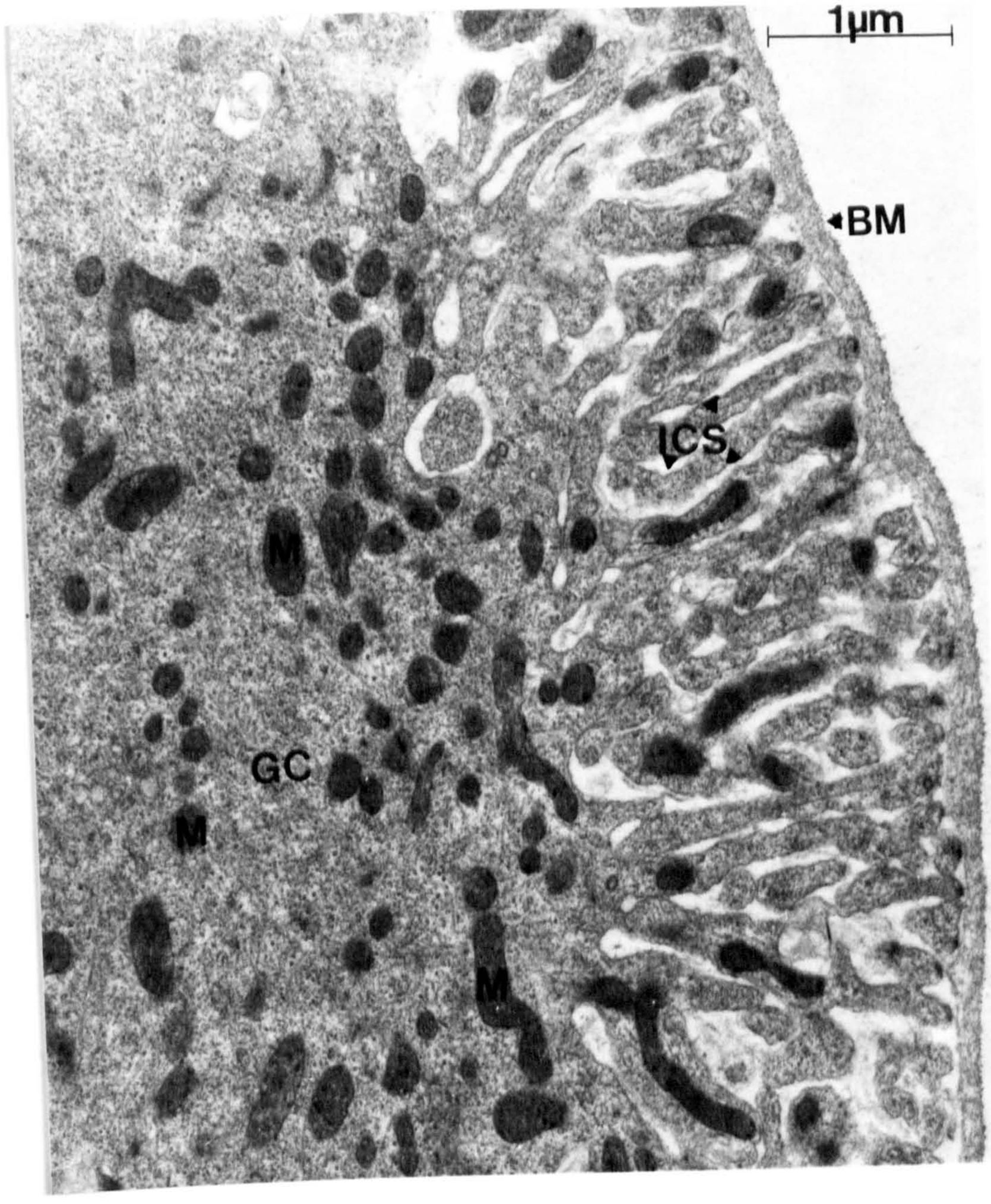


Figure 3.3 High magnification (x 20,000) electron micrograph of prothoracic gland cell containing intercellular spaces (ICS), basement membrane (BM), mitochondria (M) and granular cytoplasm (GC).

plentiful in the late last instar larvae of *Manduca sexta* (Sedlak et al., 1983). The abundance of ribosomes is suggestive of active protein production; these proteins may contribute to the reconstruction of the cell structures during pupariation (Sedlak et al., 1983).

In the peripheral cytoplasm, numerous mitochondria are evident, many of which are elongated, a feature which may correspond to active organelles. In *Bombyx mori*, mitochondrial morphology can be correlated to the ecdysteroid titre. During peaks of ecdysteroid production, mitochondria are seen as rod-like structures, which progressively change into large, inactive, so-called macromitochondria, lacking inner membranes (Beaulton, 1968). Interestingly, the presence of ER and mitochondria are consistent with the notion that microsomal and mitochondrial enzymes participate in ecdysteroid synthesis. For example, in *Manduca*, the 7-dehydrogenation of cholesterol and the C-25 hydroxylation are microsomal, but the C-22 and C-2 hydroxylations are mitochondrial (Grieneisen et al., 1993).

3.2.2 Cross-Reactivity of DHS-1-13.5 Antiserum

HPLC-RIA analyses were performed as described in Section 2.2.14. Rabbit DHS-1-13.5 antiserum (Soumoff et al., 1981) was obtained following immunization with 20-hydroxyecdysone-2-succinyl-thyroglobulin amide (from Prof. J.D. O'Connor, U.C.L.A.). It is well established that the structure of a compound (e.g. 3-dehydroecdysone), which has an alternative grouping (e.g. C-3 oxo) near to the position of the original hapten-carrier protein bridge in the immunogen (e.g. 20-hydroxyecdysone-2-hemisuccinate), can have equal or greater binding to the antiserum than that of the hapten [ecdysone] (Warren et al., 1982).

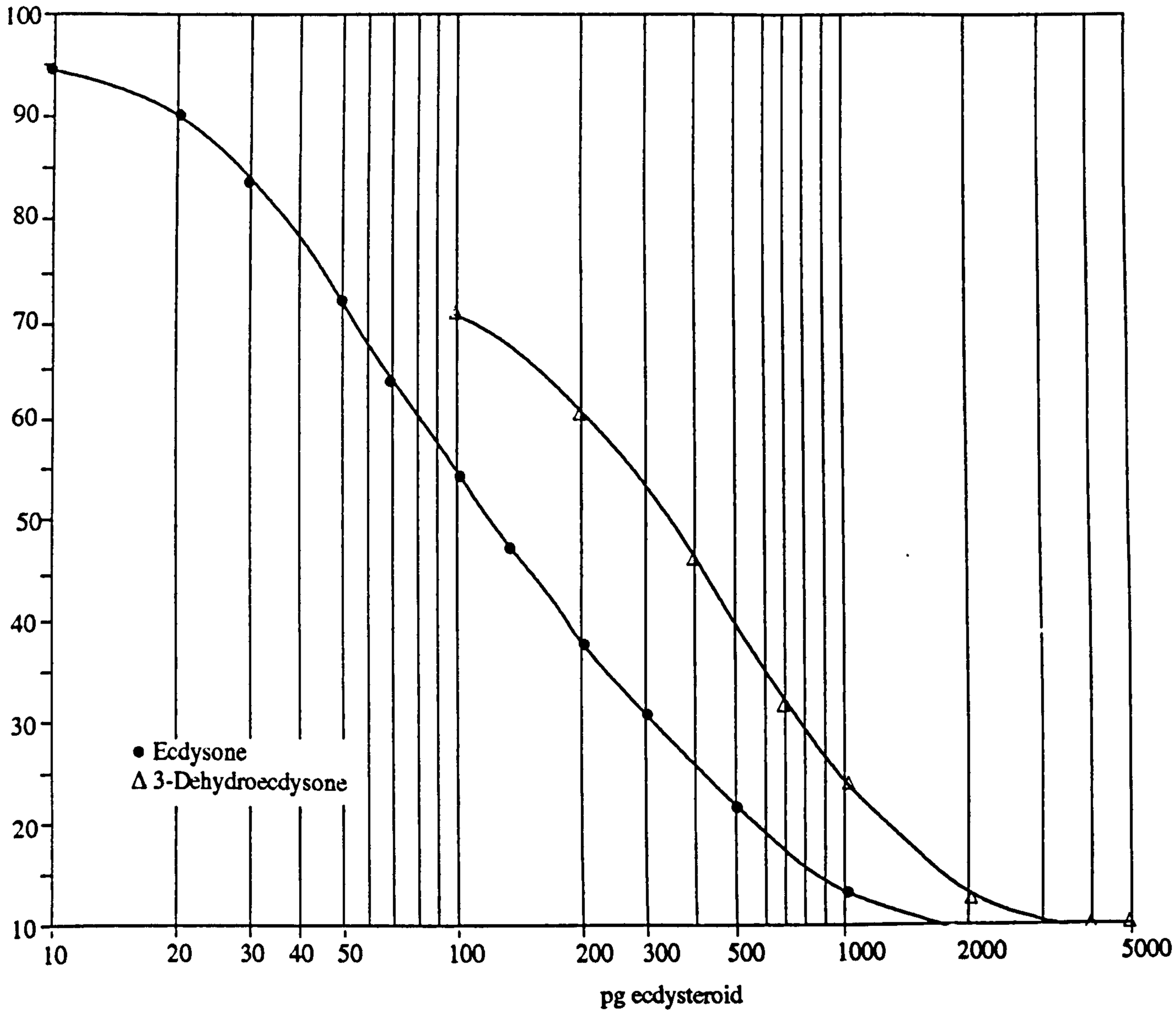


Figure 3.4. The cross-reactivity of 3-dehydroecdysone with DHS-1-13.5 antiserum. The results are expressed as the amount of radioactive ecdysteroid (cpm) bound to the antiserum (C_X) relative to the blank tubes containing no ecdysteroid (C_0), expressed as a percentage, *versus* the amount of standard ecdysteroid, plotted on a logarithmic scale.

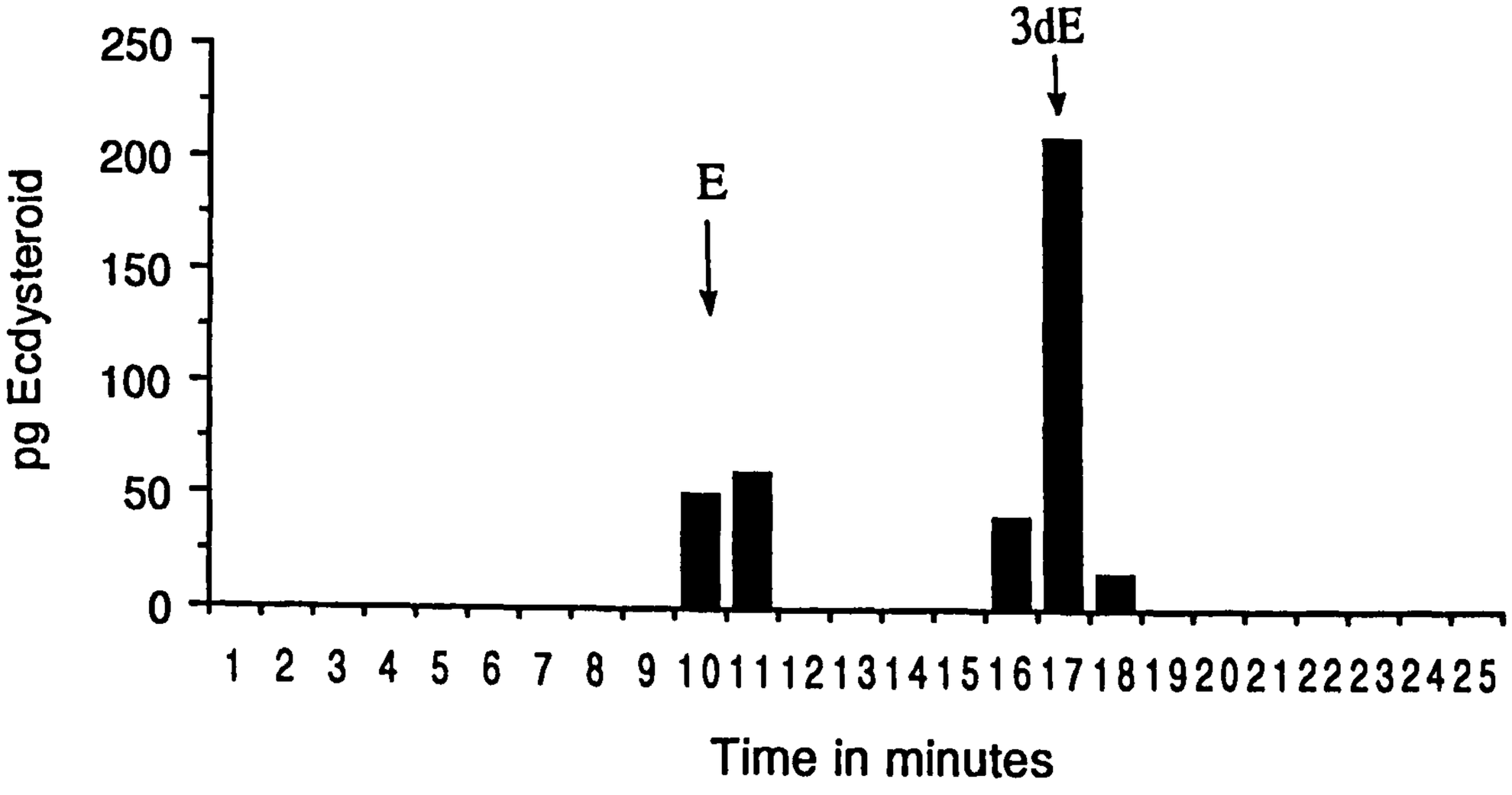


Figure 3.5. HPLC-RIA chromatogram (DHS-1-13.5 antiserum) of the free ecdysteroid fraction from the prothoracic gland secretions.The positions of authentic 3-dehydroecdysone (3dE) and ecdysone (E) are shown.

Hence, there was a need to construct cross-reactivity curves for both ecdysone and 3-dehydroecdysone. Two duplicate assays were performed and the curves are shown in figure 3.4. Cross-reactivity was calculated to be 2.36, using:

$$\text{Cross-Reactivity} = \frac{\text{Amount of 3-dehydroecdysone required for 50\% displacement of radioactivity}}{\text{Amount of ecdysone required for 50\% displacement of radioactivity}}$$

3.2.3 *In Vitro* Secretions of the Prothoracic Glands

Last instar larvae were synchronized and sacrificed at approximately 96h into the sixth instar (day 4), a point of high ecdysteroid output (Jarvis, 1991; see Chapter 4). Prothoracic glands were excised as detailed in Section 2.2.2 and, typically, 20 glands (10 pairs) were incubated in 500µl Grace's T.C. insect medium (without haemolymph) [GIBCO, Grand Island, N.Y., U.S.A], at 25°C for 24h. The reaction was terminated by the addition of 500µl methanol. Protein was sedimented at by centrifugation at 10,000g at 4°C for 10min. The resulting pellet was re-extracted twice with methanol, the supernatants combined and dried under a stream of nitrogen. Ecdysteroids were applied to a C_{18} Sep-Pak cartridge in 10%(v/v) methanol/water and sequentially eluted as in Section 2.2.9. A aliquot from the 60%(v/v) methanol/water fraction was estimated to contain 1ng ecdysteroid by RIA (DHS-1-13.5 antiserum) and applied to HPLC, using system 1 (Section 2.2.14) for elution. Fractions (1ml) were then assayed by RIA.

A typical RIA profile is shown in figure 3.5. A blank HPLC-RIA chromatogram, without injection of sample, showed negligible immuno-

reactivity. The results, (an average of 3 separate determinations) indicate that the major product of the prothoracic glands of *S. littoralis* is 3-dehydroecdysone (19.7ng / pair glands ± 2.4ng), with a lower amount of ecdysone being released (4.3ng / pair glands ± 0.5ng). Hence, the percentage of 3-dehydroecdysone secreted is 81.7 ± 3.9%.

3.2.4 Ecdysone Oxidase and 3β-Hydroxy Forming Reductase Activities of the PGs

Since the major steroid product of the prothoracic glands is 3-dehydroecdysone, the possibility that this has arisen by ecdysone oxidase action on ecdysone in the glands was examined by incubation with [^{3}H]ecdysone. Similarly, the possibilty that ecdysone secreted by the glands was derived from 3-dehydroecdysone by reduction was investigated.

10 prothoracic glands from day 4 larvae were incubated with 0.2μCi [^{3}H]ecdysone or [^{3}H]3-dehydroecdysone and, in the case of the reductase assay, 0.5mM NADH or NADPH was added. Assays were carried out in Grace's TC medium, approximately 500μl total volume. Incubations were at 37^{o}C for 18h, duplicated and repeated twice.

In both cases no discernible activity was detected. Addition of either NADH or NADPH did not result in metabolism of [^{3}H]3-dehydroecdysone by reductases.

3.2.5 Verification of the Occurrence of a 3-Dehydroecdysone 3β-Hydroxy Forming Reductase Activity in the Haemolymph

The occurrence of enzymic activity in the haemolymph for reduction of 3-dehydroecdysone (of prothoracic gland origin) to ecdysone was then examined.

Haemolymph was collected from 100 sixth instar larvae (day 3-4; approximately 20ml) and treated according to Section 2.2.3. 3-Dehydroecdysone (0.5mg) and NADPH (0.5mM) were dissolved in 0.2M sodium phosphate buffer, pH 7.4 and incubated with the haemolymph preparation at 37°C for 18h. The reaction was terminated with 4ml chilled methanol, the protein sedimented by centrifugation at 10,000g for 15min and the pellet re-extracted with methanol. The ecdysteroids were separated by C_{18} Sep-Pak (Section 2.2.9) chromatography and the 60%(v/v) methanolic fraction analyzed by reversed-phase HPLC, (system 1; Section 2.2.9). The putative ecdysone product was then recovered for further analysis: a) reversed-phase HPLC (system1); b) adsorption chromatography (system 2); c) conversion into the 2,3-acetonide derivative (Section 2.2.13) following analysis on HPLC using a linear 30min gradient of 40-100% (v/v) methanol/water at 1ml/min.

Co-chromatography of the putative ecdysone with an authentic marker was observed using both reversed-phase HPLC and adsorption HPLC, (figures 3.6a and b). After conversion of the enzymic product to an acetonide, putative ecdysone 2,3-acetonide was found to co-elute with an authentic marker, (figure 3.6c). A negative-ion fast-atom bombardment mass spectrum clearly showed the $[M-H]^-$ ion at 463, corresponding to a relative molecular mass of 464, (figure 3.7). Hence,

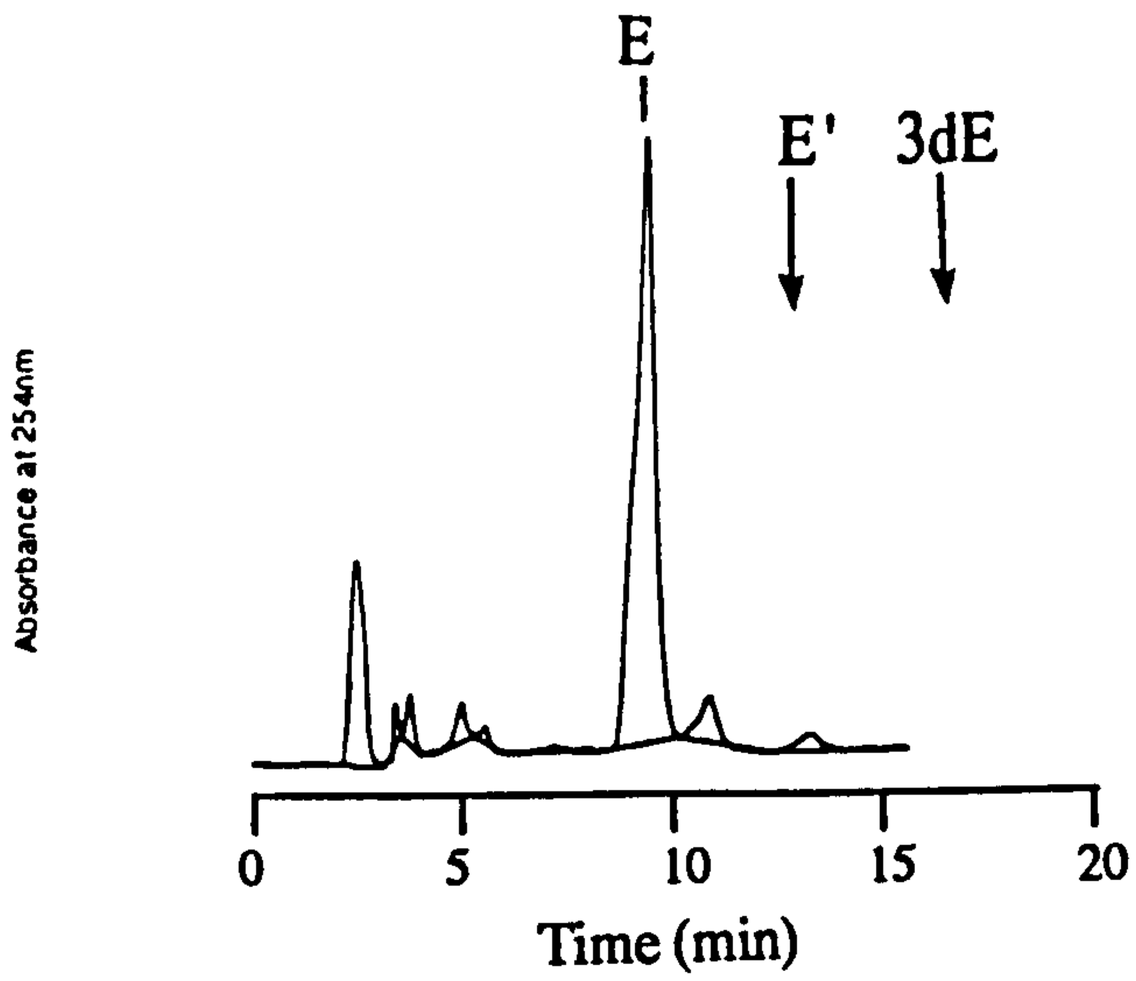


Figure 3.6a. HPLC fractionation on a reverse-phase column (system 1) of the putative ecdysone product. Authentic standards are shown: ecdysone (E), 3-epiecdysone (E') and 3-dehydroecdysone (3dE).

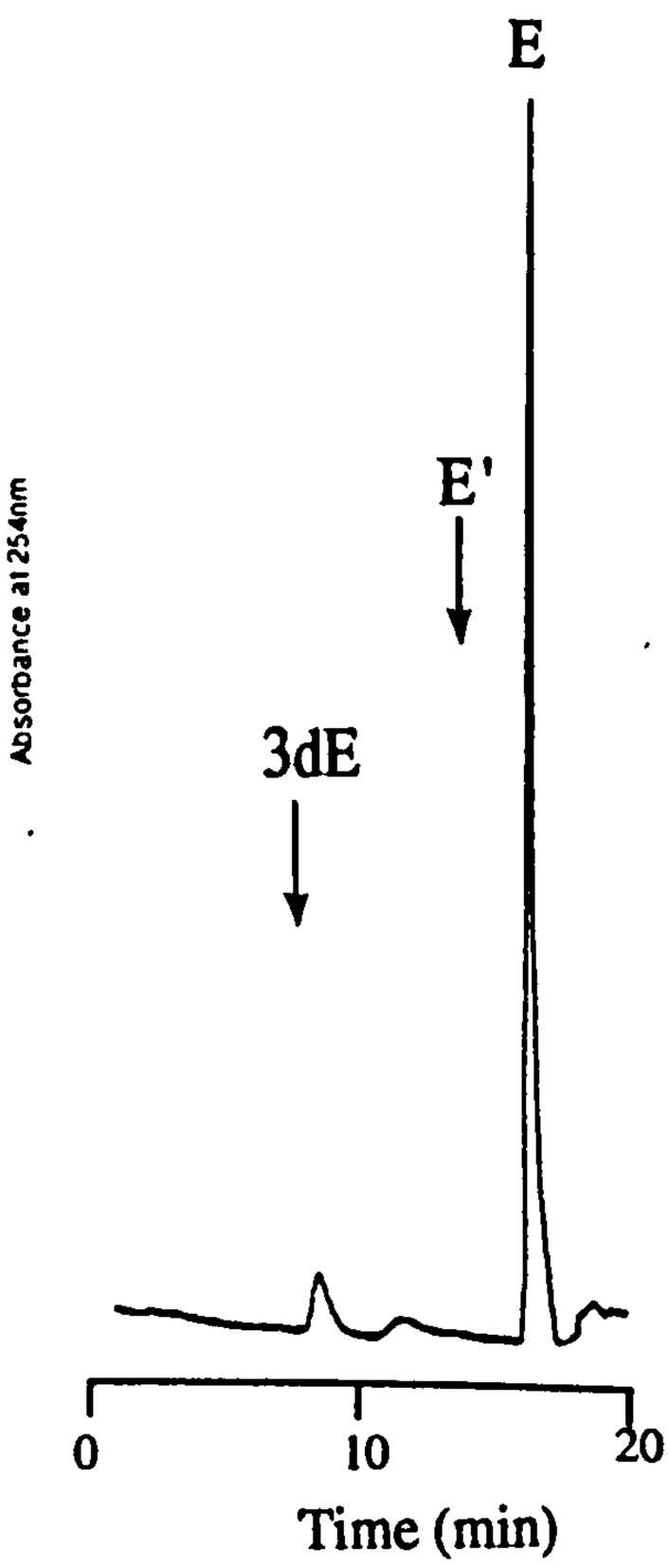


Figure 3.6b. HPLC fractionation on an adsorption column (system 2) of the putative ecdysone product. Authentic standards are shown: ecdysone (E), 3-epiecdysone (E') and 3-dehydroecdysone (3dE).

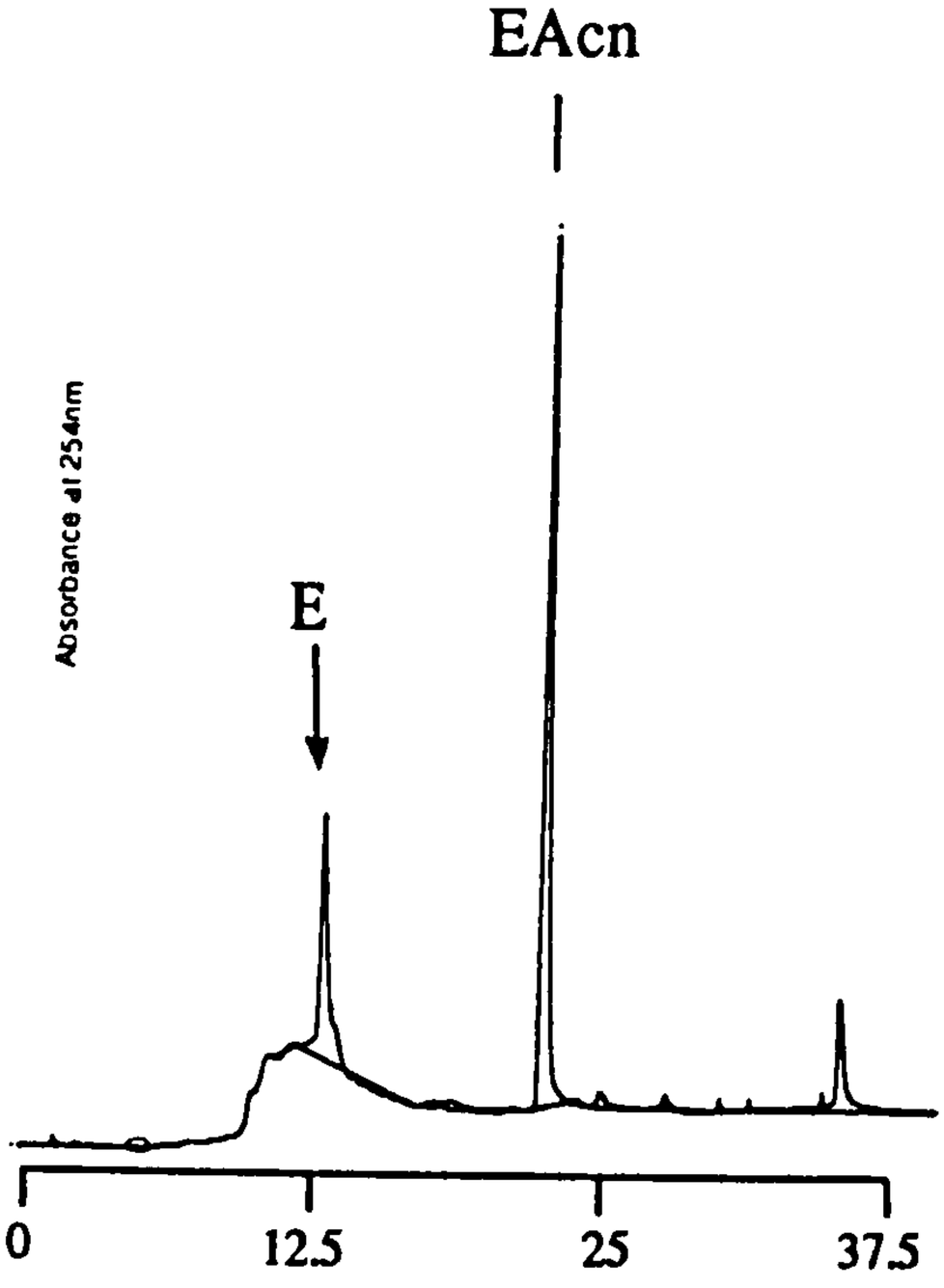


Figure 3.6c. HPLC fractionation of the putative ecdysone converted into a 2,3-acetonide derivative, using a reversed-phase column eluted with a linear grdient of 40-100%(v/v) methanol/water over 30min. Positions of authentic standards are shown: ecdysone (E) and ecdysone 2,3-acetonide (EAcn).

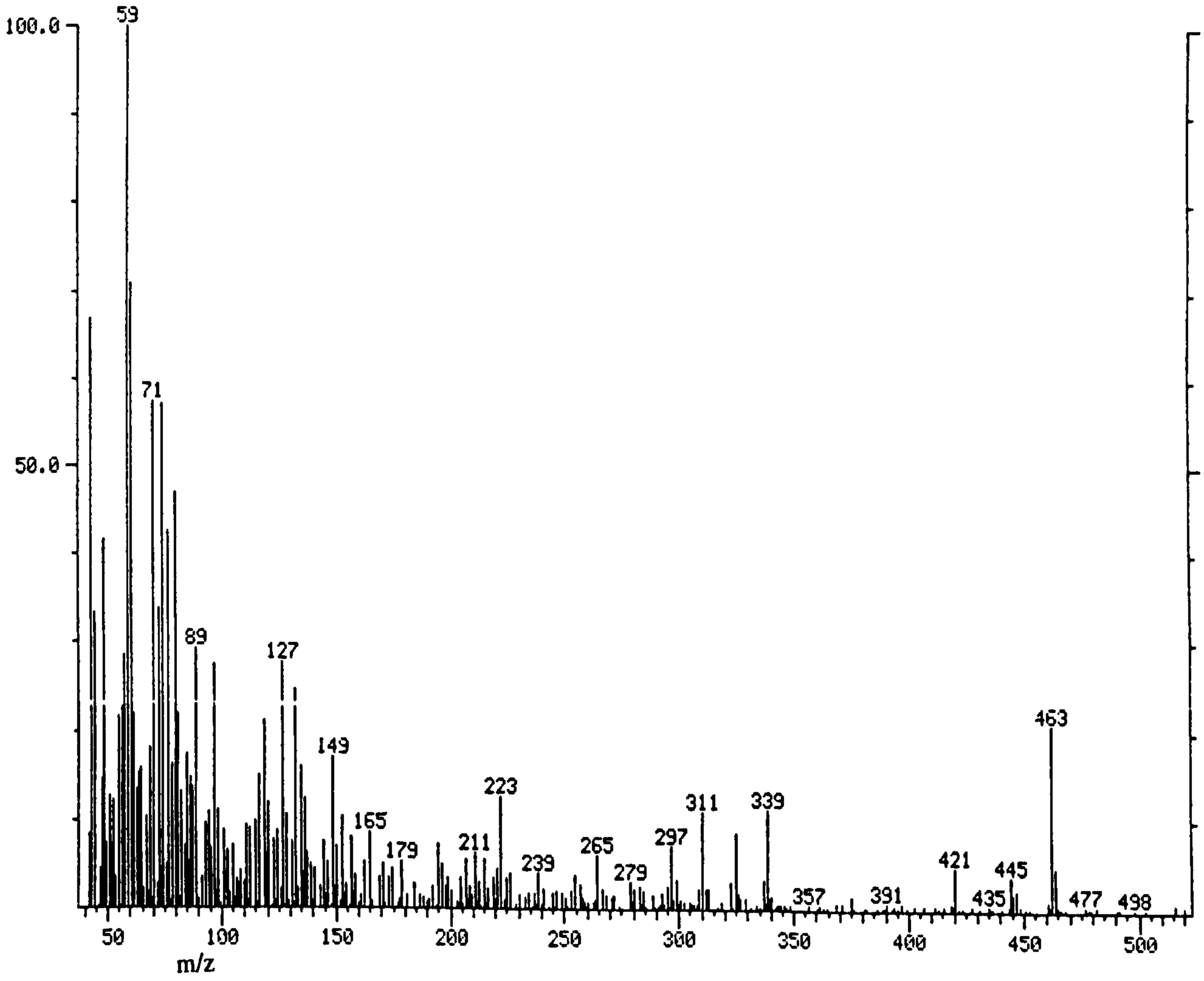


Figure 3.7. Negative-ion FAB-MS of the putative ecdysone product from reduction of 3-dehydroecdysone by haemolymph enzyme.

a factor exists in the haemolymph which can convert 3-dehydroecdysone into ecdysone.

3.2.6 Conversion of 5β-Ketodiol and 3-Oxo-5β-ketodiol in Isolated Prothoracic Glands

[^{3}H]5β-ketodiol or [^{3}H]3-oxo-5β-ketodiol (0.5μCi) were added in 10μl ethanol to 490μl Grace's Medium containing approximately 30 (day 4, VI instar) prothoracic glands, and incubated at 37°C for 18h. The products were analyzed by reversed-phase HPLC, using a C_{18} Nova-Pak column. Initially ecdysteroids were separated using a 35-100%(v/v) linear 30min gradient of methanol in water at a rate of 1ml/min. Radioactivity was monitored using an on-line flow detector. End-products (i.e. those most polar) were collected and re-analyzed using HPLC system 1. Unreacted 3-oxo-5β-ketodiol and 5β-ketodiol were also recovered and analyzed by reversed-phase HPLC using a linear gradient of 80-90% (v/v) acetonitrile in water, over 30min at 1ml/min.

Tritiated 5β-ketodiol was principally converted into a metabolite having the same retention time as ecdysone (approximately 6.0% conversion) on reversed-phase HPLC analysis using the 35-100% (v/v) methanol/ water gradient solvent system (figure 3.8). This peak was collected for re-analysis. Minor components were tentatively labelled 22-deoxyecdysone (8.5% conversion) and 2,22-dideoxyecdysone (36.7% conversion). Values represent the mean results of two investigations. Reversed-phase HPLC analysis using the 35-100% (v/v) methanol/water gradient system showed that once again, [^{3}H]-3-oxo-5β-ketodiol was converted to several metabolites, (figure 3.9). A major peak corresponding to the retention time of ecdysone/3-dehydroecdysone

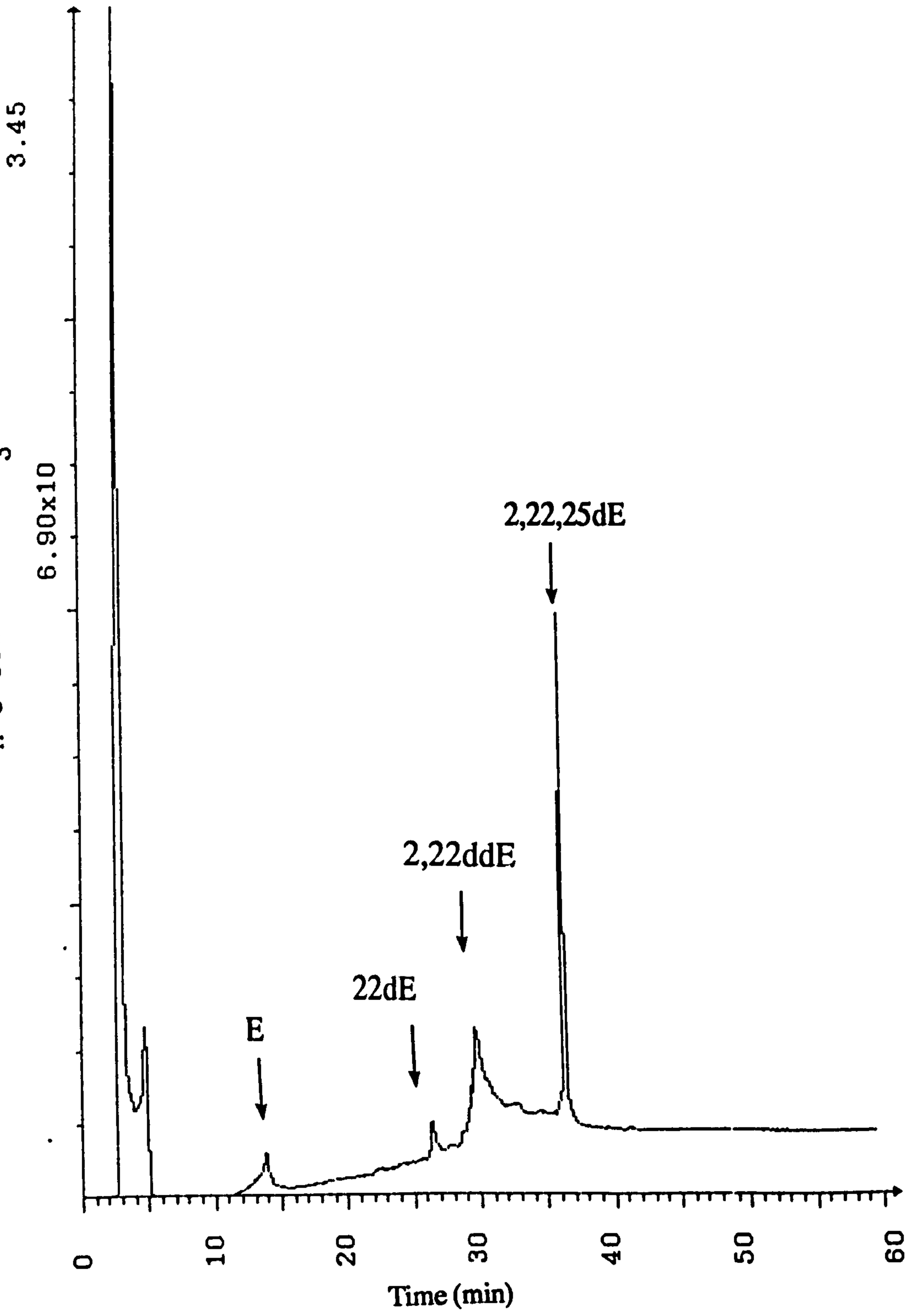


Figure 3.8. Reversed-phase HPLC fractionation [using a linear 35-100%(v/v) methanol/ water gradient over 30min] of the free ecdysteroid fraction from prothoracic glands incubated with [^{3}H]5β-ketodiol. The positions of authentic compounds are indicated: ecdysone (E), 2-deoxyecdysone (2dE), 22-deoxyecdysone (22dE), 2,22-dideoxyecdysone (2,22-ddE) and 2,22,25-trideoxyecdysone (i.e. 5β-ketodiol; 2,22,25dE).

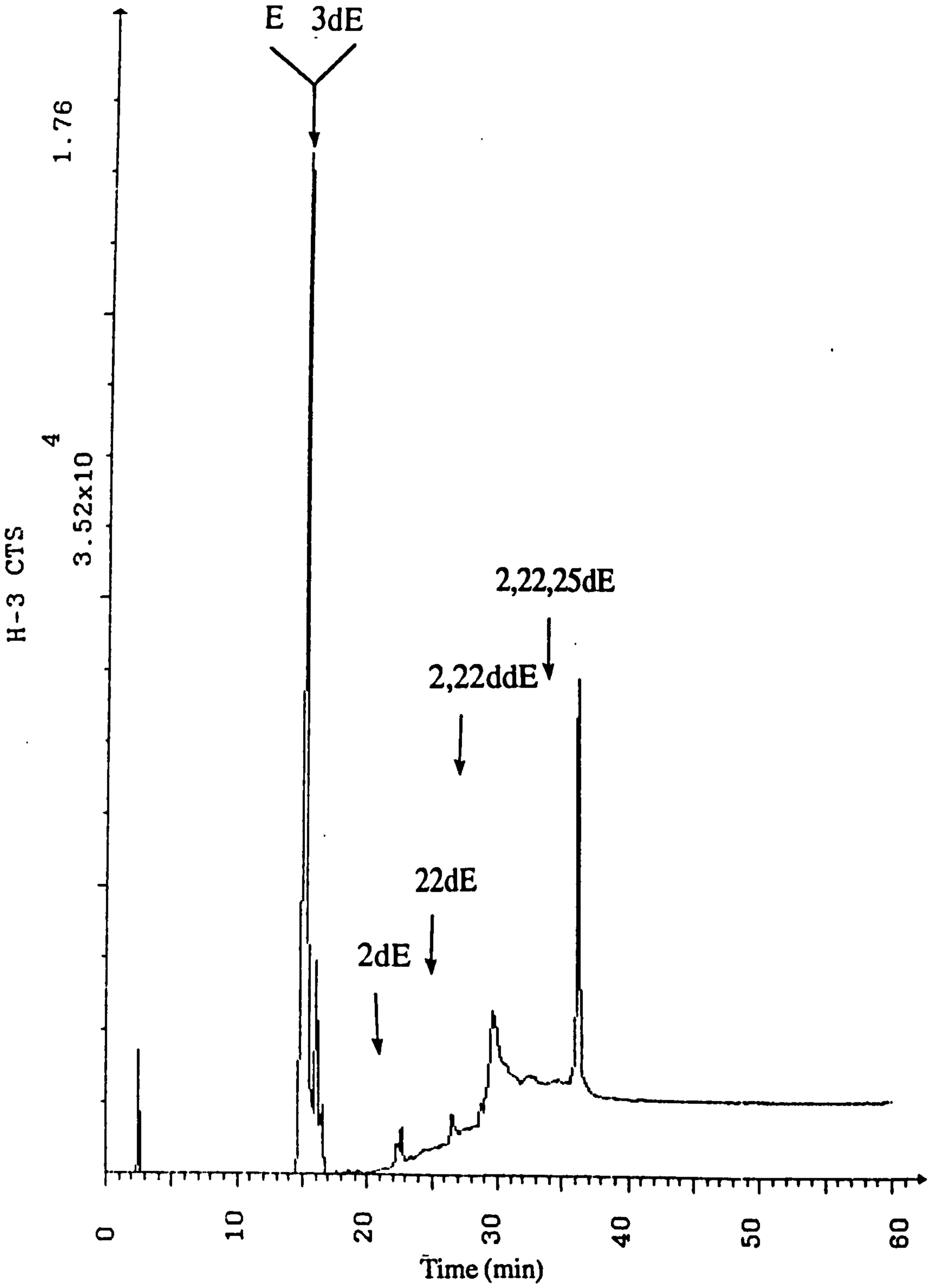


Figure 3.9. Reversed-phase HPLC fractionation [using a 35-100(v/v) methanol/ water gradient over 30min] of the free ecdysteroid fraction from prothoracic glands incubated with [^{3}H]3-oxo-5β-ketodiol. The positions of authentic compounds are indicated: 3-dehydroecdysone (3dE), ecdysone (E), 2-deoxyecdysone (2dE), 22-deoxyecdysone (22dE), 2,22-dideoxyecdysone (2,22-ddE) and 2,22,25-trideoxyecdysone (i.e. 5β-ketodiol; 2,22,25dE).

(32.5%) was noted, and was recovered for further investigation. Minor peaks eluting just after 2-deoxyecdysone (3.2%), 22-deoxyecdysone (3.9%) and 2,22-dideoxyecdysone (18.7%) were also observed. Since this solvent system was unable to fully resolve 3-oxo- and 3β-hydroxy ecdysteroids, it can only be postulated that these are 3-oxo- derivatives. Hence, further chromatographic analysis was necessary.

The major (end)products of the 3-oxo-5β-ketodiol and 5β-ketodiol experiments were re-analyzed using HPLC system 1. In the case of the 5β-ketodiol incubation, the major product, as expected, co-chromatographed with authentic ecdysone with no detectable 3-dehydroecdysone (figure 3.10a). From the 3-oxo-5β-ketodiol experiment, the products a mixture of ecdysone and 3-dehydroecdysone, in the ratio of approximately 1:6 (figure 3.10b).

The unreacted 3-oxo-5β-ketodiol and 5β-ketodiol had also been collected to ascertain whether any reduction or oxidation, respectively, at C-3 had taken place. Using a linear 30min gradient of 80-90% (v/v) acetonitrile in water, at 1ml/min, unchanged respective substrates were the only discernible peaks.

3.4 DISCUSSION

It is now a generally accepted concept that 3-dehydroecdysone is the primary secretion of Lepidopteran prothoracic glands. However, as in *S. littoralis*, it is not the sole secretion, since a lesser quantity of ecdysone is also detected (*ca.* 18%). Although in other Lepidopteran species a far greater proportion of 3-dehydroecdysone has been reported (≥95% 3-dehydroecdysone; Blais and Lafont, 1991; Warren et al., 1988) , it is by no means exceptional for the ratio to have been found to be slightly lower. In fact, some Lepidopteran species secrete

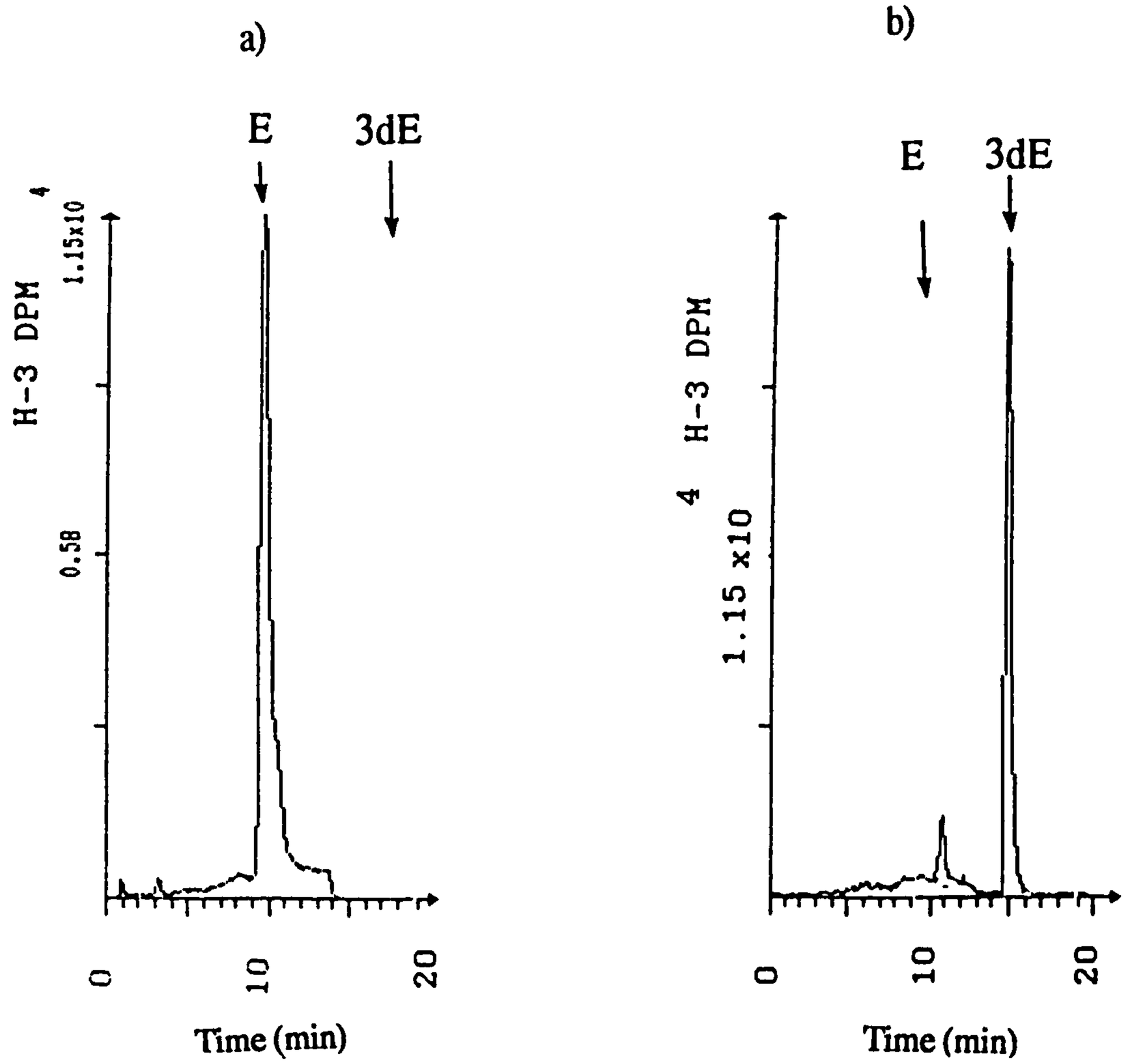


Figure 3.10. Reversed-phase HPLC of the putative ecdysone recovered from the prothoracic gland incubationwith a) [^{3}H]5β-ketodiol and b) [^{3}H]3-oxo-5β-ketodiol was re-chromatographed on system 1. Authentic ecdysteroid markers are indicated: ecdysone (E), and 3-dehydroecdysone (3dE)

only a 1:1 ratio (*Leucinia separata*) and some secrete merely traces of 3-dehydroecdysone (*Bombyx mori*; Kiriishi et al, 1990).

Hence, even within a single order, there are dramatic differences in the ecdysteroids liberated by the prothoracic glands. Physiologically speaking, these differences cannot be easily explained, although there is some evidence to support a developmental importance. Sakurai and Gilbert (cited in Sakurai et al., 1989b) noted an increase in 3-dehydroecdysone/ ecdysone ratio as the development of *Manduca sexta* progressed.

It appears that the haemolymph contains a 3β-hydroxy forming reductase (3-dehydroecdysteroid 3β−reductase), which converts 3-dehydroecdysone into ecdysone. Whether this enzyme is stage-specific or can be correlated to the products of the prothoracic glands will be discussed in Chapter 4.

In light of these data, 3-oxo-5β−ketodiol could represent a potential precursor in the biosynthetic pathway, at least in Lepidoptera, and possibly in other insects such as *Locusta migratoria* (Roussel, 1992). The glands of *S. littoralis* were capable of transforming [^{3}H]-5β−ketodiol and [^{3}H]3-oxo-5β−ketodiol into ecdysone and 3-dehydroecdysone plus ecdysone, respectively. This has important implications on the nature of intermediates of ecdysteroid synthesis, two lines of evidence pointing to 3-oxo-5β−ketodiol being a possible intermediate in *Spodoptera littoralis*: firstly, the secretion of 3-dehydroecdysone and lesser quantities of ecdysone (*in vitro*) from the prothoracic glands, and secondly, the major product of the 3-oxo-5β−ketodiol incubation is 3-dehydroecdysone plus a much lower amount of ecdysone. It must be borne in mind that incubation with [^{3}H]5β−ketodiol does not yield 3-dehydroecdysone. Therefore, it is questionable as to whether 5β−ketodiol is the usual precursor in the prothoracic glands of

Spodoptera. If this were correct, presumably the 3-oxo group of the 3-oxo Δ^4 steroid, which is thought to exist early in ecdysteroid biosynthesis, is conserved, rather than reduced, hence leading to the formation of mainly 3-oxo-5β-ketodiol, not 5β-ketodiol.

Similarly, in the prothoracic glands of *Pieris brassicae*, the primary secretion is 3-dehydroecdysone (95%), with smaller amounts of ecdysone (5%). Incubation with [^{3}H]5β-ketodiol yields only ecdysone, whereas using [^{3}H]3-oxo-5β-ketodiol, furnishes 3-dehydroecdysone and traces of ecdysone (Blais and Lafont, 1991). It is likely that 3-oxo-5β-ketodiol also represents the major precursor in *Pieris* prothoracic glands.

In contrast, the prothoracic glands of *Locusta migratoria*, which are known to secrete exclusively ecdysone *in vivo* (Hirn et al., 1979), can metabolise [^{3}H]3-oxo-5β-ketodiol to form 3-dehydroecdysone and ecdysone, in approximately equal quantities (Roussel, 1992). Obviously, 3-oxo-5β-ketodiol cannot be the usual biosynthetic precursor in this species and 5β-ketodiol is a more likely candidate.

There is always the possibility that interpretation of the results of transformation of exogenous labelled substrates can be complicated if there are any differences between their metabolism and those of endogenous compounds.

On incubation of *Spodoptera littoralis* prothoracic glands with [^{3}H]ketodiol, several peaks were resolved by reversed-phase HPLC, the most polar being ecdysone. Other peaks were tentatively labelled 2,22-dideoxyecdysone and 22-deoxyecdysone. The identities of the biosynthetic intermediates following 3-oxo-5β-ketodiol incubation with *Spodoptera* prothoracic glands can only be speculated. Unfortunately, authentic ecdysteroids were not available for analytical comparisons.

However, the peaks were slightly less polar than the 3β-hydroxy intermediates and could therefore be 3-oxoecdysteroids.

Interestingly, in the 3-oxo-5β−ketodiol experiments using *Locusta,* the putative 3-oxo derivatives could be resolved into both 3-oxo- and 3β-hydroxy-ecdysteroids, in approximately equal proportions. Moreover, there was no reduction of the 3-oxo-5β−ketodiol, (Roussel, 1992), in agreement with the results from *Spodoptera littoralis.*

The data revealed that the prothoracic glands have negligible ecdysone oxidase activity, in accordance with previous reports, (Gilbert, 1989). A 3β-hydroxy-forming reductase had been postulated to exist on the basal sheath of the prothoracic glands, converting 3-dehydroecdysone to ecdysone. However, when the glands are thoroughly washed 3-dehydroecdysone secretion is enhanced, (Warren, et al, 1988; Kiriishi, et al., 1990). This enzyme therefore is also located in the haemolymph , and *in vitro* at least, may adhere to the sheath. It is therefore not surprising that the washed *Spodoptera* glands did not possess an active 3β-reductase. The significance of these results is that no interconversion of 3-dehydroecdysone and ecdysone takes place.

Although 3-dehydroecdysteroids appear to have hormonal activity in some systems (Sommé-Martin et al., 1990; see Chapter 7), it is generally held that 3-dehydroecdysone is reduced in the haemolymph, by a protein factor, to ecdysone (See Chapter 4), and subsequently hydroxylated at C-20 in peripheral tissues to form what is conventionally regarded as the most potent moulting hormone, 20-hydroxyecdysone.

Chapter 4

Characterization and purification of a haemolymph 3-dehydroecdysone 3β-reductase enzyme

4.1 INTRODUCTION

In 1985, a haemolymph stimulatory factor (HSF) was postulated to increase the rate of secretion of ecdysone from larval and pupal prothoracic glands of *Manduca sexta* (Watson et al., 1985). The factor was found to be both heat labile and protease-sensitive, with a reported molecular weight of 30kDa, i.e. suggestive of a protein. Since then, 3-dehydroecdysone has been proved to be the major secretion from Lepidopteran prothoracic glands (Warren et al., 1988; Kiriishi et al., 1990), and hence, the HSF is believed to be not a stimulatory factor as such, but a 3-dehydroecdysone 3β-reductase, often shortened to the more informal term, 'ketoreductase'.

Addition of the protein factor to cultured prothoracic glands did not give an increased amount of ecdysteroids (Watson et al., 1985; Sakurai at al., 1989b; Kiriishi et al., 1990). However, there is an increase in H-22 immunoreactive binding. [H-22 antiserum is known to "recognise" the A-ring of ecdysteroids (Horn et al., 1976).] In fact, the protein factor enhances the rate of conversion of 3-dehydroecdysone to ecdysone, rather than actually stimulating the prothoracic glands. Preliminary characterization of the factor has only been reported for *Manduca*, and has indicated a molecular weight of 20-30kDa (Watson et al., 1985; Sakurai et al., 1989b) and a requirement for NADPH, NADH being much less effective *in vitro*. Hence, optimal conditions have yet to be established.

