METABOLISM OF ECDYSTEROIDS
IN *SPODOPTERA LITTORALIS*

Thesis submitted in accordance with the

requirements of the

UNIVERSITY OF LIVERPOOL

for the degree of

Doctor of Philosophy

by

Nicholas Peter Milner

September 1984

"All the business of war and indeed all the business of life, is to endeavor to find out what you don't know from what you do, that's what I called 'guessing what was on the other side of the hill',..."

Arthur Wellesley, Duke of Wellington
(1769-1852)

Summary

The work presented in this thesis is concerned principally with the investigation of the metabolism of moulting hormones (ecdysteroids) in the 6th larval and pupal instars of Spodoptera littoralis. The moulting hormone titres for these instars have been determined and the inactivation processes controlling the titre investigated.

The ecdysteroid titre in the haemolymph of 6th instar larvae has been determined by radioimmunoassay. The titre revealed two minor peaks followed by a major peak prior to pupation. The free ecdysteroids at the titre peaks were analysed by gas chromatography/mass spectrometry (selected ion monitoring) demonstrating that 20-hydroxyecdysone is the principal ecdysteroid present at these times.

In pupae, the changes in the titre of various ecdysteroids were monitored by h.p.l.c. The titres of ecdysone, 20-hydroxyecdysone, 3-epiecdysone, 3-epi-20-hydroxyecdysone, 26-hydroxyecdysone and 20,26-dihydroxyecdysone were established. These showed that midway through the instar considerable amounts of ecdysone and 20-hydroxyecdysone were present accompanied by smaller amounts of 26-hydroxyecdysone and 20,26-dihydroxyecdysone. Significant quantities of 3-epiecdysone and 3-epi-20-hydroxyecdysone were detected early in the instar and, indeed, during the first four days of pupal development these were the only detectable ecdysteroids present.

The metabolism of injected [^{3}H] ecdysone was investigated during the decrease in titre at the end of the 6th larval instar and during the decrease in titre midway through the pupal instar. In both cases the main metabolites were shown to be 20-hydroxyecdysone, 26-hydroxyecdysone, 20,26-dihydroxyecdysone and two highly polar ecdysteroid

metabolites which were not hydrolysable by *Helix pomatia* enzyme preparation.

The unhydrolysable highly polar ecdysteroids were further characterised by derivatisation (methylation), h.p.l.c. and mass spectrometry as ecdyson-26-oic acid and 20-hydroxyecdyson-26-oic acid. The intermediacy of 20,26-dihydroxyecdysone in the metabolism of 20-hydroxyecdysone to the 20-hydroxyecdyson-26-oic acid has also been indicated by the formation of the acid from [^{3}H] 20,26-dihydroxyecdysone injected into pupae.

The ecdysone 3-epimerisation reaction has been investigated *in vitro*. The results indicated the intermediacy of 3-dehydroecdysone in the 3-epimerisation of ecdysone, and implicated the involvement of NADPH and molecular oxygen in this reaction.

Incubation of tissue homogenates from 6th instar larvae showed that 3-epimerisation was most prominent in the midgut and 20-hydroxylation of ecdysone in the fat body. The variation in the activities *in vitro* of the 3-epimerisation and 20-hydroxylation enzymes in homogenate of tissues at different times during the 6th larval instar has been investigated. The 3-epimerisation activity in midgut *in vitro* was shown to increase dramatically midway through the instar and remained high until just prior to pupation. The fat body 20-hydroxylase activity titre showed one major peak midway through the instar accompanied by two minor peaks early and late in the instar.

FOREWARD

The work described in this thesis was carried out in the Biochemistry Department of the University of Liverpool between 1980 and 1983. During this period, the research was financed by the Science and Engineering Research Council.

I am grateful to Professor T.W. Goodwin, C.B.E., F.R.S. for giving me the opportunity to work in this Department. Many thanks are also due to all members of the laboratory 216 for their friendship, assistance and helpful discussion, and to Mrs. V. Bennett for typing this manuscript.

However, above all, I would like to express my appreciation to Dr. H.H. Rees for his enthusiastic guidance and encouragement during the course of this research and for his invaluable help in the preparation of this thesis.

Finally, I would like to thank my parents for their loving care and encouragement throughout my education, and very special thanks to Sufia for her loving support during the writing of this thesis.

CONTENTS

Page No.

Part 1 General Introduction

Introduction 1
1. Insect Endocrinology 2
2. Juvenile hormones 4
3. Occurrence of the ecdysteroids 5
4. Structure and Nomenclature of sterols and ecdysteroids 5
5. Biosynthesis of ecdysteroids 7
6. Mode of action of moulting hormones 10
7. Metabolism of moulting hormones 12
8. Objectives of this study 19

Part 2 General Experimental Techniques

A. Materials

1. Solvents 20
2. Reagents 20
3. Chromatographic materials 21
4. Ecdysteroids 21
5. Radiochemicals 21
6. Antisera 22
7. Others 22

B. Methods

1. Growth of insects 22
2. Preparation of insect diet 24
3. Administration of radioactive cholesterol and ecdysone 26
4. Extraction of insect material 27
5. Separation of ecdysteroids 28
6. Enzymic hydrolysis 28
7. Chromatographic methods 29
8. GC/MS (Gas-liquid chromatography/mass spectrometry) 31
9. Sep-pak C_{18} cartridges 33

Page No.

10. Electrophoresis 33
11. Radioimmunoassay 34
12. Mass spectrometry 37
13. Chemical synthesis of 3-dehydro and 3-epi ecdysteroids 37
14. Chemical derivatisations of ecdsyteroids 38
15. Radioassay procedures 40
16. Protein estimation 41
17. Abbreviations 42

Part 3 Investigation of the ecdysteroids in the pupae of *Spodoptera littoralis*

Section A : The metabolism of [^{3}H] ecdysone and [^{14}C] cholesterol in *S. littoralis* pupae 44

Section B : Identification of ecdysonoic acid and 20-hydroxyecdysonoic acid 58

Section C : Titres of the principal ecdysteroids during the pupal stage of male and female *S. littoralis* determined by high-performance liquid chromatography 71

Section D : Ecdysteroids associated with the ovaries of *S. littoralis* 81

Section E : Discussion of the studies on *S. littoralis* pupae 88

Part 4 Investigation of the ecdysteroids in the sixth instar larvae of *Spodoptera littoralis*

Section A : The metabolism *in vivo* of [23,24 $^{3}H_{2}$] ecdysone in the sixth instar larvae of *S. littoralis* 113

Section B : Titre and identification of ecdysteroids present in sixth instar *S. littoralis* larvae 123

Section C : Developmental variation of the ecdysone metabolising enzymes *in vitro* during the sixth instar larval stage of *S. littoralis* 140

Page No.

Section D : Investigation of the ecdysone 3-epimerisation reaction in *S.littoralis* 152

Section E : Discussion of the studies on *S.littoralis* larvae 166

Part 5 General Discussion 186

Bibliography 194

Appendices

Appendix 1 : Trivial and I.U.P.A.C. equivalent names

Appendix 2 : Mass spectra

PART 1 GENERAL INTRODUCTION

PART 1

Introduction

Of all species of animals, the insects are by far the most numerous and widespread. The diversity of an estimated 1 to 10 million species (Eisner and Wilson, 1977) enables them to occupy a wide variety of ecological niches. Despite their diversity, growth and development in all these species involves successive moults of the cuticle during their life-cycle. The class Insecta can be broadly divided into two categories; the hemimetabolous and the holometabolous insects. Hemimetabolous insects develop through a series of larval moults, accompanied by gradual development and differentiation towards the final moult into the adult form. In the holometabolous insects, no such gradual changes from larval to adult characteristics are observed. In fact,the larval stage of these insects is structurally dissimilar to the adult and often may live in an entirely different environment. A series of larval-larval moults is followed by complete metamorphosis, development into the adult form via a pupal stage, where the larval tissues are largely destroyed and adult tissues formed.

That the initiation of moulting was under hormonal control was first suggested by Kopec (1922) and subsequently proved by the work of Wigglesworth on *Rhodnius* (1934). It was not until 1954 that the hormone was isolated from the pupae of the silkworm, *Bombyx mori* (Butendandt and Karlson, 1954). Subsequently, the moulting hormone, ecdysone, was identified as a pentahydroxysterol in 1965 (Huber and Hoppe, 1965). Since then a number of ecdysone-like compounds have been isolated from insects. These were principally the products of ecdysone metabolism. It is the metabolites of the moulting hormone system which will be investigated in this study using the holometabolous insect *Spodoptera littoralis* (cotton leafworm).

1. Insect Endocrinology

The size and form of insects is limited by the cuticle which surrounds the animal. In some parts, the cuticle is soft and so can stretch and unfold, but surrounding other parts, such as the head and appendages, there are always rigid skeletal structures. Therefore for development and growth to occur, it is necessary for the cuticle to be periodically removed and replaced. This is accomplished by moulting, which involves detachment of the cuticle from the epidermis (apolysis), the formation of a new cuticle and the subsequent shedding of the old cuticle (ecdysis). It is only during this short period, when the epidermis is detached from the cuticle, that development and multiplication of the epidermal cells can take place. Therefore, growth in the insect takes place in cycles, short periods of rapid growth, alternating with longer periods of cuticle formation and more gradual growth.

Control of the moulting cycles and, therefore, of growth and development of the insect is achieved by the production of the moulting hormone. This initiates apolysis (separation of the cuticle and the epidermis), cuticle synthesis and ecdysis.

During the life cycle of insects, moulting hormone acts in concert with juvenile hormone (J.H.) to ensure the correct development of the animal. Increased levels of moulting hormone induce moulting, whereas juvenile hormone modulates this effect by determining the nature of the moult; larval-larval, larval-pupal or pupal-adult.

The secretion of both hormones is controlled by an endocrine system consisting of the brain and associated secretory glands in the head of the insect, and the prothoracic glands located in the prothorax. In response to environmental stimuli, such as photoperiod, distention of the gut and temperature, the neurosecretory cells of the pars inter-

cerebralis areas of the brain secrete a brain hormone (prothoracicotropic hormone, PTTH). PTTH is then transferred to the corpora cardiaca via nerve axons. The corpora cardiaca seems to serve as a storage organ for this secretion, from where it can be released into the haemolymph as required. Alternative sites of PTTH release have also been suggested, viz. the corpus allatum (Agui et al., 1980) and the brain itself (Ishizaki and Suzuki, 1981). Once in the haemolymph, the tropic hormone can stimulate the prothoracic gland cells to synthesise and release the moulting hormone, ecdysone. Ecdysone is believed to act principally as a prohormone, which is activated by its conversion into 20-hydroxyecdysone in certain peripheral tissues of the insect.

The type of moult the insect undergoes is determined by the level of juvenile hormone in the haemolymph. The corpora allata is responsible for the secretion of this hormone, and it is thought that its secretion is inhibited by direct nervous control from the brain and is stimulated by a neurosecretory product of cerebral origin (Scharrer, 1964). The presence of J.H. suppresses adult differentiation in favour of larval structures. In holometabolous insects, a high J.H. titre will result in a larval-larval moult, an intermediate J.H. titre in a larval-pupal moult, and in the virtual absence of J.H. a pupal-adult moult will take place. The 'classical scheme' of hormonal control of insect post-embryonic development is summarised in figure 1-1. Although in the figure the prothoracic glands are shown as the sole physiological source of ecdysone during development, ecdysone synthesis has also been shown to occur in other tissues.

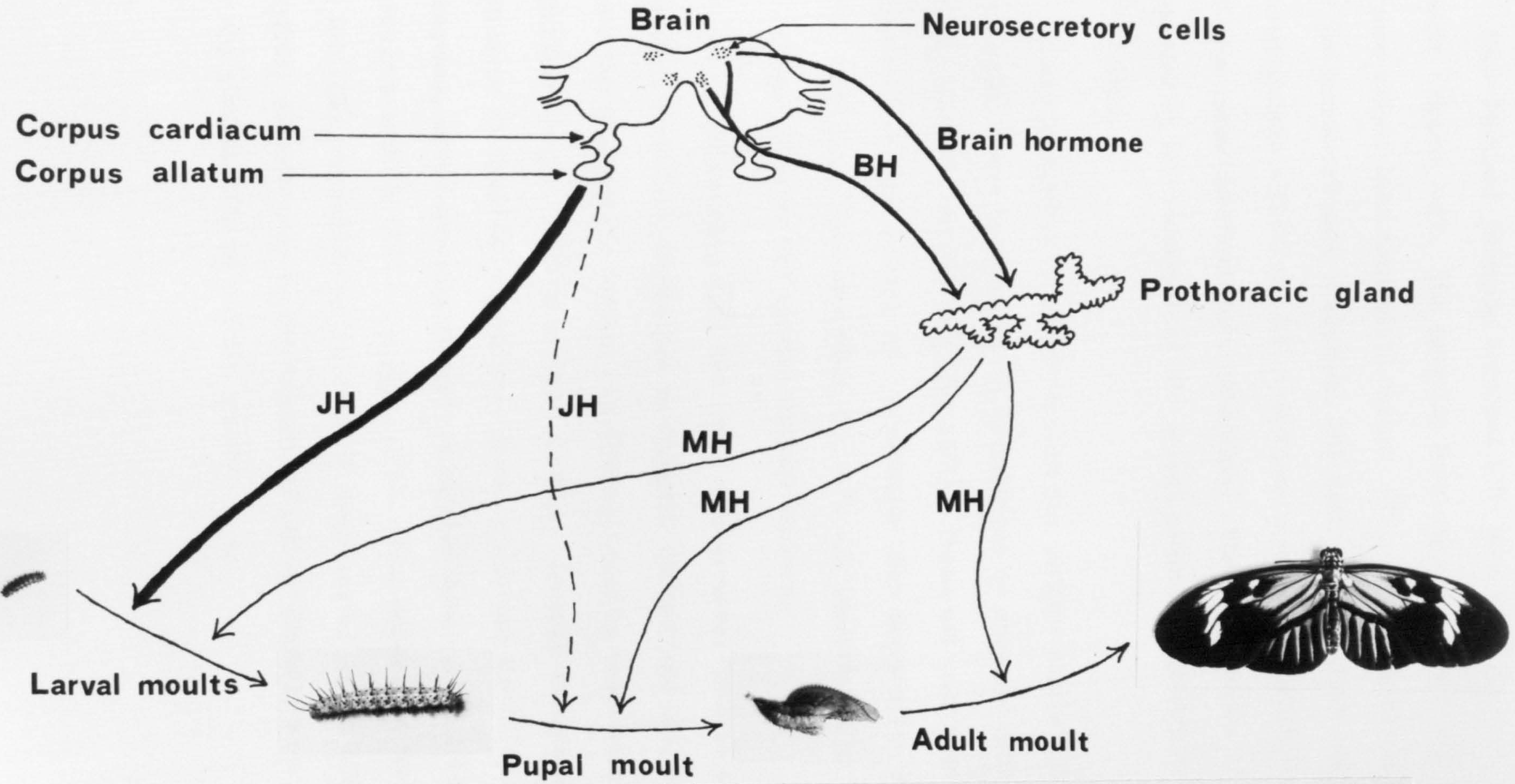


Figure 1-1: The classical scheme for the hormonal control of moulting and metamorphosis during development of a holometabolous insect.

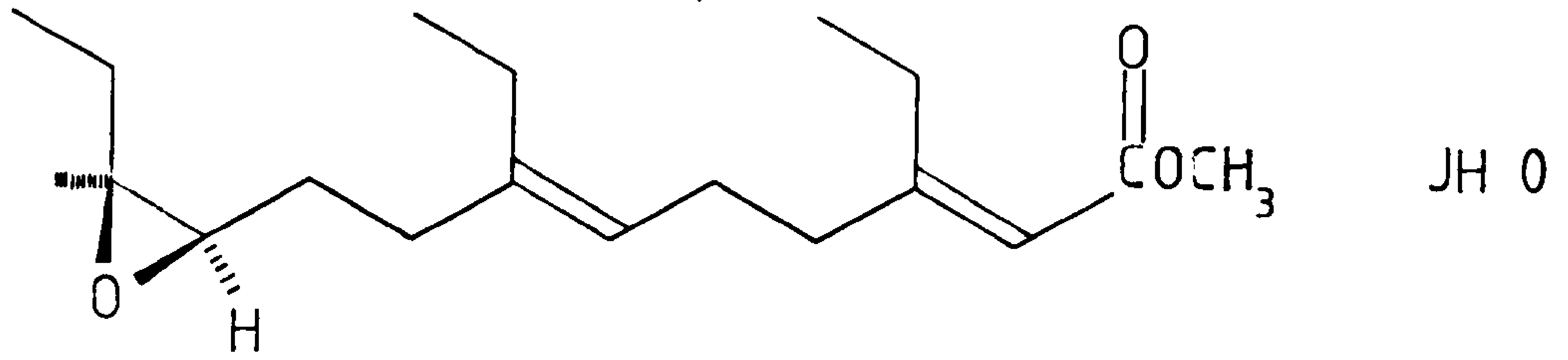
(from Rees, 1977)

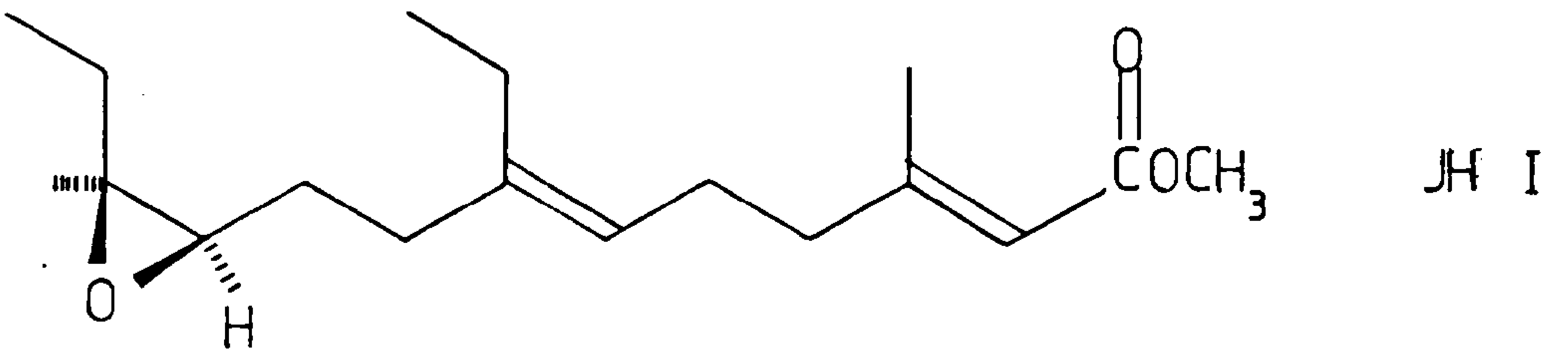
2. Juvenile hormones

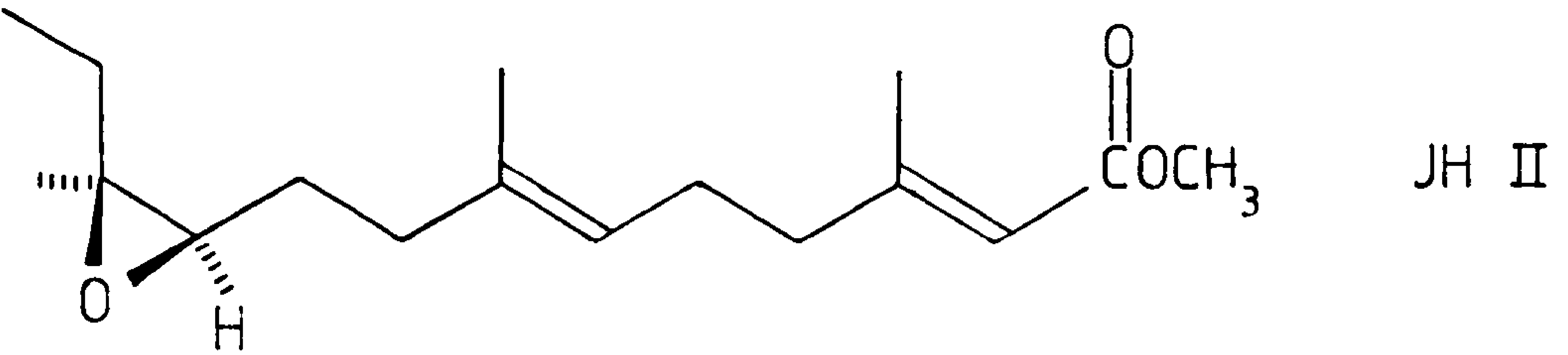
Four distinct juvenile hormones (JH) have been isolated from insects (figure 1-2). The juvenile hormones are synthesised in the corpora allata from isoprenoid units; JH1 arises from one mevalonate and two homomevalonate molecules, JH2 from two mevalonate and one homomevalonate molecules, JH3 from three mevalonate molecules, and JHO from three homomevalonate molecules. The biosynthesis is then completed by the formation of the methyl ester and epoxidation of the $\Delta^{10,11}$ bond.

Juvenile hormone is secreted from the corpora allata into the haemolymph, where the hormone is transported by specific hydrophilic carrier proteins (Kramer and Law, 1980). These not only provide transport but also protect the JH molcules from degradative enzymes. After fulfilling their function, the JH's are inactivated by the action of carboxylesterase and epoxide hydrase enzymes.

It is interesting that two types of esterases, general and JH specific, have been identified in insects (Kramer and Law, 1980). These two degradative enzymes are distinguished by their ability to hydrolyse the JH bound to carrier protein. General esterases cannot hydrolyse JH bound to the carrier protein, whereas the JH specific esterases, which are only present at certain developmental times, can hydrolyse such JH (Kramer *et al*., 1977). This shows that the JH titre is not only controlled by the rate of secretion of JH but also by the activity of esterases in the haemolymph and is possibly also affected by the availability of carrier protein.







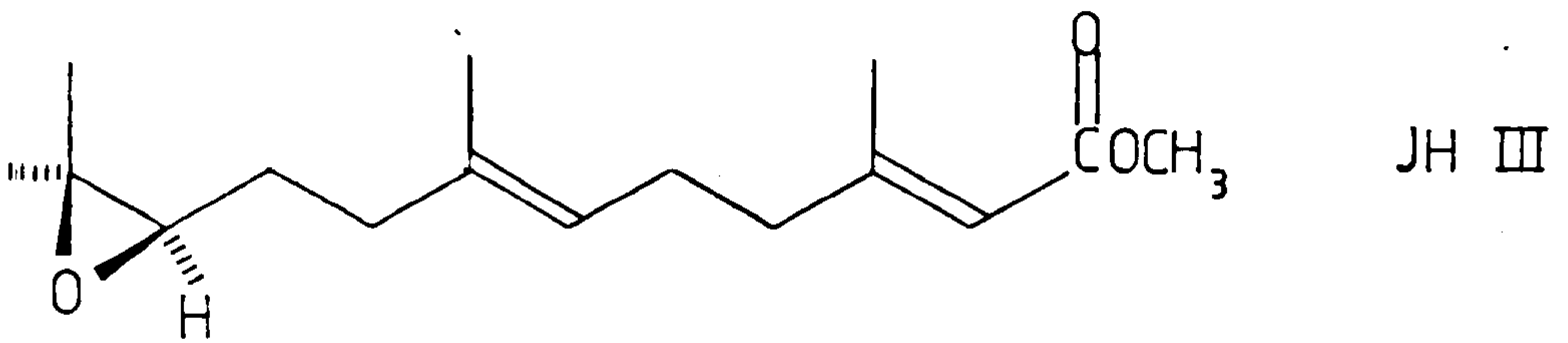


Figure 1-2 : The structure of naturally occurring juvenile hormones

3. Occurrence of the ecdysteroids

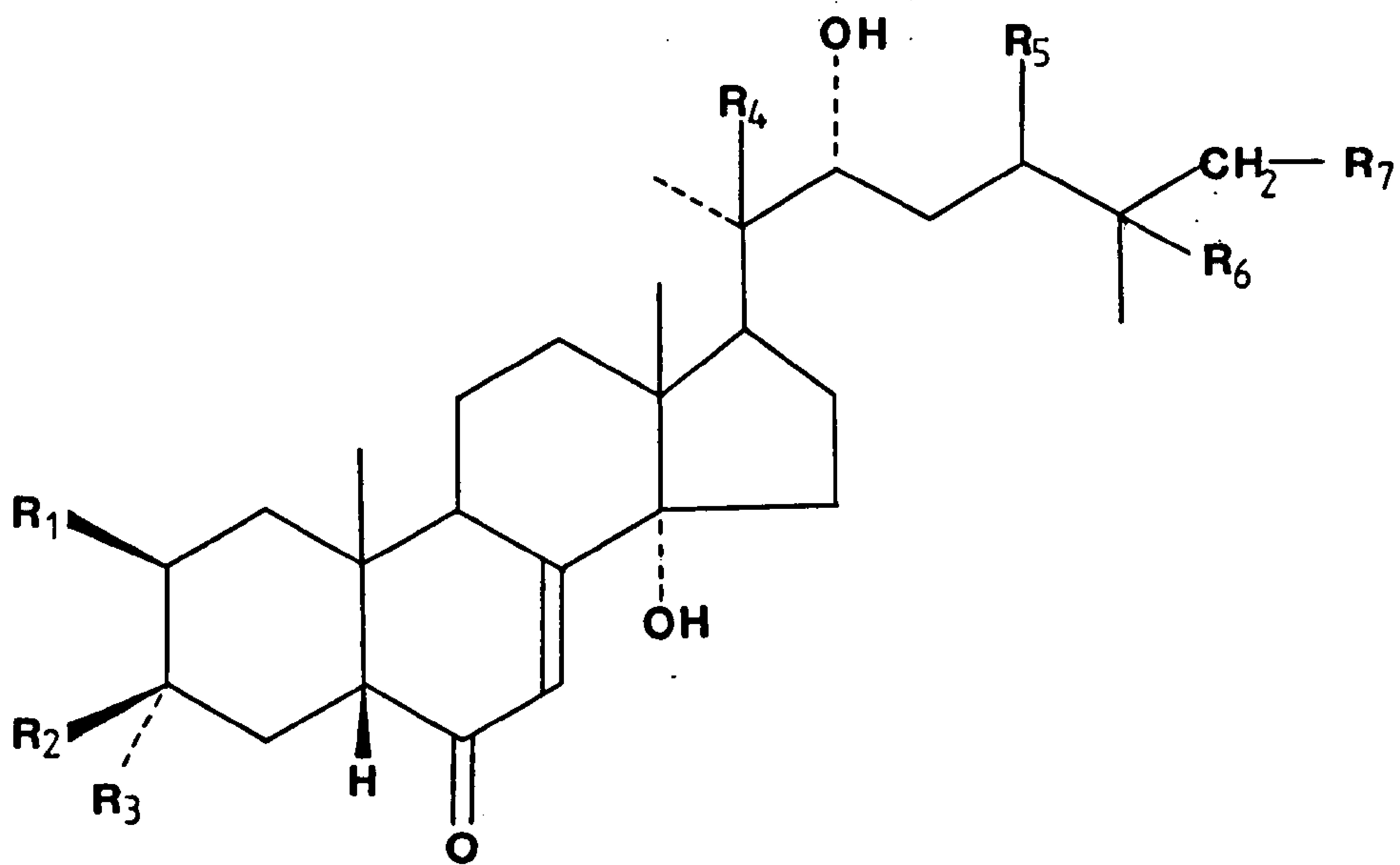
After the initial isolation of ecdysone in 1954, numerous ecdysone-type molecules, or ecdysteroids to give them their generic name (Goodwin *et al.*, 1978), were isolated from various insects and crustaceans. Indeed, ecdysteroids are widespread in Arthropoda and also have been found in some sub-groups of the Nematoda (Mendis *et al.*, 1983), Annelida (King, 1972), and Mollusca (Romer, 1979) Phyla.

20-Hydroxyecdysone was first purified by Karlson in 1956. Subsequently, an entire range of compounds related to ecdysone were discovered. The major invertebrate ecdysteroids can be divided into four main groups based on their structures: the 3-epiecdysteroids, the 26-hydroxyecdysteroids, the 2-deoxyecdysteroids and the 25-deoxy-ecdysteroids. In addition to these, Makisterone A (24-methyl-20-hydroxyecdysone) has also been found in Arthropods (Faux *et al.*, 1969; Kaplanis *et al.*, 1975). These ecdysteroid structures are shown in figure 1-3.

Ecdysteroids have not only been isolated from animals. Certain plants have been shown to contain many ecdysteroids. Indeed, plants have provided a major source of authentic material for studies on insect development and moulting hormone metabolism.

4. Structure and Nomenclature of sterols and ecdysteroids

Sterols: Although this study will concentrate on aspects of ecdysteroid metabolism, sterols are the effective precursors of ecdysteroids and provide the structural skeleton upon which ecdysteroids are based. Therefore, an explanation of the structure of these compounds is necessary.



	R1	R2	R3	R4	R5	R6	R7
Ecdysone	OH	OH	H	H	H	OH	H
20-Hydroxyecdysone	OH	OH	H	OH	H	OH	H
26-Hydroxyecdysone	OH	OH	H	H	H	OH	OH
20,26-Dihydroxyecdysone	OH	OH	H	OH	H	OH	OH
2-Deoxyecdysone	H	OH	H	H	H	OH	H
2-Deoxy-20-hydroxyecdysone	H	OH	H	OH	H	OH	H
3-Epiecdysone	OH	H	OH	H	H	OH	H
3-Epi-20-hydroxyecdysone	OH	H	OH	OH	H	OH	H
3-Epi-26-hydroxyecdysone	OH	H	OH	H	H	OH	OH
3-Epi-20,26-dihydroxyecdysone	OH	H	OH	OH	H	OH	OH
Ponasterone	OH	OH	H	OH	H	H	H
Inokosterone	OH	OH	H	OH	H	H	OH
Makisterone	OH	OH	H	OH	CH_3	OH	H

Figure 1-3 : Ecdysteroids isolated form arthropods

The basic sterol molecule consists of a ring structure containing three six-membered rings, and one five membered ring to which is attached an aliphatic side chain consisting of at least eight carbon atoms (figure 1-4). The nomenclature of structures in the thesis follows the rules recommended in the I.U.P.A.C.- I.U.B. 1971 Definitive Rules for Steroid Nomenclature (1971). This means of notation differentiates between nuclear substituents above the plane of the ring, termed 'β' and denoted by ———, and substituents below the plane, termed 'α' and denoted by ------.

Ecdysteroids: These compounds are polyhydroxylated ketosteroids possessing a 14 α-hydroxy-7-en-6-one system (α, β-unsaturated oxo group). The ecdysteroids referred to in this study all possess a cis fused A/B ring system, which effectively results in a non-planar molecule (figure 1-5). Indeed, this conformational arrangement is critical for moulting hormone activity, 5α-ecdysteroids being hormonally inactive.

The ecdysteroid side chain is linked to the tetracyclic nucleus at C-17 in the β-configuration. An asymmetric carbon centre is generated at C-20. The Sequence Rule Procedure (Cahn et al., 1966) is used to describe the stereochemistry at these points, using the R and S symbols to indicate the three dimensional positions of each substituent. Active moulting hormones all possess a 22R configuration and in 20-hydroxy-ecdysteroids a 20-hydroxy group is present producing a 20R stereochemical center.

Ecdysteroids isolated from Arthropoda which had been identified at the outset of this work are presented in figure 1-3. The I.U.P.A.C. names for the ecdysteroids referred to in this thesis are given in Appendix 1.

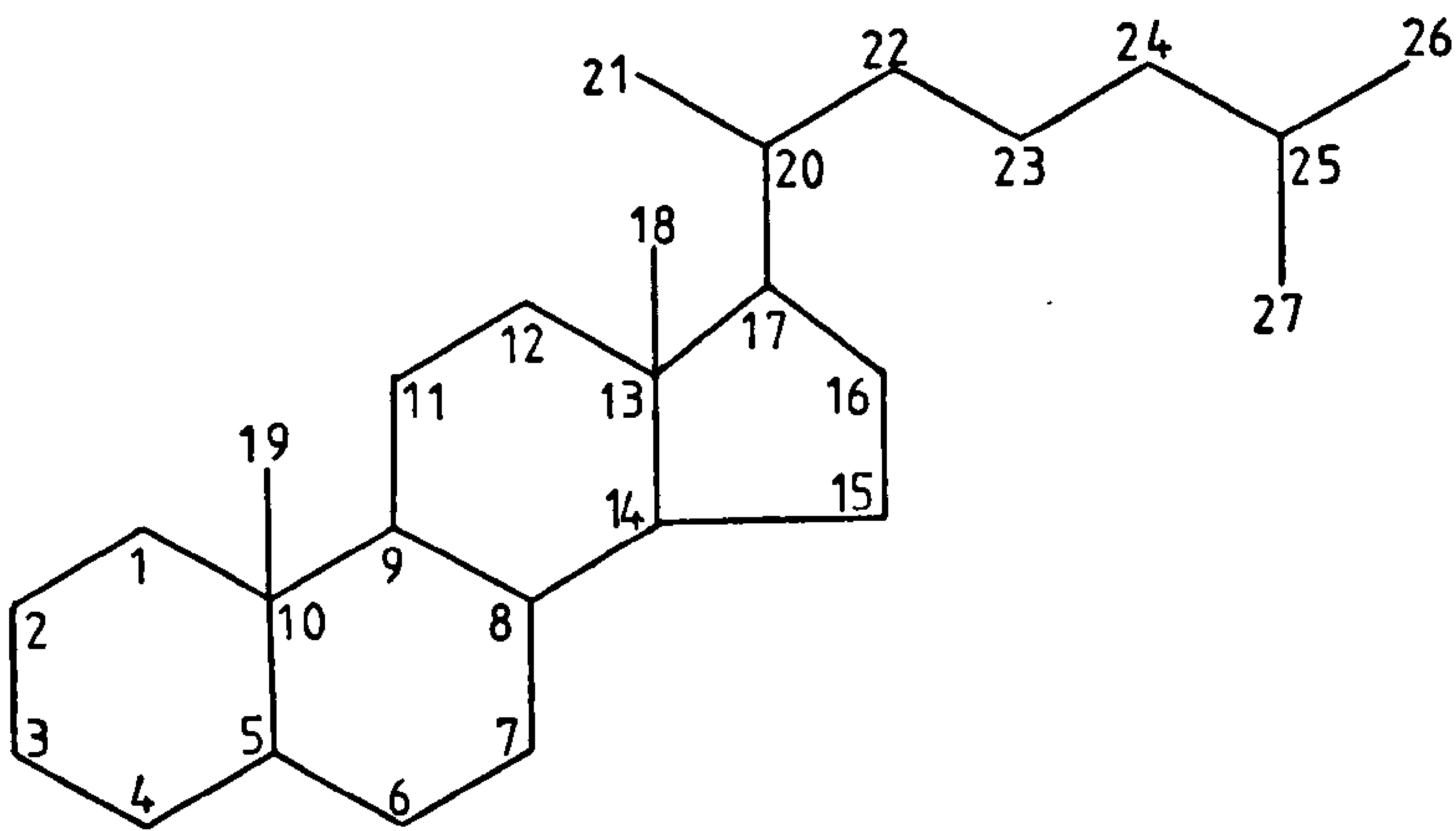


Figure 1-4 : Basic sterol skeleton and numbering

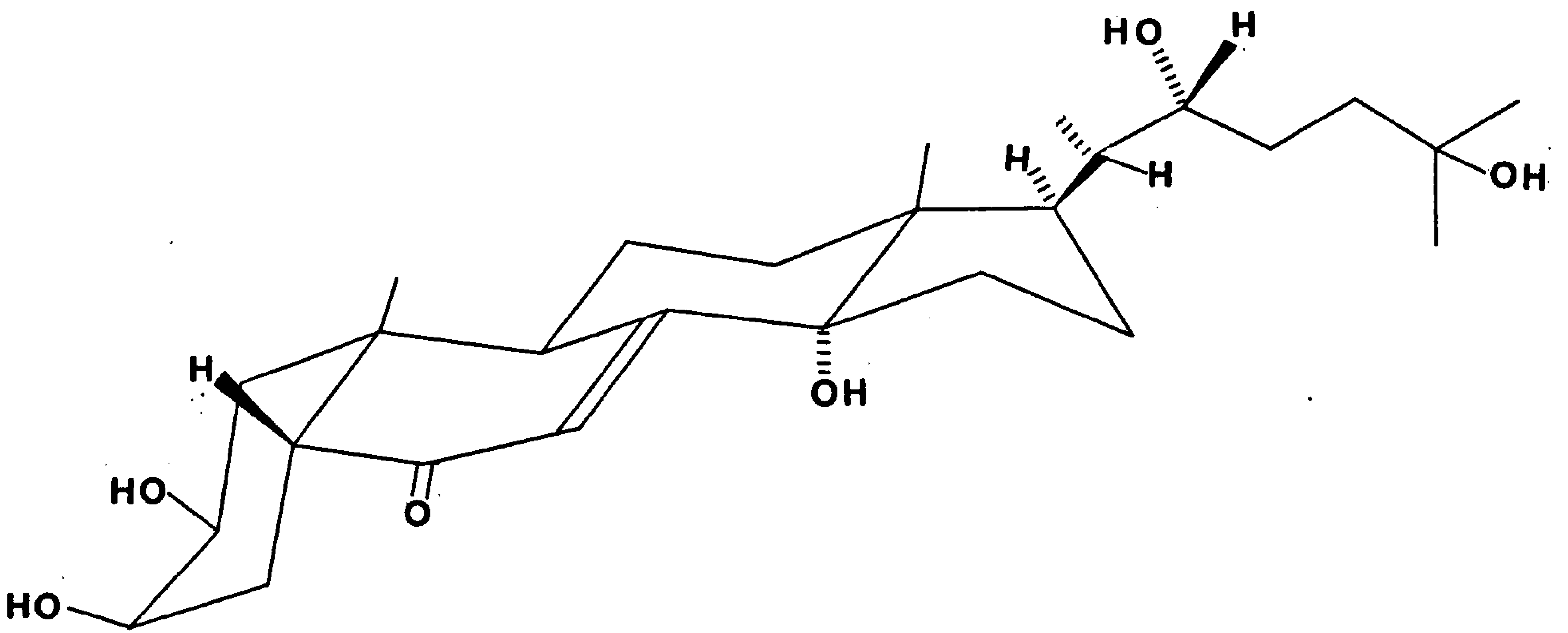


Figure 1-5 : Structure of ecdysone, showing 5β configuration and asymmetric centres

5. Biosynthesis of ecdysteroids

As previously mentioned, sterols are the precursors of ecdysone. Insects cannot synthesize sterols de novo (Clayton, 1964) and must obtain such compounds from the diet. In the case of plant-feeding insects, such as Spodoptera littoralis, sterols are obtained primarily in the form of C_{28} and C_{29} phytosterols, which are characterised by the presence of alkyl groups at C-24. Therefore, to obtain cholesterol, the dietary sterols must be dealkylated by removal of the alkane or alkene substituents from C-24 on the sterol side chain. This process is common to most phytophagous insects, although notably in Oncopeltus fasciatus (Svoboda et al., 1977), Dysdercus fasciatus (Gibson et al., 1983) and Trogoderma granarium (Svoboda et al., 1980), dealkylation of sterols is not achieved.

The conversion of [^{3}H] cholesterol into ecdysone in Calliphora was first reported in 1963 (Karlson and Hoffmeister, 1963). This conversion has also been demonstrated by the incubation in vitro of radioactive cholesterol with prothoracic glands, the principal site of larval ecdysone biosynthesis (Romer et al., 1974; Sakurai et al., 1977). The biosynthesis of ecdysone from cholesterol involves some elaboration of the ring structure before side chain hydroxylation. The sterol nuclear modifications required during formation of ecdysteroids include the introduction of a 6-oxo-Δ^7 feature and the formation of the A/B cis ring junction.

In Calliphora erythrocephala it has been demonstrated that the introduction of the Δ^7 bond occurs via the stereospecific elimination of the cis 7β and 8β hydrogens (Cook et al., 1973). The stereospecificity of the hydrogen elimination is of some significance with regard to the biosynthetic pathway of ecdysone. It has been suggested that during

double bond formation, the presence of electron-withdrawing groups attached to α-carbon atoms promotes the trans elimination of hydrogen atoms (Wilton and Akhtar, 1970). If this is correct, it would appear that the introduction of the 6-oxo group occurs after the removal of the cis 7β and 8β hydrogens.

7-Dehydrocholesterol is, therefore, a possible intermediate in the transformation of cholesterol into ecdysone. Indeed, both cholesterol and 7-dehydrocholesterol were incorporated into 20-hydroxyecdysone in *Calliphora stygia* (Horn *et al.*, 1974) and *Schistocerca gregaria* (Johnson and Rees, 1977). However, because the incorporation of these two compounds was similar and the simultaneous administration of non-radioactive 7-dehydrocholesterol with [^{3}H] cholesterol only slightly reduces the incorporation into ecdysone, it is possible that the biosynthesis of ecdysteroid does not proceed exclusively via a 7-dehydrocholesterol intermediate.

The formation of the A/B cis ring junction appears also to occur during the early stages of ecdysteroid biosynthesis. By analogy to the mechanism involved in the formation of bile acids and some 5β steroids in mammalian systems (for reviews see Samuels and Eik-Nes, 1968; Goad, 1975) a 3-oxo-Δ^{4}-steroid has been proposed as a possible intermediate in the formation of the A/B cis ring junction (Rees *et al.*, 1980). Support for this view was obtained by the incorporation of stereospecifically labelled cholesterols in *S. gregaria* (Davies *et al.*, 1981). This showed that the 3α- and 4β-hydrogens of cholesterol are eliminated during ecdysteroid formation (Davies *et al.*, 1981). As [4-^{14}C] cholest-4-en-3-one is not a 20-hydroxyecdysone precursor in the plant *Podocarpus elatus*, it is therefore possible that a 3-oxo-Δ^{4}-steroid, if involved in the biosynthesis, occurs later in the biosynthetic pathway, probably after introduction of the Δ^{7} bond.

The elucidation of the early parts of ecdysone biosynthesis, basically involving rearrangement of the sterol nucleus, remains largely uncertain and is subject to speculation rather than substantial evidence. This is essentially due to the low levels of ecdysteroids in insects which, consequently, means that intermediates do not accumulate. However, the later stages of moulting hormone synthesis, involving hydroxylation of the side chain, are much better understood.

With the availability of potential deoxyecdysteroid precursors, the hydroxylation sequence of the biosynthetic pathway of ecdysone has been investigated in a number of insect species. In the Lepidopteran, Manduca sexta 22,25-dideoxyecdysone is efficiently converted into ecdysone, 20-hydroxyecdysone, and 20,26-dihydroxyecdysone both in in vivo (Kaplanis et al., 1969, 1974), and in Malpighian tubules and fat body in vitro (King, 1972), in the in vitro systems, metabolism occurs via 22-deoxyecdysone. The efficient conversion of the triol, 22,25-dideoxyecdysone, suggests that hydroxylation at C-2 occurs before side chain hydroxylation (Svoboda et al., 1978). Indeed, in Calliphora stygia prepupae 22,25-dideoxyecdysone was more efficiently metabolized to ecdysteroids than 2,22,25-trideoxyecdysone (Thom son et al., 1971).

22-Deoxyecdysone was converted into ecdysone, 20-hydroxyecdysone and 20,26-dihydroxyecdysone in Manduca prepupae. In addition, this compound has been established as an intermediate of 22,25-dideoxyecdysone metabolism to ecdysone in fat body and Malpighian tubules and in the pupae of Manduca (Kaplanis et al., 1972). This implicates 22-hydroxylation as the ultimate step in the formation of ecdysone, thus, establishing a hydroxylation sequence of C-14, C-2, C-25 and C-22 (figure 1-6).

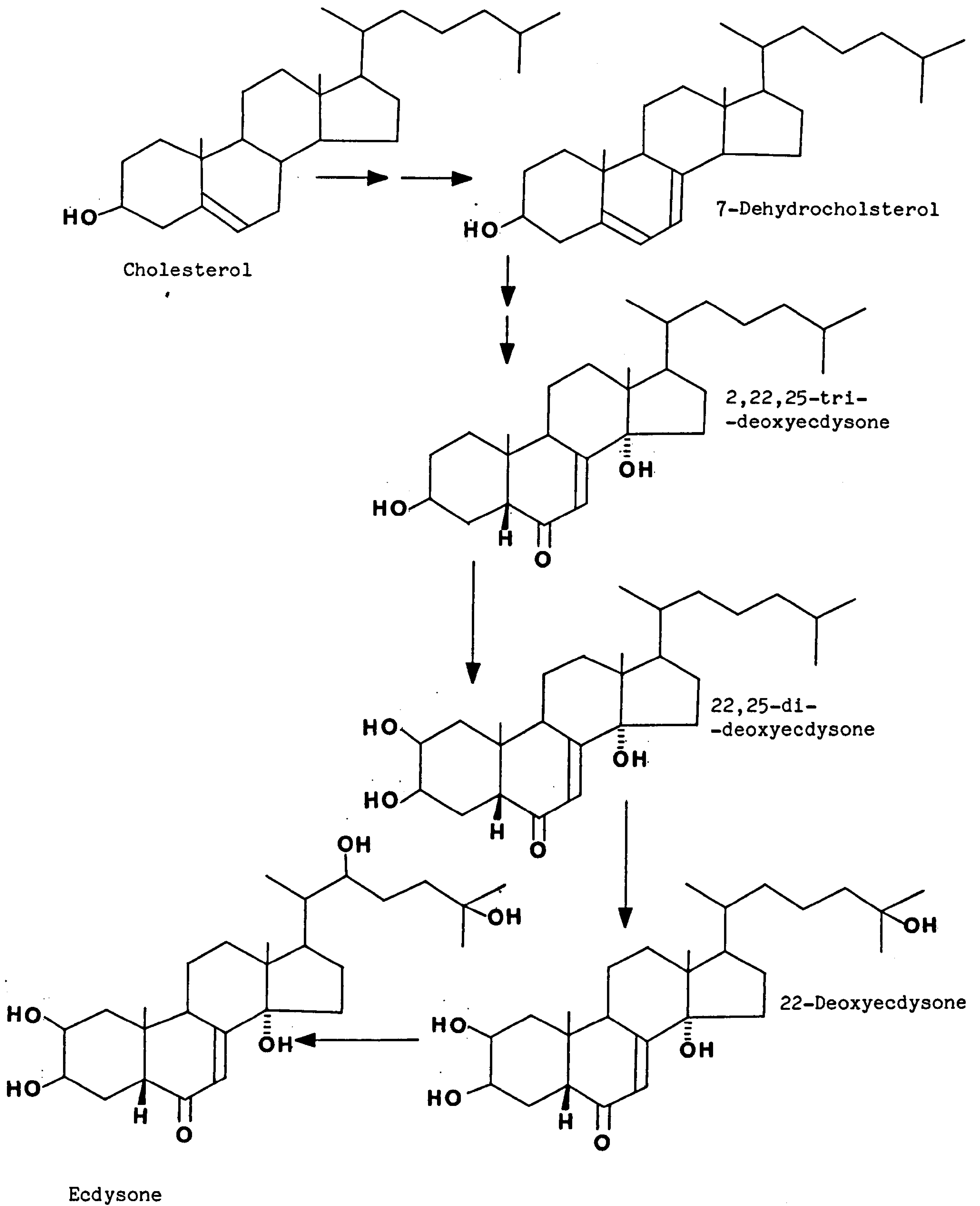


Figure 1-6 : A proposed scheme for ecdysone biosynthesis in insect larvae (Svoboda et al., 1978). Other pathways are also plausible (Rees, 1983).