If 3-dehydroecdysone is envisaged as a prohormone, and the ketoreductase as an activating enzyme present not only in the haemolymph, but also in various other organs, such as the fat body, salivary glands, suboesophageal gland, Malpighian tubules, hindgut, brain (Gelman, et al., 1991) and midgut (Milner and Rees, 1985; Nigg

et al., 1974), it is tempting to suggest two possible roles for the enzyme: a) since the ketoreductase is found in the haemolymph of insects which undergo pupal diapause, such as *Antheraea polyphemus* and *Hyalophora crecopia* (Sakurai et al., 1989b; Warren et al., 1988), it may be a required factor for breaking of diapause and b) activation. For example, if 3-dehydroecdysone is sequestered into tissues, to be activated at specific points during development by the ketoreductase, this may account for the observation of Sakurai et al., (1991) that insect parts (e.g. isolated abdomens) "moult" in the absence of prothoracic glands.

One of the most intriguing aspects of this enzyme is the fluctuating levels in the haemolymph. Although in *Manduca* the peak titre was reported by Sakurai et al. (1989b) to occur during the last day of larval life (day 8) and the first day of pupal life, another group (Watson et al., 1988) describe a slightly different developmental profile for the haemolymph stimulatory factor. In the latter case, the titre falls between day 1 and 4, then peaks on day 7, after which it falls into decline at the end of the last larval instar. The latter scheme is strongly suggestive of a role linked to the ecdysteroid titre. In *Manduca*, a small rise in ecdysteroids is observed (day 4-5), eliciting wandering and committing the insect to pupal development, whereas high ecdysteroid titres are seen on day 7-8, and stimulate the larval-pupal moult. Hence the enzyme levels peak just prior to those of circulating ecdysteroids (Watson et al., 1985). Even the results obtained by Sakurai et al. (1989b) for the reductase profile, suggest that the enzymic activity starts to rise before the increase in ecdysteroid titre on day 7, *albeit* not reaching a peak before day 8.

The work described in this chapter initially investigates the profile of the haemolymph 3-dehydroecdysone 3β-reductase during the development of the sixth instar stage of *Spodoptera littoralis*. This will

be followed by characterization of the enzyme optimal requirements to allow establishment of an assay system. The enzyme will then be purified ; availability of pure enzyme would allow further studies involving utilizing the corresponding antibodies and ultimately lead to cloning of the responsible gene.

4.2 EXPERIMENTAL AND RESULTS

4.2.1 General Methods

Haemolymph was collected and treated according to Sections 2.2.2 and 2.2.3. The enzyme preparation was dialysed in 100 volumes of 10mM Tris-HCl buffer, pH 7.4 (buffer A). The enzyme could be frozen at -20°C for at least 3 months, without any detrimental loss of activity.

Enzymic activity was assayed in 0.2M sodium phosphate buffer, pH 7.4, using enzyme preparation (100µl, typically 0.5-1.0mg protein), 0.5mM NAD(P)H (100µl) and 8.4µM unlabelled 3-dehydroecdysone (50µl). After 5-10min equilibration of the reactants and enzyme at 37°C, the reaction was initiated by addition of 3-dehydroecdysone to the NAD(P)H and enzyme preparation. Reactions were carried out at 37°C for 1h, in duplicate, using boiled enzyme preparation as the control (See Section 2.2.4.). The reaction was terminated by addition of chilled ethanol (400µl) and the protein precipitate removed by centrifugation at 9,000g for 10min. The supernatant was dried *in vacuo* the reaction products analyzed by reversed-phase HPLC, using an isocratic mobile phase of 0.1% (v/v) TFA in water/ acetonitrile [78/22 (v/v)], at a rate of 1ml/min, (system 1).

4.2.2 Ecdysteroid Titre throughout the Last Larval Instar

In the following two sections, the developmental profile of the haemolymph 3-dehydroecdysone 3β-reductase enzyme during the sixth instar will be compared with the corresponding ecdysteroid titre in the same haemolymph samples. Typically the results for each time point represent assays on haemolymph from two groups of approximately six insects. The ecdysteroid assays were carried out in triplicate on each sample, whereas duplicate enzyme assays per sample carried out.

Haemolymph was extracted from accurately synchronized sixth instar larvae [±3h], at the following time-points: 50, 76, 92, 99, 105, 107,110 and 116 hours. 10µl from 50h, 76h and 116h old larvae were taken and 1µl from the remainder. Protein was precipitated by addition of chilled ethanol (100µl) and sedimentated at 9,000g (10min). The supernatants were withdrawn and the pellet re-extracted with ethanol. Alcoholic extracts were combined and dried *in vacuo*. Individual assays were carried out using extracts from the equivalent of the following volumes of haemolymph: 10µl for the 50, 76 and 116h samples, but 1µl for the remainder. Ecdysteroid content (pg ecdysone equivalents) of the samples was estimated by RIA (Section 2.2.14) using H-22 antiserum. This antiserum is prepared by immunization with ecdysone 22-succinylthyroglobulin, and hence, is specific for the ecdysteroid nucleus (Horn et al., 1976). In view of this, ecdysone and 20-hydroxyecdsone are immunoreactive, (cross-reactivities of 1.0 and 2.05 respectively, J.M. Mercer, personal communication), but 3-dehydroecdysone shows little binding (a cross-reactivity of 21.4 compared to ecdysone; Warren and Gilbert, 1986).

The immunoreactive ecdysteroid profile during the sixth instar is given in figure 4.1a. The titre remains very low until the start of the

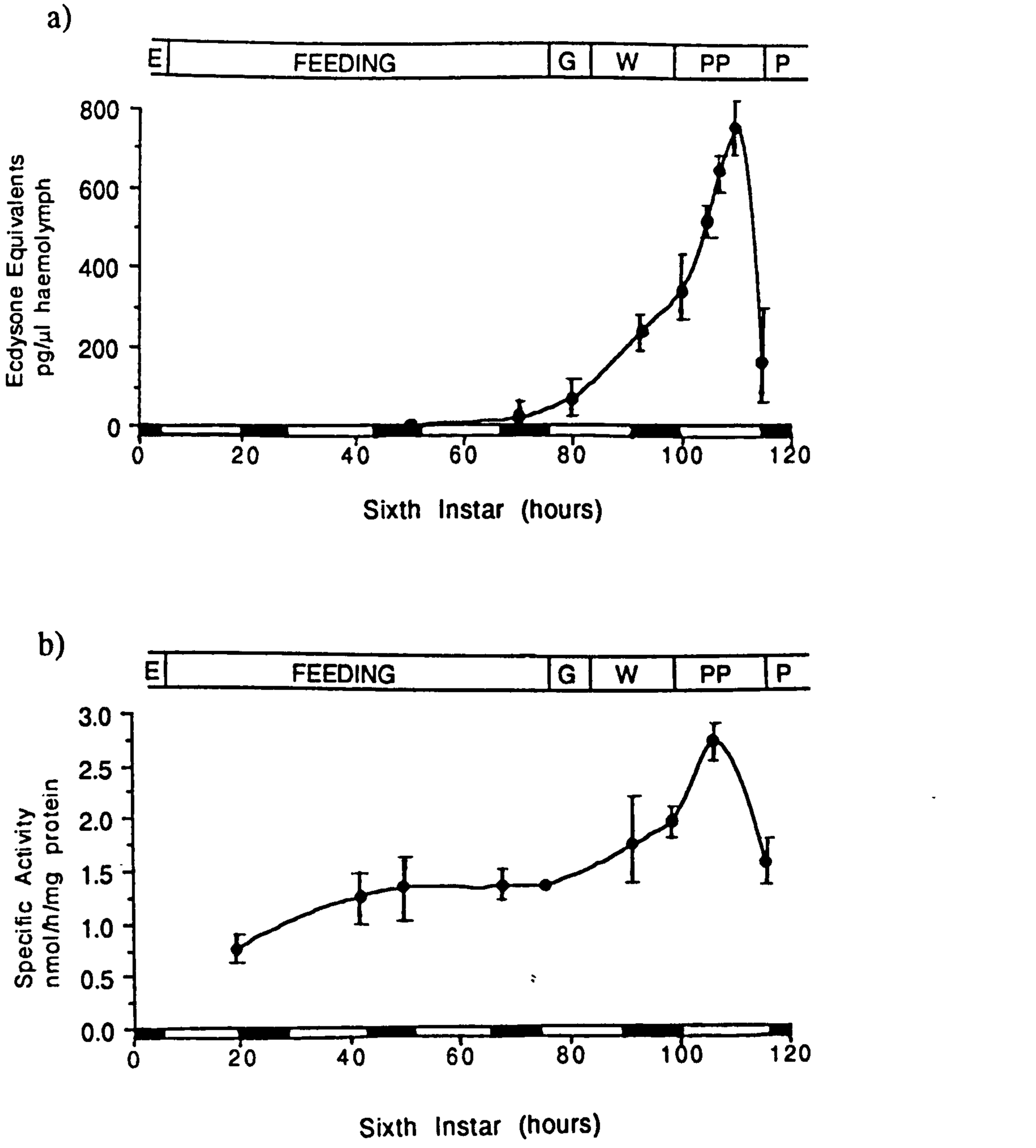


Figure 4.1a. The haemolymph ecdysteroid titre during the last larval instar of *S. littoralis*. 4.1b. The specific activity profile of haemolymph 3-dehydroecdysone 3β-reductase specific activity throughout the sixth instar. Characteristic changes during development are indicated: ecdysis (E), gut purge (G), wandering (W), pharate pupariation (PP) and pupariation (P). Boxes represent the light (photophase) and dark (scotophase) periods. Values represent the mean of two complete investigations (±SD). Where error bars are not shown, they are within the (•) symbol.

4th. photophase (76h) when it begins to rise slowly. From the time of the 5th. scotophase (92h) until the 5th. photophase (110h) there is a sharp peak (*ca* 750pg ecdysone equivalents/ µl haemolymph), which declines after 110h, during the last photophase (5th).

4.2.3 Developmental Profile of the 3-Dehydroecdysone 3β-Reductase

Sixth instar larvae were accurately synchronized (±3h) by photoperiod. Batches of approximately 5 insects were sacrificed at the following time-points (hours) 19, 41, 50, 68, 76, 92, 99, 107 and 116. The experiment was repeated with a second set of larvae of the same generation. A mean value was taken and the standard deviation calculated. The peak was found to occur at approximately 107h (±3h), i.e. in the fifth photophase (figure 4.1b).

The enzyme titre begins to rise gently early in the instar (20-40h) and stays at a relatively constant level until the 4th. photophase (80h), after which there is a linear rise until the beginning of the 5th. photophase, followed by a rapid increase reaching a peak at about 107(±3)h. There is then a sharp decline, towards the end of the 5th. photophase.

Evidently the enzyme reaches a peak just prior to that of the peak ecdysteroid titre. These results also suggest that the precise timing of the enzyme titre is physiologically important.

4.4.4 Optimization of the Activity of the 3-Dehydroecdysone 3β-Reductase

a) Cofactor Preference

Since no assay system had previously been described for the *Spodoptera* enzyme, optimal conditions had to be defined. In view of the fact that in *Manduca*, NADPH was found to be favoured over NADH, (Sakurai et al., 1989b) and that an enzyme catalysing the same reaction in *Spodoptera* midgut had a preference for NADH (Milner and Rees, 1985), the cofactor requirements of the haemolymph 3-dehydroecdysone 3β-reductase were examined. Five sets of tubes were set up in duplicate, containing the following:

Enzyme preparation	**Cofactor**	**Specific activity of the 3β-reductase (nmol/h/mg protein)**
Non-dialysed	none	4.77± 0.41
Dialysed	none	0
Dialysed	NADPH	4.54± 0.21
Dialysed	NADH	1.86± 0.02
Boiled	NAD(P)H	0

Reactions were terminated after 1h.

These data tend to suggest that there is a factor occurring naturally in the haemolymph that can act as a cosubstrate for the reaction. However, this factor must be of sufficiently low molecular weight to be removed by dialysis [or gel filtration (Sakurai et al.,

1989b)], since dialysed enzyme preparation could not to bring about the conversion, without added NAD(P)H. Presumably, the factor in the haemolymph is the latter factor.

b) Temperature

Usual assays were set up (Section 4.2.1 and using 0.5mM NADPH as cofactor). A range of temperatures was used to determine the optimal value: 20, 25, 30, 35, 36, 37, 38, 39, 40°C. The optimum was found to be 37°C, (figure 4.2), above which activity decreased, possibly owing to the start of protein denaturation.

c) pH

Incubation mixtures were set up in the usual manner (Section 4.2.1 and using 0.5mM NADPH as cofactor), but using the following buffer systems, at constant ionic strength (I=0.2) :

pH 5.1-6.4, 0.2M sodium acetate/ acetic acid

pH 6.5-7.8, 0.2M sodium phosphate

pH 8.0-9.0, 0.2M Tris/ Tris-HCl.

The solutions were made up at room temperature and the accurate pH measured at 37°C. pH 7.5 was found to be optimal (figure 4.3). However, activity did not decline as rapidly as expected in the higher pH range (8.0-9.0) and the enzyme appears to have quite a wide range over which it is active. At lower pH values, (<7.5) there was low activity.

d) Protein Concentration

The linearity of the rate of reaction with increasing amounts of enzyme protein was examined. Protein was estimated using the Lowry method, with BSA as the standard protein (Lowry et al., 1951) and appropriate volumes of enzyme preparation were added to the

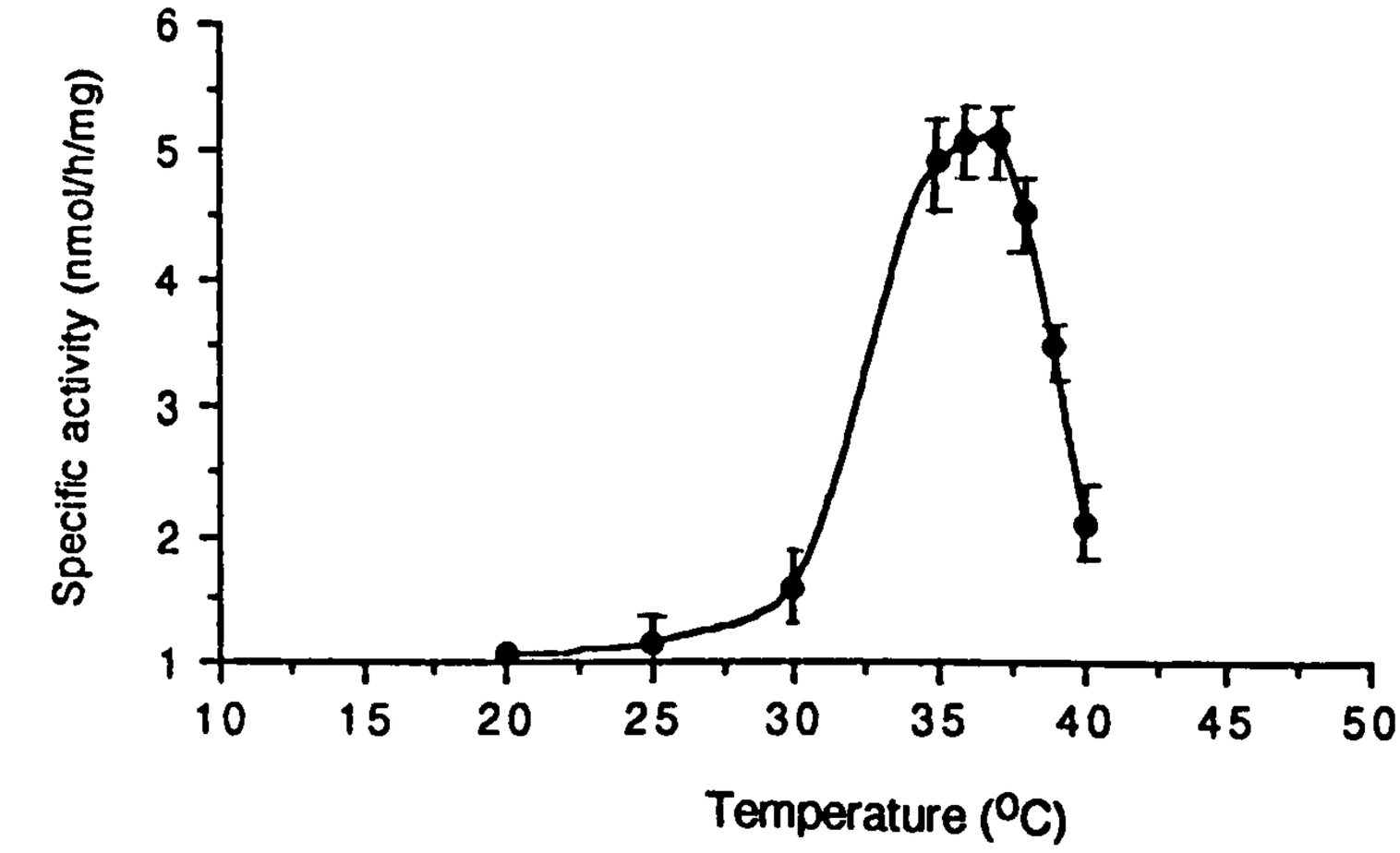


Figure 4.2. Effect of temperature on the haemolymph 3-dehydroecdysone 3β-reductase. Values represent the mean of two investigations (±SD). Where error bars are not shown, they are within the (•) symbol.

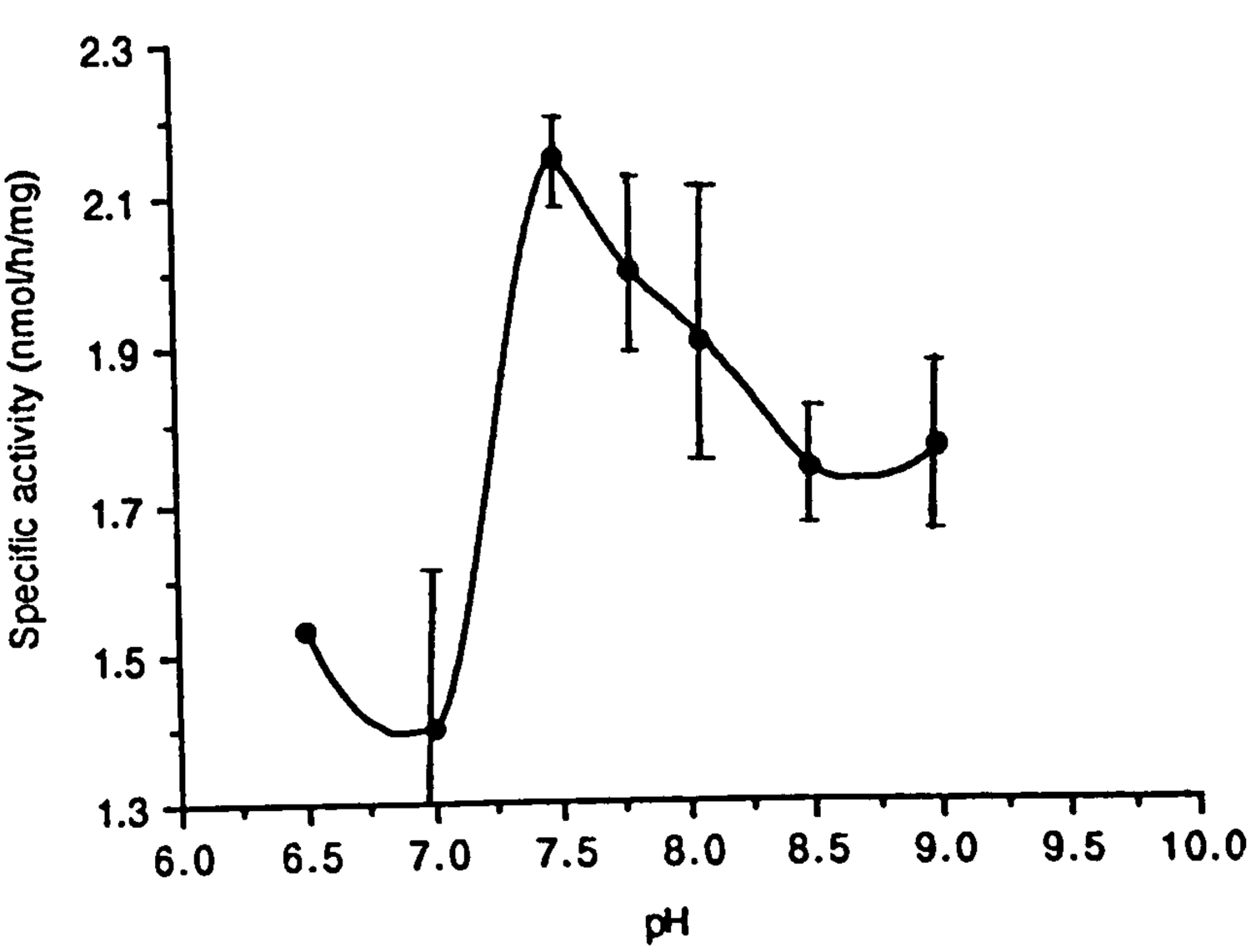


Figure 4.3. Effect of pH on the haemolymph 3-dehydroecdysone 3β-reductase. Values represent the mean of two investigations (±SD). Where error bars are not shown, they are within the (•) symbol.

incubation mixtures (0-2.0mg total protein). The concentration of NADPH (0.5mM) and 3-dehydroecdysone (8.4μM) were maintained constant. The activity of the 3-dehydroecdysone 3β-reductase was assayed and the results are presented in figure 4.4. The rate of reaction is apparently linear up to 1mg protein.

e) Reaction Time

Reaction tubes were set up as usual (as in Section 4.2.1 and using 0.5mM NADPH as cofactor), but terminated at timed intervals from 0 to 60min. Figure 4.5 clearly shows that the reaction proceeds to give a sigmoidal plot of activity *versus* time. The reaction appears to be linear between 10 and 20min, but shows a lag-time between 0 and 10min. Moreover, the exact reasons for these kinetics are unclear.

From the above combined data, a standard *in vitro* assay was now established: pH 7.4, 1.0mg protein, for 20min at 37°C and excess NADPH (0.5mM) as cofactor. (For a comparison with selected midgut enzymes, see Section 2.2.4; Chapter 6.)

4.2.5 Fractionation of the Enzyme

Haemolymph (110ml) from approximately 350 sixth instar larvae (day3-4) was extracted within 1month, cell debris and haemocytes sedimented at 15,000g (15min) and mercaptoethanol was added to a final concentration of 0.2% (v/v). The enzyme preparation was dialysed against 100 volumes of buffer A, containing 0.2%(v/v) mercaptoethanol, i.e. buffer B. The dialysate was centrifuged at 18,000g for 15min and the supernatant applied to a DEAE-cellulose column,

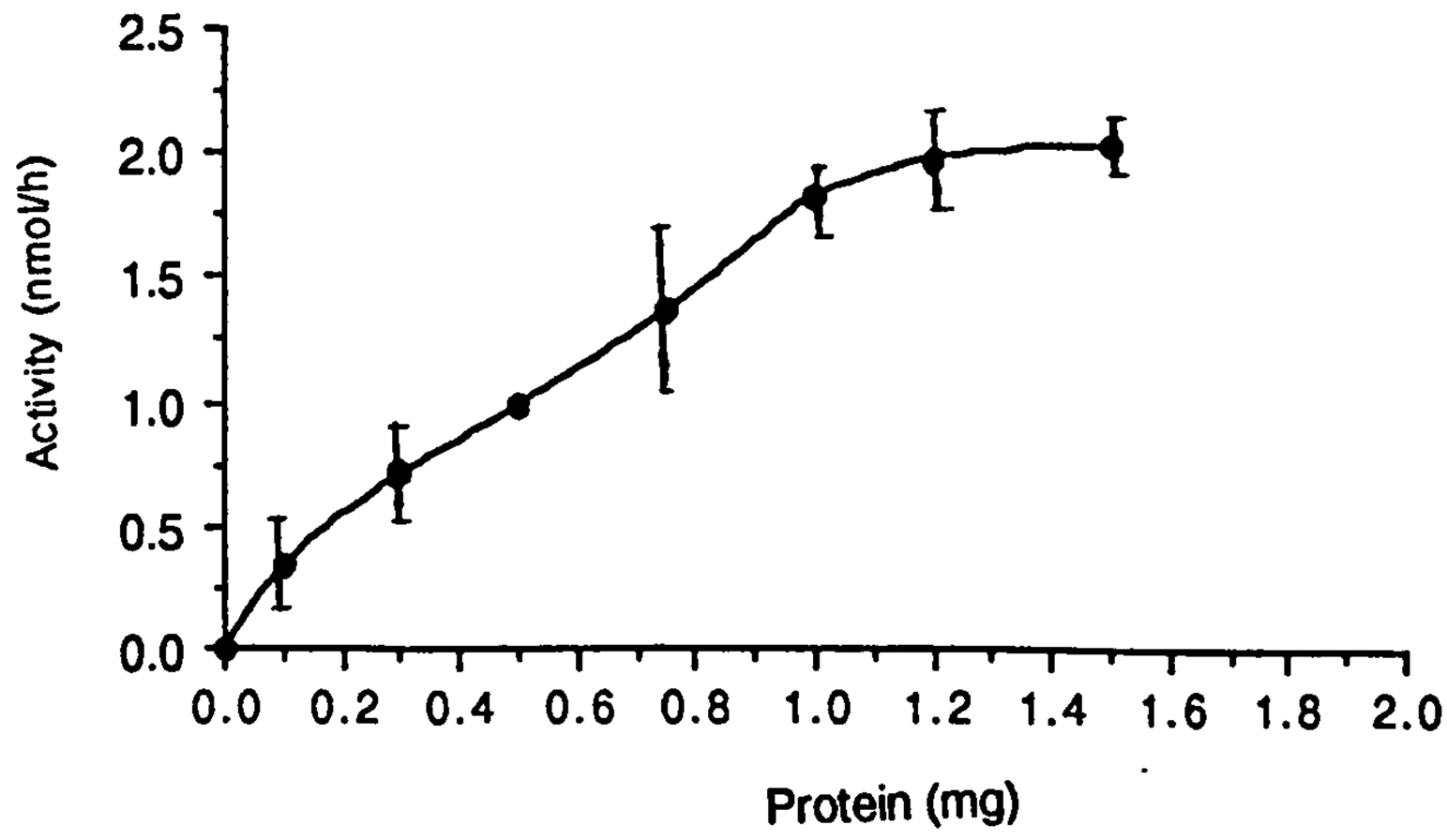


Figure 4.4. Effect of protein concentration on the rate of conversion of 3-dehydroecdysone into ecdysone by the haemolymph 3-dehydroecdysone 3β-reductase. Values represent the mean of two investigations (±SD). Where error bars are not shown, they are within the (•) symbol.

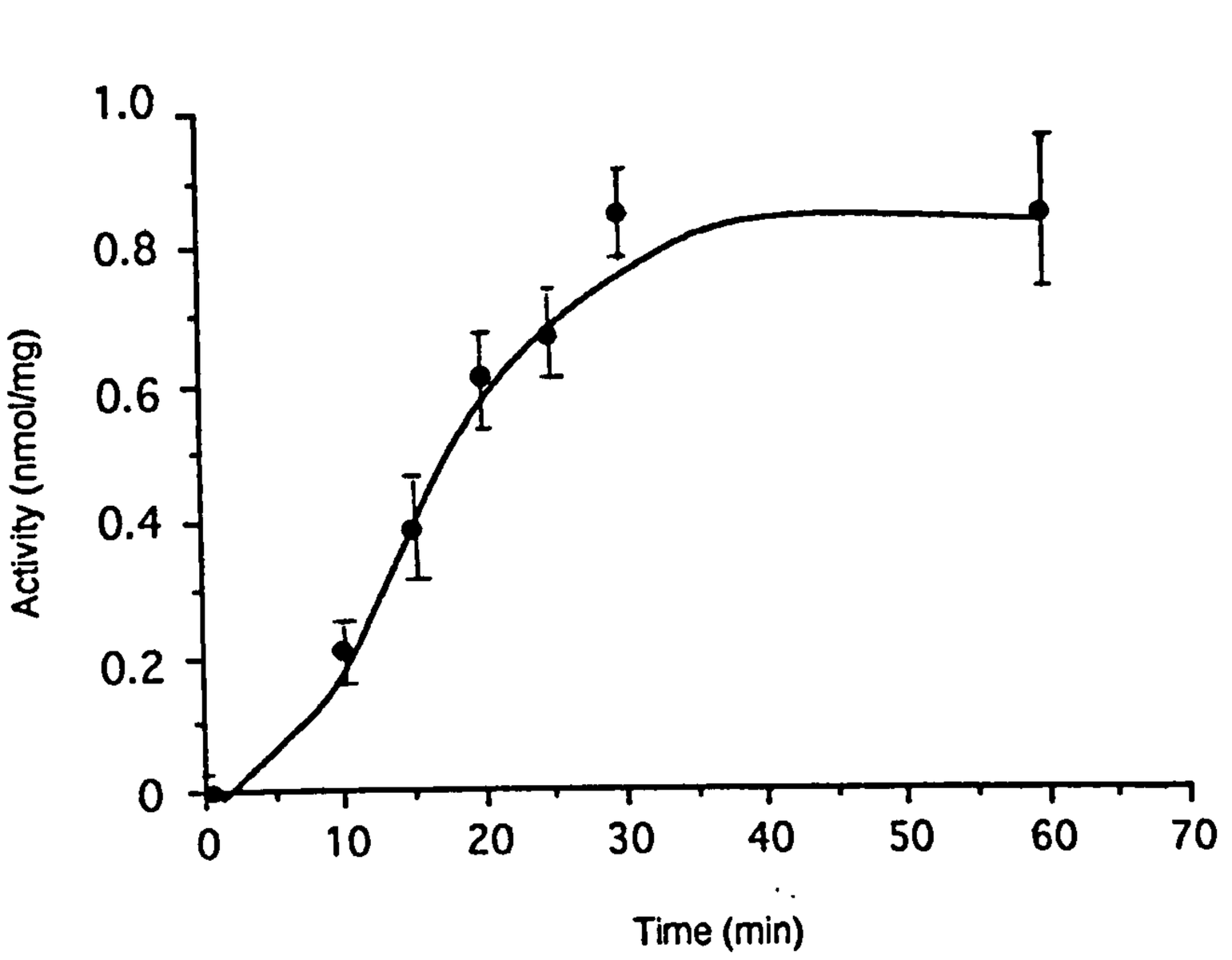


Figure 4.5. Time course of the haemolymph 3-dehydroecdysone 3β-reductase. Values represent the mean of two investigations (±SD). Where error bars are not shown, they are within the (•) symbol.

pre-equilibrated with buffer B. The enzyme was then fractionated using DEAE-cellulose, FPLC Mono-Q and FPLC Phenyl Superose columns, detailed in section 2.2.4. After each chromatography step, aliquots (typically 100-200µl) were taken at each stage for i) protein determination by the Lowry method (Lowry et al., 1951), using BSA as the standard protein and ii) SDS-PAGE (Section 2.2.5). Fractions were subsequently frozen until the assays had been carried out.

The enzyme was partially purified by sequential column chromatography (Figures 4.6a,b,c). Typical purification results are shown in Table 1; a yield of approximately 9% and purification factor of 56 were obtained. That the purification factor is rather low is perplexing, and could be due to detrimental effects of freeze-thawing.

Analysis under denaturing conditions on SDS-PAGE (stained using Coomassie Blue R) revealed the presence of a major band, assumed to be the enzyme (figure 4.7), which had a mass of 34,700Da, by comparison to a standard curve, constructed from the mobilities of the standard molecular weight markers. The molecular weight is comparable to the *Manduca* haemolymph enzyme (20-30kDa) and of the enzymes found in various organs of *Ostrinia nubilalis* (25-37kDa; Gelman et al., 1991).

4.4.6 Determination of the K_m Value and the V_{max}

3-Dehydroecdysone 3β-reductase was prepared as in Section 2.2.3 and applied to a DEAE-cellulose column (Section 2.2.5). Fractions of highest activity were pooled and dialysed. Using the new optimal assay conditions, (Section 4.2.4) 0.5mM NADPH (100µl) was added to 100µl of the fractionated enzyme preparation the assay carried out using the following final concentrations of 3-dehydroecdysone 0, 10, 20, 30, 40,

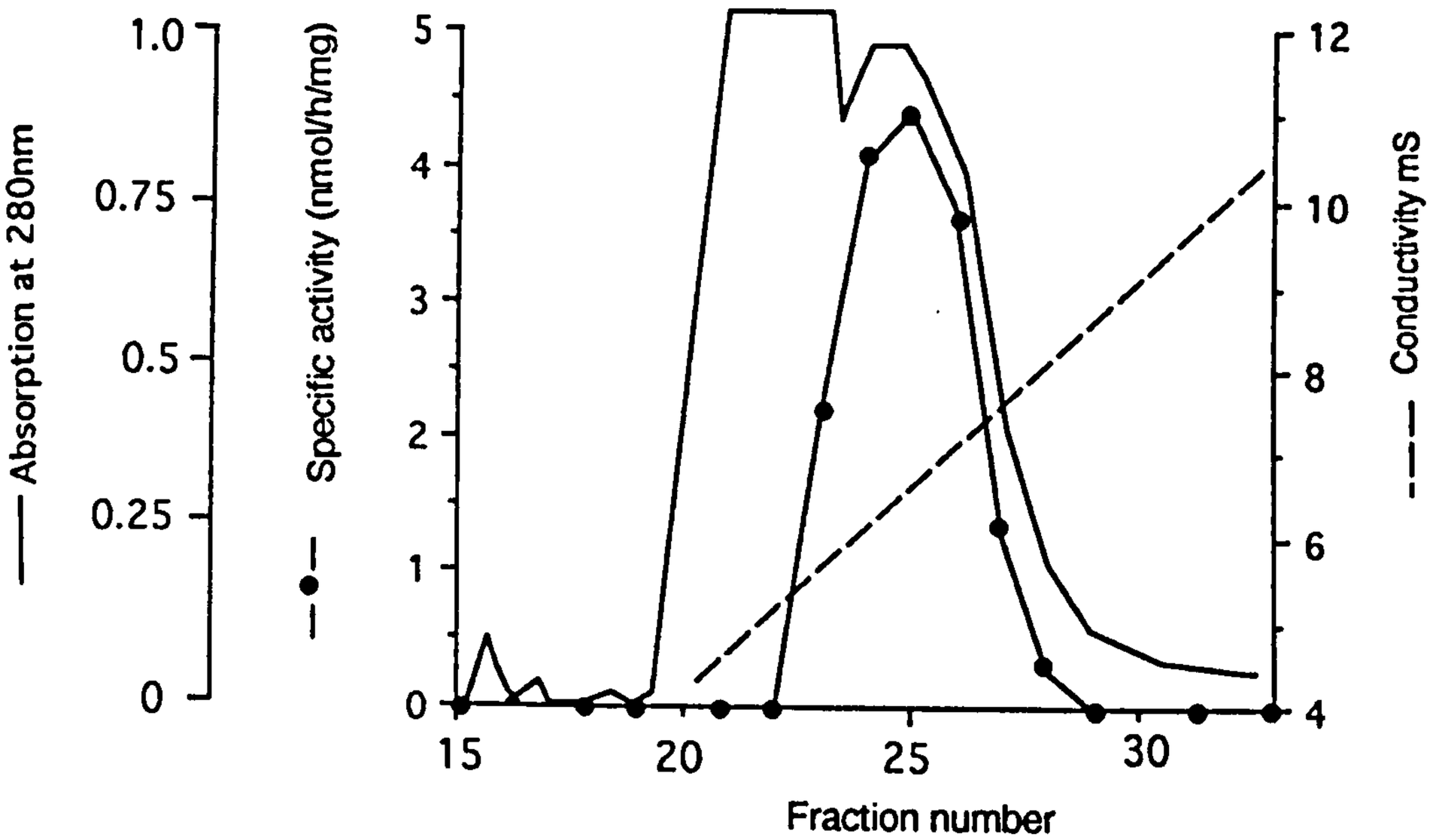


Figure 4.6a DEAE-cellulose chromatography of the 3-dehydroecdysone 3β-reductase enzyme. Elution was by means of a linear salt gradient generated by mixing buffer B and 0.5M NaCl in buffer B.

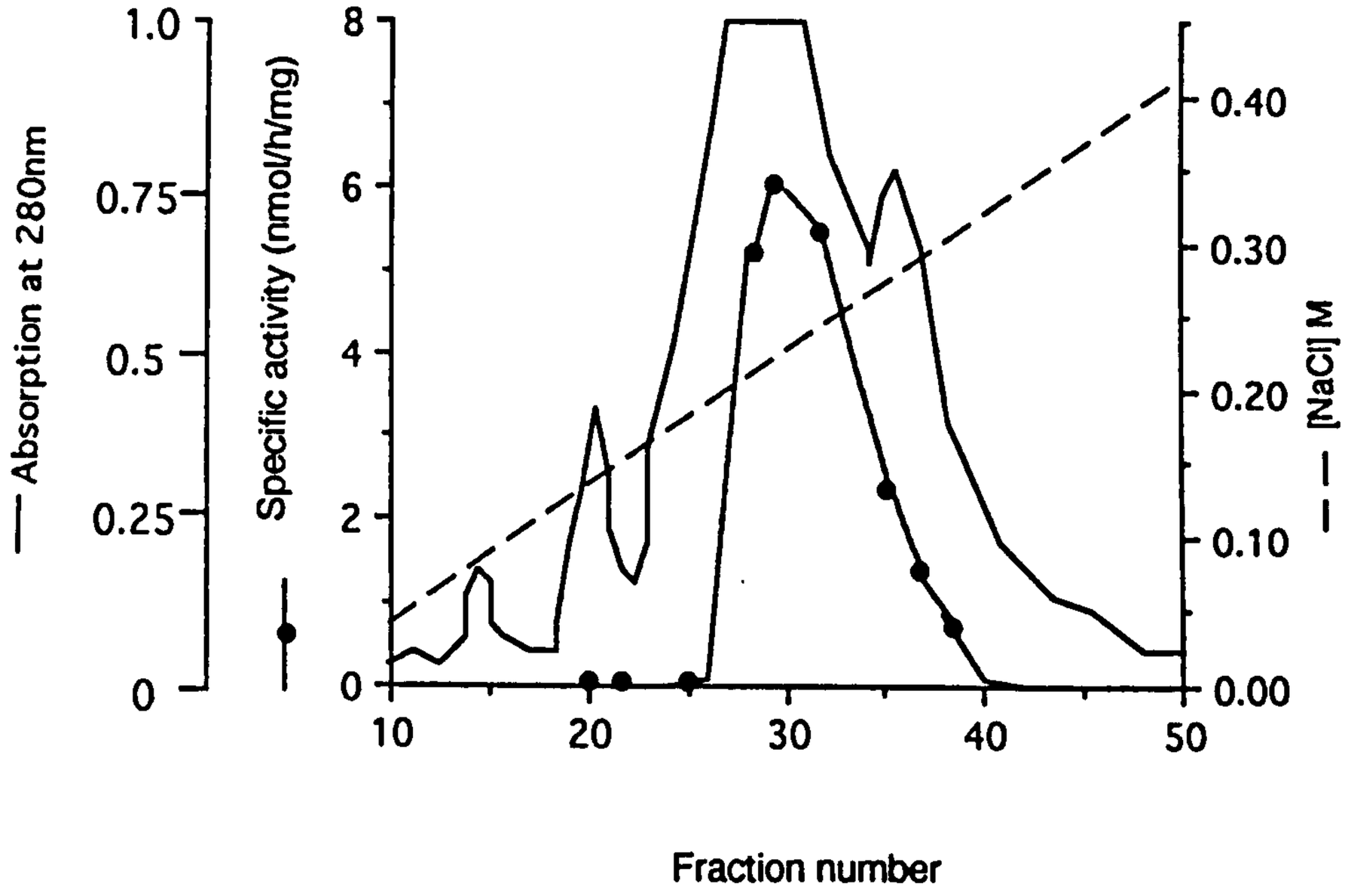


Figure 4.6b FPLC on Mono-Q of the 3-dehydroecdysone 3β-reductase. The enzyme was previously fractionated by DEAE-cellulose, applied to Mono-Q and eluted using a linear salt gradient formed by mixing buffer B and 0.5M NaCl in buffer B.

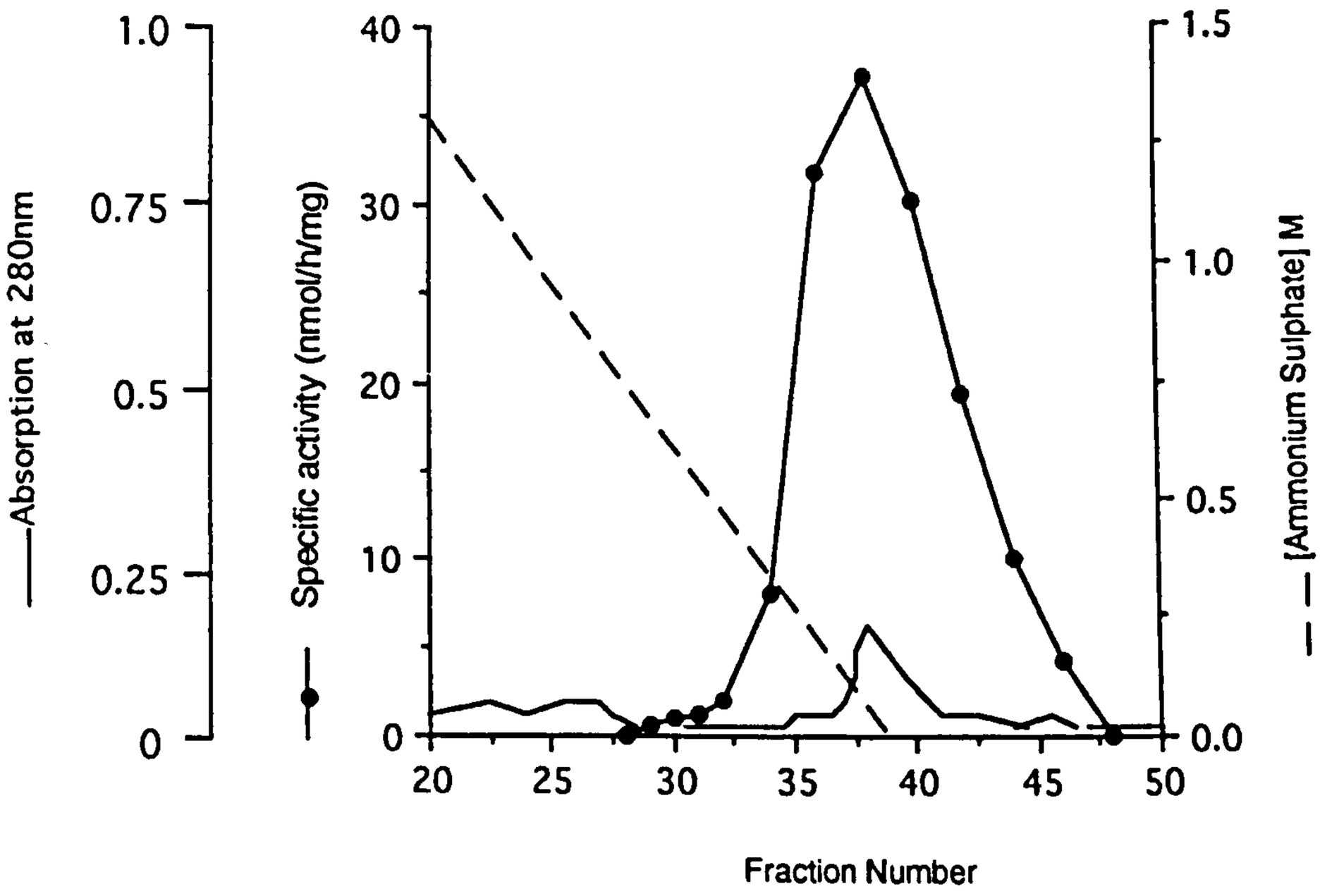


Figure 4.6c Hydrophobic interaction FPLC elution of the 3-dehydroecdysone 3β-reductase. Enzyme activity was previously fractionated on Mono-Q, applied to Phenyl Superose and eluted with a reverse gradient of ammonium sulphate in a reverse phosphate gradient generated by mixing buffer C and 10mM phosphate buffer, pH 7.5.

Step	Activity nmol/h	Protein mg	Sp. Activity nmol/h/mg	Yield %	Purification Fold
Haemo-lymph	3555	546	6.51	100	1
DEAE	1046.5	58.5	17.89	29.4	2.75
Mono-Q	444.8	13.2	67.40	12.5	10.4
Ph-Sup HIC	317.9	0.87	365.4	8.9	56.1

Table 1 The purification of haemolymph 3-dehydroecdysone 3β-reductase

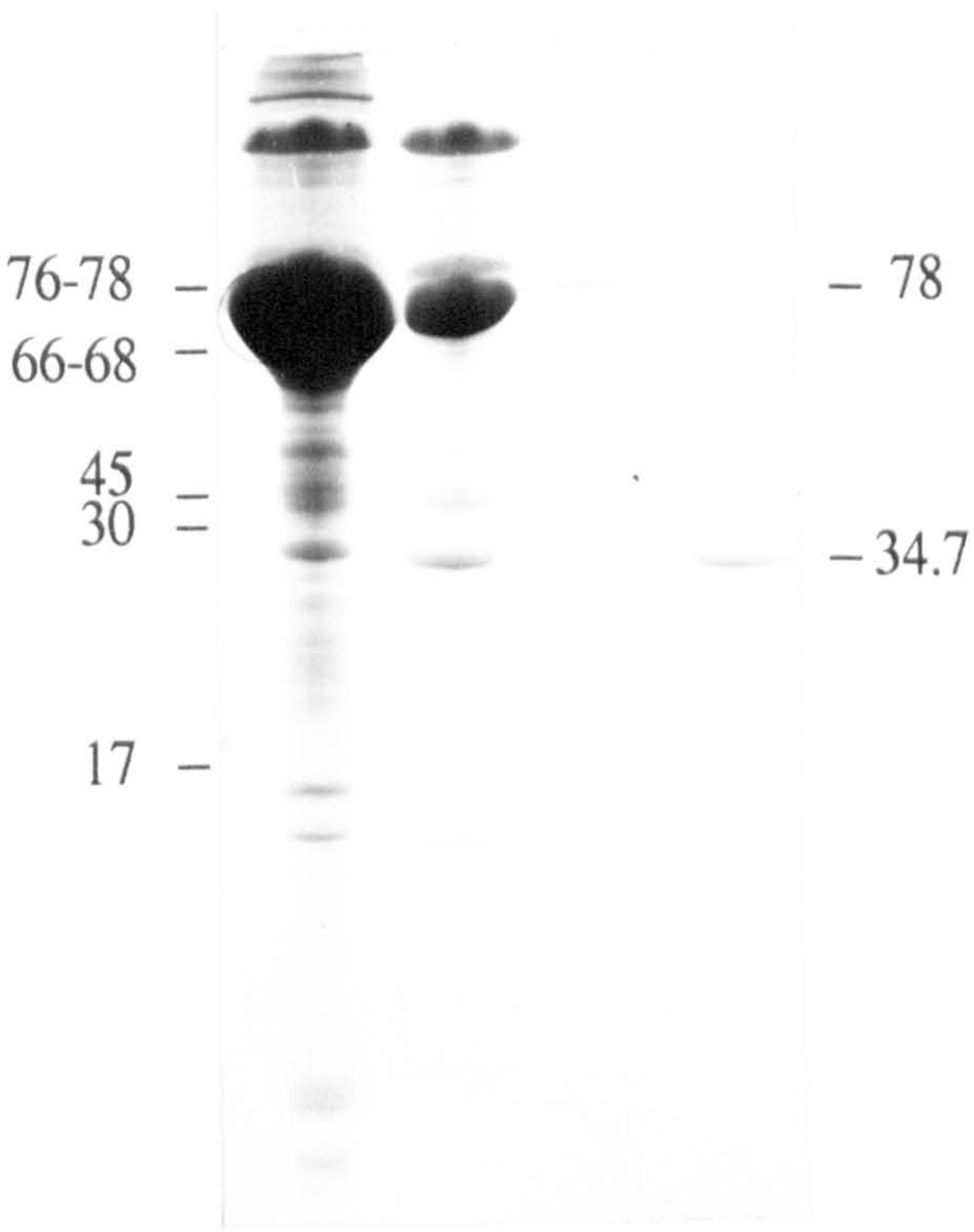


Figure 4.7. SDS-polyacrylamide gel of the haemolymph 3-dehydroecdysone 3β-reductase at each stage of its purification. The positions and molecular weights (kDa) of major polypeptide bands are indicated. The mobilities of a range of standard molecular weight markers are shown for each gel: ovtransferrin 76-78kDa; bovine serum albumin 66-68kDa; ovalbumin 45kDa; carbonic anhydrase 30kDa; myoglobin 17kDa.

Lane 1	Total haemolymph protein
Lane 2	3β-Reductase from DEAE-cellulose chromatography
Lane 3	3β-Reductase from Mono-Q FPLC
Lane 4	3β-Reductase Phenyl Superose FPLC

50 and 100μM. Kinetic parameters were estimated using the Apple Macintosh "Regression" package, which fits a best curve by iteration (figure 4.8). Values represent the mean of two complete determinations (± the standard error).

When considering enzyme kinetics, it is assumed that all of the sterol substrate is solubilized in the aqueous buffer. However, since ecdysteroids are moderately to poorly soluble in water, the constant, K_m, must be an apparent K_m.

The apparent K_m, found by was quoted as 21.4± 5.9μM and the V_{max}, under the conditions used, was 5.49± 0.54nmol/h/mg protein. The K_m value is of the same order of magnitude as the semi-purified *Manduca* midgut 3-dehydroecdysone 3β-reductases, *vis-à-vis* 17.7±10.3 μM, when NADPH is added and 15.5±3.2μM, using NADH, (Weirich et al., 1989). The V_{max} values are obviously non-comparable due to the different assay systems and purification states of the enzyme used.

4.3 DISCUSSION

From the evidence, it would seem that the *Spodoptera* haemolymph 3-dehydroecdysone 3β-reductase has some features in common with those of the *Manduca* enzyme. The peak titre is reached just prior to that of the peak in haemolymph ecdysteroid content. Such fluctuations suggest a role closely linked to the circulating levels of ecdysteroids and may represent the activation of 3-dehydroecdysone product of the prothoracic glands (prohormone) to the more hormonally active ecdysone. This could contribute to the huge surge in ecdysteroid titre at the end of the sixth instar.

The RIA determination of ecdysteroid levels was carried out using H-22 antiserum, which has low immunoreactivity against 3-

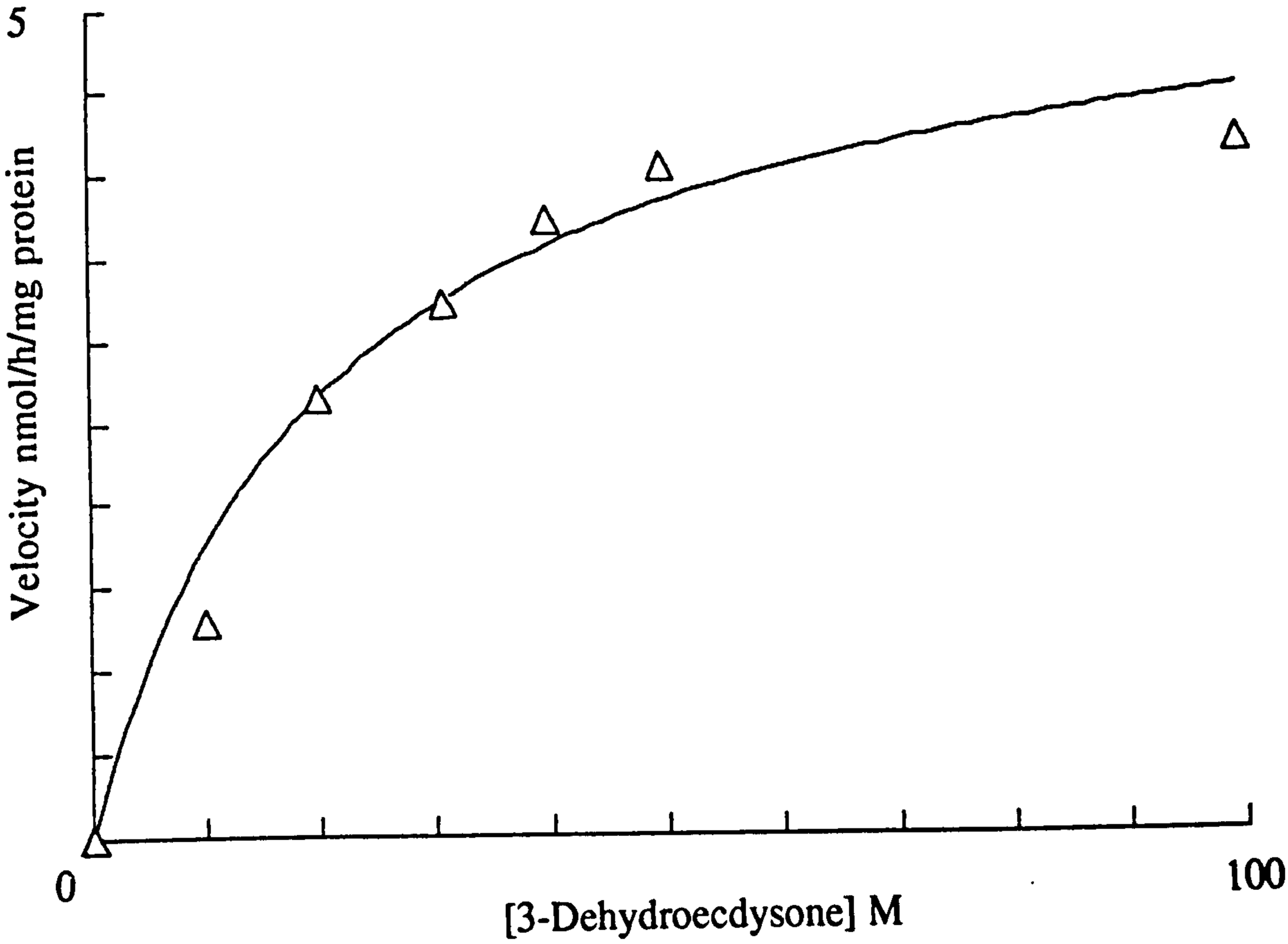


Figure 4.8. Effect of the concentration of 3-dehydroecdysone on the rate of conversion by the haemolymph 3-dehydroecdysone 3β-reductase.

dehydroecdysone. Therefore, it can be said with a high degree of certainty that 3-dehydroecdysone does not represent a significant proportion of the ecdysteroids detected. Moreover, the ecdysteroids are probably mainly ecdysone and 20-hydroxyecdysone. At the high point of haemolymph ecdysteroid titre in *Spodoptera littoralis,* Jarvis (1991) identified the component ecdysteroids as ecdysone and 20-hydroxyecdysone (in the approximate ration of 1:11.2).

The 3-dehydroecdysone 3β-reductase of *Spodoptera* has a preference for NADPH over NADH, as in *Manduca sexta* (Sakurai et al., 1989b). Whether this reflects the situation *in vivo* can only be speculated, since NADPH is usually present in biosynthetic cells or those performing the pentose phosphate pathway of monosaccharide metabolism. Since the haemolymph is not considered a major site of NADPH production, the source of such a cofactor *in vivo* is still an open question. It is conceivable that the enzyme is synthesized in an organ such as the fat body, and that the NADPH associates with the protein before being exported into the haemolymph.

A so-called haemolymph stimulatory factor has been found to be released by the fat body of *Manduca sexta* (Greutzmacher et al., 1984). There is circumstantial evidence that this is the same as the 3-dehydroecdysone 3β-reductase of the haemolymph. Both act independently of a rise in cAMP levels in the prothoracic glands (Smith, et al. 1986), are heat labile and have similar molecular weights (*ca* 30kDa). The fat body's role as a major metabolic organ, its proximity to the haemolymph and its distribution throughout the whole insect, suggests that this tissue would be well suited to the site of synthesis for the enzyme.

Other biochemical characteristics of the enzyme *in vitro* (optimal pH, temperature, time and protein linearity) can be compared to the

previously reported 3-dehydroecdysone 3β-reductase of both *Manduca* [3β-reductases (NAD(P)H have an optimal pH value of 6.5; Weirich et al., 1989] and *Spodoptera* midgut [3β-reductase (NADH) has an optimal pH of 6.5 and 3β-reductase (NADPH) has an optimal pH at 7.9; Chapter 6]. The pH is slightly above neutral (7.5) and hence, physiologically suited to the haemolymph pH of 7.4. The temperature optimum is 37°C. It should be added that most of the time courses exhibit plateaux, possibly due to the limited concentration of ecdysteroid substrate (see Chapter 6 for details of the K_m values).