It must be stressed, however, that the hydroxylation sequence may vary from species to species and depending on the developmental stage of the insect. 2-Deoxyecdysteroids are found in comparatively large amounts in many ovarian systems (Ohnishi et al., 1977, 1981; Hetru et al., 1978; Gande and Morgan, 1979; Dinan and Rees, 1981; Lagueux et al., 1981), strongly suggesting that during ovarian synthesis of ecdysone, 2-hydroxylation is the final step. Indeed, in *Locusta migratoria* ovaries a hydroxylation sequence of C-14, C-25, C-22, C-2 has been experimentally supported (Hoffman et al., 1980).

6. Mode of Action of moulting hormones

Prior to hormonal action at the molecular level in target cells, the overall moulting hormone activity in the insect is governed by synthesis, inactivation and excretion of the moulting hormone. In immature insects, ecdysone is synthesised and secreted into the haemolymph (principally from the prothoracic glands). Once in the haemolymph the moulting hormone can be transported to other tissues, possibly via carrier proteins. At peripheral tissues the ecdysteroid titres are probably controlled by conversion of ecdysone into the hormonally more active 20-hydroxyecdysone and metabolism of both compounds to inactivation products. Therefore, the response of the moulting hormone target cells is considerably influenced by these processes that effectively control the concentration and the type of ecdysteroid in the insect.

The early effect of moulting hormones at target cells has been studied by observation of the effect of ecdysone and 20-hydroxyecdysone on the giant polytene chromosomes in dipteran salivary glands. Polytene chromosomes are produced when repeated cycles of DNA replication occur without cell division. The resulting chromosome structure consists of

a lateral array of many strands of DNA, which is visible under the light microscope.

These structures are of particular interest as gene transcription as individual loci can be seen under the microscope. Before transcription of RNA can occur at any single site on the polytene chromosome the DNA is unfolded into open loops which are visible as 'puffs'. The larger and more diffuse they appear the higher the rate of transcription.

In Chironomus tentans salivary glands intense puffing activity was associated with periods of moulting and at the early stages of metamorphosis. This phenomenon of puffing was also found to be induced by ecdysone (Clever and Karlson, 1960). In salivary glands of both Chironomus (Clever and Karlson, 1960) and Drosophila melanogaster (Ashburner and Richards, 1976) the sequence of 'puffing' in response to moulting hormone was studied in some detail. Based on these observations a model to explain the puffing sequence in Drosophila was constructed (Ashburner and Richards, 1976; figure 1-7). 'Early' puffing is observed within minutes of moulting hormone administration. This puffing responds quantitatively to the concentration of the hormone, requires its continual presence, and does not require protein synthesis. The 'late puffs' appear some hours after hormone administration, these puffs show an all-or-none reaction to the hormone, require protein synthesis, and do not require continuous presence of moulting hormone but do require some previous exposure to the hormone. It was, therefore, postulated that the activated ecdysone-receptor complex induces transcription of the sites of 'early puffs' leading to the synthesis of a product (P) which is either protein or requires protein synthesis for its formation. P then induces 'late puff' activity while inhibiting 'early puffs'. In addition, the activated receptor-ecdysone complex

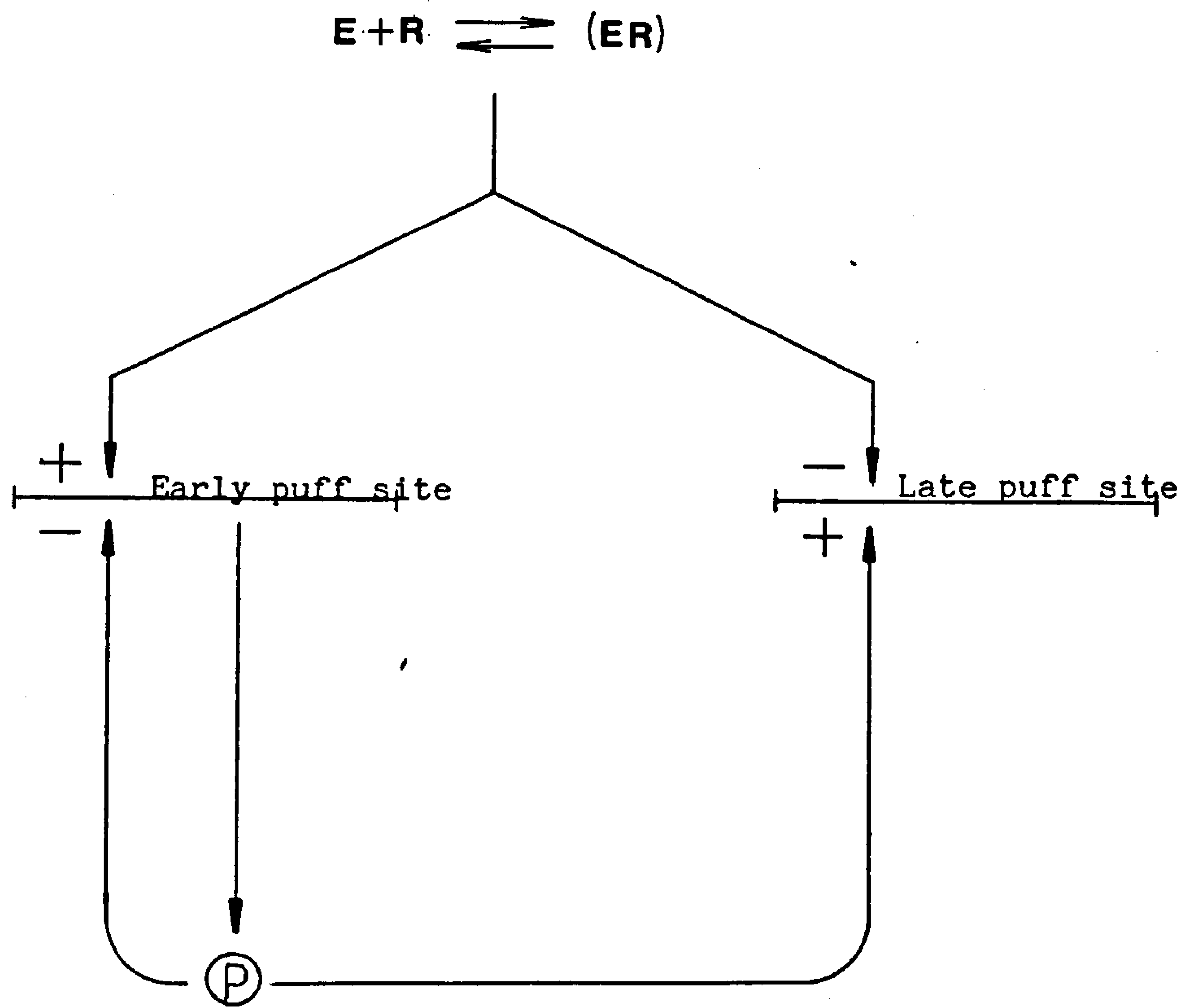


Figure 1-7 : Model for the control of polytene chromosome puffing by ecdysteroids. R : receptor, E : ecdysteroid, P : product requiring protein synthesis.

represses the 'late puff'.

This hypothesis assumes the presence of an ecdysteroid receptor in the target cell and of the direct action of the moulting hormone on the nucleus. Indeed, Gronemeyer and Pongs (1980) have shown ecdysone to be located inside the nucleus bound to chromosomal proteins, and specific ecdysteroid receptors have been shown to be present in both cytoplasm and nucleus of target tissues (Maroy et al., 1978; Yund et al., 1978).

The most crucial step in the effective action of the moulting hormone presumably involves the recognition of the ecdysteroid by the cellular receptor. 20-Hydroxyecdysone has been found to be the most active hormone for the induction of early and late puffs (Ashburner, 1971, 1972). The further metabolism of 20-hydroxyecdysone will presumably reduce ecdysteroid-receptor interaction and, therefore, significantly decrease its moulting hormone activity. However, the solubility and/or binding of the ecdysteroids to haemolymph carrier proteins will possibly also affect the moulting hormone activity. An alternative hormonal role for 20-hydroxyecdysone metabolites cannot be discounted.

7. Metabolism of moulting hormones

The rapidly fluctuating levels of hormone present in the insect are governed by both synthesis and inactivation of the hormone. Therefore, a most important aspect of the control of the endogenous ecdysteroid titre involves the metabolism of moulting hormones. On the one hand, metabolism of ecdysone to 20-hydroxyecdysone causes an increase in moulting hormone activity (Gilbert and King, 1973; Sridhara et al., 1978), whereas a series of other reactions inactivate the moulting hormones. A number of ecdysteroid inactivation compounds have been

isolated from insects, these include 26-hydroxyecdysteroids, 3-epi-ecdysteroids, 3-dehydroecdysteroids and conjugated ecdysteroids. As this thesis is principally concerned with aspects of ecdysone metabolism, the occurrence of moulting hormone metabolites in insects will now be considered in some detail.

20-Hydroxyecdysone: Although ecdysone may have direct hormonal effects, it is essentially considered as a 'pre-hormone' serving as a precursor to the active moulting hormone, 20-hydroxyecdysone (Gilbert and King, 1973; Sridhara et al., 1978). When ecdysone is injected into insects it is rapidly metabolised to 20-hydroxyecdysone (King and Sidall, 1969). Such a conversion has been reported in all insect species analysed showing that this reaction probably occurs in most species of that class. Moulting hormone is secreted from the prothoracic glands in the form of the prohormone, ecdysone (Chino et al., 1974; King et al., 1974). The 20-hydroxylation process has been shown to occur in tissues peripheral to the prothoracic glands (King, 1972). This process has been the subject of considerable study in certain peripheral tissues of Locusta migratoria, Schistocerca gregaria and Manduca sexta. The 20-hydroxylase enzyme appears to be located primarily in the Malpighian tubules and fat body of Schistocerca (Johnson and Rees, 1977). In Manduca sexta (Smith et al., 1980) and Locusta migratoria (Feyereisen and Durst, 1978) these tissues also show considerable 20-hydroxylase activity, with some activity also associated with the midgut tissue (Nigg et al., 1976; Feyereisen and Durst, 1978).

This enzyme appears to be present in both microsomal and mito-chondrial fractions in Manduca sexta fat body and midgut (Kaplanis et al., 1980) and Schistocerca gregaria fat body (tentative: Johnson and

and Rees, 1977; Greenwood, 1982), although in Locusta migratoria Malpighian tubules (Feyereisen and Durst, 1978) 20-hydroxylase is attributed to the microsomal subcellular compartment only.

Recent studies in these insect species have shown that the enzyme has a requirement for molecular oxygen and NADPH (Feyereisen and Durst, 1978; Johnson and Rees, 1977; Smith *et al.*, 1979). The involvement of cytochrome P-450 in this enzymic system has also been established. These results indicate that this enzyme system has similar characteristics to vertebrate steroid hydroxylase systems.

The kinetic properties of the 20-hydroxylase enzyme in the insect tissues, show notable differences. That of the fat body is characterised by a low Km and low Vmax (Bollenbacher *et al.*, 1977) whereas the enzyme associated with midgut or Malpighian tubules tissues shows a high Km value and a high Vmax (Meyer *et al.*, 1978; Johnson and Rees, 1977). This suggests that these tissues have different roles in the control of ecdysone metabolism in the insects.

The stereochemistry of the 20-hydroxyecdysone molecule is characterised by a 20*R* configuration. This shows that it is derived from the cholesterol and ecdysone molecules by the direct replacement of the C-20 hydrogen.

26-Hydroxyecdysteroids: Both 20,26-dihydroxyecdysone (Thompson *et al.*, 1967) and 26-hydroxyecdysone (Kaplanis *et al.*, 1973) were first isolated and characterised from *Manduca sexta* pupae and eggs, respectively. Their involvement in the ecdysone metabolic pathway was confirmed by radioactive tracer experiments. 26-Hydroxylation of 20-hydroxyecdysone was demonstrated in *Calliphora vicina* (Greenwood and Russell, 1978) and 20,26-dihydroxyecdysone was shown to be one of a number of hydroxylated

metabolites of labelled 22,25-dideoxyecdysone in Manduca (King, 1972). In the latter study 26-hydroxylation was also demonstrated to take place in isolated fat body and Malpighian tubules.

26-Hydroxyecdysone and 20,26-dihydroxyecdysone were found as metabolites of radiolabelled ecdysone in Calliphora (Koolman et al., 1979) and Pieris brassicae (Lafont et al., 1980). In addition, 26-hydroxyecdysone can also be converted into 20,26-dihydroxyecdysone (Lafont et al., 1980), demonstrating that side chain hydroxylation does not necessarily follow an ordered sequence. The molecules derived from 26-hydroxylation of ecdysone possess C-25 and C-26 hydroxyl groups. These compounds can therefore exist in the 25R and 25S isomeric forms. Indeed both these stereoisomers of 26-hydroxyecdysone have been tentatively identified as metabolites of radiolabelled ecdysone (Lafont et al., 1980).

As the 26-hydroxyecdysteroids possess approximately 10% of the activity of 20-hydroxyecdysone (Bergamasco and Horn, 1980) they must be considered as moulting hormone inactivation products. However, a possible alternative endocrine function of these ecdysteroids cannot be discounted.

3-Dehydroecdysteroids: 3-Dehydroecdysone (Karlson et al., 1972) and 3-dehydro-20-hydroxyecdysone (Koolman and Spindler, 1977), synthesised by incubation in vitro of ecdysone and 20-hydroxyecdysone, respectively, with Calliphora vicina ecdysone oxidase preparations, have been well characterised in that insect. In a survey of several insect species, the occurrence of ecdysone oxidase has been investigated (Koolman, 1978). The enzyme was shown to occur principally in Dipteran species, although some activity was also detected in Daphnis nerei (Lepidoptera) pupae.

However, it must be noted that in the survey, silica t.l.c. was employed for the enzyme assay. As it has been shown that 3-dehydroecdysone co-chromatographs with ecdysteroid 3 (or 2)-acetates on silica t.l.c. (Isaac et al., 1981), the identification of the products remains to be confirmed.

The formation in vivo of 3-dehydroecdysteroids in radioactive tracer experiments has been reported in several insect species (for review see Koolman, 1982). However, once again, the metabolites were often only identified by silica t.l.c.

3-Epiecdysteroids: 3-Epiecdysteroids differ from the corresponding ecdysteroids only in the configuration of the hydroxy group at C-3. This change in structure considerably reduces the moulting hormone activity of the ecdysteroid. 3-Epiecdysone (10-20%) and 3-epi-20-hydroxyecdysone (7-10%) both show considerably less activity than 20-hydroxyecdysone in the housefly moulting hormone bioassay.

A 3-epiecdysteroid, 3-epi-20-hydroxyecdysone, was first isolated and conclusively characterised from Manduca sexta meconium (Thompson et al., 1974). Subsequently, the 3-epimers of ecdysone, 20-hydroxyecdysone and 20,26-dihydroxyecdysone were all isolated from Manduca sexta pupae (Kaplanis et al., 1979). In that study ecdysteroids were investigated five days after the peak titre of moulting hormone activity in Manduca pupae. The relative abundance of 3-epiecdysteroids at that stage of pupal development lead to the suggestion that 3-epimerisation was a means of inactivation of the more active ecdysteroids.

3-Epiecdysone is also a product of ecdysone metabolism in an in vitro midgut enzyme system from Manduca sexta (Nigg et al., 1974). The enzyme was found in the post-microsomal supernatant fraction and

was shown to be dependant on NADH or NADPH and oxygen (Meyer et al., 1979). It was proposed that the epimerisation of ecdysteroids occurs via a 3-dehydroecdysone intermediate, and Koolman (1976) suggested that the 3-epimerase system could in fact be composed of ecdysone oxidase and a 3-dehydroecdysone reductase. Indeed, when radiolabelled 3-dehydroecdysone was injected into Calliphora vicina an ecdysone-like substance was produced that appeared to be 3-epiecdysone (Koolman, 1980).

Ecdysteroid conjugates: In a number of insect species the formation of ecdysteroid conjugates is a major route of inactivation and excretion of moulting hormones. Large amounts of conjugates have been found in the faeces of insects (e.g. King, 1970, 1972; Weinheimer and Romer, 1977). Various types of conjugate have been reported in the literature: sulphate esters (Koolman et al., 1973), phosphate esters (Sannasi and Karlson, 1974) and glucoside esters (Heinrich and Hoffmeister, 1970). However, in most cases, the identification of these compounds relied solely on enzymic cleavage of the conjugates. As the enzyme preparations have been shown to be highly contaminated with other hydrolytic activity, this means of identification may be insufficient.

The ovaries and eggs of insects have provided an excellent source of ecdysteroid conjugates (for review see Hoffman et al., 1980). Indeed, it is from these sources that ecdysteroid conjugates have been characterised by physicochemical means: C-22 phosphates (Isaac et al., 1983; Tsoupras et al., 1982b) C-3 phosphates and 22-adenosinemono-phosphates (Tsoupras et al., 1982a). It is possible that in these systems, conjugates may serve as a storage form of ecdysteroids, and that hydrolysis of these compounds may be one important factor in the

control of the moulting hormone level during embryogenesis (Isaac *et al.*, 1983).

Conjugates have also been characterised from insects during post-embryonic development. The ecdysteroid conjugates, 3-(or 2-)acetate 22-phosphate and 3-(or 2-) phosphate derivatives of 20-hydroxyecdysone have been identified as metabolites of 20-hydroxyecdysone incubated *in vitro* with gut-Malpighian tubule complexes from the last instar larvae of *Locusta* (Tsoupras *et al.*, 1983).

Other metabolites: In addition to the metabolites already described, the presence of a non-hydrolysable polar ecdysteroid metabolite(s) has been reported in a number of insects: *Manduca sexta* (Moriyama *et al.*, 1970), *Locusta migratoria* (Koolman *et al.*, 1973), *Calliphora vicina* (Sannasi and Karlson, 1974), *Pieris brassicae* (Lafont *et al.*, 1980). The compound from *Locusta* was shown to behave as a carboxylic acid in electrophoresis (Koolman *et al.*, 1973) and the one from *Pieris* showed a pK of 4-5, typical of weak acids (Lafont *et al.*, 1980). Such compounds were therefore suggested to be ecdysteroid-26-oic acids and indeed, this has been confirmed recently (see Part 3, section B).

Side chain cleavage of 20-hydroxyecdysone to give posterone has been demonstrated in prepupae of *Calliphora stygia* (Galbraith *et al.*, 1969) and *Bombyx mori* larvae (Hikino *et al.*, 1975). However, these were only found to constitute minor metabolic pathways.

8. Objectives of this study

This study was aimed principally to investigate the metabolism of ecdysone in the Lepidopteran, *Spodoptera littoralis*. The elucidation of the pathways of inactivation of the active moulting hormones, ecdysone and 20-hydroxyecdysone, was of particular importance. It was also endeavoured to monitor the conversion of ecdysone into the more active 20-hydroxyecdysone and the inactivation of these compounds during the development of the insect. These processes were investigated throughout the developmental stages surrounding metamorphosis (i.e. final larval and pupal stages), thus providing a system where the various metabolic pathways could be monitored and compared during the time when the insect undergoes considerable morphological and physiological change.

PART 2 GENERAL EXPERIMENTAL TECHNIQUES

PART 2

General Experimental Techniques

A. Materials

1. Solvents

Butan-1-ol; Fisons Ltd.

Chloroform; A.R. (May and Baker)

1,2-Dichloroethane; H.p.l.c. grade (Rathburn Chemicals Ltd.)

Ethanol was redistilled

Ethyl Acetate was redistilled

Hexane; Koch Light Laboratories

Isopropanol; A. Gallenkamp and Co.

Methanol was redistilled

Methanol; H.p.l.c. grade (Rathburn Chemicals Ltd.)

Toluene; Koch Light Laboratories

Water; Glass distilled

Acetonitrile; H.p.l.c. grade (Rathburn Chemicals Ltd.)

2. Reagents

Insect diet ingredients:

Agar; Difco Laboratories, Detroit, Michigan, U.S.A.

Casein; B.D.H. Laboratories Ltd.

Wheat Germ (Bemax); Boots Chemist

Dried active yeast; Distillers Co. Ltd., Surrey, U.K.

Corn Oil; bought locally

Streptomycin; Sigma Chemical Co., Poole, Dorset, U.K.

Aureomycin; Cyanamid

Reagents

Diazomethane kindly synthesised by Mr. A. Price and Mr. M. Jolly using an Aldrich Diazald Kit (Aldrich Chemical Co. Ltd.)

Dicyclohexylcarbodiimide; B.D.H. Laboratories Ltd.

Dimethylaminopyridine; Aldrich Chemical Co. Ltd.

Sodium borohydride; B.D.H. Laboratories Ltd.

Glacial Acetic acid; Fisons Ltd.

Platinum IV oxide; B.D.H. Laboratories

Tween 20; Koch Light Laboratories

Activated Charcoal; Sigma Chemical Co.

N-trimethylsilyl Imidazole (TSIM); Pierce Chemical Co., Rockford, Illinois, U.S.A.

3. Chomatographic adsorbents

Silica gel (70-230 mesh, Kieselgel 60) used for column chromatography was obtained from E. Merck, Darmstadt, Germany.

Kieselgel G, Kieselgel GF_{254} (nach Stahl, Type 60) used for t.l.c. were obtained from E. Merck, Darmstadt, Germany

Celite; Koch Light Laboratories Ltd.

Sep-pak cartridges; Waters Associates Ltd.

Partisil-ODS3; Whatman, Maidstone, U.K.

APS-Hypersil; Shandon-Southern Products Ltd., Runcorn, U.K.

Partisil-SAX; Whatman, Maidstone, U.K.

4. Ecdysteroids

Ecdysone; Simes, Milan, Italy

20-Hydroxyecdysone was a gift from Dr. G.B. Russell, D.S.I.R., New Zealand

5. Radiochemicals

[23,24-$^3H_2$(N)] Ecdysone ($\simeq$ 53.6 Ci/mMol); New England Nuclear, Boston, Mass., U.S.A.

[$1\alpha,2\alpha$-$^3H_2$] Cholesterol (50 Ci/mMol); Amersham International, Amersham, Bucks., U.K.

[4-^{14}C] Cholesterol (50 mCi/mMol); Amersham International

6. Antisera

ICT-1 Antiserum was a kind gift from Professor K.D. Spindler, Zoologisches Institute der Techischen Hochsule, Darmstadt, Germany.

DHS 1-15 Antiserum was a kind gift from Professor J.D. O'Connor, University of California, Los Angeles, California, U.S.A.

7. Others

Aryl sulphatase enzyme preparation (prepared from *Helix pomatia*) was purchased from Sigma London Chemical Co. Ltd.

8. *Spodoptera littoralis* (Boisd.)

The insecticide-susceptible laboratory strain of *S.littoralis* (Lepidoptera) used in this work was obtained from I.C.I., Jealotts Hill Research Station, Berkshire, and from the NERC Unit of Invertebrate Pathology, Forestry Institute, University of Oxford.

B. Methods

1. Growth of insects (Clarke, 1981)

A culture of *Spodoptera littoralis* (Cotton leafworm) was reared under licence from the Ministry of Agriculture, Fisheries and Food. The insects were reared in a locked isolation insectary, maintained at a constant temperature of 25°C and 60-70% relative humidity. Lighting was operated on a 16hr "daylight", 8hr "dark" cycle.

Adult moths were kept in waste paper bins lined with tissue paper with lace netting covering the top, held in place with elastic bands. Adults were fed on a 2% (w/v) sucrose solution which was absorbed onto cotton wool in a petri dish. No more than 20 males and 20 females were kept in one bin. Eggs were laid by females in batches on the underside of the netting or on the paper tissue lining. Eggs were washed in 0.2% (v/v) household bleach for exactly 2 minutes, to reduce the risk of virus infection, gently blotted dry, and transferred to a small, transparent, plastic sandwich box (6 cm x 11.5 cm x 17 cm) with a tight fitting lid. The box was lined with tissue paper and contained a small strip of diet. Eggs could be brushed off the lace netting using a small paintbrush, or removed directly on the tissue paper which was cut from the adult cage lining. After approximately 3 days, the 1st instar larvae hatched and fed on the diet contained in the box. When the larvae reached the 3rd instar, they were transferred to a larger transparent plastic box (10 cm x 15 cm x 30 cm) which had two holes in the lid covered with lace netting. This box was also lined with tissue paper, and contained a large strip of diet. No more than 50 insects were kept in these larger boxes. During the 6th and final instar the larvae grew rapidly for 3 days then stopped eating. They decreased in size and weight and after 6 days, they shed their old cuticle and pupated. After pupation the cuticle was soft and almost transparent but after several hours it tanned and hardened to form the mature pupa which neither ate nor excreted but was undergoing extensive metamorphic activity. After 9 days the pupae were soaked in 2% (v/v) bleach for 10 minutes to reduce the risk of viral infection, blotted dry, sexed and

transferred to the adult cages where they emerged and continued the life cycle. Pupae were sexed by studying the rear abdomen on which the male pupae have 2 small swellings (the male genital aperture). Larval insects were transferred to clean boxes containing new strips of diet, on Mondays, Wednesdays and Fridays, after which the boxes were soaked in 2% bleach for an hour before thorough cleaning.

Under these conditions the complete life cycle took approximately 6 weeks. The duration of each stage is shown in Table 2-1.

2. Preparation of insect diet

An artificial agar-based diet was prepared as described by McKinley (1970). Agar (75g) was placed in a double boiler and 2250 ml of glass distilled water added to the inner compartment. The boiler was heated on a gas ring, and the contents stirred occasionally. When the agar had boiled for 30 mins, it was removed from the heat and allowed to cool to 80°C.

While the agar was boiling the following ingredients were mixed together using a mechanical mixer.

Ingredient mix

Casein	132g
Wheat Germ (Bemax)	250.5g
Wesson Salts	37.5g
Sitosterol	3.75g
Active dried yeast	57g
Methyl-4-hydroxybenzoate	3.75g
Sugar	117g

Table 2-1: Life cycle of *S. littoralis* under laboratory conditions (Clarke 1981)

Stage	Duration of instar (days)	Approx. length of insect (mm)	Head capsule (mean width mm)
Eggs	3	0.5	-
1st larval	3	1.0	0.28
2nd larval	3	2.0	0.44
3rd larval	3	5.0	0.68
4th larval	3	10.0	1.17
5th larval	4	20-25	1.69
6th larval	6	25-35	2.51
Pupa	10	15.0	-
Adult	7	-	-

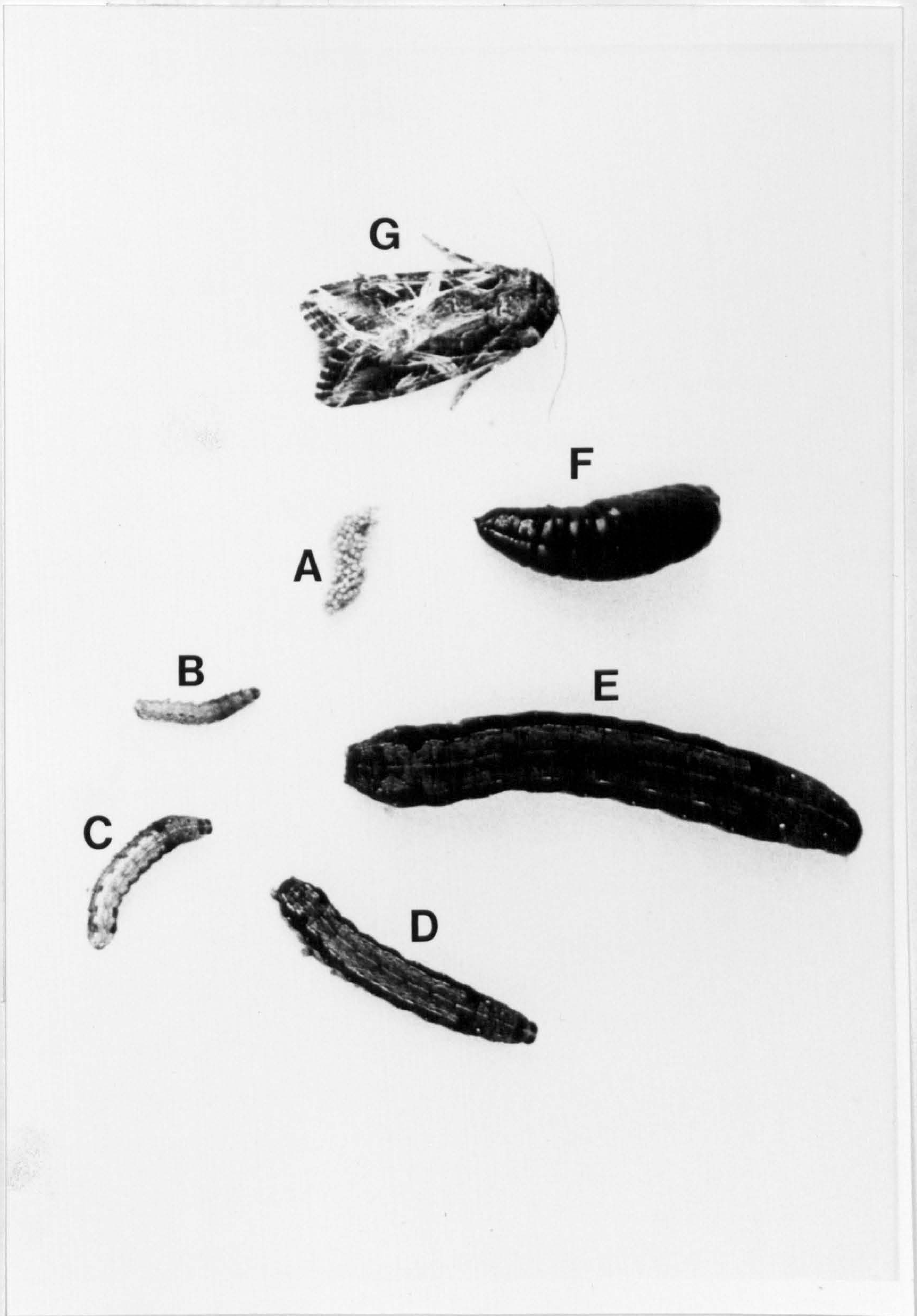


Plate 2-1 : Various stages of *Spodoptera littoralis* life cycle

A:Eggs
B:3rd larval instar
C:4th larval instar
D:5th larval instar
E:6th larval instar
F:Pupa
G:Adult

Sorbic acid	6g
Glass distilled water	1,200 ml
4M Potassium hydroxide	18.75ml
Corn Oil	7.5 ml
10% (v/v) Formaldehyde soln.	16.5 ml

The agar was then mixed well with the "ingredient mix" (637.5g) and choline chloride (3.75g) and the "antibiotic plus vitamin mix" (22.5g) finally mixed into the total mix, which was poured out into 5 polythene storage containers.

When the diet had set, the sealed lids were put on the containers and the diet stored in the cold room at 4°C until used. The diet was generally not stored for more than three weeks.

<u>Wesson Salt Mix</u>

Dipotassium hydrogen phosphate	129g
Calcium carbonate	120g
Sodium chloride	67g
Magnesium Sulphate	40.8g
Calcium hydrogen phosphate	30g
Ferric citrate	11g
Manganese tetrahydrate	2g
Potassium iodide	0,32g
Copper sulphate	0.12g
Zinc chloride	0.10g

<u>Vitamin mix</u>

Nicotinic acid	5g
Calcium pantothenate	5g
Riboflavin (B_2)	2.5g
Aneurine hydrochloride (B_1)	1.25g

Pyridoxine hydrochloride (B_6)	1.25g
Folic acid	1.25g
D Biotin	0.1g
Cyanocobalamine	0.01g

Antibiotic plus vitamin mix

Vitamin mix	2g
Streptomycin	4g
Aureomycin	36g
Ascorbic acid	80g

3. Administration of Radioactive cholesterol and ecdysone

Radioactive cholesterol and ecdysone were administered to insects both in the larval and pupal forms, by injection. Ecdysone solutions to be injected were prepared in insect 'Dipteran' Ringer solution (130 mM NaCl, 4.6mM KCl, 1.9mM $CaCl_2$; Bodenstein, 1946). [23 24-^{3}H] Ecdysone was rigorously purified prior to use. The radioactively labelled ecdysone (the amount being dependant upon the experiment) was applied to a 2g silicic acid column in a small volume (usually 0.5 ml) of chloroform/methanol (19:1 $^v/v$). The column was then sequentially developed with 20 ml aliquots of 0, 8, 15 and 20% ($^v/v$) methanol in chloroform, the 15% methanol in chloroform fraction containing the purified [23,24-^{3}H] ecdysone. The purity of the labelled ecdysone after column chromatography was further ascertained by t.l.c. of a small aliquot followed by radioscanning (0.25mm Kieselgel GF254; developed once with $CHCl_3/CH_3OH$ [4:1]). In cases where minor contaminants were discovered the ecdysone was further purified by this t.l.c. system, and the band co-migrating with authentic ecdysone eluted.

Cholesterol solutions were prepared by emulsifying with ten times the amount of Tween 20 in insect 'Dipteran' Ringer solution.

Each insect was injected with 5 µl of solution, under the cuticle in the thorax region of the larvae, and ventrally between the 1st and 2nd abdominal segments in the pupae. Immediately the syringe was withdrawn, the wound was covered with molten, low melting point paraffin wax, which set hard. Losses due to bleeding were minimal and further development of the insects was not affected by this treatment.

4. Extraction of insect material

Insects to be extracted were first macerated in 100 ml 70% ($^{v}/v$) methanol/water using a Polytron homogeniser for 3 minutes. The homogenate was then centrifuged to separate the extract from the residue, which was re-extracted twice in 70% methanol/water. The residue was further re-extracted three times with methanol and all the extracts combined. If cholesterol and other sterols were required for analysis the residue was further extracted three times with 50% dichloromethane/methanol.

The combined extracts were then evaporated to dryness and the extract partitioned between counter- saturated 70% methanol/water and hexane. The hexane phase was re-extracted once with 70% methanol/water. The 70% methanol/water phase was re-extracted once with hexane.

The 70% methanol/water phase contained the ecdysteroids and the hexane phase contained sterols.

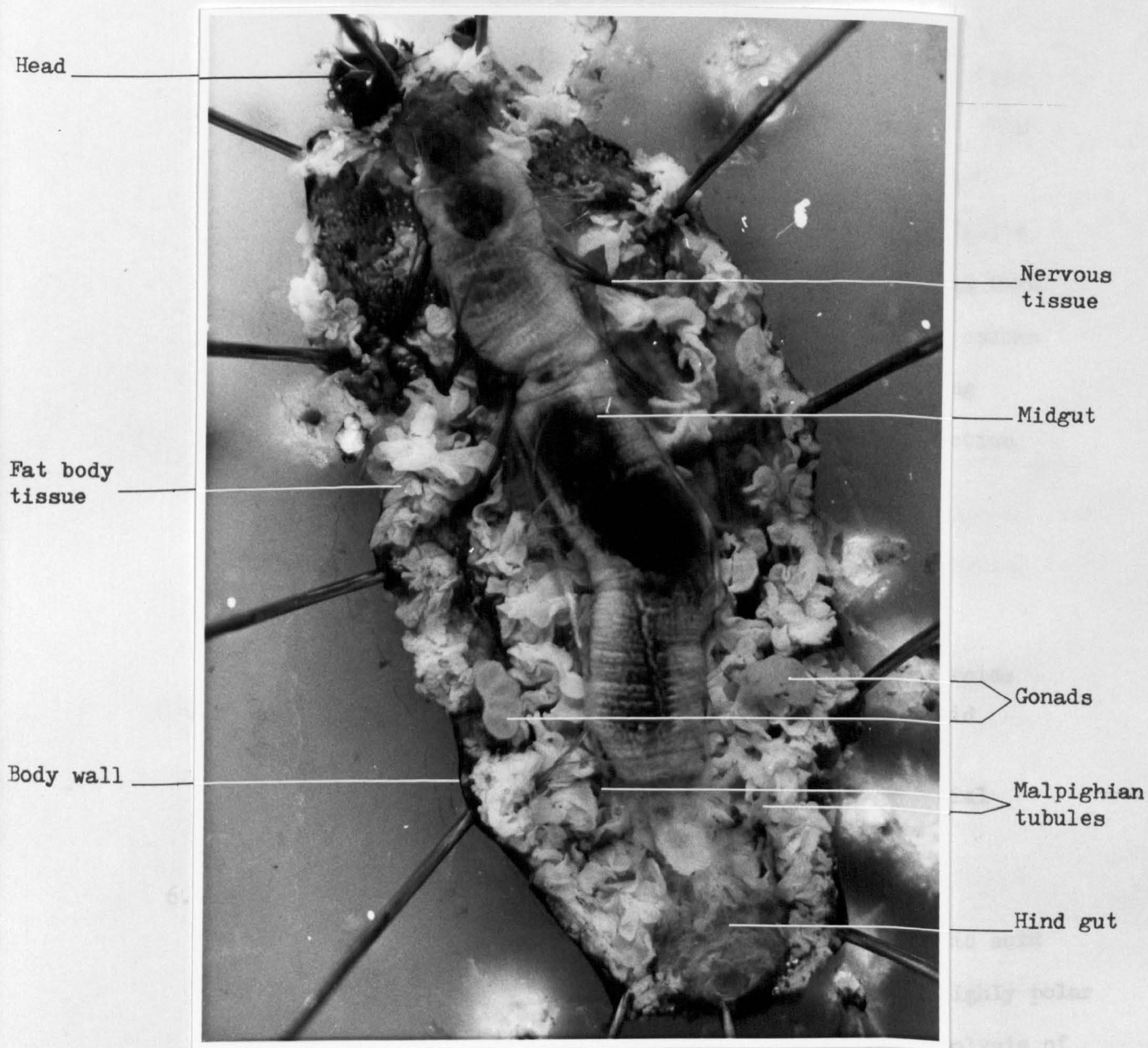


Plate 2-2 : Internal anatomy of *Spodoptera littoralis* sixth instar larvae (3rd day, male).

5. Separation of free and highly polar ecdysteroids

This separation involves the use of a silica column (70-230 mesh, Kieselgel 60 reinst) which was prepared as a slurry in chloroform (10g/100mg sample). The sample was loaded onto the column coated on "Celite". The use of Celite (a calcinated diatomaceous earth) allows both free ecdysteroids and the more polar ecdysteroid conjugates to be applied to the column; the conjugates are virtually insoluble in relatively non-polar solvents. In this procedure the sample was coated onto Celite by evaporating to dryness with the Celite. The Celite was then suspended in chloroform and loaded onto the column. The column was then developed in a stepwise manner with the following solvents (Dinan and Rees, 1981). The volume of each fraction was ten times the weight of adsorbant employed.

Eluting solvent	Compound eluted
Chloroform	Sterols
30% (v/v) Methanol/Chloroform	Free ecdysteroids
80% (v/v) Methanol/Chloroform	Highly polar ecdysteroids (including ecdysteroid conjugates).
Methanol	Residual polar material

6. Enzymic hydrolysis

The 80% (^{v}/v) methanol/chloroform fraction from silicic acid column chromatography of ecdysteroid extracts contains highly polar material including ecdysteroid conjugates. Possible hydrolysis of such conjugates was explored using a number of enzyme preparations: aryl sulphatase (a crude *Helix pomatia* preparation), alkaline phosphatase (crude intestinal mucosa preparation), acid

phosphatase (crude potato preparation). 1mg of the enzyme preparation was dissolved in 1ml of the appropriate buffer (aryl sulphatase in 0.2M sodium acetate, pH 5.9; alkaline phosphatase in 0.2M Tris [hydroxymethyl] methylamine, pH 8.9; acid phosphatase in 0.2M sodium acetate, pH 5.9) and was added to the conjugate dissolved in an equal volume of the respective buffers. The mixture was incubated at 37°C for 16 hrs and the reaction stopped by the addition of ethanol (8 ml).

The products of the reaction (the ecdysteroids released from conjugation) were then separated from the unhydrolysable highly polar ecdysteroids by silicic acid column chromatography as previously described (p. 28).

The overall ecdysteroid isolation procedure, which separates the free ecdysteroids (see p.28), the ecdysteroids released from conjugation and the unhydrolysable highly polar ecdysteroids is summarised in figure 2-1.

7. Chromatographic methods

a. Thin-layer chromatography

Thin-layer chromatography of ecdysteroids was performed using either 0.5 mm or 0.25 mm thick Kieselgel GF254 plates for preparative and analytical purposes, respectively. Before use, the plates were washed once in methanol and then activated at 100°C for 1 hour.

Free ecdysteroids were applied to the plates in methanol and the chromatogram was then developed once with methanol/chloroform (1:4, v/v). Ecdysteroids were visualized under u.v. light (254 nm) and radioactivity was monitored by scanning the plates,

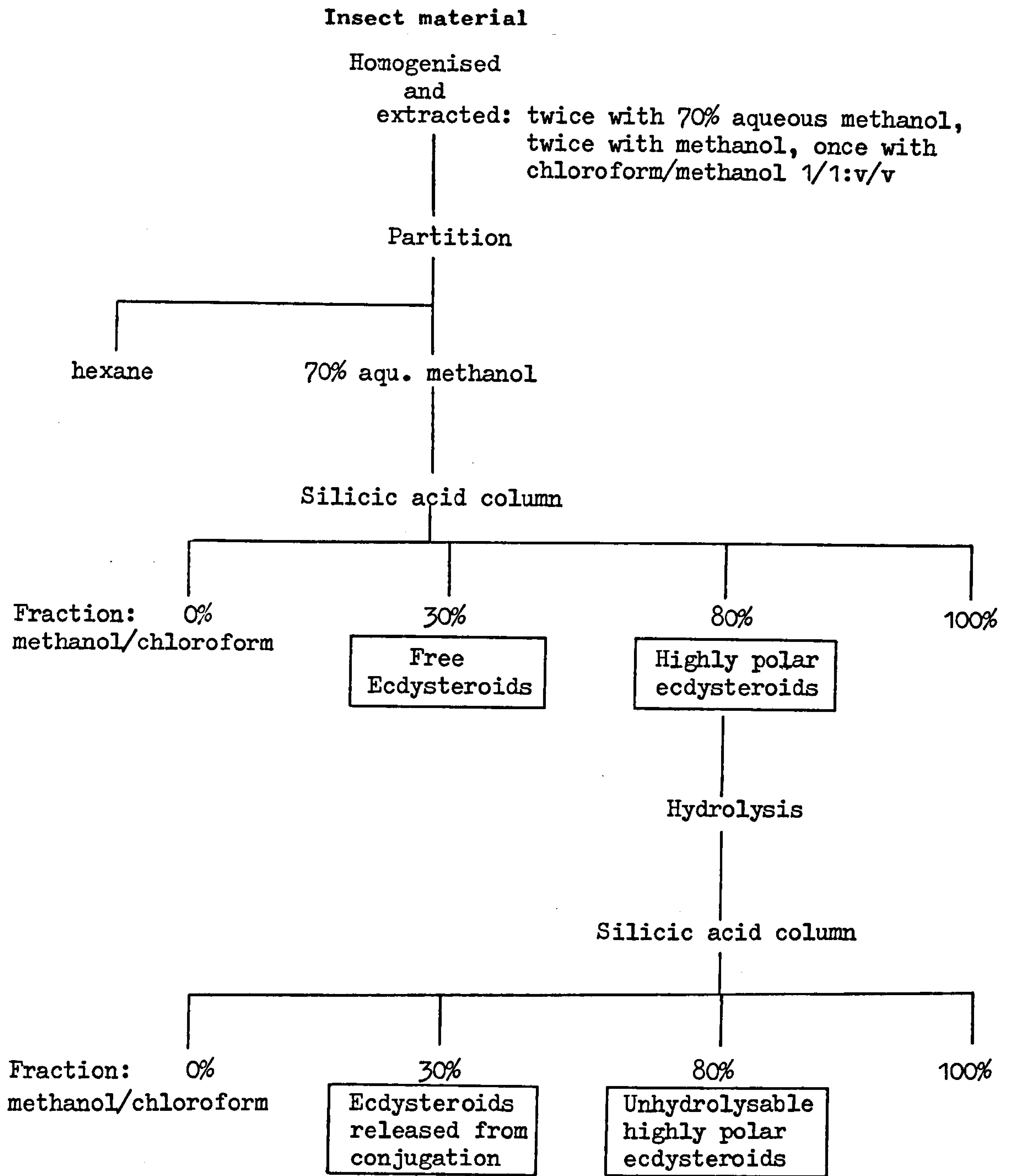


Figure 2-1: Summary of a typical ecdysteroid extraction procedure for *Spodoptera littoralis* pupae or larvae

or by radioassaying 1cm bands of silica scraped from the plate.

For chromatography of very polar metabolites of ecdysone the plates were developed once with an ethyl acetate/ethanol/water (80/20/10, v/v) solvent system.

b. High-performance liquid chromatography

A Waters Associates high-performance liquid chromatography system was used incorporating model M6000A pumps, a single pump for isocratic elution, and two pumps in conjunction with a solvent programmer (model 660) for elution by solvent gradient. The samples were introduced onto the column via either a Waters Associates U6K injector or a Rheodyne Model 7125 injector. For ecdysteroid detection the eluent from the column was passed through either a Perkin-Elmer LC-55 variable wavelength detector set at 242 nm, or, for more sensitive work, a Waters Model 440 fixed wavelength (254 nm) detector was used. Radioactivity was monitored by collecting eluent fractions and assaying these by scintillation counting.

Three types of reversed-phase columns were employed; Partisil ODS-3 (particle size 10 µm, 25 cm x 4.6 mm i.d.; Whatman), Altex Ultrasphere ODS (particle size 5 µm, 15 cm x 4.6 mm i.d.), and a radial compression module ODS column (particle size 5 µm, 10 cm x 5 mm; Waters Associates). Adsorption chromatography employed APS-Hypersil columns (aminopropyl silicone, 25 cm x 4.6 mm i.d.; Shandon-Southern Products) and for anion exchange chromatography Partisil-SAX (strong anion exchange column, 25 cm x 4.6 mm i.d.; Whatman) columns were used. For reversed-phase applications a guard column was used, these were dry packed with Whatman

CO:Pell ODS (octadecyl silica bonded to glass beads).

Apart from the Altex Ultrasphere ODS and radial compression module ODS columns which were purchased ready packed, the columns were packed under pressure in the laboratory using the following procedures.

Partisil ODS-3 - A slurry of 4g of Partisil ODS-3 was made up in 150 ml of propan-2-ol and, using a Haskel fluid pump (Haskel Engineering and Supply Co., Burbank, Calif. U.S.A.), was packed into a steel column (25 cm x 4.6 mm i.d.) using 500 ml of propan-2-ol as packing solvent. Initially 100 ml of solvent was pumped at 85 lb/in^2, then the pressure was increased to 100 lb/in^2 for the following 400 ml of solvent. The packed column was then made ready for use by equilibrating the column with methanol.