It is rather interesting that the 3-dehydroecdysone 3β-reductase reaction does not progress linearly with respect to time, but appears to have a lag period between 0 and 10min (although this can only be postulated, since it is dependent on the first time-point, figure 4.5). It is possible that this enzyme is hysteretic. Hysteresis describes the action of an enzyme which responds slowly (in terms of some kinetic or physical characteristic) to a rapid change in substrate or cosubstrate concentration (Frieden, 1970). Such slow changes result in a lag in the response of the enzyme. Several means of activation, depending upon the enzyme, are possible, e.g. (co)substrate induced isomerization of the enzyme, displacement by other ligands [(co)substrate], or polymerization and depolymerization. The time of the lag may also be dependent on temperature, buffer and pH.

In the case of the 3-dehydroecdysone 3β-reductase, pre-equilibration did not remove or decrease the lag period. It is therefore necessary to sequentially change other factors during the equilibration period, to assess which factor(s) is (are) required to activate the enzyme. However, it must be borne in mind that examination of the changes in kinetics or ligand binding characteristics may indicate changes which may not be metabolically significant *in vivo*.

Significant purification of the enzyme was achieved. As alluded to previously, the molecular weight was of the same order as those found in *Manduca* (Sakurai et al., 1989b; Watson et al., 1985) and *Ostrinia* (Gelman et al., 1991), the latter examples being determined by gel exclusion chromatography. Therefore, it is assumed that the published values would represent the total molecular weight, rather than that of a subunit. The determination of the molecular weight of the reductase from *Spodoptera* was under the denaturing conditions of SDS-PAGE, hence this would not necessarily be indicative of the total molecular weight (although highly likely), but could be that of a subunit.

The 3-dehydroecdysone 3β-reductase would appear to be rather hydrophobic. This is borne out by the fact that the protein elutes at the end of the reversed ammonium sulphate gradient (i.e. at low salt concentration) from the hydrophobic interaction column, (figure 4.6c). Since this enzyme has to bind to a steroid molecule, which, although polyhydroxylated, is still only moderately soluble in an aqueous solution, it could be postulated that the enzyme provides a microenvironment conducive to substrate binding.

A crude measure of an enzymes's affinity for its substrate is often quoted as the K_m value. In the case of this reductase, the value may be regarded as high (21.4± 5.9μM), compared to the peak haemolymph ecdysteroid titre (1.6± 0.1μM). However, this further signifies the great differences between the *in vitro* potential of a semi-purified enzyme, using optimal *in vitro* conditions, and of the *in vivo* situation, which is in a state of constant flux.

The discovery of the role of the reductase in ecdysone production has added a new dimension to the control of moulting hormone levels in Lepidoptera. The ability of several other organs to convert 3-dehydroecdysone into ecdysone is of particular importance. Gelman et

al. (1991) have described that various organs of *Ostrinia nubilalis* (Lepidoptera) possess enzyme activity. Similarly, a reductase activity is found in the midgut of *Manduca sexta* (Mayer et al., 1979) and *Spodoptera littoralis* (Milner and Rees, 1985; Chapters 5 and 6) and the enzyme may also be present in the embryonic stage of *Lymantria dispar* (Kelly, et al., 1990). Thus, the reduction of 3-dehydroecdysone to ecdysone may also occur in late embryonic development, in a similar manner to that of post-embryonic life.

In conclusion, the importance of the 3-dehydroecdysone 3β-reductase has only recently been realized and will remain a major area of interest for some time.

Chapter 5

Verification of the ecdysone epimerization and phosphorylation reactions in the midgut cytosol of *Spodoptera littoralis*

5.1 INTRODUCTION

The midgut of mid to late sixth (last) instar larvae of *Spodoptera littoralis* is actively involved in ecdysone metabolism. A small degree of conversion of ecdysone into 20-hydroxyecdysone takes place in the mitochondria (Chen, unpublished results) and, additionally, a relatively active cytosolic system, converting metabolites to their respective 3-epimers is present. 3-Epimerization causes a substantial reduction in moulting hormone activity (Nigg et al., 1974; Mayer et al., 1979; Lafont and Koolman, 1984). Activation and inactivation reactions are compartmentalized and the enzymes, products and cofactors in the following discussion are located in the cytosolic post-microsomal supernatant.

The first step in the epimerization of ecdysteroid is the irreversible dehydrogenation of (20-hydroxy)ecdysone to 3-dehydro(20-hydroxy)ecdysone, yielding hydrogen peroxide as the secondary product. The former are regarded as catabolites of low hormonal activity, having only $^1/_{10}$-$^1/_{15}$ the activity of the parent compound, as found by the *Calliphora* assay (Koolman and Spindler, 1977) and in two different *Drosophila* chromosome puff assays (Richards, 1978). Interestingly, the physiological significance of 3-dehydroecdysteroids is not fully understood: do they act as inactivation products? Are they formed from injested phytoecdysteroids? (Beydon et al., 1987) or do they act as hormones themselves? (Spindler et al., 1977; Sommé-Martin et al., 1990). It is possible that 3-dehydroecdysteroids perform all three functions, at selected phases of development.

It is well known that 3-dehydroecdysteroids do not appreciably accumulate in the midgut *in vivo,* but are converted into 3α-hydroxy epimers. These compounds which have been most notably detected

during embryogenesis and in the gut tissue (Kaplanis et al., 1979; Kaplanis et al., 1980; Lafont et al., 1980) and represent, along with conjugates (and ecdyson 26-oic acids), major inactivation products of ecdysone (Koolman, 1982). *In vitro* epimerization in the midgut cytosol of *Manduca sexta* was first demonstrated by Nigg et al., in 1974, the 3-epimers being found to be much less active than the corresponding 3β-hydroxyecdysteroids in the *Musca* bioassay (Nigg et al., 1974; Kaplanis et al., 1979). Since 3α-ecdysone cannot be converted back into a 3β-metabolite, 3α-epimers represent an irreversible loss of hormonal activity. However, 3-dehydroecdysteroids can be converted back into their 3β-hydroxy parent compounds and, therefore, could be regarded as temporary inactive forms.

In summary therefore, 3-dehydroecdysone can be either converted into a 3α- or 3β-hydroxy-ecdysone. The cofactors of these reactions require investigation: in *Spodoptera littoralis*, the 3α-hydroxy-forming reductase has preference for NADPH, whereas there is a preference of NADH for the 3β-hydroxy forming one (Milner and Rees, 1985); the converse is true for *Manduca sexta* (Weirich et al., 1989). These metabolites may be further inactivated by the action of phosphotransferase enzymes. Ecdysteroid conjugates have long been recognized as important metabolites of the moulting hormones, ecdysone and 20-hydroxyecdysone. The nature of the conjugate moiety and its position in the ecdysteroid molecule can depend upon species and the developmental stage of the insect. Many ecdysteroid conjugates have been isolated from ovaries and eggs of various species (for review see Hoffmann, et al., 1980), where they are believed to act as storage forms, for donation from the mother to the egg, and later hydrolysed in the embryo. However many conjugates may also be considered inactivation products (Lafont and Koolman, 1984). It is the latter case that will be

examined, i.e. the phosphorylation of the metabolites of epimerization in the midgut. Although a plethora of information exists on ovarian and egg ecdysteroid phosphates, little has been recorded on the midgut phosphates, and even less on the enzymes furnishing these reactions (Weirich et al., 1986).

The aims of this chapter are then two-fold: firstly the cosubstrate requisites will be addressed, with particular emphasis on the reductases. Secondly, to fully establish the major (and minor) products of epimerization and phosphorylation.

5.2 EXPERIMENTAL AND RESULTS

5.2.1 Assay Procedure

Dialysed enzyme preparation (100µl) was added to a mixture of 8.4µM ecdysteroid containing 200,000dpm of tritiated substrate (50µl) and the appropriate cofactor dissolved in 0.2M sodium phosphate buffer, pH 7.4 (100µl). In the case of the ecdysone oxidase, no cofactor was required, for the 3-dehydroecdysone 3β-reductase 0.5mM NADH was added and for the 3-dehydroecdysone 3α-reductase 0.5mM NADPH was used. 2mM ATP and 10mM $MgCl_2$ were added to the phosphotransferase incubations.

Reactions were carried out at 37°C for 1hour and terminated by addition of chilled methanol (400µl). The protein was sedimented at 9,000g for 10min at 4°C and the pellet re-extracted with methanol. The supernatants were combined and dried under a stream of nitrogen. Samples were desalted by C18 Sep-pak (Section 2.2.9), free ecdysteroids eluted in the 60%(v/v) methanol/water fraction and polar conjugates in the 30%(v/v) methanol/water fraction. Analysis of the reaction products

was by HPLC (Section 2.2.9), typically using a reversed-phase column: (i) isocratic elution at 1ml/min with 0.1%(v/v) TFA in water/ acetonitrile [78:22 (v/v)] (system 1), for free ecdysteroids or (ii) a linear 30min gradient at 1ml/min of 20-70%(v/v) methanol/ 20mM citrate buffer, pH 6.5 (system 3), for polar conjugates. For the verification of reaction products, two other HPLC systems were employed: adsorption chromatography on an aminopropyl silica column, developing with 8%(v/v) methanol in 1,2-dichloroethane at 1ml/min (system 2) and reversed-phase chromatography using a linear 60min gradient at 1ml/min, (system 4).

5.2.2 Cofactor Requirements

a) The Reductases

The following reactions were set up using 8.4µM 3-dehydroecdysone containing 0.5µCi (50µl) and dialysed enzyme preparation (100µl), together with one of the following:

i) no cofactor

ii) 0.5mM NADPH

iii) 0.5mM NADH

Analysis of the products revealed the following:

i) No conversion of 3-dehydroecdysone was found using a mixture which contained no additional cofactor.

ii) When 0.5mM NADPH was added to the incubation mixture, it was apparently utilized by a 3-dehydroecdysone 3α-reductase enzyme, yielding a product which co-chromatographed with 3-epiecdysone. A lesser quantity of (putative) ecdysone was also detected, due to the

presence of a 3-dehydroecdysone 3β-reductase, using NADPH (figure 5.1a).

iii) By adding NADH to the reaction, the major product was found to be (putative) ecdysone and a smaller amount of (putative) 3-epiecdysone (figure 5.1b).

b) The Phosphotransferases

The following incubations were set up using 8.4μM ecdysone containing 200,000dpm [$^3H_2$]ecdysone (50μl) and enzyme preparation (100μl):

i) non-dialysed enzyme preparation + no cofactor

ii) dialysed enzyme preparation + no cofactor

iii) dialysed enzyme preparation + 10mM $MgCl_2$

iv) dialysed enzyme preparation + 2mM ATP

v) dialysed enzyme preparation + 10mM $MgCl_2$ + 2mM ATP

i) When non-dialysed enzyme preparation was used in the incubation, endogenous cofactors were sufficient to bring about 30.5% conversion into a metabolite having the same retention time as 3-epiecdysone (HPLC system 1) and 7.5% conversion into a metabolite having the same retention time as ecdysone 22-phosphate, (HPLC system 3; figures 5.2a and b). The putative ecdysone 22-phosphate was hydrolyzed by a crude arylsulphatase preparation (*ex. Helix pomatia,* Section 2.2.10), and ecdysone was revealed to be the only free ecdysteroid (system 1). That 3-dehydroecdysone was not detected was not surprising, as it is known that epimerase action rapidly converts any 3-dehydroecdysone into 3-epiecdysone (Nigg et al., 1974).

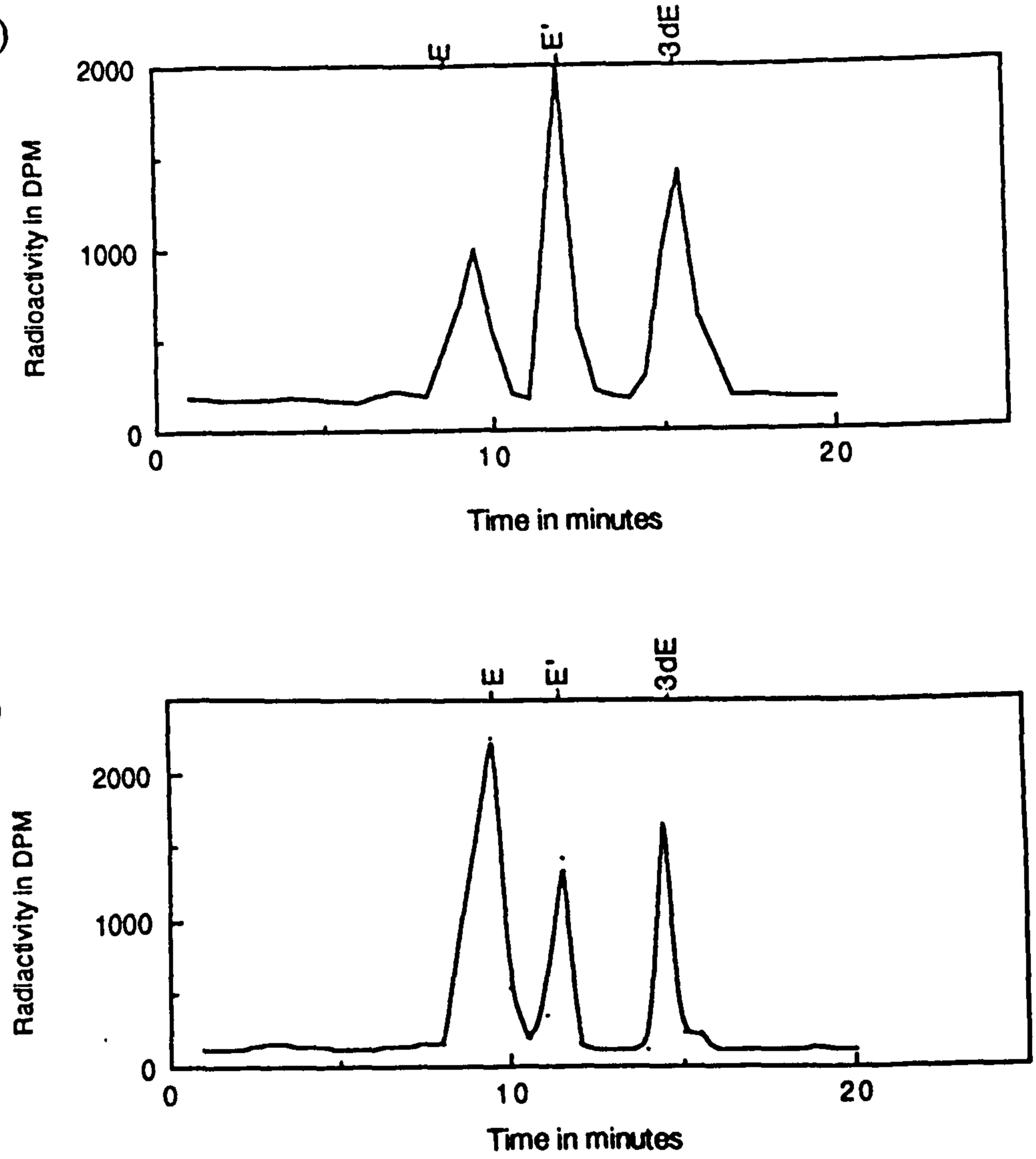
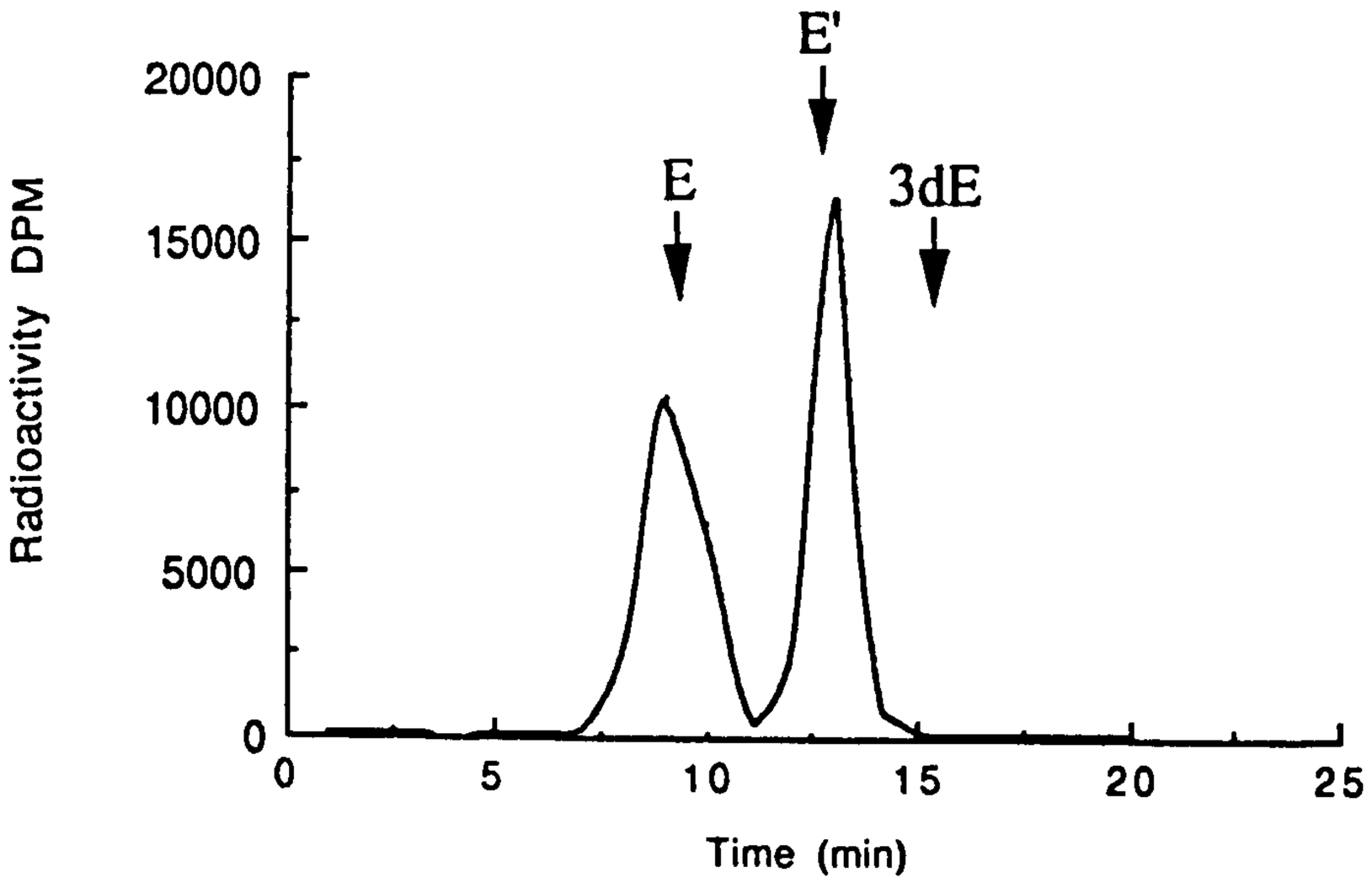


Figure 5.1. Separation of [^{3}H]ecdysteroid products by reversed-phase HPLC. 3-Dehydroecdysone (3dE) and 3-epiecdysone (E') were separated from the starting substrate, 3-dehydroecdysone (E) using an acetonitrile/ TFA isocratic gradient for elution, system 1. a) Using 0.5mM NADPH and b) 0.5mM NADH in the incubation mixture. The chromatography was monitored by collecting fractions at 1min intervals for determination of radioactivity.



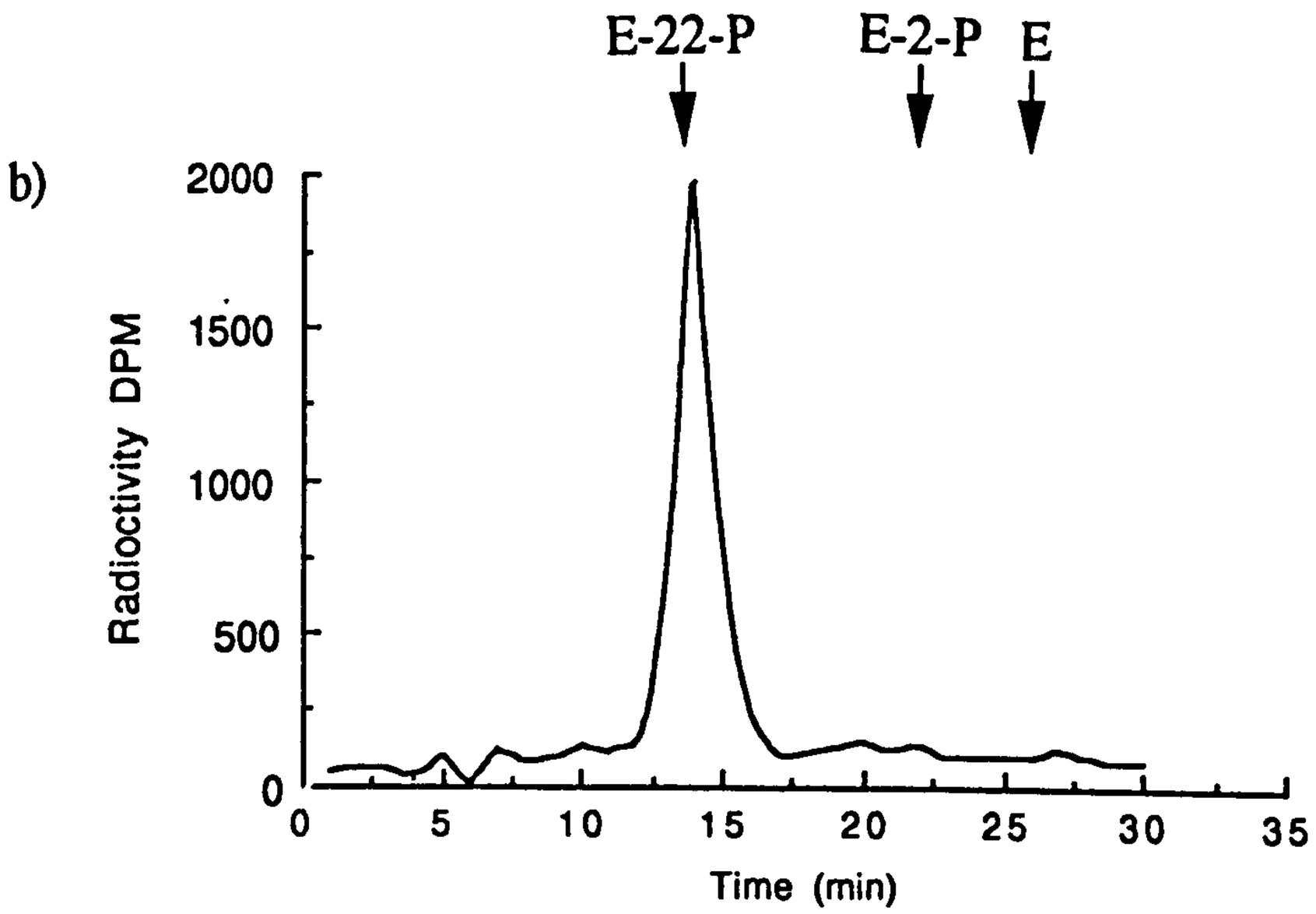


Figure 5.2. Reversed-phase HPLC fractionation of the products obtained from incubation of non-dialysed cytosol with [^{3}H]ecdysone. a) analysis of the 60%(v/v) methanol/water Sep-pak fraction, using acetontrile/TFA isocratic elution, (system 1); and b) analysis of the 30%(v/v) methanol/water Sep-pak fraction, eluted with a methanol/citrate buffer gradient (system 3). The positions of authentic compounds are indicated: ecdysone (E), 3-epiecdysone (E^1), 3-dehydroecdysone (3dE), ecdysone 2-phosphate (E-2-P) and ecdysone 22-phosphate (E-22-P).

ii) However, if a similar reaction was carried out using a dialysed preparation and no additional cofactors, conversion of ecdysone to 3-dehydroecdysone (*ca.* 15%) was observed.

Only in the latter set of reactions (v), containing ATP and $MgCl_2$, was activity found (100% relative conversion) and the products were tentatively assigned as ecdysone 2-phosphate and ecdysone 22-phosphate, by co-chromatography on reversed-phase HPLC (system 3). When no cofactor was added (ii) or $MgCl_2$ alone (iii), no conversion was observed. If ATP was the added as the sole cofactor (iv), then 30% relative conversion into phosphates was found. Therefore, both ATP and $MgCl_2$ are required.

c) Anaerobic Reactions

Ecdysone oxidase has previously been reported to require molecular oxygen in the blow-fly, *Calliphora vicina* (Koolman and Karlson, 1978). This experiment was to investigate the oxygen dependence of the oxidase in *S. littoralis* and to find out if other midgut enzymes are affected by anaerobic conditions.

Incubation mixtures were set up as usual (Section 5.2.1) and transferred to a heating block warmed to 37°C. Vials were stoppered using "Suba" seals, nitrogen was continuously flushed through the apparatus, which had previosly been alternately evacuated and filled with nitrogen several times. Any dissolved oxygen was removed by a glucose oxidase-peroxidase system containg ABTS dye [2,2'azino-bis(ethylbenzthiazoline-6-sulphonic acid)], bought as a kit from Boehringer Mannheim GmbH, Germany. Peroxidase (0.4U), glucose oxidase (0.5U), glucose (0.5mM) and ABTS (0.5mg) were added to each anaerobic incubation. Control tubes for the oxidase assay, (containing

boiled enzyme preparation, ecdysone, boiled glucose oxidase and peroxidase and ABTS dye) were also set up. Oxygen combined with the glucose to form gluconate and hydrogen peroxide, and the latter was converted to a dark green complex when acted upon by the dye (Shimizu, 1973):

$$\text{Glucose} + O_2 \longrightarrow \text{Gluconate} + H_2O_2$$

$$H_2O_2 + \text{ABTS} \longrightarrow H_2O + \text{Coloured Complex}$$

Reactions were carried out for 1hour (the maximal velocity of the glucose oxidase-peroxidase system was known to be attained after 15min). Of the enzymes tested, 3-dehydroecdysone 3β- and 3α-reductases, ecdysone 2- and 22-phosphotransferases and ecdysone oxidase, only the latter was affected by the removal of molecular oxygen (Table 1). The ecdysone oxidase was found to have a mean specific activity of 4.62± 0.31nmol/h/mg protein. Upon removal of oxygen, oxidase activity diminished to 0.85± 0.19nmol/h/mg protein, a reduction of approximately 82%. There are two possible explanations for some activity being found under anaerobic conditions: either there is a cytosolic factor present which can act as a hydrogen acceptor, or that removal of oxygen was not 100% efficient.

5.2.3 Verification of the Reaction Products

The usual assays were set up (Section 5.2.1), but incubations included 50µg (0.43mM) of the appropriate non-labelled ecdysteroid dissolved (500µl) in 0.2M sodium phosphate buffer, pH 7.4.

Table 1. The effect of anaerobic conditions on enzymic activity in the midgut cytosol.

	Specific activity [a] nmol/h/mg		
Enzyme	Aerobic	Anaerobic	% change
ecdysone oxidase	4.62	0.85	-82
3β-reductase	8.64	8.83	+2
3α-reductase	9.19	8.68	-5
ecdysone 2-phosphotransferase	4.65	4.43	-5
ecdysone 22-phosphotransferase	5.12	5.23	+2

[a] This data represents the mean of two incubations

a) Product of the Ecdysone Oxidase Assay

Ecdysteroids were analyzed by reversed-phase and adsorption HPLC, (systems 1 and 2). Putative 3-dehydroecdysone was shown to co-chromatograph with authentic standard (figures 5.3 a and b). To substantiate the identity of the product, approximately 5.0µg of recovered "3-dehydroecdysone" was chemically reduced using sodium borohydride (Section 2.2.13). The major reduction product was found to co-chromatograph with authentic 3-epiecdysone, on reversed-phase (system 1; figure 5.3c) with a small peak corresponding to ecdysone.

Furthermore, a negative ion fast atom bombardment mass spectrum (Section 2.2.11) revealed a prominent ion at m/z 461$[M-H]^-$, corresponding to a relative molecular mass of 462 (figure 5.4).

b) The 3-Dehydroecdysone 3α-Reductase (NADPH) Assay

Using reversed-phase and adsorption HPLC (systems 1 and 2), the major product was found to co-chromatograph with authentic 3-epiecdysone (figures 5.5 a and b, respectively). Negative ion FAB-MS confirmed that the product had a relative molecular mass of 464, since a peak of m/z 463 $[M-H]^-$ was obtained (figure 5.6).

A minor peak corresponding to ecdysone on HPLC system 1 had previously been shown (figure 5.2a).

c) The 3-Dehydroecdysone 3β-Reductase (NADH) Assay

The major product was proved to be ecdysone by reversed-phase and adsorption HPLC, (systems 1 and 2; figures 5.7a and b respectively) and by negative ion FAB-MS, which revealed an ion at m/z 463$[M-H]^-$, confirming a relative molecular mass of 464 (figure 5.8).

A minor product had been found to co-elute with standard 3-epiecdysone, using reversed-phase HPLC (system 1; figure 5.1b).

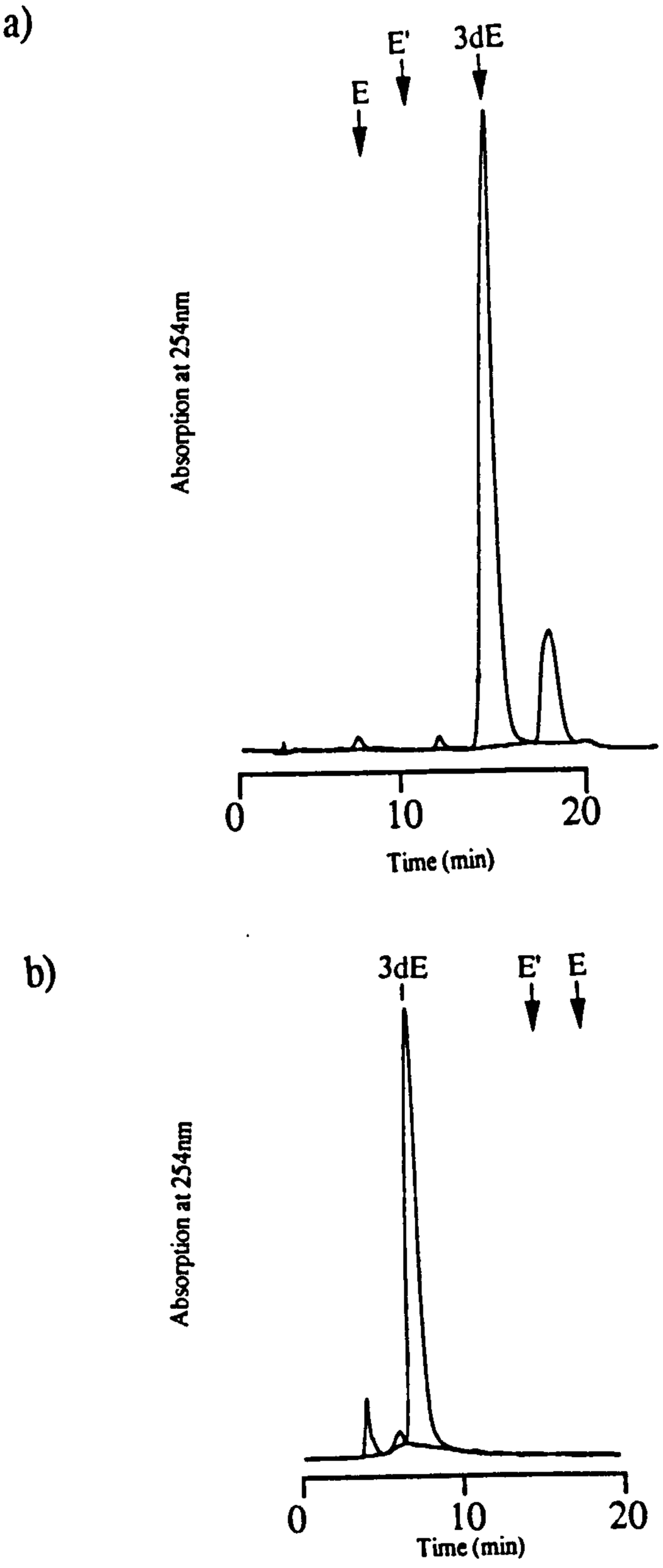


Figure 5.3. HPLC fractionation of the putative 3-dehydroecdysone product, using a) reversed-phase column, eluted isocratically with acetonitrile/TFA isocratic elution (system 1) and b) an aminopropyl silica column, eluted isocratically with methanol/ 1,2-dichloroethane (system 2). The positions of authentic ecdysteroids are shown, ecdysone (E), 3-dehydroecdysone (3dE) and 3-epiecdysone (E').

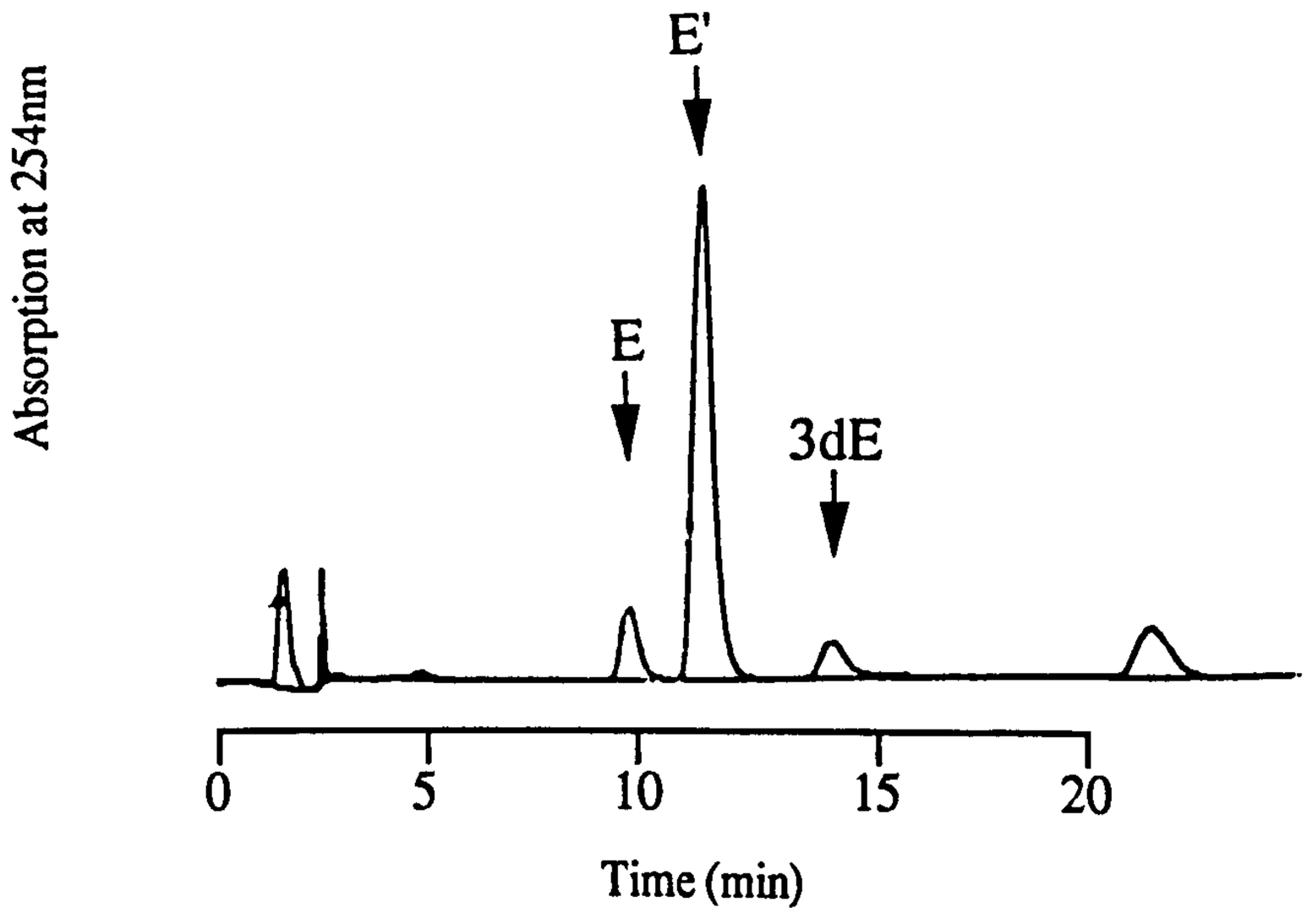


Figure 5.3c. Putative 3-dehydroecdysone was chemically converted into 3-epiecdysone and fractionated by C_{18} Sep-pak chromatography. The free ecdysteroid fraction was analyzed on a reversed-phase column by acetonitrile/TFA isocratic elution (system 1). The positions of authentic ecdysone (E), 3-epiecdysone (E') and 3-dehydroecdysone (3dE) are indicated.

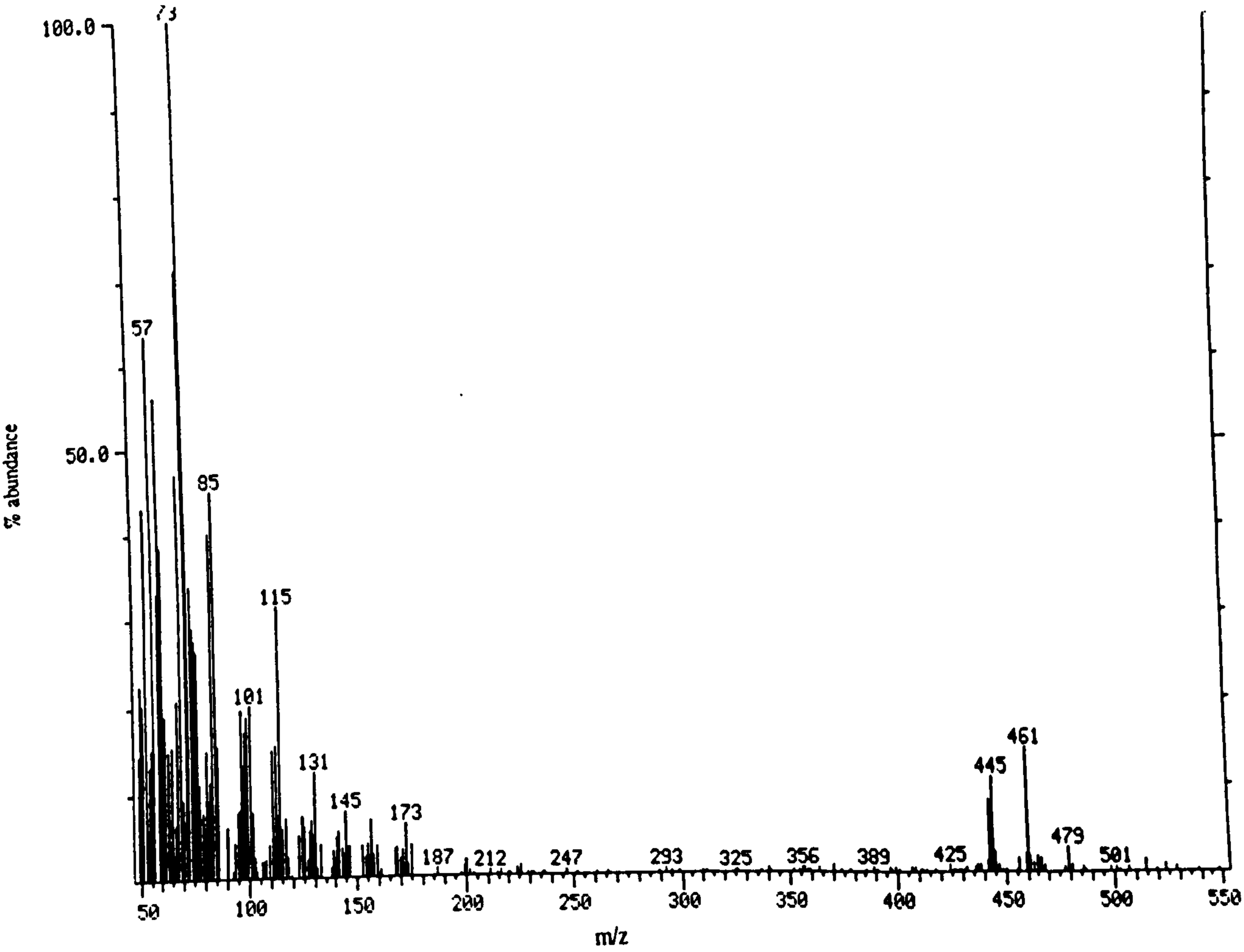
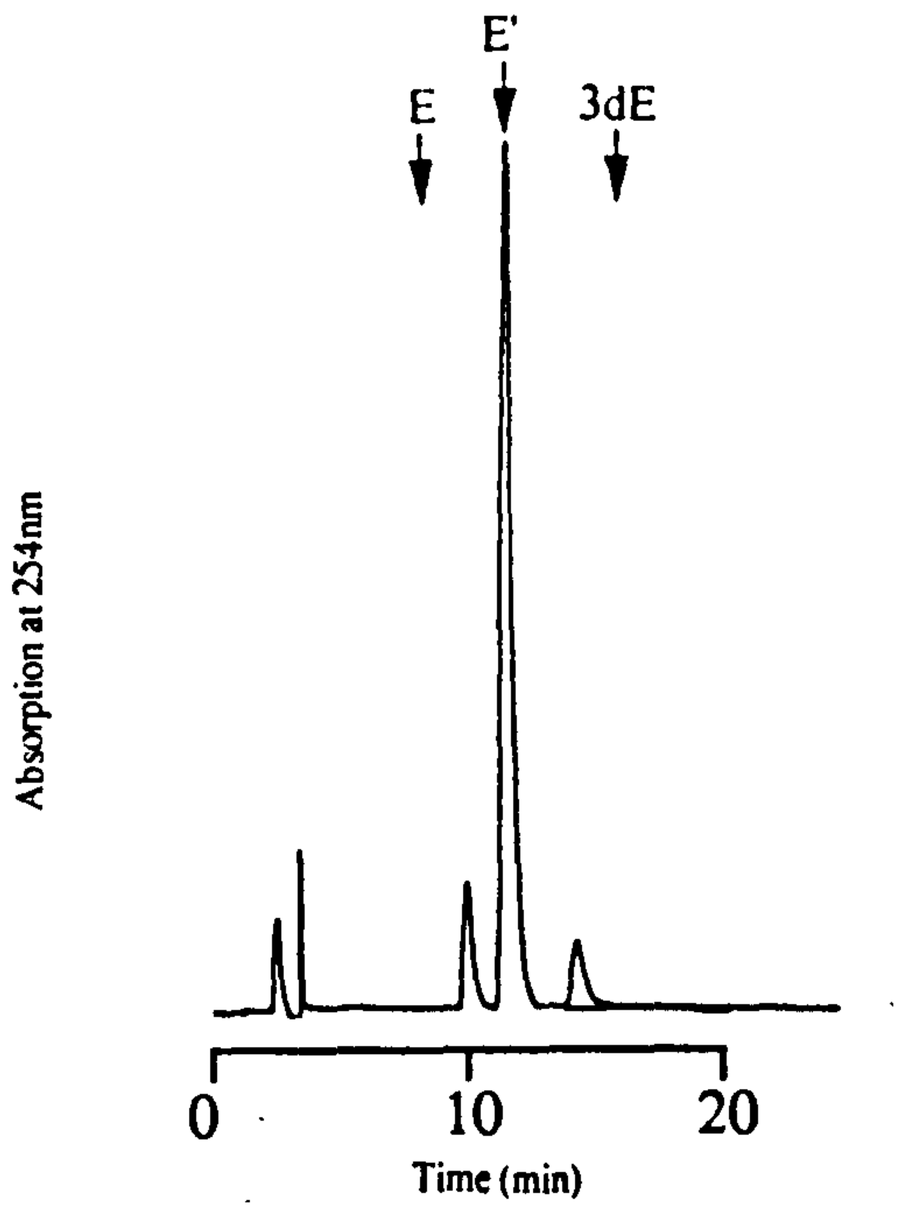


Figure 5.4 The negative-ion FAB mass specrtrum of putative 3-dehydroecdysone. The $[M-H]^-$ ion is at m/z 461.

a)



b)

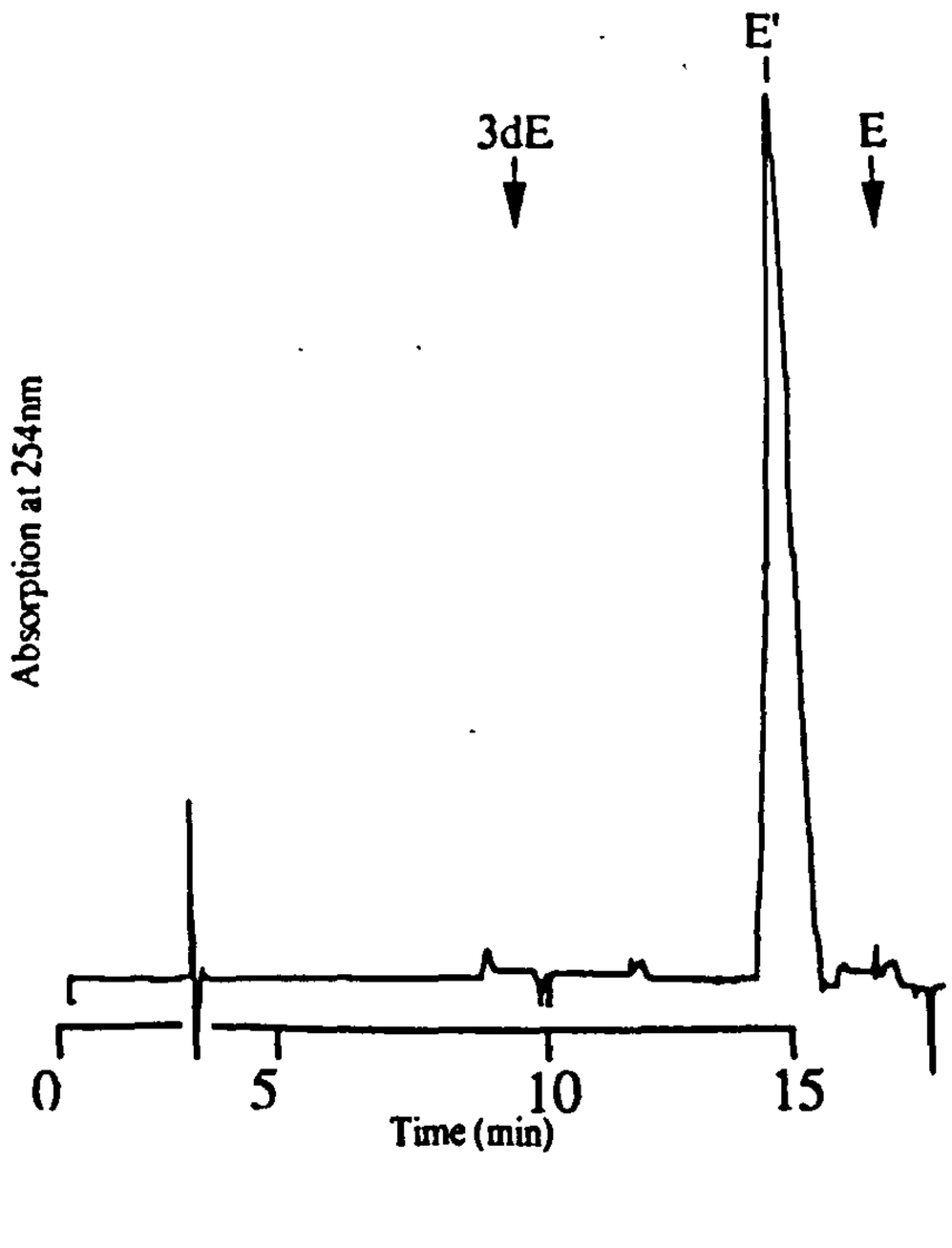


Figure 5.5. HPLC analysis of the major product of the 3-dehydroecdysone 3α-reductase (NADPH) assay, using a) a reversed-phase column eluted isocratically with acetonitrile/TFA (system 1) and b) an aminopropyl silica column isocratically eluted using methanol/ 1,2-dichloroethane (system 2). The positions of authentic compounds are indicated: ecdysone (E), 3-dehydroecdysone (3dE) and 3-epiecdysone (E^1).

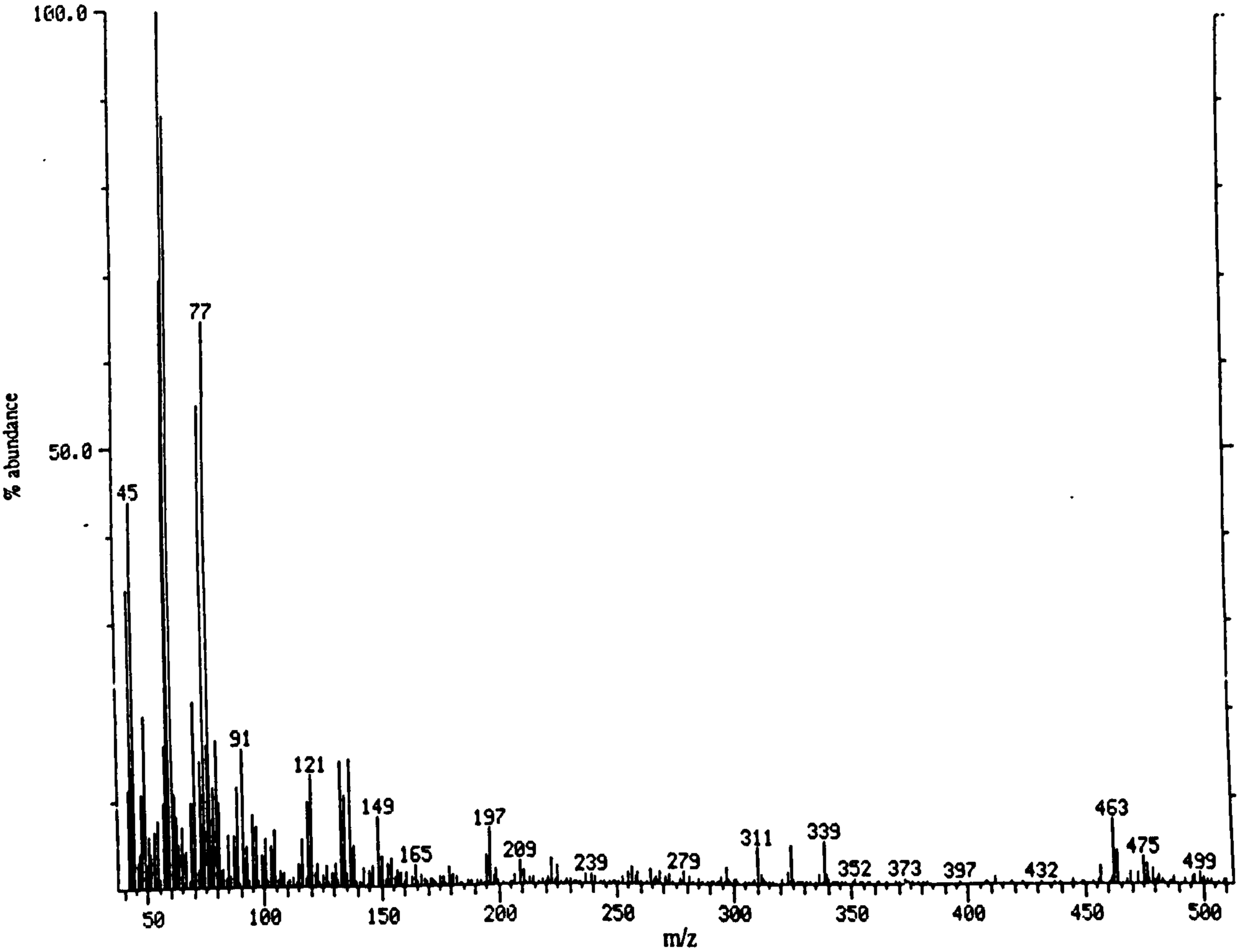
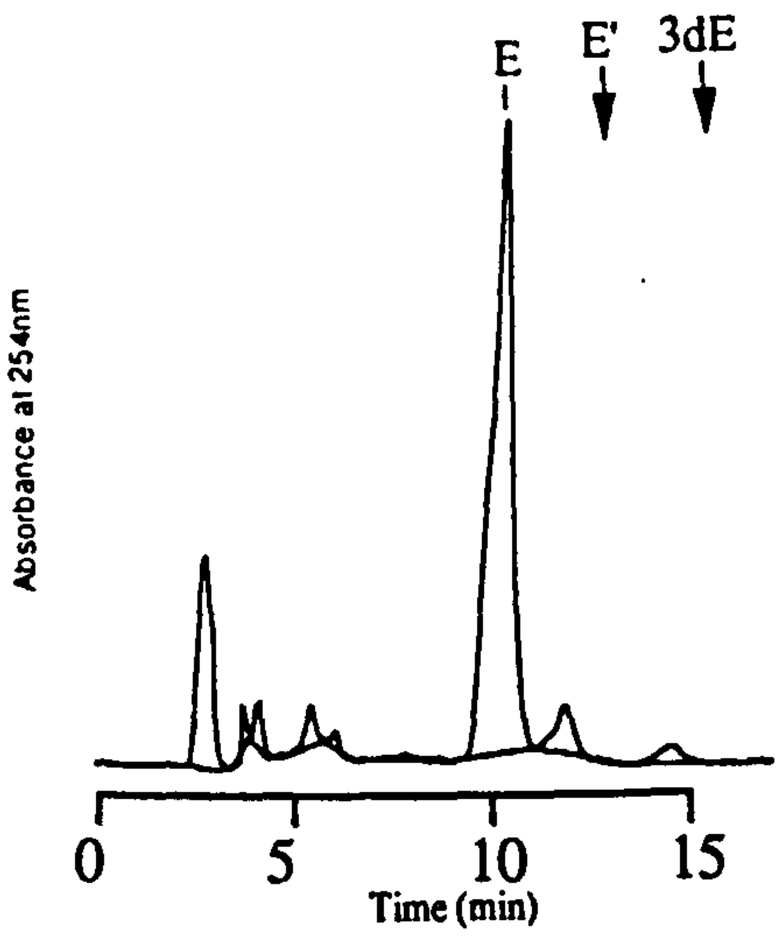


Figure 5.6 Negative-ion FAB mass spectrum of putative 3-epiecdysone. The $[M-H]^-$ ion is at m/z 463.

a)



b)

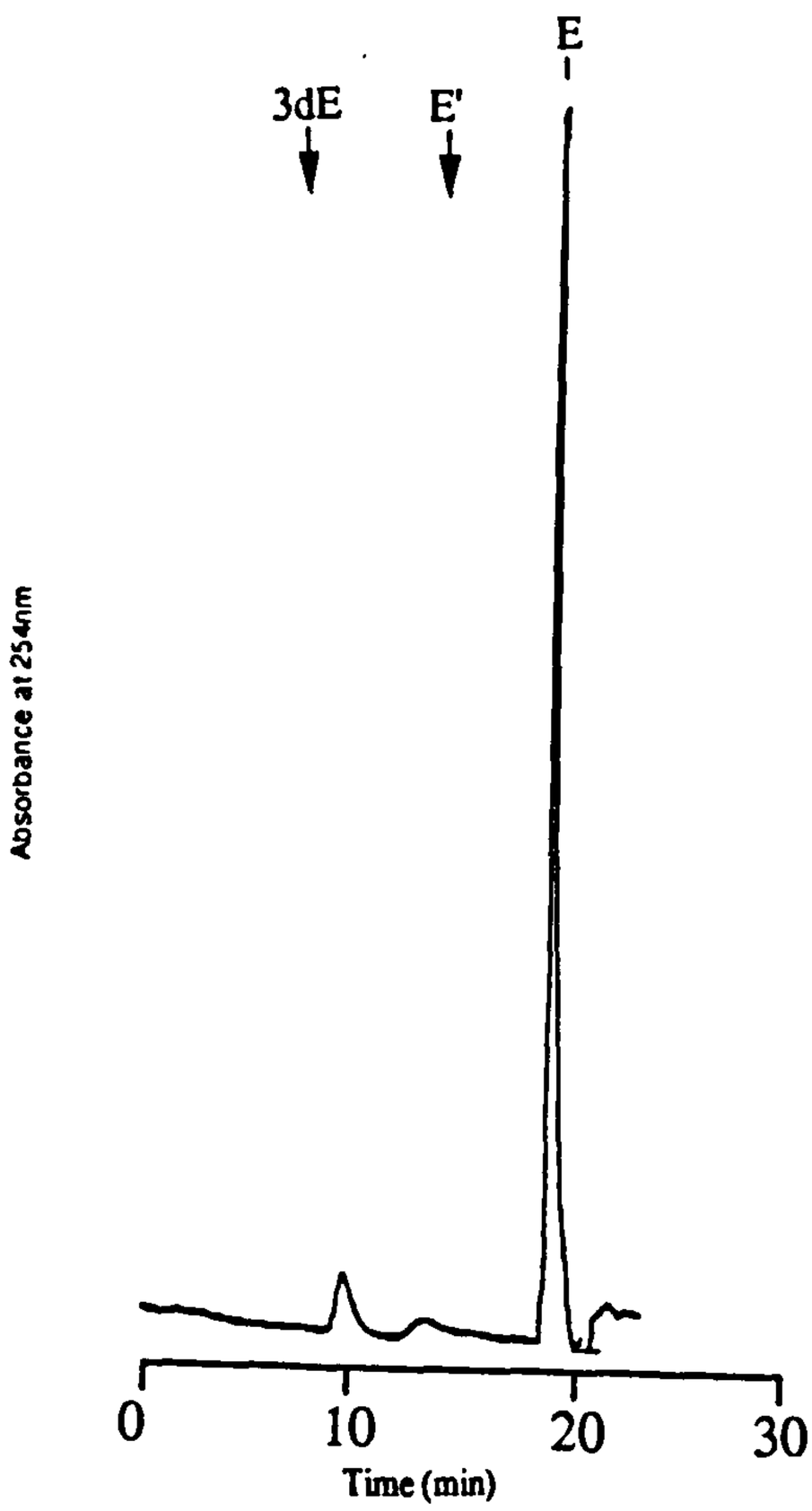


Figure 5.7. HPLC analysis of the product of the 3-dehydroecdysone 3β-reductase assay using a) a reversed-phase column eluted isocratically with acetonitrile/ TFA (system 1) and b) an aminopropyl silica column, eluted isocratically with methanol/1,2-dichloroethane (system 2). The positions of authentic marker ecdysteroids are shown: 3-dehydroecdysone (3dE), 3-epiecdysone (E') and ecdysone (E).