APS-Hypersil - A slurry of 3.8g APS-Hypersil packing material was made up in 150 ml 95% methanol/acetone. This was packed into a steel column (25 cm x 4.6 mm i.d.) using 100% acetone. The first 100 ml of packing solvent was pumped at 85 lb/in^2 and the following 400 ml at 100 lb/in^2. The column was then equilibrated first in methanol, then in dichloroethane before use.

8. GC/MS (Gas-liquid chromatography/mass spectrometry)

For gas-liquid chromatographic analysis it is necessary to trimethylsilylate ecdysteroids to produce molecules of sufficient volatility. The derivatisation required is described in section 14c of this chapter, (see p. 39).

The ecdysteroid trimethylsilylethers were separated on a Pye-Unicam 204 gas-liquid chromatograph using the following

column and conditions: column, 1.5m x 2 mm glass column packed with 1% (w/w) OV-1 silicone phase on Gas Chrom Q (100-120 mesh) and silanized before use; injector temperature, 300°C; column, 280°C; inlet pressure of helium carrier gas, 2 Kg cm^{-2}. The retention times of ecdysteroid TMS ethers were measured relative to the internal standard Makisterone A TMS-ether.

For detection of the TMS ethers, the chromatogram was linked via a single-stage all glass jet separator (temperature 280°C) to a VG Micromass 7070F mass spectrometer coupled to a Finnigan Incos data system. Selected ion monitoring (SIM) during elution from the gas chromatograph was achieved by rapid switching of the accelerating voltage (Mendis et al., 1983). Two ions were monitored; m/z 561 and m/z 567.

For quantification of ecdysteroids during SIM a series of standardised mixtures of ecdysone and 20-hydroxyecdysone were also submitted for GC/MS. To both the standard mixture and biological samples a known amount of Makisterone A (5ng) was added prior to trimethylsilylation, thus acting as an internal standard. The peak areas corresponding to ecdysone and 20-hydroxyecdysone were computed and expressed relative to the peak area corresponding to Makisterone A (5ng). Thus, by reference to the chromatograms of standard mixtures the quantities of ecdysone and 20-hydroxyecdysone in the biological samples were calculated.

9. Sep-pak C_{18} Cartridges

For the rapid purification or desalting of samples prior to HPLC, GLC or radioimmunoassay, Sep-pak C_{18} cartridges (purchased from Waters Associates) were often used. The cartridges take the form of small plastic 'mini-columns' which are packed with C_{18} reverse-phase packing material similar to that used in HPLC columns. The cartridges are designed to fit onto the end of standard disposable plastic syringes for elution. The procedure for using the cartridges in the purification of ecdysteroids and ecdysteroid conjugates has been developed previously in this laboratory (Greenwood, 1981) and is summarised below.

The cartridge was first flushed with 4 ml methanol to wet the packing material. 10 ml of water was then passed through the cartridge to activate the packing material. The sample to be purified was then dissolved in 1 ml 10% methanol/water ($^{v}/v$) and loaded onto the cartridge. A further 3 ml of 10% methanol/water was then passed through the cartridge. Conjugates and polar material were then eluted with 4 ml 25-30% methanol/water and 'free' ecdysteroids with 4 ml 60% methanol/water. Less polar material was subsequently eluted by passing methanol (4 ml) and, then, hexane (4 ml) through the cartridge. The fractions were then collected and evaporated to dryness under vacuum prior to further purification steps.

10. Electrophoresis (cellulose acetate)

A Shandon paper electrophoresis tank, filled with 0.25M sodium phosphate pH 7 buffer, was used connected to a Shandon

power pack set at 100 volts (2mA). The radioactive sample was applied in a minimum volume (4μl) of buffer to the centre of a 15 cm cellulose acetate strip, and the voltage applied for 6½ hours.

The developed cellulose acetate strip was then assayed by cutting the strip into 0.25 cm strips and each of these assayed for radioactivity.

11. Radioimmunoassay (R.I.A.)

a. Antisera

Two different antisera were used during the course of this research. Both were produced by administration of hapten-thyroglobulin conjugates to rabbits. The first, ICT-1 (Spindler et al., 1978) used inokosterone-26-oic acid coupled, via the 26 position, to thyroglobulin. The resulting antiserum was more specific for the nucleus of the ecdysteroid molecule than the side chain. The optimum dilution for this antiserum was $^{1}/500$ (Clarke, 1980), giving 47% binding of the [^{3}H] ecdysone employed.

Alternatively, the DHS 1-15 antiserum (Soumoff et al., 1981) was occasionally utilised. This antiserum was produced by using 20-hydroxyecdysone 2-hemisuccinate as hapten, conjugated to thyroglobulin via the hemisuccinate moiety. This yields an antiserum having more specificity for the ecdysteroid side chain rather than the A and B rings of the nucleus. The optimum dilution for this antiserum was found to be $^{1}/500$ (50% binding).

b. Assay procedure

A series of 20 tubes were used for compiling a standard curve. Into each tube a known amount of ecdysone solution (from 0 to 920pg)

was added in duplicates. A further 30 tubes were utilised for the biological samples (15 duplicates) to be analysed.

All 50 samples were then evaporated to dryness in the tubes. To these, 100 µl of [23,24-$^3H_2$] ecdysone solution in 0.1M borate buffer pH 8.4 (4,000 c.p.m. 80 Ci/mMol) was added and each tube vortex mixed. 100 µl of the antiserum, suitably diluted in borate buffer, was then added to each tube and gently mixed. Control tubes containing 100 µl [23,24-$^3H_2$] ecdysone and 100 µl borate buffer were prepared. All the tubes were then incubated overnight at 4°C.

After incubation, 1.0 ml of activated dextran-coated charcoal suspension in borate buffer (1g Norvit A charcoal and 10 mg dextran T40 mixed in borate buffer (1000 ml) for at least 24 hours prior to use) was added to each tube and mixed. After exactly 10 minutes, the tubes were centrifuged at 2000 xg for 10 minutes at 4°C. This separated the ecdysteroids not bound to the anti-bodies (which sediment with the activated charcoal) from the ecdysteroids bound by the antiserum (which remain in solution). 1 ml of the supernatant was then removed from each tube and radioassayed in 'Aquasol' scintillation fluid (4 ml).

c. Analysis of results

The results of the radioassay represent the amount of [23, 24, $^3H_2$] ecdysone bound to the antiserum (expressed in c.p.m.)

The results from the ecdysone standards tubes are then collated to produce a standard curve, which can be expressed graphically by plotting c.p.m. versus the amount of standard ecdysone. Alternatively, an improved graphic representation can be achieved

by plotting $^{Co}/Cx$ (Co: c.p.m. when ecdysone = 0 pg, Cx:c.p.m. when ecdysone = Xpg) versus amount of standard ecdysone in pg (X), which produces a straight line rather than a curve.

The results from the biological samples can then be calculated by reading from the standard curve. The most accurate results are obtained for samples containing 50 to 150 pg of ecdysone equivalents. Whenever possible, the amount of biological sample to be assayed was calculated so to provide approximately 100 pg ecdysone equivalents of immunoreactive material.

d. Validity tests

It is conceivable that during such a sensitive assay, dealing with sub-nanogram levels of ecdysteroids, non-ecdysteroidal components of a biological sample could have some enhancing or inhibitory effects on the binding of the antibody to ecdysteroids. In order to verify that these effects were not influencing the results of the assay, validity tests were employed.

For this test 100 pg ecdysone equivalents of a biological sample was mixed with 80 pg of ecdysone standard (in duplicate samples). The sample was then subjected to radioimmunoassay along with a biological sample not supplemented with ecdysone standard. The results of the assay were then compared. If there is no interference the biological sample without 80pg ecdysone standard should read 100 pg and the biological sample with 80 pg ecdysone standard should read 180 pg. If the difference between the two results was not 80 ng then it must be assumed that some degree of interference was occurring.

12. Mass Spectrometry

Mass spectra were obtained using a VG 70-70F micromass mass spectrometer operated by Dr. M. Rose and Mr. M. Prescott of the Biochemistry Department, Liverpool University.

Mass spectra are given in appendix 2.

13. Chemical synthesis of 3-dehydro and 3-epi-ecdysteroids (Dinan and Rees, 1978)

a. 3-Dehydro-20-hydroxyecdysone: Platinum IV oxide (145 mg) in glacial acetic acid (5 ml) was reduced with hydrogen, and the reduced platinum formed was washed several times with distilled water.

20-Hydroxyecdysone (140 mg) was dissolved in warm distilled water (100 ml), the solution cooled to room temperature, and the reduced platinum added. A gentle stream of oxygen was bubbled continuously through the reaction mixture, which was stirred at room temperature. The progress of the reaction was monitored periodically by thin-layer chromatography. The best yield was obtained after 3½ hours. The reaction was stopped by the addition of methanol (300 ml), the mixture centrifuged and after removal of the supernatant the catalyst pellet was washed twice with dichloromethane/methanol (1/1: v/v). The combined supernatants were then evaporated to dryness under vacuum. The crude reaction products were dissolved in 1 ml of methanol and separated by t.l.c. with dichloromethane/methanol (4/1:v/v) for development, yielding 37mg of 3-dehydro-20-hydroxyecdysone.

b. 3-Dehydroecdysone: The same procedure was used for synthesising 3-dehydroecdysone. 100 mg of ecdysone were used, yielding 31 mg of 3-dehydroecdysone.

c. <u>3-Epi-20-hydroxyecdysone</u>: 3-dehydro-20-hydroxyecdysone (17 mg) in 2 ml of dry ethanol/tetrahydrofuran was treated with $NaBH_4$ (10 mg) and the mixture left at room temperature for 10 minutes. The reaction was then stopped by the addition of one drop of glacial acetic acid and the mixture subjected to t.l.c. with dichloromethane/methanol (4/1:v/v) for development. This system does not clearly separate 20-hydroxyecdysone from the corresponding 3-epimer, but does separate these reaction products from 3-dehydro-20-hydroxyecdysone. The region corresponding to 20-hydroxyecdysone and its 3-epimer was eluted in 1/1:v/v dichloromethane/methanol. This was then applied to t.l.c. plates for continuous elution in 90/15 chloroform/methanol ($^v/v$) for 2¼ hours. Two close u.v. absorbing bands were discernible. The most polar co-chromatographed with authentic 20-hydroxyecdysone. The two bands were eluted, yielding 7 mg of 3-epi 20-hydroxyecdysone and 4 mg of 20-hydroxyecdysone.

d. <u>3-Epiecdysone</u>: The same reaction was performed on 3-dehydroecdysone (15 mg) yielding 6 mg of 3-epiecdysone and 14 mg of ecdysone.

All these products were then further purified by h.p.l.c., and characterised by mass spectrometry and co-chromatography with authentic material on ODS-3 reversed-phase and APS-Hypersil h.p.l.c.

14. <u>Chemical derivatisations of ecdysteroids</u>

a. <u>Methylation by diazomethane</u>. This method was used for the methylation of ecdysteroid-26-oic acids.

The sample was dissolved in 750 µl of methanol and treated with a solution of diazomethane in ether until the yellow colour, characteristic of diazomethane, persisted in the solution. A ratio of methanol to ether of at least 3:1 was maintained to ensure that the ecdysteroids remained in solution. After 15 minutes at room temperature, the reaction was stopped by the addition of a few drops of 0.1M acetic acid in ether until the yellow colour disappeared. The solvents were then evaporated to dryness and the products analysed by h.p.l.c.

b. <u>Methylation method employed for the methylation of cholic acid (Neises and Steglich, 1978)</u>

Cholic acid (5mg) was dissolved in anhydrous methanol (2ml) to which dimethylaminopyridine (DMAP; 11mg) and dicyclohexylcarbodiimide (DCC; 11 mg) were added at room temperature and the mixture stirred for 60 minutes. The solvent was then removed by evaporation under vacuum and the products analysed by t.l.c.

Although cholic acid was efficiently methylated by this method, methylation of ecdysteroid acids was not accomplished. The reason for this is obscure.

c. <u>Silylation of ecdysteroids.</u> Trimethylsilylethers of ecdysteroids for gas liquid chromatographic analysis were prepared using N-trimethylsilylimidazole (TSIM) by a method based on observations of Wilson <u>et al</u>. (1980) and Bielby <u>et al</u>. (1980).

The sample to be silylated, was evaporated to dryness under dry nitrogen in a Reactivial and desiccated under vacuum

for a minimum of 2 hours. TSIM (70 µl) was then added in a glove box filled with dry nitrogen, the Reactivial tightly capped under dry nitrogen and heated for 18 hours at 140°C. The reaction was stopped by the addition of hexane/ethyl acetate (7/3: v/v) and the ecdysteroid TMS ethers isolated by silicic acid column chromatography eluting with hexane/ethyl acetate (7/3:v/v) (Mendis et al., 1983).

15. Radioassay procedures

a. Liquid scintillation counting

Early in the research programme radioactivity of samples was assayed on Intertechnique 3 channel scintillation spectrometers, models ABAC SL30 and ABAC SL40. Radiolabelled samples in 20 ml vials were assayed in 10 ml of Dioxan based scintillant containing 50g naphthalene and 15g of butyl PBD (5-[4-biphenyl]-2-[4-t-butyl phenyl]-1-oxa-3,4-diazole) per litre of dioxan.

^{14}C and ^{3}H activities are expressed in dpm. This was achieved automatically using the computer interfaced with the scintillation counter to subtract background and correct for efficiency and chemical quenching, by means of an external standard source. Corrections for efficiency and chemical quenching were made from quench curves prepared using ^{3}H and ^{14}C n-hexadecane standards and quenching with increasing amounts of carbon tetrachloride. During the majority of the research programme, a Beckmann LS6800 was available and was extensively used for radioactive assays. This instrument did not require quench curves for efficiency calculations as this was achieved by using H# values to determine d.p.m.

b. Thin-layer chromatogram radioscanning

Radioscanning was performed using a Panax RTLS-1A fitted with a P7900A ratemeter. Settings for time constant, Geiger dead-time and slit width were kept the same throughout at 10 seconds, 200 micro-seconds and 2 min, respectively. Plates were normally scanned over 20 mins (for a 20 cm x 20 cm plate). Range settings were determined by the level of radioactivity present on the plate but were generally between 3 and 30 counts per second.

16. Protein estimation - the tannin micromethod for protein determination (Mejbaum-Katzenellenbogen *et al.*, 1958).

Reagents

Tannin: Phenol (2ml) was mixed with 1M HCl (98ml) at 80°C, to which 10g of tannin was added. The mixture was kept warm until all the tannin was dissolved. The solution was then stored in a brown bottle away from sunlight.

Gum Arabic: 0.1g gum arabic (acacia) was dissolved in 100 ml of warm water.

Standard protein solution: (100 µg/ml). Bovine serum albumin was dissolved in 0.9% NaCl solution and made up to 100 ml.

Test procedure

1. 0.25 ml of protein solution was placed in a 5 ml test tube and warmed in a water bath at 30°C for 5 minutes.
2. 0.25 ml of Tannin reagent (pre-warmed at 30°C) was then added and mixed.
3. The mixture was incubated at 30°C for 10 minutes.
4. 2 ml of gum arabic solution was added.

5. The mixture was then cooled to room temperature and the optical density (650 nm) determined using a Unicam SP1800 spectrophotometer.

For each set of biological samples assayed, a set of standard protein solutions (10µg-100µg) were also assayed to produce a standard curve, from which protein content of the biological samples could be determined.

17. Abbreviations

In some instances in this thesis names of ecdysteroids were abbreviated using the following:

E	Ecdysone
20E	20-Hydroxyecdysone
26E	26-Hydroxyecdysone
20,26E	20,26-Dihydroxyecdysone
3eE	3-Epiecdysone
3e20E	3-Epi-20-hydroxyecdysone
3dE	3-Dehydroecdysone
3d20E	3-Dehydro-20-hydroxyecdysone
2dOE	2-Deoxyecdysone
2dO20E	2-Deoxy-20-hydroxyecdysone
Eacid	Ecdyson-26-oic acid
20E acid	20-Hydroxyecdyson-26-oic acid
E-2P	Ecdysone-2-phosphate
20E-2P	20-Hydroxyecdysone-2-phosphate
E-22P	Ecdysone-22-phosphate
20E-22P	20-Hydroxyecdysone-22-phosphate

PART 3 INVESTIGATION OF THE ECDYSTEROIDS IN THE PUPAE OF *SPODOPTERA LITTORALIS*

PART 3

Investigation of the ecdysteroids in the pupae of *Spodoptera littoralis*

		Page No.
Section A	The metabolism of [^{3}H] ecdysone and [^{14}C] cholesterol in *S. littoralis* pupae	44
Section B	Identification of ecdysonoic acid and 20-hydroxyecdysonoic acid	58
Section C	Titres of the principal ecdysteroids during the pupal stage of male and female *S. littoralis* determined by high-performance liquid chromatography	71
Section D	Ecdysteroids associated with the ovaries of *S. littoralis*	81
Section E	Discussion of the studies on *S.littoralis* pupae	88

PART 3 SECTION A

PART 3 SECTION A

The metabolism of [^{3}H] ecdysone and [^{14}C] cholesterol in S. littoralis pupae

Page No.

Introduction

[^{3}H] Ecdysone metabolism 46

[^{14}C] Cholesterol metabolism 47

Experimental and Results

High-performance li quid chromatography systems used in this section 49

1. Metabolism *in vivo* of [23,24 $^3H_2$] ecdysone in the pupae of *S. littoralis* 49

   [23,24 $^3H_2$] Ecdysone injection and extraction 49

   Analysis of the free ecdysteroids 50

   Analysis of the highly polar ecdysteroids 50

   Overall pattern of metabolites 51

2. The metabolism *in vivo* of [^{14}C] cholesterol in pupae of *S. littoralis* 52

   The metabolism *in vivo* of [^{14}C] cholesterol in female pupae (from day 2 to day 6 of pupal development) 52

      Injection of [4-^{14}C] cholesterol and extraction 52

      Analysis of free ecdysteroids 52

      Analysis of the highly polar ecdysteroid fraction 53

      Overall pattern of metabolites 53

   Metabolism *in vivo* of [^{14}C] cholesterol in male pupae (from day 2 to day 9 of pupal development) 54

Page No.

Injection of [4-^{14}C] cholesterol and extraction 54

Overall pattern of metabolites 55

3. Investigation of [^{14}C] cholesterol metabolites detected during *in vivo* incubations that could not be identified as ecdysteroids 55

PART 3 SECTION A

The metabolism of [^{3}H] ecdysone and [^{14}C] cholesterol in *S. littoralis* pupae

INTRODUCTION

[^{3}H] Ecdysone metabolism

To gain information concerning ecdysone inactivation in pupae, the metabolism of injected exogenous [^{3}H] ecdysone was investigated. This served as a qualitative, if not quantitative, analysis of the types of inactivation products formed from ecdysone and, therefore, also served as a basis for further quantitative studies.

The first radiotracer experiments using injected [^{3}H] ecdysone in insects demonstrated that ecdysone is metabolised to 20-hydroxy-ecdysone (King and Siddall, 1969) and to other, then unidentified, more polar compounds (Moriyama *et al.*, 1970). Since then radiotracer experiments have yielded a wide range of ecdysone metabolites consisting of mainly 20 and 26 hydroxlated ecdysteroids, 3-epiecdysteroids and conjugated ecdysteroids: 20,26-dihydroxyecdysone (Greenwood and Russell, 1978), 26-hydroxyecdysone (Koolman *et al.*, 1980), 3-epiecdysone, 3-epi-20-hydroxyecdysone, 3-epi-20,26 dihydroxyecdysone (Lafont *et al.*, 1980). Indeed, 20 and 26 hydroxylated and 3-epimerised ecdysteroids have all been found to occur in the lepidopterans, *Manduca sexta* (Kaplanis *et al.*, 1979) and *Pieris brassicae* (Lafont *et al.*, 1980). Incorporation of [^{3}H] ecdysone into highly polar metabolites has also been observed. Preliminary characterisation of these compounds has suggested that these are sulphates (Koolman *et al.*, 1973), glucosides (Kaplanis *et al.*, 1974), phosphates (Sannasi and Karlson, 1974) and acidic derivatives of ecdysteroids (Koolman *et al.*, 1973) (for further discussion see Part 1).

The free ecdysteroid titre determined by radioimmunoassay in the male and female pupae had previously been determined (Clarke, 1981, see

figure 3A-1). The moulting hormone titre consists of one major peak at day 5 in males, but two major peaks at day 6 and day 8½ in females. The second female peak is suggested to be associated with the ovaries (Clarke, 1981). The earlier female peak and the male peak, are assumed to be due to prothoracic gland synthesis of ecdysone, although direct experimental evidence for this is lacking. The metabolism *in vivo* of [^{3}H] ecdysone was initially investigated at this early pupal stage of development.

As the peaks of immunoreactivity are followed by a rapid decline, it must be assumed that it is at this stage that metabolism of ecdysone is most active. Therefore, [^{3}H] ecdysone was injected at the peak titres (day 5 in the males, day 6 in the females) and the pupae extracted after the decline in titre (day 7).

[^{14}C] Cholesterol metabolism

The conversion *in vivo* of [^{3}H] cholesterol into insect moulting hormone was first demonstrated by Karlson and Hoffmeister (1963) in *C.erythrocephala*, where an 0.0001% incorporation of substrate into ecdysone was observed. Subsequently, Willig *et al*. (1971) demonstrated that [4-^{14}C] cholesterol was also incorporated into 20-hydroxyecdysone as well as into ecdysone. The percentage conversion of radioactive labelled cholesterol into ecdysteroids is low in insect larvae (generally less than 0.12%; Johnson and Rees, (1977)), although incorporation in ovarian systems is considerably higher (Dinan and Rees, 1981). The reason for these low incorporation figures is that moulting hormones occur only in small amounts and are synthesised from a large cholesterol pool. However, the fact that radioactive cholesterol can be incorporated, enables labelled ecdysteroids to be produced *in vivo* in quantities representative of the endogenous ecdysteroids synthesised in the insect.

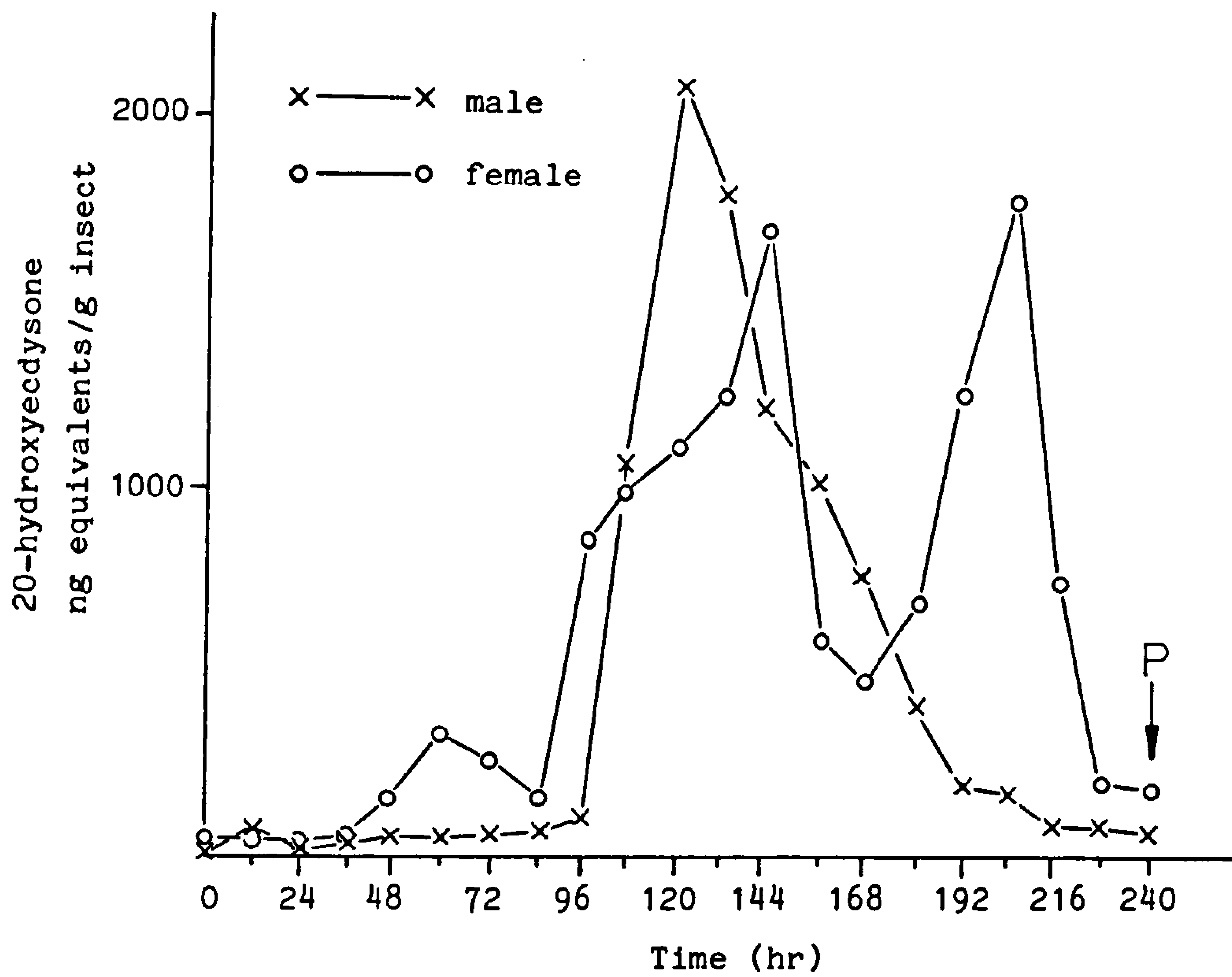


Figure 3A-1: Ecdysteroid titre curve for S. littoralis pupae (ICT-1 antiserum) P= pupation

This provides a more physiological picture of the ecdysteroid complement than would be provided by the metabolism of exogenous [^{3}H] ecdysone. Of course, the incorporation will always be dependent on the rate at which labelled cholesterol reaches the site of ecdysone synthesis.

In the present work, the times of injection and the duration of the *in vivo* incubation of [^{14}C] cholesterol were again based on the immunoreactive ecdysteroid titre (figure 3A-1). The stage at which maximum synthesis occurs should correspond to the rapid increase in the titre up to the peaks, at day 6 in the female, and day 5 in the male. For effective biosynthesis of ecdysone it must also be ensured that cholesterol reaches the site of synthesis before active synthesis occurs. Therefore, [^{14}C] cholesterol was injected at day 2 of female pupal development to allow the substrate to reach the site of ecdysone biosynthesis by the time active synthesis starts at day 3½ (see figure 3A-1). The cholesterol was incubated for 4 days until day 6 of female pupal development when the peak immunoreactive ecdysteroid titre is attained. This experiment gives valuable information on the nature of ecdysteroids present during times of active synthesis. However, it is also desirable to trace the fate of [^{14}C] cholesterol over a period of extensive metabolism of ecdysone. To this end, [^{14}C] cholesterol was injected at day 2 of male pupal development and allowed to incubate for a further 7 days sacrificing pupae at day 9 of development. Thus, the fate of the radioactivity can be monitored over a period of extensive metabolism of ecdysone (from day 5 to day 9 of development: see figure 3A-1).

EXPERIMENTAL AND RESULTS

High-performance liquid chromatography systems used in this section

System 1: Two Partisil ODS-3 reversed-phase columns in series eluted with methanol/water (9:11, v/v) at 1.5 ml/min.

System 2: One Partisil ODS-3 reversed-phase column eluted at 2 ml/min with a linear gradient (30 minutes) of methanol in water (1:19, v/v) changing to methanol in water (7:3, v/v).

System 3: An aminopropyl silicone (APS-Hypersil) column eluted with either 6 or 8% (v/v) methanol in dichloroethane at 2 ml/min.

System 4: A Partisil-ODS-3 reversed-phase column eluted at 2 ml/min with a linear gradient (30 mins) of acetonitrile in 0.02M Tris-perchlorate buffer pH 7, (1:9, v/v) changing to acetonitrile in 0.02M Tris-perchlorate buffer (2:3, v/v).

System 5: A Partisil-OD3 reversed-phase column eluted at 2 ml/min with a linear gradient (30 mins) of 0.02M Tris-perchlorate buffer pH 7 changing to acetonitrile.

1. Metabolism *in vivo* of [23,24 $^3H_2$] ecdysone in the pupae of *S.littoralis*

[23,24 $^3H_2$] Ecdysone injection and extraction

Male and female pupae were to be treated separately, and so 2 groups of *S. littoralis* pupae, 17 females (4.19g) and 10 males (2.26g) were synchronized at pupation and prepared for injection after 5 days (males) and 6 days (females) of pupal development.

Each pupa was injected with 0.54 μCi of [23,24 $^3H_2$] ecdysone (1.6 Ci/mmol). After 24 hours of further development under normal conditions, the insects were sacrificed. The males and females were extracted in

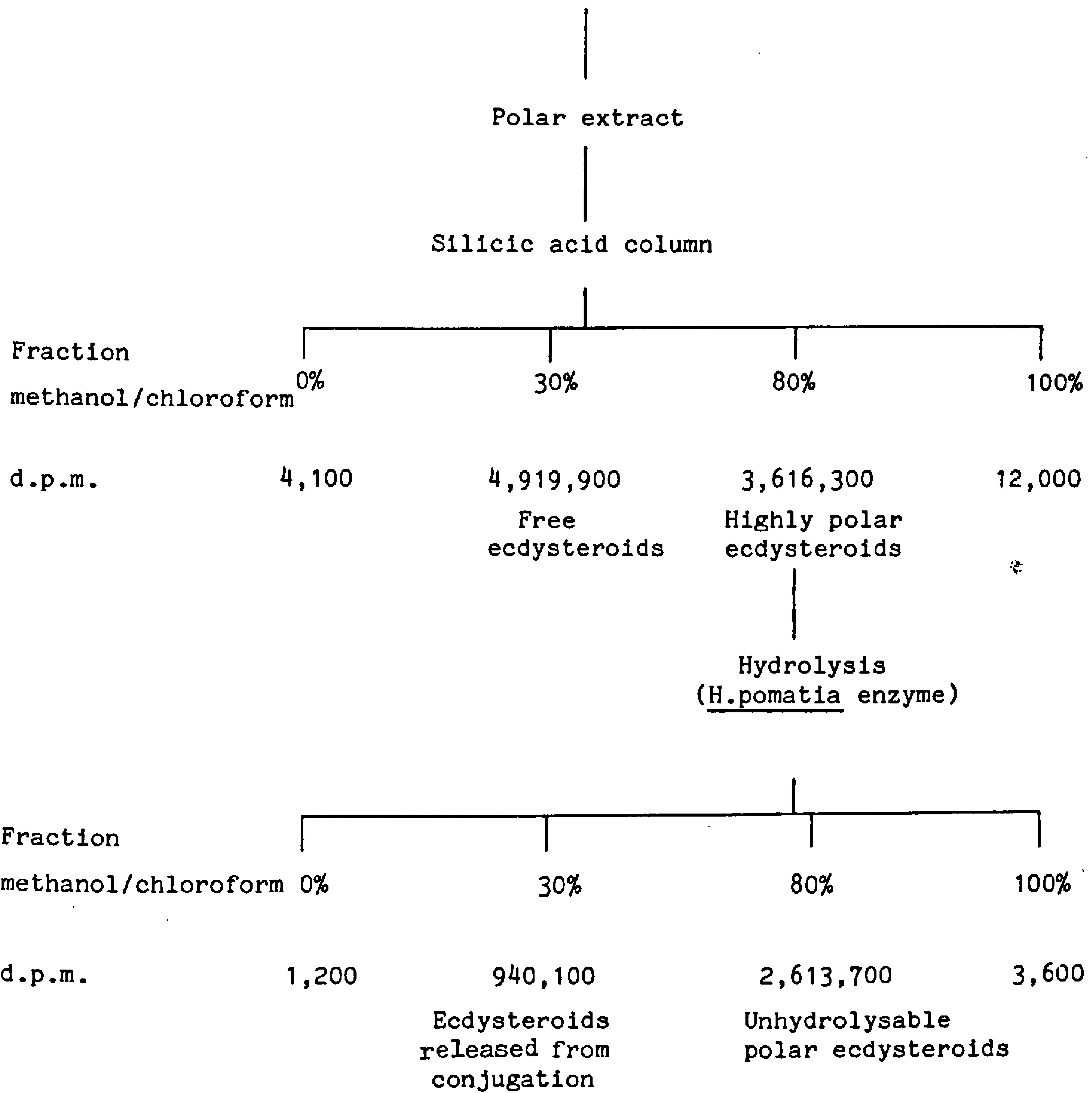


Figure 3A-2 : Summary of the $[^3H]$ ecdysone metabolite distribution in pupae. The distribution of metabolites in both male and female pupae was very similar and numbers represented here have been calculated from the distribution in male pupae.

separate groups by the method described in Part 2. The distribution of the radioactive metabolites after separation on a silicic acid column are shown in figure 3A-2.

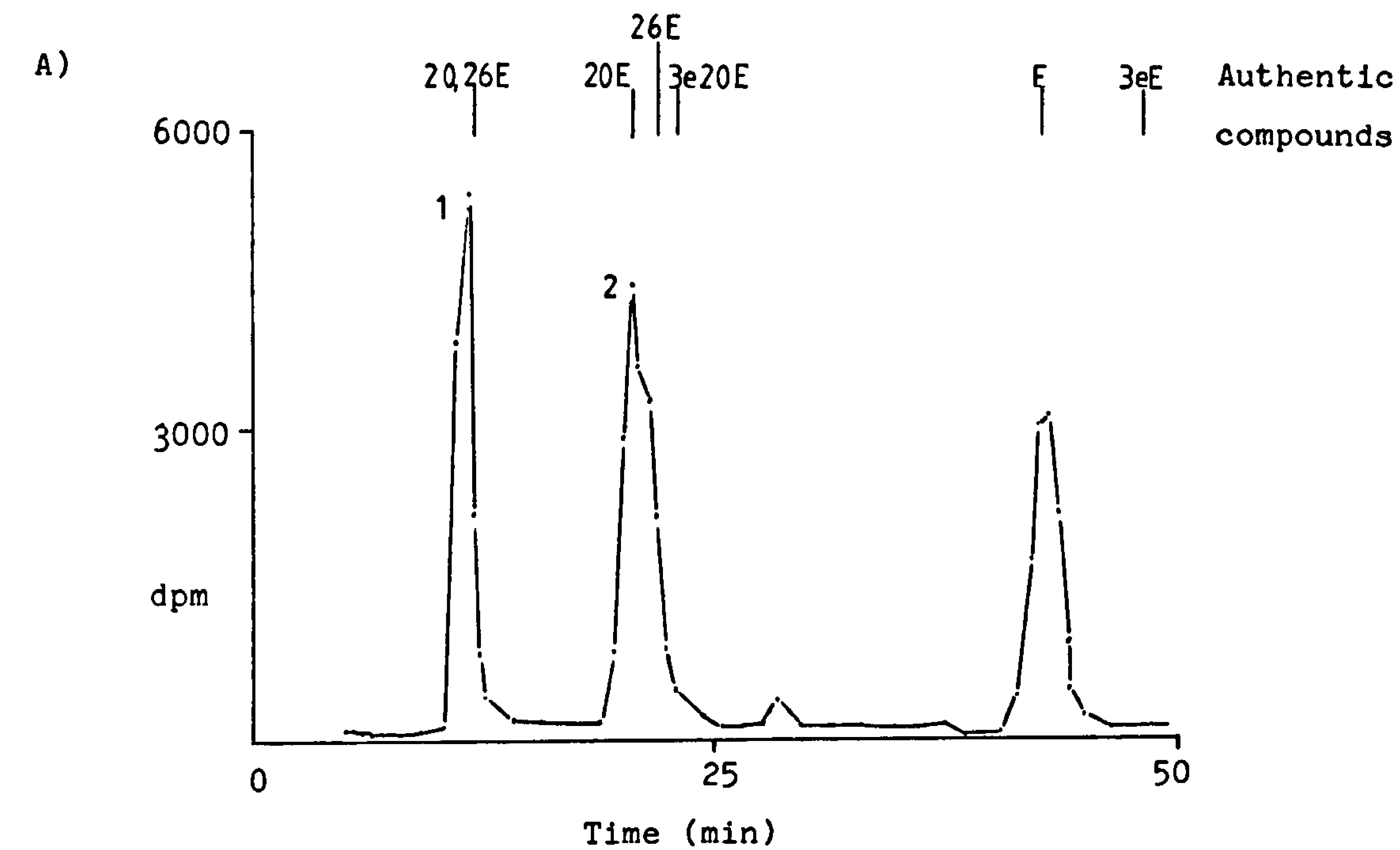
<u>Analysis of the free ecdysteroids (30% methanol/chloroform column fraction)</u>

The [^{3}H] metabolites in this fraction in male and female pupae were analysed using h.p.l.c. system 1 (figure 3A-3). Authentic markers were used to identify the metabolites and radioactivity monitored by scintillation counting. The chromatograms for both fractions (male and female) are shown in figure 3A-3. By co-chromatography of these compounds, collected from reversed-phase h.p.l.c., with authentic material on an APS-Hypersil h.p.l.c. column (system 3), their identities were confirmed as 20,26-dihydroxyecdysone (figure 3A-4(a)) 20-hydroxyecdysone, and 26-hydroxyecdysone (figure 3A-4(b)).

<u>Analysis of the highly polar ecdysteroids (80% methanol/chloroform column fraction)</u>

To identify the ecdysteroid moieties of any conjugates, the highly polar fractions were treated with the <u>Helix pomatia</u> enzyme preparation (crude aryl sulphatase enzyme preparation) and the products of the hydrolysis were separated by silicic acid column chromatography.

The 30% methanol/chloroform fractions (containing ecdysteroids released from conjugation by hydrolysis) were analysed by h.p.l.c. system 1. This fraction from both male and female pupae produced similar elution profiles (the fraction from the male pupae is represented in figure 3A-5). The peaks of radioactivity corresponding to 20,26-dihydroxyecdysone, 20-hydroxyecdysone, 26-hydroxyecdysone, ecdysone, and 3-epiecdysone were collected and re-chromatographed on h.p.l.c. system 3



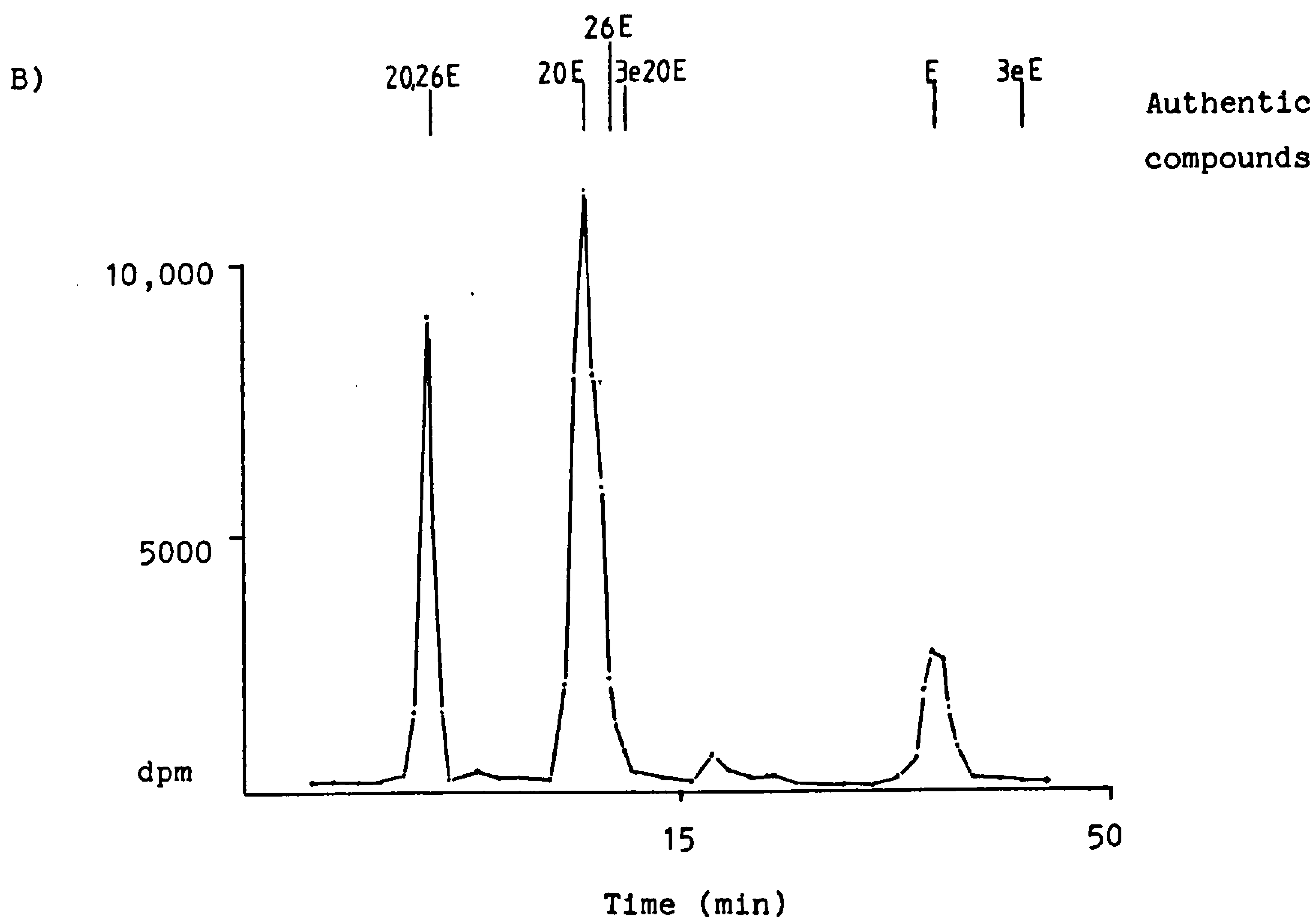


Figure 3A-3 : H.p.l.c. analysis of the free ecdysteroid metabolites of [^{3}H] ecdysone in female (A) and male (B) insects, using system 1. Authentic markers were used to identify the peaks.

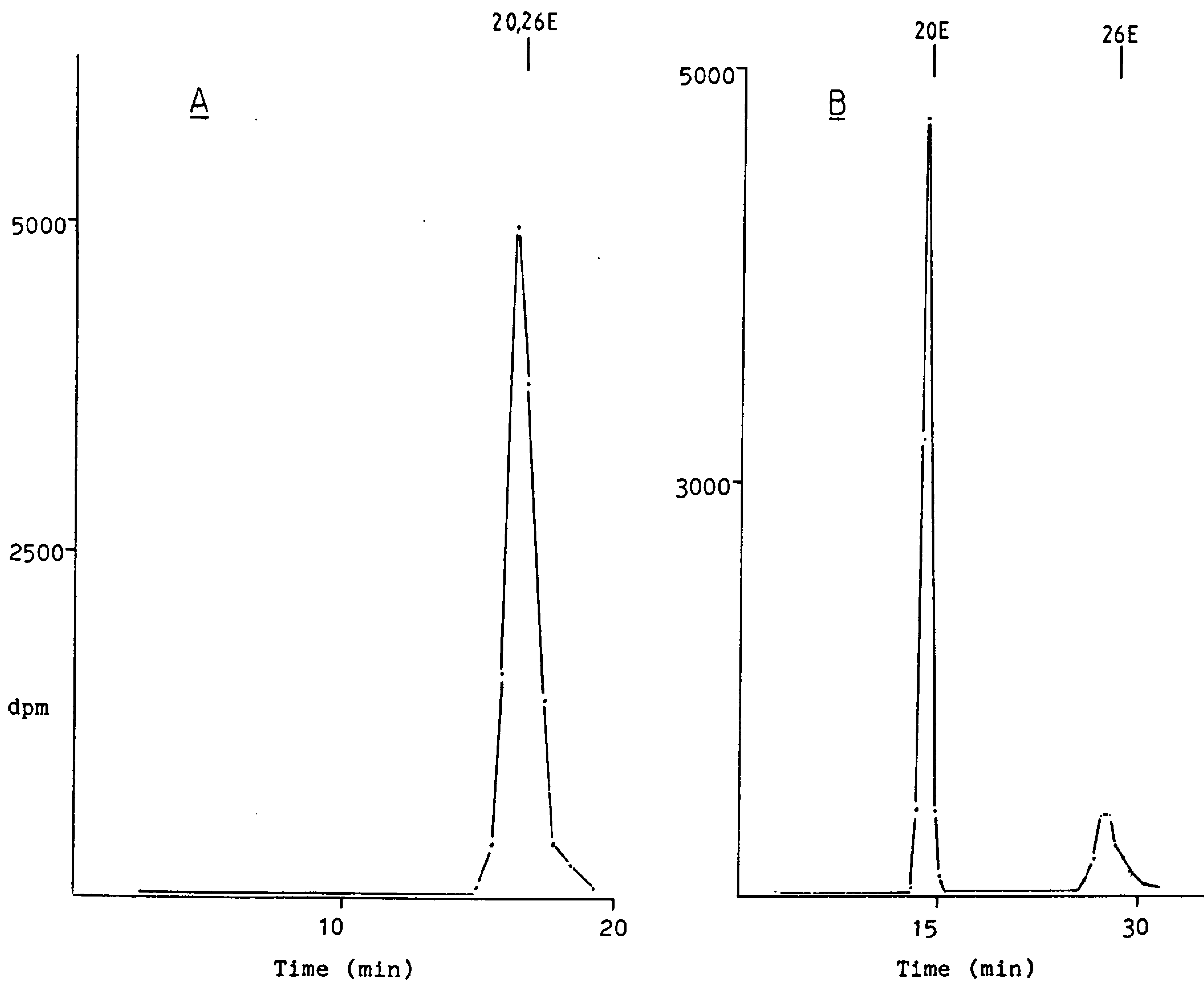


Figure 3A-4 : A) Re-chromatography of 20,26-dihydroxyecdysone [peak 1, figure 3A-3(A)] on h.p.l.c. using system 3 (8% methanol in dichloroethane).

B) Re-chromatography of 20-hydroxyecdysone and 26-hydroxyecdysone [peak 2, figure 3A-3(A)] on h.p.l.c. using system 3 (6% methanol in dichloroethane).

Authentic markers were used to identify the peaks of radioactivity.