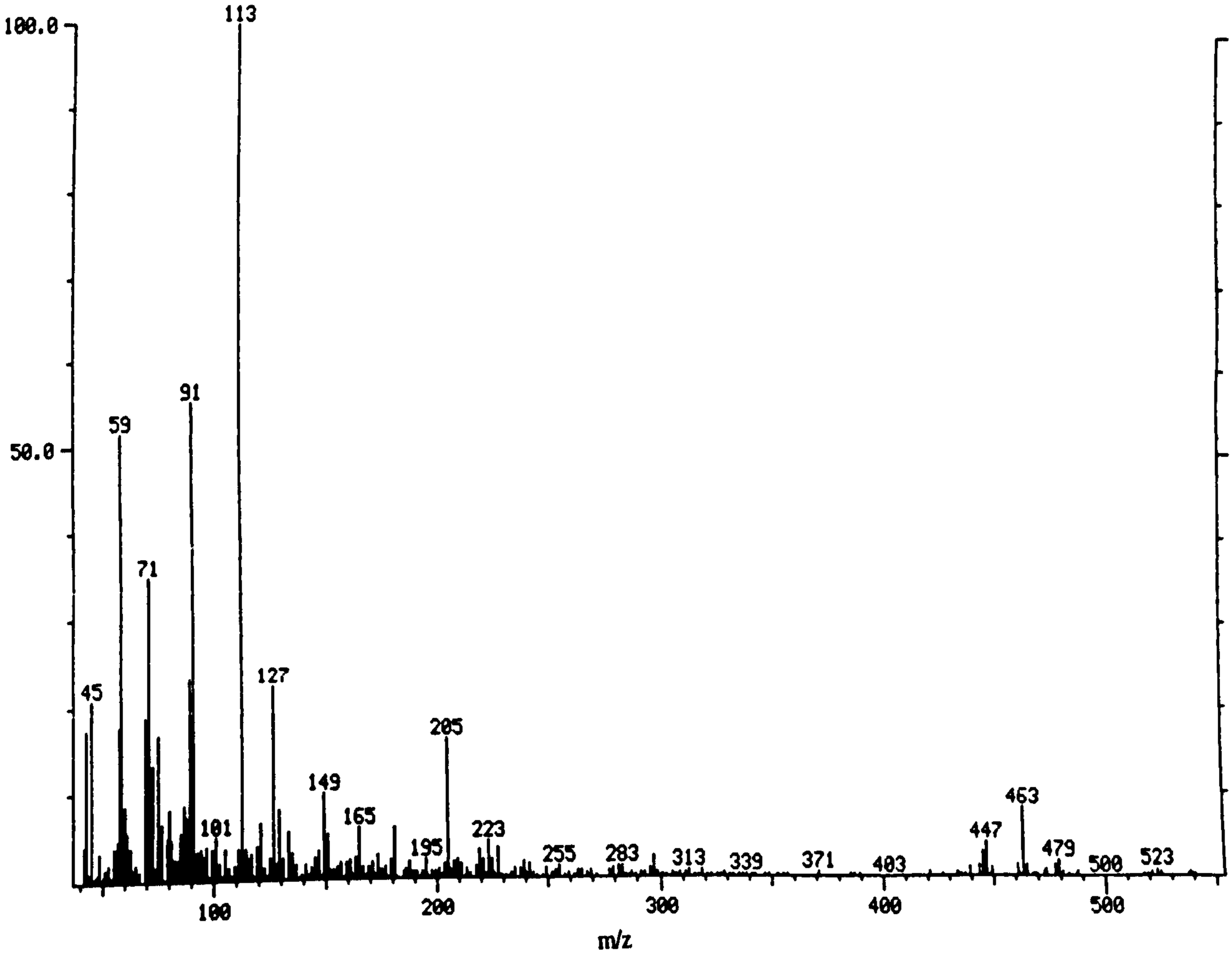
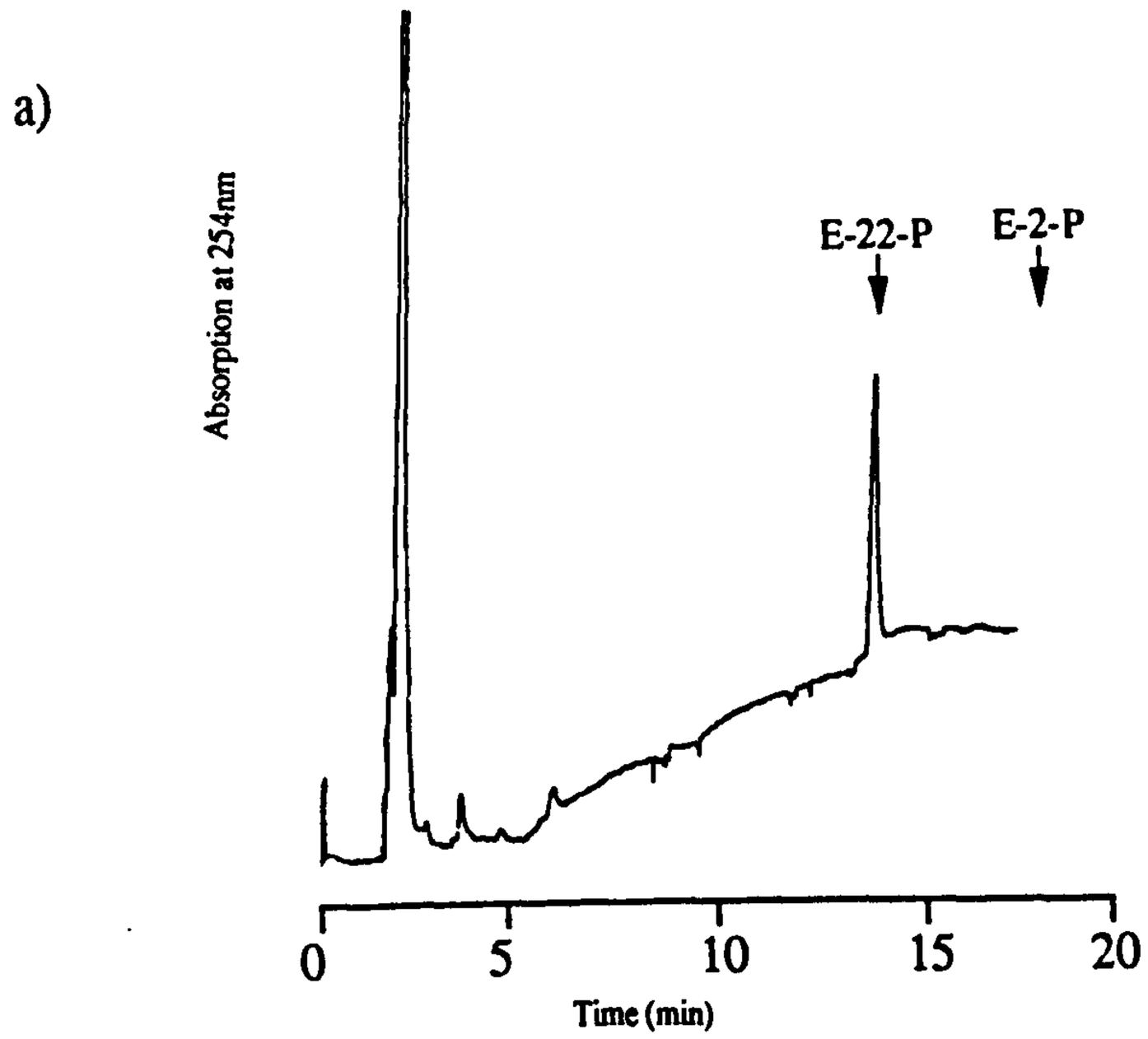


Figure 5.8 The negative-ion FAB mass spectrum of the putative ecdysone. The $[M-H]^-$ ion is at m/z 463.

d) The Products of the ATP: Ecdysone 2- and 22-Phosphotransferases

Using ATP and $MgCl_2$ as the cofactors, in the usual assay (Section 5.2.1), the products were collected and re-analyzed using HPLC systems 3 and 4 (figures 5.9 a and b, figures 5.10 a and b, repectively). In both cases, the most polar metabolite was found to co-chromatograph with ecdysone 22-phosphate and the less polar one with ecdysone 2-phosphate. However, this was not a full confirmation of the positional isomers of the phosphates and, hence, the following strategy was devised, using chemical acetonide synthesis (figure 5.11a).

To aid in the identification using acetonides, putative 20-hydroxyecdysone phosphates were enzymatically synthesized (figure 5.11b). The phosphates of 20-hydroxyecdysone and ecdysone were further converted into acetonides chemically (Figures 5.12a,b and 5.13a,b, respectively). Acetonides of ecdysone and 20-hydroexyecdysone were also made as chromatographic markers. Obviously acetonide formation makes the phosphates less polar. Since acetonide production requires a *cis* vicinal diol, the presence of a phosphate at C-2 or C-3, or in the case of 20-hydroxyecdysone, at C-20 or C-22, would block derivative formation. The products were analysed by reversed-phase HPLC. Enzymic hydrolysis of the products then allows chromatographic comparison of the resulting acetonides with the authentic compounds. The strategy is outlined in figure 5.11b.



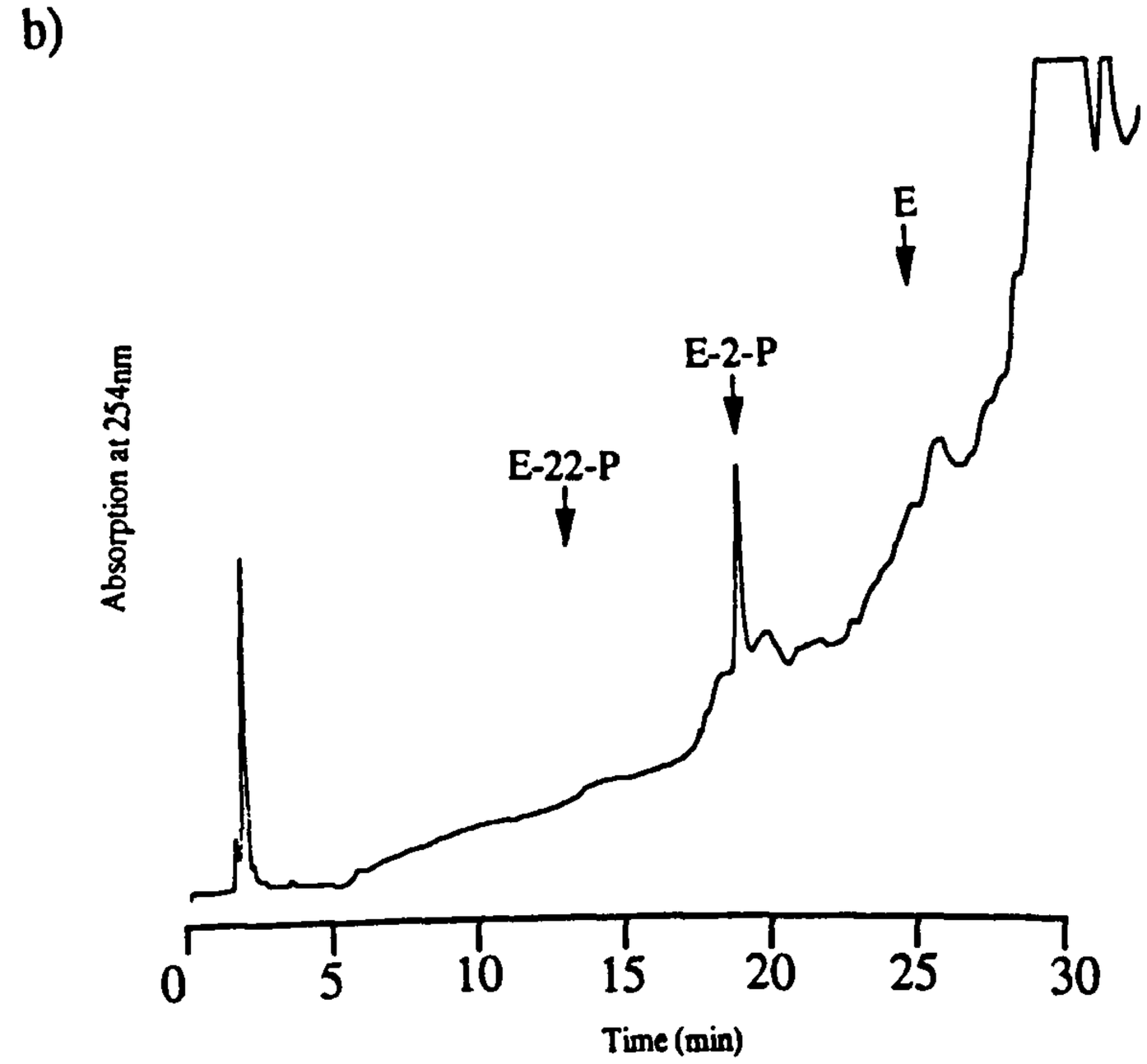


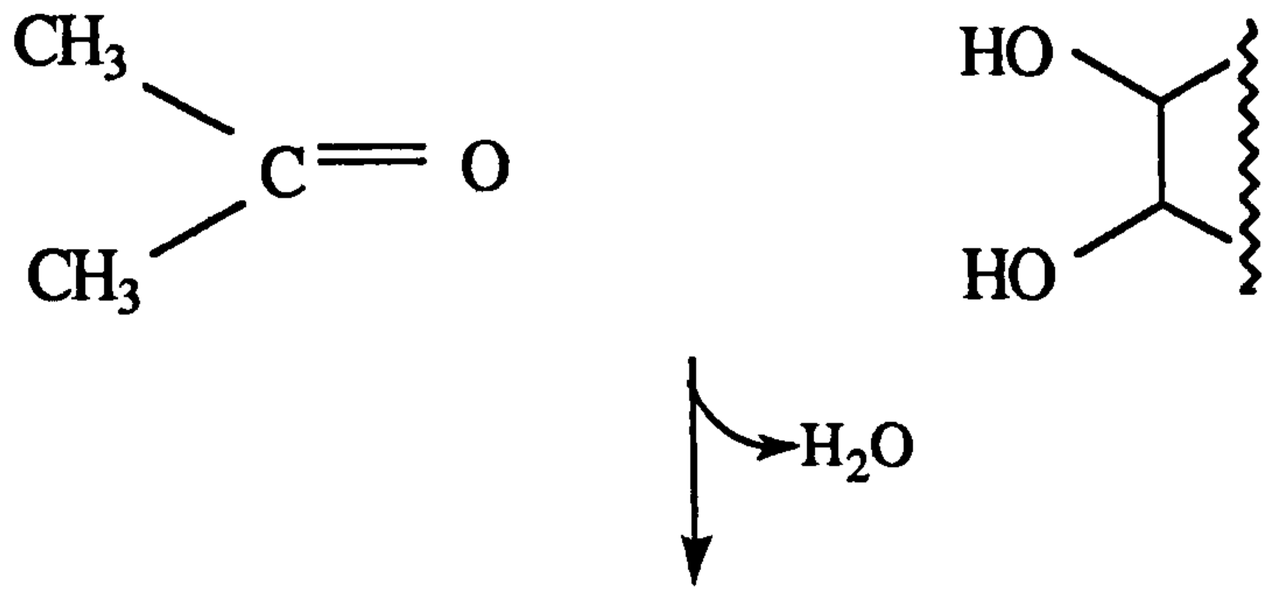
Figure 5.9. Reversed-phase HPLC analysis of the polar ecdysteroids produced in the phosphotransferase assay, using a gradient of methanol/sodium citrate buffer, pH 6.5, for elution (system 3). a) Putative ecdysone 22-phosphate (E-22-P) and b) putative ecdysone 2-phosphate (E-2-P). The positions of authentic ecdysteroids are shown.

a)

b)

Figure 5.10. Reversed-phase HPLC analysis of the polar ecdysteroids from the phosphotransferase assay using acetonitrile/ Tris-$HClO_4$ buffer, pH 7.5, gradient (system 4) for elution. a) Putative ecdysone 22-phosphate (E-22-P) and b) putative ecdysone 2-phosphate (E-2-P). The positions of elution of authentic ecdysteroids are indicated.

Figure 5.11a. Acetonide reaction



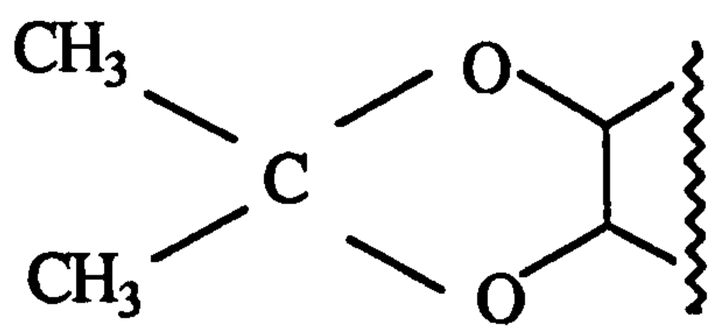


Figure 5.11b. Predicted sequence of events

i) 20-hydroxy-ecdysone 2-phosphate —(acetonide formation)→ ecdysone 20,22-acetonide 2-phosphate —(enzymic hydrolysis)→ ecdysone 20,22-acetonide

ii) 20-hydroxy-ecdysone 22-phosphate → 20-hydroxyecdysone 2,3-acetonide 22-phosphate → 20-hydroxy-ecdysone 2,3-acetonide

iii) ecdysone 22-phosphate → ecdysone 2,3-acetonide 22-phosphate → ecdysone 2,3-acetonide

iv) ecdysone 2-phosphate ⟶✕ acetonide

Figures 5.12 and 5.13 clearly indicate how the phospho-esters, upon acetonide formation, were converted into more hydrophobic ecdysteroids (Table 2). The retention time of the "ecdysone 2-phosphate", after treatment with acetonide reagents, remained the same (figure 5.13b), since an acetonide could not be formed (the molecule is blocked at C-2 or C-3).

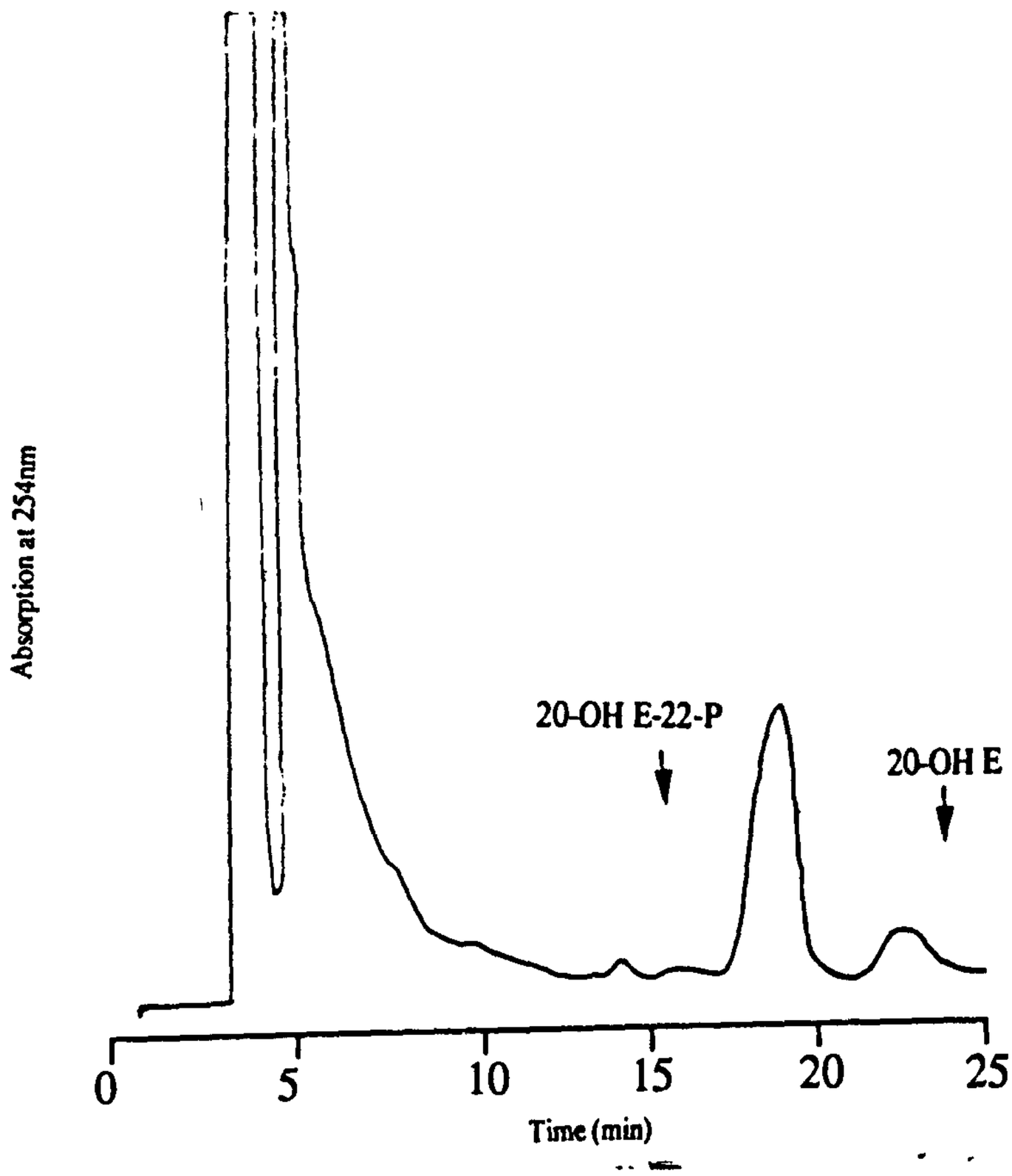
The phospho-acetonides were then hydrolyzed using a crude arylsulphatase preparation from *Helix pomatia* (Section 2.2.10), to give free acetonides (figures 5.14 a, b and c). Samples were analyzed using reversed-phase HPLC (system 3) and the retention times are given, for comparison, in Table 2.

It would appear from the data that a) 20-hydroxyecdysone 20,22-acetonide is formed [figure 5.11b, sequence (i)] suggesting that the C-2 or C-3 hydroxyl group is blocked.b) 20-hydroxyecdysone 2,3-acetonide is also synthesized [figure 5.11b, sequence (ii)], suggesting that the C-22 hydroxyl group can be blocked; phosphorylation at the tertiary C-20 is unlikely. Furthermore, since ecdysone does not have a C-20 hydroxyl group, the only possible phospho-acetonide metabolite would be ecdysone 22-phosphate 2,3-acetonide [figure 5.11b, sequence (iii)], which would be hydrolyzed to ecdysone 2,3-acetonide, as was shown in figure 5.14c. The fact that no diacetonide of 20-hydroxyecdysone was produced suggested that there were no phosphates at positions other than C-2, -3, -20 and -22.

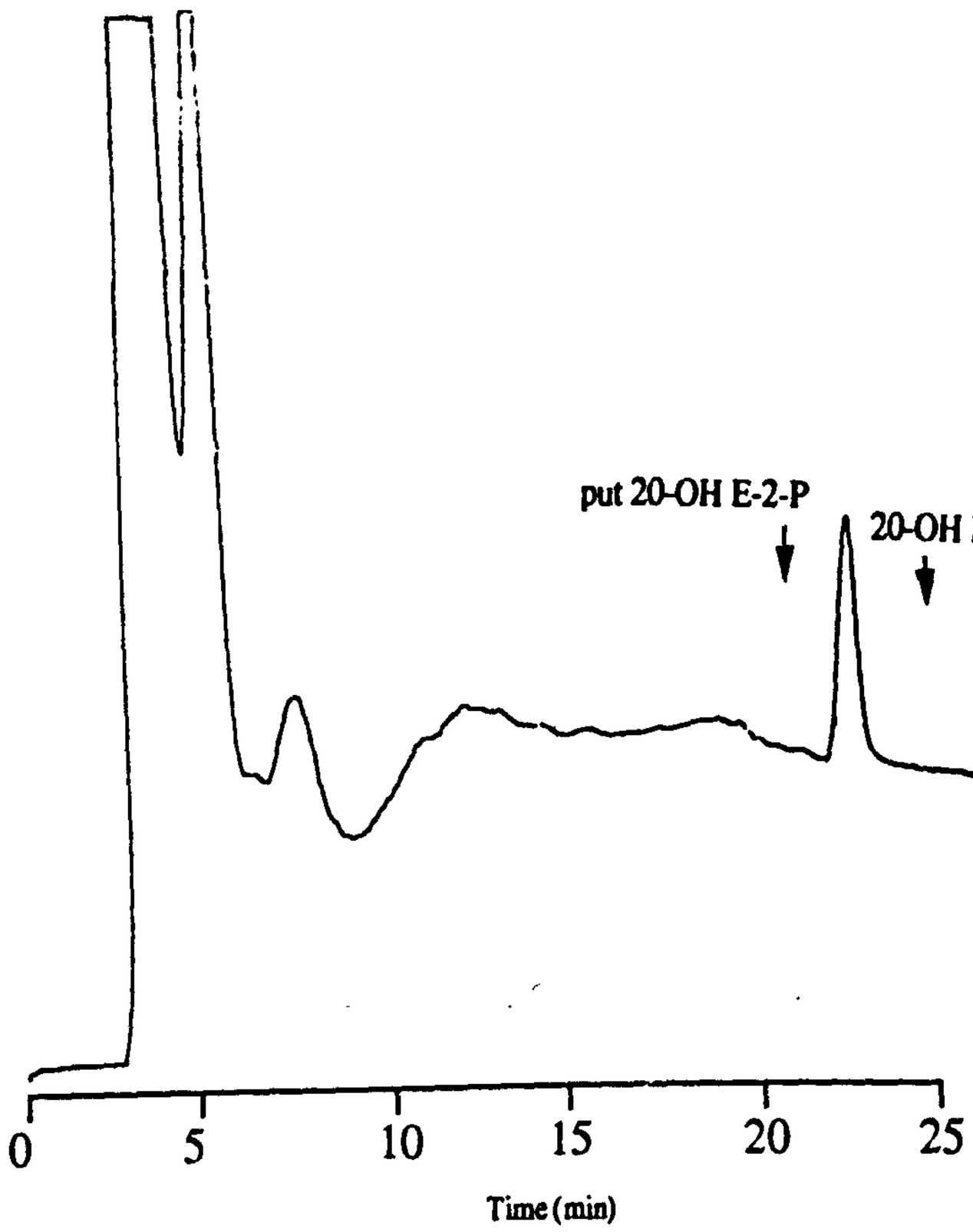
Thus the products formed in these transformations were in conformance with those expected in figure 5.11b. Hence, the parent ecdysteroid may be phosphorylated at either C-22 or C-2/ C-3. However, it must be borne in mind that there was co-chromatography of an ecdysone metabolite with ecdysone 2-phosphate; by analogy with ecdysteroid 2- and 3-acetates (Rees and Isaac, 1985), ecdysone 3-

Figure 5.12. The acetonides of putative 20-hydroexyecdysone phosphates were chemically synthesized and analyzed by reverse-phase HPLC, using a methanol/ sodium citrate buffer, pH 6.5, gradient (system 3) for elution. a) Acetonide of putative 20-hydroxyecdysone 22-phosphate (20-OH E-22-P) and b) acetonide of putative 20-hydroexyecdysone 2-phosphate. The positions of authentic compounds are shown: ecdysone (E), 20-hydroxyecdysone (20-OH E), 20-OH E-22-P as well as putative 20-hydroxyecdysone 2-phosphate (put. 20-OH E-2-P).

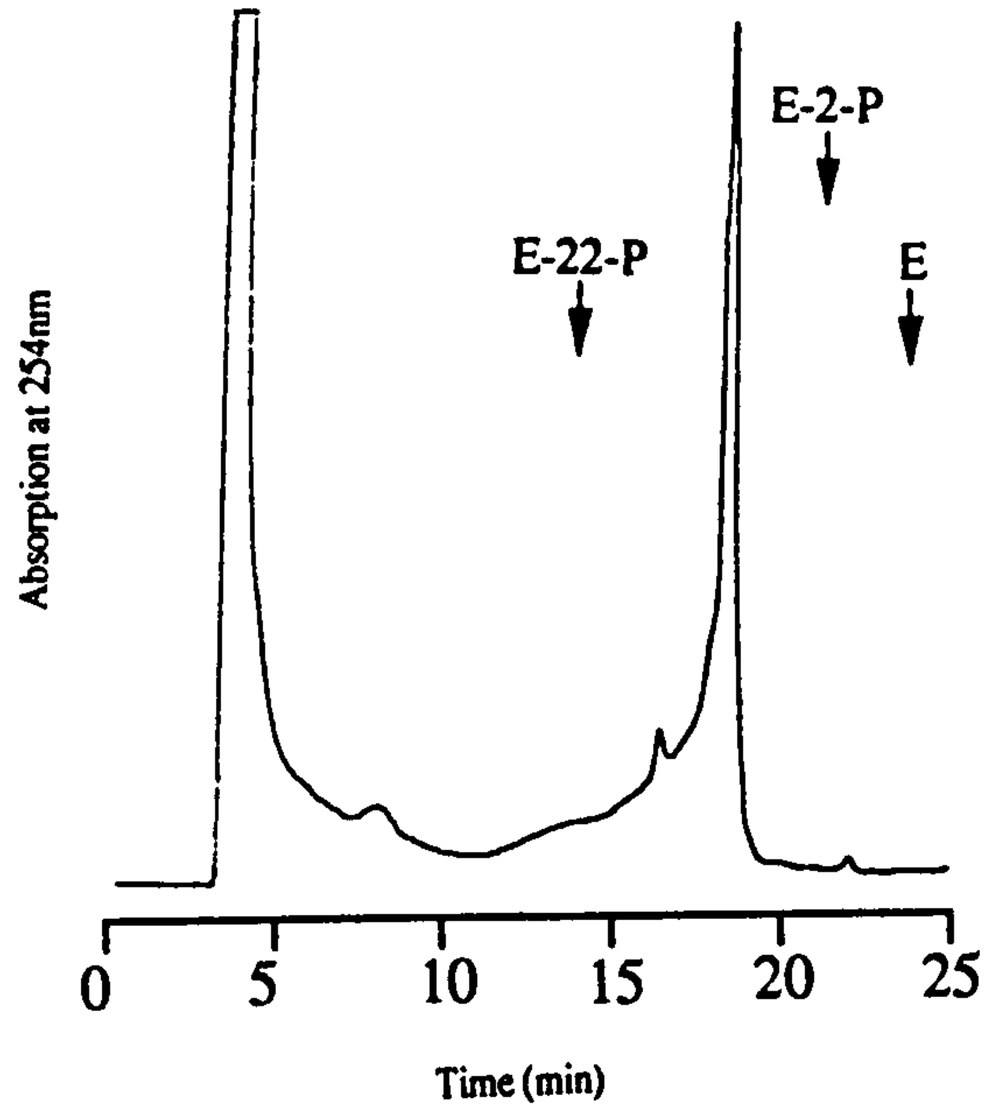
a)


Absorption at 254nm
20-OH E-22-P
20-OH E
0
5
10
15
20
25
Time (min)

b)


Absorption at 254nm
put 20-OH E-2-P
20-OH E
0
5
10
15
20
25
Time (min)

a)



b)

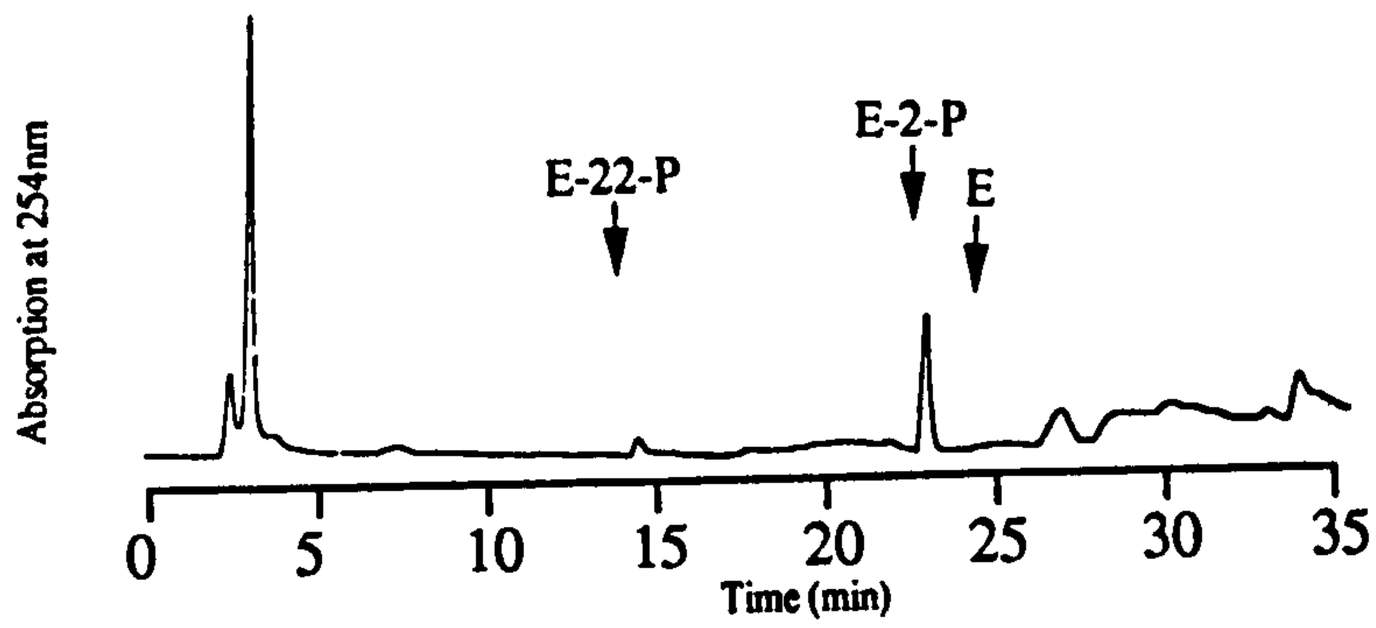


Figure 5.13. The putative ecdysone phosphates (ecdysone 22-phosphate and ecdysone 2-phosphate) were subjected to acetonide formation and the products analyzed by a methanol/citrate buffer, pH 6.5, gradient (system 3) for elution. a) Acetonide of putative ecdysone 22-phosphate and b) the effect of acetonide reagents on putative ecdysone 2-phosphate. The positions of authentic compounds are indicated, ecdysone (E), ecdysone 22-phosphate (E-22-P), ecdysone 2-phosphate and (E-2-P).

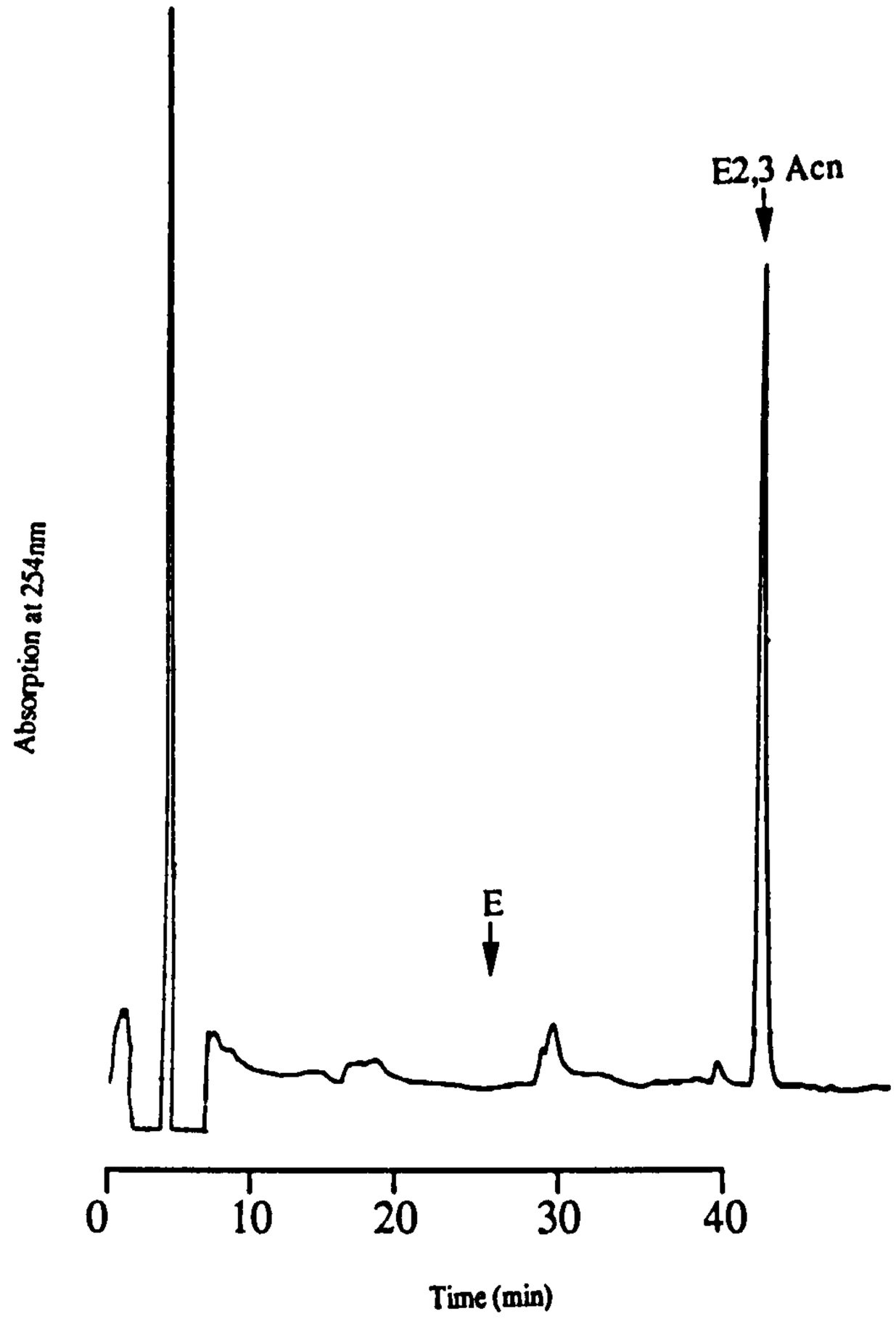
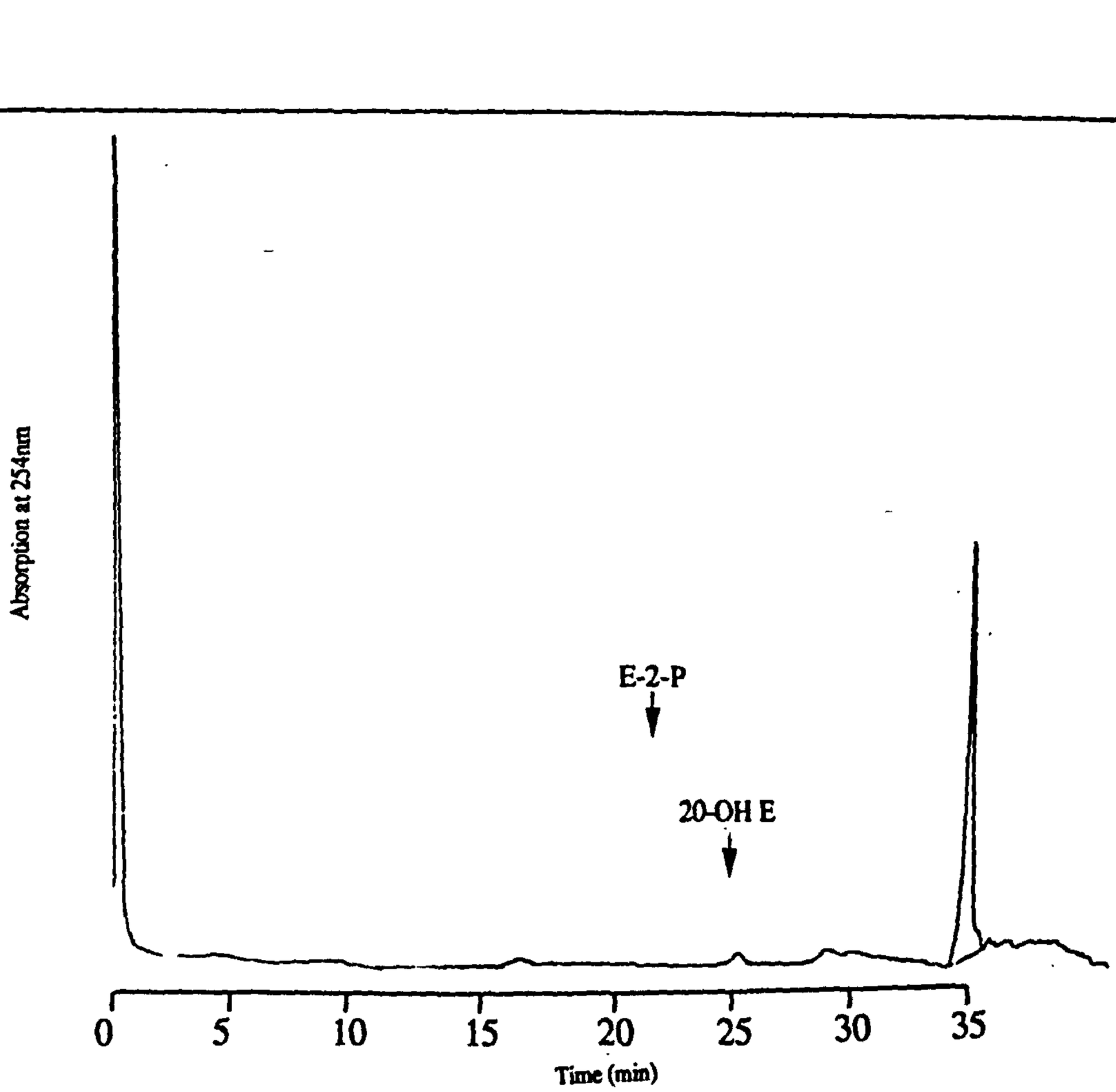


Figure 5.14. Reversed-phase HPLC fractionation using a methanol/ citrate buffer, pH 6.5, gradient (system 3) for elution, of the ecdysteroids released [60%(v/v) Sep-pak fraction] upon hydrolysis of the polar conjugated acetonides, with *H. pomatia* arylsulphatase from acetonides of a) 20-hydroxyecdysone 22-phosphate, b) 20-hydroxyecdysone 2-phosphate and c) ecdysone 22-phosphate. The positions of authentic ecdysone (E), 20-hydroxyecdysone (20-OH E), ecdysone 2-phosphate (E-2-P), ecdysone 22-phosphate (E-22-P), 20-hydroxyecdysone 22-phosphate (20-OH E-22-P) and ecdysone 2,3-acetonide (E2,3 Acn) are shown.

b)


Absorption at 254nm
E-2-P
20-OH E
0
5
10
15
20
25
30
35
Time (min)

c)

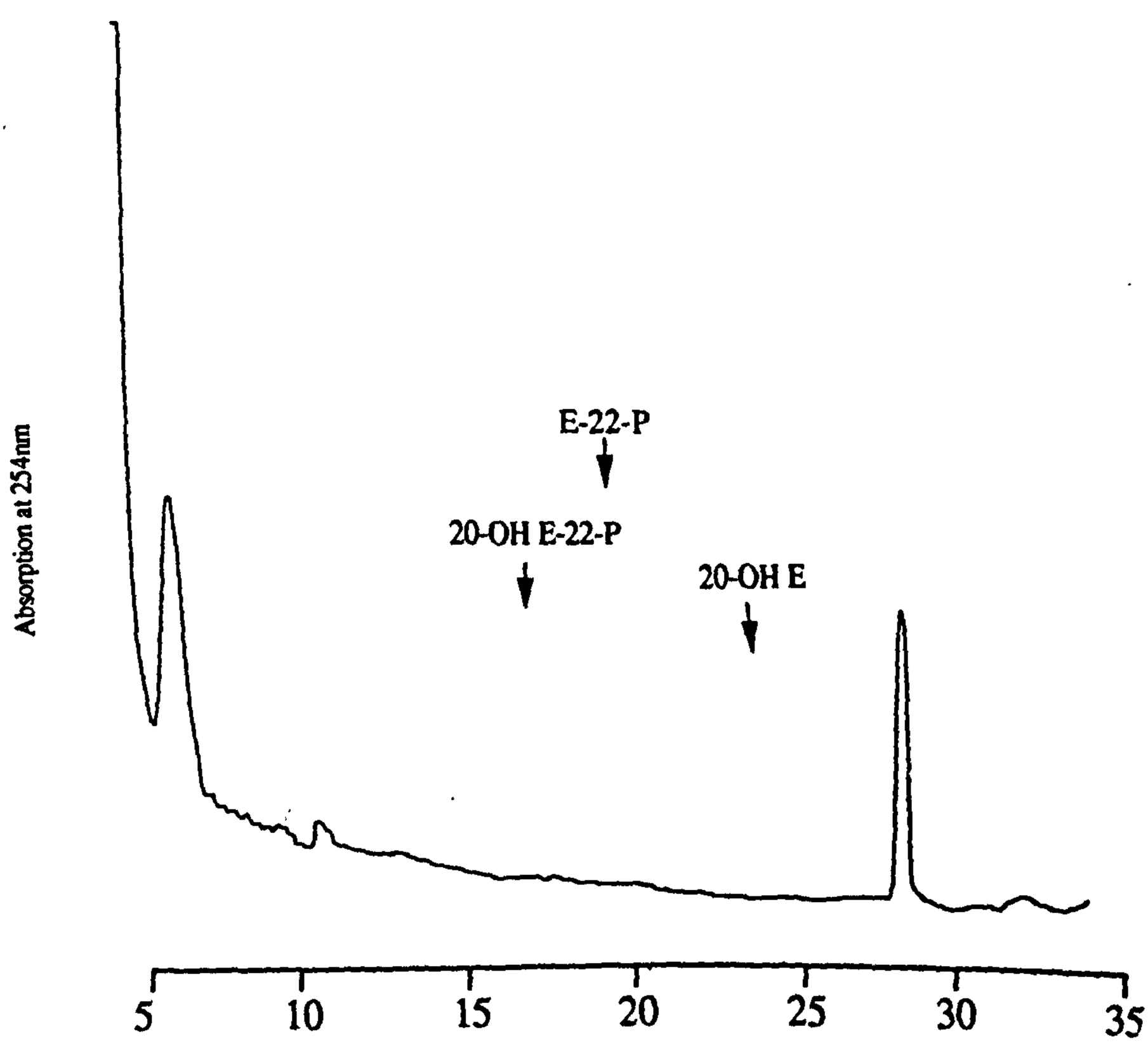

Absorption at 254nm
E-22-P
20-OH E-22-P
20-OH E
5
10
15
20
25
30
35
Time (min)

Table 2. A comparison of the retention times on reversed-phase HPLC (system3) of the ecdysteroid phosphates, phospho-acetonides and acetonides

Ecdysteroid	Retention time(min)	Figure
Authentic ecdysteroid samples		
Ecdysone	27.1	
20-Hydroxyecdysone	24.7	
Phosphate samples of enzymic origin		
Ecdysone 2-phosphate	23.3	5.9,5.10
Ecdysone 22-phosphate	17.1	5.9,5.10
20-Hydroxyecdysone 2-phosphate	21.7	
20-Hydroxyecdysone 22-phosphate	15.6	
Phospho-acetonides		
20-Hydroxyecdysone 22-phosphate 2,3 acetonide	18.0	5.12a
20-Hydroxyecdysone 2-phosphate 20,22-acetonide	23.2	5.12b
Ecdysone 22-phosphate 2,3-acetonide	18.6	5.13a
Hydrolysis Products (acetonides)		
20-Hydroxyecdysone 2,3-acetonide	28.6	5.14a
20-Hydroxyecdysone 20,22-acetonide	33.1	5.14b
Ecdysone 2,3-acetonide	41.9	5.14c

phosphate would be expected to be more polar than the 2-phosphate and would be resolved by reversed-phase HPLC. The evidence, although cicumstantial, suggests that there is phosphorylation at C-2, but not C-3.

Further investigations showed that ecdysteroids altered at the C-3 position could be enzymically phosphorylated. Rather conveniently, these are also metabolites of the epimerization system, namely 3-dehydroecdysone and 3-epiecdysone. The two ecdysteroids were used as potential substrates (figure 5.15 a and b) and the products compared to ecdysone phosphates by reversed-phase HPLC (figure 5.15c).

In all three cases, two products were formed. The putative phosphates of 3-epiecdysone and 3-dehydroecdysone were slightly less polar than those of ecdysone. By analogy with the ecdysone phosphates, it could be argued that these represent esterification at C-2 and C-22 hydroxyl groups, since phosphorylation cannot take place at C-3 in 3-dehydroecdysone. Conversions were typically 8% conversion into 3-epiecdysone 22-phosphate and 16% conversion into 3-epiecdysone 2-phosphate; 6% into ecdysone 22-phosphate and 14% into ecdysone 2-phosphate; 7% into 3-dehydroecdysone 22-phosphate and 12% conversion into 3-dehydroecdysone 2-phosphate (mean of two incubations).

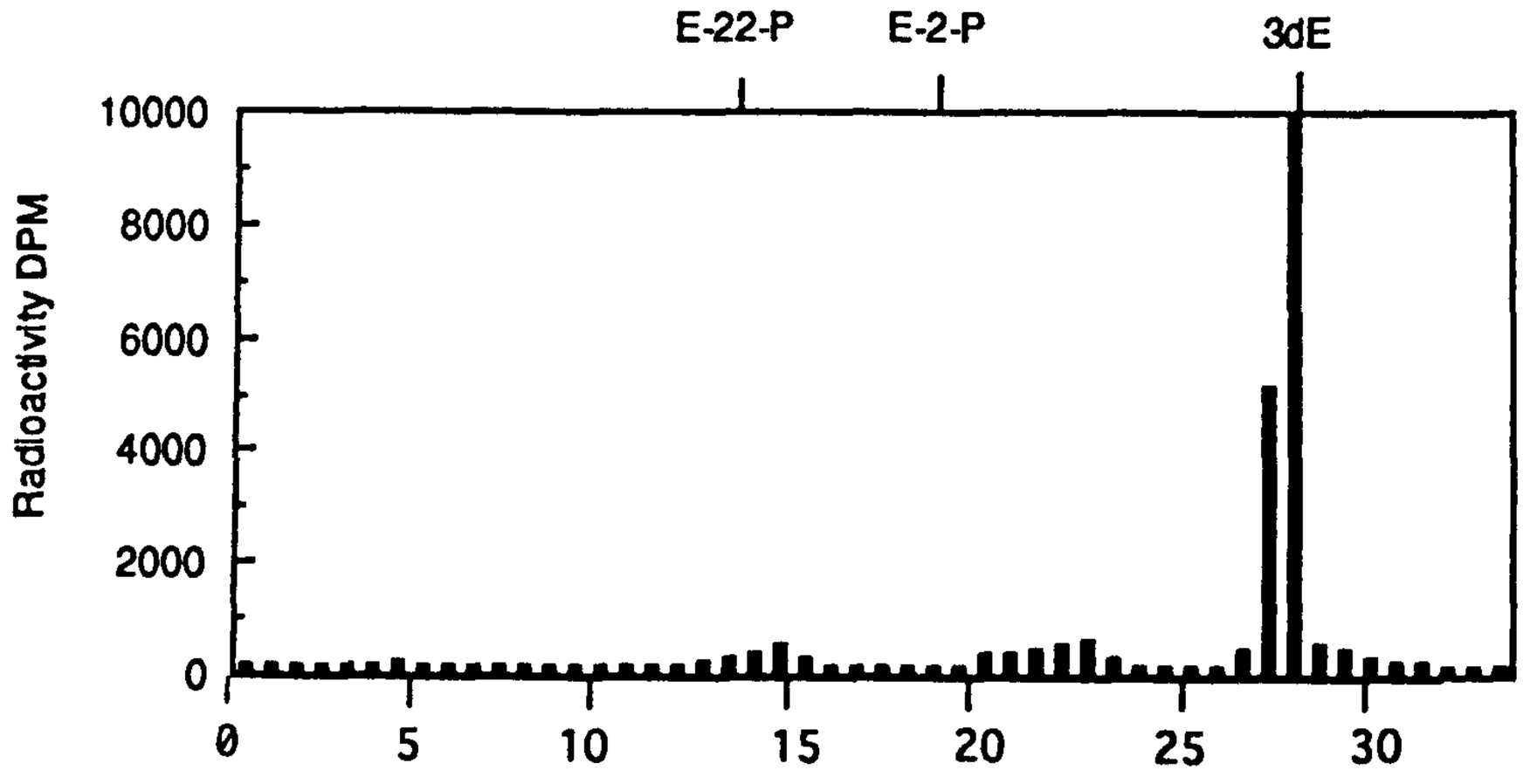
Upon hydrolysis of these putative phosphates by a crude arylsulphatase preparation, the parent ecdysteroid moieties were recovered.