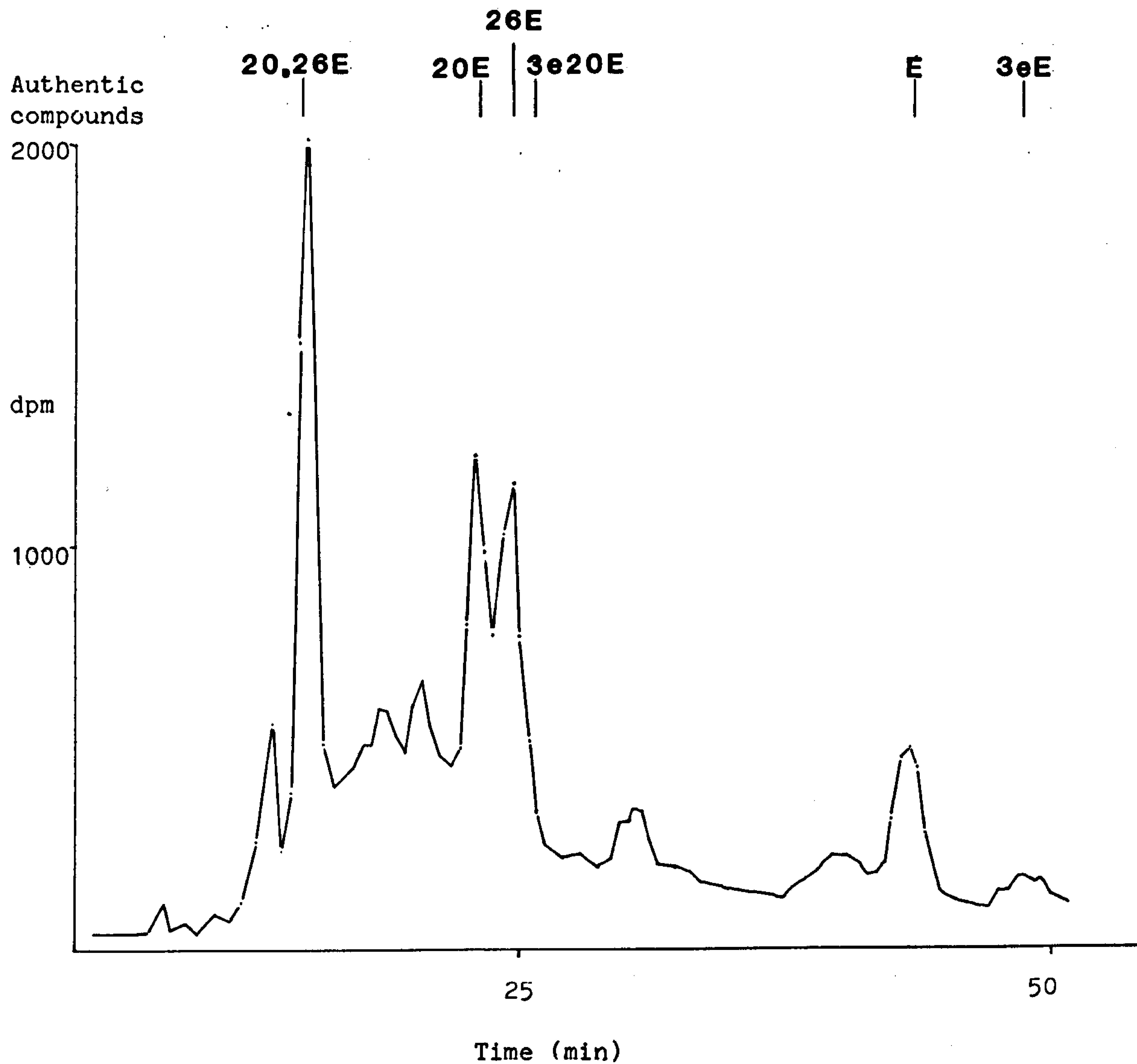


Figure 3A-5 : H.p.l.c. analysis of the [^{3}H] ecdysteroids released from the highly polar ecdysteroid fraction (males) by the *Helix* enzyme.

(adsorption chromatography) with authentic ecdysteroids as markers, and indeed this confirmed their identities.

Only a small proportion of the highly polar metabolites subjected to hydrolysis were hydrolysed (less than 30%), the majority remaining as unhydrolysable polar metabolites (eluting from a silicic acid column in the 80% methanol/chloroform fraction) (figure 3A-2). This fraction was chromatographed using h.p.l.c. system 2, revealing the presence of two polar compounds (figure 3A-6) subsequently identified as 20 hydroxyecdysone-26-oic acid and ecdysone-26-oic acid (see Part 3 section B).

Overall pattern of metabolites

A summary of the quantitative analysis of the metabolites formed is shown in Table 3A-1. Both male and female pupae demonstrated similar ecdysteroid metabolites, the only significant difference being that slightly more conjugation occurred in the male pupae, perhaps only as a result of the longer incubation time of [^{3}H] ecdysone in male pupae (48 hours as opposed to 24 hours as with the females).

The most active metabolic pathways proved to be 20- and 26-hydroxylation leading to 20-hydroxyecdysone, 26-hydroxyecdysone and 20,26-dihydroxyecdysone (figure 3A-3). Much labelled ecdysone (almost one third) was further metabolised to the 26-oic acids of ecdysone and 20-hydroxyecedysone (figure 3A-6).

In addition to the analysis of the highly polar ecdysteroid fractions by hydrolysis using the Helix pomatia enzyme, these fractions were also analysed by the use of two phosphatase enzymes: acid phosphatase (crude potato preparation) and alkaline phosphatase (crude calf intestinal mucosa preparation). The percentage hydrolysis and the quantities of each ecdysteroid released from conjugation by these enzymes were almost

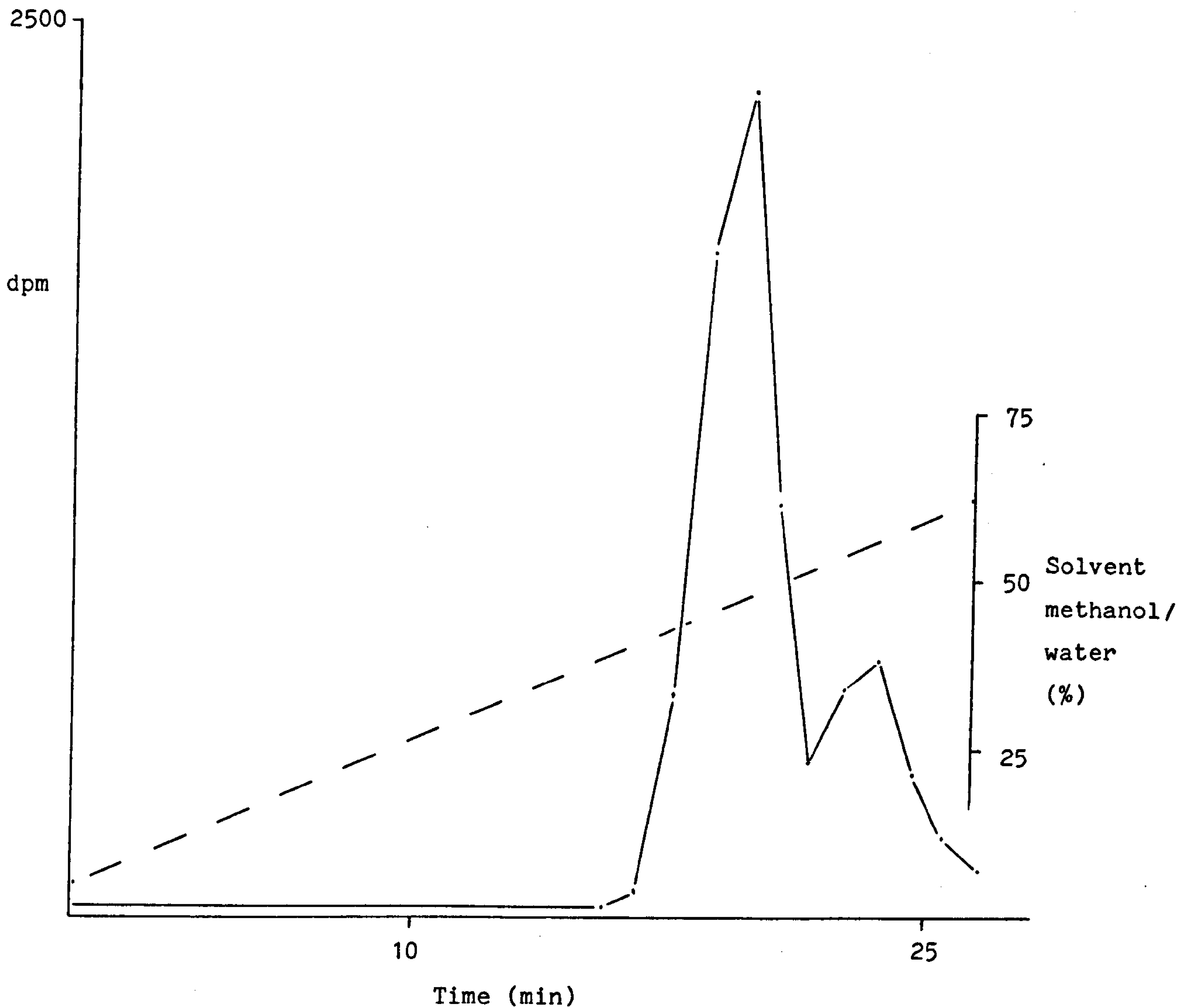


Figure 3A-6 : H.p.l.c. of the [^{3}H] unhydrolysable polar ecdysteroid fraction (day 7 female pupae) using system 2, after metabolism in vivo of [^{3}H] ecdysone. The unhydrolysable polar ecdysteroids were subsequently identified as 20-hydroxyecdyson-26-oic acid (major peak) and ecdyson-26-oic acid (see Part 3, section B).

Table 3A-1 : Summary of the [^{3}H] ecdysteroid metabolites isolated from male (day 7) and female (day 6) pupae after metabolism *in vivo* of [^{3}H] ecdysone.

	Male	Female
FREE ECDYSTEROIDS	4,919,900 dpm	10,912,300 dpm
Ecdysone	13.8	25.1
20-Hydroxyecdysone	36.2	28.0
26-Hydroxyecdysone	20.8	11.8
20,26-Dihydroxyecdysone	24.25	24.5
ECDYSTEROID CONJUGATES*	940,100 dpm	793,500 dpm
Ecdysone	6.5	15.9
20-Hydroxyecdysone	16.3	10.6
26-Hydroxyecdysone	18.9	24.4
20,26-Dihydroxyecdysone	24.7	44.3
UNHYDROLYSABLE POLAR ECDYSTEROIDS	2,613,700 dpm	6,112,300 dpm
1. 20-Hydroxyecdyson-26-oic acid †	67.9	59.7
2. Ecdyson-26-oic acid †	24.9	25.2

Numbers refer to the percentage of radioactivity recovered from reverse-phase h.p.l.c. in various compounds.

*Ecdysteroids released from conjugation by *H. pomatia* enzyme hydrolysis.

†Compounds subsequently identified (see Part 3, section B).

identical to those obtained from hydrolysis by *Helix pomatia* (aryl sulphatase) enzyme preparation. This emphasises the crude nature of these enzyme preparations. Indeed, the *Helix pomatia* aryl sulphatase preparation has been found to contain phosphatase activity (Isaac *et al.*, 1982).

2. The metabolism *in vivo* of [^{14}C] cholesterol in pupae of *S. littoralis*

The metabolism *in vivo* of [^{14}C] cholesterol in female pupae (from day 2 to day 6 of pupal development)

Injection of [4-^{14}C] cholesterol and extraction

Twelve female pupae (day 2) were injected with 12 µCi of [4-^{14}C] cholesterol (50 mCi/mmol) by the method described in Part 2. The pupae were then reared under normal conditions for a further 4 days and then sacrificed. The insects were then extracted and purified by silicic acid column chromatography (for method see Part 2). The distribution of radioactivity after silicic acid column chromatography is represented in Table 3A-2.

Analysis of the free ecdysteroids (30% methanol/chloroform fraction)

The [^{14}C] cholesterol metabolites in the free ecdysteroid fraction were analysed by h.p.l.c. system 1 (figure 3A-7). Authentic markers were used to identify the radioactive metabolites by co-chromatography. The [^{14}C] compounds identified are similar to those formed during [^{3}H] ecdysone metabolism. These compounds were collected from the reversed-phase system and re-chromatographed with authentic markers on h.p.l.c. system 3 (APS-Hypersil, adsorption column). Their identities were confirmed in this way and it also allowed the separation of 26-hydroxyecdysone from 20-hydroxyecdysone (figure 3A-8) which have similar

Table 3A-2 : Distribution of radioactivity after Hexane/aqueous methanol partition and silicic acid column chromatography of an extract from female pupae injected with 12 µCi [^{14}C] cholesterol and extracted at day 6.

	Fraction	dpm
	Hexane fraction	16,610,000 (Hexane fraction + 0% (Chloroform))
Silicic acid column (Methanol/chloroform fraction)	0% (Chloroform)	
	30%	125,200
	80%	41,900
	100% (methanol)	5,500

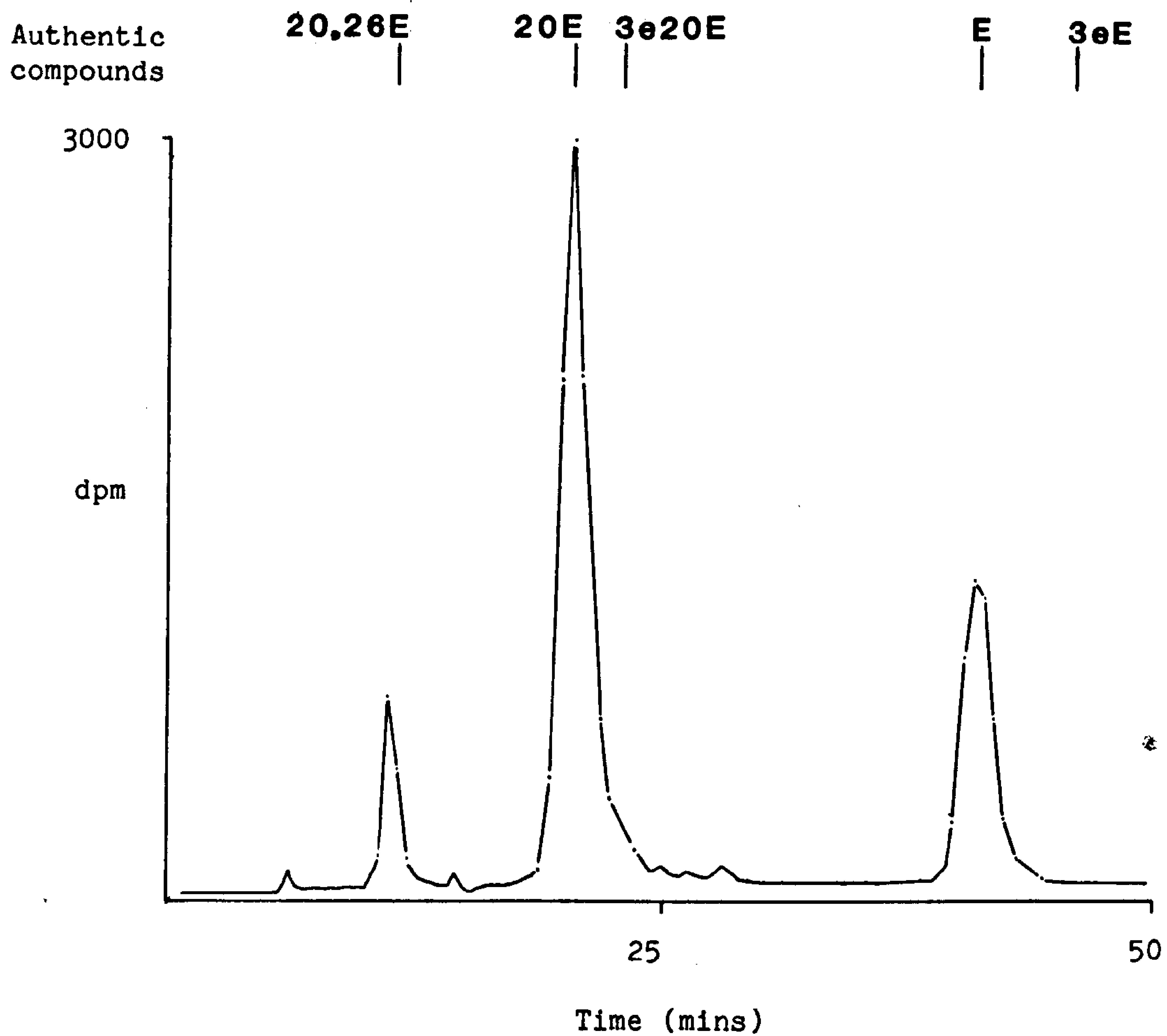


Figure 3A-7 : H.p.l.c. analysis (system 1) of the [^{14}C] free ecdysteroid fraction from day 6 female pupae, after administration of [^{14}C] cholesterol. Authentic marker compounds were used to identify the peaks of radioactivity.

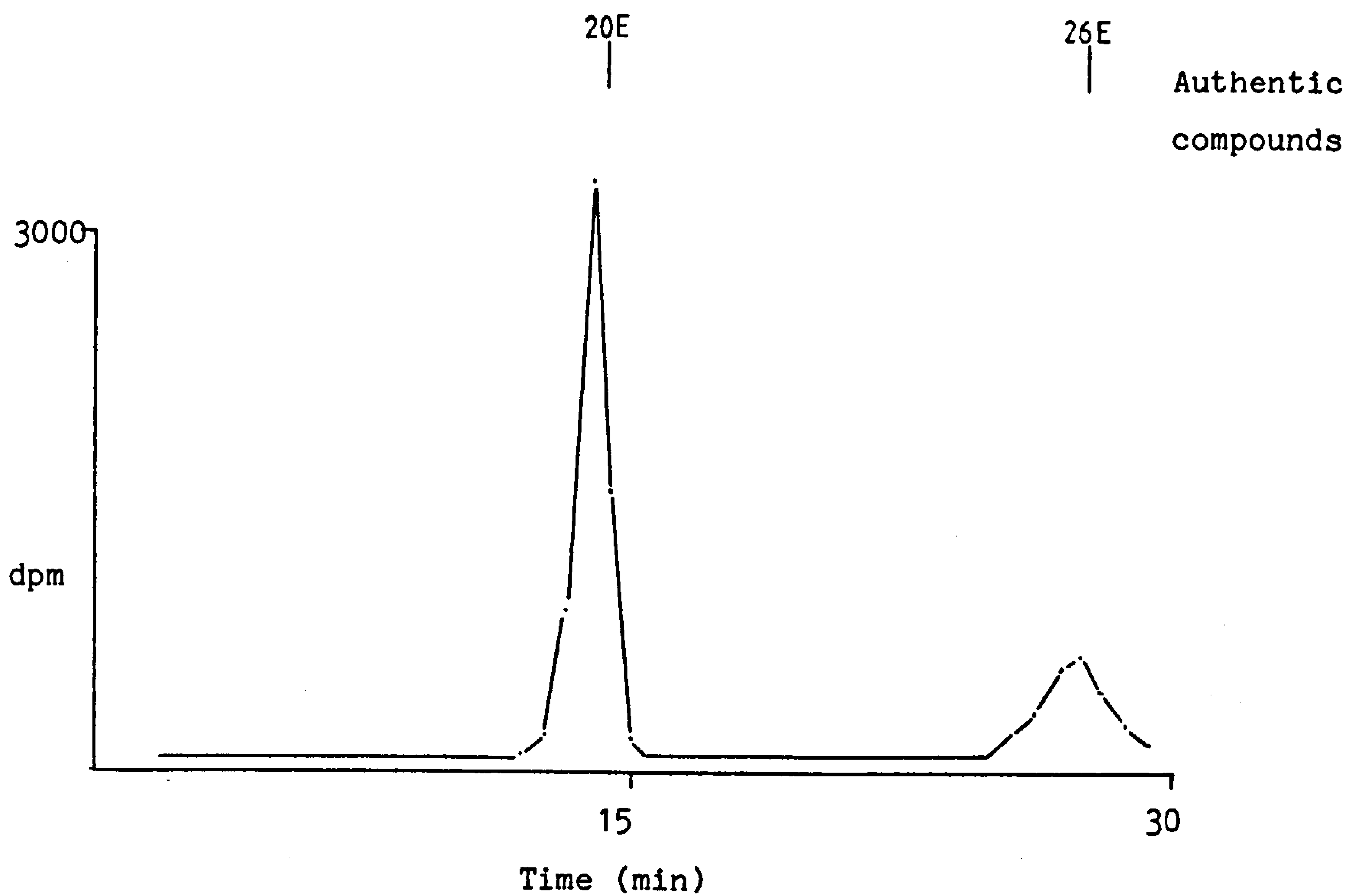


Figure 3A-8 : H.p.l.c. analysis of 20-hydroxyecdysone and 26-hydroxyecdysone (peak 2, ex figure 3A-7) from day 6 female pupae [^{14}C] free ecdysteroid fraction using system 3 (one APS-hypersil column with isocratic elution at 2 ml/min with 6% methanol in dichloroethane.

chromatographic behaviour on reversed-phase h.p.l.c. (see figure 3A-7).

Analysis of the highly polar ecdysteroid fraction (80% methanol/chloroform)

The metabolites in this fraction were analysed using h.p.l.c. system 4 (figure 3A-9). Characterised [^{3}H] 20-hydroxyecdyson-26-oic acid and ecdyson-26-oic acid (ex pupal [^{3}H] ecdysone injections, see Part 3, section B) were co-chromatographed as markers. [^{14}C] apparently co-chromatographs with marker ecdysonoic acid and 20-hydroxyecdysonoic acid but no other major peaks are apparent.

Overall pattern of metabolites

The amounts of characterised ecdysteroids found _in vivo_ from 12 µCi [^{14}C] cholesterol are represented in Table 3A-3 and indicate that the ecdysteroids represent 0.32% of the radioactivity recovered in the initial aqueous methanolic extract. These results also effectively demonstrate that the radioactive ecdysteroids recovered from both the 30% and 80% methanol/chloroform column fractions do not account for the total radioactivity in each of these fractions (cf. Tables 3A-2 and 3A-3). The 30% methanol/chloroform fraction contains 125,200 dpm [^{14}C] (Table 3A-2) but only 51,600 dpm were eluted from h.p.l.c. (system 1) as [^{14}C] ecdysteroids. Most of the remaining radioactivity could be recovered by following the h.p.l.c. run with 100% methanol elution (recovering a further 61,000 dpm). The 80% methanol/chloroform fraction contains 41,900 dpm [^{14}C] (Table 3A-2) but a mere 2,500 dpm was recovered as 20 hydroxyecdysone-26-oic acid and ecdyson-26-oic acid. Throughout this work this phenomenon has been present in all _in vivo_ radioactive cholesterol incorporation experiments performed with _S. littoralis,_ and is dealt with in more detail towards the end of this section (p.55).

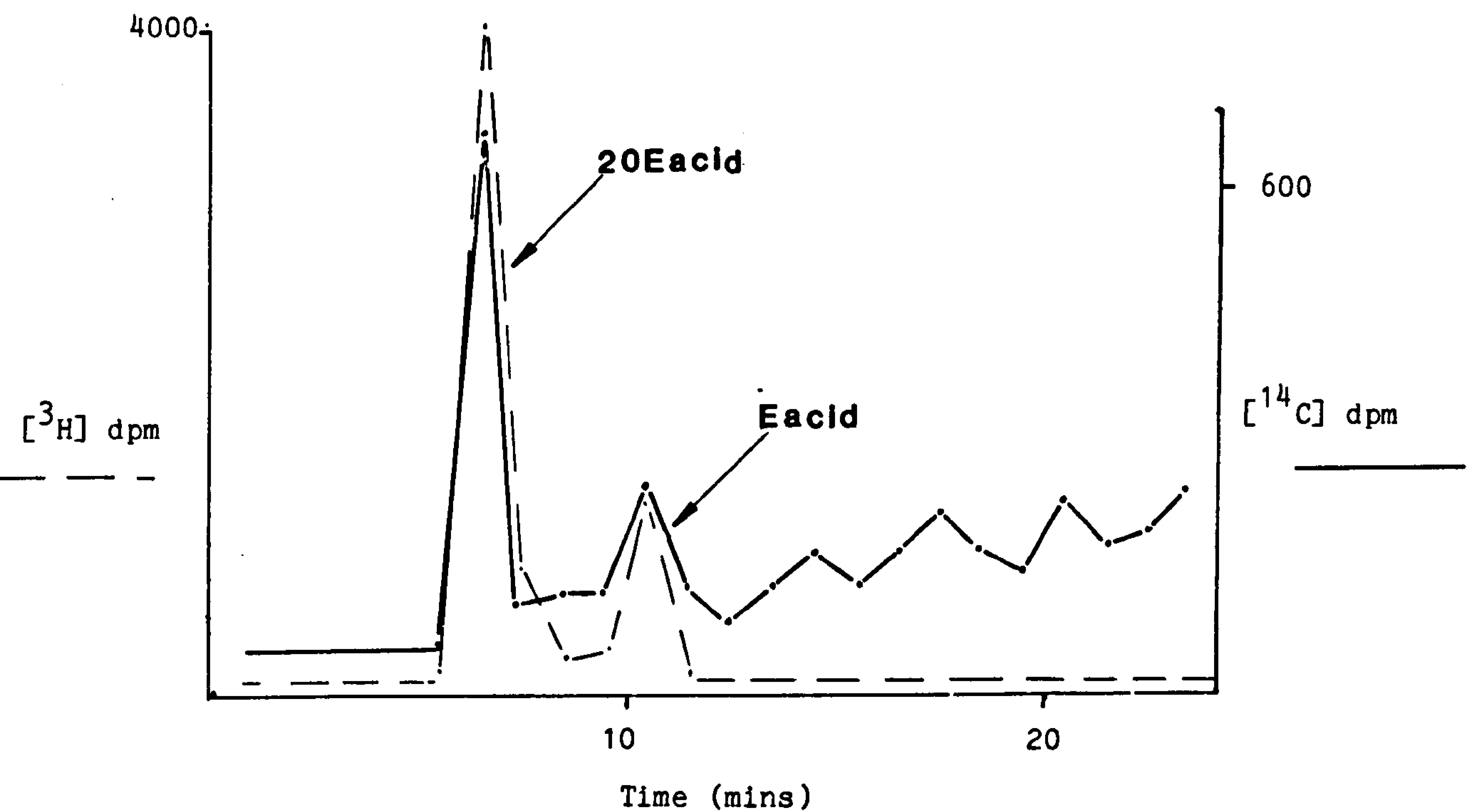


Figure 3A-9 : H.p.l.c. analysis of the [^{14}C] unhydrolysable polar ecdsyteroid fraction from day 6 female pupae using system 4. Characterised [^{3}H] 20-hydroxyecdyson-26-oic acid and ecdyson-26-oic acid (see Part 3, section B) were used as markers for identification by co-chromatography.

Table 3A-3 : Summary of the [^{14}C] ecdysteroids found in day 6 female pupae after metabolism of 12 μCi [^{14}C] cholesterol administered on day 2.

Ecdysteroid identified	dpm
Ecdysone	14,400
20-hydroxyecdysone	24,600
26-hydroxyecdysone	7,200
20,26-dihydroxyecdysone	5,400
Ecdyson-26-oic acid	300
20-hydroxyecdyson-26-oic acid	2,200
Total	54,100

Total radioactivity recovered in the initial aqueous methanolic extract : 16,877,100

Total radioactivity recovered in the form of ecdysteroids : 54,100

The total incorporation into ecdysteroids is 0.32% of the radioactivity recovered in the initial extract.

The ecdysteroids produced from this incubation (Table 3A-3) are similar to those formed in the foregoing [^{3}H] ecdysone incubations *in vivo*. [^{14}C] 20-hydroxyecdysone is the principal ecdysteroid synthesised, with [^{14}C] ecdysone and the 26-hydroxylated ecdysteroids, [^{14}C] 26-hydroxyecdysone and [^{14}C] 20,26-dihydroxyecdysone, also being produced. A small amount of highly polar ecdysteroid was also present in the form of 20-hydroxyecdyson-26-oic acid, together with a trace of ecdyson-26-oic acid.

Metabolism *in vivo* of [^{14}C] cholesterol in male pupae (from day 2 to day 9 of pupal development)

Injection of [4-^{14}C] cholesterol and extraction

Fifteen male pupae (day 2) were injected with 15 µCi of [4-^{14}C] cholesterol (50 mCi/mmol) and the pupae reared under normal conditions for a further 7 days before sacrifice. The insects were then extracted and the extract fractionated by silicic acid column chromatography. The distribution of radioactivity after silicic acid column chromatography is represented in figure 3A-10.

As with previous radioactive metabolite analyses the highly polar fraction (80% methanol/chloroform fraction), having been first analysed by h.p.l.c. (figure 3A-12), was hydrolysed by *Helix pomatia* enzyme preparation. The distribution of the radioactivity after hydrolysis is represented in figure 3A-10.

The four ecdysteroid fractions from these purification steps (i.e. the free ecdysteroid fraction, the highly polar ecdysteroid fraction, the fraction containing ecdysteroids released from conjugation, and the unhydrolysable polar ecdysteroid fraction) were analysed using h.p.l.c. system 4 (see figures 3A-11 to 3A-14).

[^{14}C] cholesterol (15 μCi)
injected into 15 male pupae

Aqueous methanol
extract 16,699,300 dpm

Partition

Hexane
14,343,100 dpm

70% Aqueous methanol
1,806,200 dpm

Silicic acid column

Fraction methanol/ chloroform:	0%	30%	80%	100%
dpm	521,200	396,800	735,500	103,300
		Free Ecdysteroids	Highly polar ecdysteroids	

hydrolysis
(*H.pomatia* enzyme)

Fraction methanol/ chloroform :	0%	30%	80%	100%
dpm	27,200	121,200	639,400	12,400
		Ecdyst's released from conjugation	Unhydrolysable polar metabolites	

Figure 3A-10 : Summary of the distribution of [^{14}C] radioactivity in day 9 male pupae administered with [^{14}C] cholesterol at day 2.

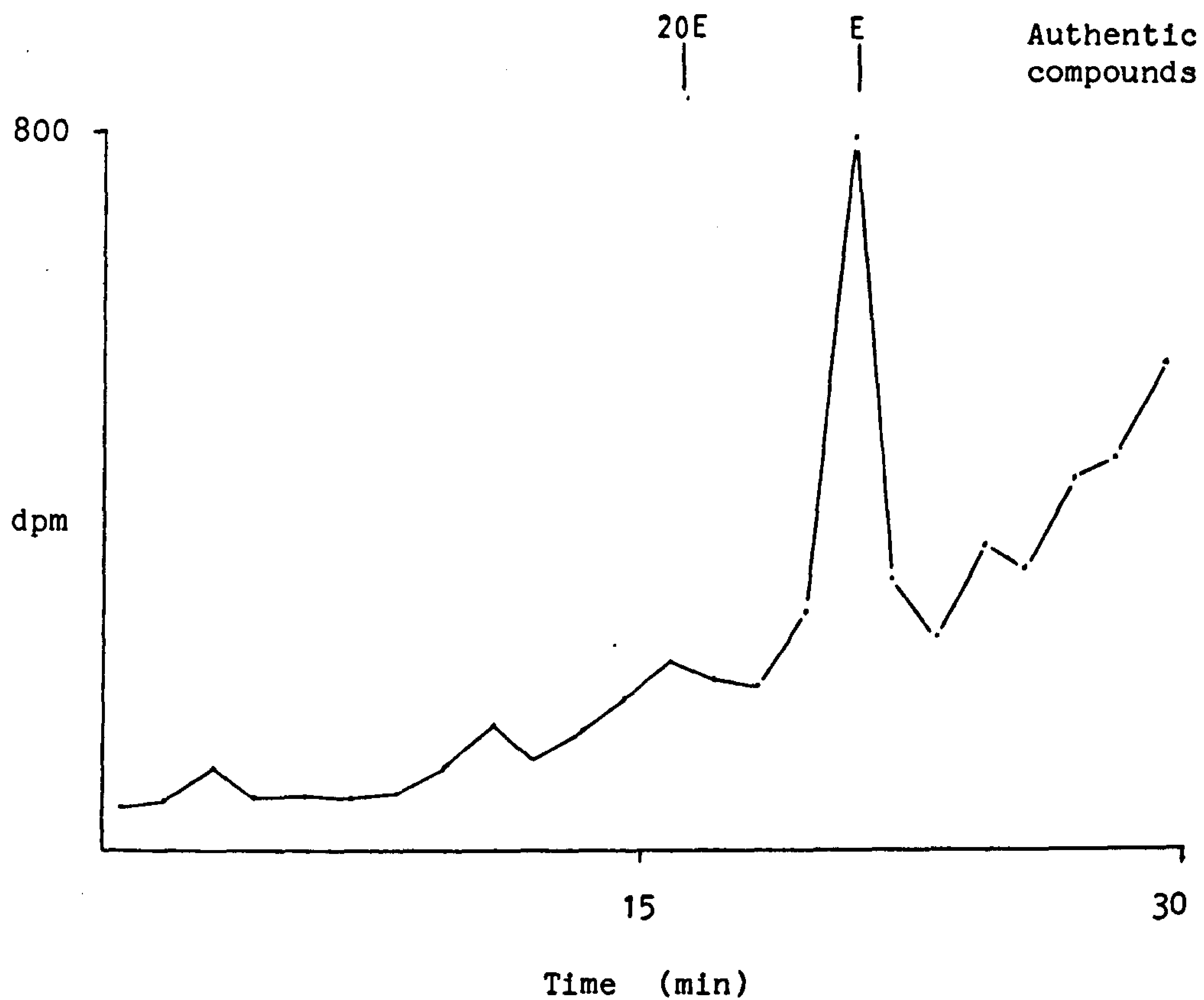


Figure 3A-11 : H.p.l.c. analysis (system 4) of the [^{14}C] free ecdysteroid fraction from day 9 male pupae, after administration of [^{14}C] cholesterol at day 2.

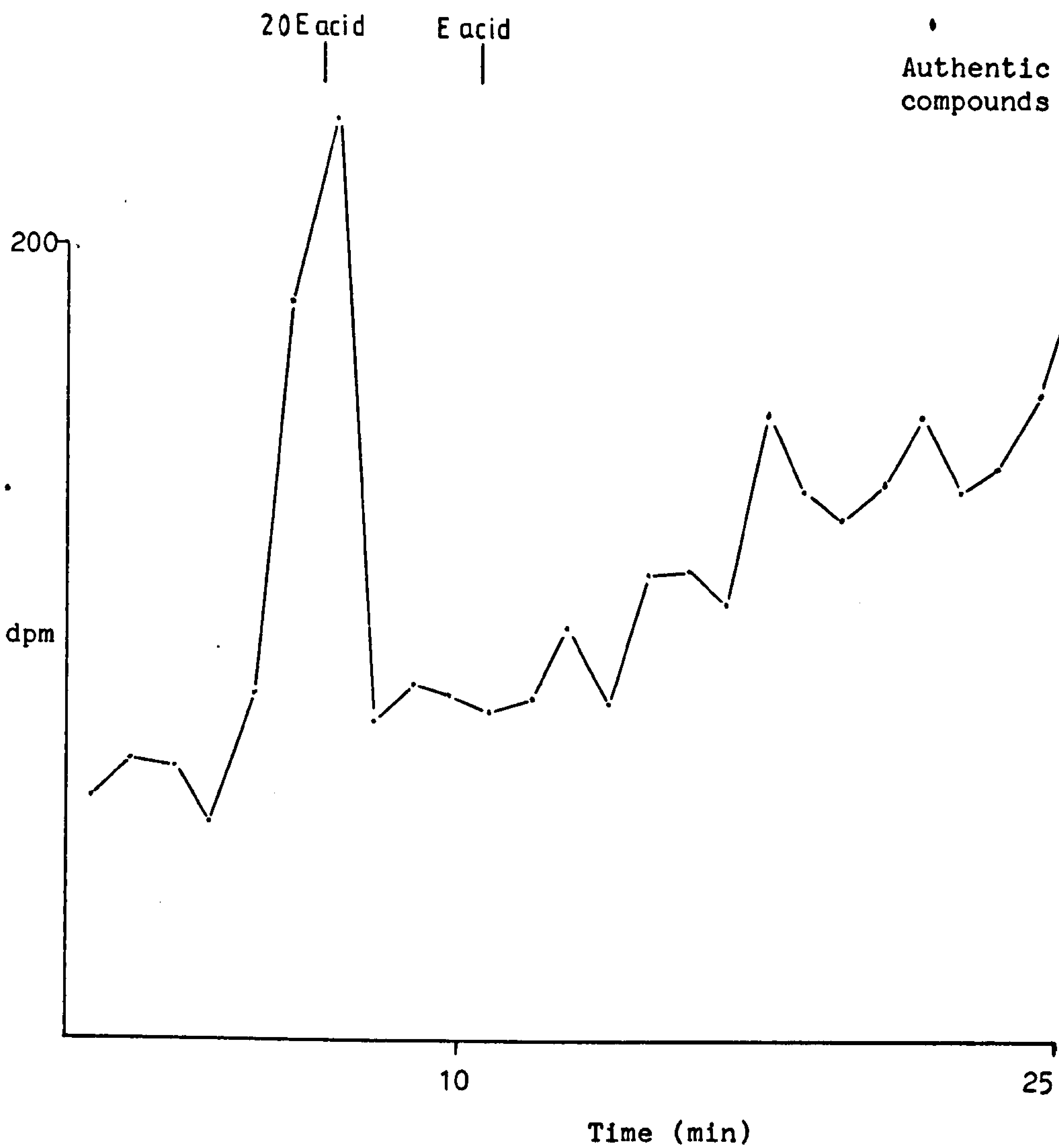


Figure 3A-12 : H.p.l.c. (using system 4) of the [^{14}C] highly polar ecdysteroid fraction extracted from day 9 male pupae of S. littoralis, following administration of [^{14}C] cholesterol at day 2.

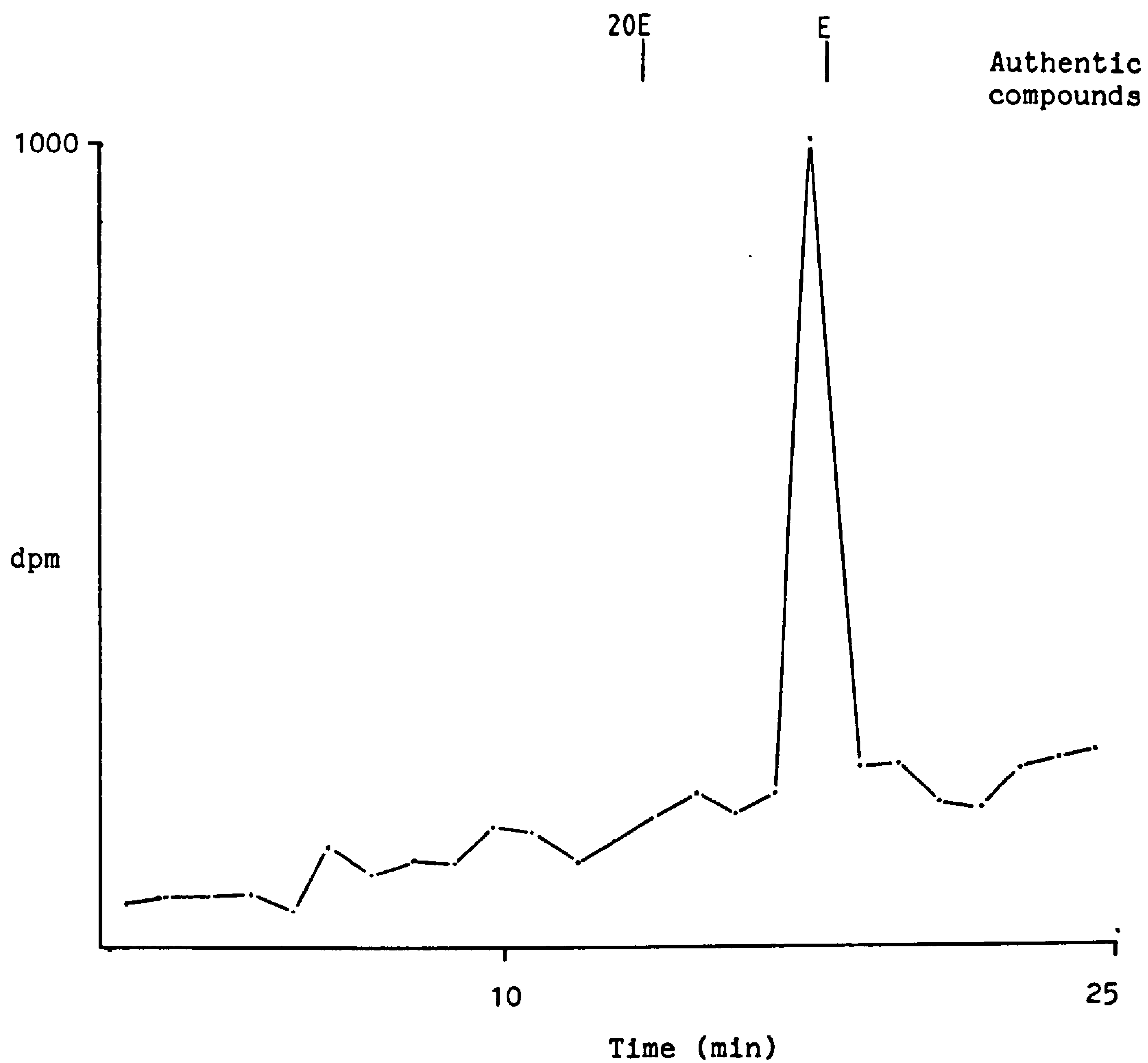


Figure 3A-13 : H.p.l.c. analysis (system 4)of the [^{14}C] ecdsyteroids released from conjugation by the H.pomatia enzyme preparation.

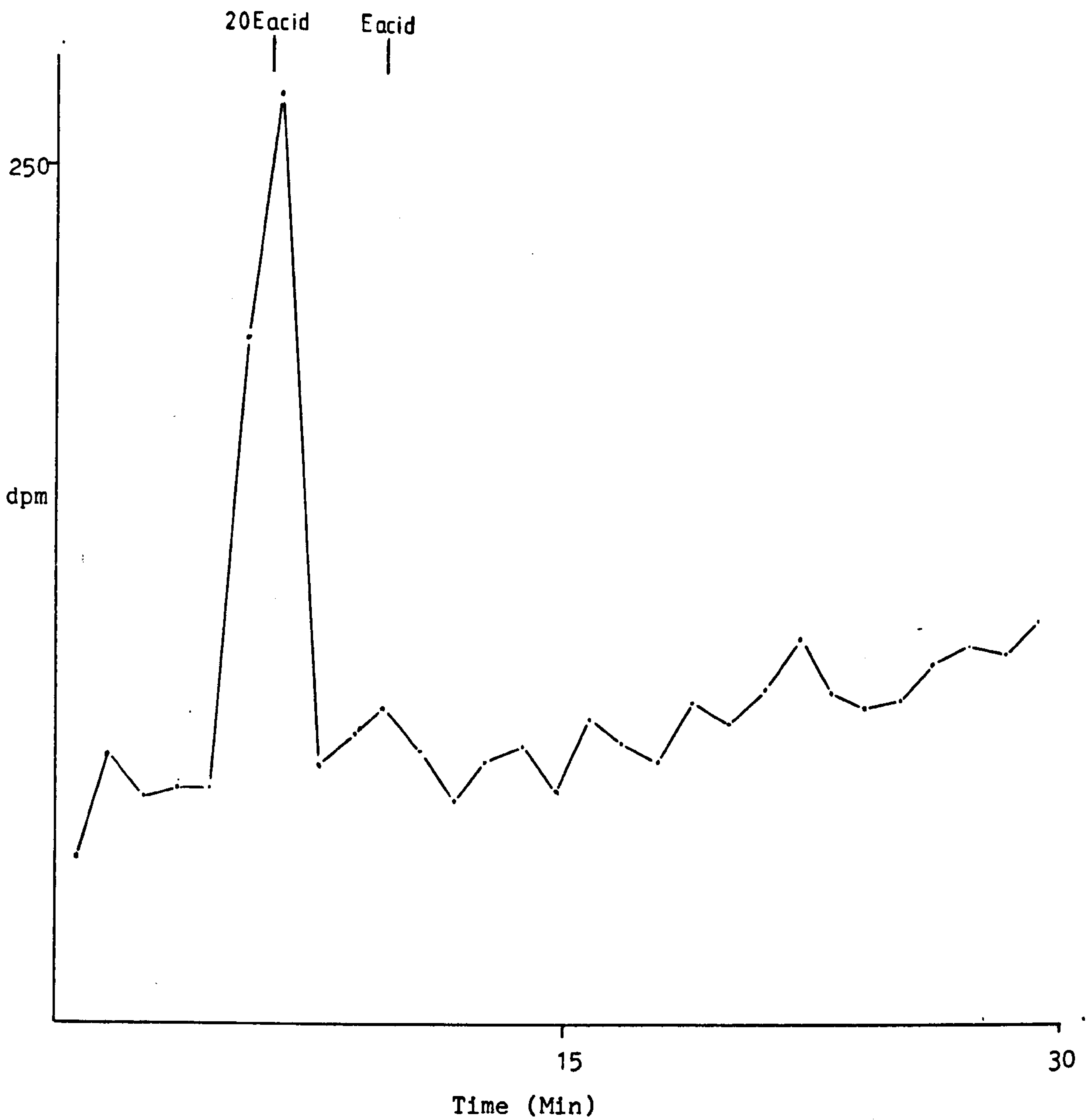


Figure 3A-14 : H.p.l.c. analysis (system 4) of the unhydrolysable [^{14}C] polar ecdysteroids extracted from day 9 male pupae of S. littoralis. The ecdysonoic acid markers used were isolated from S. gregaria.

Overall pattern of metabolites

The summary of the quantities of [^{14}C] ecdysteroids formed from [^{14}C] cholesterol are shown in Table 3A- 4.

It is apparent once again, that the amounts of ecdysteroids recovered do not account for the total radioactivity contained in each silicic acid column fraction, and this facet of the experiment will be considered in the next sub-section.

The radioactive ecdysteroid complement of this incubation shows a quite different composition from that of the ecdysteroids found at day 6 of pupal development (Table 3A-3). Firstly, in the present case, the proportion of recovered radioactivity that is in the form of ecdysteroids is much lower (0.19% compared to 0.32%) (cf. Tables 3A-3 and 3A-4), but also the only free ecdysteroid (figure 3A-11) and, indeed, the only conjugated ecdysteroid (figure 3A-13) is ecdysone. However, a high proportion of the ecdysteroid metabolites is found in the form of 20-hydroxyecdyson-26-oic acid (figure 3A-12).

3. Investigation of [^{14}C] cholesterol metabolites detected during in vivo incubations that could not be identified as ecdysteroids

Both the [^{14}C] cholesterol incubations previously described produced radioactive components, eluting in the 30% and 80% methanol/chloroform fractions from silicic acid column chromatography, that cannot be accounted for by known [^{14}C] ecdysteroids alone.

Both the free ecdysteroid and highly polar ecdysteroid fraction (30% and 80% methanol/chloroform fractions from silicic acid column chromatography) were further analysed using h.p.l.c. system 5 (gradient elution from 0 to 100% acetonitrile) which ensures elution of most

Table 3A-4 : Summary of the [^{14}C] ecdysteroids found in day 9 male pupae following administration of 15 μCi [^{14}C] cholesterol at day 2.

^{14}C Cholesterol injected = 15 μCi

Ecdysteroid identified	dpm
Ecdysone	15000
20-hydroxyecdyson-26-oic acid	6600
Ecdysone-conjugate	2800
Total	24400

Total radioactivity recovered in the initial aqueous methanol extract : 16,699,300

Total radioactivity recovered in ecdysteroids : 24,400

The total incorporation into ecdysteroids is 0.19% of the radioactivity recovered in the initial extract.

metabolites, even as non-polar as cholesterol (figure 3A-13). This analysis revealed that the majority of the radioactive material in each of these fractions consists of cholesterol metabolites displaying apparent non-polar characteristics, of only slightly greater polarity than cholesterol (figure 3A-15). Further characterisation of these compounds was achieved by thin-layer chromatography using a chloroform in methanol (80:15, v/v) solvent system with continuous elution ($1\frac{1}{2}$ hours) (figure 3A-16). The resulting radiochromatogram shows that the [^{14}C] compounds from both fractions, although displaying similar characteristics on reversed-phase h.p.l.c., differ greatly in their behaviour on silica t.l.c. The compound from the 30% methanol/chloroform fraction chromatographs at the solvent front (non-polar) and the compound from the 80% methanol/chloroform fraction moves only slightly off the origin (highly polar).

The 30% and 80% methanol/chloroform fractions _after_ enzymic hydrolysis of the highly polar fraction (figure 3A-10) were also subjected to t.l.c. (figure 3A-17). The results indicated that most of the radioactive compounds in the highly polar fraction were not affected by enzymic hydrolysis (figure 3A-10), although some non-polar products eluted in the 30% methanol fraction [figure 3A-17(a)]. This indicated that at least some of the unknown [^{14}C] metabolite(s) could have been a hydrolysable conjugate of some description.

In an attempt to obtain mass of the unknown metabolites, an extract of 525, day 9 pupae, was separated into the same silicic acid chromatographic fractions as the pupal _in vivo_ [^{14}C] cholesterol incubations and the fractions chromatographed on h.p.l.c. with the appropriate [^{14}C] fractions. The chromatography was monitored by following the U.V. absorbance at 254 nm (which detects ecdysteroid-type molecules) and by

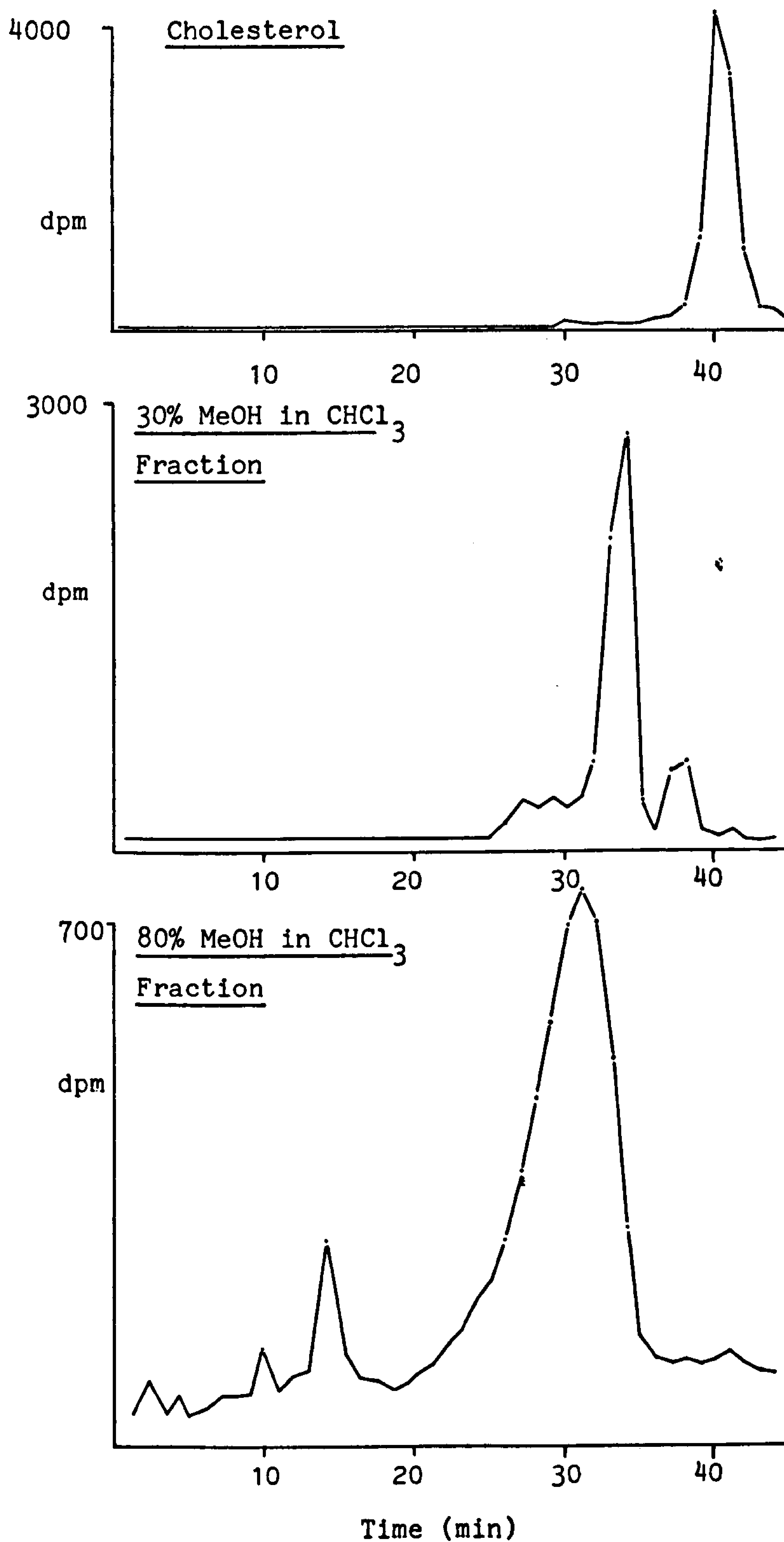
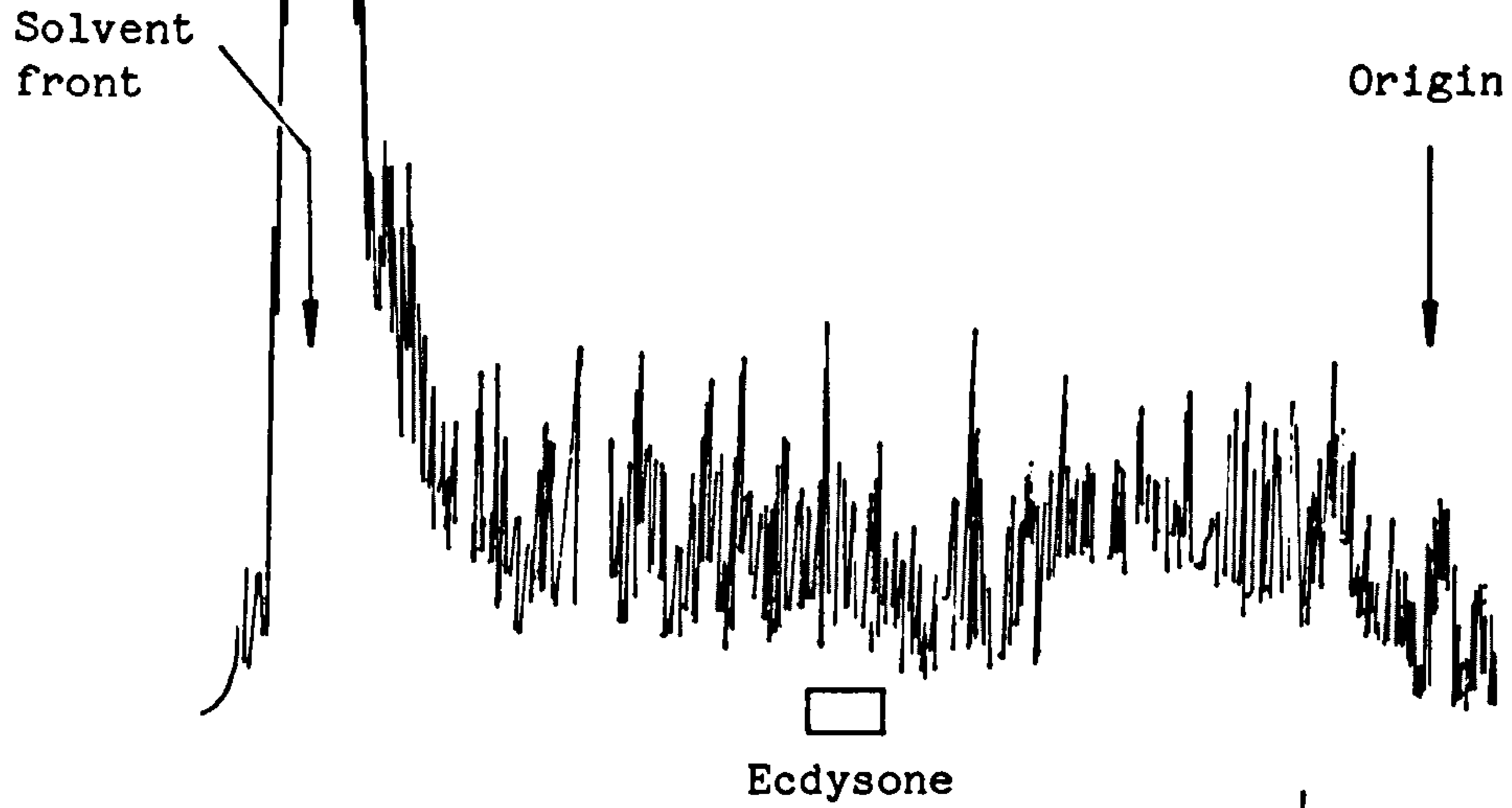


Figure 3A-15 : H.p.l.c. analysis (system 5) of the 30% and 80% methanol/chloroform fractions from silicic acid column chromatography of day 9 pupal extracts after administration of [^{14}C] cholesterol at day 2. H.p.l.c. analysis of cholesterol is also represented as a reference compound.

a) $30\%\ MeOH/CHCl_3$ fraction



b) $80\%\ MeOH/CHCl_3$ fraction

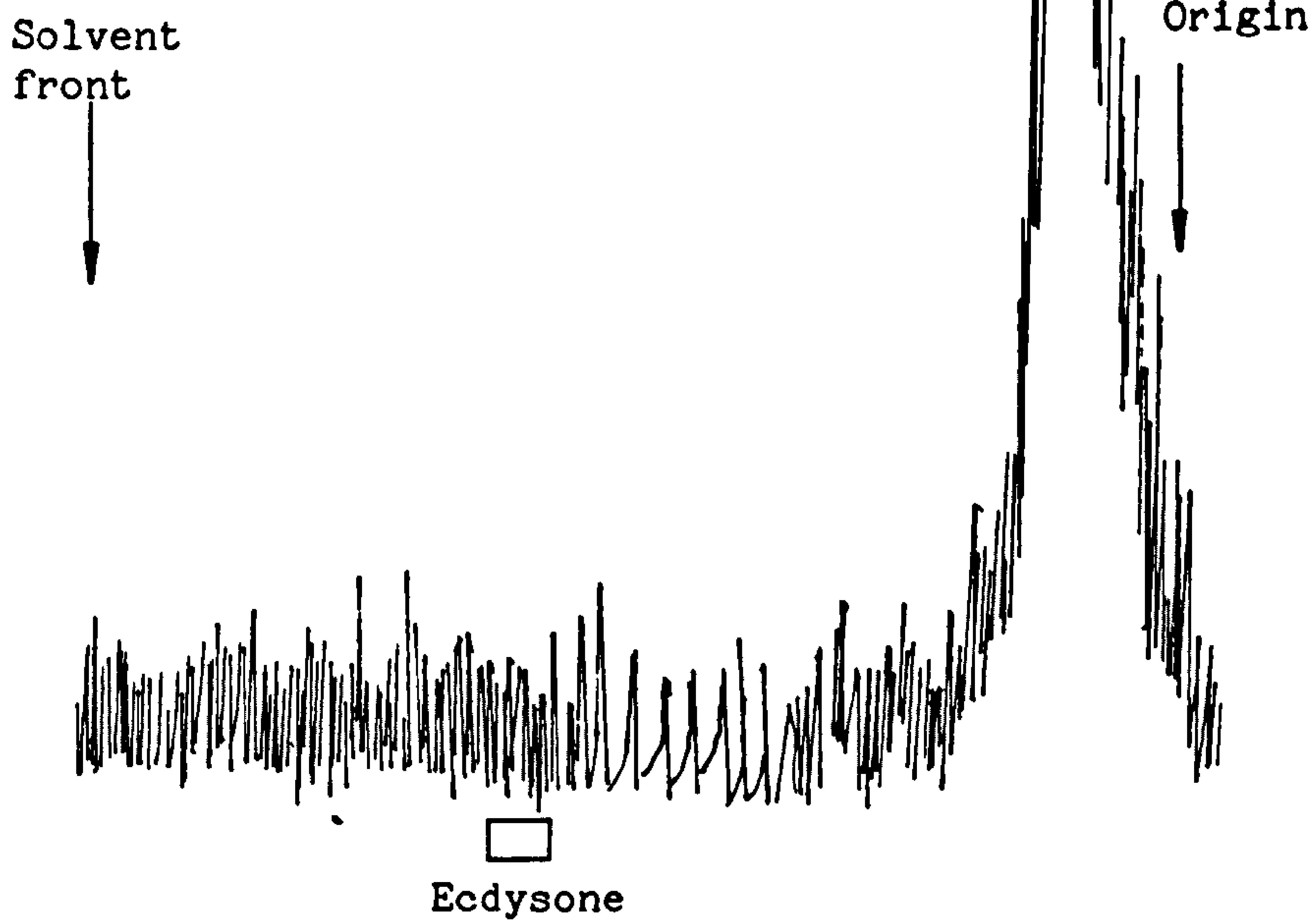
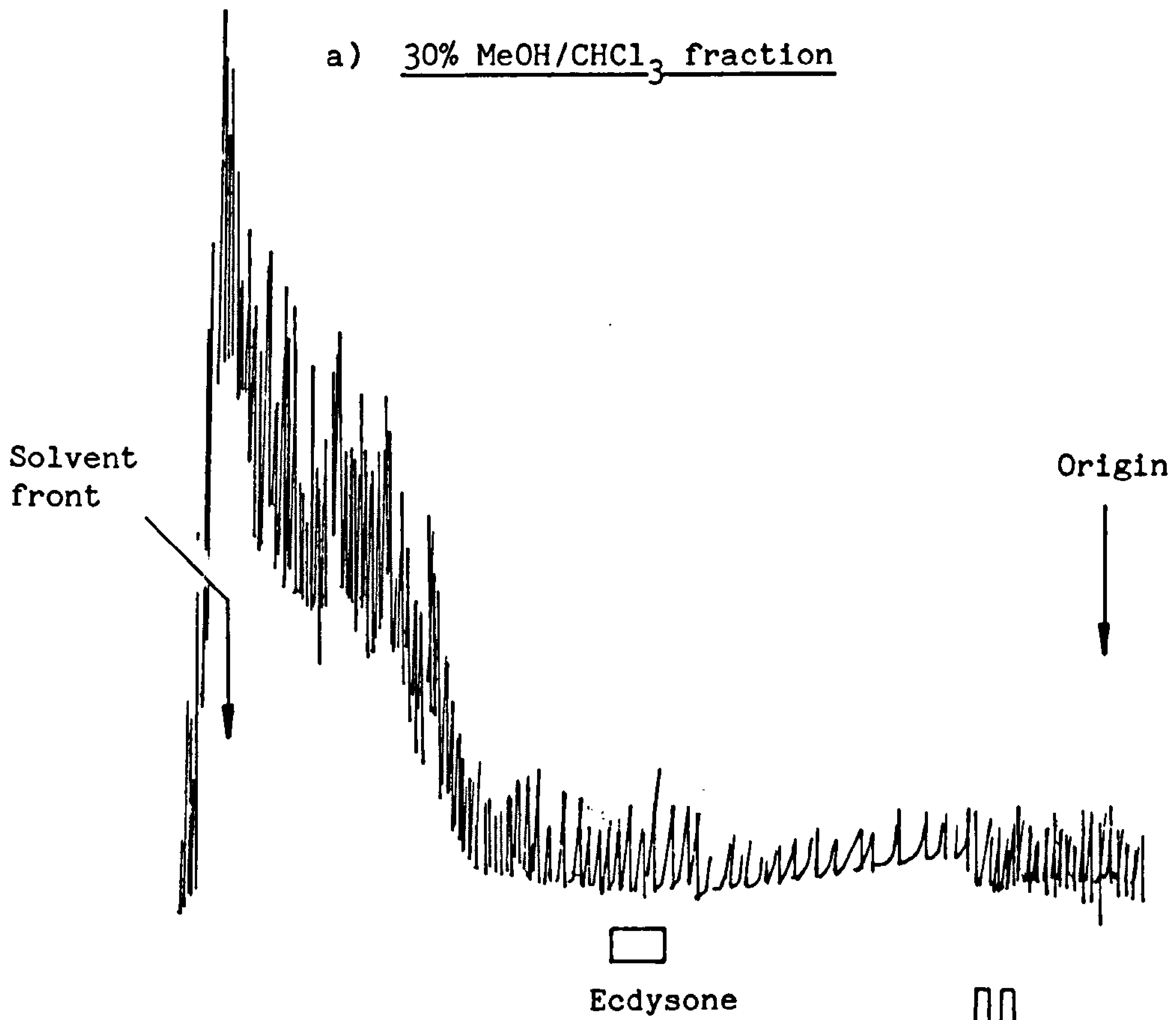


Figure 3A-16 : Silica t.l.c. radiochromatogram of the 30% and 80% methanol/chloroform fractions from silicic acid column chromatography of day 9 male pupal extracts after administration of [^{14}C] cholesterol at day 2.



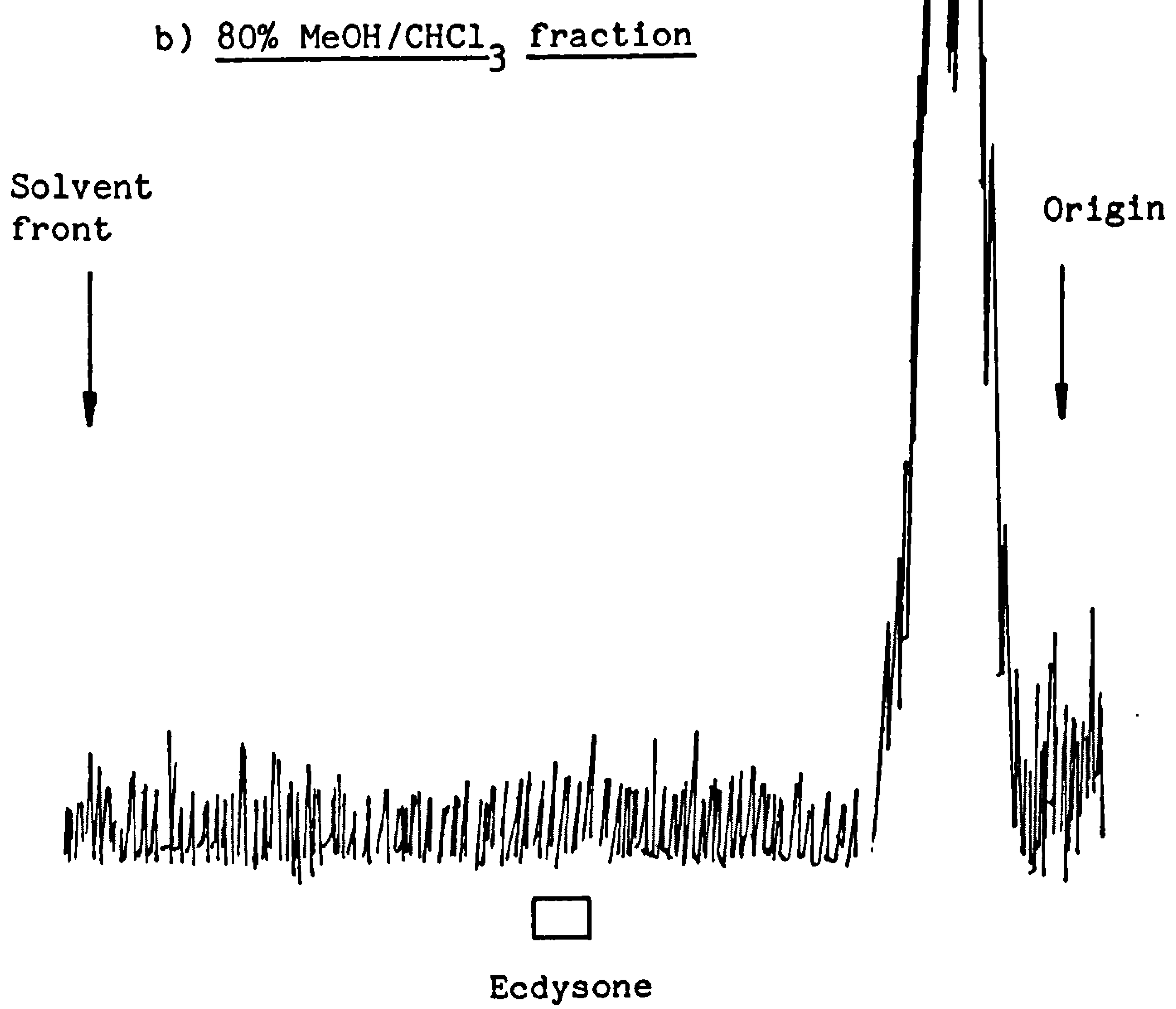


Figure 3A-17 : Silica t.l.c. radiochromatogram of the [^{14}C] 30% and 80% methanol/chloroform fractions from silicic acid column chromatography after hydrolysis of the highly polar ecdysteroid fraction by *Helix pomatia* enzyme

radioassay of 1 ml fractions (figure 3A-18). This analysis revealed that none of the radioactive peaks, in any fraction, co-chromatographed with U.V.-absorbing peaks of the pupal mass extract. This indicates that either the unknown compounds do not absorb U.V. light at 254 nm, and therefore are not ecdysteroidal, or that the [^{14}C] metabolites are not metabolites of endogenous cholesterol, and are only produced by injection of exogenous radioactive cholesterol. In addition to this, it must be noted that none of these metabolites are produced from incubations of [^{3}H] ecdysone *in vivo* (see p. 50 and p. 51). The foregoing findings suggest that these [^{14}C] metabolites of cholesterol are not part of any moulting hormone metabolic pathway. For this reason the identification of these compounds was pursued no further.

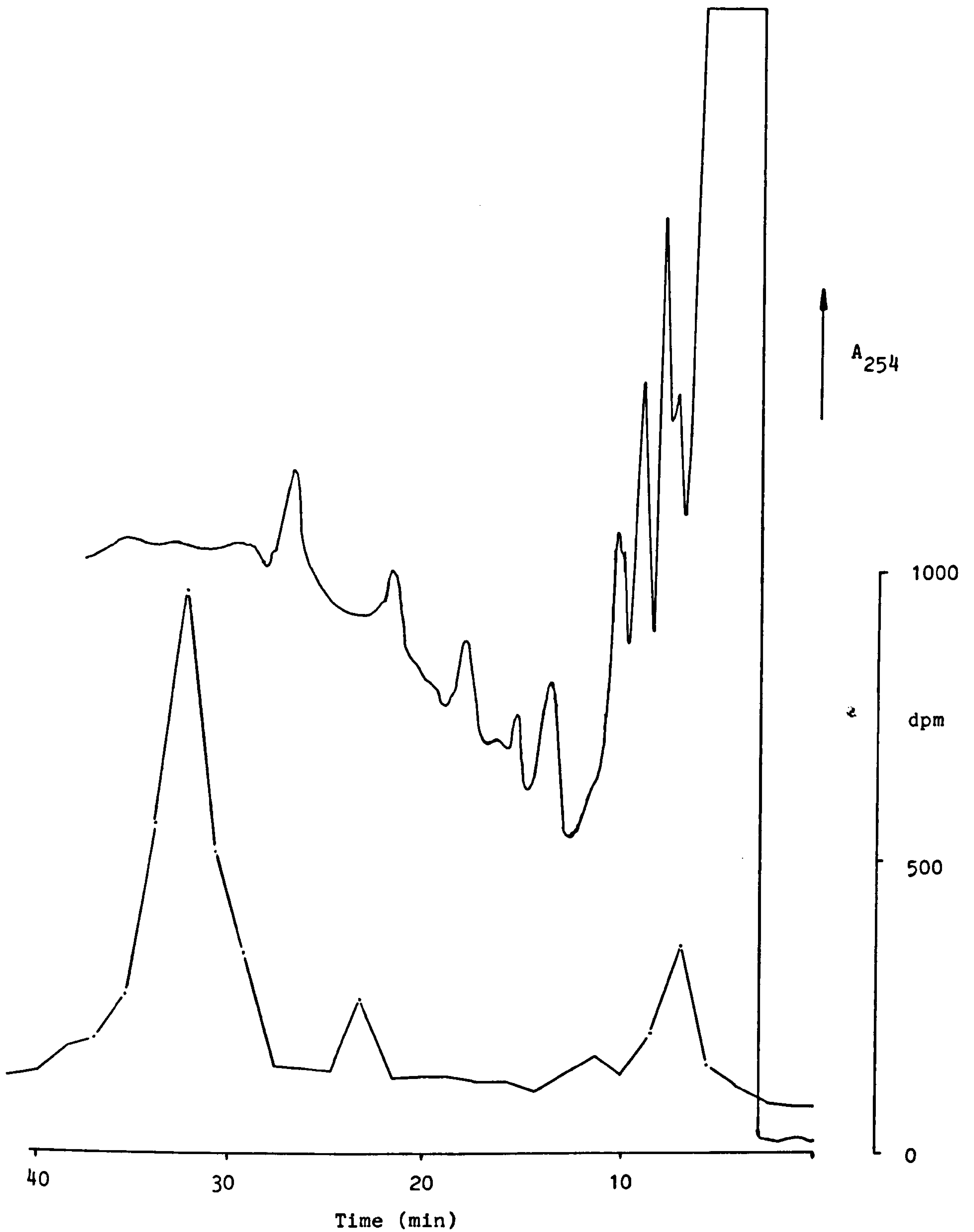


Figure 3A-18 : H.p.l.c. analysis (system 5) of the 80% methanol/chloroform fraction from silicic acid column chromatography of an extract from 525 male pupae. The same silicic acid column fraction isolated from pupae after incubation *in vivo* with [^{14}C] cholesterol was co-injected for reference purposes.

PART 3 SECTION B

PART 3 SECTION B

Identification of ecdysonoic acid and 20-hydroxyecdysonoic acid

Page No.

Introduction 59

Experimental and results

High-performance liquid chromatographic systems used in this section 61

Characterisation of the unhydrolysable polar fraction from the metabolism *in vivo* of [^{3}H] ecdysone in *S. littoralis* pupae 62

The metabolism *in vivo* of [^{3}H] 20,26-dihydroxyecdysone 63

Methylation of the pupative 20-hydroxyecdyson-26-oic acid (U.P.1) 65

Isolation of mass of polar ecdysteroids U.P.1 and U.P.2 66

Large scale extraction of the unhydrolysable polar compounds U.P.1 and U.P.2 from day 9 pupae 67

Methylation of the unhydrolysable polar fractions with diazomethane 68

Characterisation of U.P.1 and U.P.2 by mass spectrometry [negative ion fast atom bombardment (FAB)] 69

Structure of U.P.1 and U.P.2 70

PART 3 SECTION B

Identification of ecdysonoic acid and 20-hydroxyecdysonoic acid

INTRODUCTION

In a number of insect species, it has been reported that appreciable amounts of highly polar ecdysteroid fractions were not hydrolysed by Helix pomatia hydrolases. The first report of such an unhydrolysable compound was made in 1970 (Moriyama et al.) with 2% of injected [^{3}H] ecdysone being metabolised to an unhydrolysable polar compound in larvae of Bombyx mori. Since then, similar compounds have been reported on a number of occasions in a variety of insects: Locusta migratoria (Koolman et al., 1973), Calliphora erythrocephala (Young, 1976), Tenebrio molitor (Weinheimer and Romer, 1977), Sarcophaga peregrina (Moribayashi et al., 1978), Pieris brassicae (Lafont et al., 1980), Schistocerca gregaria eggs (Dinan and Rees, 1981).

Thorough characterisation of these compounds has only recently been undertaken. Koolman et al. (1973) first suggested, on the basis of electrophoretic behaviour, that the unhydrolysable polar compound from Locusta (Ic) could have a carboxylic function in the ecdysteroid side-chain. However, it was not until 1980 (Lafont et al.) that unhydrolysable polar metabolites received a thorough analysis. In the pharate pupae and pupae of P. brassicae (Lafont et al., 1980) the majority of polar metabolites from injected [^{3}H] ecdysone were in the form of unhydrolysable polar metabolites. By h.p.l.c. the unhydrolysable fraction from Pieris was resolved into two pairs of polar compounds (P_1 and P_2). The pK of these compounds was evaluated at 4-5 (analogous to that of a carboxylic acid) and methylation of these compounds by diazomethane resulted in a large decrease in polarity of these compounds. In addition, it was noted

that methylated P_1 chromatographed close to 20-hydroxyecdysone. It was therefore postulated, that the P_1 pair could be the 3α and 3β epimers of a 20-hydroxyecdysone 26-carboxylic acid, and the P_2 pair the corresponding derivatives of ecdysone. Indeed, injection of $[^3H]$ 20-hydroxyecdysone or $[^3H]$ ecdysone into Pieris pupae yielded the P_1 pair, or the P_1 plus P_2 pairs, respectively (Beydon et al., 1981). It has also been verified that P_1 and P_2 were true endogenous ecdysteroid metabolites and not mere catabolites of injected $[^3H]$ hormone, by $[^3H]$ cholesterol incorporation experiments in Pieris (Beydon et al., 1981).

In this study of ecdysone metabolites in S.littoralis pupae, analogous unhydrolysable compounds have been isolated (see Section A). The major aim of the work described in this section was to unequivocally identify these compounds which constitute a high proportion of ecdysone metabolites in this insect.

The metabolism in vivo of both $[^{14}C]$ cholesterol and $[^3H]$ ecdysone in pupae (Part 3, Section A) and larvae (Part 4, Section A) all yielded two unhydrolysable polar compounds, which remained unidentified after the full analysis of the other metabolites by h.p.l.c. The highest yield of these compounds was observed during the pupal metabolism of $[^3H]$ ecdysone injected at day 5 (male) or day 6 (female) and the insects sacrificed at day 7 (see Section A). Therefore, the $[^3H]$ unhydrolysable polar metabolites from that experiment were chosen for further characterisation.

EXPERIMENTAL AND RESULTS

High-performance liquid chromatographic systems

System 1: a Partisil-ODS 3 reversed phase column eluted at a flow rate of 2 ml/min with a linear gradient (30 minutes) of methanol in water changing from (1:19 v/v) to (7:3, v/v).

System 2: an Ultrasphere-ODS reversed phase column eluted at a flow rate of 1 ml/min with a linear gradient (20 minutes) of methanol in water changing from (7:13, v/v) to (7:3, v/v).

System 3: a Partisil-SAX (anion exchange) column eluted isocratically (2 ml/min) with 0.01M sodium phosphate buffer pH 5.8.

System 4: a Partisil ODS-3 reversed phase column eluted at a flow rate of 1 ml/min with a linear gradient (30 min) of acetonitrile in 0.02M Tris perchlorate buffer pH 7 changing from (1:10, v/v) to (2:3, v/v).

System 5: a Partisil ODS-3 reversed phase column eluted at a flow rate of 2 ml/min with a linear gradient (30 min) of methanol in 0.02M sodium citrate buffer pH 6.5 changing from (1:9, v/v) to (7:3, v/v).

System 6: a Partisil ODS-3 reversed phase column eluted at a flow rate of 2 ml/min with a linear gradient (40 min) of methanol in water changing from (1:19, v/v) to (1:1, v/v).

System 7: an APS-Hypersil column eluted isocratically (2 ml/min) with 7.5% methanol in dichloroethane.

System 8: an APS-Hypersil column eluted isocratically (2 ml/min) with 5.5% methanol in dichloroethane.

Characterisation of the unhydrolysable polar fraction from the metabolism *in vivo* of [^{3}H] ecdysone in *S. Littoralis* pupae

The [^{3}H] unhydrolysable polar ecdysteroid fraction was characterised by a combination of techniques. In addition to h.p.l.c. analysis of this fraction, which was performed during routine analysis of [^{3}H] ecdysone metabolites (see Section A), thin-layer chromatography and electrophoresis were also used for a preliminary investigation of the properties of these compounds.

H.p.l.c. analysis: H.p.l.c. using system 1 revealed the characteristic profile typical of the unhydrolysable polar fraction, consisting of two peaks of similar polarity, both being more polar than ecdysone and 20-hydroxyecdysone. For reference purposes these two compounds will be referred to as U.P.1 (unhydrolysable polar one) and U.P.2 (see figure 3B-1).

T.l.c. analysis (figure 3B-2): continuous elution for 1½ hours with an ethylacetate/ethanol/water (20:80:10, v/v/v) solvent system, followed by scanning of the plate for radioactivity revealed the presence of one major radioactive band (Rf 0.38) and a minor band (Rf 0.26).

Cellulose acetate electrophoresis (figure 3B-3) was performed in 0.25M sodium phosphate buffer (pH 7) with 100 volts applied for 6½ hours. The strip was then cut into sections for radioassay in liquid scintillation cocktail (Aquasol). The radioactive material migrated slowly towards the anode, behaviour typical of a weak acid as demonstrated by the migration of cholic acid.

Anion-exchange chromatography: H.p.l.c. using a Partisil-SAX column (system 3) was attempted, but U.P.1 and U.P.2 were only retained on the column marginally longer than the void volume. Therefore, this means

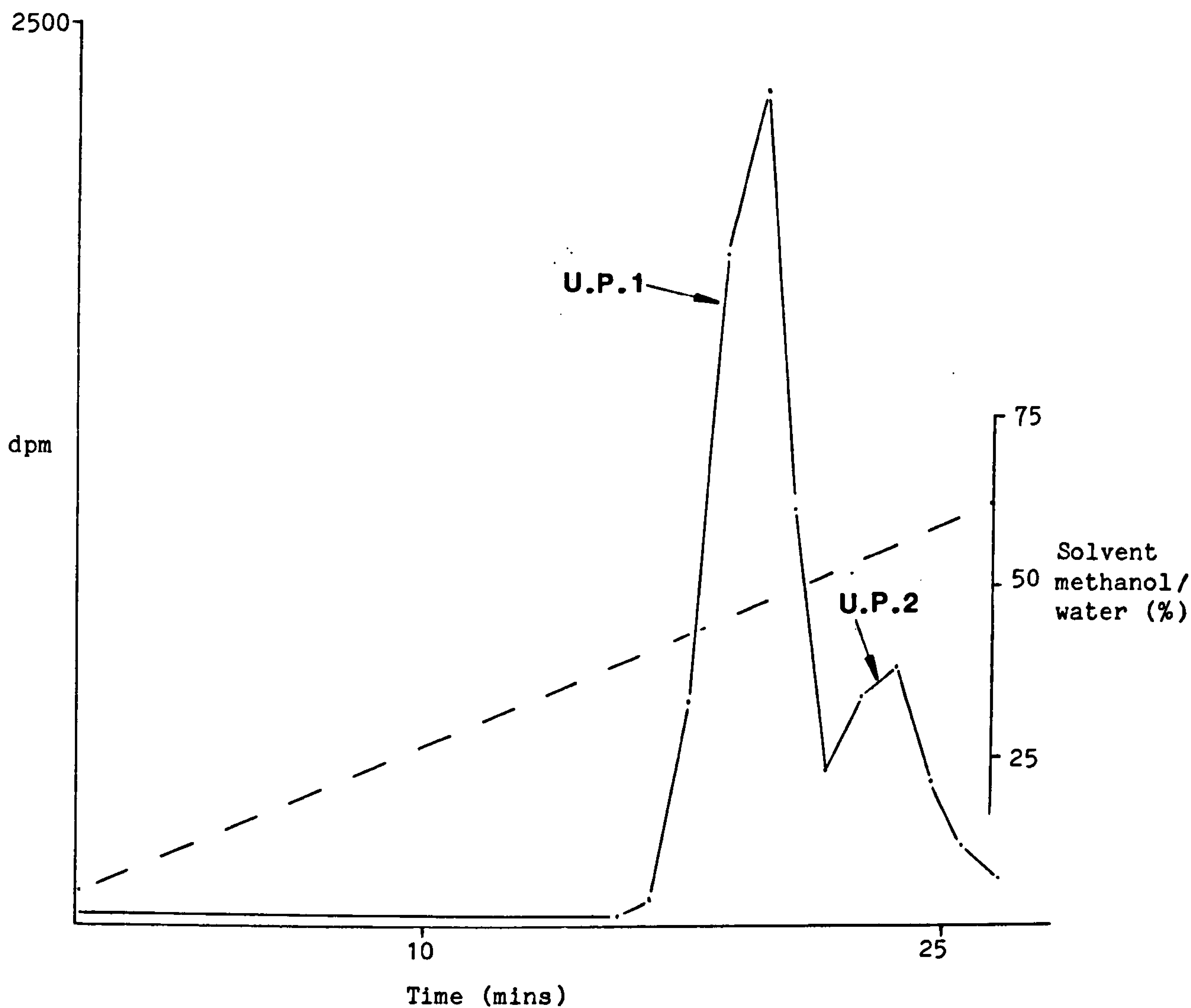


Figure 3B-1 : H.p.l.c. analysis (system 1) of the [^{3}H] unhydrolysable polar ecdysteroid fraction from silicic acid column chromatography. The metabolites were extracted from S. littoralis pupae after injection of [^{3}H] ecdysone (part 3, section A).

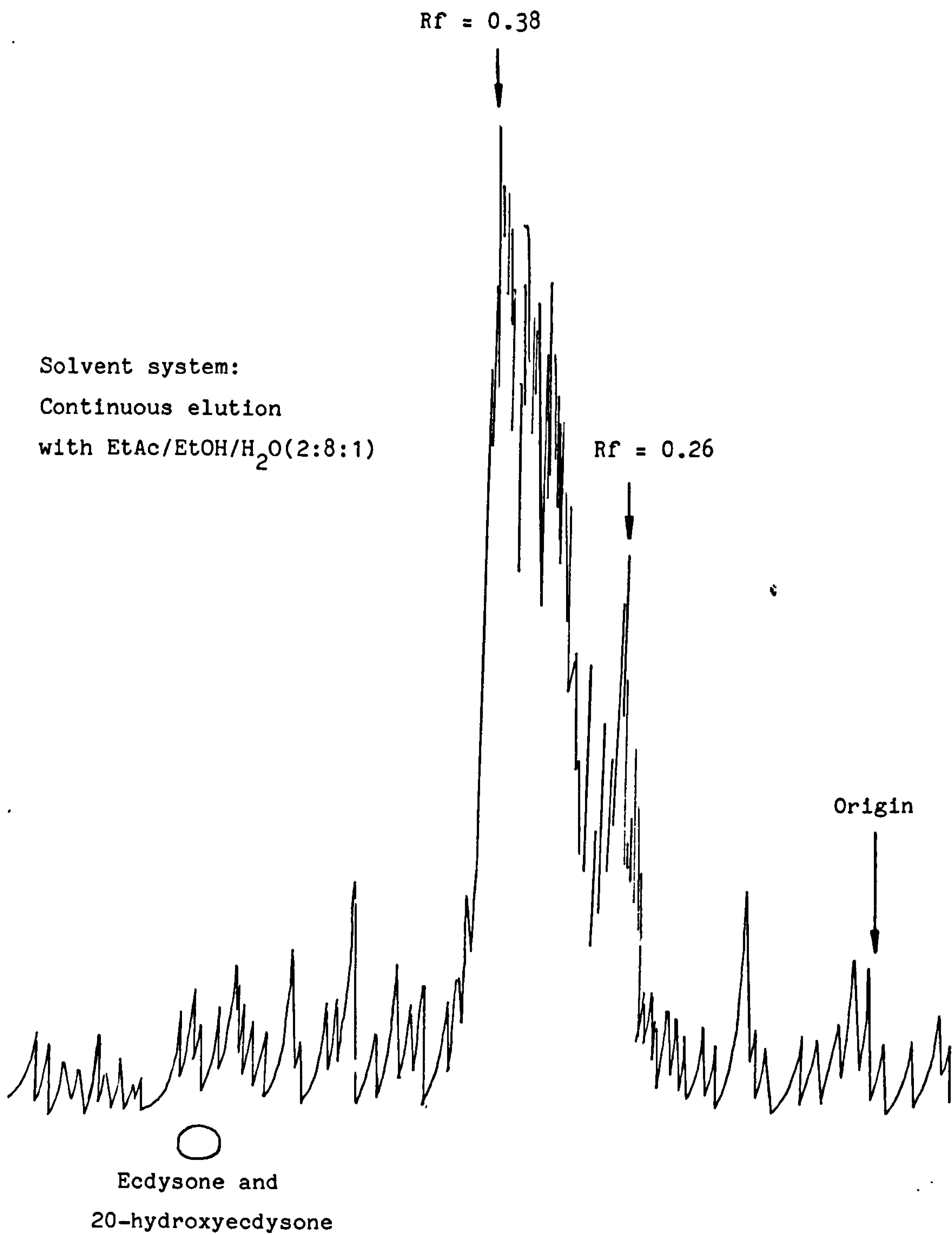


Figure 3B-2 : Silica t.l.c. radiochromatogram of the [^{3}H] unhydrolysable polar ecdysteroid fraction (from silicic acid column chromatography).

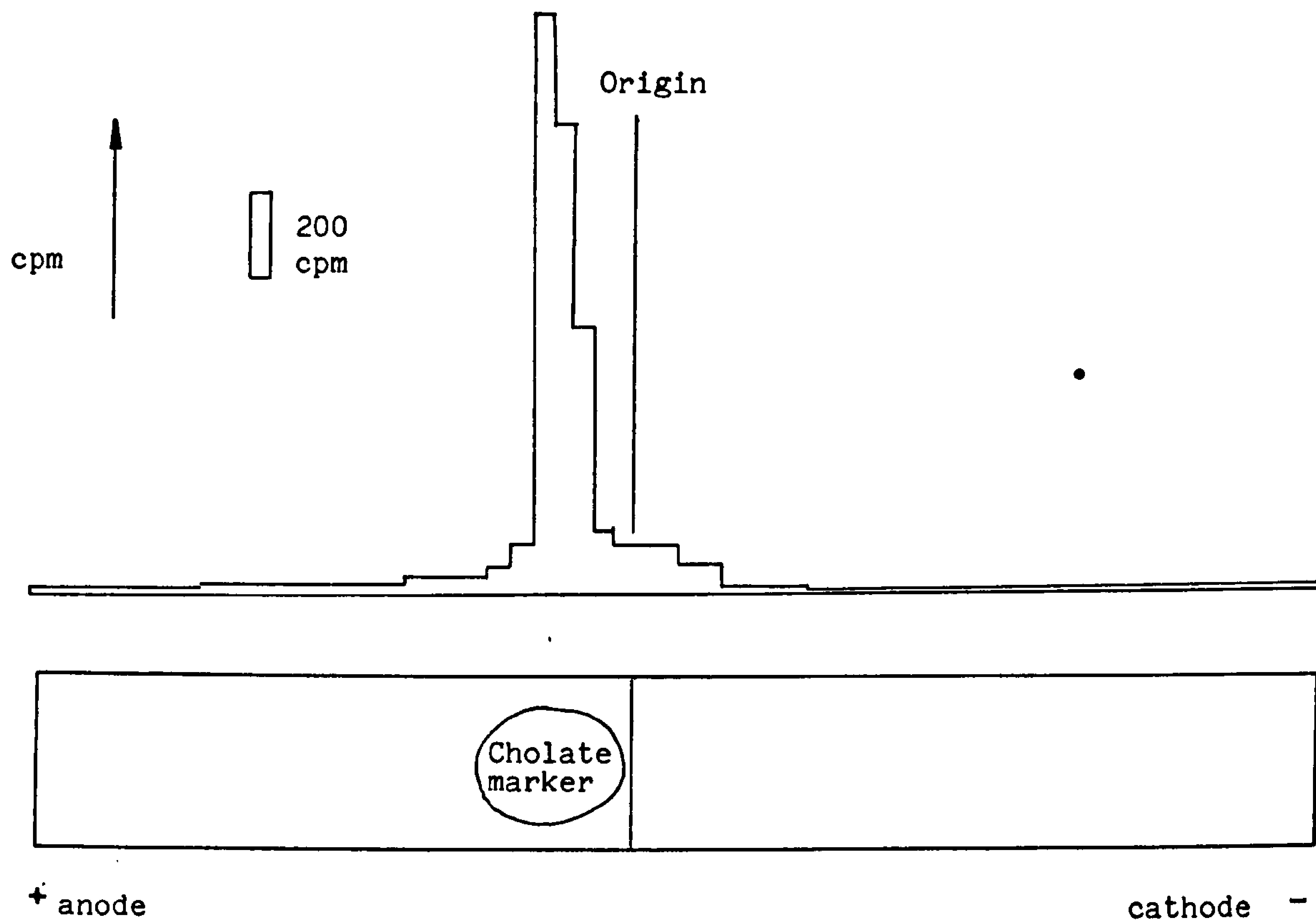


Figure 3B-3 : Cellulose acetate electrophoresis of the [^{3}H] unhydrolysable highly polar ecdysteroid fraction, using a cellulose acetate strip (15 cm x 2 cm)with the voltage set at 100v for 6½ hours.

of analysis could not be used effectively. However, this behaviour on anion-exchange does indicate that the U.P. compounds are not highly charged as are compounds such as ecdysteroid phosphates and sulphates.

The metabolism *in vivo* of [^{3}H] 20,26-dihydroxyecdysone

From the metabolism *in vivo* of [^{3}H] ecdysone (section A) it can be seen that ecdysone is metabolised via 20- and 26-hydroxylation to 20,26-dihydroxyecdysone, and is also metabolised to the more polar compounds U.P.1 and U.P.2. It would, therefore, be conceivable that 20,26-dihydroxyecdysone could constitute an intermediate in the formation of U.P.1 and/or U.P.2.

To investigate the possible intermediacy of 20,26-dihydroxyecdysone in the metabolism of [^{3}H] ecdysone to the unhydrolysable compounds, day 6 female pupae were injected with [^{3}H] 20,26-dihydroxyecdysone and the insects sacrificed after 24 hours. The [^{3}H] 20,26-dihydroxyecdysone used in this experiment was purified by h.p.l.c. from the [^{3}H] products of previous *in vivo* [^{3}H] ecdysone incubations (section A), and its purity verified by h.p.l.c. on both reversed-phase and adsorption columns.