5.3 DISCUSSION

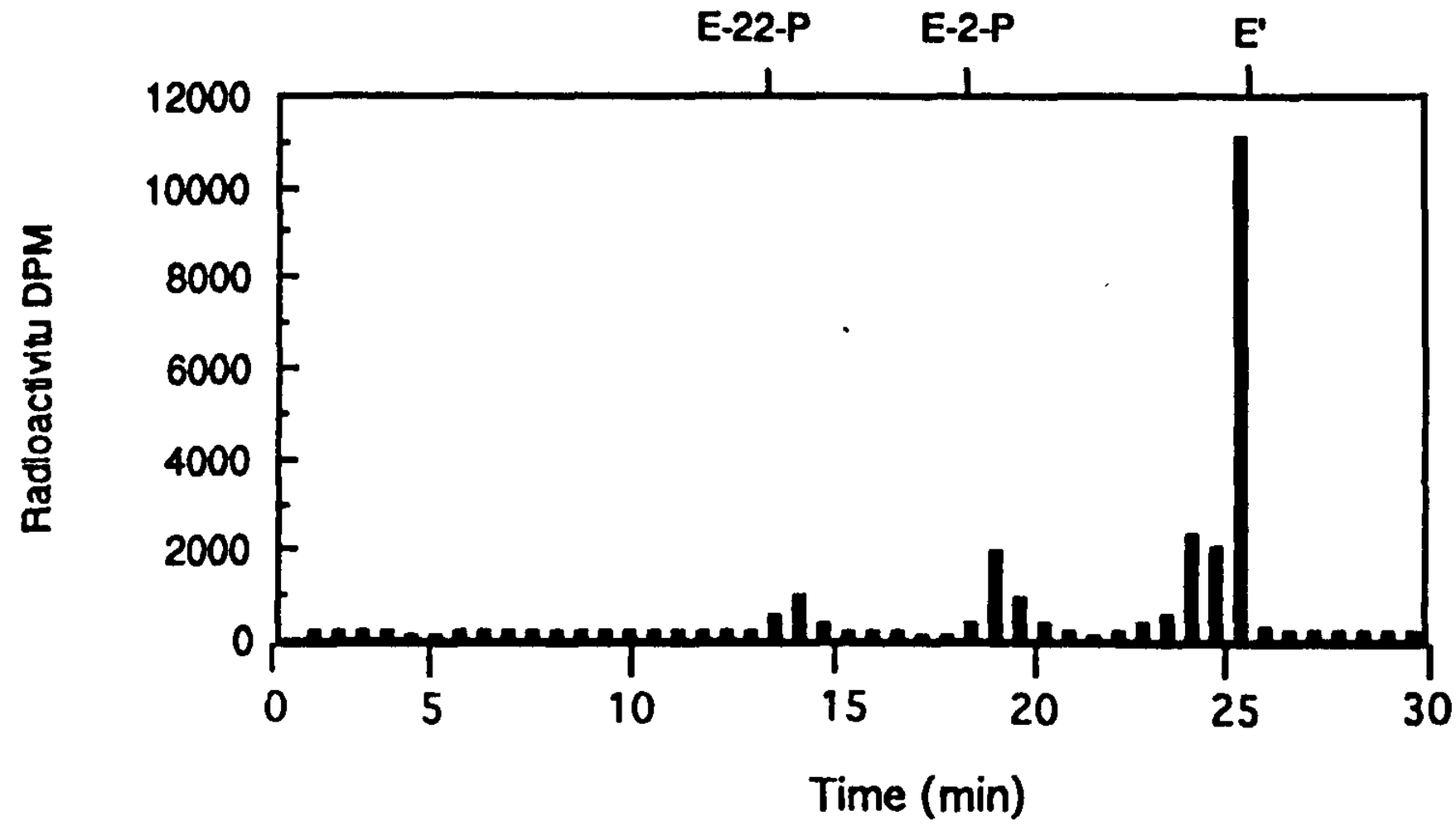
The cofactor requirements of the reductases have already been discussed for *S. littoralis* and these most recent results confirm that the crude 3-dehydroecdysone 3β-reductase, *in vitro*, has preference for

Figure 5.15 Reversed-phase HPLC fractionation using a methanol/citrate buffer, pH 6.5 gradient of the polar ecdysteroids from phosphotransferase assays using a) 3-dehydroecdysone (3dE), b) 3-epiecdysone (E) and c) ecdysone (E) as substrates. The positions of authentic E, E-22-P (ecdysone 22-phosphate), E-2-P (ecdysone 2-phosphate), 3dE, and E are indicated.

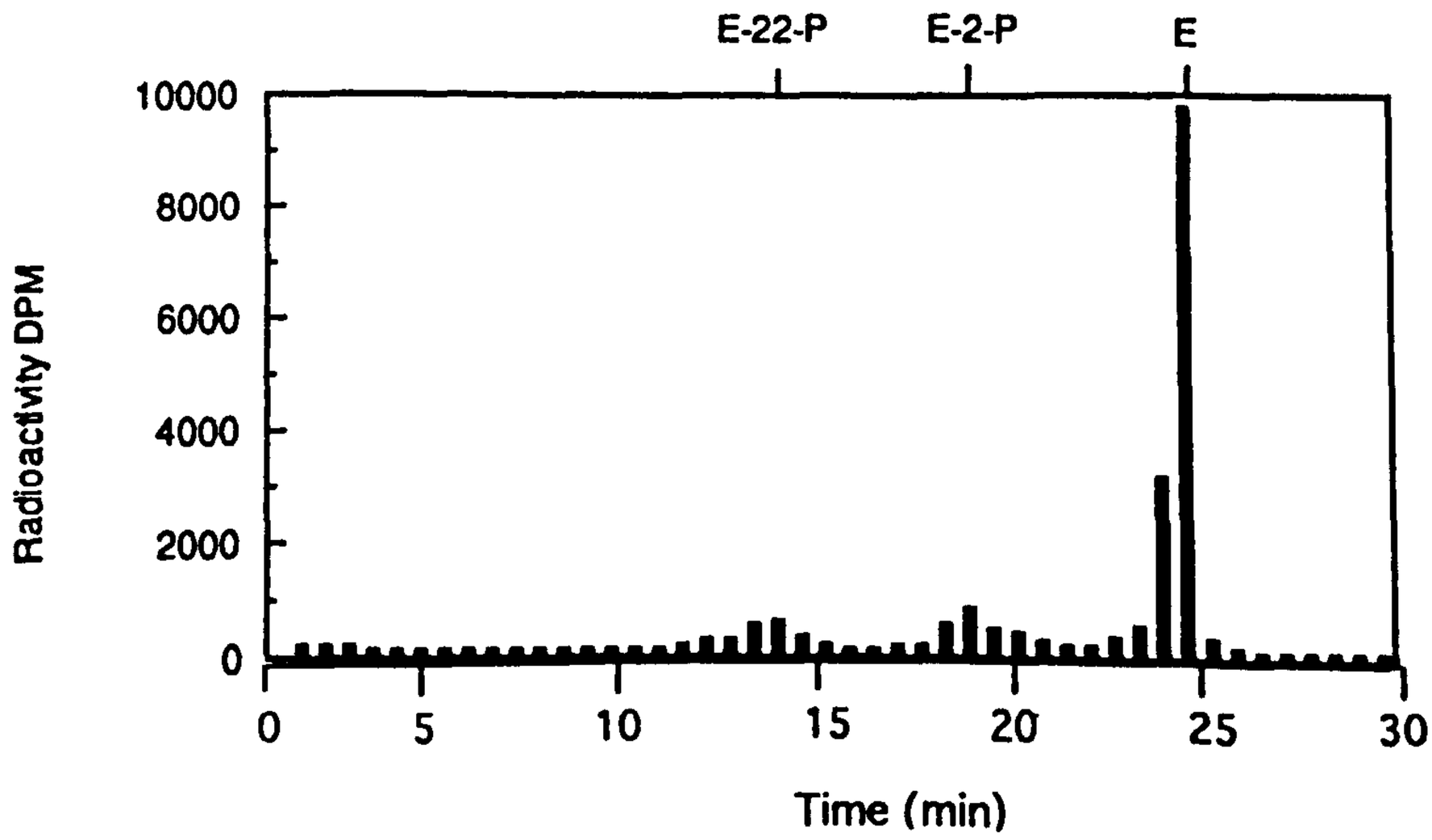
a)


E-22-P
E-2-P
3dE
Radioactivity DPM
10000
8000
6000
4000
2000
0
0
5
10
15
20
25
30
Time (min)

b)


E-22-P
E-2-P
E'
Radioactivity DPM
12000
10000
8000
6000
4000
2000
0
0
5
10
15
20
25
30
Time (min)

c)


E-22-P
E-2-P
E
Radioactivity DPM
10000
8000
6000
4000
2000
0
0
5
10
15
20
25
30
Time (min)

NADH and the 3α-reductase for NADPH. Activity is also detected when the alternative cofactor is used. The ratio of NADH to NADPH is also an interesting aspect of this work. It is generally tissues of biosynthesis (e.g. those performing the pentose phosphate pathway) which contain a higher proportion of NADPH. In rat liver, the concentrations of NADH, NAD^+, NADPH and $NADP^+$ have been determined using an indirect method (Veech et al., 1987), using reactions which are known to be near equilibration, in certain cellular compartments. For example, lactate dehydrogenase is present exclusively in the cytosol and in sufficiently high concentrations that the reaction is near euilibrium. Therefore, the equilibriation constant, K, approximates to 1 and, the ratio of [lactate]/[pyruvate], is equivalent to [NADH]/ $[NAD^+]$. Similarly, the conversion of pyruvate to malate has been studied in order to deduce the $[NADP^+]$/ [NADPH] ratio.In the cytosol of rat liver, and presumably other tissues, the $[NAD^+]$/ [NADH] ratio is about 1000, whereas that for the $[NADP^+]$/ NADPH is about 0.01 (Krebs, 1973). It has therefore been assumed that the cells of the midgut contain a higher percentage of NADPH, as oxidized cofactor. The question that must now be raised is the significance of the 3-dehdroecdysone 3β-reductase, which has preference for NADH. It is possible that this enzyme plays only a minor role *in vivo*.

That ATP and $MgCl_2$ are required for phosphotransfer has been well-documented by several workers (Yang and Wilkinson, 1973; Weirich et al., 1986; Kabbouh and Rees, 1991a). In the case of the ATP: 2-deoxyecdysone 22-phosphotransferase in the follicle cells of *Schistocerca gregaria*, GTP was found to be far less effective than ATP, hence, the nature of the nucleotide is important. Magnesium was an efficient divalent cation and stimulated phosphotransferase activity in the presence of ATP. However, high Mg^{2+} concentrations (>10mM) were

found to have inhibitory effects on 2-deoxyecdysone 22-phosphotransferase in follicle cell preparations (Kabbouh and Rees, 1991a).

Exclusion of molecular oxygen has already been found to prevent the ecdysone oxidase reaction (Koolman and Karlson, 1978). This was confirmed by the anaerobic experiments for the *Spodoptera littoralis* enzyme, but an apparent slow turnover rate was observed with the exclusion of oxygen. Whether this was due to incomplete removal of oxygen or whether molecular oxygen is not be the only species capable of acting as a hydrogen acceptor, cannot be ascertained. In fact, several inorganic dyes can be substituted for oxygen in the oxidase reaction: O_2< $K_3[Fe(CN)_6]$ < 2,6-dichloroindophenol (Koolman and Karlson, 1978). It is also possible that there may be a natural oxidizing agent, besides oxygen, present in some insects, which accounts for the minimal activity in an oxygen limited environment. Surprisingly, under anaerobic conditions, the ecdysone oxidase activity of *Pieris brassicae* remains unaffected (Blais and Lafont, 1984).

There is substantial evidence as to the authenticity of the products of the epimerization system. 3-Dehydroecdysteroids were initially mistaken for 3-acetates, when analyzed by TLC and certain HPLC systems (Lafont et al., 1980) and hence their identification necessitates additional experiments. Since several physico-chemical analyses were undertaken, the product of the ecdysone oxidase reaction is confirmed as being 3-dehydroecdysone. In the case of the reductases, 3-epiecdysone was fully resolved from ecdysone, by both HPLC systems employed (1 and 2). It should be noted that 3-dehydroecdyosne and ecdysone were used as substrates in this and subsequent studies, despite 20-hydroxyecdysone being the major metabolite *in vivo* (ecdysone can be purchased in radioactive form, whereas 20-hydroxyecdysone has to be

synthesized enzymically). Apart from kinetic analyses, the findings reported in Chapters 5 and 6 should be equally applicable to the 20-hydroxyecdysteroids

By the use of two complementary HPLC systems alone, the phosphate isomers produced by *Spodoptera littoralis* cytosol have been found to co-chromatograph with ecdysone-2- and 22-phosphate authentic markers. In *Manduca sexta,* incubation with ecdysone under aerobic conditions yielded 4 distinct phospho-conjugate peaks, but using 3-epiecdysone, 2 peaks were observed (Weirich et al., 1986). Unfortunately, none of these metabolites could be fully identified. In this case, it has been postulated that the hydroxyl groups at C-2, C-3 and C-22 may be phosphorylated and that the enzymes may in fact be stereospecific, hence, the reduced number of conjugates found in the 3-epimer incubations. Of particular note is the fact that ecdysone, 3-dehydroecdysone and 3-epiecdysone all have the capacity to be phosphorylated in *Spodoptera* midgut cytosol preparations. By analogy with the ecdysone metabolites of this enzyme system, 3-dehydroecdysone phosphorylation is expected to be on C-22 and C-2, not C-3.

When ecdysone is added to a non-dialysed enzyme preparation, 3-epiecdysone and small amounts of ecdysone 22-phosphate are produced. It is possible that ecdysone 2-phosphate is also formed, but in insufficient quantities to detect. Interestingly, upon hydrolysis of the ecdysteroid 22-phosphate, only ecdysone was released; this may reflect a) exogenous ecdysone is present in greater quantities than the enzymically formed 3-epiecdysone, and is therefore phosphorylated to a greater degree or b) ecdysone is preferentially phosphorylated *in vivo* and exogenous 3-epiecdysone is only phosphorylated in the absence of ecdysone.

In his thesis, Milner (1984) described how the primary faecal ecdysteroid of early last instar larvae was 20-hydroxyecdysone 22-phosphate, with a smaller quantity of ecdysone 22-phosphate and even lower levels of ecdysone 2-phosphate, but in later larvae (pre-pupal stage), ecdyson- and 20-hydroxyecdyson-26-oic acids are excreted. No 3α-epimers or conjugates were detected. The modification of the ecdysteroid is apparently important for its excretion, and, although the mechanism has not been explored, unchanged free and conjugated forms have also been found to be excreted by *Locusta migratoria* (Hoffmann et al., 1974).

Evidently 2- and 22-phosphates are synthesized by the midgut, a situation which is different from that in locust ovaries, where the 22-phosphate derivatives are formed (Isaac et al., 1983). One must now pose the question whether phosphorylation at C-2 and C-22 are species and/or tissue specific.

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Chapter 6

Characterization and partial purification of the enzymes of epimerization and phosphorylation in the midgut cytosol of *Spodoptera littoralis*

6.1 INTRODUCTION

In 1974, Nigg et al., discovered a 3-epimerization system in the high-speed supernatant (85,000g) of *Manduca sexta* midgut, converting ecdysone into 3-epiecdysone. Removal of NAD(P)H prevented the reaction going to completion, but a build-up of 3-dehydroecdysone suggested it was the intermediate (Chapter 1, figure 1.10). The first enzyme of the sequence, ecdysone oxidase, has been characterized in several midgut homogenates (for review see Koolman, 1985), including *Manduca* (Weirich et al., 1989, 1991) and *Calliphora vicina* (Koolman and Karlson, 1975, 1978). Furthermore, in the latter case, ecdysone oxidase was partially purified and its specificity for several steroids established.

With regards to the reductases, work on the epimerase (3-dehydroecdysone 3α-reductase), was expanded by Mayer et al. in 1979, who initiated a cofactor specificity study and described preliminary kinetic parameters. The sequence shown in figure 6.1a was determined by isotope dilution experiments. Milner and Rees (1985) used ecdysone combined with either [^{3}H]ecdysone or [^{3}H]3-dehydroecdysone and found that the recovered [^{3}H]3-epiecdysone had a higher specific activity when the incubation contained [^{3}H]3-dehydroecdysone rather than [^{3}H]ecdysone. Using kinetic analysis, Weirich et al., (1989) found that the conversion of 3-dehydroecdysone into 3-epiecdysone, depending on various substrate and cosubstrate concentrations, was 13 to 100 times faster than the rate of conversion of ecdysone into 3-dehydroecdysone. In each case, the scheme in figure 6.1a is more compatible than figure 6.1b.

It is important to reiterate that the addition of 3-dehydroecdysone and NADPH to a cytosolic enzyme preparation from *Spodoptera*

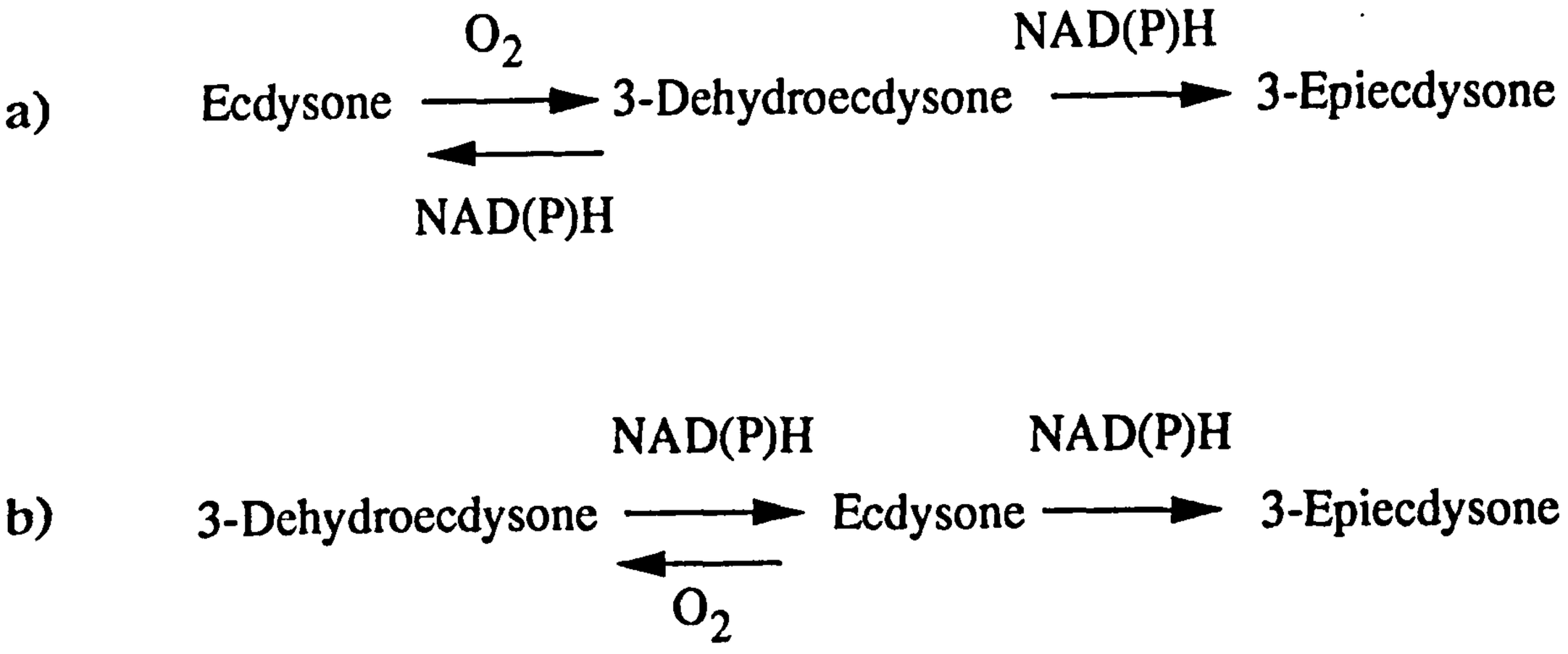


Figure 6.1 (a) and (b). Possible metabolic sequences in the midgut cytosolic fraction via the intermediacy of a) 3-dehydroecdysone and b) ecdysone.

littoralis resulted in primarily 3-epiecdysone and addition of NADH yielded predominantly ecdysone (Milner and Rees, 1985; Chapter 5). As mentioned in Section 1.7.2, this contrasts with the *Manduca* system, which requires mainly NADH for 3α-epimer production and NADPH for 3β-hydroxyecdysteroid synthesis. It became apparent that there was potentially five enzymic activities in the epimerization reaction of the midgut [ecdysone oxidase, 3-dehydroecdysone 3α-reductase (NADPH), 3-dehydroecdysone 3α-reductase (NADH), 3-dehydroecdysone 3β-reductase (NADPH) and 3-dehydroecdysone 3β-reductase (NADH)]. For simplicity these activities are considered separately in this chapter. Recently, efficient assay systems for the epimerization enzymes have been developed in *Manduca sexta* (Weirich et al., 1989, 1991), describing optimal pH, protein linearity and cosubstrate/substrate kinetics.

Other ecdysteroid inactivating enzymes are known to exist in the the midgut, among these are the cytosolic phosphotransferases. Little is known of these enzymes, and indeed, our knowledge is quite preliminary of ecdysteroid phosphotransferases in general. It has previously been alluded to that in *Manduca*, a midgut cytosol incubation using ATP, Mg^{2+} ions and (3-epi)ecdysone resulted in a variety of phosphoesters, none of which were further identified (Weirich et al., 1986). Dialysed cytosolic enzyme preparation from *Spodoptera littoralis* has already been shown to contain phosphotransferase activity, when supplemented with ATP and Mg^{2+}, furnishing ecdysone 2- and 22-phosphates (Chapter 5).

In this chapter, a characterization of all the enzymes catalysing the foregoing oxidation/reduction reactions is described. Firstly, in order to study the midgut enzymes, it was important to determine how their biological activities fluctuate throughout the period of interest, i.e. the

final instar. From the titres of the enzymes, it was possible to suggest a scheme for the significance of the changing enzymic activities in relation to the changes in ecdysteroid titre.

Previously, the cofactor requirements and the product(s) of the reactions were verified (Chapter 5); a more in-depth study of the enzyme requirements shall now be presented, with respect to the basic features of optimal enzyme action: pH, temperature, reaction time, protein and substrate concentrations, such that standard assay systems could be defined.

To begin to understand the behaviour of an enzyme in a complex system (e.g. the cell), it is usually studied in as simple a system as possible, containing buffer, substrate and cofactors. In this vein, the enzymes were partially purified and the significance of cofactor specificities discussed once more in light of the new findings.

The 3-dehydroecdysone 3β-reductase was assayed in the presence of air. It could be argued that ecdysone oxidase would convert some ecdysone produced back into 3-dehydroecdysone. Even by repeated evacuation and making use of the glucose oxidase/ peroxidase system under an atmosphere of nitrogen, (Section 5.2.2) residual oxidase activity was still found (18% relative conversion). Thus, in measuring the changes in 3β-reductase activity during the sixth instar (Section 6.2.1), it would have been difficult to totally eliminate ecdysone oxidase activity by the above system. Furthermore, attempts to undertake such manipulations might also lead to inconsistencies in 3β-reductase activty. Therefore, the 3β-reductase activities assayed in air in the enzymic profile are "apparent" values. However, they should still reflect the qualitative changes in the profile. [Nevertheless, in the previous chapter, (refer to Table 1) it was found that 3β-reductase activity is only increased by 2% under anaerobis].

The assay used for the ecdysone oxidase is satisfactory, since dialysed enzyme preparation has been used throughout, NAD(P)H was removed, resulting in negligible 3β-reductase activity.

In the case of determination of the properties of the ecdysone oxidase and 3-dehydroecdysone 3β-reductase (pH and temperature optima, protein and time linearities; Section 6.2.2), enzyme was collected at 28h for the oxidase, a time of low 3β-reductase activity (Refer to figure 6.2) and at 100h for the 3β-reductase, i.e. when oxidase activity had diminished. Furthermore, in all of the kinetic studies, enzymes were fractionated by DEAE-cellulose, hence, removing oxidase activity from that of the 3β-reductase.

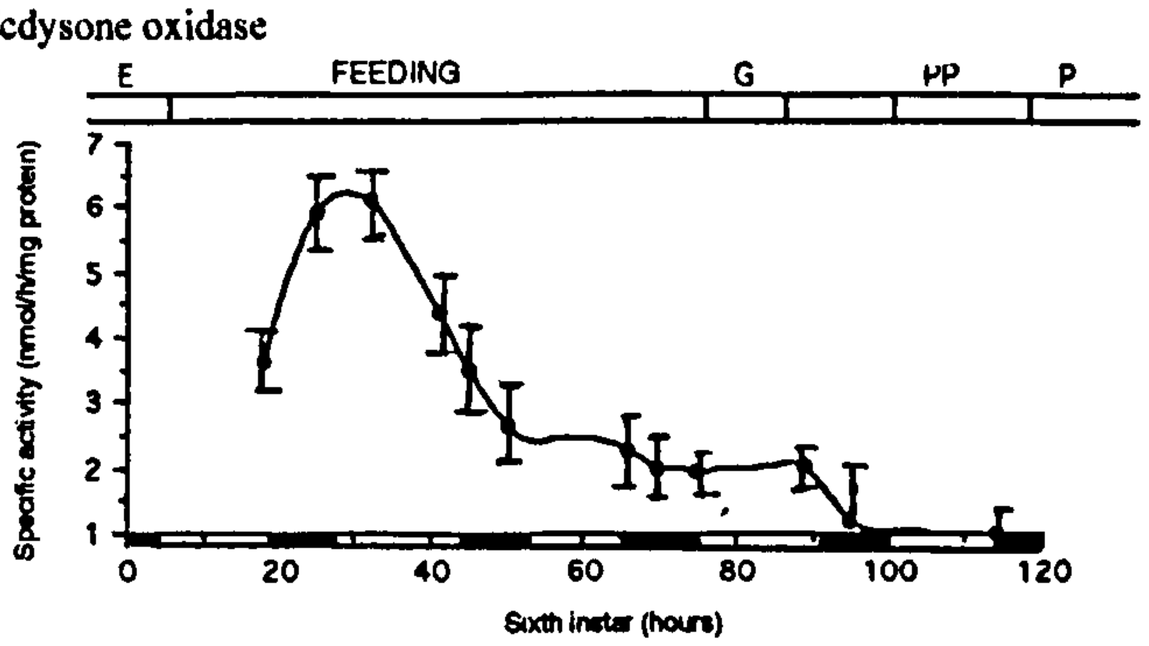
6.2 EXPERIMENTAL AND RESULTS

6.2.1 Developmental Profiles of the Enzymes

Insects were accurately synchronized (±3h) by photoperiod at the 5th./6th. instar moult and batches of approximately 10 insects were sacrificed at the following time-points; ecdysone oxidase and 3-dehydroecdysone 3α-reductase: 18, 25, 31, 41, 45, 50, 66, 89, 114 hours; 3-dehydroecdysone 3β-reductase: additionally 70, 75, 95h; phosphotransferases: 20, 27, 35, 45, 51, 59, 69, 75, 83, 93, 99, 107, 120h. Post-microsomal supernatants of the midgut were obtained (Section 2.2.3) and the enzymes assayed according to Section 5.2.1. The experiment was carried out three times using different generations of insects.

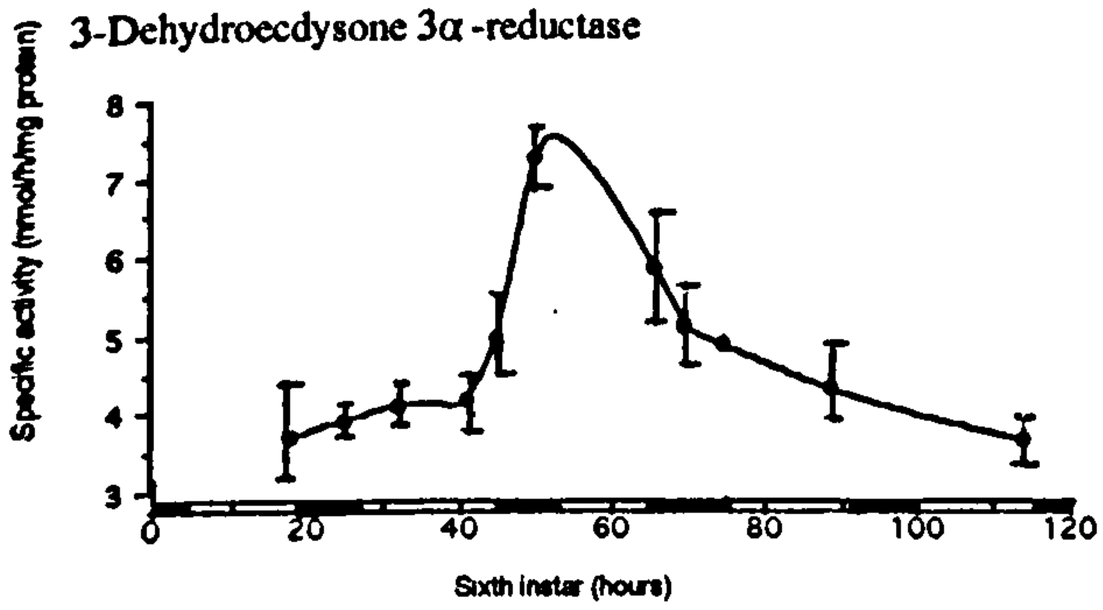
The qualitative and quantitative profiles of the specific activities of the enzymes during the sixth instar are shown in figure 6.2a-e. The ecdysone oxidase activity reached a peak at approximately 31±3h, in the

a) Ecdysone oxidase

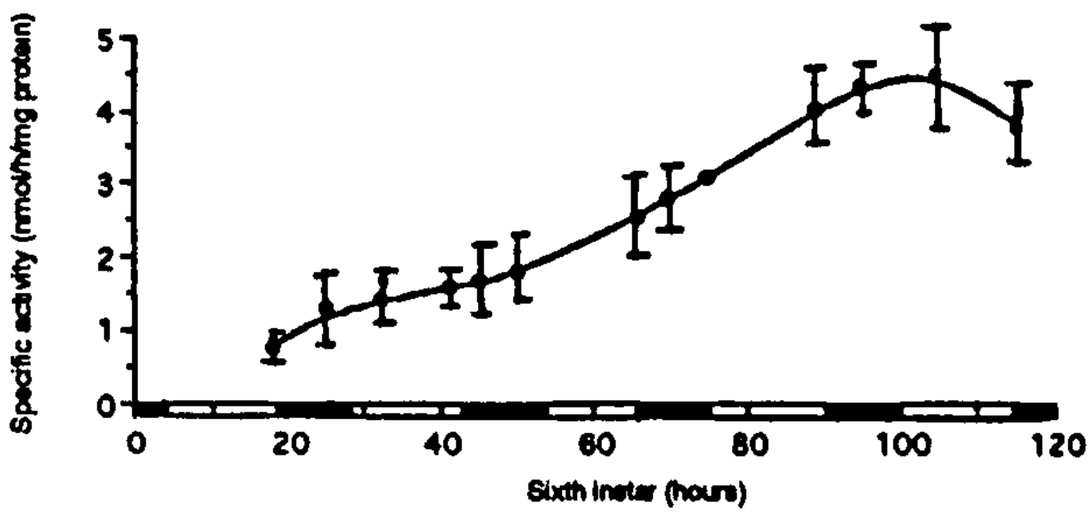


b)

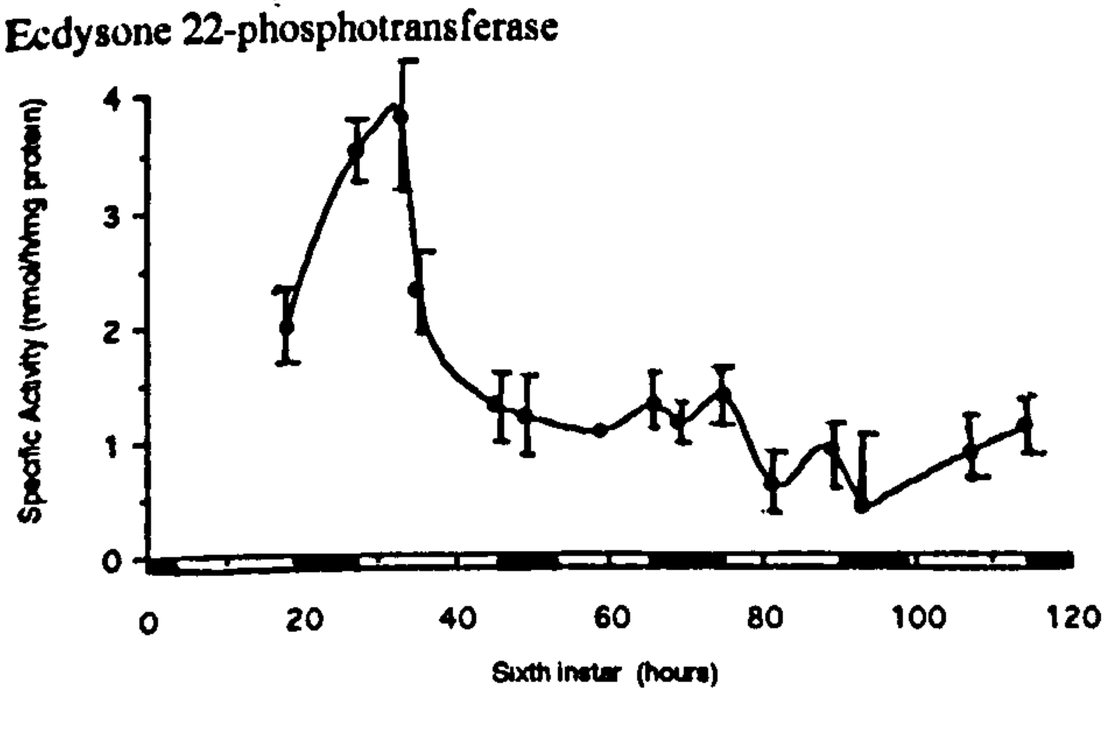
3-Dehydroecdysone 3α -reductase



c) 3-Dehydroecdysone 3β -reductase



d) Ecdysone 22-phosphotransferase



e) Ecdysone 2-phosphotransferase

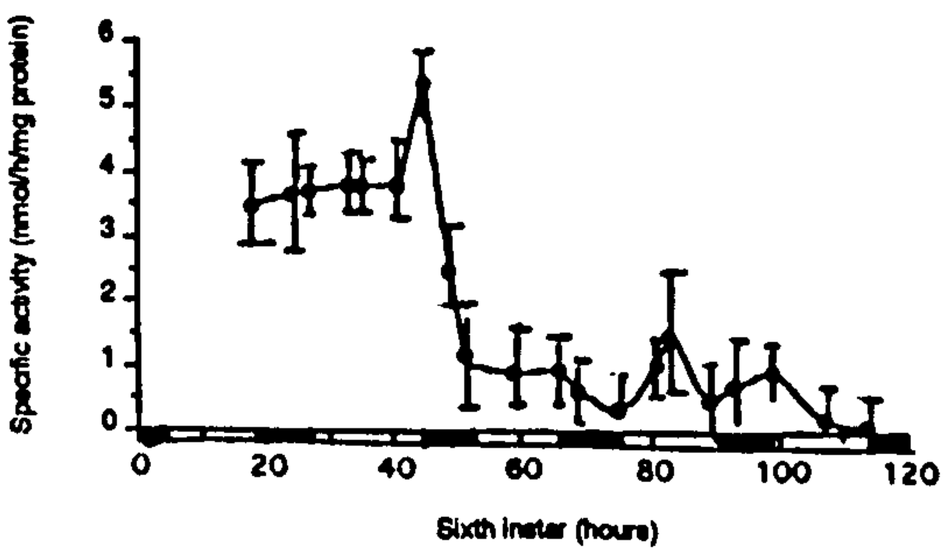


Figure 6.2. Midgut enzyme activity profiles during the sixth (final) instar of Spodoptera littoralis. Boxes on the abscissa refer to the light and dark phases and insects were synchronized by photoperiod (±3h). Each point represents the mean of 3 separate investigations (each assay being carried out in duplicate) and bars represent the standard deviation from the mean. Where bars are not shown, the SD is within the • symbol. Various developmental markers are shown ecdysis (E), feeding, wandering (W), pharate pupal (PP) and pupariation (P).

second photophase. Enzymic activity delines to a basal level at around 50h and remains at low, but constant activity until 90h, when the titre falls to indistinguishable levels by 100h.

The 3-dehydroecdysone 3α-reductase (assayed using NADPH) has a rather intriguing profile. During the first 40h of the instar, activity is at a low plateau but reaches a peak at about 55h. There then follows a steady decline in activity, until the end of the instar, when at 114h, activity falls to a basal level. The reason for such a pattern of peaks and troughs is largely unclear.

In contrast, the 3-dehydroecdysone 3β-reductase (NADH) is highest in the fifth photophase. There is a steady rise in "apparent" enzymic activity throughout the instar, until a peak is reached at 107±3h, after which the level drops. It is quite interesting that this closely resembles the profile given in Chapter 4 for the haemolymph 3-dehydroecdysone 3β-reductase, suggesting that these enzymes may have a common control mechanism.

The 22-phosphotransferase enzyme reached a peak early in the instar at 27±3h. In the case of the 2-phosphotransferase enzyme, activity was relatively high until about 45h, when it declined. For both enzymes, activity was maintained at a comparatively low level later in the instar. Once again, the significance of these data is not easily explained.

6.2.2 Optimal Conditions for Enzymic Activity

Reaction conditions for maximal activity were ascertained by individually changing the following parameters. The reductases were assayed using both NADH and NADPH. The assays utilized dialysed cytosol as a source of enzymes and were carried out in duplicate as in Chapter 5, Section 5.2.1. Values represent the mean of two separate

incubations (±SD). All batches of incubations included control tubes containing boiled enzyme preparation. The results for these incubations (generally <3% conversion) were subtracted from the experimental values.

i) The Concentration of Protein

0-2.0mg/ml protein used to find the range of protein concentrations over which the enzymic activities were linear.

ii) pH Optima

Incubation mixtures were carried out using the following buffers (Long, 1961), at constant ionic strength (I=0.2):

pH 5.1-6.4, 0.2M sodium acetate/acetic acid;

pH 6.5-7.8, 0.2M sodium phosphate;

pH 8.0-9.1, Tris/Tris-HCl.

Accurate pH measurements were taken at 37°C, i.e. the incubation temperature.

iii) Temperature optima

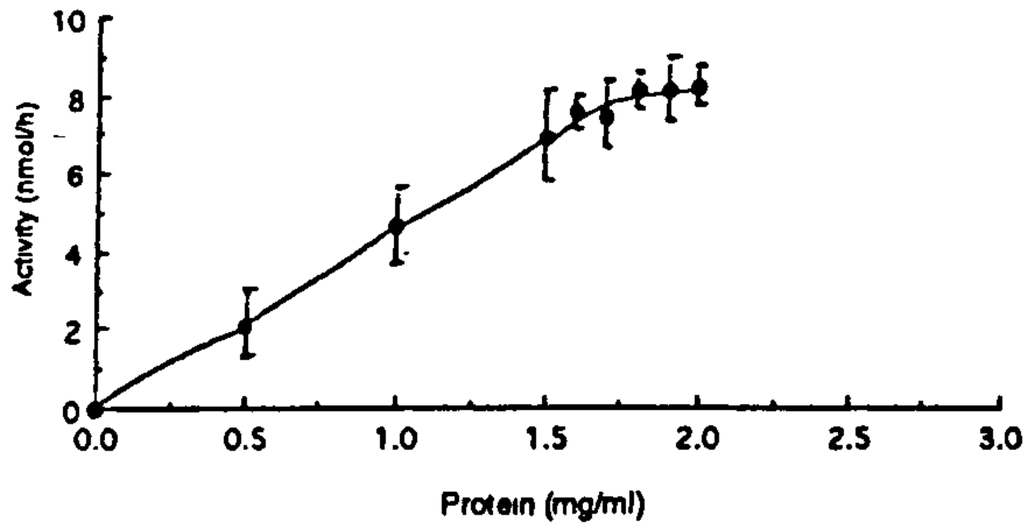
A temperature range of 20°-50°C was used to determine peak values.

iv) Reaction time

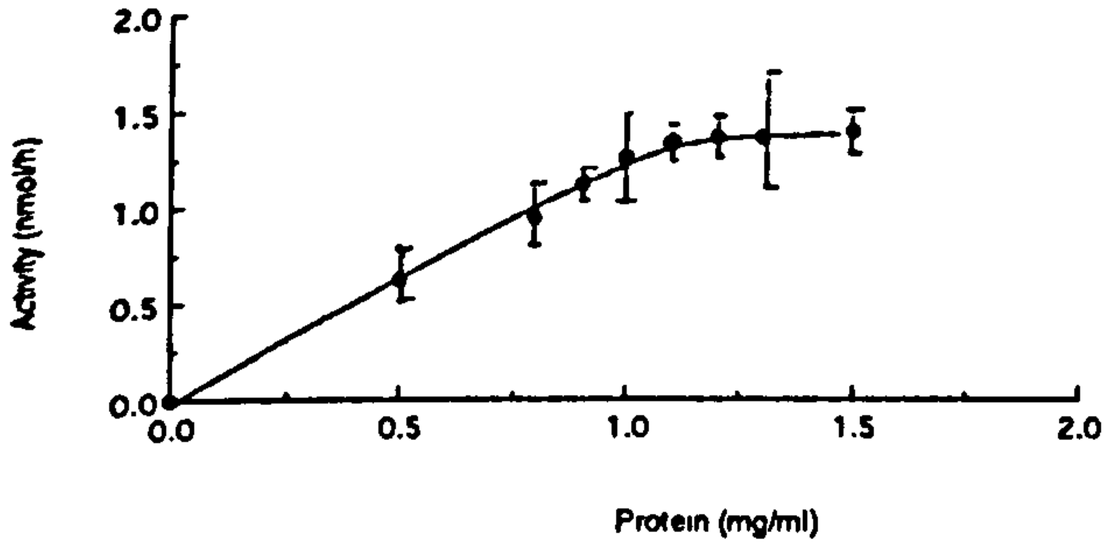
Reactions were stopped at timed intervals from 0-120min.

The optimal conditions were derived from the results given in in figures 6.3, 6.4, 6.5 and 6.6 and are summarized in Table 1. These conditions were then used in all subsequent work. It is interesting to note that the reductases display quite different sets of results and that

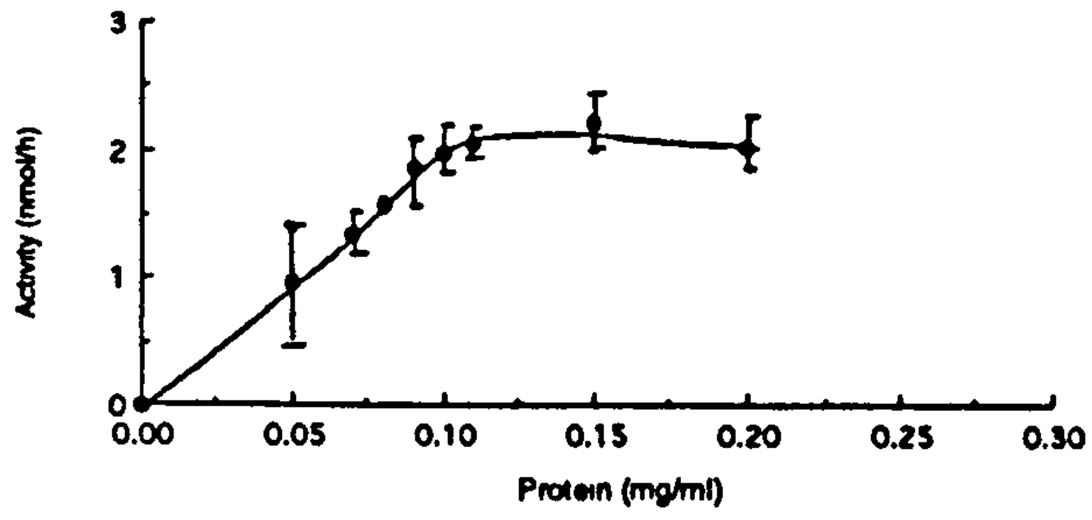
a) Ecdysone oxidase



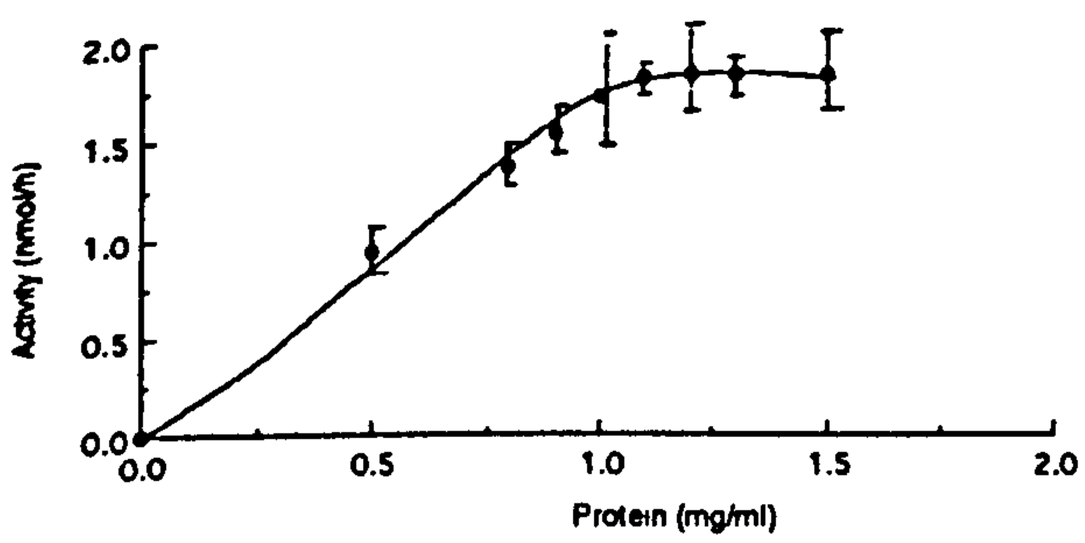
b) 3-Dehydroecdysone 3α-reductase (NADH)



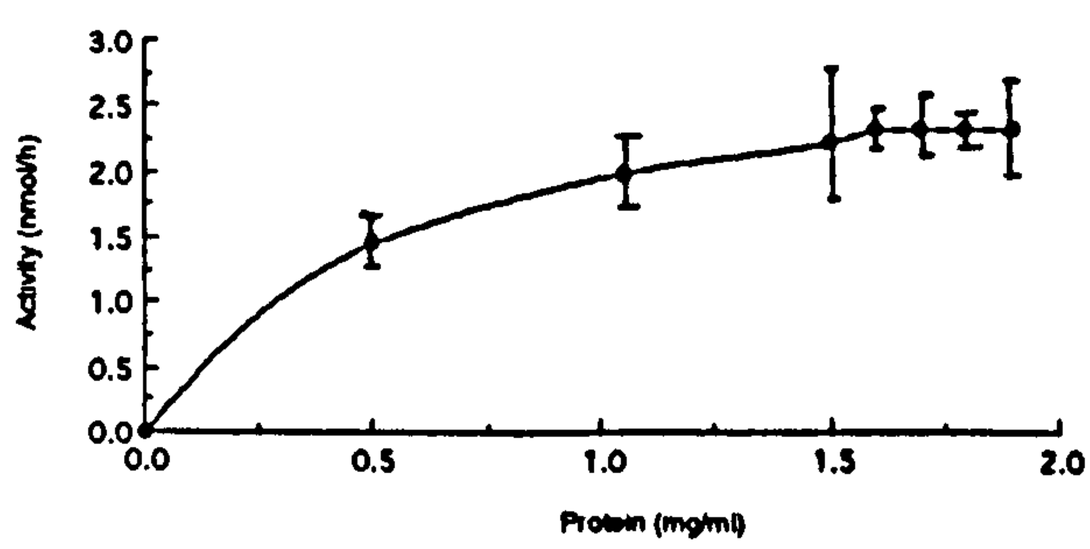
c) 3-Dehydroecdysone 3α-reductase (NADPH)



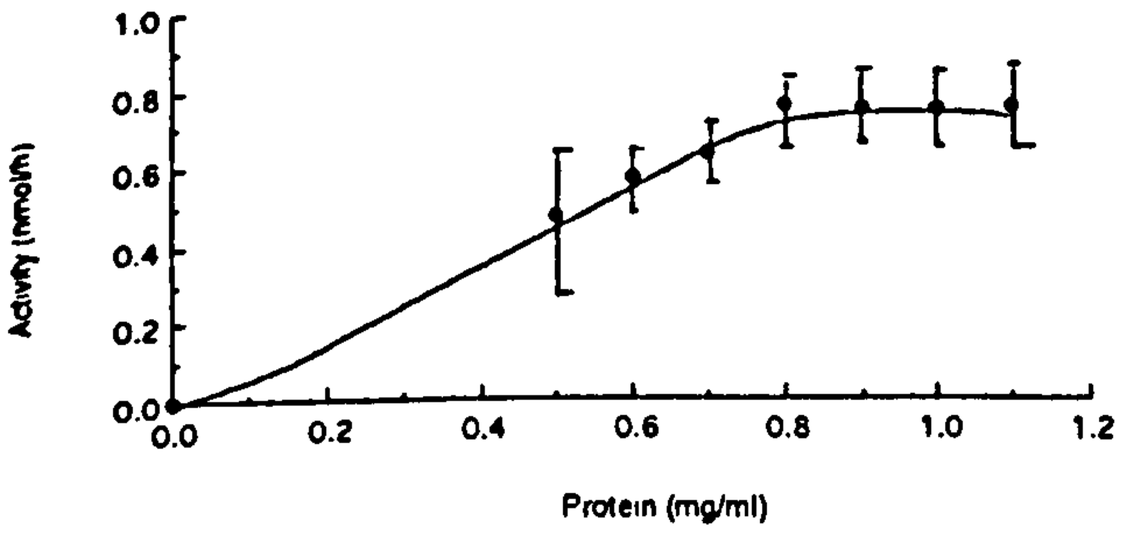
d) 3-Dehydroecdysone 3β-reductase (NADH)



e) 3-Dehydroecdysone 3β- reductase (NADPH)



f) Ecdysone 22-phosphotransferase



g) Ecdysone 2-phosphotransferase

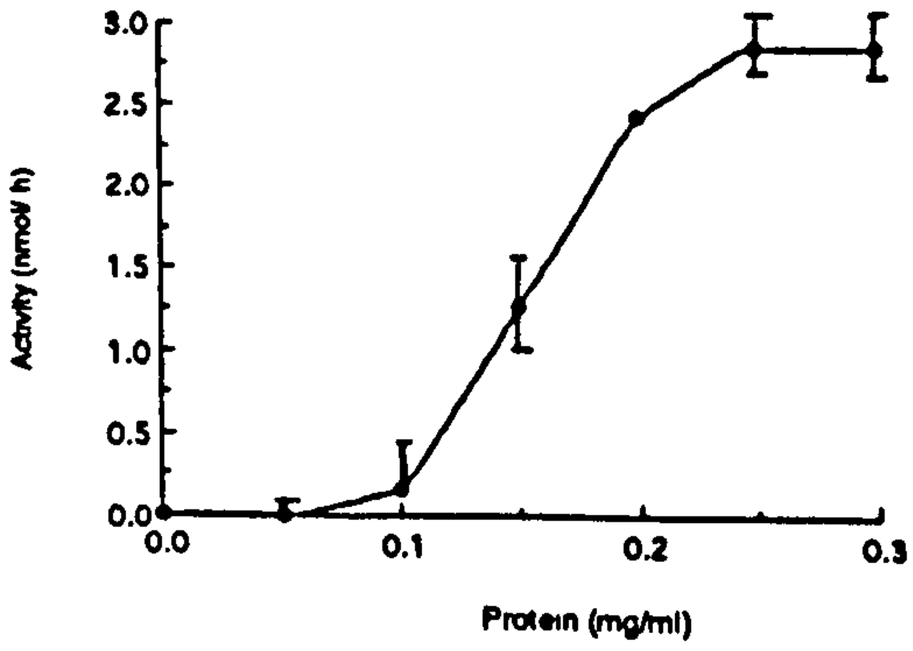
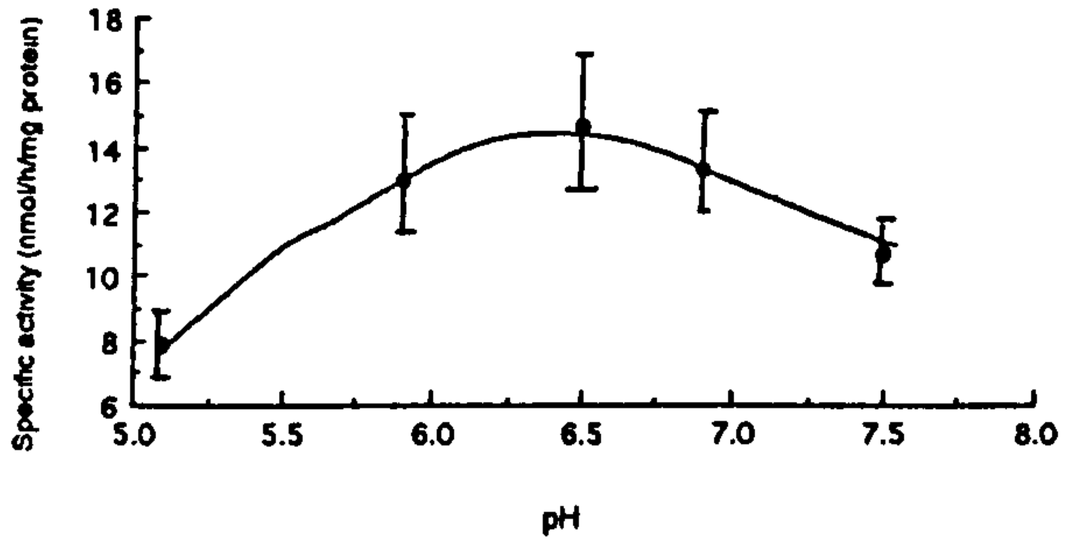


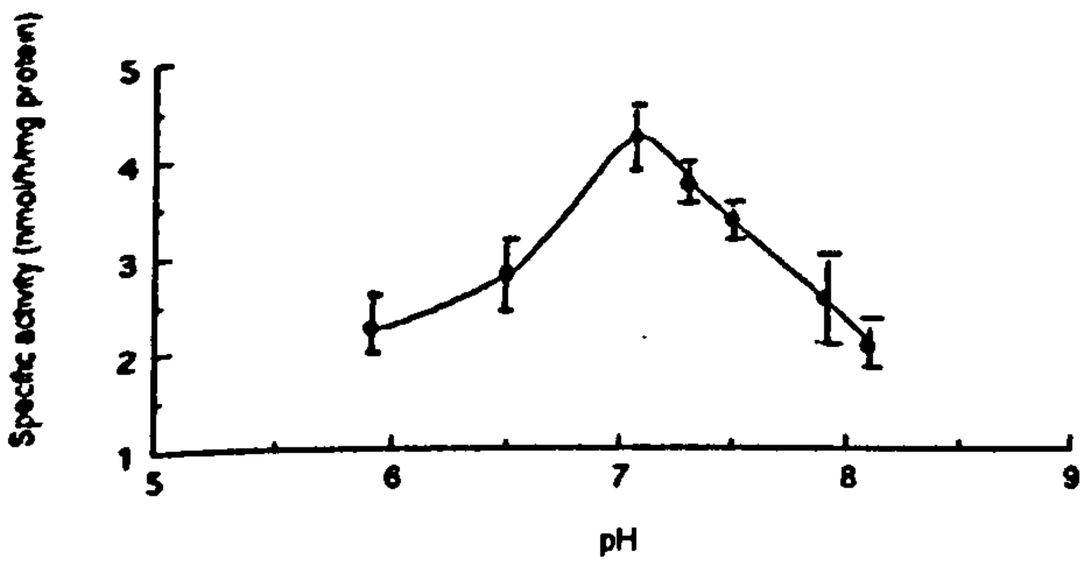
Figure 6.3. Effect of protein concentration. Incubation mixtures were set up using 0-2.0mg/ml protein. Concentrations of ecdysteroid (8.4μM) and cofactors were maintained constant. Points represent the mean of two determinations (± SD). Where bars are not shown, the SD is within the • symbol.

a) Ecdysone oxidase

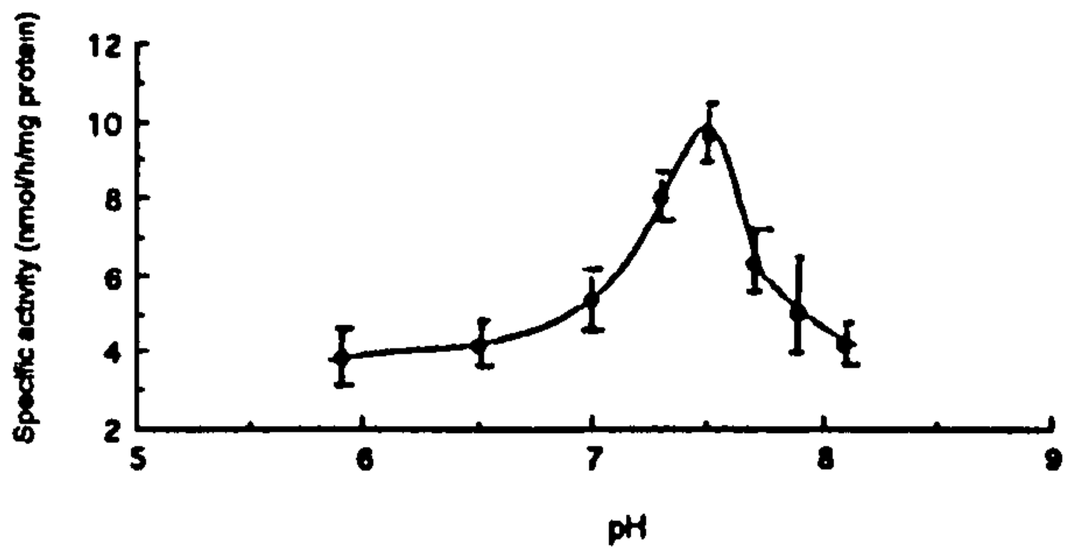


OPTIMAL PH 7.4

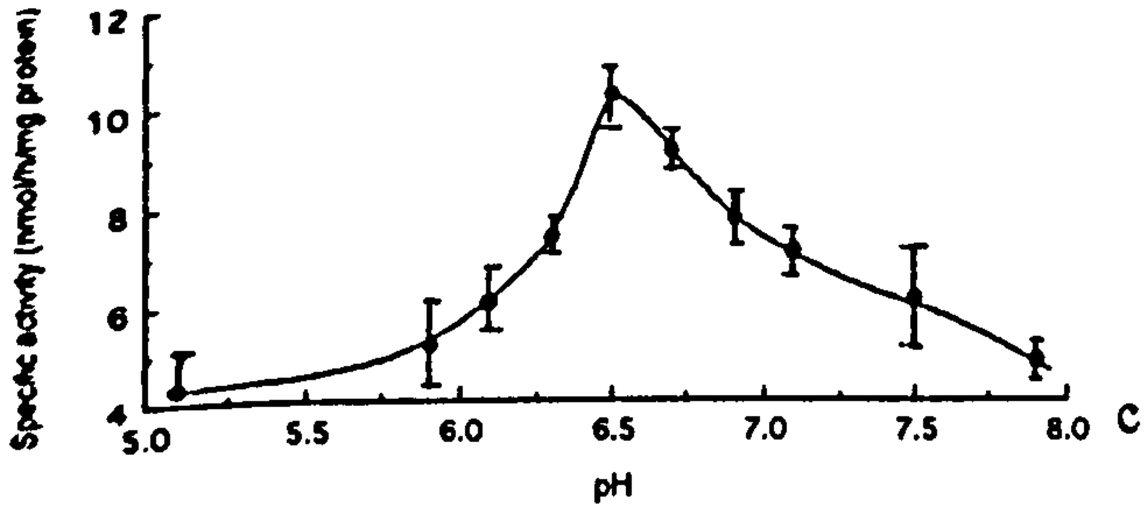
b) 3-Dehydroecdysone 3α-reductase (NADH)



c) 3-Dehydroecdysone 3α-reductase (NADPH)