Six day 6 female pupae were injected with 200,000 dpm of [^{3}H] 20,26-dihydroxyecdysone and then reared under normal conditions for 24 hours. The pupae were then extracted and purified by silicic acid column chromatography (as described in Part 2). The free ecdysteroid fraction (30% methanol/chloroform silicic acid column fraction; 86,800 dpm) was analysed by h.p.l.c. system 2 (figure 3B-4) showing that the only radioactive compound in this fraction is the unmetabolised [^{3}H] 20,26-dihydroxyecdysone. The highly polar fraction (80% methanol/chloroform fraction; 42,800 dpm) was subjected to enzymic hydrolysis by the *Helix pomatia* enzyme preparation and the products of the hydrolysis separated from any unhydrolysable polar products by a second silicic acid column

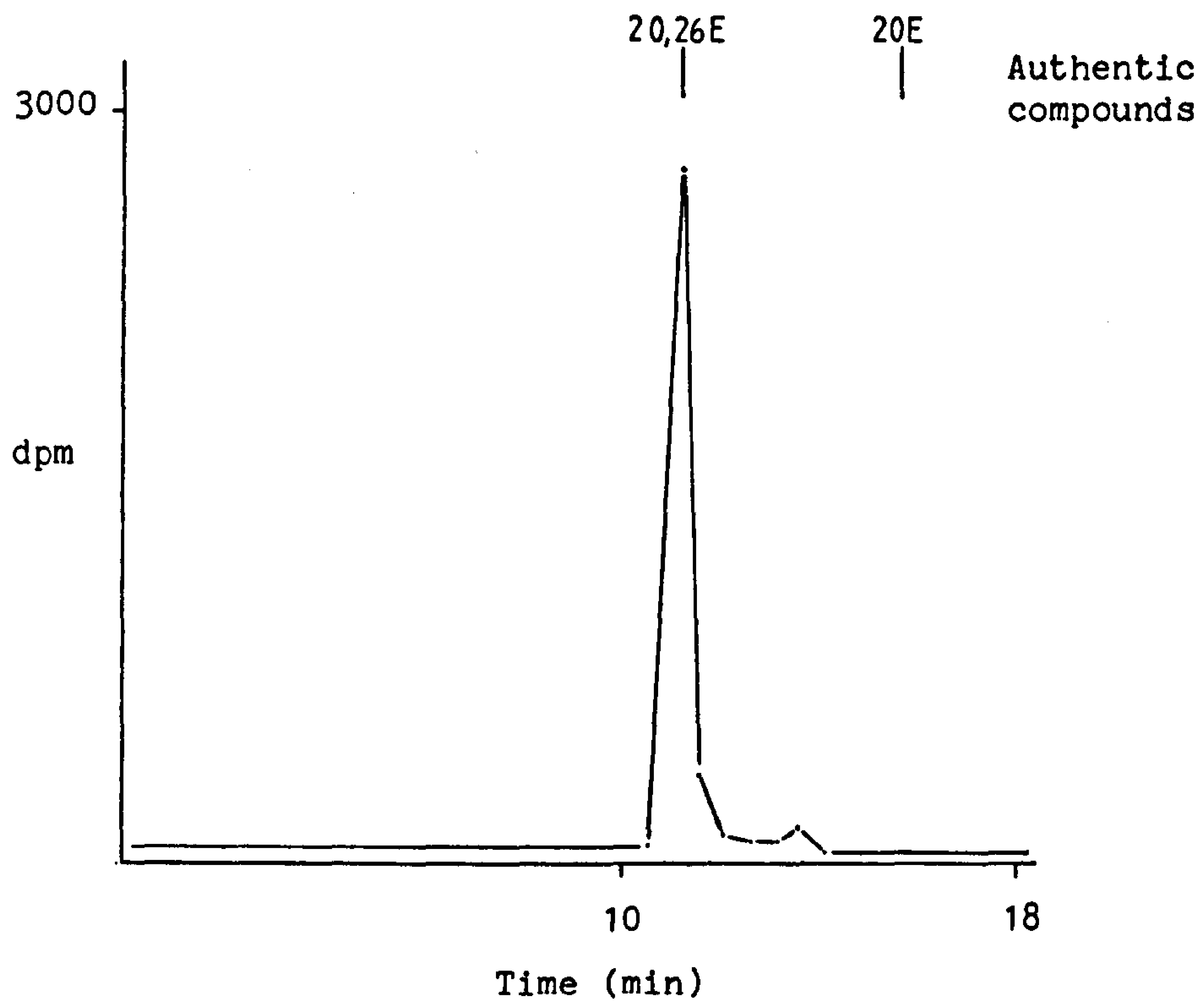


Figure 3B-4: H.p.l.c. analysis (system 2) of the [^{3}H] free ecdysteroid fraction from silicic acid column chromatography, after metabolism *in vivo* of [^{3}H] 20,26-dihydroxyecdysone in pupae.

chromatography (see Part 2). The resulting fractions [the 30% methanol/chloroform fraction containing ecdysteroids released by hydrolysis (8,400 dpm) and 80% methanol/chloroform fraction containing unhydrolysable polar compounds (22,500 dpm)] were analysed by h.p.l.c. The released ecdysteroids analysed by system 2 (figure 3B-5) show that the majority of the conjugated ecdysteroids were in the form of 20,26-dihydroxyecdysone conjugates. The unhydrolysable polar fraction, analysed by h.p.l.c. system 1 (figure 3B-6) reveals a single peak at retention time 19 minutes, which chromatographs with U.P.1. This indicates that 20,26-dihydroxyecdysone is an intermediate in the formation of one of the polar compounds. Furthermore, it would tend to suggest that U.P.1 is formed from 20,26-dihydroxyecdysone whereas the less polar U.P.2 is an analogous metabolite of 26-hydroxyecdysone.

The unhydrolysable compound formed during the metabolism of 20,26-dihydroxyecdysone _in vivo_ was further characterised by t.l.c. and electrophoresis. T.l.c. analysis (figure 3B-7) was carried out using continuous elution for 1½ hours with an ethyl acetate/ethanol/water (20:80:10, v/v/v) solvent system. The developed plate was scraped in 1 cm bands and radioassayed. One radioactive peak chromatographing at Rf = 0.35 was observed.

Cellulose acetate electrophoresis (figure 3B-8): The same system as used earlier was employed (100 volts applied for 6½ hours in 0.25M sodium phosphate buffer pH 7). The radioactivity displayed the same behaviour as U.P.1 and U.P.2 formed from [^{3}H] ecdysone (i.e. migrates slowly towards the anode).

This series of analyses (t.l.c., electrophoresis and h.p.l.c), thus confirms that the polar product U.P.1 produced from [^{3}H] ecdysone metabolism _in vivo_ is identical to the polar product formed from [^{3}H]

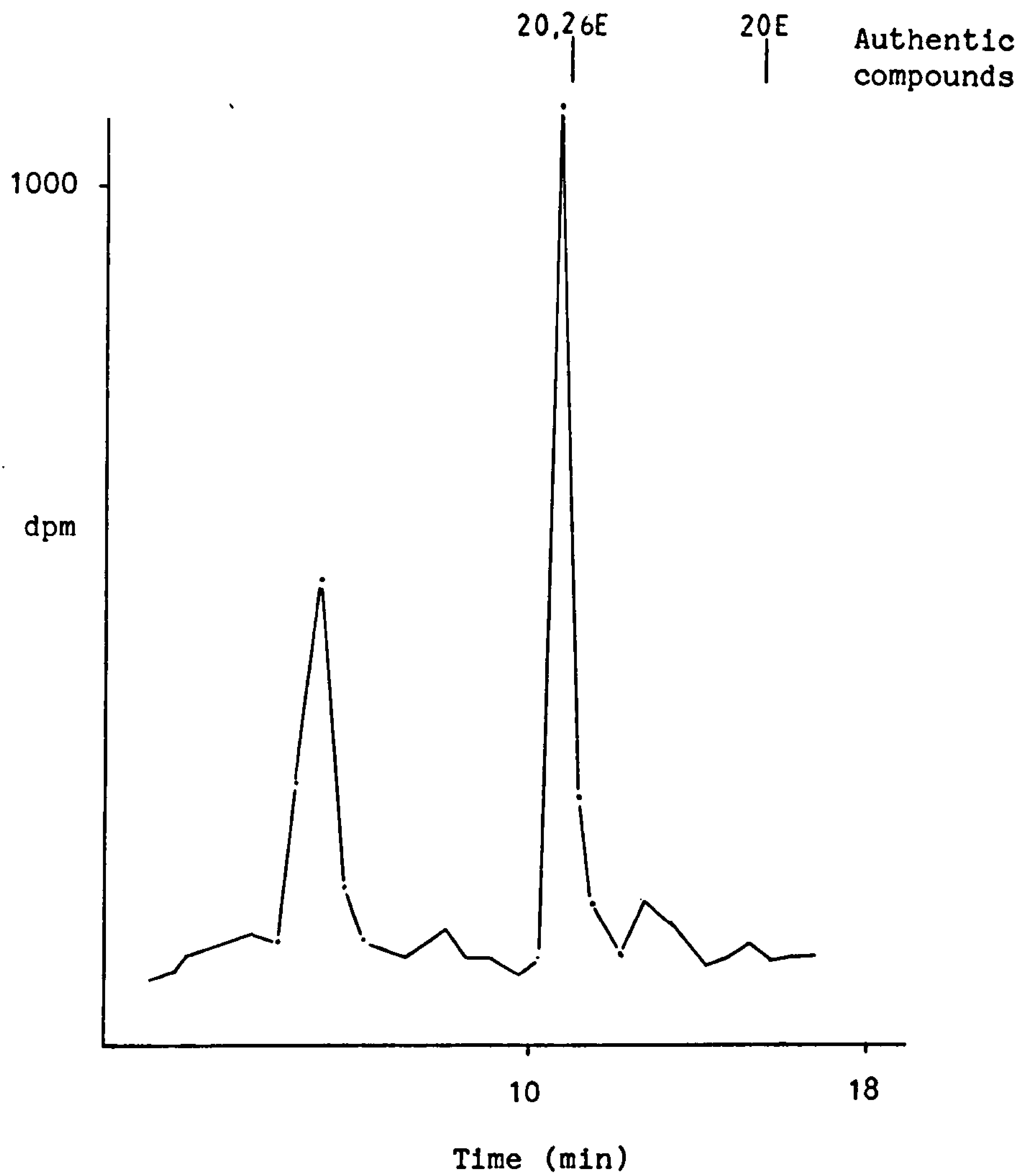


Figure 3B-5 : H.p.l.c. analysis (system 2) of the [^{3}H] ecdysteroids released by the H.pomatia enzyme from conjugates produced by metabolism in vivo of [^{3}H] 20,26-dihydroxyecdysone in pupae.

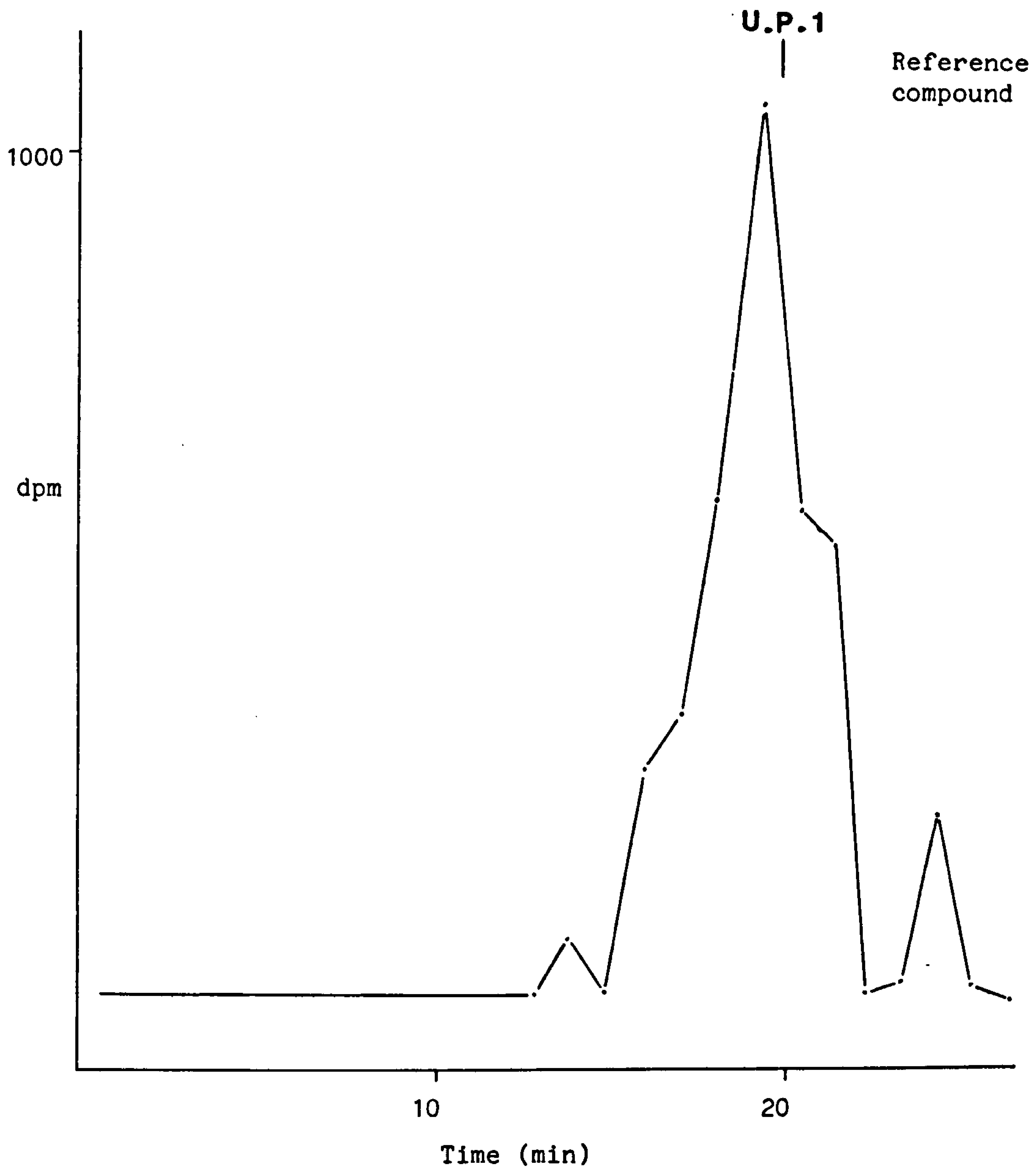


Figure 3B-6 : H.p.l.c. analysis (system 1) of the [^{3}H] unhydrolysable polar ecdysteroid fraction (from silicic acid column chromatography), produced by metabolism *in vivo* of [^{3}H] 20,26-dihydroxyecdysone in pupae. The [^{3}H] UP.1 produced from the metabolism *in vivo* of [^{3}H] ecdysone in pupae (see fig. 3B-1) was used as a reference compound, and was chromatographed on the same system just prior to the present anslysis.

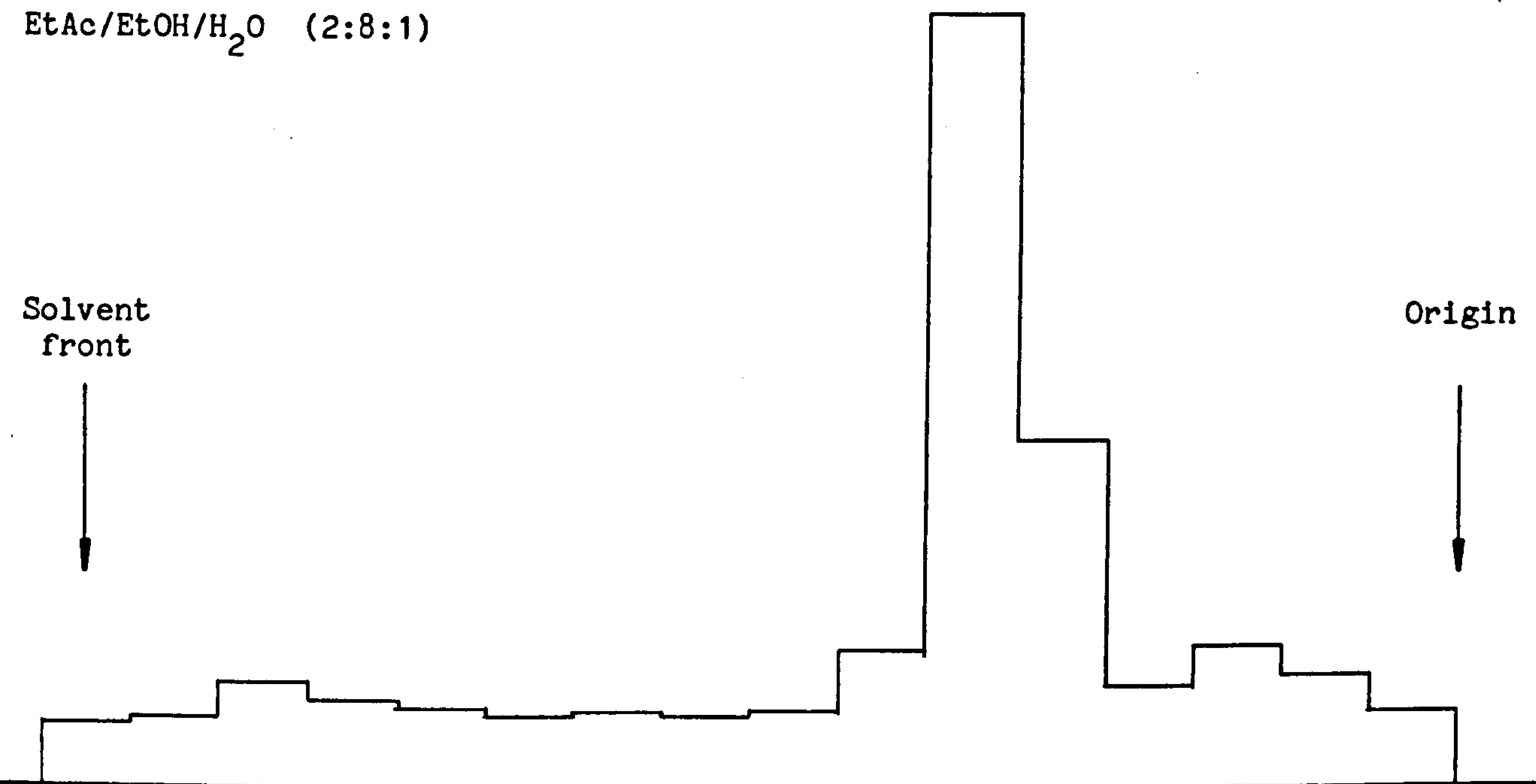


Figure 3B-7 : Silica t.l.c. radiochromatogram of the [^{3}H] unhydrolysable polar ecdysteroid fraction (from silicic acid column chromatography) formed by metabolism *in vivo* of [^{3}H] 20,26-dihydroxyecdysone in pupae.

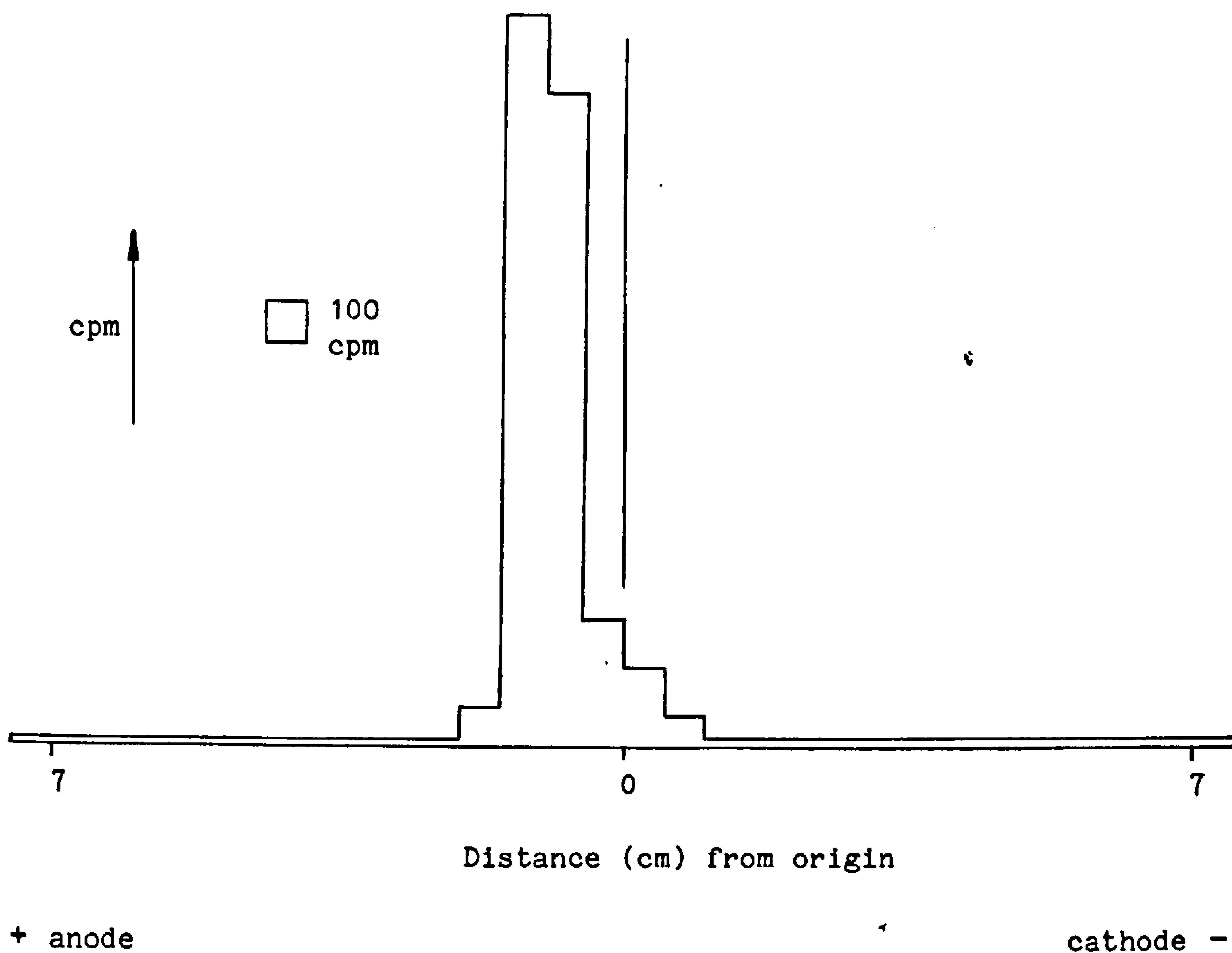


Figure 3B-8 : Cellulose acetate electrophoresis of the [^{3}H] unhydrolysable highly polar ecdysteroid fraction formed by metabolism *in vivo* of [^{3}H] 20,26-dihydroxyecdysone in pupae.

20,26-dihydroxyecdysone metabolism. This implies that 20,26-dihydroxy-ecdysone is indeed an intermediate in the formation of U.P.1. It would also appear, according to the cellulose acetate electrophoresis behaviour, that U.P.1 may be a carboxylic acid derivative of 20,26-dihydroxy-ecdysone with U.P.2 being an analogous derivative of 26-hydroxyecdysone. The addition of a carboxylic acid grouping on the ecdysteroid molecule would presumably result from oxidation of a primary hydroxyl group (i.e. at the C-26 position). At this stage, the evidence was consistant with the possibility that the unknown compounds were 20-hydroxyecdyson-26-oic acid* (U.P.1) and ecdyson-26-oic acid* (U.P.2).

Methylation of the putative 20-hydroxyecdyson-26-oic acid (U.P.1)

The evidence for the structure of U.P.1 has been strongly in favour of a 26-oic derivative of 20-hydroxyecdysone. Therefore, to further characterise this compound it was methylated.

Two methylation techniques were attempted, the first involved the method described by Neises and Steglich (1978) (see Part 2). Although this reaction methylated cholic acid to methyl cholate (characterised by mass spectrometry, chemical ionisation, isobutane , m/z, 387 $[M+H-2H_2O]^+$, 369$[M+H-3H_2O]^+$, 355 $[M+H-2H_2O-CH_3OH]^+$, 337 $[M+H-3H_2O-CH_3OH]^+$) no reaction with U.P.1 was achieved. The reason for this is uncertain.

The second methylation procedure employed diazomethane as the methylating agent (see Part 2). The products of this reaction were analysed by reversed-phase h.p.l.c. system 5 (figure 3B-9), which shows the production of one major peak eluting at a similar retention time to 20-hydroxyecdysone. This chromatographic behaviour is consistent with

* semi-trivial names given to 2β, 3β, 14α,20R, 22R, 25-hexahydroxy-5β-cholest-7-en-6-on-26-oic acid and 2β, 3β, 14α, 22R, 25 pentahydroxy-5β-cholest-7-en-6-on-26 oic acid.

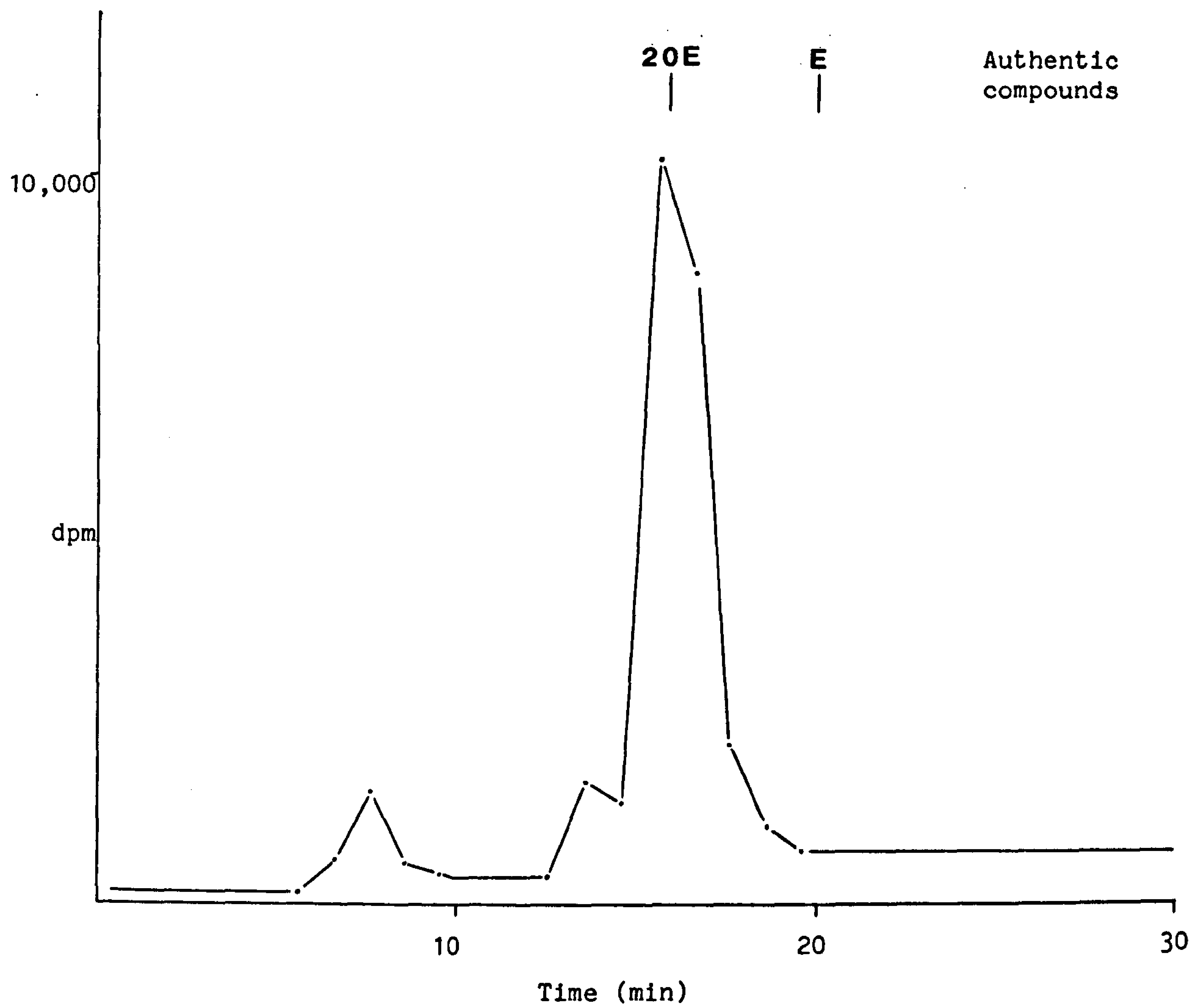


Figure 3B-9 : H.p.l.c. analysis (system 5) of the methylated unhydrolysable polar ecdysteroid, U.P.1.

the assumption that this compound is the methyl ester of 20-hydroxyecdysone, with the effect of the carboxylic group of U.P.1 being nullified by its methylation, thus producing a molecule of similar polarity to 20-hydroxyecdysone.

Chromatography using an aminopropyl column is not possible with these unhydrolysable compounds, as no elution of radioactivity is observed. This is presumably due to very strong interaction between the amino groups of the column and the proposed carboxylic acid group of the unhydrolysable polar compounds. However, as would be expected, no such problems occur with the methyl esters of these compounds and the developed chromatogram using h.p.l.c. system 7 (figure 3B-10) reveals that once again the methyl ester chromatographs close to 20-hydroxyecdysone.

Isolation of mass of polar ecdysteroids U.P.1 and U.P.2

For further physico-chemical characterisation of these unknown compounds it was necessary to isolate large quantities of U.P.1 and U.P.2. This was achieved by extraction of 525 day 9 male pupae. At the same time, ten day 5 male pupae were injected with 10 µCi of [23, 24 $^3H_2$] ecdysone (1.6 Ci/mmol) and the insects sacrificed at day 9 of pupal development, to produce [^{3}H] polar metabolites present at this stage of pupal development. The pupae incubated with [^{3}H] ecdysone were extracted and purified by the same procedure as used in section A. The unhydrolysable polar ecdysteroid fraction [80% methanol/chloroform fraction, after hydrolysis of the polar ecdysteroids (see figure 2-1)]

was analysed by h.p.l.c. system 4 (figure 3B-11). As expected, both [^{3}H] U.P.1 and [^{3}H] U.P.2 were produced with little else being present.

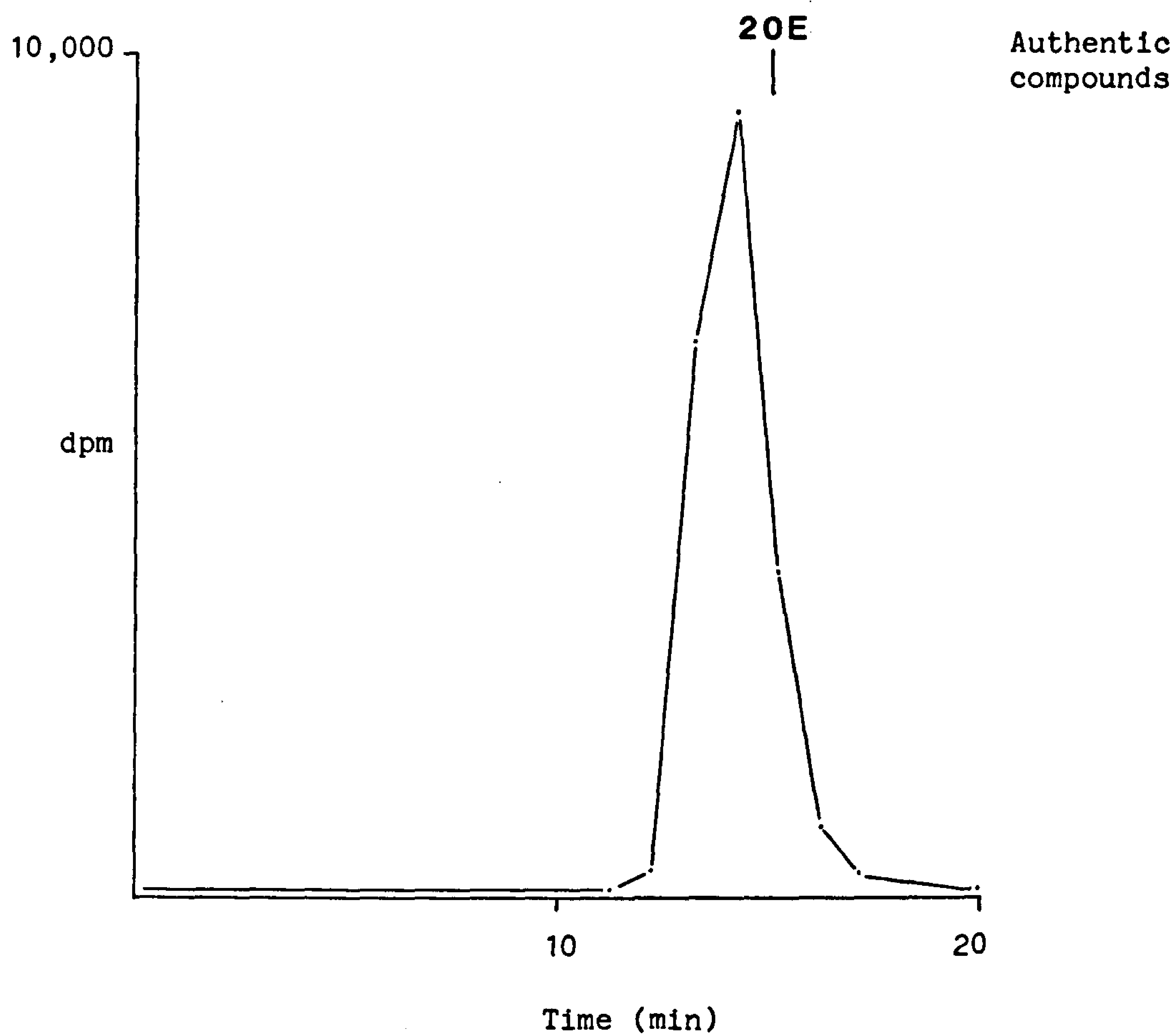


Figure 3B-10 : H.p.l.c. analysis (system 7) of the methylated unhydrolysable polar ecdysteroid , U.P.1.

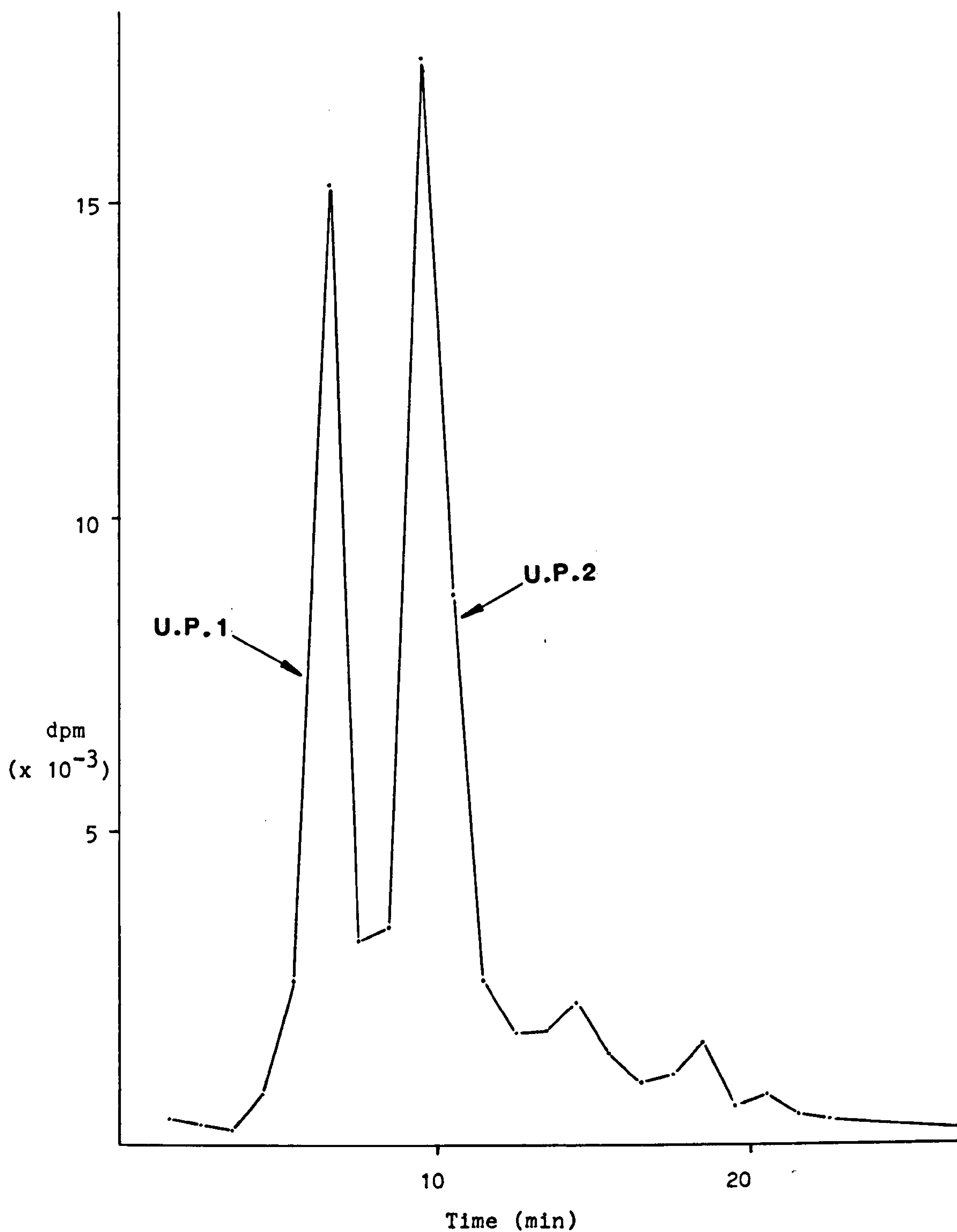


Figure 3B-11 : H.p.l.c. analysis (system 4) of the [^{3}H] polar ecdysteroids isolated from day 9 *S. littoralis* pupae after metabolism *in vivo* of [^{3}H] ecdysone.

<u>Large scale extraction of the unhydrolysable polar compounds U.P.1 and U.P.2 from day 9 pupae</u>

525 (116.4g) day 9 male pupae were extracted as described in Part 2 and the highly polar ecdysteroids (figure 2-1) further purified by silicic acid column chromatography (using two 20g columns). The highly polar ecdysteroid fraction was then further purified by h.p.l.c. using the radioactive metabolites [^{3}H] U.P.1 and [^{3}H] U.P.2 as markers.

The polar fraction was first subjected to reversed-phase chromatography using h.p.l.c. system 5 (figure 3B-12) and the peaks corresponding to U.P.1 and U.P.2 isolated and rechromatographed on the same h.p.l.c. system. The samples of U.P.1 and U.P.2, isolated in this way, were then desalted by reversed-phase SEP-PAK (see Part 2), and subjected to a second h.p.l.c. purification on system 6.

The quantities of U.P.1 and U.P.2 isolated by this procedure were estimated by U.V. peak area detected at 254 nm (based on peak areas of standard ecdysone solutions). The quantity of U.P.1 was approximately 27 μg with the amount of U.P.2 being in the order of 1 μg. These quantities are sufficient for characterisation by mass spectrometry and methylation, but not for Fourier transformer n.m.r. studies. Therefore, to obtain the required quantities for a thorough physico-chemical analysis another source of these compounds was required.

<u>Extraction of the unhydrolysable polar fraction from developing eggs of *S. gregaria* (performed by Dr. R.E. Isaac)</u>

High concentrations of ecdysteroids have been found in newly laid eggs of the desert locust, *Schistocerca gregaria*, mainly as phosphate esters (Isaac *et al.*, 1982). During the later stages of embryogenesis the composition of the ecdysteroids changes, characterised by the appearance of ecdysone acetate conjugates (Isaac *et al.*, 1981) and

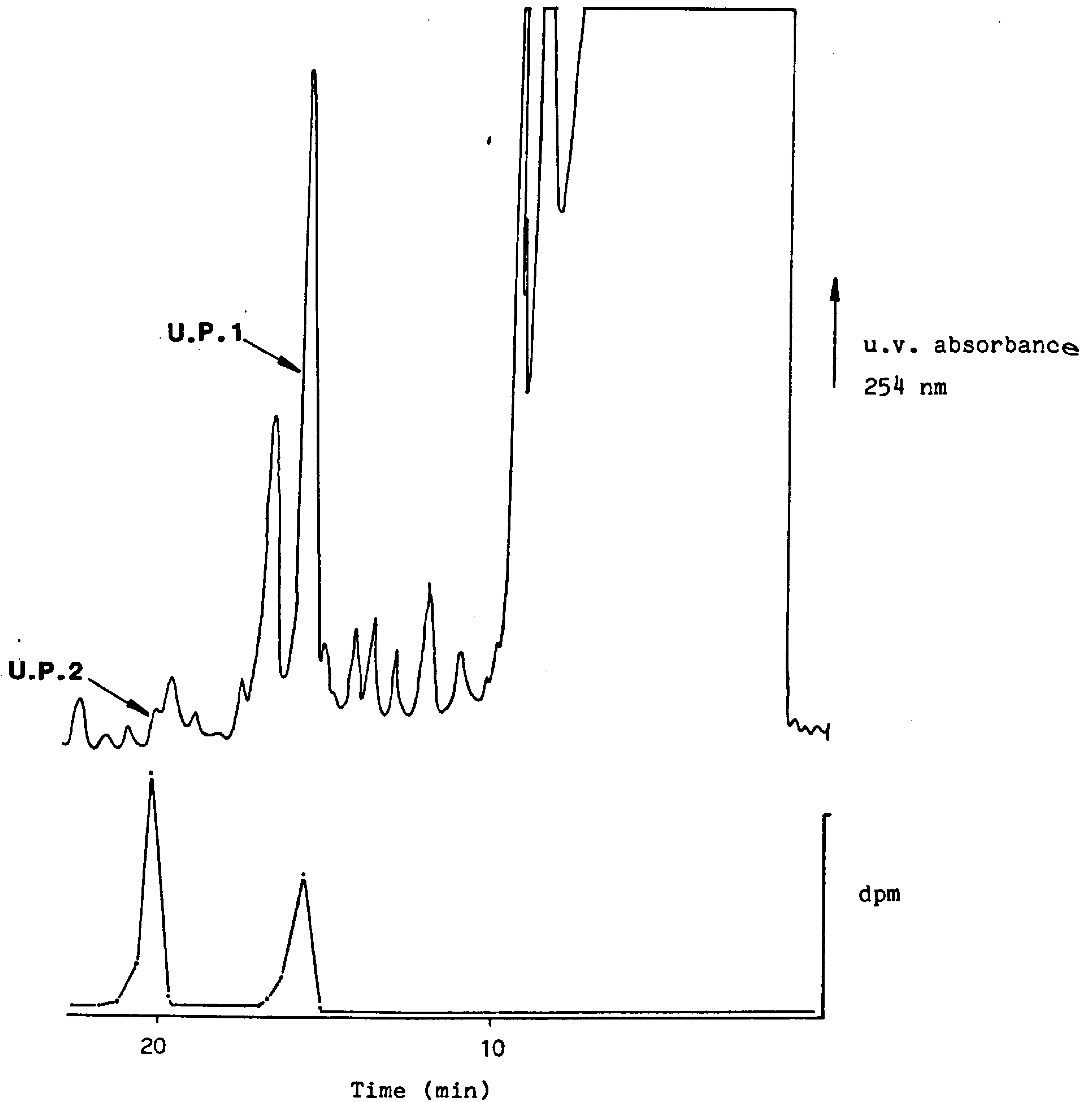


Figure 3B-12 : H.p.l.c. analysis (system 5) of the highly polar ecdysteroid fraction from the extraction of day 9 *S. littoralis* pupae. To identify the u.v. peaks corresponding to U.P.1. and U.P.2. [^{3}H] highly polar ecdysteroids isolated from pupae after metabolism *in vivo* of [^{3}H] ecdysone were used for comparison in a separate chromatographic run.

unhydrolysable polar ecdysteroids (Dinan and Rees, 1981). Therefore, eggs at a late stage of embryogenesis were used for the isolation of unhydrolysable polar ecdysteroids.

The polar ecdysteroid fraction from an extract of day 15-16 eggs (120g) was obtained by the extraction procedure described in Part 2, and was chromatographed on reversed-phase chromatography (system 5). Two u.v. absorbing compounds, unaffected by incubation with Helix enzyme, were identified by co-chromatography with [^{3}H] unhydrolysable polar compounds U.P.1 and U.P.2 (figure 3B-13), previously isolated and characterised from *S. littoralis*. These two compounds were then further purified by re-chromatography on h.p.l.c.system 5. The compounds were then desalted on reversed-phase SEP-PAK cartridges before chromatography on reversed-phase h.p.l.c. system 6. U.V spectra of the two compounds showed an absorbance maximum at 242 nm, characteristic of ecdysteroids, and indicated a yield of 300 μg for each of the compounds isolated.

Methylation of the unhydrolysable polar fractions with diazomethane

The following metabolites were methylated by treatment with diazomethane (see Part 2): U.P.1 and U.P.2 from the extraction of day 9 male *S. littoralis* pupae, U.P.1 and U.P.2 from the metabolism *in vivo* of [^{3}H] ecdysone in *S. littoralis* male pupae, and U.P.1 and U.P.2 from the extraction of day 15-16 eggs of *S. gregaria*. (Isaac *et al*., 1983).

The products of the methylation of U.P.1 from all three sources were analysed by h.p.l.c. system 5 (figure 3B-14). The results demonstrate, in all three cases, the conversion of U.P.1 to a less polar compound chromatographing close to authentic 20-hydroxyecdysone.

The methylated unhydrolysable polar compound U.P.2 was also analysed on the same h.p.l.c. system. Insufficient mass was present in the

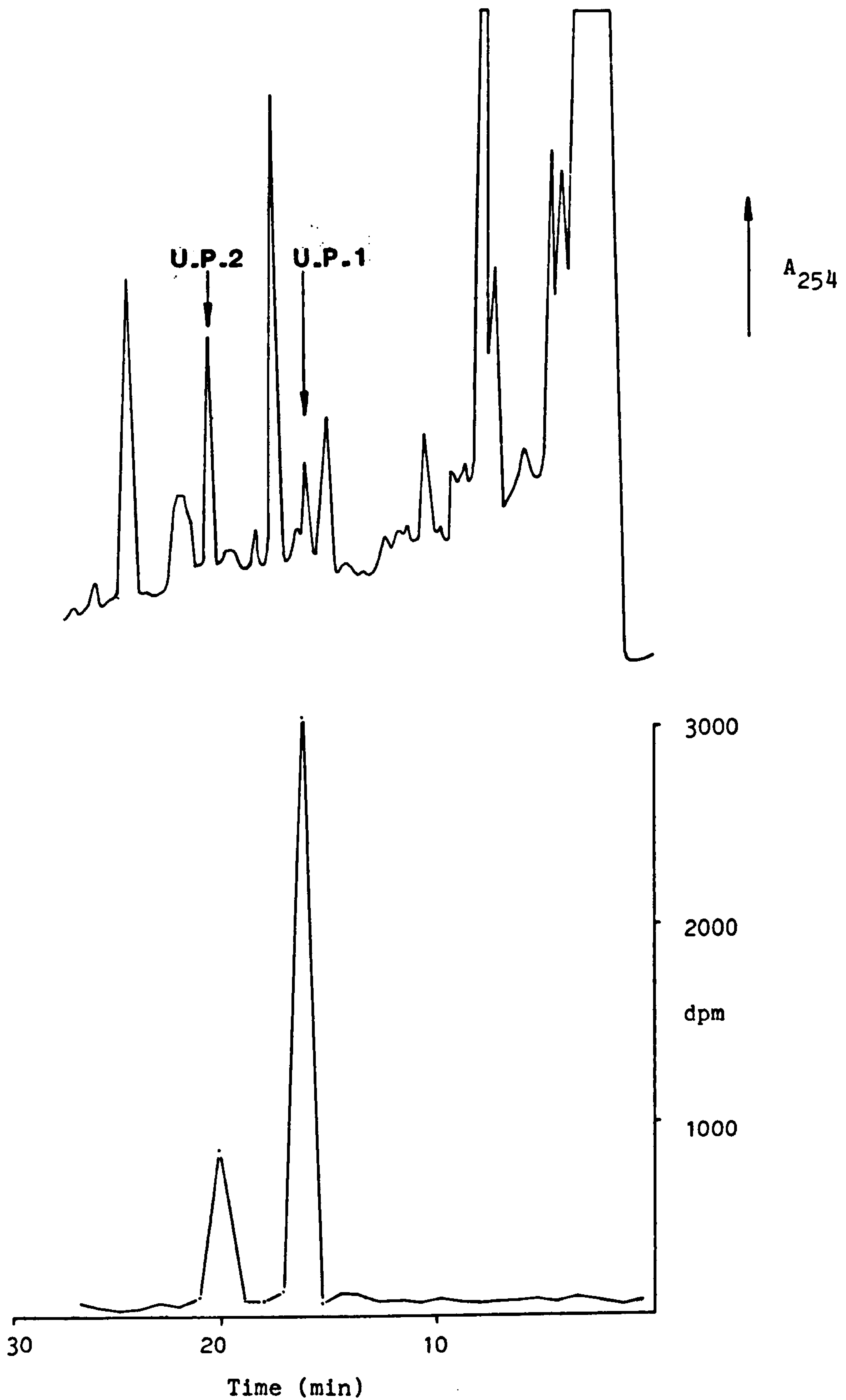
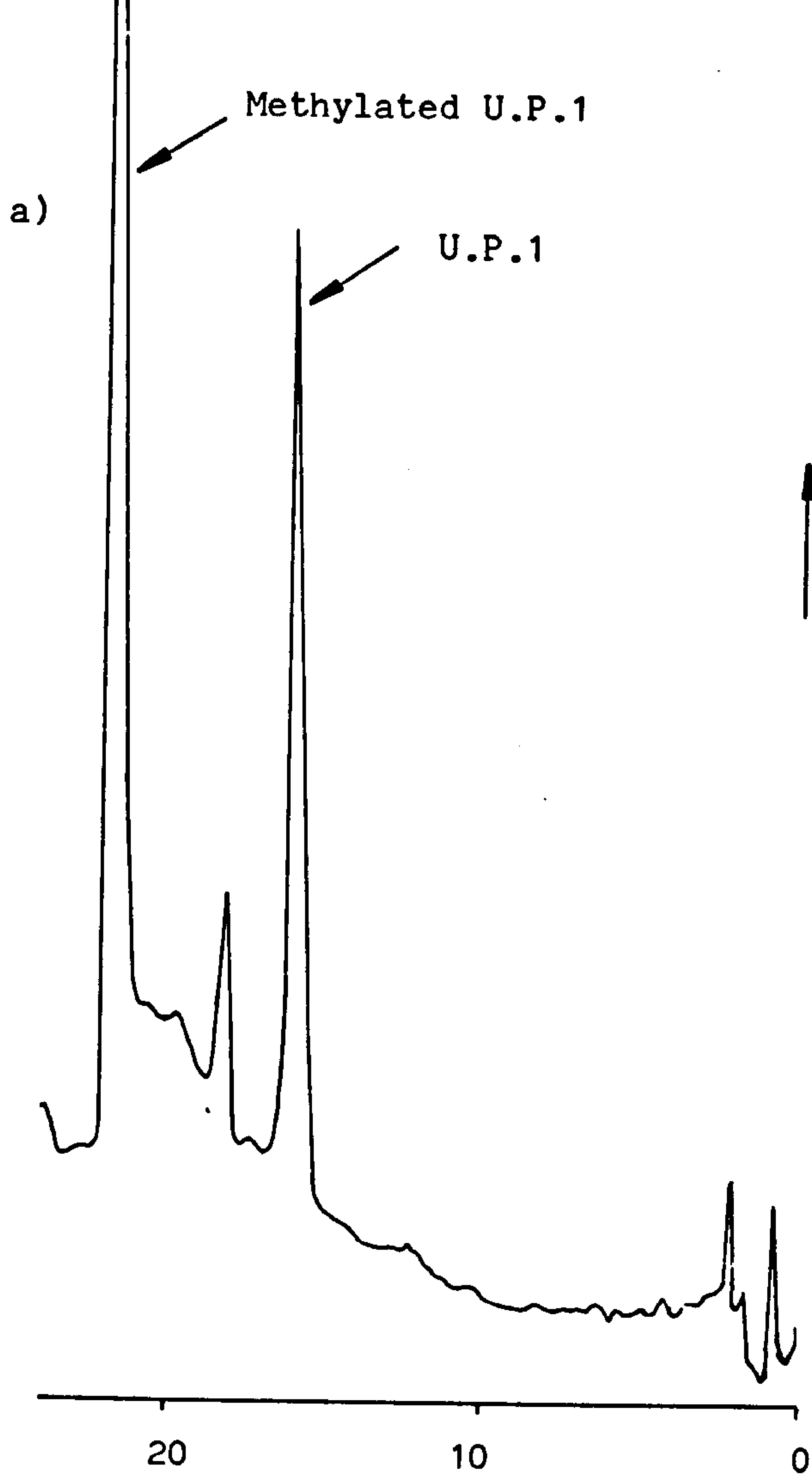
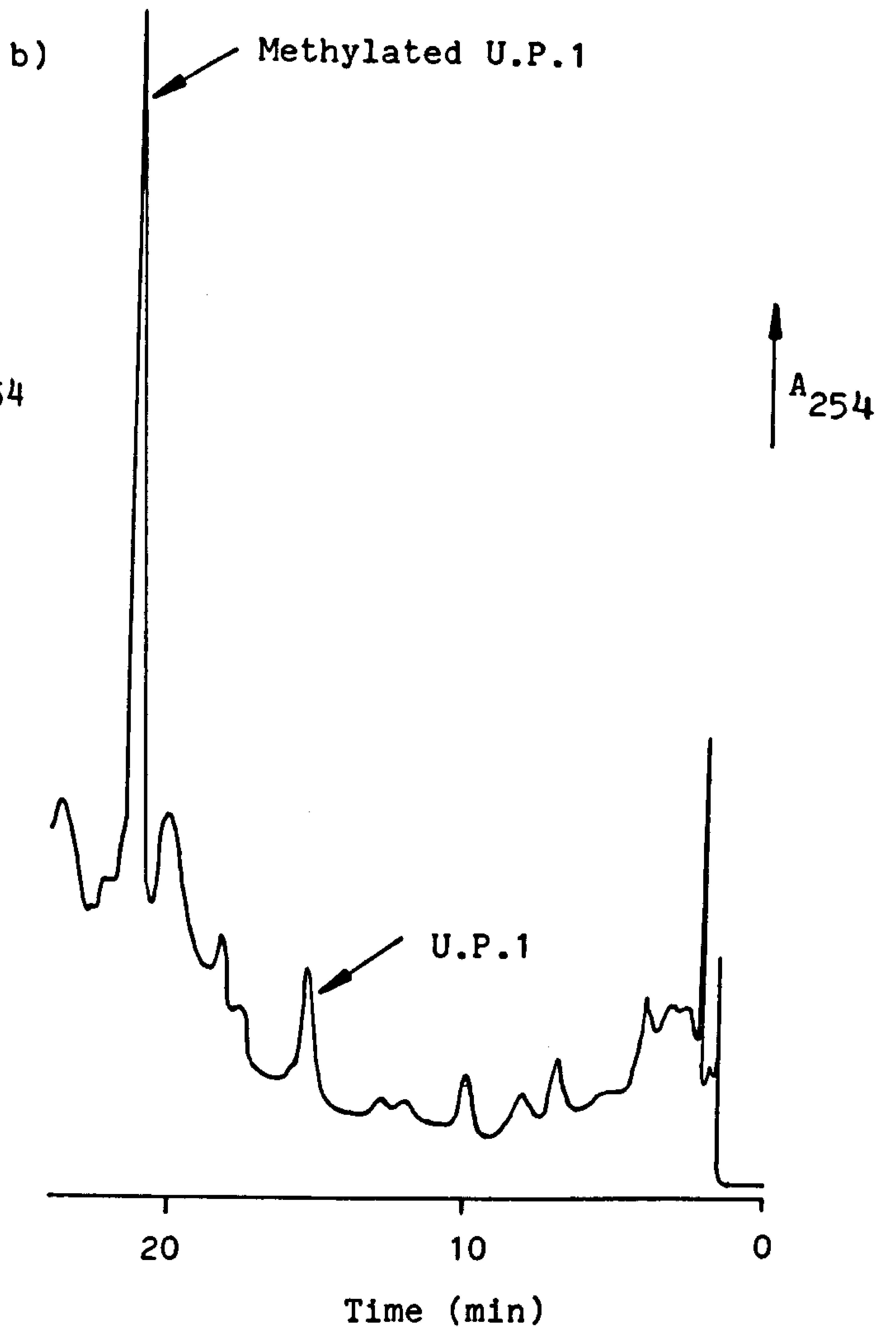


Figure 3B-13 : H.p.l.c. analysis (system 5) of the highly polar ecdysteroid fraction from *S. gregaria* eggs compared to the h.p.l.c. separation of [^{3}H] U.P.1 and U.P.2 from *S. littoralis*.





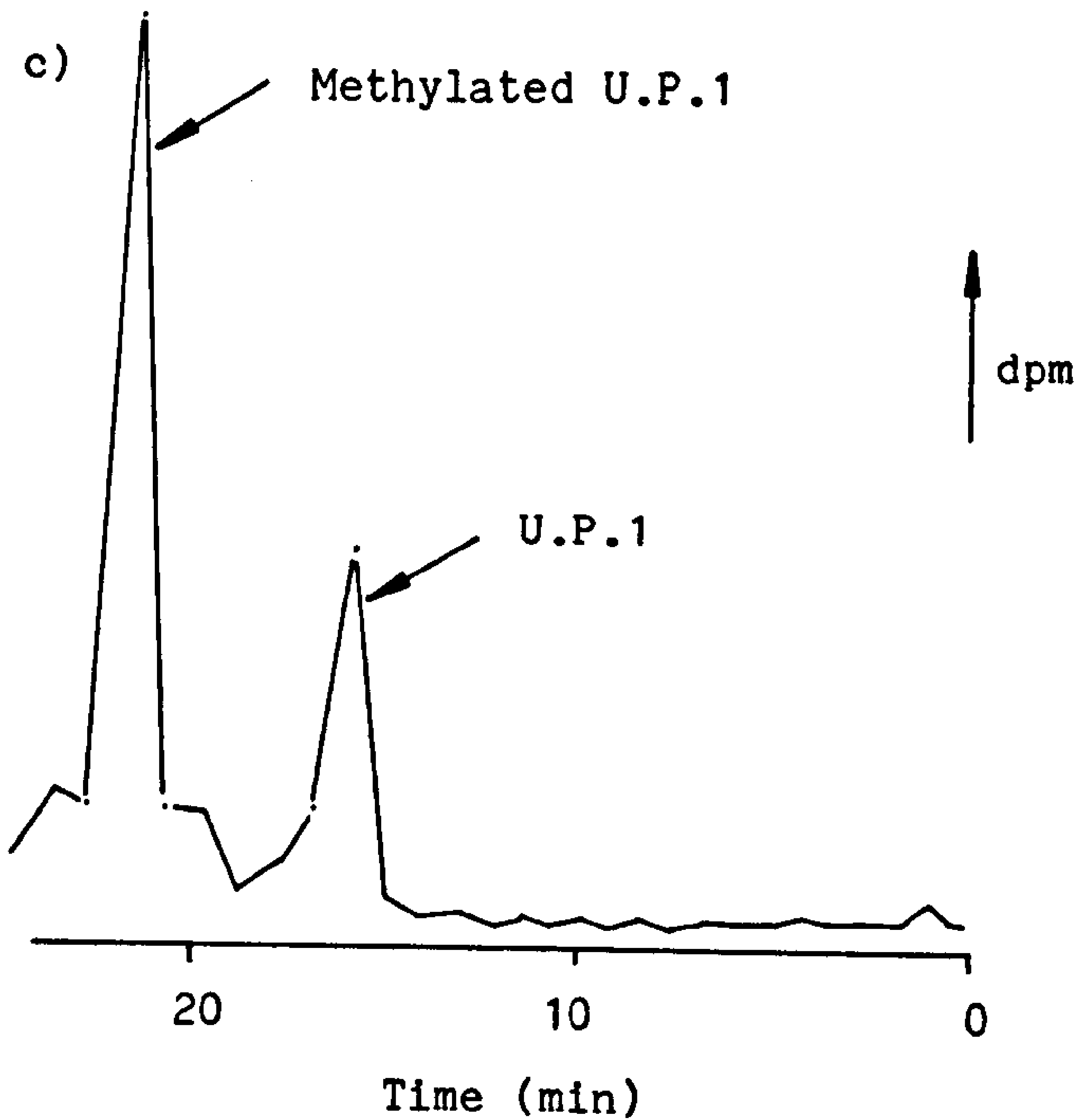


Figure 3B-14 : H.p.l.c. analysis (system 5) of methylated U.P.1 as extracted from a) *S.gregaria* eggs b) *S.littoralis* pupae c) metabolism of [^{3}H] ecdysone in *S.littoralis* pupae

S. littoralis pupal extract for conclusive detection by u.v. However, the U.P.2 from the S. gregaria egg extract and the S. littoralis [^{3}H] U.P.2 were both successfully methylated and analysed by reversed-phase h.p.l.c. (figure 3B-15). Once again U.P.2 was converted into a compound of similar polarity to ecdysone.

For thorough chromatographic characterisation by h.p.l.c. it is desirable to verify chromatographic behaviour on at least two systems. As explained earlier in this section U.P.1 and U.P.2 cannot be effectively chromatographed using anion exchange or amino propyl columns, but their methylated derivatives, possessing no carboxyl group, can be chromatographed on an APS-Hypersil column. The U.P.1 methyl ester samples were analysed by h.p.l.c. system 7 (figure 3B-16), and the U.P.2 methyl esters analysed by h.p.l.c. system 8 (figure 3B-17). It is apparent that each sample of the methyl esters of U.P.1 and U.P.2 co-chromatograph respectively. The chromatographic behaviour of these compounds is once again very similar to that of ecdysone in the case of U.P.2 methyl ester, and 20-hydroxyecdysone in the case of U.P.1 methyl ester.

Characterisation of U.P.1 and U.P.2 by mass spectrometry [negative ion fast atom bombardment (FAB)]

Sufficient material for mass spectrometry of U.P.1 and U.P.2 and their corresponding methyl esters was isolated from the S.gregaria eggs extract. From S.littoralis pupae enough material was obtained for a similar analysis of U.P.1 and U.P.1-methyl ester.

The negative ion FAB mass spectra of U.P.1 and U.P.2 (Isaac et al., 1983) isolated from S.gregaria produced abundant $[M-H]^-$ ions at m/z 509 and 493, respectively, indicating molecular weights of 510 for U.P.1 and 494 for U.P.2. This was also confirmed by the presence of low abundance ions corresponding to $[M-H]^-$ of the sodium salts (m/z 531 for

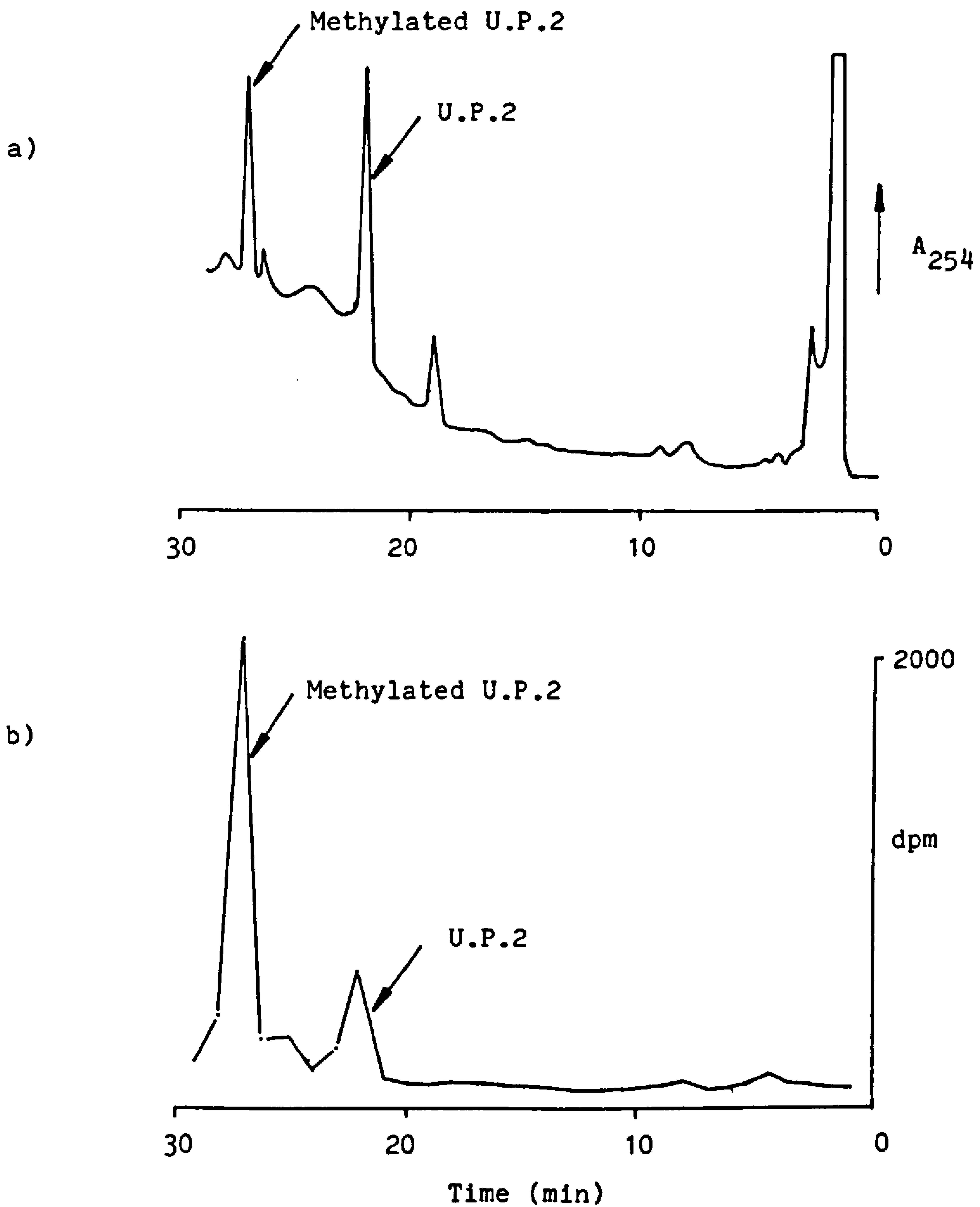


Figure 3B-15 : Reversed-phase h.p.l.c. analysis (system 5) of methylated U.P.2 as extracted from a) *S. gregaria* eggs b) metabolism *in vivo* of [^{3}H] ecdysone in *S.littoralis* pupae.

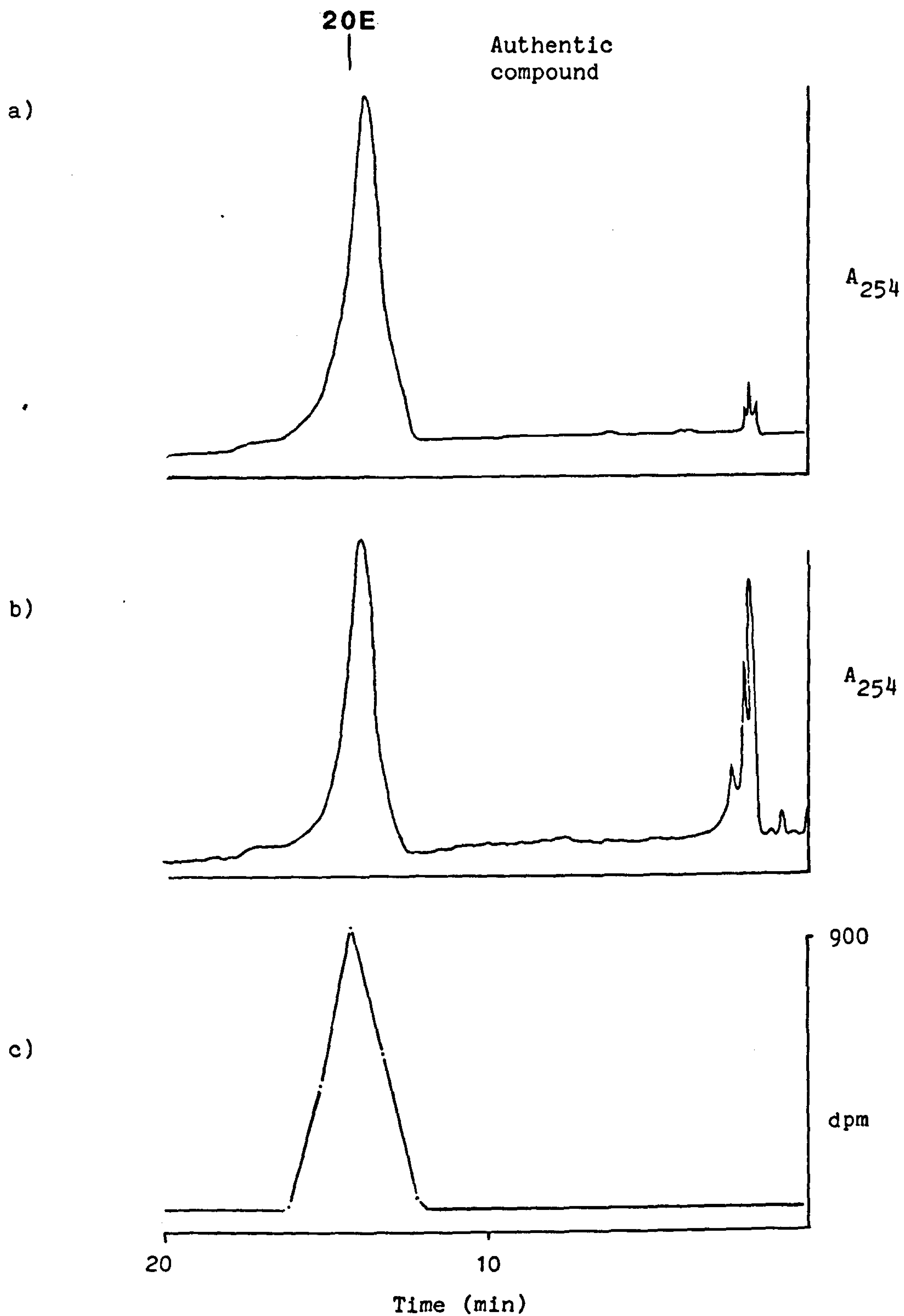


Figure 3B-16 : Adsorption h.p.l.c. analysis (system 7) of methylated U.P.1. as extracted from a) *S.gregaria* eggs b) *S.littoralis* pupae c) metabolism *in vivo* of [^{3}H] ecdysone in *S.littoralis* pupae.

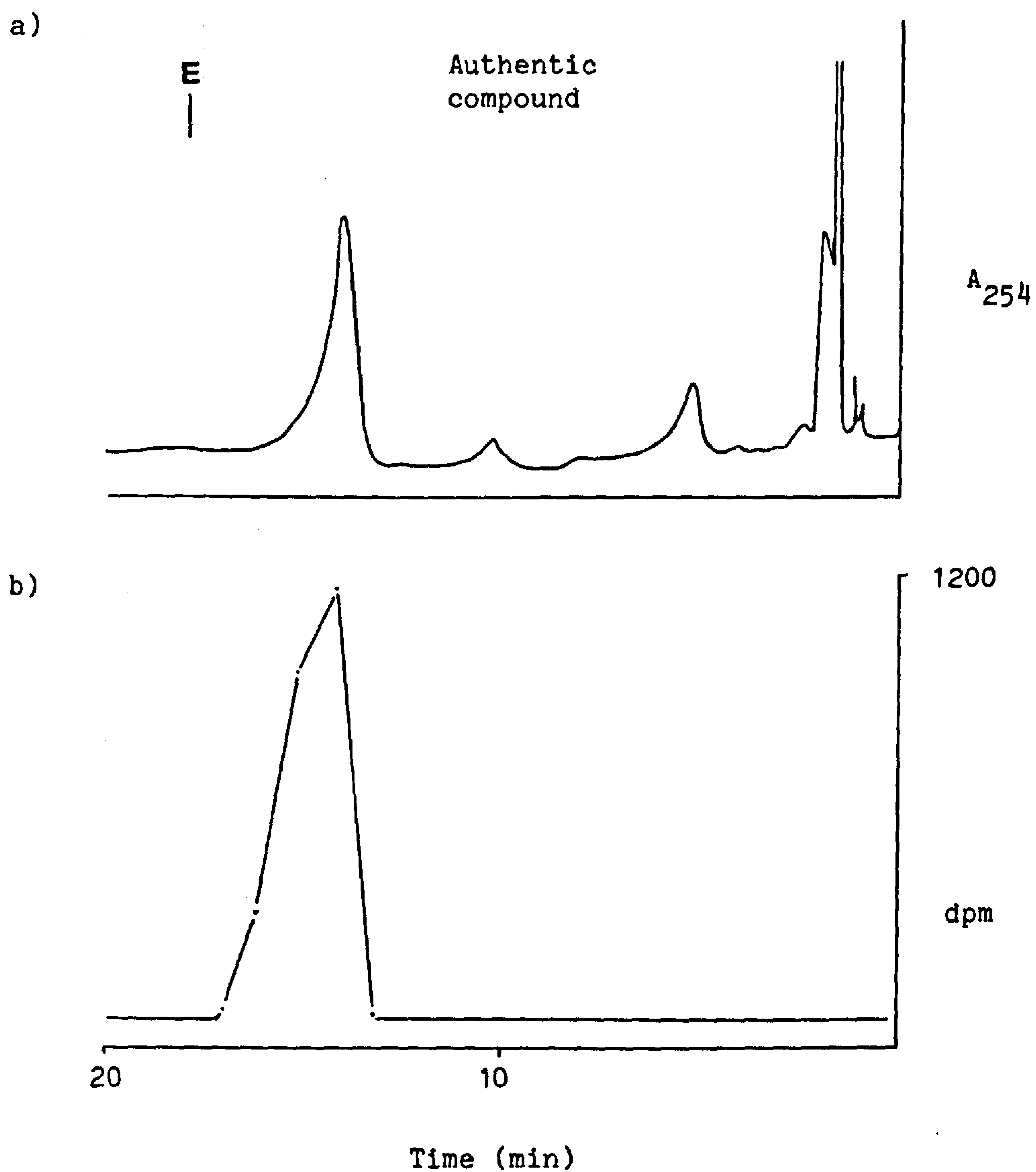


Figure 3B-17 : Adsorption h.p.l.c. analysis (system 7) of methylated U.P.2. as extracted from a) *S.gregaria* eggs b) metabolism *in vivo* of [^{3}H] ecdysone in *S. littoralis* pupae.

U.P.1, m/z 515 for U.P.2) and the glycerol adducts (m/z 601 for U.P.1, m/z 585 for U.P.2).

Although the background ions in the negative ion FAB mass spectra of U.P.1 from S.littoralis pupae were more abundant than with the S.gregaria sample, significant ions corresponding to $[M-H]^-$ (m/z 509) and $[M-H]^-$ of the sodium salt (m/z 531) were observed (see Appendix 2).

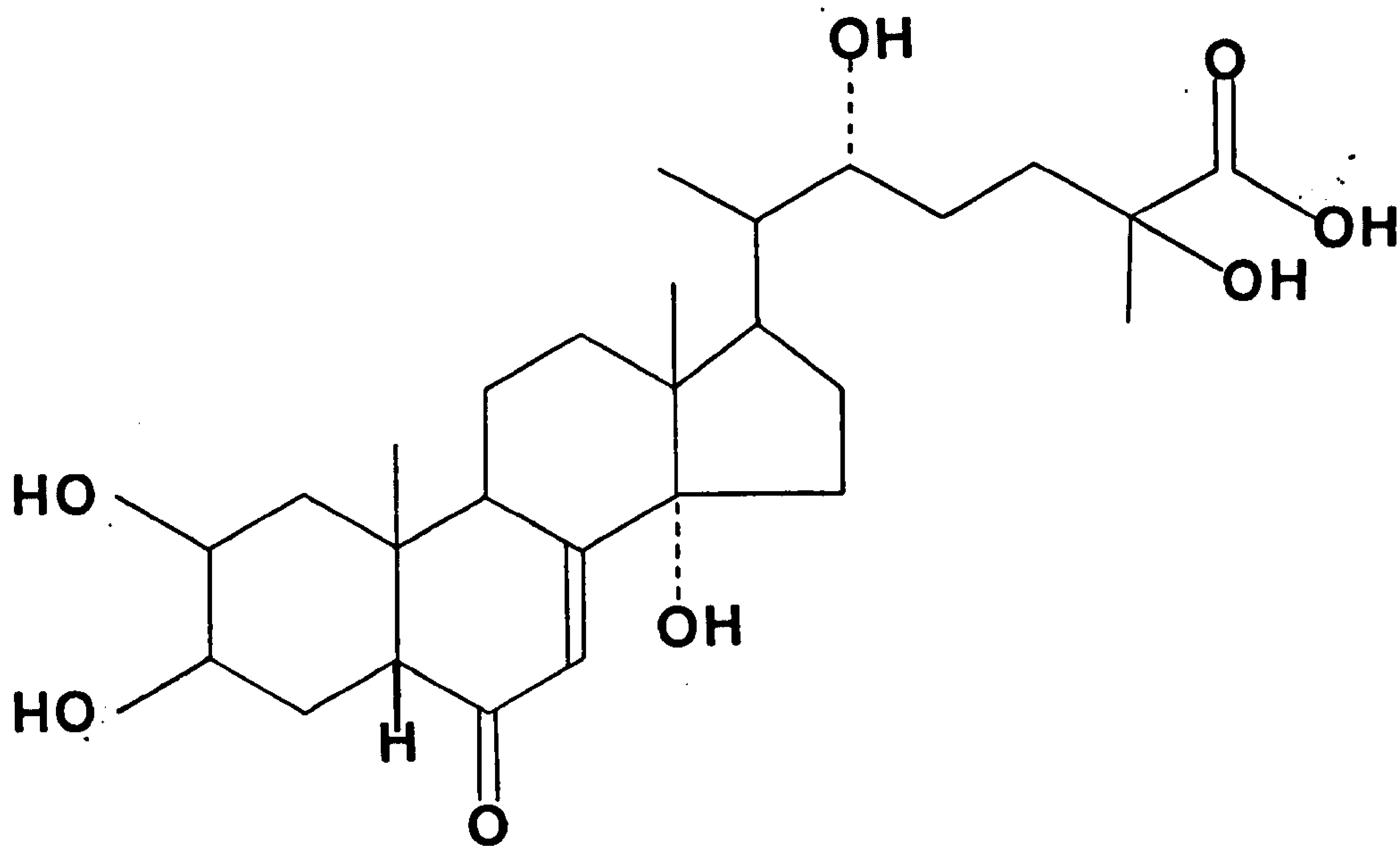
The negative ion FAB mass spectrum of the methylated U.P.1 and U.P.2 as extracted from S.gregaria gave $[M-H-MeOH]^-$ ions at m/z 491 and m/z 475, respectively and less abundant $[M-H]^-$ ions (m/z 523, m/z 507) and glycerol adducts $[M-H+ glycerol]^-$ (m/z 615, m/z 599).

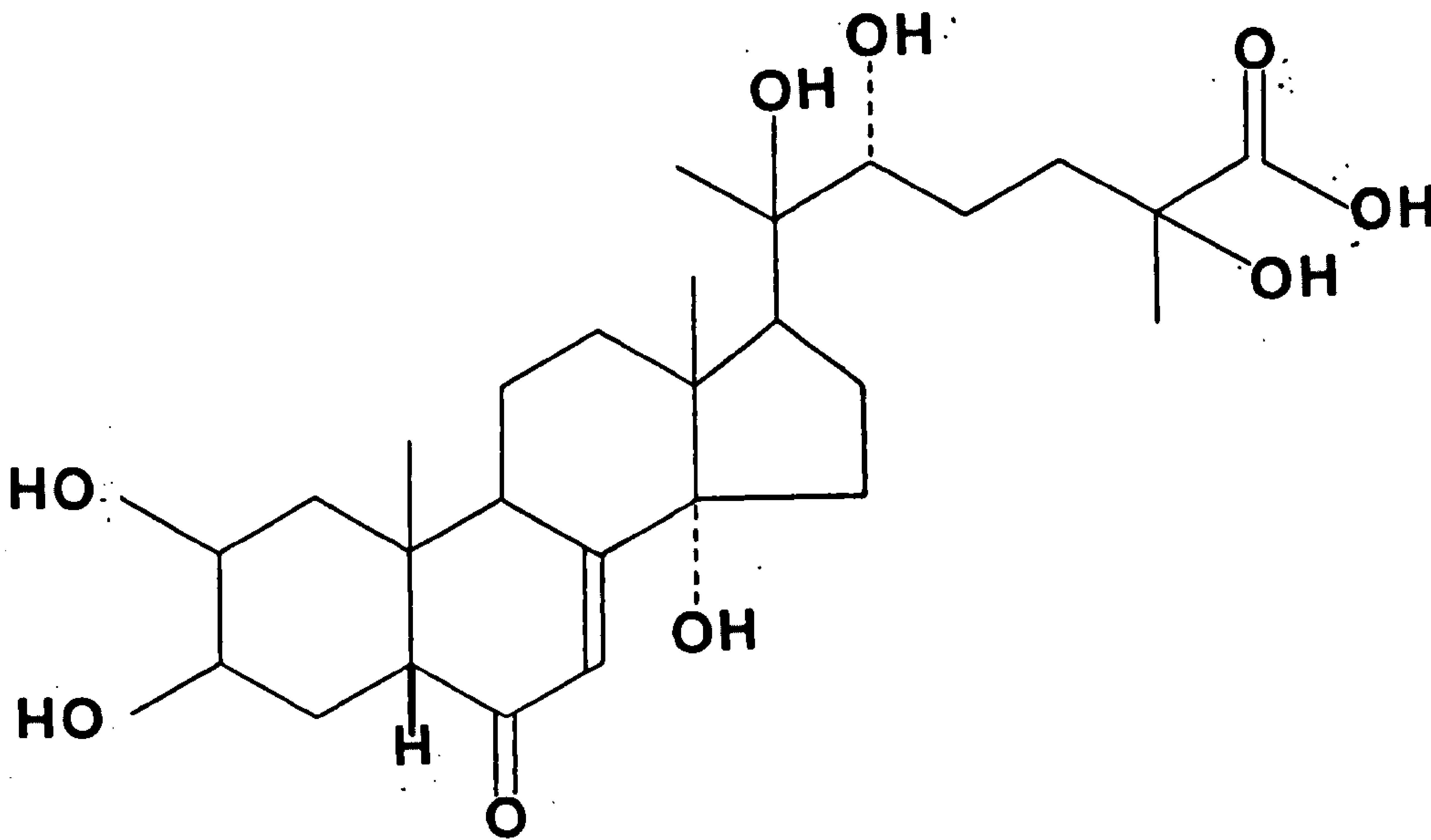
The negative ion FAB mass spectrum of methylated U.P.1 from S.littoralis pupae gave a prominent $[M-H-MeoH]^-$ ion at m/z 491 and $[M-H]^-$ (m/z 523) and $[M-H+glycerol]^-$ (m/z 615) ions (see Appendix 2).

U.P.1 and U.P.2 from S.gregaria eggs were also characterised by p.m.r. spectroscopy (Isaac et al., 1983).

Structure of U.P.1 and U.P.2

The work described in this section has unequivocally established the structures of the unhydrolysable polar metabolites of ecdysone in S.littoralis pupae as ecdyson-26-oic acid (figure 3B-18) and 20-hydroxy-ecdyson-26-oic acid (figure 3B-18). This indicates that these compounds constitute the end products of ecdysone metabolism via 20 and 26 hydroxylation and further oxidation at C-26 to 26-carboxylic acids (for further discussion see Part 3 section E).





<u>Figure 3B-18</u> : Structures of ecdyson-26-oic acid (U.P.2) and 20-hydroxyecdyson-26-oic acid (U.P.1).

PART 3 SECTION C

PART 3 SECTION C

Titres of the principal ecdysteroids during the pupal stage of male and female *S. littoralis* determined by high-performance liquid chromatography

Page No.

Introduction 72

Experimental and results

Preparation of biological samples for high-performance liquid chromatography 74

Quantitative analysis of the ecdysteroids in each sample by high-performance liquid chromatography 74

Investigation of the later stages of the female pupal titre by radioimmunoassay 76

High-performance liquid chromatography radioimmunoassay (h.p.l.c.-RIA) analysis of a pupal titre sample 78

PART 3 SECTION C

Titres of the principal ecdysteroids during the pupal stage of male and female *S. littoralis* determined by high-performance liquid chromatography

INTRODUCTION

The earliest reports of moulting hormone titres determined by bioassay in insect tissues were for *Calliphora* (Karlson and Shaaya, 1964) and *Bombyx mori* (Shaaya and Karlson, 1965). However, the development of more recent techniques has provided more accurate and convenient methods for ecdysteroid measurements. Many ecdysteroid titres in a variety of insects and crustaceans have been determined by using radioimmunoassay (RIA). This method provides a sensitive assay capable of detecting picogram levels of ecdysteroid that can be measured in relatively crude extracts (Borst and O'Connor, 1974; DeReggi *et al.*, 1975). The major drawback with this method lies in its inability to differentiate between the various species of ecdysteroid molecules. This can be overcome to some extent by the separation of different ecdysteroids by t.l.c. prior to R.I.A. (Bollenbacher *et al.*, 1975; Calvez *et al.*, 1976). Measurements have been made on *Pieris brassicae* using coupled GC/MS(SIM) [Gas liquid chromatography/Mass spectrometry (selected ion monitoring)] (Lafont *et al.*, 1975), which provides sensitivity and specificity. However, this method is expensive and requires a lengthy and crucial derivatisation process (Morgan and Poole, 1976), which is not suitable for the analysis of a large number of samples.

High-performance liquid chromatography in conjunction with an U.V. monitor has been used to good effect in the determination of ecdysone and 20-hydroxyecdysone titres in *Heliothis zea* (Holman and Meola, 1978).

This method, while not being as sensitive as R I.A. or GC/MS (SIM) requires no derivatisation and offers the possibility of analysis of a number of individual ecdysteroids. For these reasons, h.p.l.c. with U,V. monitoring of the effluent was chosen for the quantitative analysis of ecdysteroids during the pupal stage of S. littoralis for determination of a pupal titre curve for ecdysone, 20-hydroxyecdysone, 3-epi-ecdysone, 3-epi-20-hydroxyecdysone, 26-hydroxyecdysone, and 20,26-dihydroxyecdysone in both males and females.

The pupal stage of S. littoralis is particularly amenable to the study of ecdysteroid metabolites. Not only are the levels of ecdysteroids considerably higher than during larval development, but the pupa provides a closed system, so that all metabolites remain in the insect.

A pupal titre of ecdysteroids in female and male pupae of S.littoralis has been determined by R.I A. (Clarke, 1981; figure 3C-1). This revealed the presence of one major ecdysteroid peak in the male and two major peaks in the female. The additional peak in the female titre is thought to be associated possibly with ovarian synthesis of ecdysteroids. In the present work the titre of individual ecdysteroids during the pupal stage has been determined by h.p.l.c. to provide further information. Since a much lower titre of ecdysteroids was observed during the second peak in females compared to the R.I.A. titre, further investigation of that peak in females was undertaken.

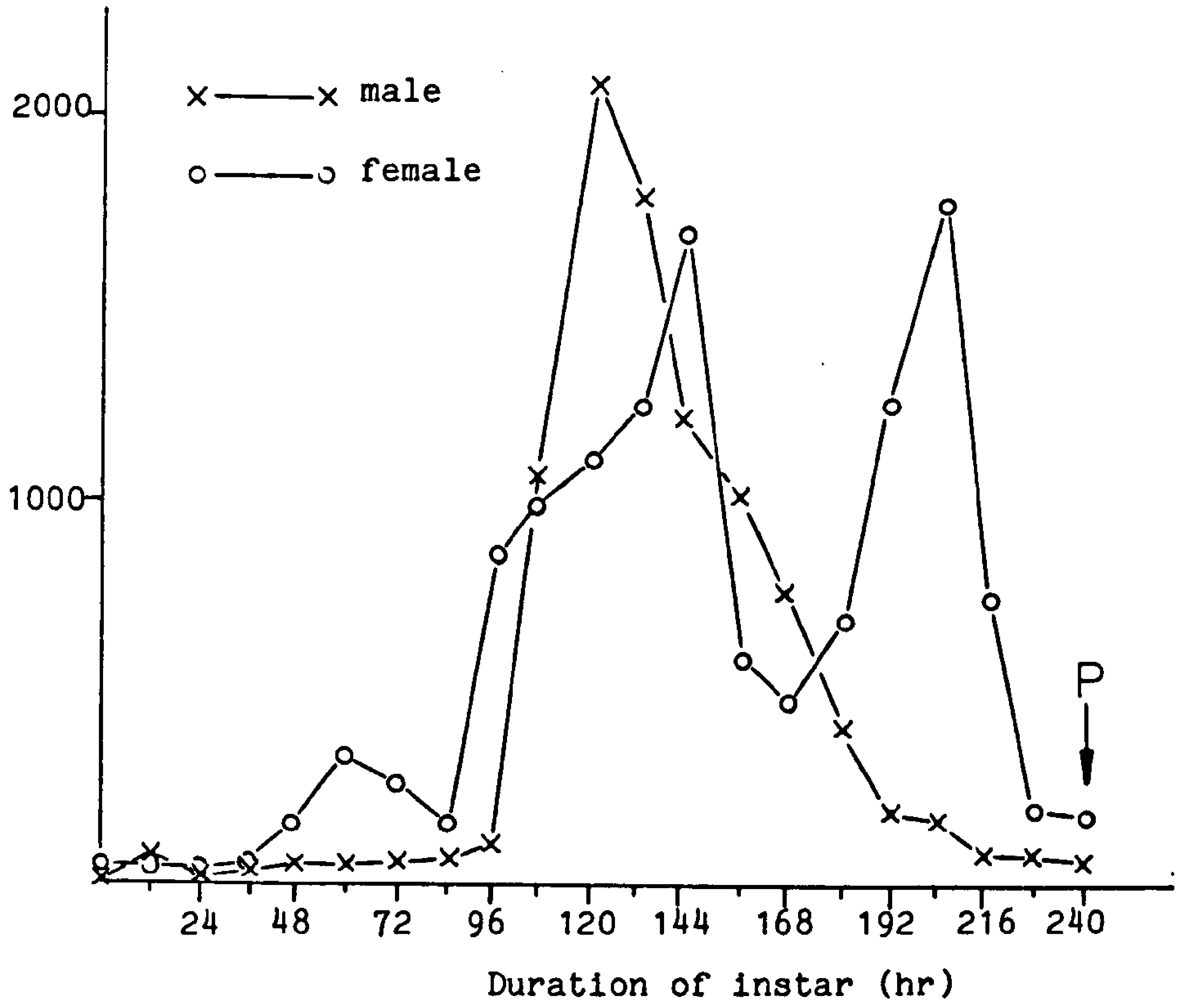


Figure 3C-1 : Ecdysteroid titre curve for S. littoralis pupae (ICT-1). P : pupation.

EXPERIMENTAL AND RESULTS

Preparation of biological samples for high-performance liquid chromatography

Batches of approximately 10 male and female pupae taken at 12 hour intervals during pupal development were extracted and fractionated on a silicic acid column by G.S. Clarke. The free ecdysteroid fraction (30% methanol/chloroform fraction from silicic acid column chromatography) was then further purified by reversed-phase SEP-PAK (see General Experimental Techniques, Part 2). SEP-PAK purification of free ecdysteroids was verified for efficiency and recovery (see Part 4, Section B) demonstrating that ecdysteroids of polarity ranging from ecdysone to 20,26-dihydroxyecdysone can be purified at more than 90% recovery. The purified samples were then dissolved in methanol and held in a freezer until analysis.