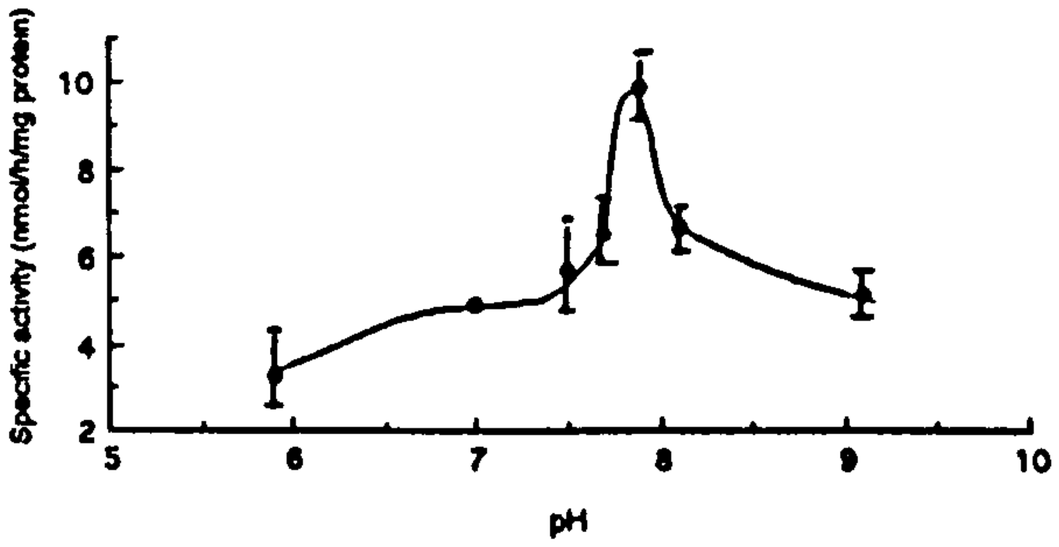


d) 3-Dehydroecdysone 3β-reductase (NADH)

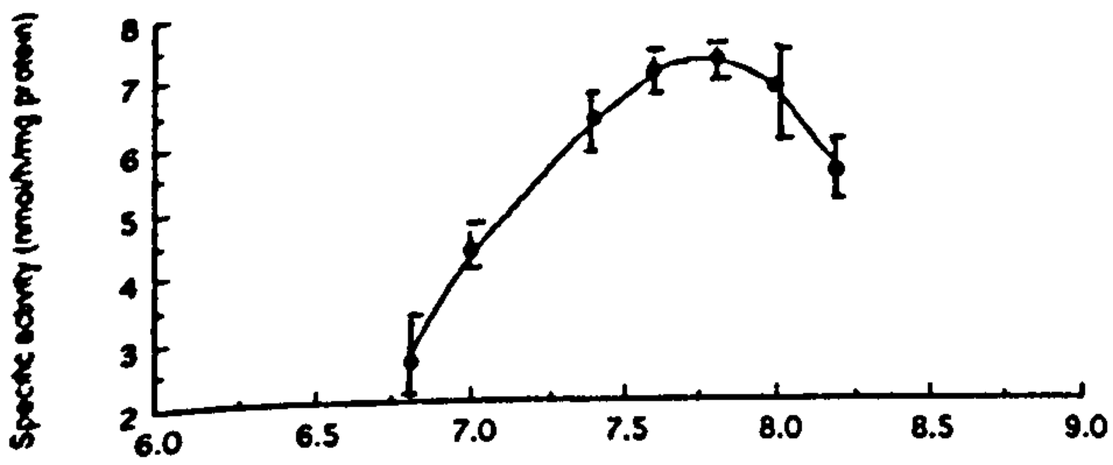


C

e) 3-Dehydroecdysone 3β-reductase (NADPH)



f) Ecdysone 22-phosphotransferase



g) Ecdysone 2-phosphotransferase

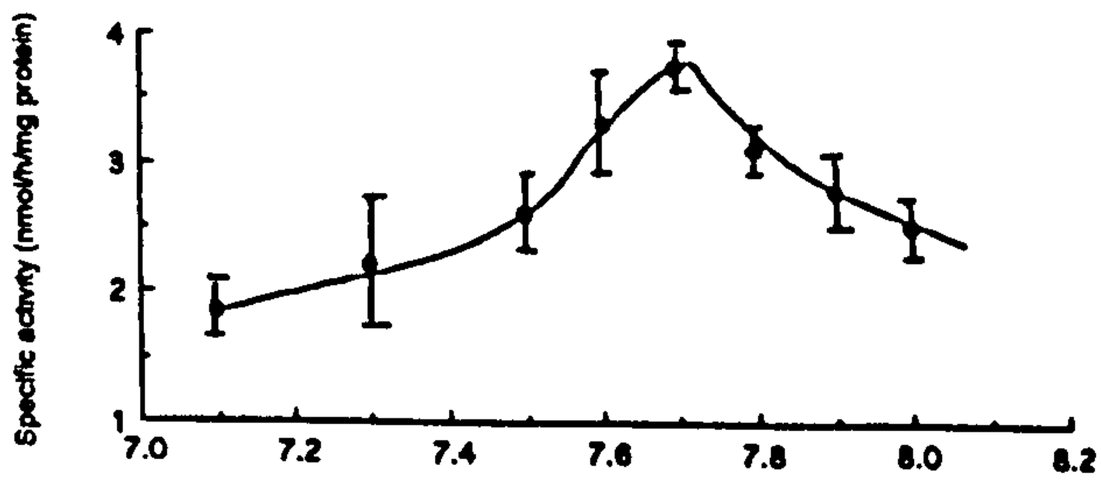
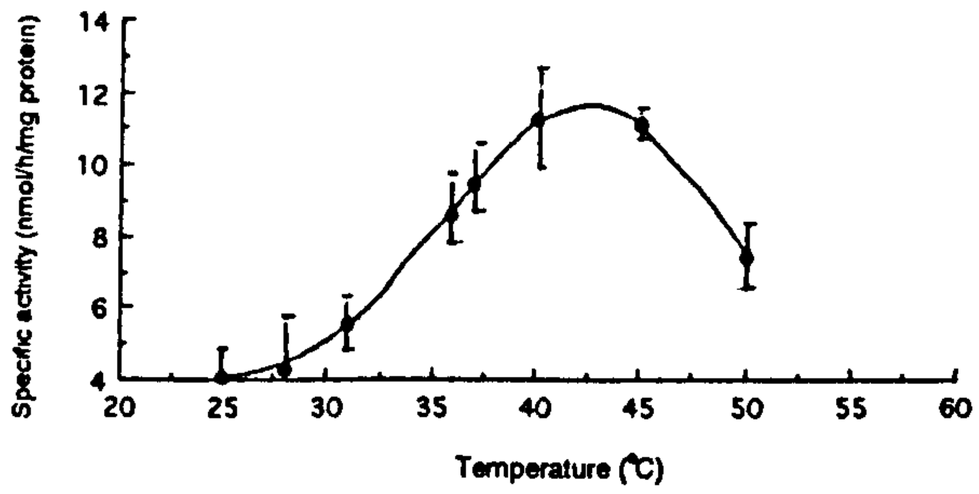
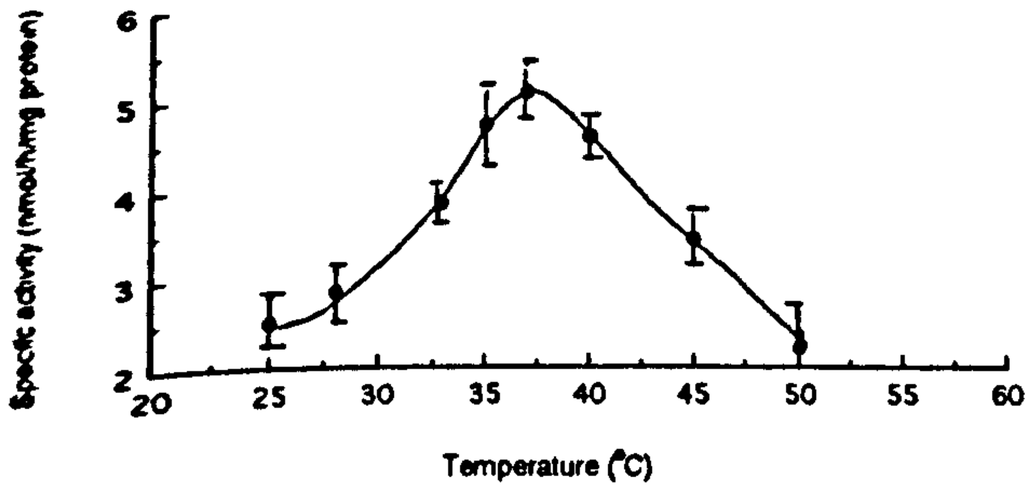


Figure 6.4. Effect of pH. Incubation mixtures contained 8.4μM ecdysteroid, 0.1-1.0mg/ml protein and the appropriate cofactor(s) in 250μl of buffer. Buffers were sodium acetate/ acetic acid, for pH 5.1-6.4, sodium phosphate for pH 6.5-7.8 and Tris/Tris-HCl for pH 8.0-9.1, at constant ionic strength. Points represent the mean of two determinations (± SD). Where bars are not shown, the SD is within the • symbol.

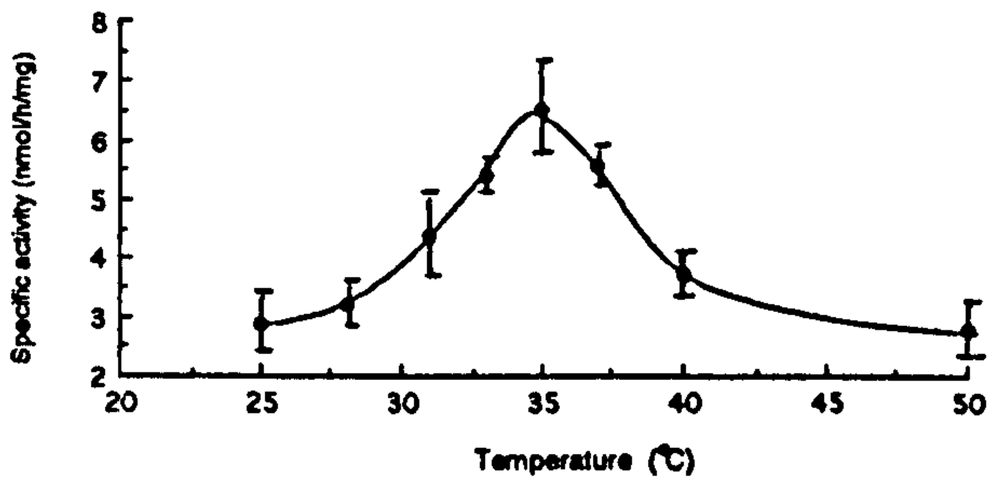
a) Ecdysone oxidase



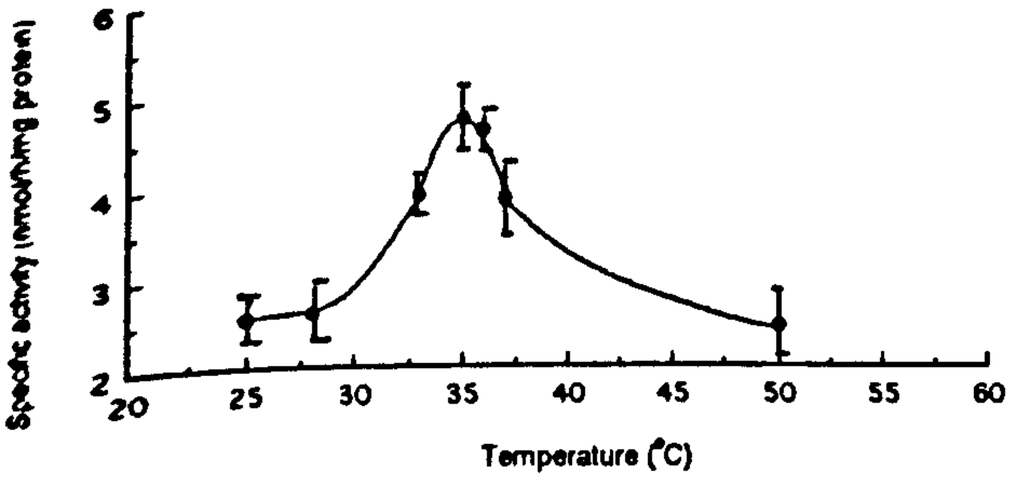
b) 3-Dehydroecdysone 3α-reductase (NADH)



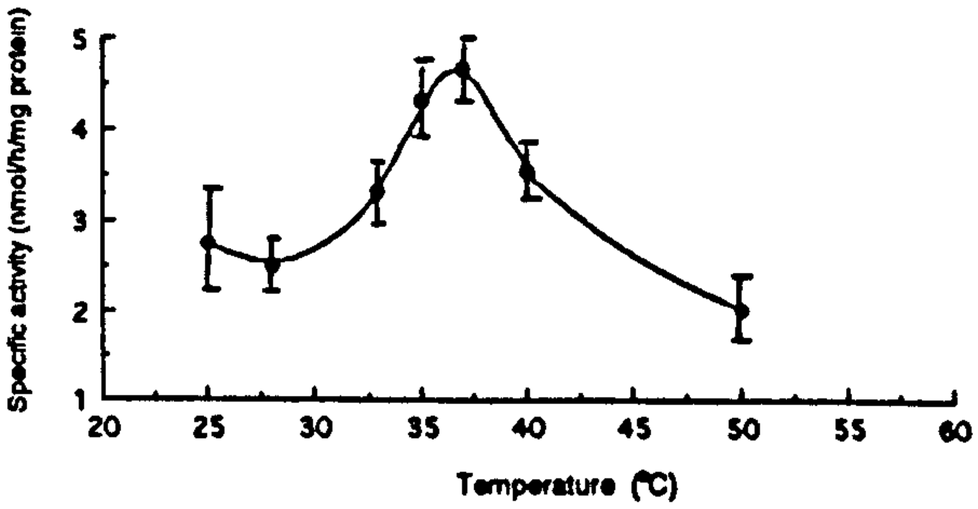
c) 3-Dehydroecdysone 3α-reductase (NADPH)



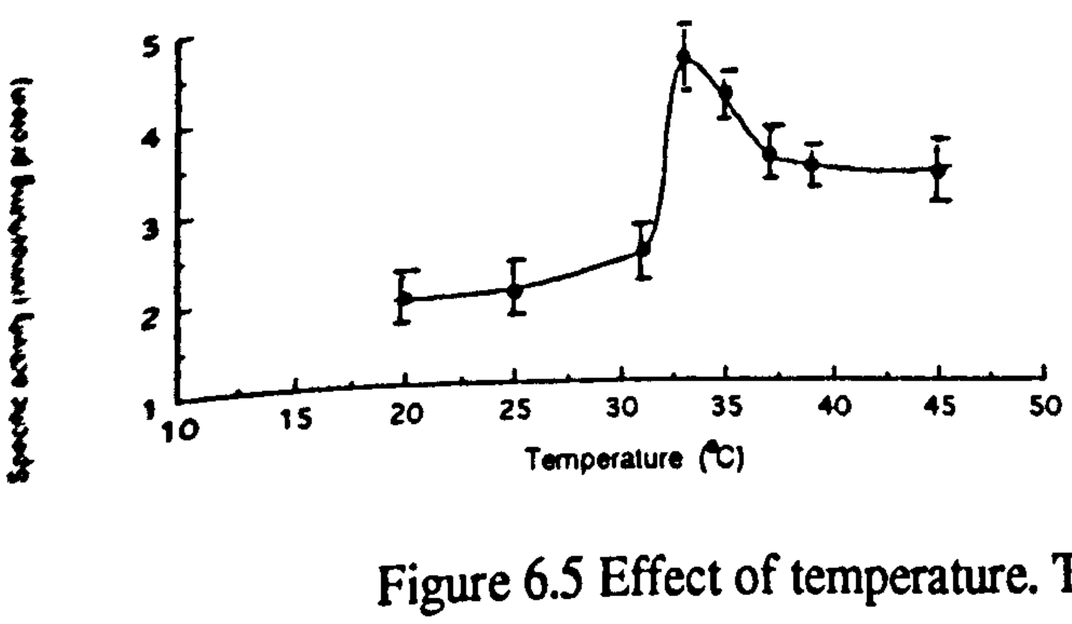
d) 3-Dehydroecdysone 3b-reductase (NADH)



e) 3-Dehydroecdysone 3β-reductase (NADPH)



f) Ecdysone 22-phosphotransferase



g) Ecdysone 2-phosphotransferase

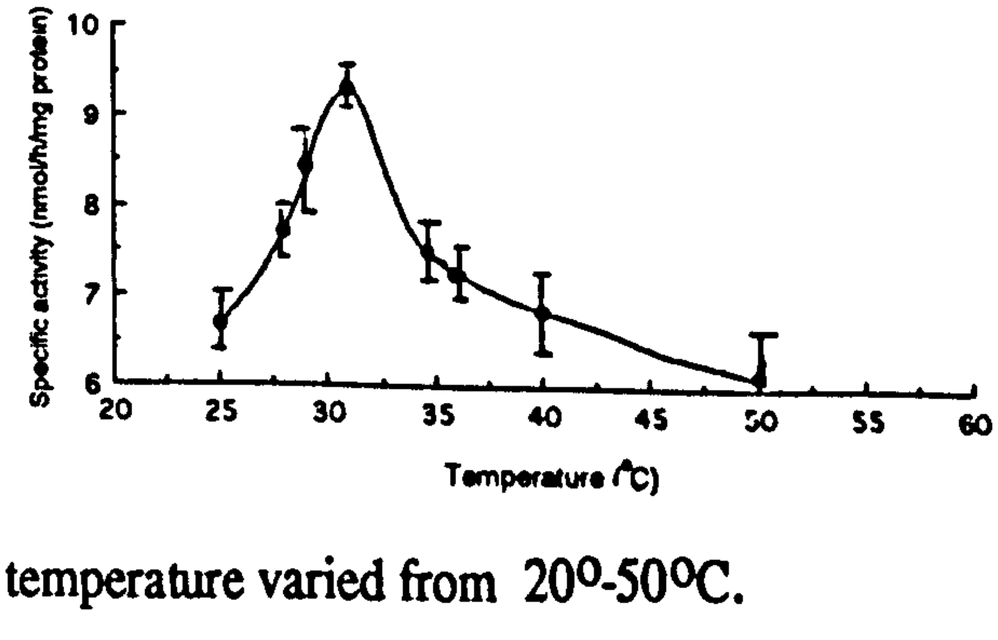
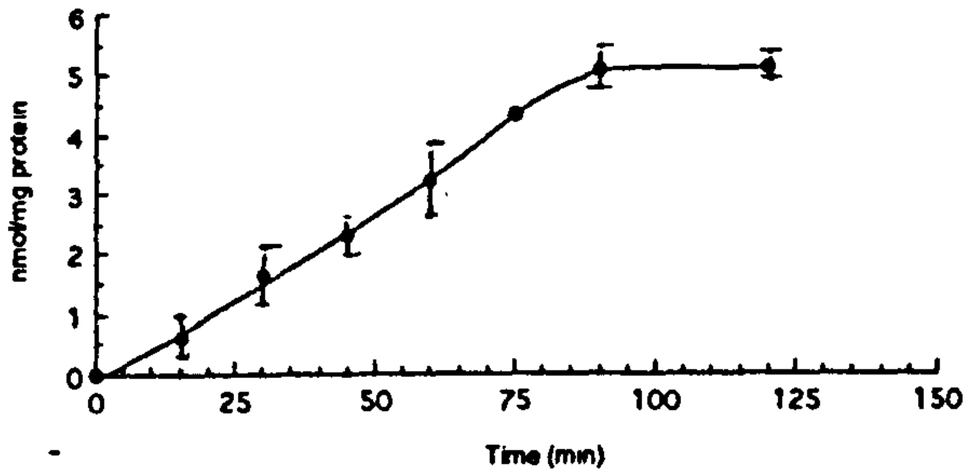
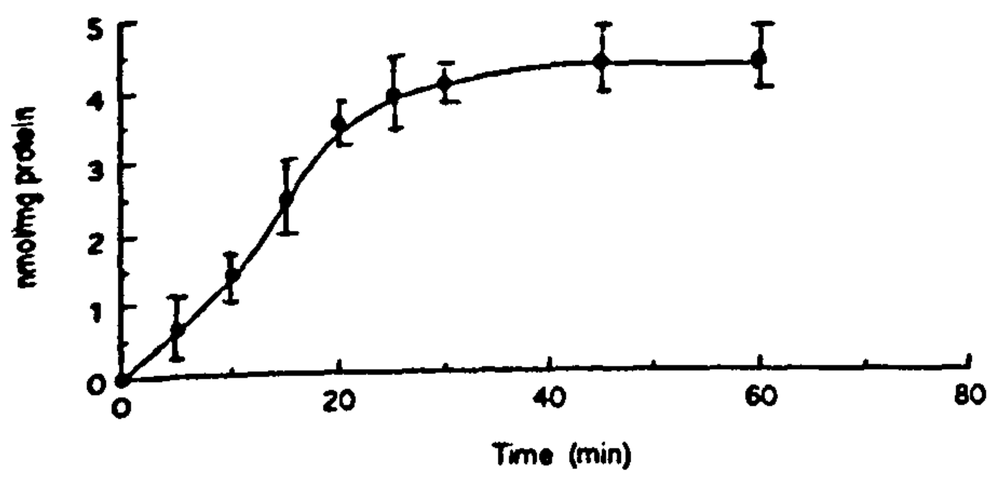


Figure 6.5 Effect of temperature. The incubation temperature varied from 20°-50°C. Points represent the mean of two determinations (± SD). Where bars are not shown, the SD is within the • symbol.

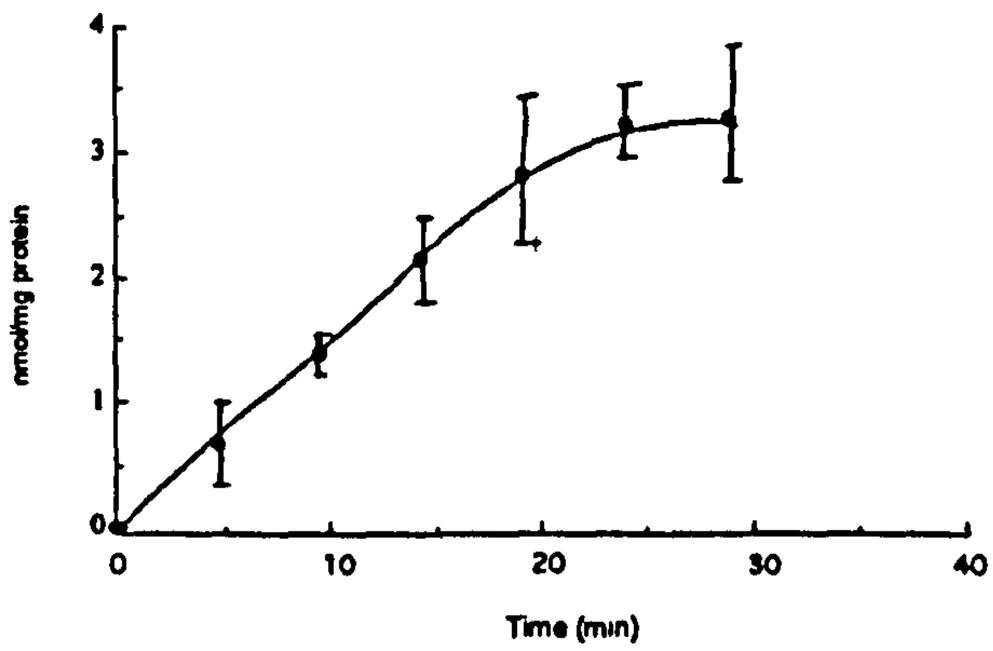
a) Ecdysone oxidase



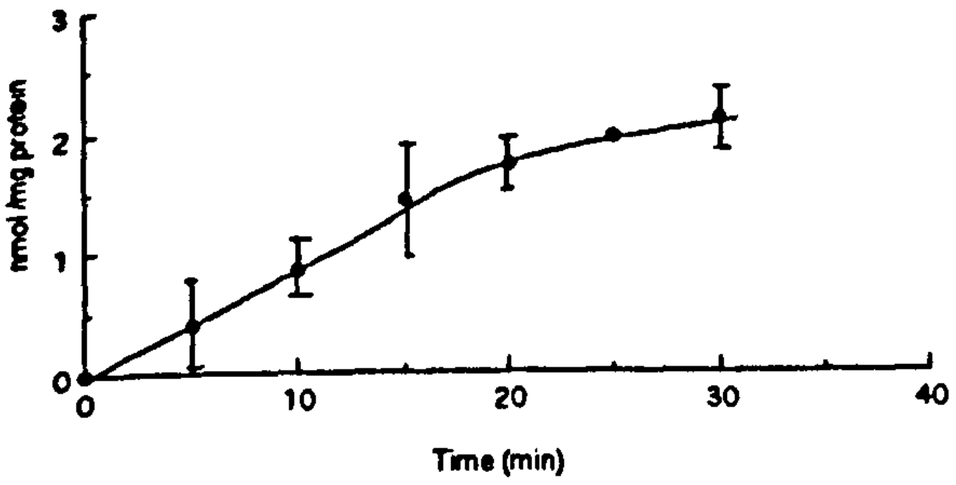
b) 3-Dehydroecdysone 3α-reductase (NADH)



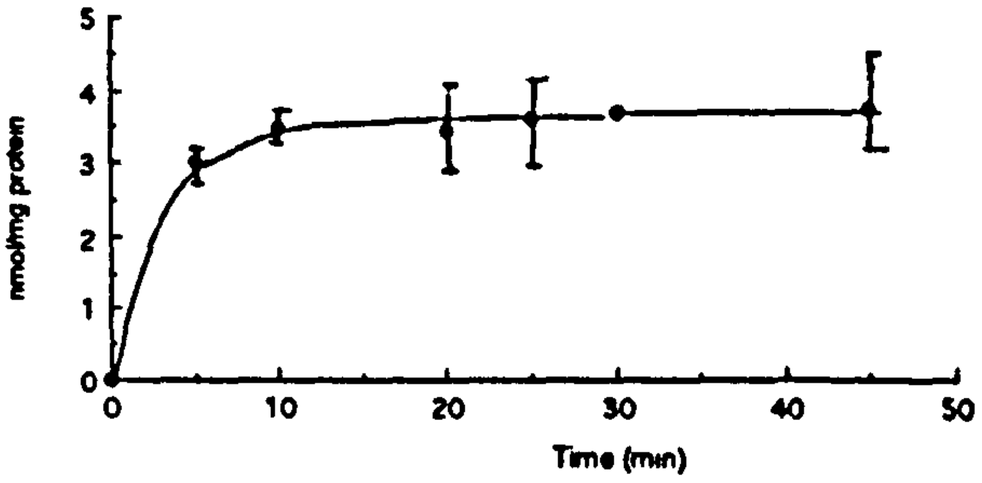
c) 3-Dehydroecdysone 3α-reductase (NADPH)



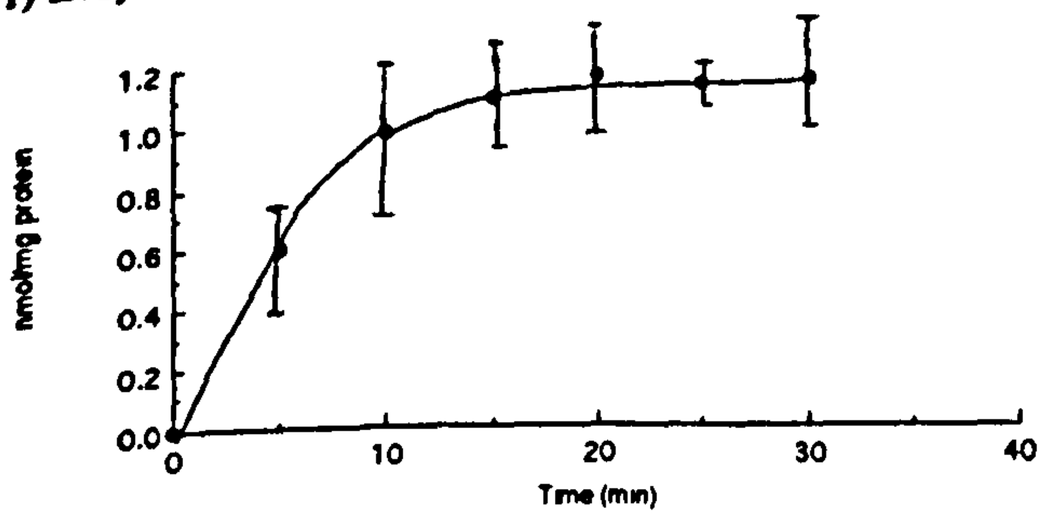
d) 3-Dehydroecdysone 3β-reductase (NADH)



d) 3-Dehydroecdysone 3β-reductase (NADPH)



f) Ecdysone 22-phosphotransferase



g) Ecdysone 2-phosphotransferase

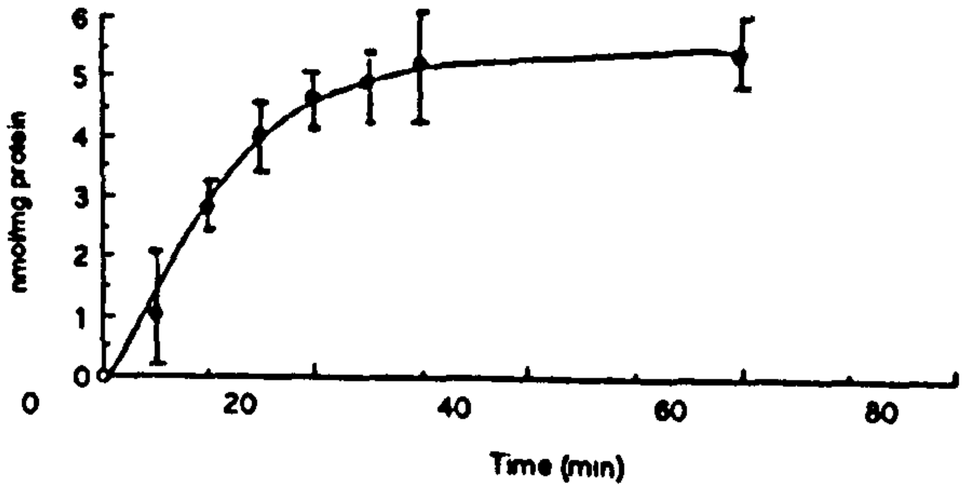


Figure 6.6 Effect of the time of incubation. Incubations were stopped by addition of methanol at timed intervals from 0-120min. Values are the mean of two investigations (±SD). Where bars are not shown, the SD is within the • symbol.

Table 1 Optimal reaction conditions for the midgut enzymes

Enzyme	Substrate	Cofactor in assay	max. protein[a] (mg/ml)	approx. pH	Temperature °C	max. linear reaction time (min)
Ecdysone oxidase	Ecdysone	O_2	1.7	6.5	40	90
3α-Reductase[b] (NADH)	3-Dehydroecdysone	0.5mM NADH	1.2	7.1	37	20
3α-Reductase[b] (NADPH)	3-Dehydroecdysone	0.5mM NADPH	0.1	7.5	35	20
3β-Reductase[c] (NADH)	3-Dehydroecdysone	0.5mM NADH	1.0	6.5	35	20
3β-Reductase[c] (NADPH)	3-Dehydroecdysone	0.5mM NADPH	0.5	7.9	37	<5
Ecdysone 22-phosphotransferase[d]	Ecdysone	10mM $MgCl_2$ 2mM ATP	0.7	7.8	33	10
Ecdysone 2-phosphtransferase[e]	Ecdysone	10mM $MgCl_2$ 2mM ATP	<0.5	7.6	33	15

[a] maximum concentration of protein in a dialysed cytosolic preparation of enzyme yielding linear rates of reaction

[b] full name: 3-dehydroecdysone 3 α-reductase

[c] full name: 3-dehydroecdysone 3 β-reductase

[d] full name: ATP:ecdysone 22-phosphotransferase

[e] full name: ATP:ecdysone 2-phosphotransferase

there is a difference in the optimal parameters, depending upon the cofactor used.

6.2.3 Fractionation of the Midgut Enzymes

A dialysed enzyme preparation from approximately 750 *S. littoralis* midguts (Section 2.2.3) was subjected to sequential column chromatography, specifically ion-exchange (DEAE-cellulose, followed by Mono-Q FPLC) and hydrophobic interaction chromatography (Phenyl Superose FPLC), as detailed in Section 2.2.5. Initially every other tube was assayed, but after location of enzymic activities, all relevant tubes were assayed. The appropriate tubes were then combined and desalted by dialysis in order to determine the enzymic activity accurately. Purification tables were constructed from the results (Tables 2a-e).

Fractionation by DEAE-cellulose (figure 6.7) partially resolved four peaks of enzyme activity, although there was appreciable overlapping of the oxidase and phosphotransferase activities (yielding ecdysone 2-phosphate). Unfortunately, no activity corresponding to the ecdysone 22- phosphotransferase was found, probably due to the effects of dilution and/or freeze-thawing.

The oxidase and phosphotransferase region were then separated into single peaks of activity by Mono-Q anion exchange FPLC, eluting at approximately 0.05M and 0.25M NaCl, respectively (figure 6.8a) and subsequent Phenyl Superose hydrophobic interaction (HIC) FPLC allowed further purification and determined the relative hydrophobicities of the proteins (figure 6.9 a and b). It is not surprising that the phosphotransferase, requiring the transfer of the highly polar

Table 2. Purification of enzymes.

a) Ecdysone oxidase

Stage	Protein (mg)	Total activity nmol/h	Specific activity nmol/h/mg	Yield (%)	Purification (fold)
Cytosol	968.6	2484	2.56	100	1.0
DEAE-cel	90.0	626.3	6.96	25.2	2.72
Mono-Q FPLC	2.0	20.2	10.1	0.81	3.94
Phenyl Superose FPLC	0.24	10.5	43.88	0.42	17.1

b) 3-Dehdyroecdysone3α-reductase (peak 1, from Mono-Q FPLC, assayed using NADPH)

Stage	Protein (mg)	Total activity nmol/h	Specific activity nmol/h/mg	Yield (%)	Purification (fold)
Cytosol	968.6	2400	2.48	100	1.0
DEAE-cel	67.2	506.8	7.54	21.1	3.04
Mono-Q FPLC	2.8	20.4	7.29	0.85	2.94
Phenyl Superose FPLC	0.23	15.1	65.6	0.63	26.5

c) 3-Dehydroecdysone 3α-reductase (peak 2, from Mono-Q FPLC, assayed using NADPH)

Stage	Protein (mg)	Total activity nmol/h	Specific activity nmol/h/mg	Yield (%)	Purification (fold)
Cytosol	968.6	2400	2.48	100	1.0
DEAE-cel	67.2	506.8	7.54	21.1	3.04
Mono-Q FPLC	3.6	22.8	6.33	0.95	2.55
Phenyl Superose FPLC	0.30	11.4	38.0	0.48	15.3

Table 2 continued. Purification of enzymes

d) 3-Dehydroecdysone 3β-reductase (peak 1, from Mono-Q FPLC, assayed using NADH)

Stage	Protein (mg)	Total activity nmol/h	Specific activity nmol/h/mg	Yield (%)	Purification (fold)
Cytosol	968.6	1999	2.06	100	1.0
DEAE-cel	78.8	596.3	7.57	29.8	3.67
Mono-Q FPLC	1.8	21.35	11.86	1.07	5.76
Phenyl Superose FPLC	0.24	8.6	35.83	0.4	17.39

e) 3-Dehydroecdysone 3β-reductase (peak 2, from Mono-Q FPLC, assayed using NADH)

Stage	Protein (mg)	Total activity nmol/h	Specific activity nmol/h/mg	Yield (%)	Purification (fold)
Cytosol	968.6	1999	2.06	100	1.0
DEAE-cel	78.8	596.3	7.57	29.8	3.67
Mono-Q FPLC	1.0	8.7	8.7	0.44	4.18
Phenyl Superose FPLC	0.39	5.4	18.0	0.25	8.65

f) Ecdysone 2-phosphotransferase

Stage	Protein (mg)	Total activity nmol/h	Specific activity nmol/h/mg	Yield (%)	Purification (fold)
Cytosol	968.6	2484	2.56	100	1.0
DEAE-cel	90.0	626.3	6.96	25.2	2.72
Mono-Q FPLC	1.8	15.6	8.67	0.62	3.39
Phenyl Superose FPLC	0.28	14.1	50.35	0.57	19.7

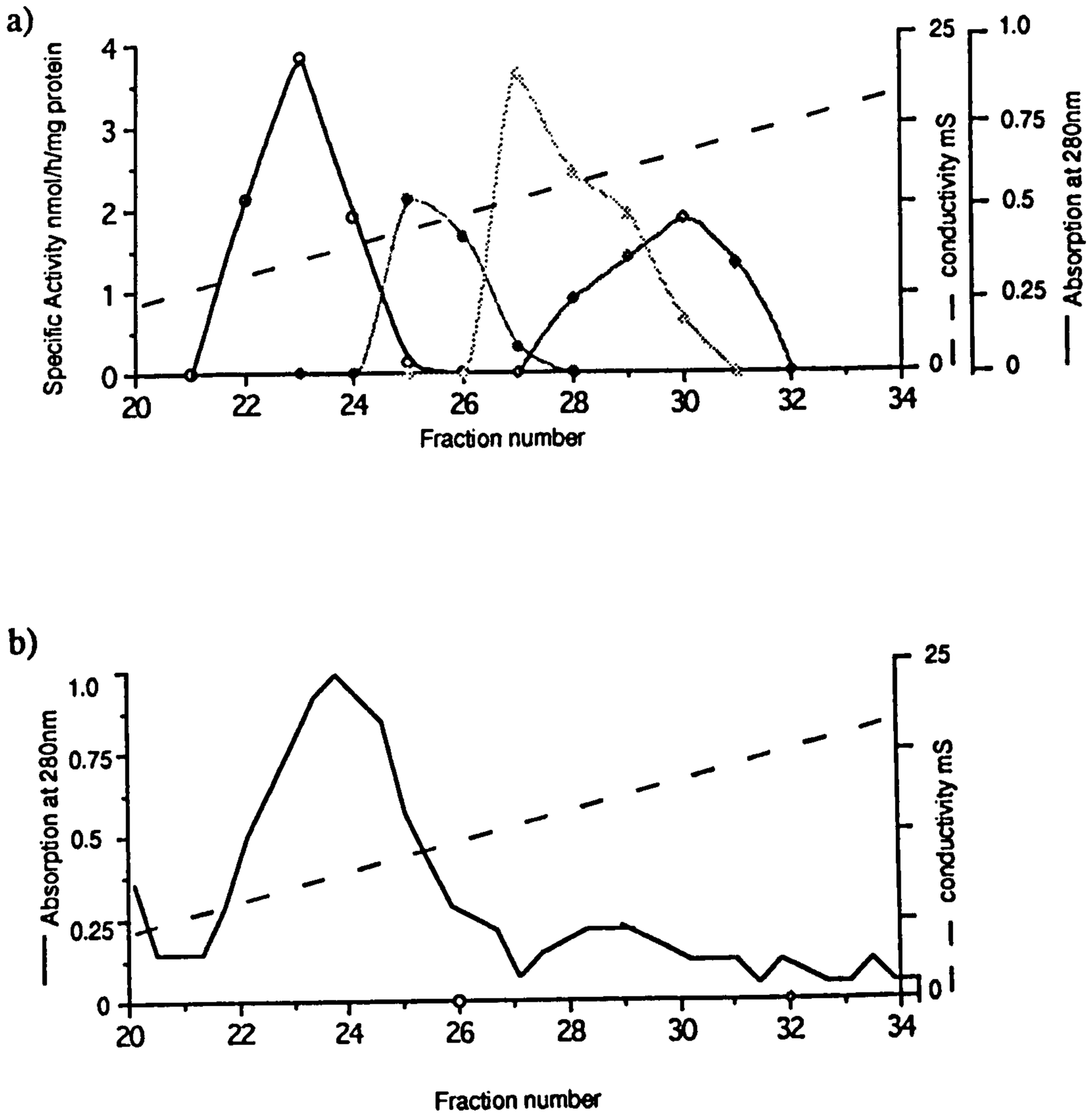


Figure 6.7.Elution profile of dailysed midgut cytosol on DEAE-cellulose using an eluent of 10mM Tris-HCl, pH 7.4, with a 0-0.5M NaCl linear gradient (440ml total) and collection of 8.0ml fractions. a)Enzymes eluted in the sequence: 3-dehydroecdysone 3α-reductase (○), 3-dehydroecdysone 3β-reductase (●), ecdysone oxidase (◁)and ecdysone 2-phosphotransferase (▵). Activity was estimated prior to the removal of salt from the fractions. b) Protein was monitored by absorption at 280nm.

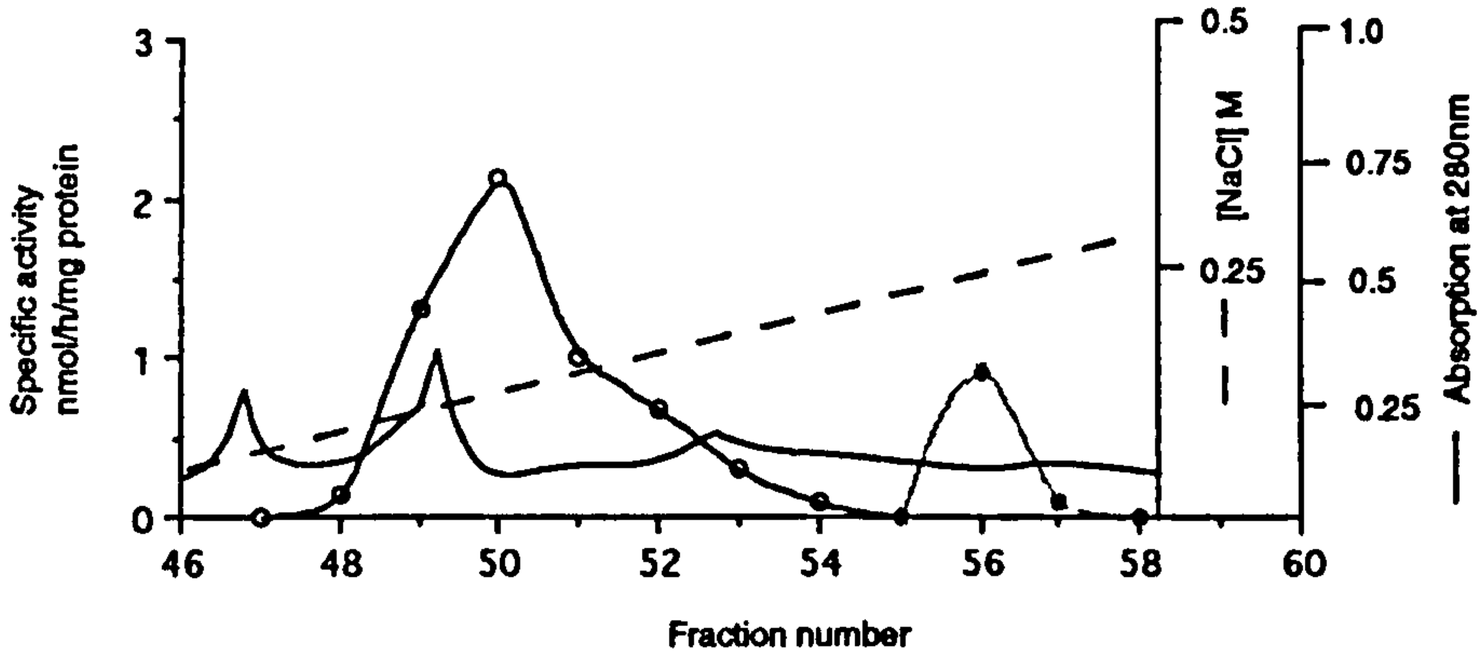


Figure 6.8a. Elution profile on Mono-Q FPLC of ecdysone oxidase (∘) and ecdysone 2-phosphotransferase (•) fractions 27-31 from DEAE-cellulose chromatography. A linear gradient of 0-0.5M NaCl in 10mM Tris-HCl (60ml) was employed and 0.5ml fractions were collected. Enzymic activity was estimated prior to removal of salt from the fractions.

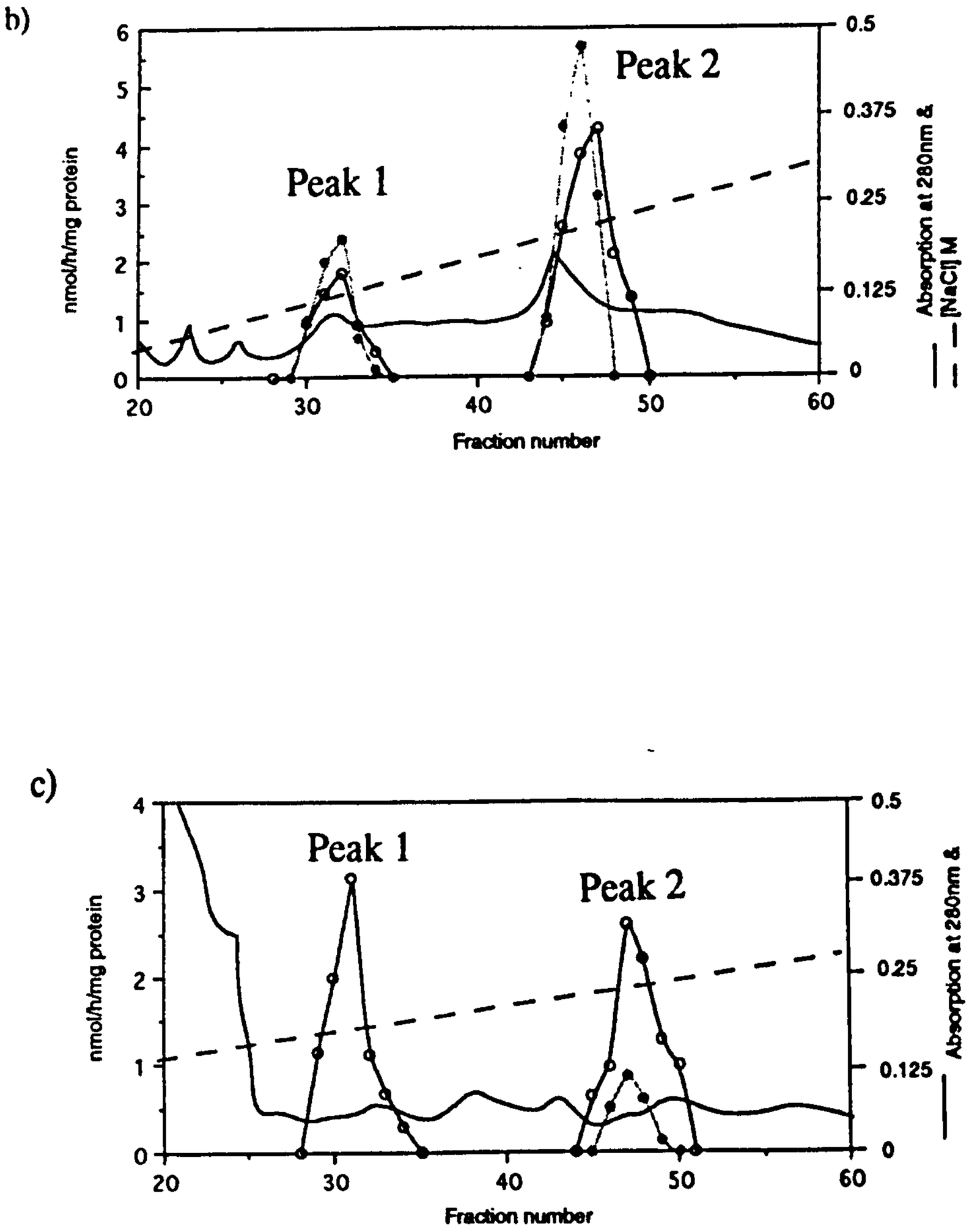


Figure 6.8. Elution profile on Mono-Q FPLC of the appropriate DEAE-cellulose fractions of b) 3-dehydroecdysone 3α-reductase (fractions 22-24) and c) 3-dehydroecdysone 3β-reductase (fractions 25-26). The enzymes were eluted using a 0-0.5M NaCl gradient in 10mM Tris-HCl, over 60ml and 0.5ml fractions were collected. Fractions were assayed using 0.5mM NADH (∘) and 0.5mM NADPH (·). Enzymic activity was assayed prior to the removal of salt from the fractions.

phosphate group, has more exposed hydrophilic residues and elutes at higher (NH4)2SO4 concentrations than the other enzymes of interest.

The 3-dehydroecdysone 3α- and 3β-reductases were adequately separated by DEAE (figure 6.7), and individually applied to Mono-Q FPLC (figures 6.8 b and c). Fractions were tested for activity using 3-dehydroecdysone and either NADH or NADPH; minor activities [3-dehydroecdysone 3α-reductase (NADH) and 3β-reductase (NADPH)] were not detected by DEAE-cellulose. It is interesting to note, however, that at least two 3α-hydroxy-forming reductases and two 3β-hydroxy-forming reductases were resolved by Mono-Q FPLC (figure 6.8b and c).

The elution profile for the 3-dehydroecdysone 3α-reductase from Mono-Q FPLC revealed two peaks of activity when the fractions were assayed using NADH and two when NADPH was used, the first peak in each case were coincident. The first peak (NADH and NADPH) eluted at approximately 0.12M NaCl, showed slightly more activity with NADPH than NADH. The second peak, eluting at approximately 0.2M salt was NADPH-dependent. This was poorly resolved from what could be considered to be peak 3, which almost coelutes with peak 2, and is NADH-dependent. Slightly higher activity was observed in the NADPH-dependent enzymes; this contrasts with crude dialysed post-microsomal preparations, whereby the preference for NADPH was much stronger. By pooling the fractions of peaks 2 and 3, and applying to a Phenyl Superose column, only one peak was present (figure 6.9d). Similarly, only a single peak was observed for peak 1 after Phenyl Superose FPLC (figure 6.9c).

The 3-dehydroecdysone 3β-reductase fractions from Mono-Q revealed two peaks having NADH preference (eluting at 0.16M and 0.25M NaCl) and one peak being NADPH-dependent, the latter co-

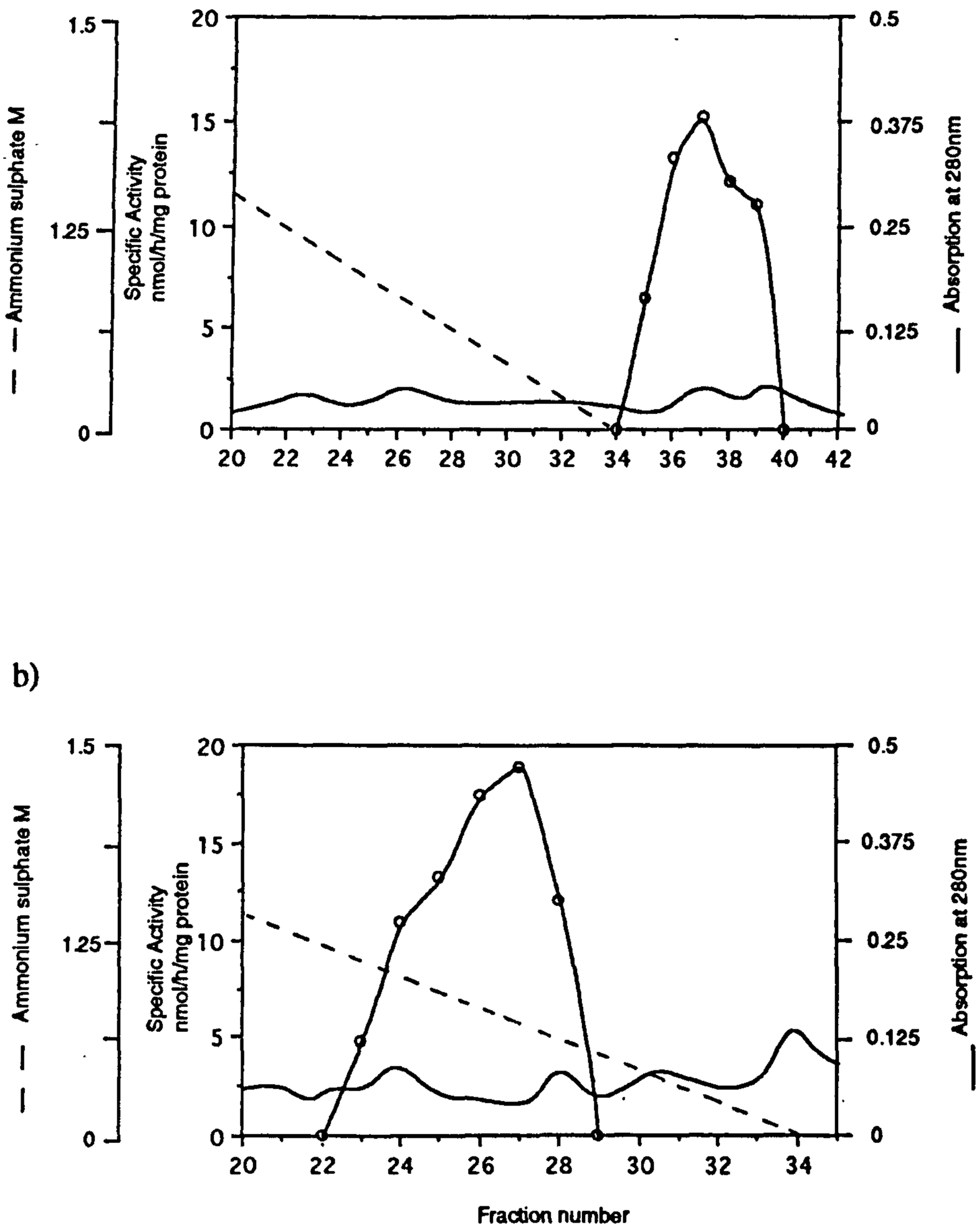


Figure 6.9. Elution pattern of the appropriate Mono-Q FPLC fractions [for (a) fractions 49-52; for (b) fractions 56-57] applied to a Phenyl Superose FPLC column of a) ecdysone oxidase, b) ecdysone 2-phosphotransferase. Elution was by a linear 17ml gradient of buffer C against 10mM sodium phosphate buffer, pH 7.5, at approximately 0.5ml/min, with collection of 0.5ml fractions. Enzymic activity was estimated before removal of ammonium sulphate from the fractions; protein was estimated by absorption at 280nm.

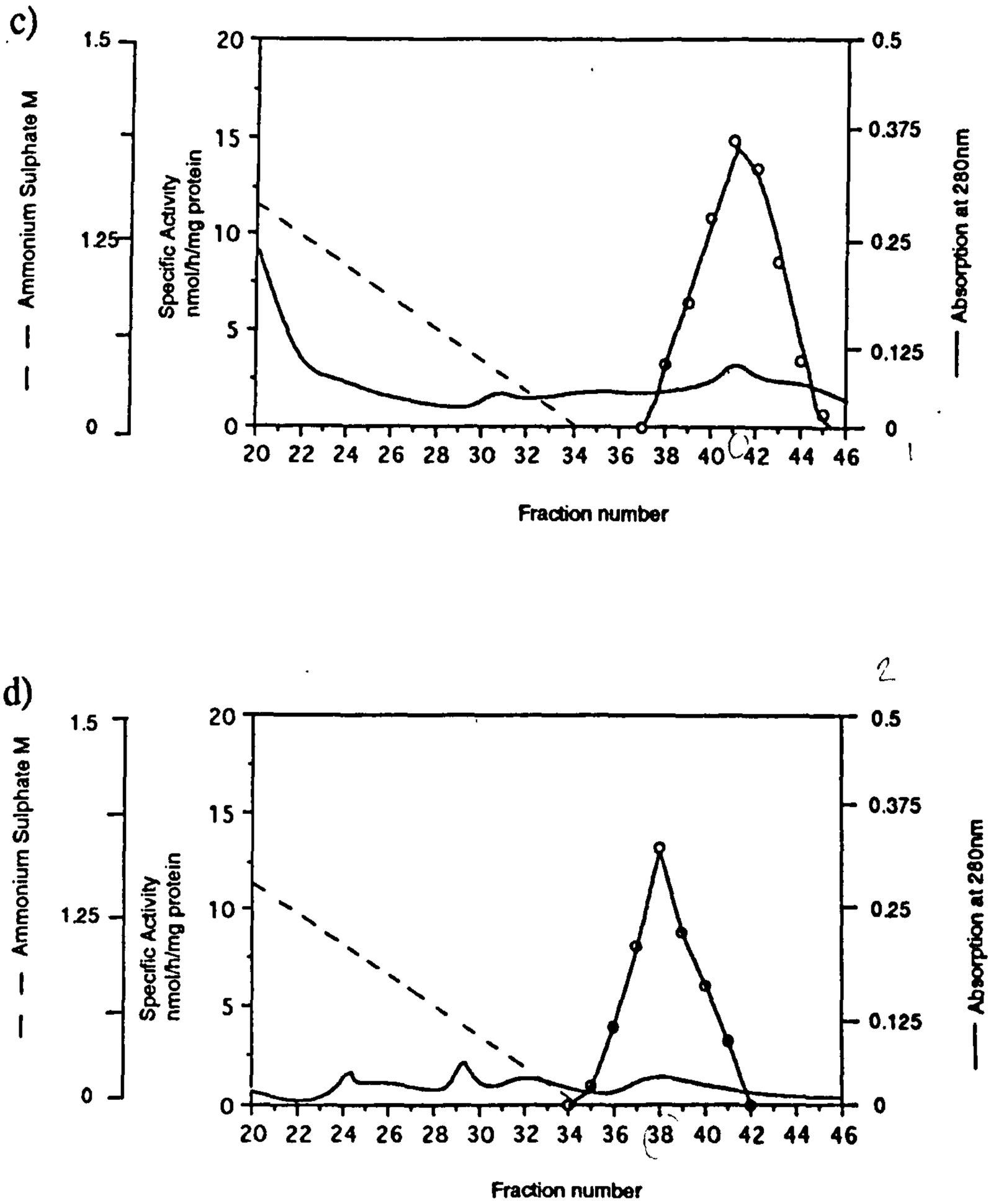
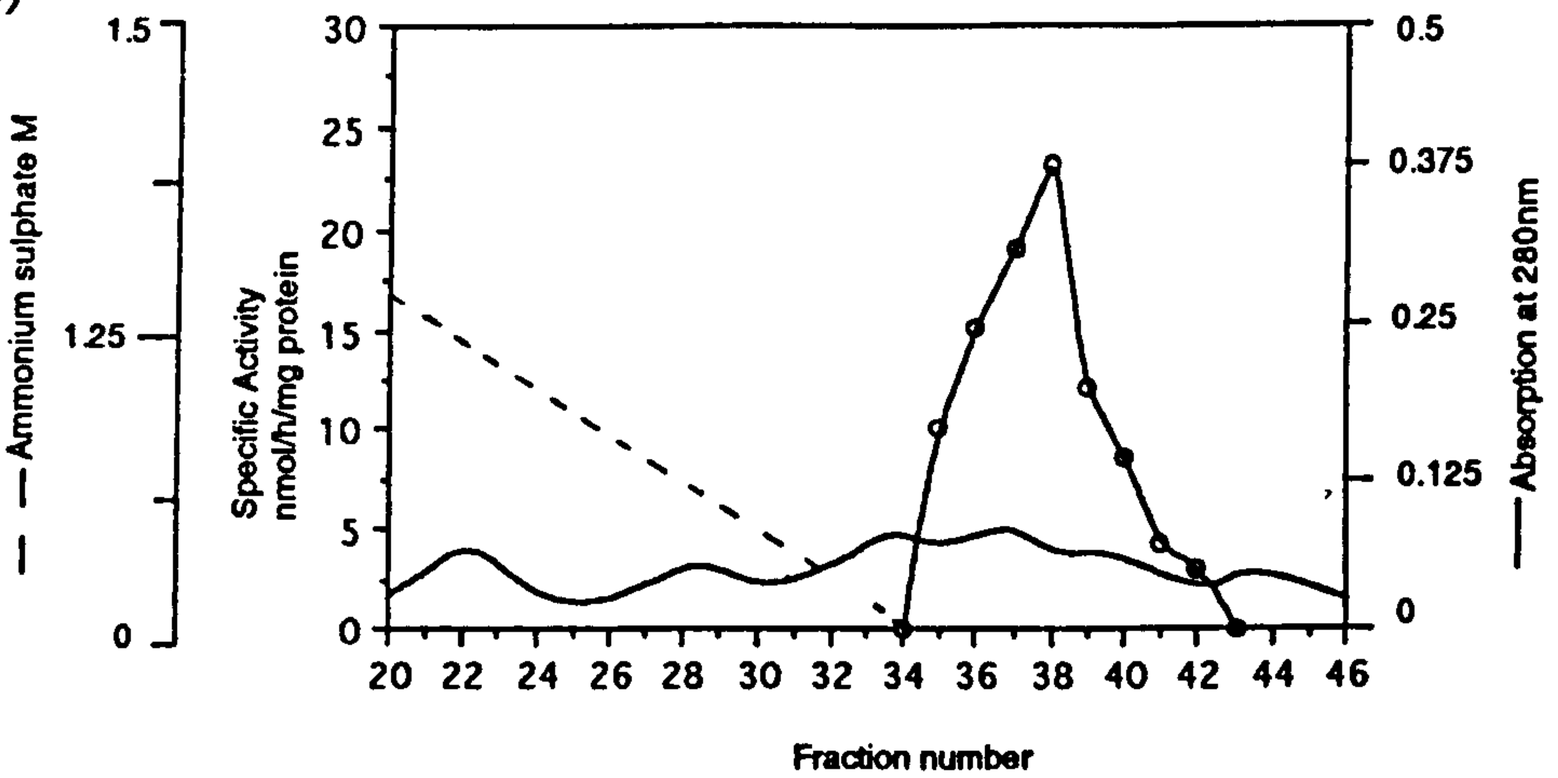


Figure 6.9. Elution profile on Phenyl Superose FPLC of c) 3-dehydroecdysone 3α-reductase [peak 1, from figure 6.8b (fractions 31-34] assayed using NADPH and d) 3-dehydroecdysone 3α-reductase [peak 2, from figure 6.8b (fractions44-49)] assayed using NADPH.

e)



f)

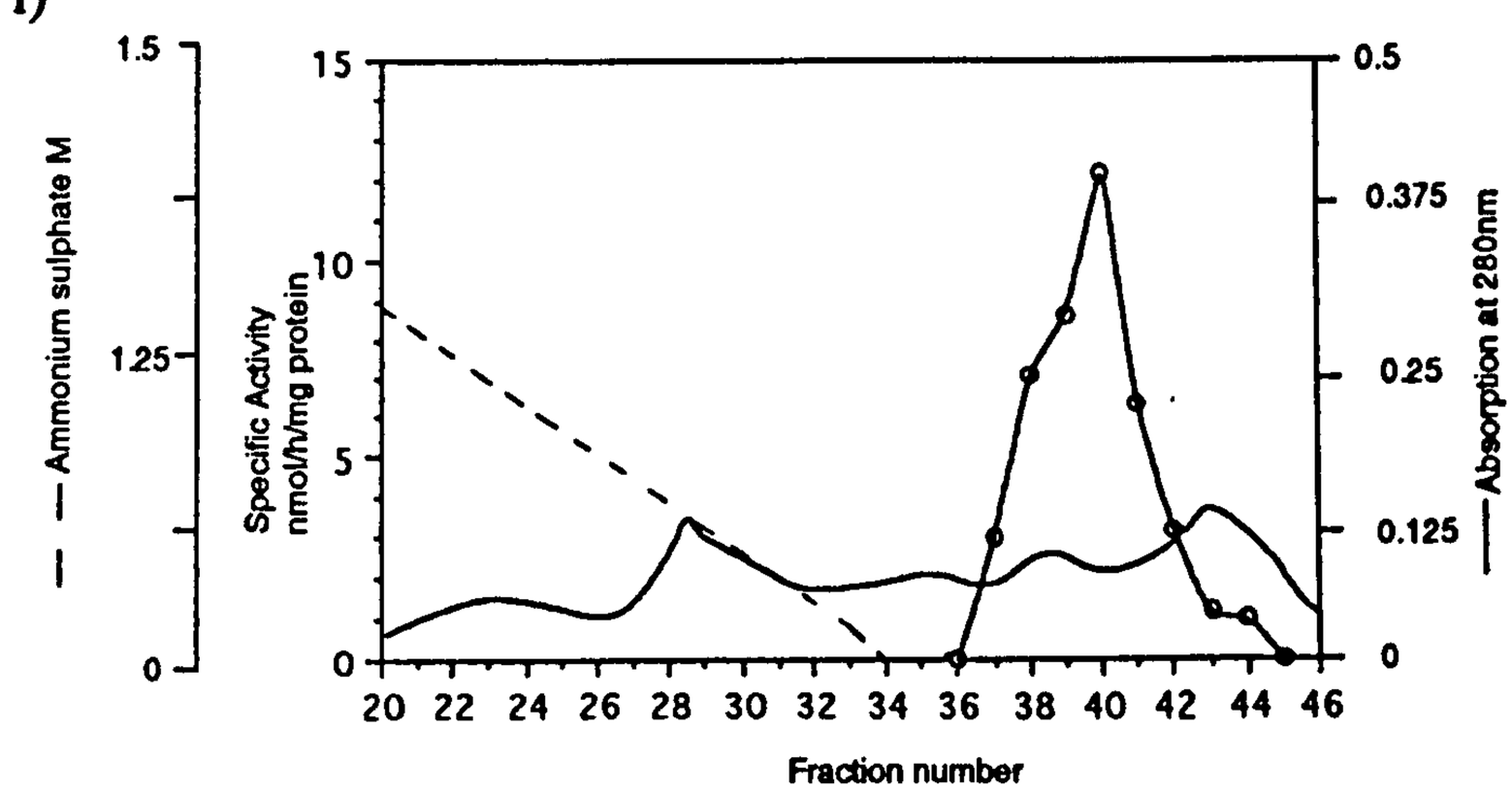


Figure 6.9. Elution profile on Phenyl Superose FPLC of e) 3-dehydroecdysone 3β-reductase [peak 1, from figure 6.8c (fractions 30-35)] assayed using NADH and f) 3-dehydroecdysone 3β-reductase [peak 2, from figure 6.8c (fractions 45-50)] assayed using NADH).

eluting with the second NADH-dependent peak. Interestingly, the 3-dehydroecdysone 3β-reductases maintain their strong preference for NADH, as in the dialysed crude cytosolic preparations.

The relative hydrophobicity of the enzymes was determined by their elution from Phenyl Superose FPLC (figures 6.9 c-f). The 3α-reductase (peak 2 from Mono-Q FPLC; figure 6.9d) and the 3β-reductase (peak 1 from Mono-Q FPLC; figure 6.9e) had highest specific activities in fraction 38, whereas the 3β-reductase (peak 2 from Mono-Q FPLC; figure 6.9f) had highest specific activity in fraction 40 and the 3α-reductase (peak 1 from Mono-Q FPLC; figure 6.9c) in fraction 41. Hence, there may be slight differences in the hydrophobicities of the exposed portion of the proteins. In contrast, the oxidase had a high specific activity in tube 37 and, therefore, is very slightly more hydrophilic (figure 6.9a). The phosphotransferase was appreciably more hydrophilic than the other enzymes, since it had a peak specific activity in fraction 27 (figure 6.9b).

The purification data for each enzyme are shown in tables 2a-e. SDS-polyacrylamide gels at various stages of purification are shown in figures 6.10 a-e. SDS-polyacrylamide gels of the enzymes were used to analyse qualitatively the degree of purification of the enzymes. Gels were originally stained with Coomassie Blue R in the case of the cytosol, DEAE-cellulose and Mono-Q fractions, but since little protein was recovered from the Phenyl Superose, the gels were silver stained (Section 2.2.7).

The gels were found to have the following features: a) ecdysone oxidase: several bands were observed in the Phenyl Superose lane, including two major polypeptides of molecular weight 54.3kDa and 12.9kDa. Other minor bands were evident, and, hence, further purification and electrophoresis (e.g. native PAGE) of the enzyme is

Figure 6.10. SDS-polyacrylamide gels of a) ecdysone oxidase and b) ecdysone 2-phosphotransferase, at each stage of their purification. The positions and molecular weights (kDa) of major polypeptide bands are indicated. The mobilities of a range of standard molecular weight markers are shown for each gel: ovtransferrin 76-78kDa; bovine serum albumin 66-68kDa; ovalbumin 45kDa; carbonic anhydrase 30kDa; myoglobin 17kDa; cytochrome c 12kDa. Gels were silver stained and photographed the same day.

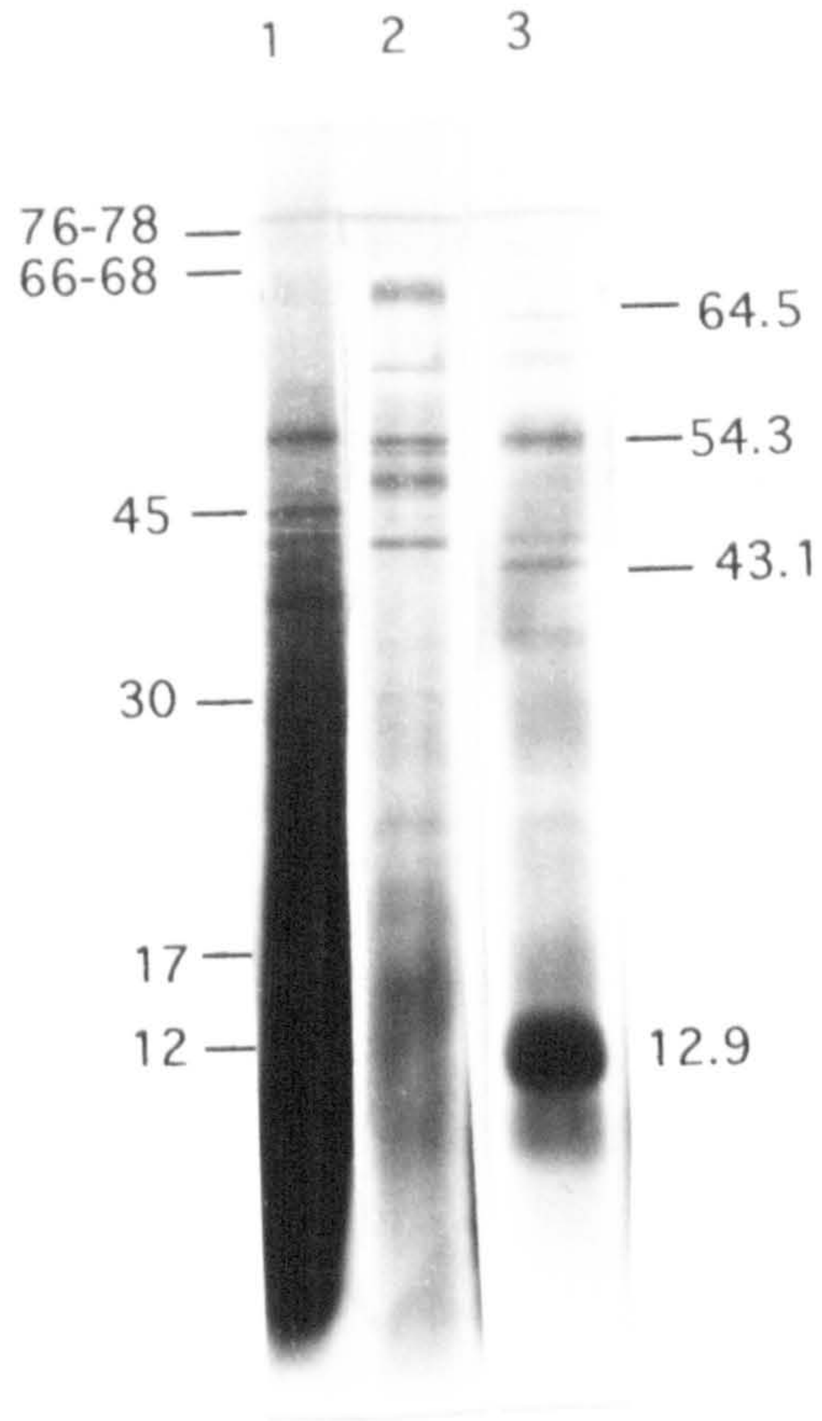
a)

Lane 1 ecdysone oxidase from DEAE-cellulose chromatography
Lane 2 ecdysone oxidase from Mono-Q chromatography
Lane 3 ecdysone oxidase from Phenyl Superose chromatography

b)

Lane 1 total midgut cytosol protein
Lane 2 ecdysone 2-phosphotransferase from DEAE-cellulose chromatography
Lane 3 ecdysone 2-phosphotransferase from Mono-Q chromatography
Lane 4 ecdysone 2-phosphotransferase Phenyl Superose FPLC, fractions
Lane 5 ecdysone 2-phosphotransferase Phenyl Superose FPLC, fractions (repeat of lane 4)

a)


1
2
3
76-78
66-68
45
30
17
12
64.5
54.3
43.1
12.9

b)

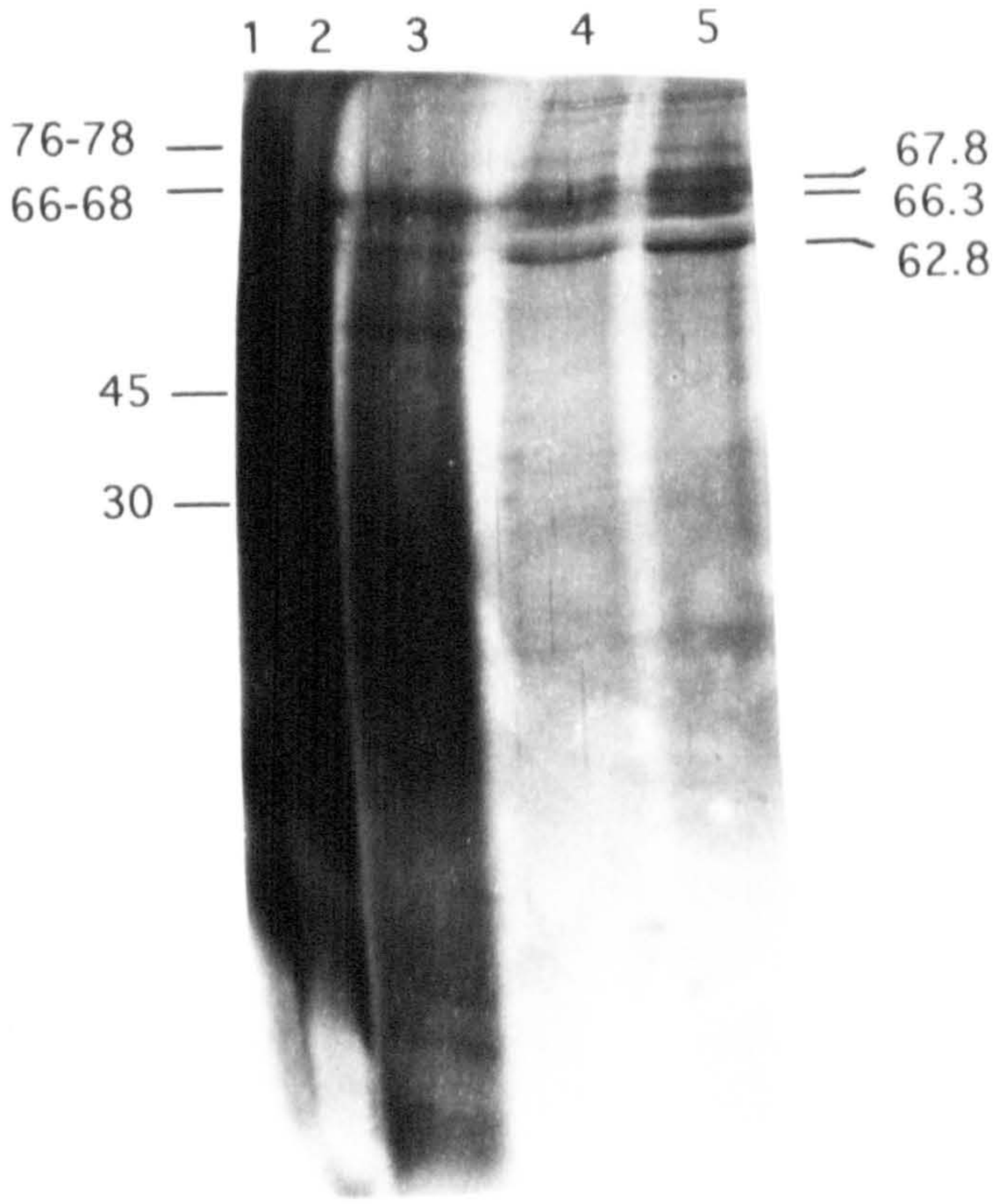

1
2
3
4
5
76-78
66-68
45
30
67.8
66.3
62.8

Figure 6.10. SDS-polyacrylamide gels of c) 3-dehydroecdysone 3β-reductase and b) 3-dehydroecdysone 3α-reductase, at each stage of their purification. The positions and molecular weights (kDa) of major polypeptide bands are indicated. The mobilities of a range of standard molecular weight markers are shown for each gel: ovtransferrin 76-78kDa; bovine serum albumin 66-68kDa; ovalbumin 45kDa; carbonic anhydrase 30kDa; myoglobin 17kDa; cytochrome c 12kDa. Gels were silver stained and photographed the same day.

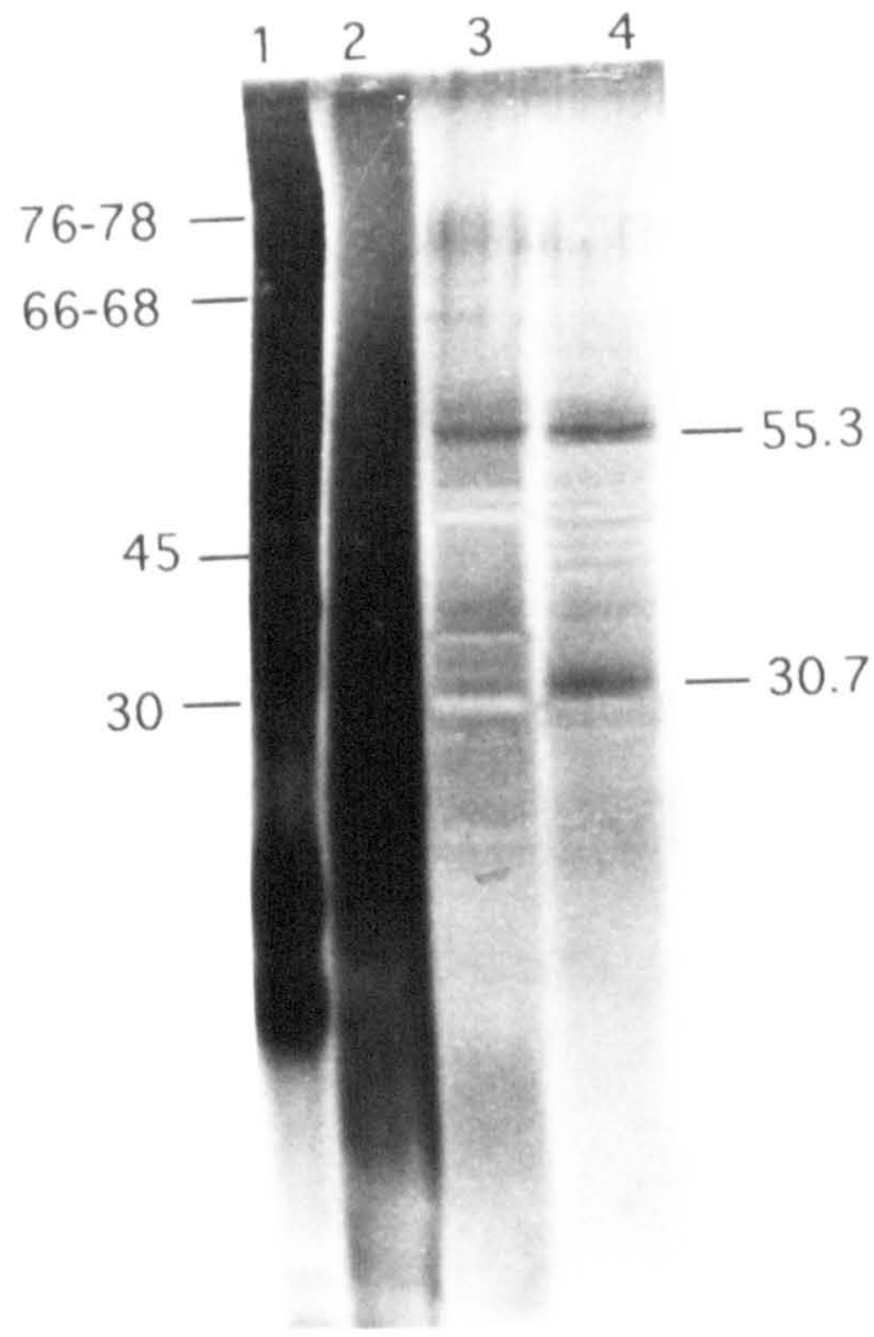
c)

Lane 1	3α-reductase from DEAE-cellulose chromatography
Lane 2	3α-reductase from Mono-Q chromatography
Lane 3	3α-reductase from Phenyl Superose chromatography "peak 1"
Lane 4	3α-reductase from Phenyl Superose chromatography "peak 2"

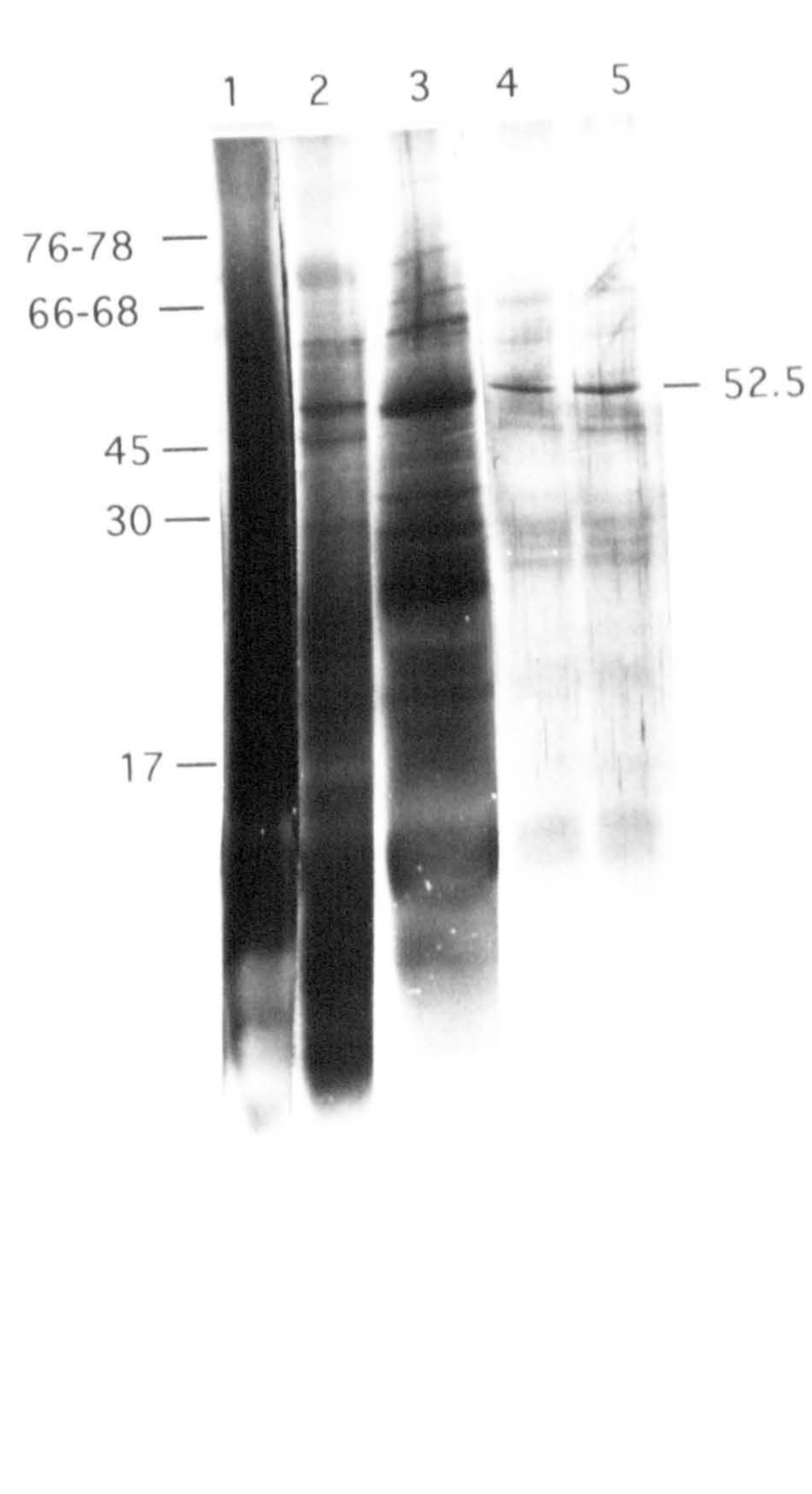
d)

Lane 1	Midgut cytosol
Lane 2	3β-reductase from DEAE-cellulose chromatography
Lane 3	3β-reductase from Mono-Q chromatography
Lane 4	3β-reductase from Phenyl Superose chromatography "peak 1"
Lane 5	3β-reductase from Phenyl Superose chromatography "peak 2"

c)



d)



required, to deduce whether these represent subunits or impurities; b) ecdysone 2-phosphotransferase: a main band of 62.8kDa was observed. Minor bands of higher molecular weight were also evident (between 66.3-67.8kDa); c) 3-dehydroecdysone 3α-reductase: both peaks 1 and 2 (from figure 6.8a, Mono-Q FPLC) after Phenyl Superose chromatography had a major band of 55.3kDa and several minor bands. Similarly, peak 2 (from figure 6.8a) Phenyl Superose fractions also had a major band of 55.3kDa. Additionally, a band of 30.7kDa was observed in peak 2 fractions; d) 3-dehydroecdysone 3β-reductase (from figure 6.8b, Mono-Q FPLC) after Phenyl Superose FPLC had a major band of 52.5kDa and contained some impurities of lower intensity staining.

6.2.4 Effect of Salt Concentration on Enzyme Activity

During the fractionation of the enzymes by column chromatography, NaCl gradients were used in ion-exchange, and the proteins tended to be eluted when the concentration of salt was moderately high. To examine whether or not the salt affects the enzyme activities final salt concentrations of 0.1M and 0.2M NaCl were added to the dialysed post-microsomal enzyme preparation prior to incubation with substrate and cofactor(s), using the standard assays according to Section 2.2.4 and this Chapter.

Sodium chloride was shown to have a profound effect on enzymic activity. Final NaCl concentrations of 0.1M and 0.2M were chosen by consideration of the salt concentrations as the enzymes elute from the ion-exchange columns. Generally enzyme activity was reduced (Table 3), when either salt concentration was used. The 3-dehydroecdysone 3α-reductase was largely unaffected, whereas the corresponding NADPH-activity was reduced by nearly half at either concentration. In stark

Table 3. The effect of sodium chloride on enzymic activity in dialysed cytosol (final column) and after re-dialysis to remove the salt.

	RELATIVE ACTIVITIES (%)			
Enzyme	No salt added	0.1M NaCl	0.2M NaCl	0.2M salt added; enzyme dialysed
Ecdysone oxidase	100	38.5	29.1	93.2
3α-Reductase (NADH)	100	97.1	88.3	88.3
3α-Reductase (NADPH)	100	55.7	53.1	99.5
3β-Reductase (NADH)	100	45.9	30.0	111
3β-Reductase (NADPH)	100	310	326	118
Ecdysone 22-phosphotransferase	100	50.5	76.7	108
Ecdysone 2-phosphotransferase	100	58.7	50.0	94.2

contrast, the 3-dehydroecdysone 3β-reductase (NADPH) underwent an activation which increased the activity by 3-fold using 0.1M and 3.3-fold with 0.2M NaCl.

It would seem that the protein has undergone a change of conformation, which may alter the active centre of the enzyme molecule and result in a change both in cofactor preference, and, at the same time, strongly increase the overall activity (Table 3). Previous assays (Section 5.2.1) in the absence of salt showed that the 3β-reductase had a strong preference for NADH over NADPH. Similar effects were observed in *Manduca sexta* 3α-reductase enzymes (Weirich et al., 1991). To confirm these were reversible changes of conformation, incubations were carried out to investigate the reversibility of the loss in activity. For this, 0.2M salt was added, but the enzyme preparation was dialysed against 10mM Tris-HCl, pH7.4, for 18h, before assay (Table 3).

The results indicated that in all cases virtually all of the activity could be restored to the control values after dialysis; small depreciations in activity were noticed in some cases, when the dialysed enzyme was used However, this may have been due to residual salt, and not to any permanent modification of enzyme structure.

6.2.5 Kinetic Parameters

Enzymes were fractionated by DEAE-cellulose anion-exchange chromatography (see Section 2.2.5), fractions of highest activity were pooled (figure 6.7) and then dialysed to remove salt prior to kinetic analysis. Increasing substrate concentrations of 10-200μM were added to the reaction mixtures.

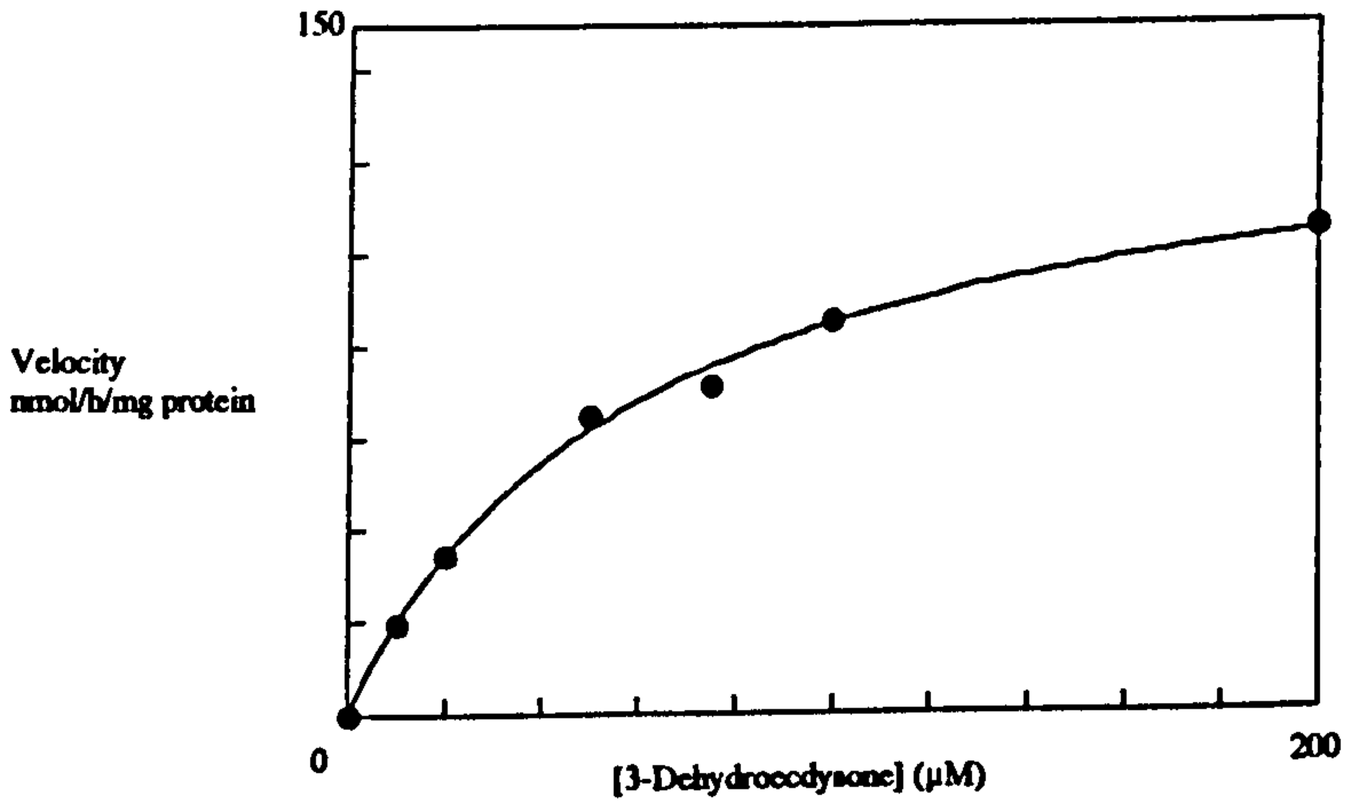
The apparent Km values were obtained using the Apple MacIntosh "Regression" computer program, which fits the best hyperbola by iteration, (figures 6.11 a-f; summary in Table 4). Except in the case of the K_m value for the 3α-reductase (NADH), the orders of magnitude of the epimerization enzymes were comparable to those described for the *Manduca sexta* enzymes, which were all in the range 10-30μM, except for the NADPH-dependent 3α-hydroxy-forming reductase, which had a value of 2μM (Weirich et al., 1989). For comparison, other reported K_m vales are: the ecdysone oxidase of *Calliphora vicina*, 31μM (Koolman and Karlson, 1975) and the enzymic inactivation of ecdysone through to 3-epiecdysone in *Manduca sexta*, 17.0 ±1.4μM (Mayer et al., 1979).

The lowest V_{max} value of the epimerization sequence was noted for the ecdysone oxidase, (33.1± 1.8nmol/h/mg). This result is in agreement with that obtained in *M. sexta,* whereby the ecdysone oxidase is the slowest enzyme of the pathway (Weirich et al., 1989). However, the NADH-dependent 3β-reductase also has comparatively low V_{max} (40.1± 0.9nmol/h/mg), and since this enzyme has previously been found to have a preference for NADH in crude dialysed cytosolic preparations, i.e it would have been thought to have a higher V_{max} than the activity measured using NADPH. In reality however, the 3β-reductase (NADPH) has the highest V_{max} of the sequence (199.7± 15.5nmol/h/mg). In the case of the 3α-hydroxy-forming reductases a similar situation is observed, the NADPH-dependent activity having a somewhat lower V_{max} than that of its NADH-dependent counter-part.

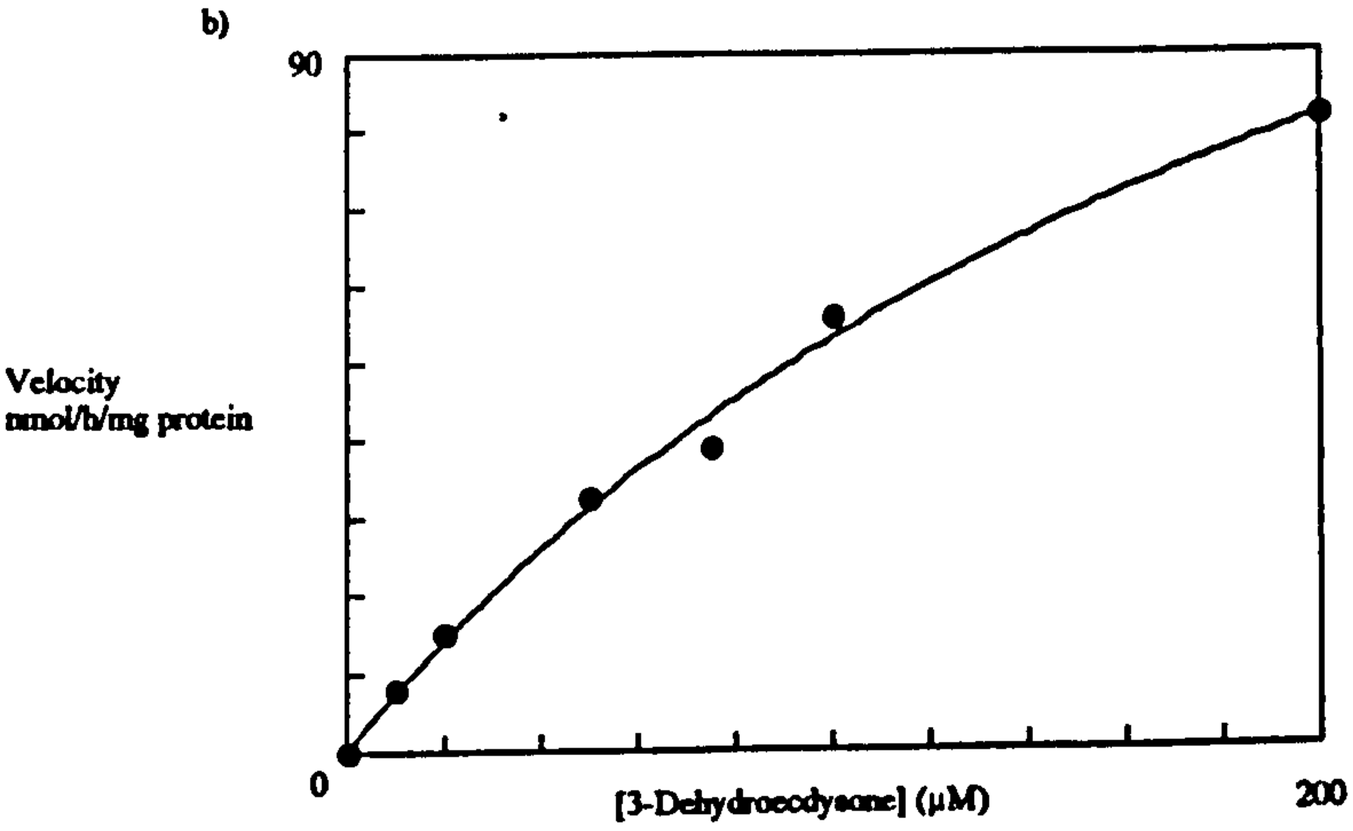
In contrast, the K_m(substrate)values reflect the cofactor preferences for *S. littoralis* in the midgut cytosol (Section 5.2.1). The 3β-hydroxy forming reductase utilising NADH has a lower K_m value than the same enzyme utilizing NADPH (24.7± 2.0μM compared with

Figure 6.11. Velocity *versus* substrate concentration plots for a) 3-dehydroecdysone 3α-reductase (NADH), b) 3-dehydroecdysone 3α-reductase (NADPH), c) 3-dehydroecdysone 3β-reductase (NADH), d) 3-dehydroecdysone 3β-reductase (NADPH), e) ecdysone oxidase and f) 2-phosphotransferase. Incubation mixtures contained the substrates ecdysone (e,f) or 3-dehydroecdysone (a-d), 0.5mM NADH (a,c) or 0.5mM NADPH (b,d) and semi-purified dialysed enzymes from the appropriate DEAE-cellulose fractions. Assays were carried out at using the optimal conditions described in Table 1.

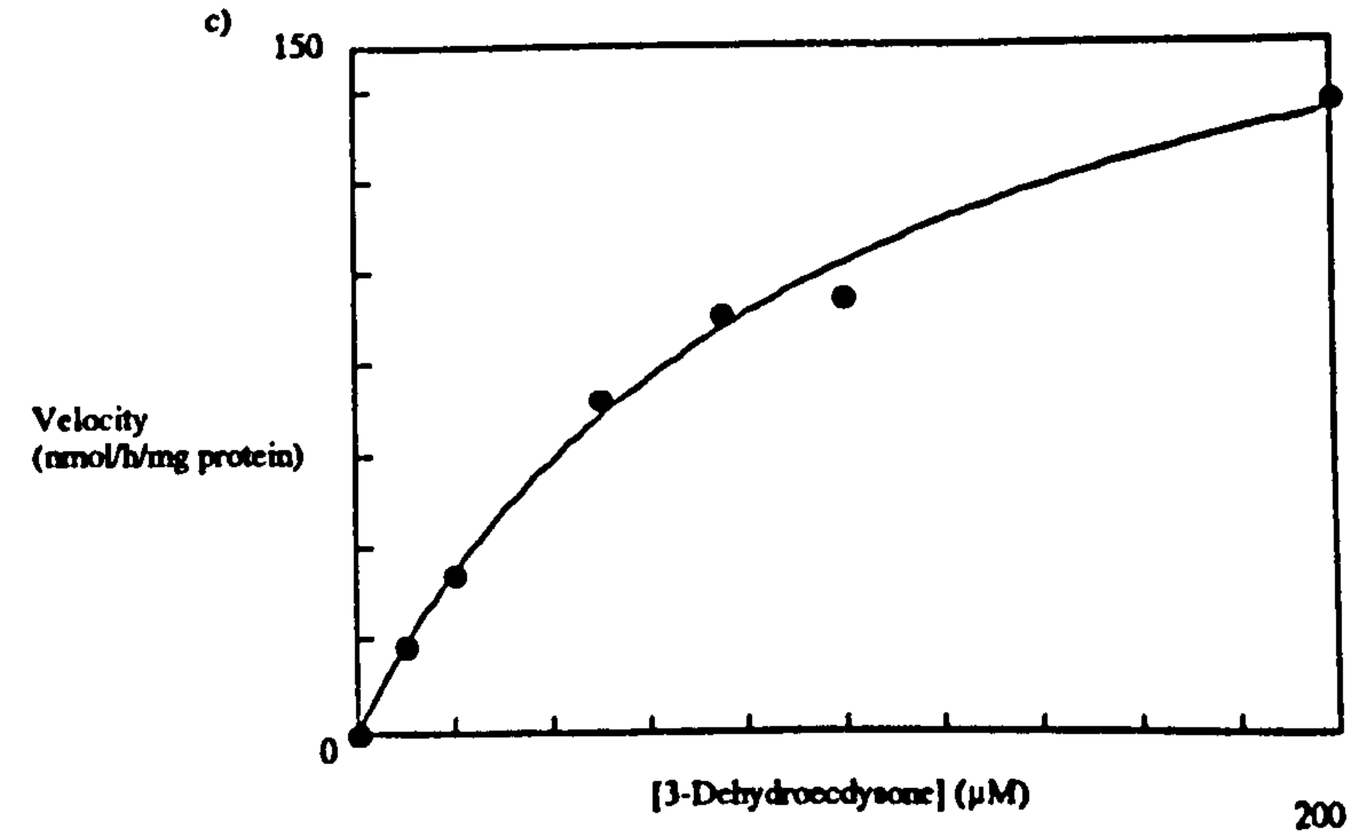
a) –


150
Velocity
nmol/h/mg protein
0
200
[3-Dehydroecdysone] (μM)

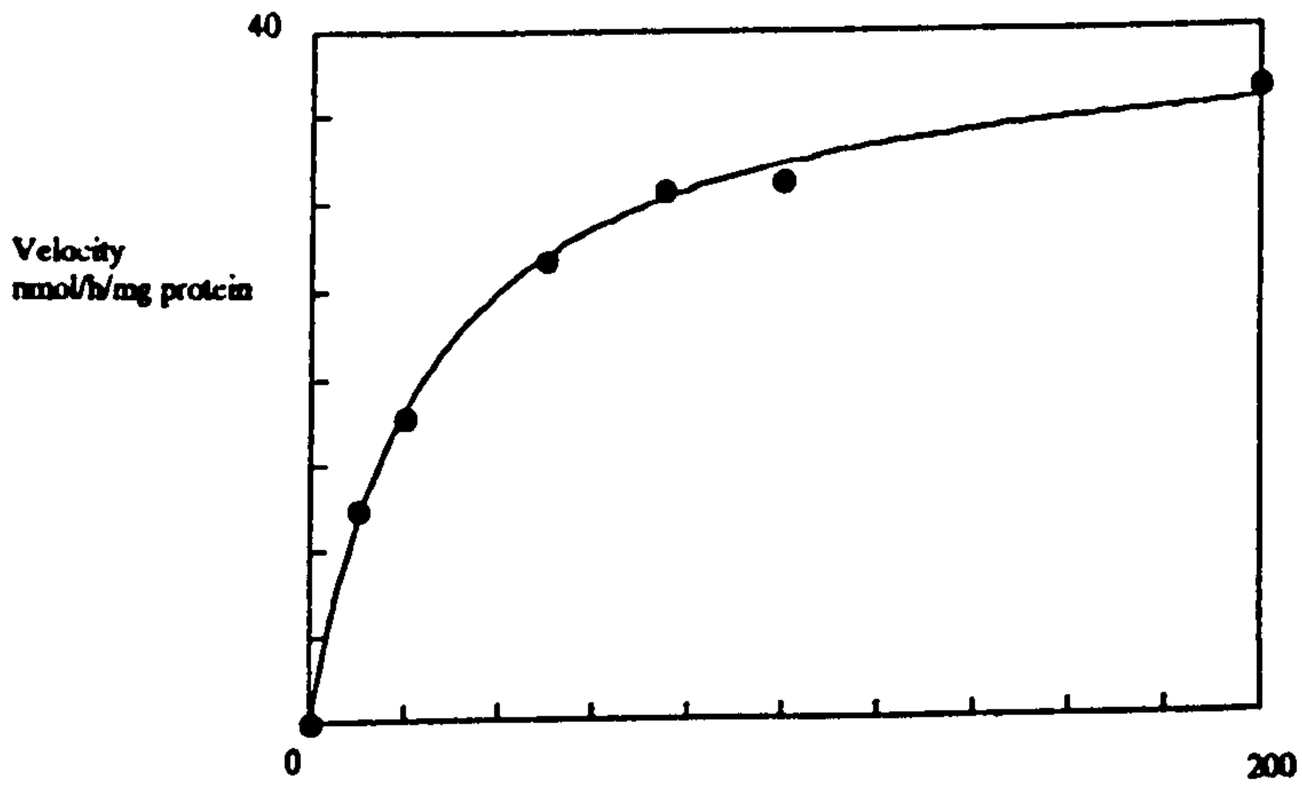
b)


90
Velocity
nmol/h/mg protein
0
[3-Dehydroecdysone] (μM)
200

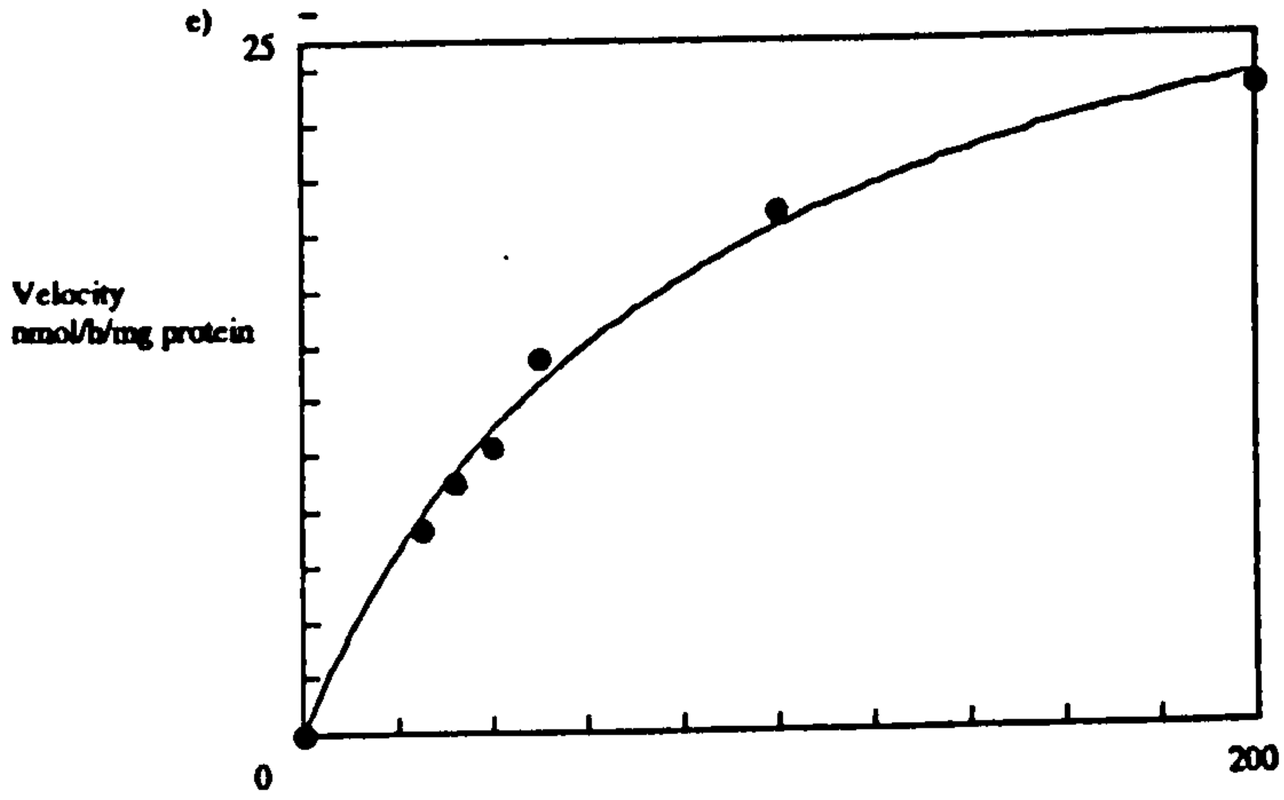
c)

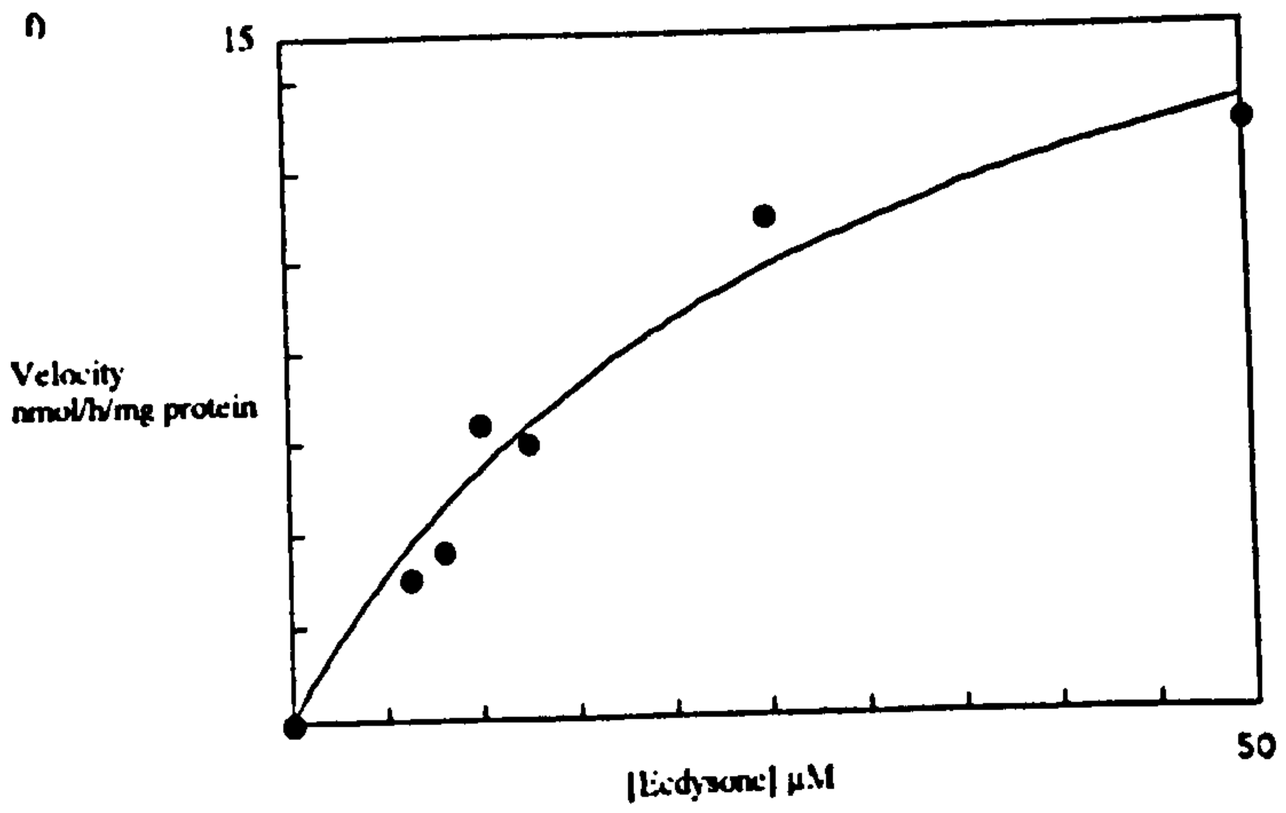

150
Velocity
(nmol/h/mg protein)
0
[3-Dehydroecdysone] (μM)
200

d)


40
Velocity
nmol/h/mg protein
0
200
[3-Dehydroecdysone] (μM)

e)


25
Velocity
nmol/h/mg protein
0
200
[Ecdysone] (μM)


0
15
Velocity
nmol/h/mg protein
50
[Ecdysone] μM

Enzyme	K_m (μM)	V_{max} (nmol/h/mg)
Ecdysone oxidase	20.4± 2.3	33.1± 1.8
3β-Reductase (NADH)	24.4± 2.0	40.1± 0.9
3β-Reductase (NADPH)	95.9± 15.4	199.7± 15.5
3α-Reductase (NADH)	229.7± 47.5	175.2± 23.3
3α-Reductase (NADPH)	58.1± 6.2	134.3± 5.7
2-Phospho-transferase	21.0±3.2	28.5±8.2

Table 4. A summary of the K_m and V_{max} values of the midgut enzymes from a semi-purified preparation (dialysed DEAE-cellulose fractions). Each datum represents the mean of two separate investigations (± S.E.M.).

95.9±15.4μM) whereas the NADPH-requiring 3α-reductase has a lower value than the NADH-requiring one (58.1± 6.2μM cf 229.7± 47.5μM). Whether or not the correct cofactor can ensure more efficient substrate binding can only be speculated.

The ecdysone 2-phosphotransferase was found to have a K_m value of 21.0±3.3μM and a V_{max} of 28.5±8.2nmol/h/mg protein. Therefore, the K_m value compares well with the values for the epimerization enzymes (Table 4), being of the same order of magnitude as all but the 3α-reductase (NADH). The V_{max} value, however, is comparatively low and may represent a rate limiting reaction in the irreversible inactivation process.

6.3 DISCUSSION

By comparison of enzyme activity profiles during the last (sixth) larval instar we gain a possible insight into the interactions of these enzyme systems (figure 6.2). Whilst feeding, the endogenous titre of haemolymph ecdysone and 20-hydroxyecdysone is low (Chapter 4, figure 4.1). The ecdysone oxidase reaches a maximum during this feeding phase and presumably ecdysteroids are inactivated either by production of 3-dehydro metabolites, having only approximately 3/10-1/15 of the activity of their parent compounds (as determined by the *Calliphora* bioassay (Karlson et al., 1972), or possibly by further reduction in activity via epimerization and/or phosphoesterifcation, since the 3α-reductase and phosphotransferases also exhibit a peak during feeding.

That there are peaks of enzymic activity for these inactivating enzymes during feeding, when the endogenous pool of active moulting hormone is low, suggests that they may play a role in the deactivation of

ingested ecdysteroids from the diet. However, it was expected that the enzyme titre would not peak, but plateau, if the activity is linked to feeding. At the cessation of feeding and the onset of wandering, the levels of 3β-hydroxy forming enzymes in the gut and in the haemolymph increase (*S. littoralis*, Chapter 4; *M. sexta* Sakurai et al., 1989b; Watson et al., 1985) and the levels of the other enzymes fall. It is tempting to suggest that the 3β-hydroxy forming enzymes in some way contribute to the huge surge in 20-hydroxyecdysone levels, since they peak just prior to the rise in active hormone titre. In this manner the 3β-reductases may be important in activation, rather than in futile cycling of ecdysone and 3-dehydroecdysone, as discussed by Weirich et al. (1989). It is also plausible that 3-dehydroecdysone is sequestered by the tissue and then re-activated when necessary. However, it is also noteworthy from the results of Chapter 4 that the haemolymph and midgut cytosol 3-dehydroecdysone 3β-reductase enzymes also appear to be different. The molecular weight of the midgut enzyme is 52.5kDa, compared with 34.7kDa for the haemolymph one; the pH optima are 6.5 and 7.9 for the migut and haemolymph enzymes respectively, suggesting that there may be subtle differences in the amino acids at the active site; the elution profiles from Mono-Q and Phenyl Superose FPLC are identical (using peak 1 from the Mono-Q profile, figure 6.8b), but the elution from DEAE-cellulose is slightly earlier for the haemolymph enzyme. This may be due to the fact that the midgut 3β-reductase activity detected in the DEAE-fractions can be attributed to two active polypeptides (peaks 1 and 2, figure 6.8b).

It is interesting to note that fat body ecdysone 20-monooxygenase, converting ecdysone to 20-hydroxyecdysone, is at highest levels at around 72±3hours (Chen, et al. 1992) i.e. when the inactivating enzymes

are at low levels, but before the 3β-reductases have reached high activity.

That the 3-dehydroecdysone 3α- and 3β-reductases have quite dissimilar profiles, i.e. that they do not peak concomittantly, implies that they have different roles to play in ecdysteroid metabolism. Early in the instar, as ecdysone oxidase activity is peaking, ecdysone would be inactivated to form 3-dehydroecdysone. Shortly afterwards but prior to gut purge and wandering in the larvae, any 3-dehydroecdysone would be preferentially converted into 3-epiecdysone, subsequently phosphorylated and probably excreted. However, several hours later, during the transition to pharate pupae, 3-dehydroecdysone is primarily cycled back to ecdysone. The controlling mechanisms for the activation and inactivation of the enzymes in the gut are not known, but are more than likely environmentally and developmentally linked.

The epimerization enzymes (ecdysone oxidase in conjunction with 3α- and 3β-reductases) act at optimal pH values of 6.5-7.9. In the case of the reductases, when NADPH is used as cofactor, the pH optimum is slightly higher than that of the incubations with NADH, (3α-hydroxy forming reductases, pH 7.5 and 7.1 respectively; 3β-hydroxy forming reductases, 7.9 and 7.5 respectively).

Once the optimum parameters for a particular enzyme had been determined, an *in vitro* assay system could be designed for maximum rate of activity. It must be emphasized that the *in vitro* systems employed are quite dissimilar from *in vivo* conditions, since they are not subject to the complex fluctuations in the microenvironment found *in situ* in the midgut cell. It is with these thoughts in mind that apparent K_m values were determined, all of which were found to be in the μM range. This may seem quite high, in view of the fact that in *Spodoptera littoralis*, the peak ecdysteroid titre during the sixth instar only reaches

1.6μM ecdysone equivalents (Chapter 4) and the K_m values for the enzymes examined range from 20.4-229.7μM. Therefore, what is being determined is the *in vitro* potential of these enzymes and not any quantitative reflection of the action of the enzyme in the organism.

How can the physiological condition be assessed? If it is assumed that the NADPH concentration is 100,000 times that of NADH *in vivo* (see Chapter 5, Discussion), it is logical to predict that the 3-dehydroecdysone 3β-reductase (NADH) has only minor activity in the cell. Moreover, since this reductase was found to have low activity with NADPH as cofactor (Chapters 5 and 6), its significance in ecdysteroid metabolism in the midgut is minimal. Hence, it would appear that the majority of the 3-dehydroecdysone entering the midgut cell is inactivated, producing 3-epiecdysone.

However, rather than picturing this sequence as "static" and stoichiometric, it may be more accurate to imagine fluctuating hormone and enzyme titres in a "dynamic" and complex set of reactions.

It could be postulated that since the K_m and V_{max} values for the enzymes are subject to changes depending upon which cofactor is chosen, the NADH/ NADPH ratio may in fact represent a mechanism by which the enzymes are controlled and, moreover, determine whether the 3-dehydroecdysone is converted into 3-epiecdysone (an irreversible loss of hormonal activity) or cycled back to ecdysone (an increase of hormonal activity). In this case it would have been useful to have studied the kinetics of the enzymes with increasing concentration of cofactor.

The enzymes studied are of low abundance in the insects, and it was clear that even more material would have to be used to further this work (currently 750 insects were sacrificed). In *S. littoralis* there is a problem of the collection of adequate crude enzyme for the purification in the shortest time span. However, the enzymes were all adequately

purified such that their molecular weights/subunit molecular weights could be deduced. The reported molecular weights of various 3-dehydroecdysone 3β-reductases on the basis of gel filtration, are between 27-37kDa (see Section 4.2.5; Watson et al., 1985; Sakurai et al., 1989b; Gelman et al., 1991), with that of the midgut 3β-reductase enzyme of *Spodoptera littoralis* (52.5kDa), being outside this range. The latter enzyme may, therefore, be quite a different protein. In the case of ecdysone oxidase, the only reported molecular weight is for the *Calliphora vicina* enzyme (250kDa; Koolman and Karlson, 1975). This is significantly larger than the polypeptides found by SDS-PAGE for the *Spodoptera* enzyme. In the latter case, several bands were found in the Phenyl Superose fractions, the major ones being 54.3kDa and 12.9kDa. The SDS-PAGE analysis of the enzyme may have revealed that a) several subunits exist and/or b) several impurities remain in the oxidase fractions. Obviously, in all cases, it is quite possible that some of the bands may be due to fragments of protein, which are the result of protease action.

It would, of course, be extremely instructive to recover enough of the enzymes in order to perform an investigation of substrate specificity. In the case of the reductases this is most important, since it is necessary to deduce whether or not all the reductase activities are specific for ecdysteroid metabolism or for other steroid substrates. Ecdysone oxidase specificity has already been reported for the *Calliphora vicina* enzyme (Koolman and Karlson, 1978). The enzyme can also oxidize 20-hydroxyecdysone (Koolman and Spindler, 1977); a number of other ecdysteroids have been tested as potential substrates. Two phytoecdysteroids, inokosterone and makisterone (figure 6.12), have a higher rate of reaction than ecdysone, but cholesterol and 7-dehydrocholesterol are not oxidized. Surprisingly, 22,25-

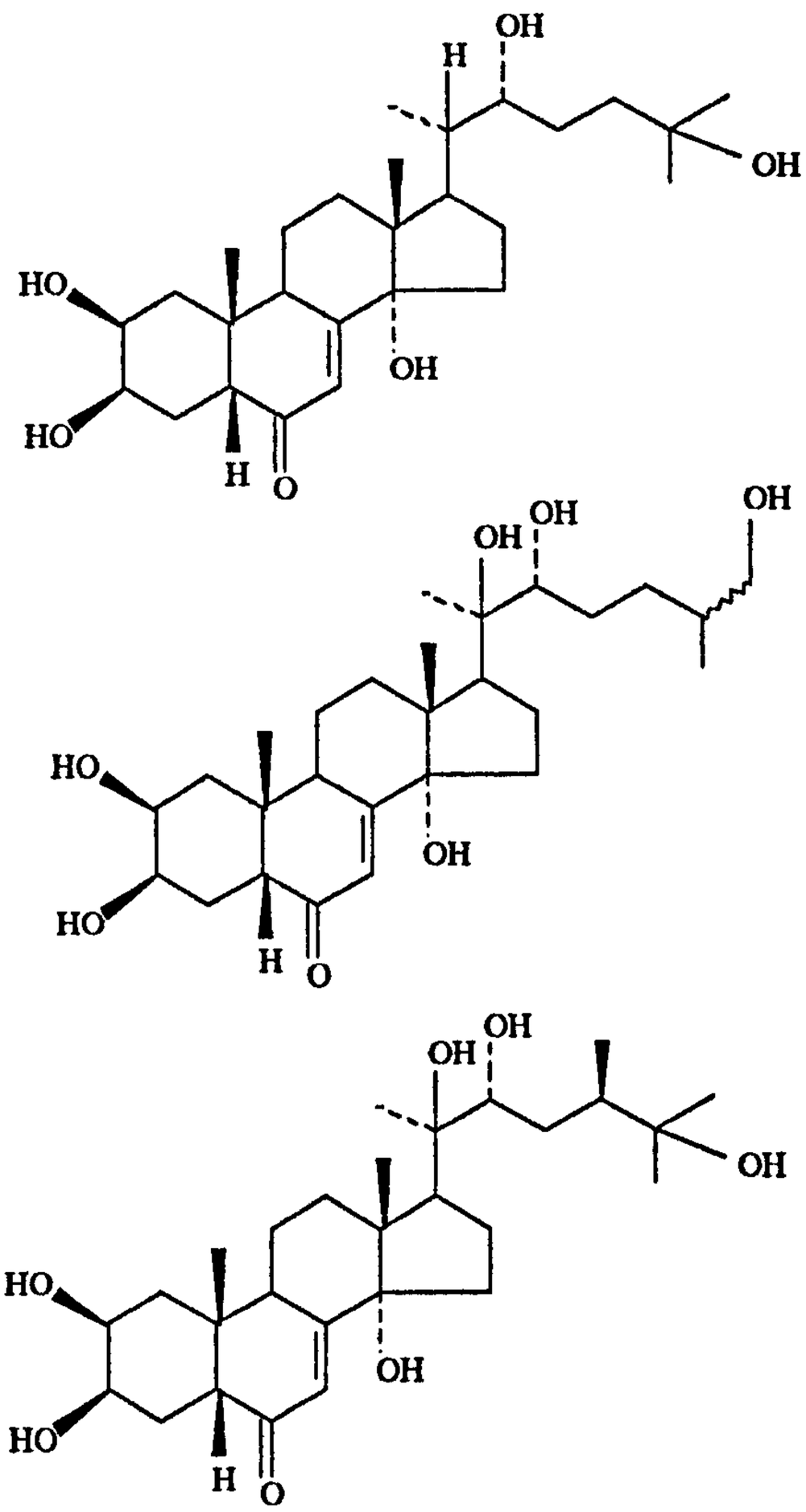


Figure 6.12. The structures of a) ecdysone, b) inokosterone and c) makisterone A.

dideoxyecdysone is not a valid substrate, indicating that the enzyme must require one or both of C-22 and C-25 to be hydroxylated and /or to be more polar than the C-H bond.

Chapter 7

General Discussion

7.1 OVERVIEW

The aims of the project were stated as being firstly, to characterize the ecdysteroid secretions from the prothoracic glands of *Spodoptera littoralis*. Since this was found to be primarily 3-dehydroecdysone (82%), it was possible that the 3-oxo group was introduced at an early stage of the biosynthetic pathway. Thus, it was intriguing to to perform a preliminary investigation as to whether 3-oxo-5β-ketodiol could be an intermediate of ecdysone/3-dehydroecdysone biosynthesis in the prothoracic glands. Consequently, 3-oxo-5β-ketodiol was implicated as being a potential precursor of 3-dehydroecdysone, although this has not been thoroughly confirmed. On the other hand, 5β-ketodiol could not be converted into 3-dehydroecdysone, but rather into ecdysone alone, suggesting it is not a viable precursor.

For a full verification of 3-oxo-5β-ketodiol as an intermediate in the pathway, six criteria should be fulfilled (Rees, 1985). It should be: a) isolated from the prothoracic glands and subjected to chemical and physical structural analysis; b) formed from the distal presursor, cholesterol; c) converted into the ultimate product, 3-dehydroecdysone; d) formed from a proximal precursor (whose structure has yet to be identified); e) converted into the proposed proximal product along the biosynthetic pathway. This would take a considerable amount of work, since there are apparently several divergent branched pathways, following the production of 3-oxo-5β-ketodiol; and f) each enzymic step of 3-oxo-5β-ketodiol production and subsequent metabolism should be demonstrated directly. Clearly, our current knowledge of the pathway is too fragmentary to allow all these idealistic criteria to be satisfied.

The second aim of the project was to characterize selected enzymes of ecdysteroid metabolism in the midgut of *Spodoptera littoralis*, specifically, those involved in the 3-epimerization and phosphorylation sequences. Additionally, the 3-dehydroecdysone 3β-reductase of the haemolymph was characterized and compared to the enzyme catalysing the same reaction in the midgut. The haemolymph enzyme reduces the 3-dehydroecdysone product of the prothoracic glands.

After a full confirmation of the enzymic products (Chapter 5), developmental profiles of the various enzymes during the sixth larval instar gave an insight into the complexity of the timing of the epimerization and phosphorylation reactions in the midgut. It was also rather interesting that the 3-dehydroecdysone 3β-reductase enzymes of the midgut and the haemolymph displayed similar profiles, suggesting a common function. In light of the ecdysteroid titre during the last larval instar, which reaches a peak when the enzymes of activation are high, it was proposed that these enzymes activate 3-dehydroecdysone to produce ecdysone. As expected, the two 3-dehydroecdysone 3β-reductases reach peak titres when the titres of the inactivating enzymes are low (Chapters 4 and 6).

In more detailed experiments, enzymic activities were optimized with respect to cofactor requirements, pH, reaction time, protein concentration and temperature (Chapters 4 and 6). Attempts were then made to purify the enzymes and in the case of the midgut, although appreciable purification was achieved, the yields were so low that the protein had to be visualized on SDS-PAGE by the use of silver staining [the method used was sensitive enough to detect 0.5-1ng of protein (Ochs et al., 1981)]. Evidently more efficient methods for enzyme purification need to be developed and it would be beneficial to use at

least 5-6 times as much starting cytosolic preparation. The elution profiles of the enzymes are now known for DEAE-cellulose chromatography, Mono-Q and Phenyl Superose FPLC, hence, this would now decrease the time spent on assaying the eluted fractions, and reduce the time for the enzymes to be exposed to any protease action.

It was disappointing that the enzyme assays could not be made less time consuming. It was not possible to monitor the oxidation of NAD(P)H for the reductases (at 340nm), since the amount of oxidized cofactor was at low concentration, compared to that of the excess NAD(P)H added. For ecdysone oxidase, ABTS dye was added, to theoretically combine with the side product, hydrogen peroxide, and form a green complex, which is detected at 414nm. However, the assay was not sensitive enough to detect the coloured complex formation.

Purification of the haemolymph 3-dehydroecdysone 3β-reductase was more successful, probably because each step could be performed much more quickly, since only one enzyme had to be located. Alternatively, the haemolymph preparation, although dialysed, was not appreciably diluted with buffer, theoretically leading to the formation of sharper elution peaks. Unfortunately, not enough protein was recovered to sequence the N-terminal amino acids of the polypeptide chain. Initially, the protein was found to be N-teminally blocked and insufficient sample remained for further analysis, e.g. by the use of trypic digests. It would be very interesting to sequence this enzyme in the future, especially since the protein was found to be quite hydrophobic (as deduced from its late elution from the Phenyl Superose FPLC).

Obviously with a reasonable amount of protein at hand, there would be many opportunties for the further study of the enzyme. One quite important feature of these enzymes which was not examined, was

the specificity for the substrate. Ecdysone oxidase of *Calliphora vicina* is specific for ecdysteroids that are rather polar and/or contain hydroxy groups in the side chain (Koolman and Karlson, 1978). The substrate specificities of the reductases and phosphotranferases are not known. Exogenous ecdysteroids, which are added to the incubation as substrates, may be metabolised in a non-specific manner. It is particularly important to bear this in mind when considering the four reductase enzyme peaks obtained by Mono-Q FPLC. It could be the case that one or both of the 3-dehydroecdysone 3α- and 3β-reductases are non-specific enzyme activities.

It has recently been shown that whole larval homogenates of *Calliphora vicina* contain a protein which cross-reacts with an antibody raised against mouse liver 3α-hydroxysteroid reductase. This suggests that there may be structural homologies between the 3α-hydroxysteroid reductases in insects and vertebrates. Furthermore, a mixture of ecdysteroids was found to inhibit the 3α-hydroxysteroid reductase of *Calliphora*, suggesting a role for the enzyme in ecdysteroid metabolism (Oppermann et al., 1992). Moreover, the 3α-hydroxysteroid reductase may be the so-called 3α-hydroxy-forming reductase of the epimerization system in the insect midgut. This is obviously of considerable interest; future work on the comparison of insect and vertebrate 3α- and 3β-hydroxysteroid reductases may prove enlightening for the development of novel insecticides, since much more is known about the vertebrate systems (for review see Penning et al., 1986; Chapman and Sauer, 1979).

Another point to remember is that the midgut phosphotransferases produce ecdysteroid 2- and 22-phosphates, although the reason why two such mechanisms exist for ecdysteroid inactivation is not known. Whether one or both of these enzymes is specific for endogenous or

exogenous ecdysteroids (injested phytoecdysteroids) is uncertain. Other phosphotransferases are known to exist in the midgut cytosol, for example there is an active system able to phosphorylate p-nitrophenol, which has been found in several insect species (Yang and Wilkinson, 1973). The latter phosphotransferase belongs to the same class of enzymes as those in this study, i.e. those with a secondary alcohol group as acceptor (EC 2.7.1). Rather confusingly, the latter enzyme substrate specificity has not been investigated. It is not unreasonable to suggest that p-nitrophenol may be phosphorylated by one, or both, of the phosphotransferases in this investigation.

7.2INACTIVATION AND EXCRETION OF ECDYSTEROIDS

It is generally believed that the terminal ecdysteroid inactivation products (3α-epimers and phospho-conjugates in the systems studied, but also ecdyson-26-oic acids and phospho-acetates in other systems) are excreted. Unchanged ecdysone, 20-hydroxyecdysone, along with 3α-epimers, ecdyson-26-oic acids and various conjugates have been identified in larval faeces (see Lafont and Koolman, 1984). Furthermore, studies of [^{3}H]ecdysone excretion in *Locusta migratoria* and *Calliphora vicina* revealed that the rate of excretion is slowest during the peaks in ecdysteroid titre (Hoffmann et al., 1974; Koolman et al., 1985) and that the ecdysteroid composition of the excreta depends upon the developmental stage. It would be interesting to initiate such a study in *Spodoptera littoralis* larvae and to correlate the findings to the sixth instar developmental enzyme titres. Several of the inactivation products of the midgut (specifically the phosphates) are thought to prevent reabsorption of the ecdysteroid moiety and, therefore, regulate

endogenous ecdysteroid levels (Modde, et al., 1984; Warren and Gilbert, 1986).

The excretion of ecdysteroids, particularly the highly charged polar conjugates, presents a problem. They do not appear suited to passive diffusion across the plasma membrane, due to their bulk and polarity. It is quite possible that membrane-mediated modes of transport exist, for example utilizing transport proteins. Several lines of evidence point to an active form of free ecdysteroid passage. Characteristics of membrane mediated transport include a) saturation kinetics, b) temperature sensitivity, c) specificity for ligand, d) involvement of metabolic energy (Höfer, 1981), e) high affinity for the ligand and f) counter-flux (Rao et al., 1976). Not all of these criteria have been fulfilled for transport of free ecdysteroids, but in models used thus far, active transport has been implicated in *Sarcophaga peregrina* (Natori and Ohtaki, 1976) and *Drosophila melanogaster* (O'Connor, 1985). [^{3}H]Ecdysone, encapsulated in a liposome made of phospholipids and cholesterol, was better able to cross the membrane than vertebrate-type steroids (Miles and Dinan, 1989). Further, the passage of the ecdysteroid, ponasterone A, was sensitive to a sulphydryl-group inhibitor (pCMPS). This infers that diffusion is not the only method of entry into a target cell.

7.3 THE CONTROL OF ECDYSTEROID LEVELS

Of course, each ecdysteroid is subject to fluctuations in titre, depending upon the developmental stage and physiological situation. These levels must be strictly controlled and are increased by biosynthesis in the prothoracic glands (and gonads) and secretion from the prothoracic glands. Levels are decreased by catabolism and

excretion. Preliminary work by Karlson and Bode (1969) suggested that the rate of inactivation of ecdysone was low when the moulting hormone titre was high and *vice versa*. This was superceded by more sophisticated experiments (Koolman, 1981), whereby it was shown in *Calliphora* larvae that the conversion of [^{3}H]ecdysone into [^{3}H]20-hydroxyecdysone is much faster than the catabolism of [^{3}H]20-hydroxyecdysone, owing to a high 20-monooxygenase activity and resulting in a concomitantly high 20-hydroxyecdysone titre. However, it was also found that ecdysone 20-monooxygenase activity in *Manduca sexta* and *Calliphora vicina* declines at pupariation, i.e. when endogenous 20-hydroxyecdysone is rising rapidly (Smith et al., 1980; Koolman, 1981). This can be explained by earlier work done on *Calliphora* by Young (1976a,b). The data showed that during puparium formation, the rate of conversion of [^{3}H]ecdysone into [^{3}H]20-hydroxyecdysone decreases, but the half-life of 20-hydroxyecdysone increases by more than 10-fold. Since the endogenous titre of 20-hydroxyecdysone rises at this time, it would appear that the inactivation (catabolism) of 20-hydroxyecdysone is more important to its regulation than monooxygenase activity.

Loss of ecdysteroid is usually by excretion, and interestingly, the rate of excretion of unchanged injected [^{3}H]20-hydroxyecdysone in *Locusta migratoria*, declines at the time of the peak of moulting hormone titre (Hoffmann et al., 1974). In *Spodoptera littoralis*, it was found that inactivating enzymes (ecdysone oxidase, 3-dehydroecdysone 3α-reductase and ecdysone phosphotransferases) are at low activity during the highest levels of moulting hormone, but activating enzymes (midgut and haemolymph 3-dehydroecdysone 3β-reductases) are at high activity. Hence, differences may occur due to species specificities.

Although the interplay between the enzymic activities may play a part in regulating the individual levels of ecdysteroids, or at least the ratio of the substrate ecdysteroid/product ecdysteroid, the factors controlling the enzymes can only be speculated, using other enzymes of ecdysteroid metabolism as examples. Most work on the regulation of ecdysteroid enzymes has concentrated on ecdysone 20-monooxygenase.

In *Locusta migratoria*, the microsomal 20-monooxygenase from Malpighian tubules is thought to be induced by its substrate, ecdysone (Feyereisen and Hoffmann, 1977). A similar phenomenon has been observed in the case of the mitochondrial monooxygenase from *Manduca sexta* gut (Keough et al., 1989).

An interesting perspective on the *Manduca* haemolymph 3-dehydroecdysone 3β-reductase (or stimulatory factor, as it was originally known) is that the titre of the factor fluctuates during development in such a way, that it is suggestive of being regulated by juvenile hormone (Watson et al., 1988). Additionally, treatment of final instar *Manduca* with 7S-hydroprene, an analogue of JH, elevates the titre of the factor. In untreated last instar larvae, the level of the factor is high at the start of the last instar (days 0-1) and falls between days 1 and 2, remaining low until day 4, then increasing to reach a peak on day 7. The titre then declines at the end of the instar. The JH titre is similarly high at the beginning of the last instar, and then falls between days 1 and 2, remains low until day 5, and increases prior to the larval-pupal moult. Similarly, in *Spodoptera*, the haemolymph (and midgut) 3β-reductase profiles are closely related to the haemolymph ecdysteroid titre (chapter 4). With such a strong resemblance between the JH, stimulatory factor and ecdysteroid profiles, it could be envisaged that JH promotes the synthesis/release/activation of the haemolymph (and midgut) 3β-reductases. This would indirectly increase the titre of

haemolymph ecdysone, via the conversion of 3-dehydroecdysone into ecdysone. Although the origin of the 3-dehydroecdysone 3β-reductase is uncertain, the fat body has been demonstrated in *Manduca* to be the source of the 'haemolymph stimulatory factor'(Greutzmacher et al., 1984), now believed to be synonomous with the 3β-reductase enzyme.

In *Spodoptera*, the JH titre has also been found to follow the same pattern as in *Manduca* (Zimowska et al., 1989). Although the insects (*Spodoptera*) were maintained under similar conditions to those used in this work, correlation of the JH titre with the current enzyme and ecdysteroid profiles during development is difficult, since some key time points were not examined in the former study. The JH titre reached a peak between 99-103h and the haemolymph reductase at 107(±3)h. Unfortunately, JH assays were not undertaken at time-points between 103-110h. It would have been fascinating to compare the JH titre for later time-points with the haemolymph and midgut reductases. Therefore, it can only be speculated that JH may also be important in regulating 3β-reductase levels in *Spodoptera* (note: Zimowska et al. use t=0 at the end of the first scotophase; in this study, t=0 is at the midpoint of the first scotophase, i.e. 3h before Zimowska et al. In this discussion, quoted times have been normalised to our timing system). A point to note is that there is a high titre of ecdysteroids at the beginning of the last larval instar in *Manduca*, whereas in *Spodoptera*, the titre is low. Interestingly, the JH titre in *Spodoptera* is high at the start of the instar (as in *Manduca)*, but the reasons for this discrepancy are, at this time, unknown.

The fine details of this apparent JH control of the 3β-reductase at the molecular level would prove illuminating for this aspect of ecdysteroid metabolism. For example, it is known that JH prevents the

cAMP-dependent phosphorylation of a 34kDa protein in the prothoracic glands; this inhibits the effects of PTTH (Rountree et al., 1987).

7.4 3-DEHYDROECDYSONE AND ITS ROLE IN METABOLISM

In this study, the 3-dehydroecdysteroid 3β-reductases are regarded as ecdysteroid activating enzymes. This assumes that (20-hydroxy)ecdysone is more hormonally active than 3-dehydro(20-hydroxy)ecdysone, as is generally accepted. However, a report by Sommé-Martin et al. (1990) suggested the exciting possibility that 3-dehydro-20-hydroxyecdysone may not only serve as an intermediate on a hormone inactivation pathway, but as an active hormone in its own right and may be the true major active metabolite. Using an ecdysone-less mutant strain of *Drosophila melanogaster*, *ecd-1*, in which the fat body polypeptide P1 was not expressed, P1 production could be induced by treatment with exogenous ecdysteroids. The biological activity of a variety of ecdysteroids was assayed by the ability to induce P1 gene transcription in the fat body. For both *in vivo* and *in vitro* studies, 3-dehydro-20-hydroxyecdysone was found to enhance transcription more efficiently than 20-hydroxyecdysone. Interestingly, the *in vivo* activity of 3-dehydro-20-hydroxyecdysone was the highest of all the ecdysteroids tested, at physiological and higher concentrations. Weak biological activity was found with 3-epi-20-hydroxyecdysone, 3-epi-20-hydroxyecdysone 3-phosphate and 20,26-dihydroxyecdysone *in vivo*, but these ecdysteroids were completely inactive *in vitro*, possibly due to their poor membrane permeability in cultured cells. In view of these results, formation of 3-dehydro-20-hydroxyecdysone can not be regarded solely as part of an inactivation pathway. Furthermore, in

Drosophila (*in vitro* and *in vivo*), the 3-dehydro-20-hydroxyecdysone was not converted into 20-hydroxyecdysone and the activity was due to the former compound itself. As 3-dehydro-20-hydroxyecdysone is present in significant quantities in *Drosophila*, it may represent a physiologically active ecdysone metabolite, at least in this species.

Clearly, this presents an anomaly to the central dogma of ecdysteroid action. Unfortunately, further studies have not been published, but it would be interesting to examine whether the P1 gene is induced by different ecdysteroids in a manner dependent upon the developmental stage of the insect and, moreover, whether P1 gene induction by 3-dehydro-20-hydroxyecdysone is exclusive to *Drosophila* or is a facet of ecdysteroid action not previously studied. However, until more evidence becomes available regarding the capacity of 3-dehydro-20-hydroxyecdysone for gene induction, it is generally held that it is less active than its 3β-hydroxy counterpart.

Using the *Calliphora* pupariation bioassay (Karlson and Shaaya, 1964), 3-dehydroecdysteroids were found to be $^{1}/_{10}$-$^{1}/_{15}$ as active as their 3β-hydroxy parent compounds (Spindler et al., 1977). Additionally, two different chromosome puff assays have also indicated that 3-dehydroecdysone is much less active than ecdysone (Spindler et al., 1977; Richards, 1978). Interestingly, Spindler et al. (1977) found that 3-dehydro-20-hydroxyecdysone was able to induce puffing in *Drosophila* salivary glands, which was comparable to that of 20-hydroxyecdysone (in agreement with the later findings of Sommé-Martin et al., 1990).

The importance of 3-dehydroecdysteroids became apparent in two main aspects of this study. Firstly, as the primary secretion of the *Spodoptera* prothoracic glands, produced from 3-oxo-5β-ketodiol. Secondly, as an intermediate in the inactivation (epimerization) of (20-

hydroxy)ecdysone in the midgut. None of these distinctive roles are compatible with 3-dehydro-20-hydroxyecdysone being more hormonally active than 20-hydroxyecdysone.

7.5 CONCLUSION

In conclusion, this study has only acted as a preliminary to more detailed studies of a fascinating aspect of ecdysteroid metabolism- the enzymology of ecdysteroid activation and inactivation. As previously outlined, many questions remain unanswered, for example the control and specificity of the enzymes. Much worthwhile work has yet to be done, comparative studies between Lepidoptera and other insect orders are still in the early stages, and a comparison of these enzymes in other Arthropods has yet to be initiated.

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Appendices

Appendix A

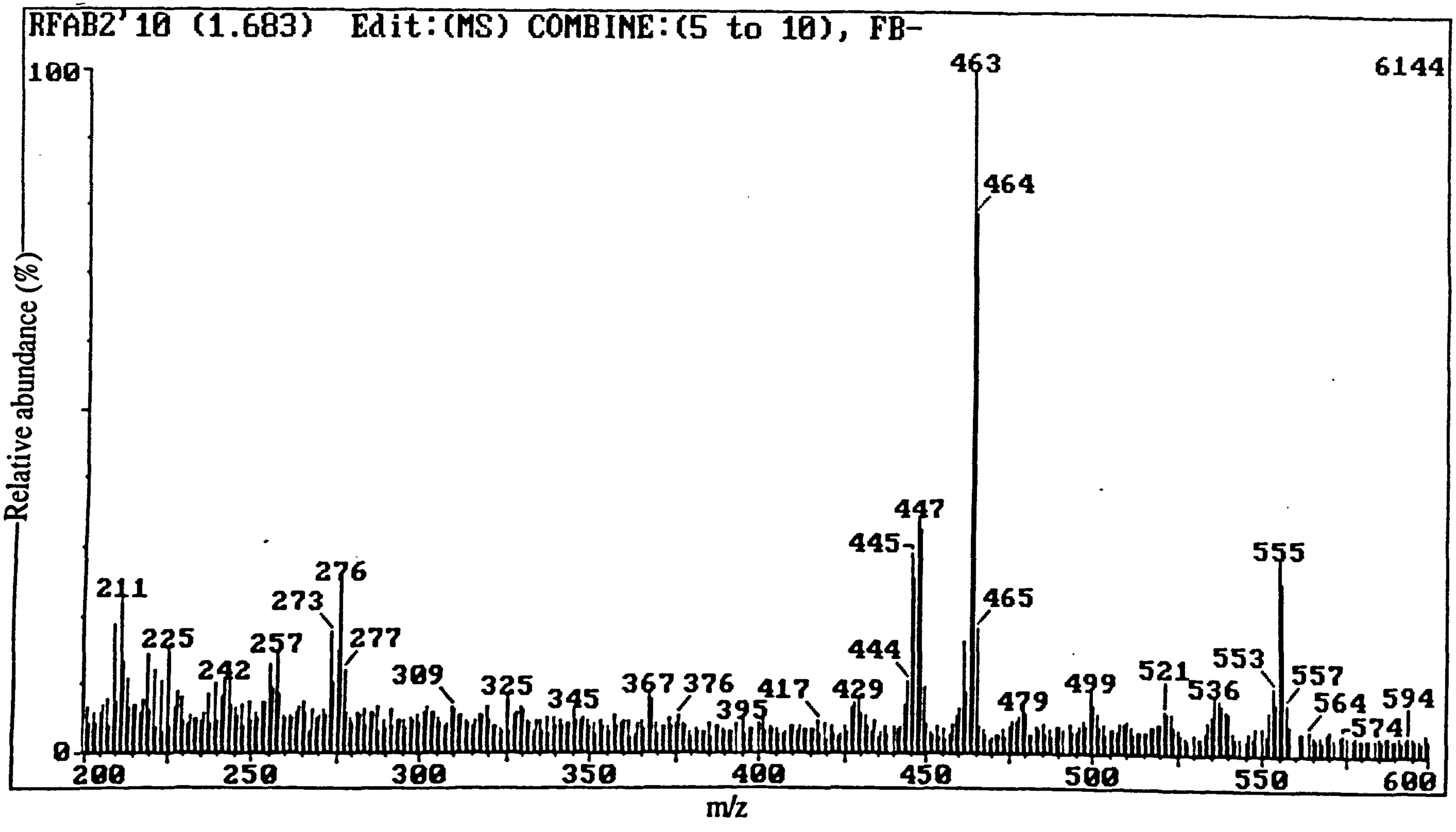
Mass Spectra

Spectrum 1 Ecdysone

Spectrum 2 3-Dehydroecdysone

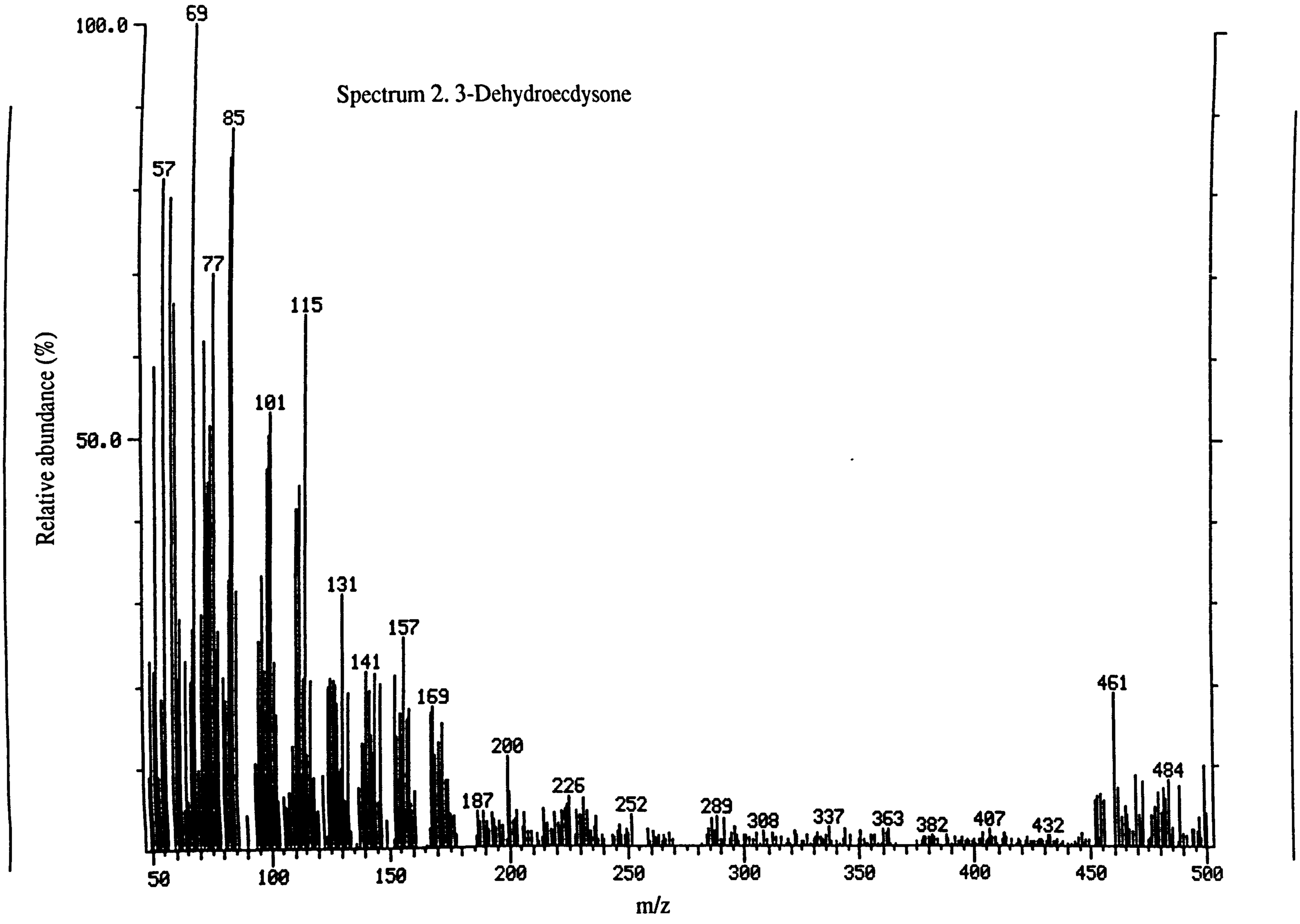
Spectrum 3 3-Epiecdysone

Spectrum 1. Ecdysone

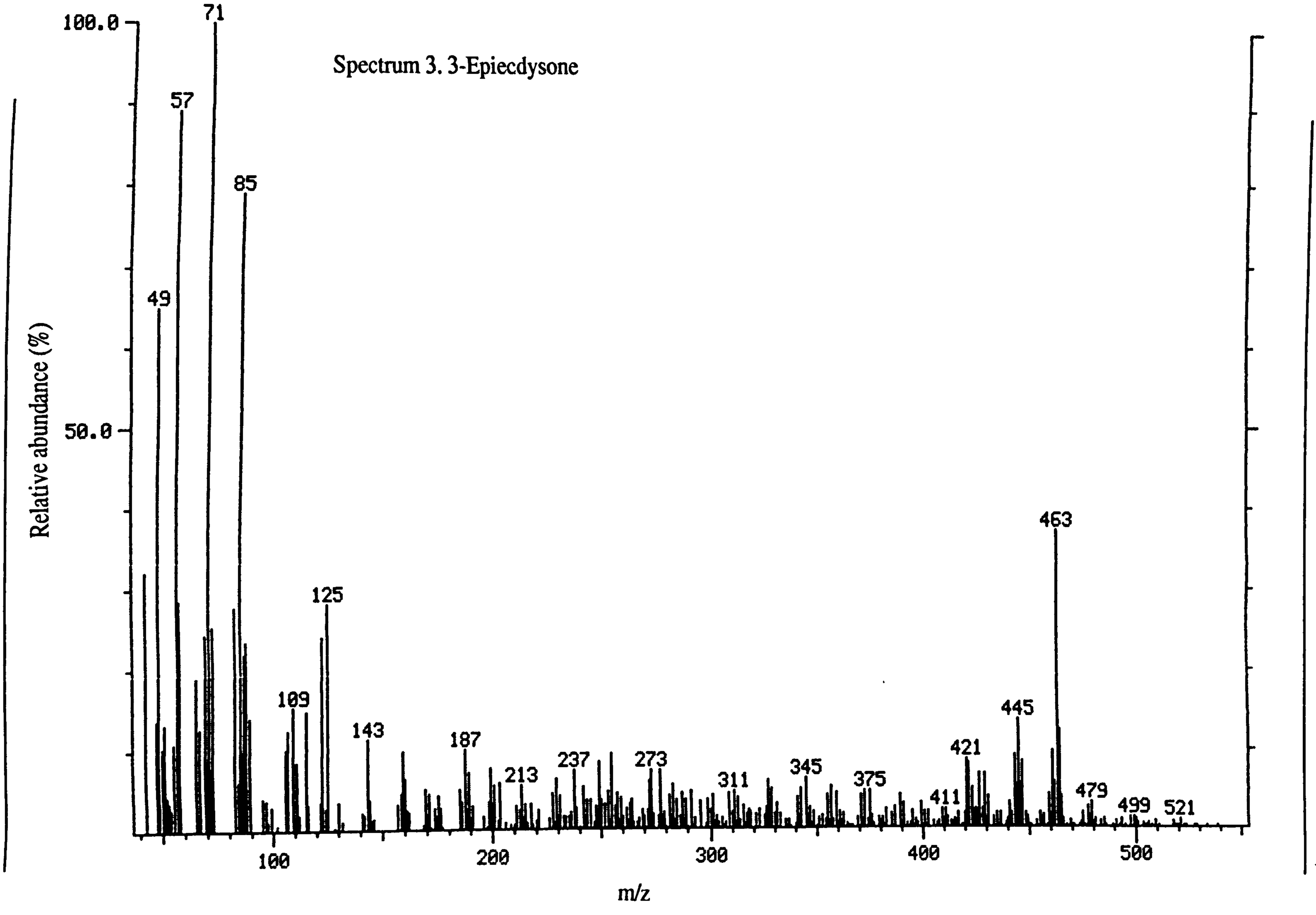


Appendix A

Spectrum 2. 3-Dehydroecdysone



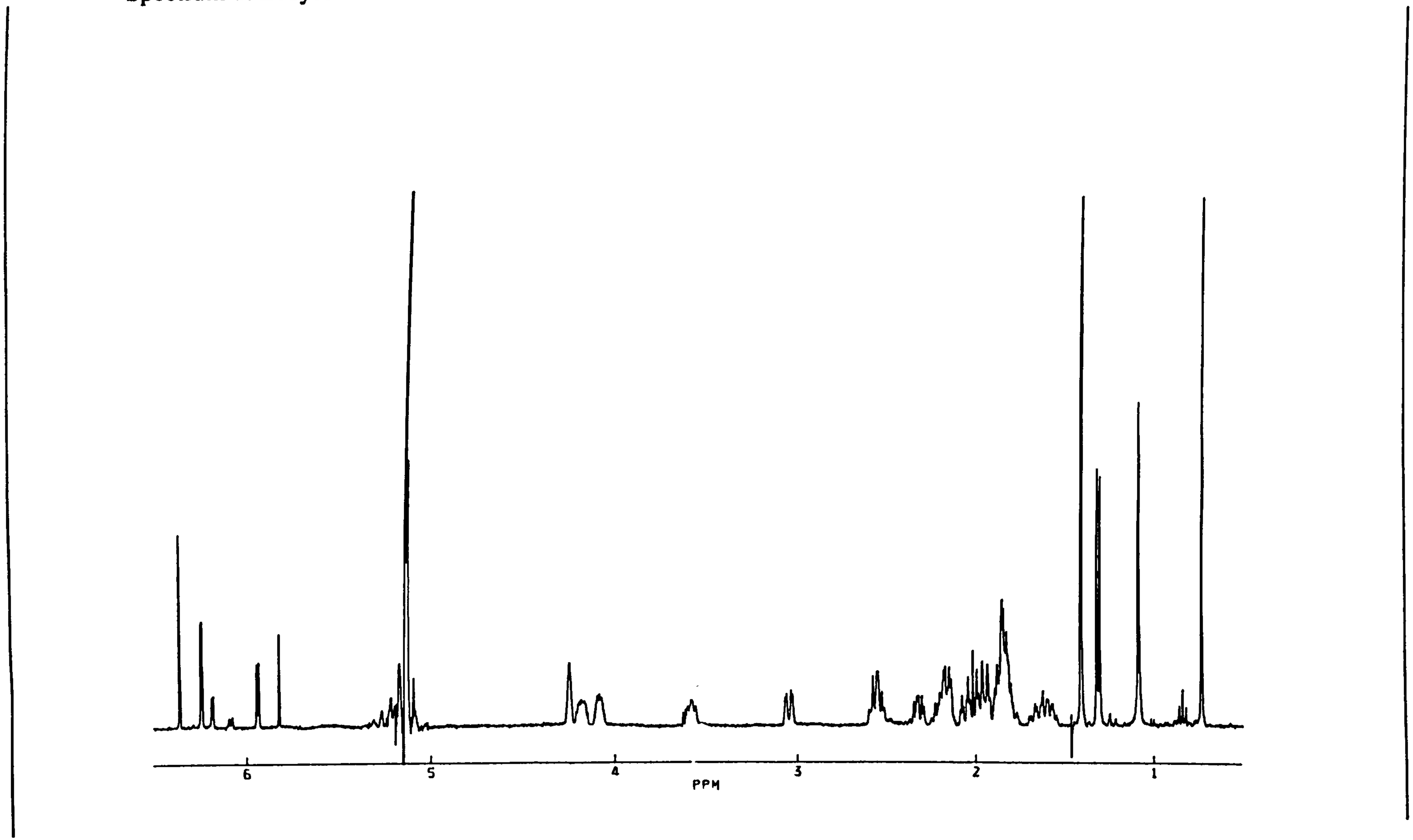
Spectrum 3. 3-Epiecdysone



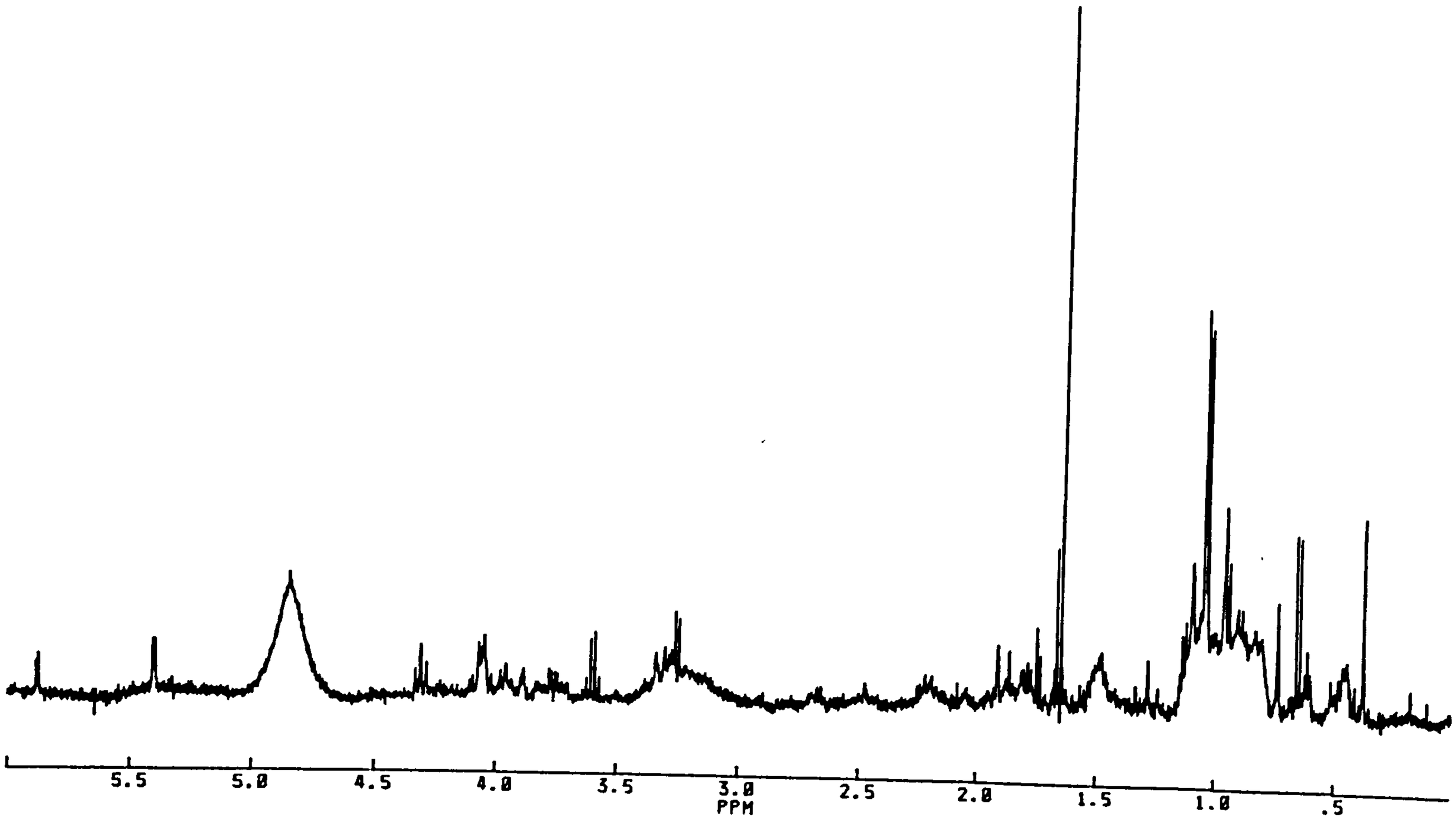
NMR Spectra

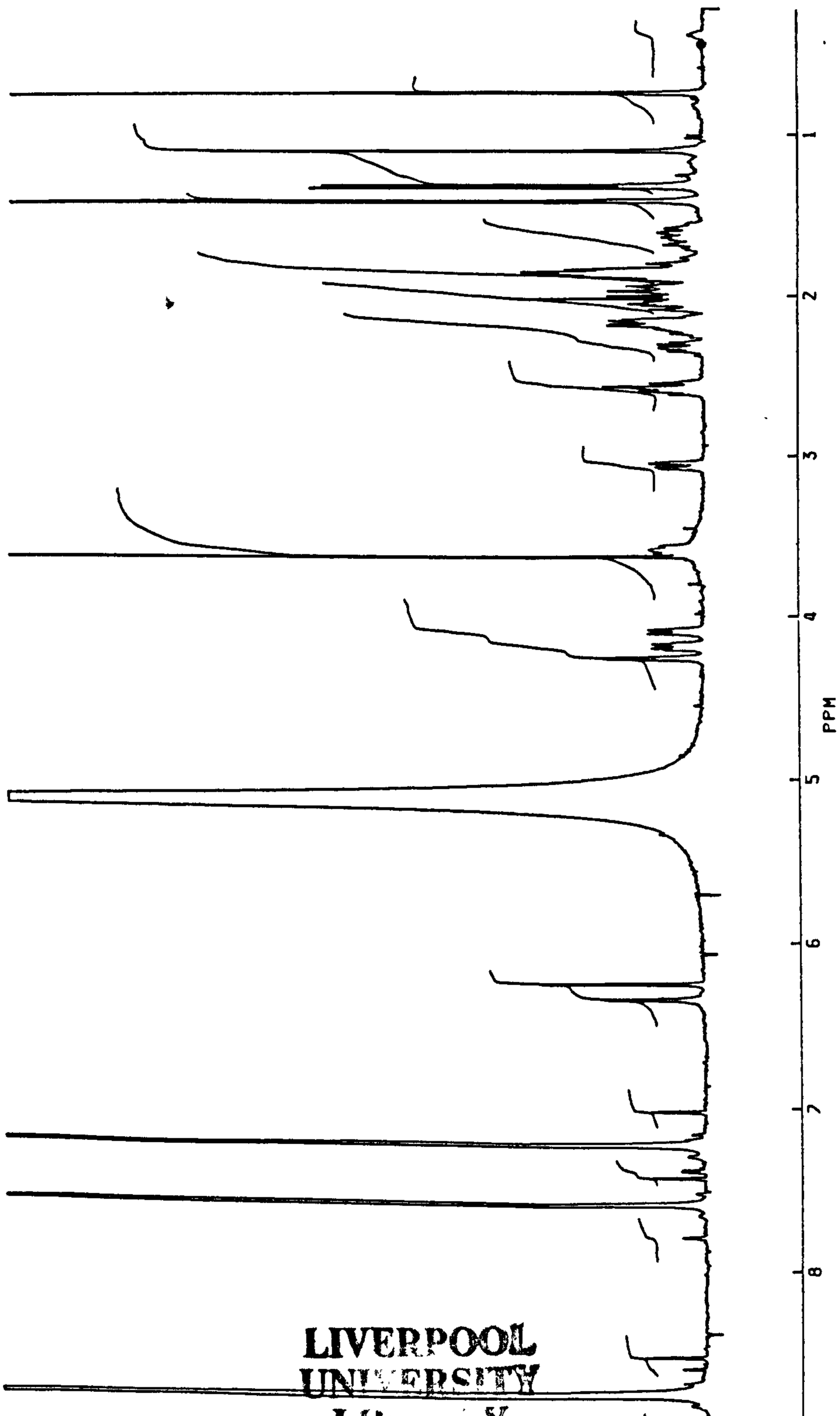
Spectrum 1	Ecdysone
Spectrum 2	3-Dehydroecdysone
Spectrum 3	3-Epiecdysone

Spectrum 1. Ecdysone



Spectrum 2. 3-Dehydroecdysone





Spectrum 3. 3-Epiecdysone