Quantitative analysis of the ecdysteroids in each sample by high-performance liquid chromatography

Analysis for ecdysone, 3-epiecdysone and 20-hydroxyecdysone was carried out on two Partisil-ODS3 reversed-phase columns in series (25 cm x 4.6 mm i.d.) eluting isocratically with 48.8% ($^{v}/v$) methanol in water at a flow rate of 1.5 ml/minute. The samples were dissolved in methanol (120 µl) and 20 µl injected in all cases. A typical chromatogram is shown in figure 3C-2. Between each analysis the column was washed with methanol in order to clean the column. The intact samples were also analysed for 20-hydroxyecdysone, 3-epi 20-hydroxyecdysone, 26-hydroxyecdysone, 20-26-dihydroxyecdysone, ecdysone and 3-epiecdysone on an APS-Hypersil (aminopropyl) column eluted under isocratic conditions with 6.5% ($^{v}/v$) methanol in dichloroethane at a flow rate of 2 ml/min. A typical chromatogram is shown in figure 3C-3. Between each analysis

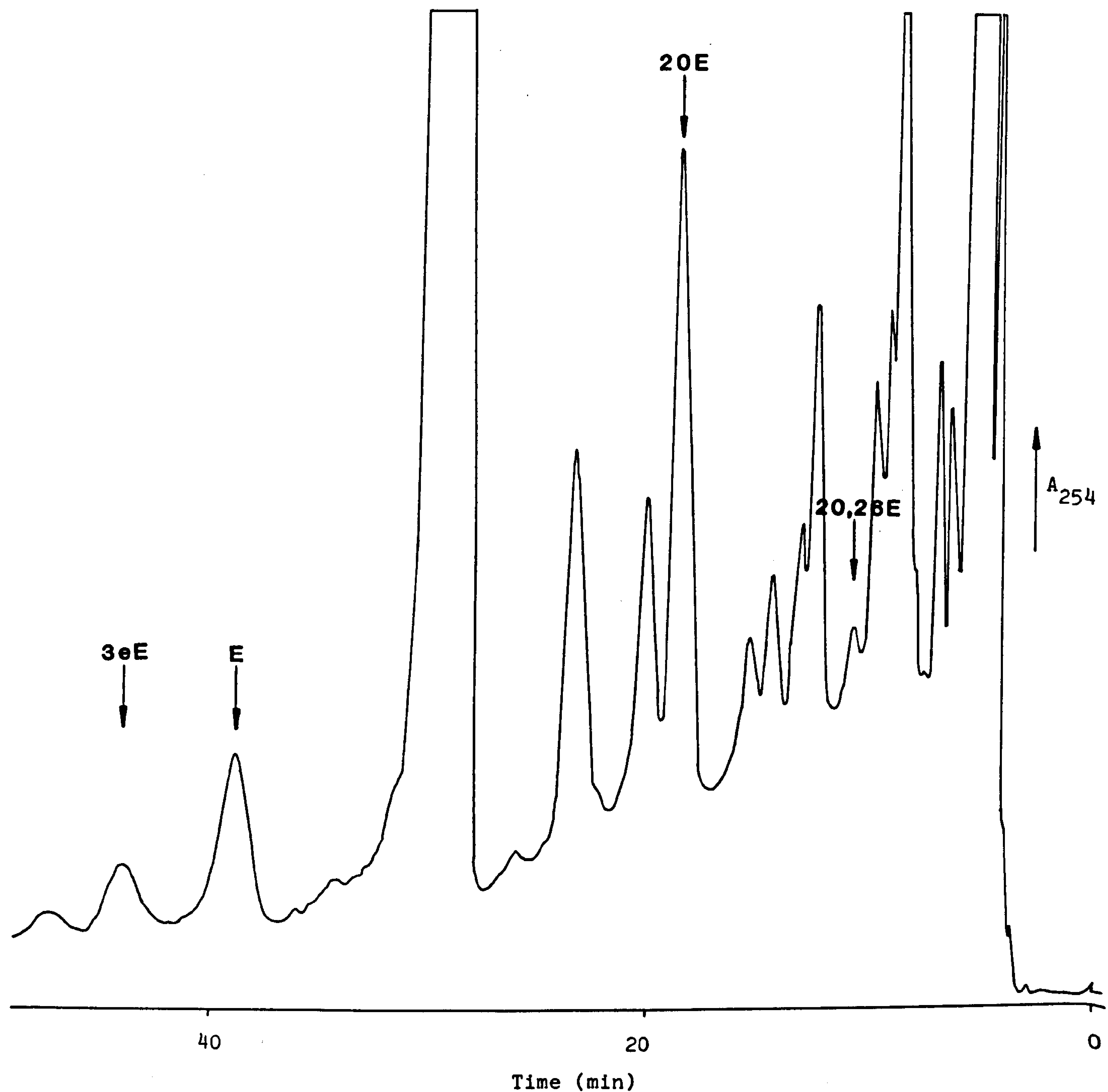


Figure 3C-2 : A typical reversed-phase chromatogram of a free ecdysteroid fraction from pupae. Two Partisil-ODS-3 reversed-phase columns in series (25 cm x 4.6 mm i.d.) were eluted under isocratic conditions with 48.8% ($^v/v$) methanol in water at a flow rate of 1.5 ml/minute. Peaks of interest were identified by chromatography of authentic compounds prior to chromatography of the sample. The positions of authentic ecdysteroids are marked.

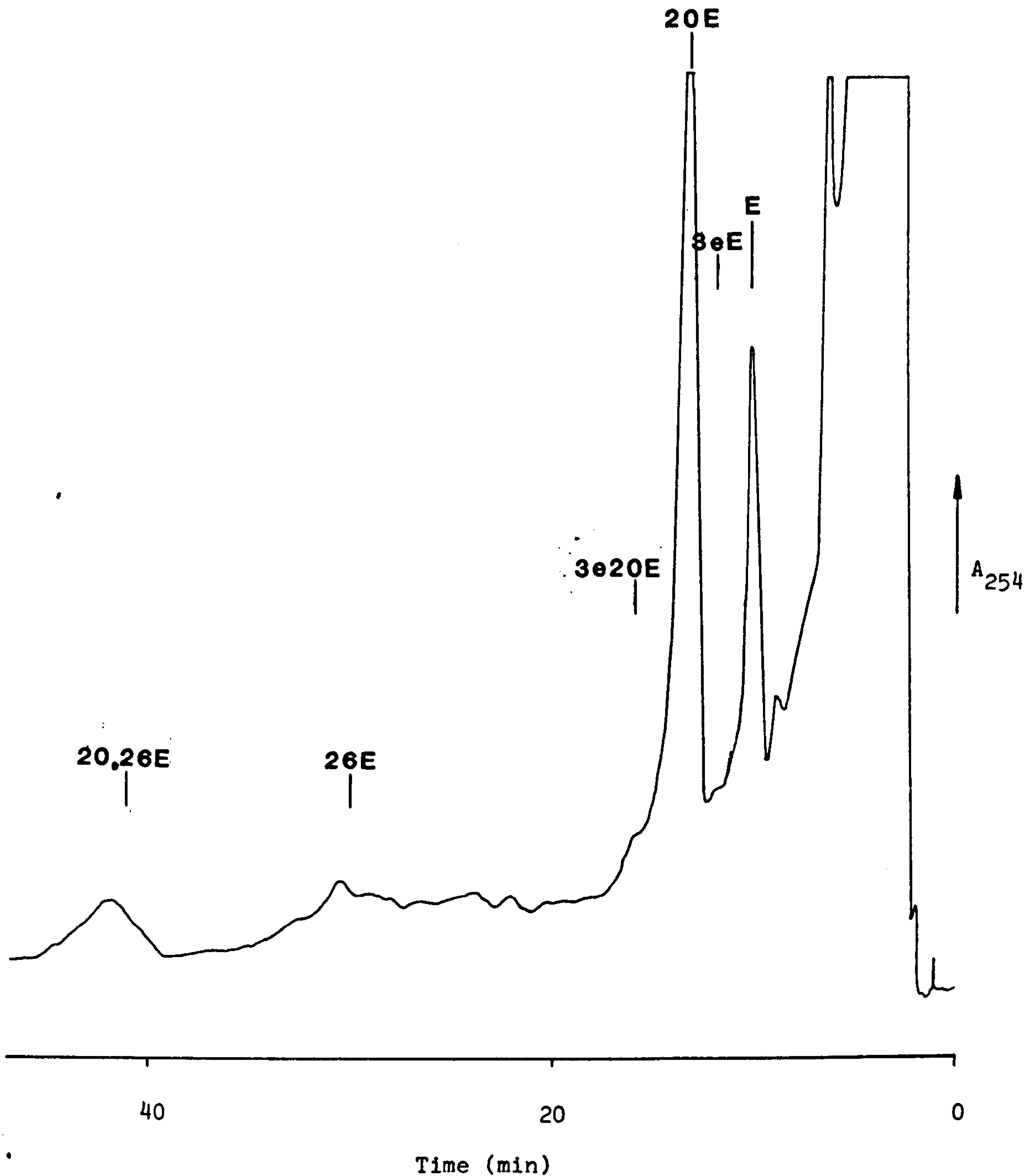


Figure 3C-3 : A typical high-performance liquid chromatogram of a pupal free ecdysteroid sample on an APS-Hypersil (aminopropyl) column (adsorption) eluted under isocratic conditions with 6.5% ($^{v}/v$) methanol in dichloroethane at a flow rate of 2 ml/min. Peaks of interest were identified by chromatography of authentic compounds prior to chromatography of the sample.

the column was washed with 10% ($^{v}/v$) methanol in dichloroethane.

Standard curves for both h.p.l.c. systems were prepared using ecdysone and 20-hydroxyecdysone standards by plotting peak area (as measured by planimetry) versus ng of ecdysteroid (figure 3C-4).

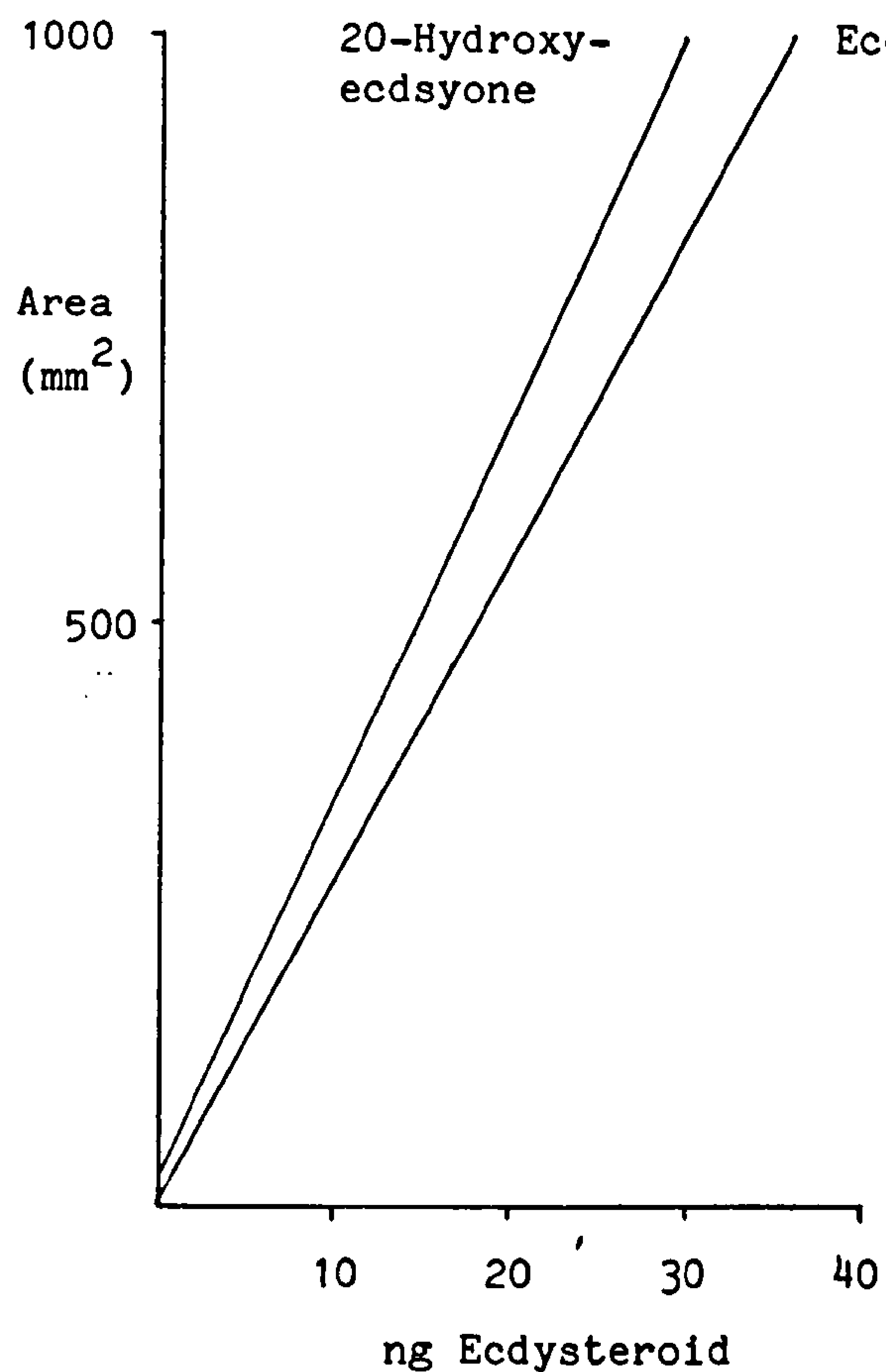
All the samples were analysed once on each h.p.l.c. system. The titres for the principal pupal ecdysteroids, ecdysone, 20-hydroxyecdysone and also for 3-epiecdysone were obtained from the reversed-phase h.p.l.c. analysis. However, this system was unsuitable for other ecdysteroids due to interfering U.V. absorbing compounds. The titres for 3-epi-20-hydroxyecdysone, 26-hydroxyecdysone and 20,26-dihydroxyecdysone were obtained by adsorption h.p.l.c. (aminopropyl column).

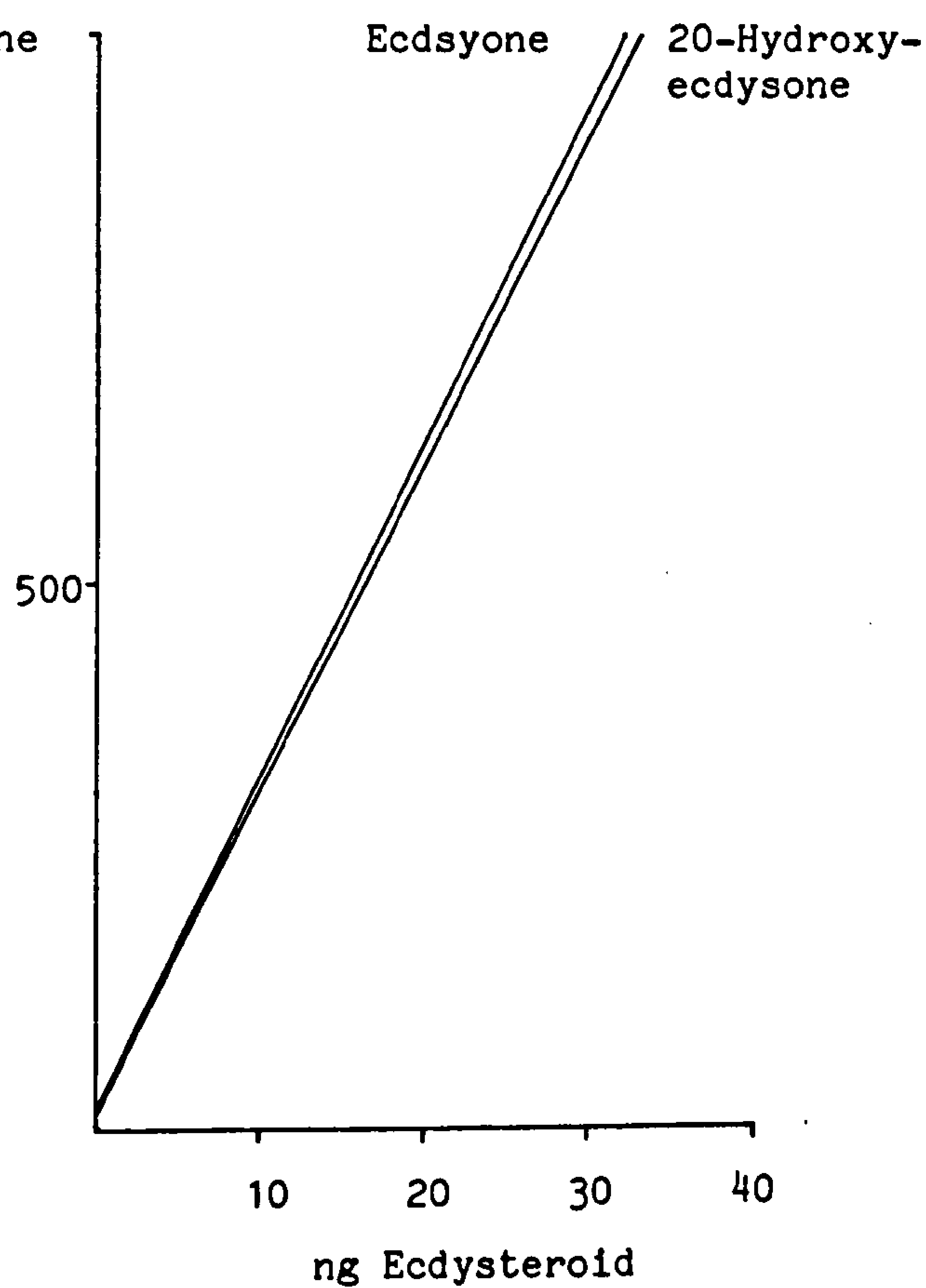
The recovery from high-performance liquid chromatography was assessed by addition of ecdysone (15 ng) to pupal samples (3 samples) containing undetectable quantities of endogenous ecdysone. These samples were then analysed by h.p.l.c. and showed a 90-93% recovery using reversed-phase systems and a 84-92% recovery using an APS-Hypersil column.

Figures 3C-5 and 3C-6 illustrate the titre curves of the ecdysteroids in male pupae, figures 3C-7 and 3C-8 show the ecdysteroid titres in female pupae. Samples were analysed at 12 hour intervals for ecdysone, 3-epiecdysone, 20-hydroxyecdysone 3-epi-20-hydroxyecdysone, 26-hydroxyecdysone and 20,26-dihydroxyecdysone. The resulting titres have, as expected, a similar profile to the RIA-titres compiled by Clarke (1981) (figure 3C-1), although the total ecdysteroid titres by h.p.l.c. analysis are much lower than those expressed by R.I.A. (for further discussion see Section E, p.100). In addition to this, the relative size of the second peak in the female titre curve is markedly lower. This aspect of the titre was, therefore, further investigated.

a) <u>Reversed-phase h.p.l.c.</u>

b) <u>Adsorption h.p.l.c.</u>





<u>Figure 3C-4</u>: Standard curves for ecdysone and 20-hydroxyecdysone (areas as measured by planimetry) on both reversed-phase and adsorption (APS-Hypersil) h.p.l.c., monitoring the u.v. absorbance.

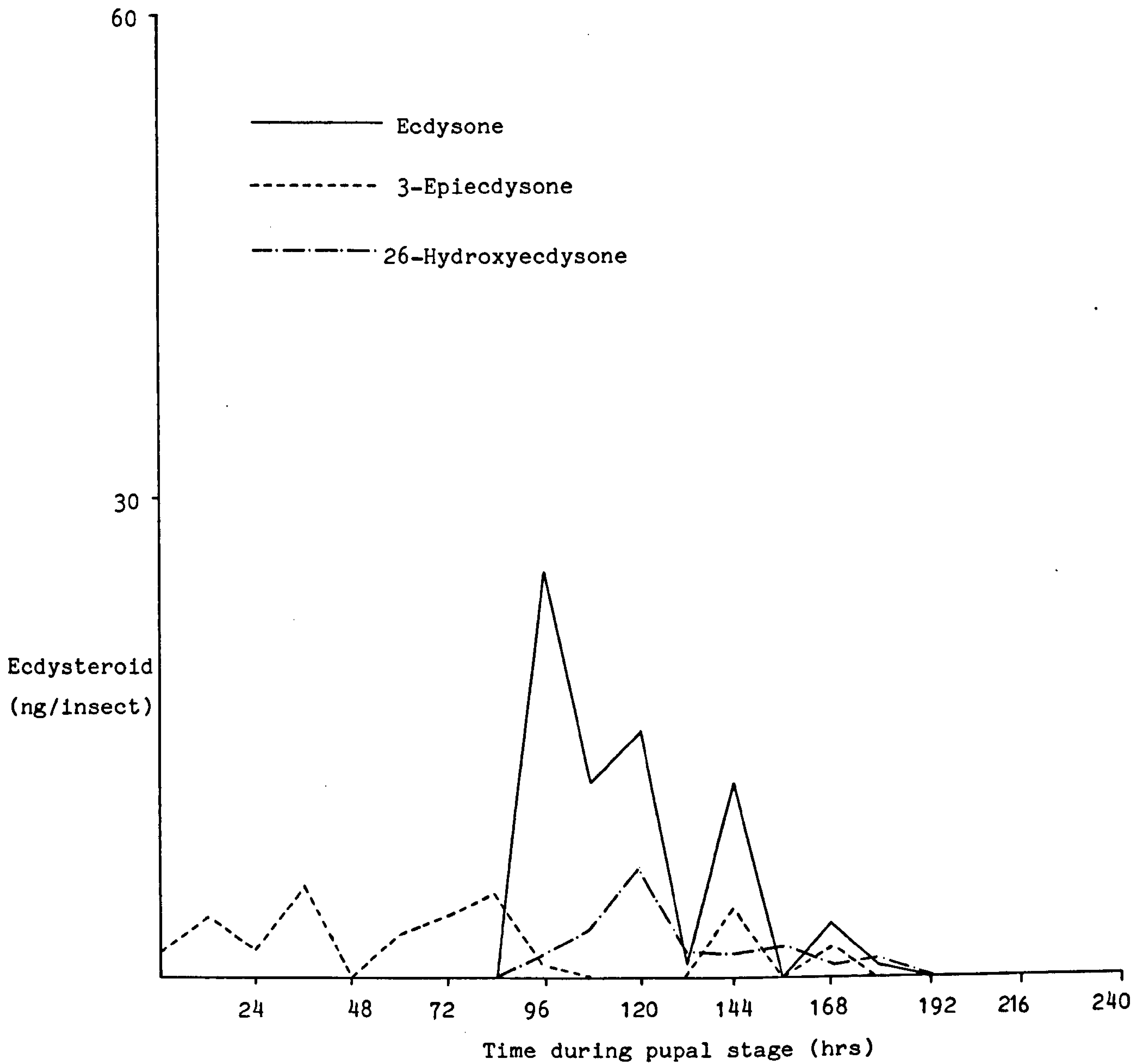


Figure 3C-5 : Titres determined by h.p.l.c. of ecdysone, 26-hydroxyecdysone and 3-epiecdysone during the pupal stage of male *S. littoralis*.

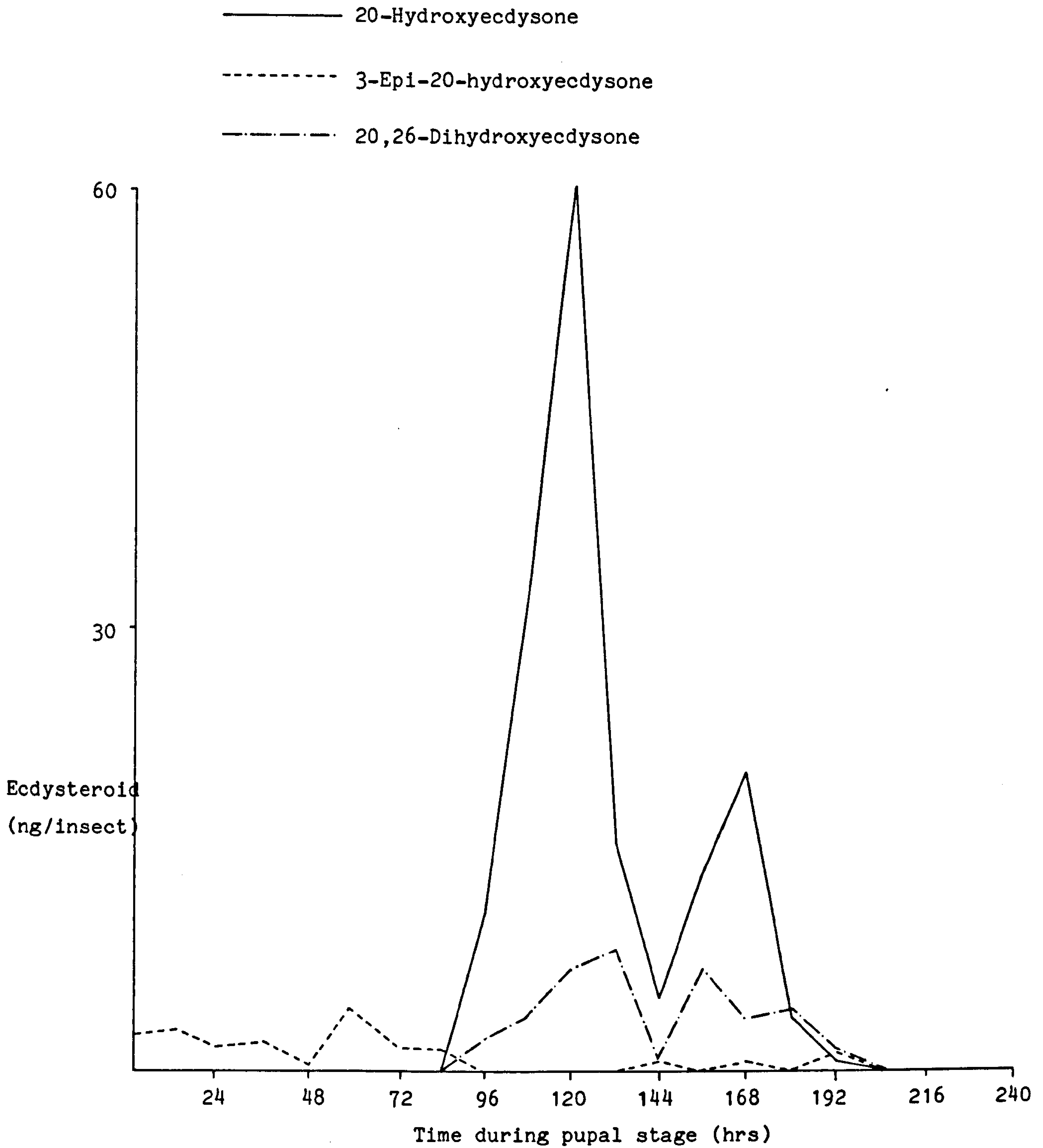


Figure 3C-6 : Titre determined by h.p.l.c. of 20-hydroxyedysone, 20,26-dihydroxyecdysone and 3-epi-20-hydroxyecdysone during the pupal stage of male S. littoralis.

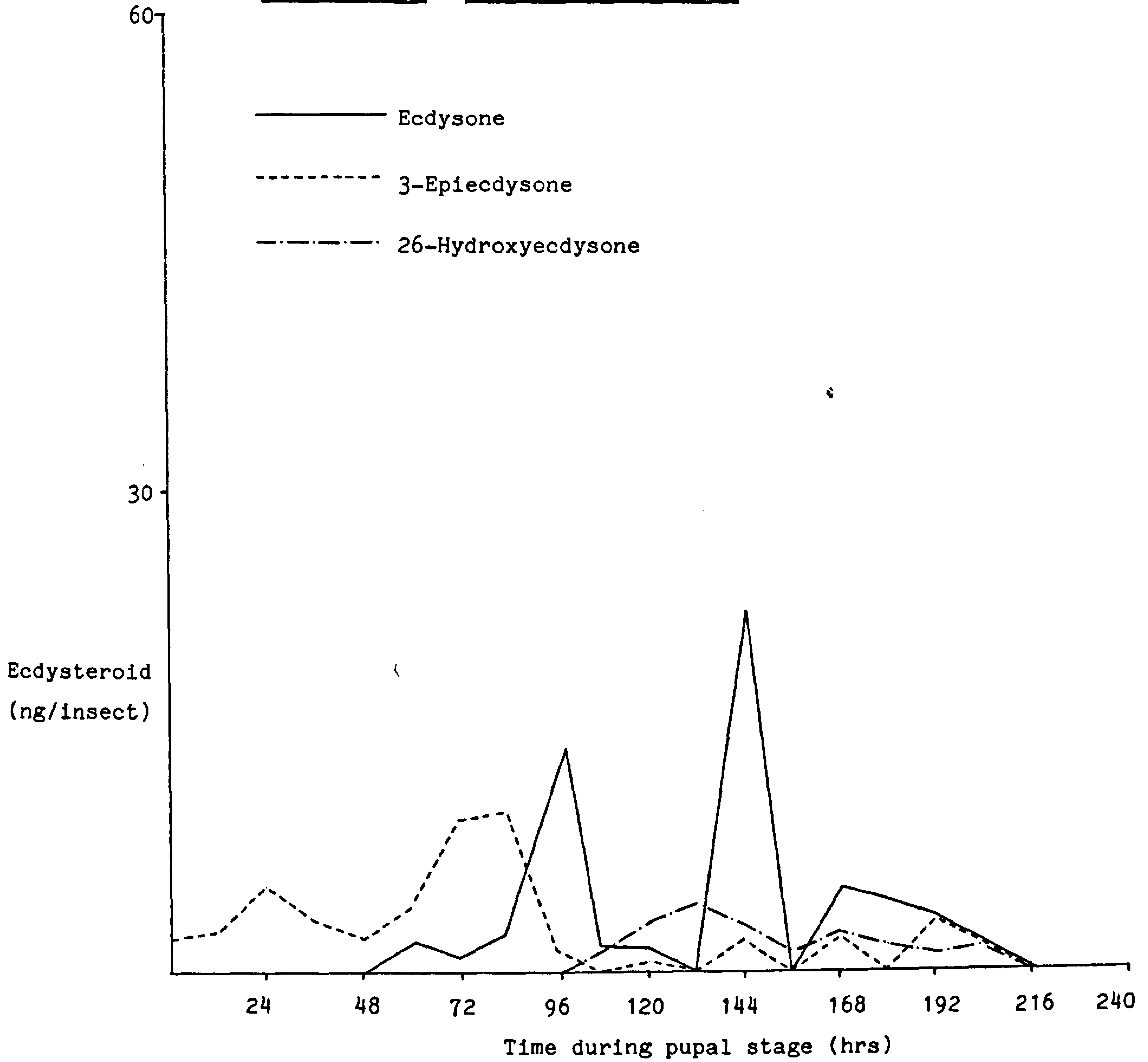


Figure 3C-7 : Titre determined by h.p.l.c. of ecdysone, 26-hydroxyecdysone and 3-epiecdysone during the pupal stage of female S.littoralis

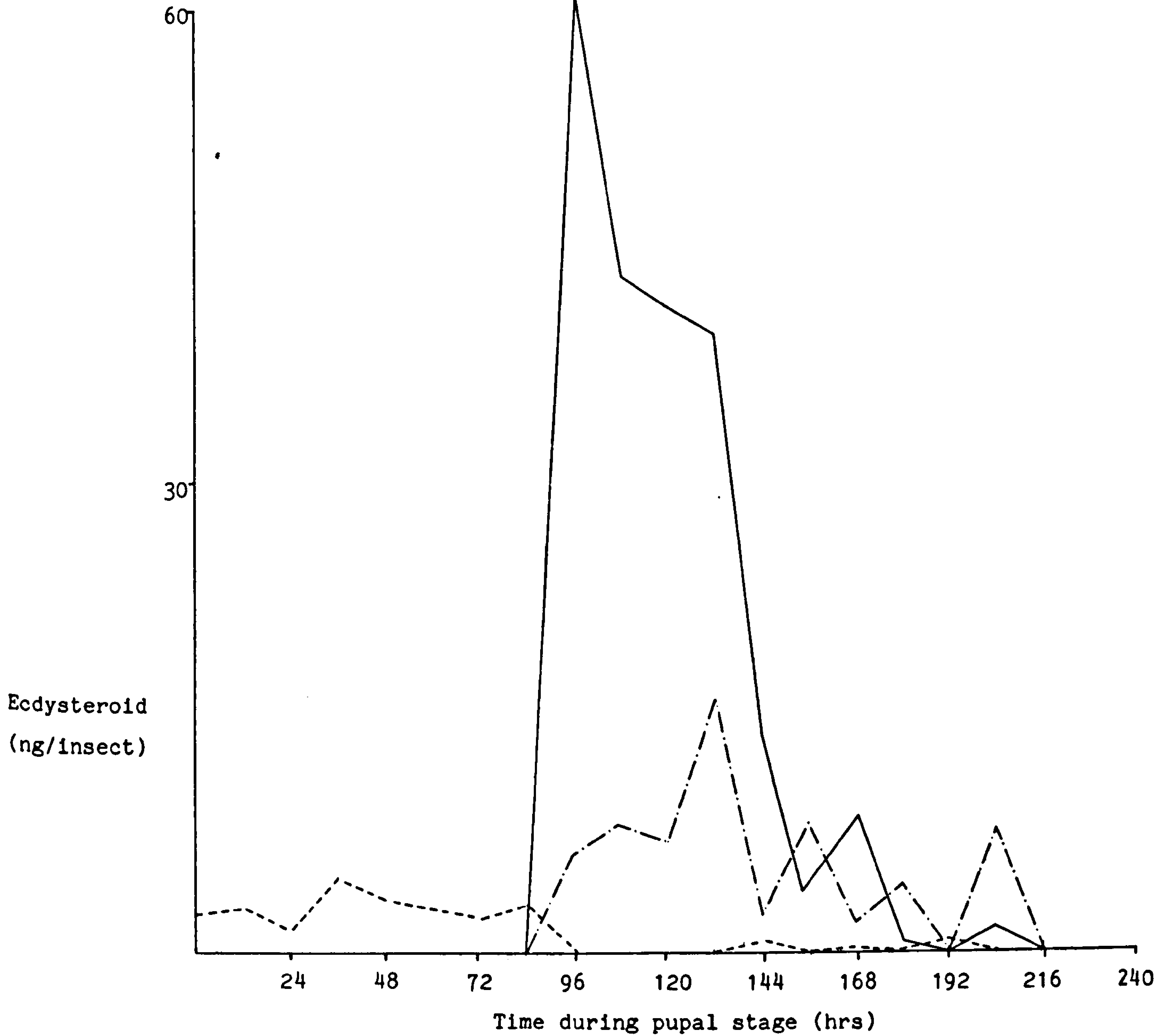


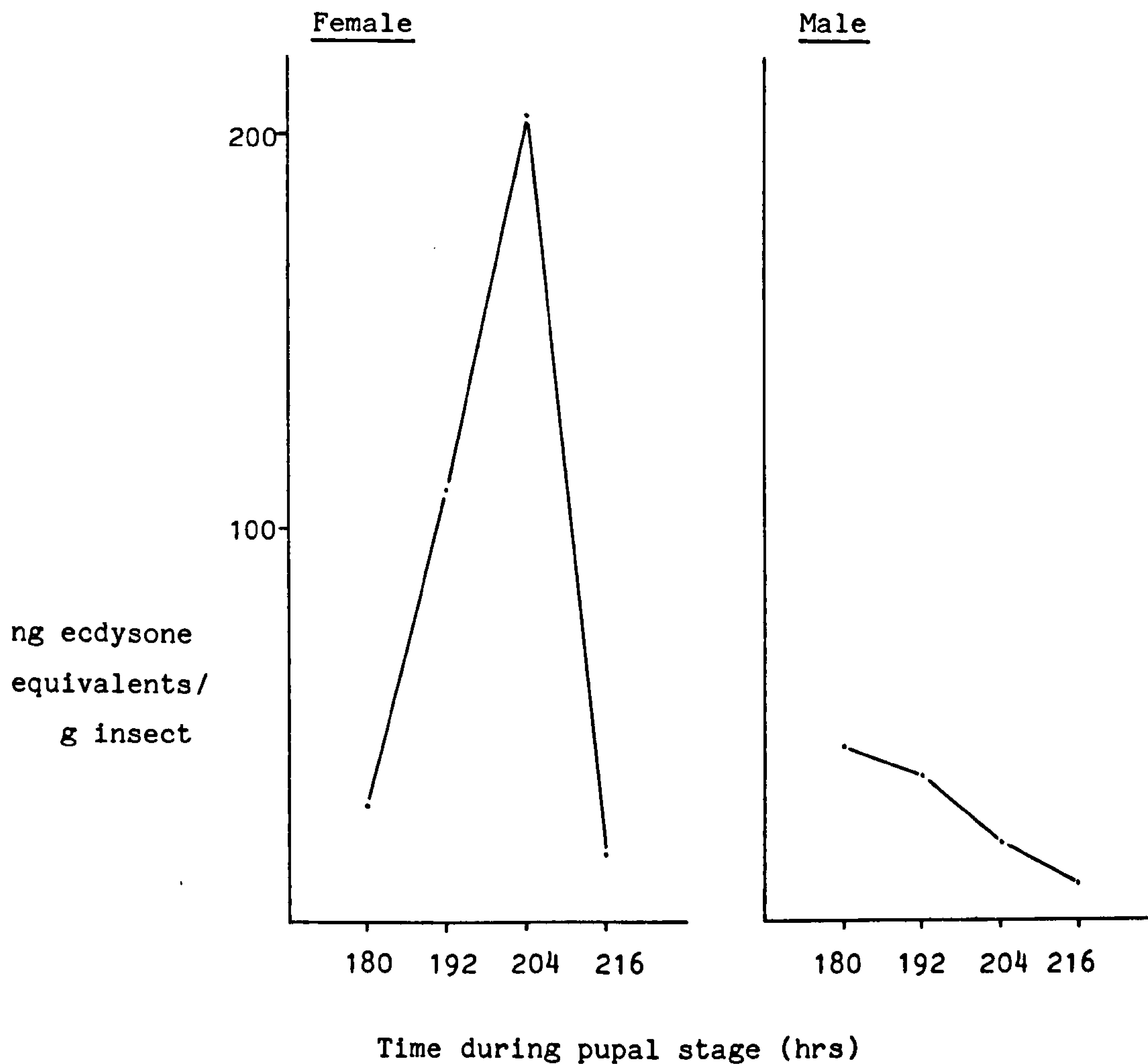
Figure 3C-8 : Titres determined by h.p.l.c. of 20-hydroxyecdysone, 20,26-dihydroxyecdysone and 3-epi-20-hydroxyecdysone during the pupal stage of female *S.littoralis*.

Investigation of the later stages of the female pupal titre by radioimmunoassay

Samples, as used for the h.p.l.c. analysis, representing 180, 192, 204 and 216 hrs of pupal development in male and female pupae were analysed by radioimmunoassay using ICT-1 antiserum (see General Experimental Techniques) and an additional sample, 120 hrs male pupae, used as a control. The results are represented in figure 3C-9 showing that there is indeed a peak of ecdysteroid immunoreactivity at 204 hrs in the female pupae. However, the peak corresponds to only 210 ng ecdysone equivalents/g insect as compared to 1700 ng 20-hydroxyecdysone equivalents/g insect (approximately 700 ng ecdysone equivalents), detected by Clarke (1981) (figure 3C-1). However the control sample (120 hrs, male pupae) shows reasonable agreement with R.I.A. results previously determined, presently 1100 ng ecdysone equivalents/g insect by RIA after SEP-PAK purification (figure 3C-9) compared to 2160 ng 20-hydroxyecdysone equivalents/g insect (approximately 870 ng ecdysone equivalents/g insect) by RIA, before SEP-PAK purification (Clarke, 1981; figure 3A-1).

It must be noted that the samples used for h.p.l.c. in the present work had been purified by an additional step, reversed-phase SEP-PAK. Therefore, it is conceivable that some immuno-reactive material (not necessarily ecdysteroids) could have been removed during this stage of purification. In order to verify this possibility the non-ecdysteroid fraction from SEP-PAK chromatography (0%, 30%, 100% methanol/water fractions) were also subjected to radioimmunoassay. However no immuno-reactive material was detected in any of the fractions.

In order to confirm the results already obtained, the R.I.A. analysis of the later stages of the female pupal titre was repeated using new samples of insects. Groups of five female pupae were



Control

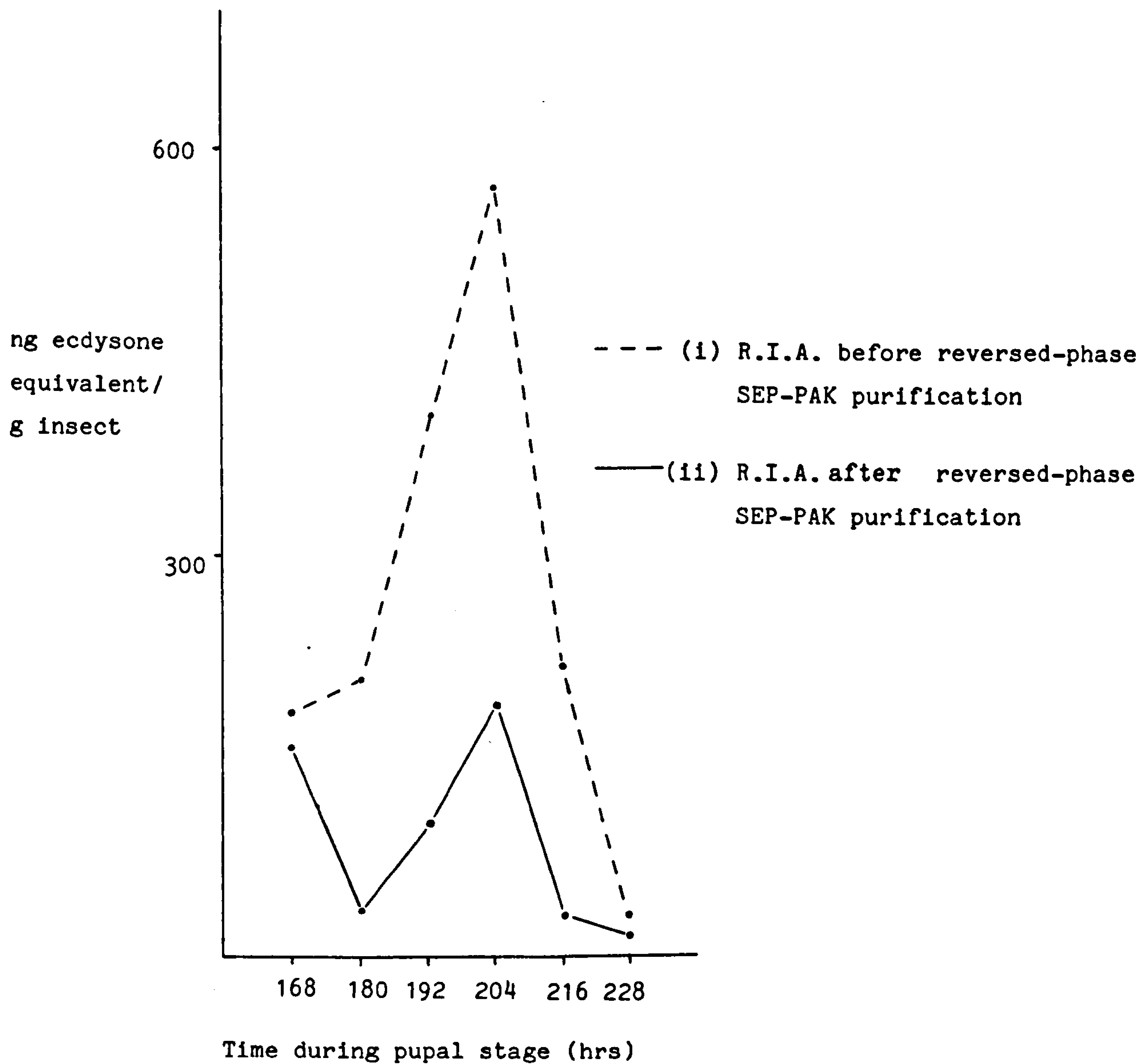
Male pupae - 120 hrs : 1100 ng ecdysone equivalents/g insect

Figure 3C-9 : Ecdysteroid titre (by R.I.A.) of the later stages of development in male and female pupae, including the result of the R.I.A. analysis of a control sample taken from earlier pupal development (120 hrs, male pupae). Each point represents the average of two measurements.

synchronised and sacrificed at 168 hrs, 180 hrs, 192 hrs, 204 hrs, 216 hrs and 230 hrs, and in addition to these, a group of female pupae were sacrificed at 108 hrs of pupal development (as a control from early pupal development). Each group was extracted and purified by silicic acid column chromatography. Half of each of the samples were then purified by reversed-phase SEP-PAK, and R.I.A. analysis performed on the samples (i) without SEP-PAK purification and (ii) with SEP-PAK purification (figure 3C-10).

This analysis confirms the results shown in figure 3C-9, and the amounts of ecdysteroid detected by R.I.A. at this stage of female pupal development were markedly affected by purification by reversed-phase SEP-PAK. However, this is apparently not the case at other stages of pupal development; SEP-PAK purification does not significantly reduce the R.I.A. response at 108 hrs of female pupal development (see figure 3C-10) or at 120hrs of male pupal development (see figure 3C-9). Therefore, it does seem that this sharp reduction in immunoreactivity after reversed-phase SEP-PAK is confined to the samples from the female pupae at the later stages of development (i.e. during the R.I.A. titre peak believed to be associated with ovarian synthesis of ecdysteroids). As no immunoreactive material was identified in the non-ecdysteroid fractions from reversed-phase SEP-PAK it must be assumed that the higher immunoreactivity observed before SEP-PAK purification was due to the presence of some interfering material which artificially enhanced the immunoreactive response of the sample at that stage of development.

Even after purification on SEP-PAK the quantities of ecdysteroids detected by R.I.A. are considerably higher than the ecdysteroid levels detected by h.p.l.c. whether it be in the early or later stages of development (Table 3C-1). In fact measurements by RIA detects up to



<u>R.I.A. analysis of 108 hrs female pupal extract:</u>

before reversed-phase SEP-PAK purification = 457 ng

after reversed-phase SEP-PAK purification = 422 ng

<u>Figure 3C-10</u> : R.I.A. titre of the later stages of female pupal development as analysed before and after purification by reversed-phase SEP-PAK. Each point represents the average of two measurements.

Table 3C-1 : Comparison of quantitative analyses by R.I.A. and h.p.l.c. of pupal samples after purification by silicic acid column chromatography and reversed-phase SEP-PAK.

Pupae		ng/gm insect		Ratio
Sex	Stage (hr from pupation)	R.I.A. Ecdysone equivalent	H.p.l.c.	ng RIA / ng h.p.l.c.
Male	120 hr	1100	307	3.58
	180 hr	55	34	1.62
	192 hr	38	12	3.17
Female	108 hr	422	150	2.81
	192 hr	105	29	3.62
	204 hr	210	55	3.82

4 times more ecdysteroid than by h.p.l.c. detection. This phenomenon could be explained by the possible high cross reactivity of the antibody with some species of ecdysteroid such as 26-hydroxylated ecdysteroids, and/or could be due to the presence of, as yet, unidentified compound(s) cross reacting with the antibody. Indeed, these possibilities were further investigated by h.p.l.c.-R.I.A.

High-performance liquid chromatography-radioimmunoassay (h.p.l.c.-R.I.A.) analysis of a pupal titre sample (168 hrs female)

The sample chosen for this analysis (168 hrs female) contains a wide range of ecdysteroids (ng/insect); ecdysone (5 ng), 20-hydroxy-ecdsyone (9 ng), 26-hydroxyecdysone (2 ng) 20,26 dihydroxyecdysone (2 ng), and 3-epi-ecdysone (1.5 ng) as detected and quantified by h.p.l.c. analysis. By R.I A. the total ecdysteroid was also determined (38 ng ecdysone equivalents/insect). This sample (5 insect equivalents, 192 ng ecdysone equivalents by RIA, 97.5 ng by h.p.l.c. analysis) was injected onto two methanol-washed reversed-phase Partisil ODS-3 columns in series developed under isocratic conditions with 49% methanol/water, v/v at a flow rate of 1.5 ml/min. The areas of the chromatogram corresponding to 20,26-dihydroxyecdysone, 20-hydroxyecdysone 26-hydroxyecdysone, ecdysone and 3-epi-ecdysone were collected separately, as were the intervening non-ecdysteroid areas, and the column finally washed with methanol (20 ml) which was also collected (figure 3C-11). The collected samples were then subjected to radioimmunoassay, and the results compared to the quantities calculated from h.p.l.c. analysis (peak area at 254 nm absorbance).

Table 3C-2 illustrates the results obtained by the two methods. The antiserum used for the RIA analysis (ICT-1) is highly specific for the ecdysone nucleus. Indeed, any change of a ligand in ring A of the

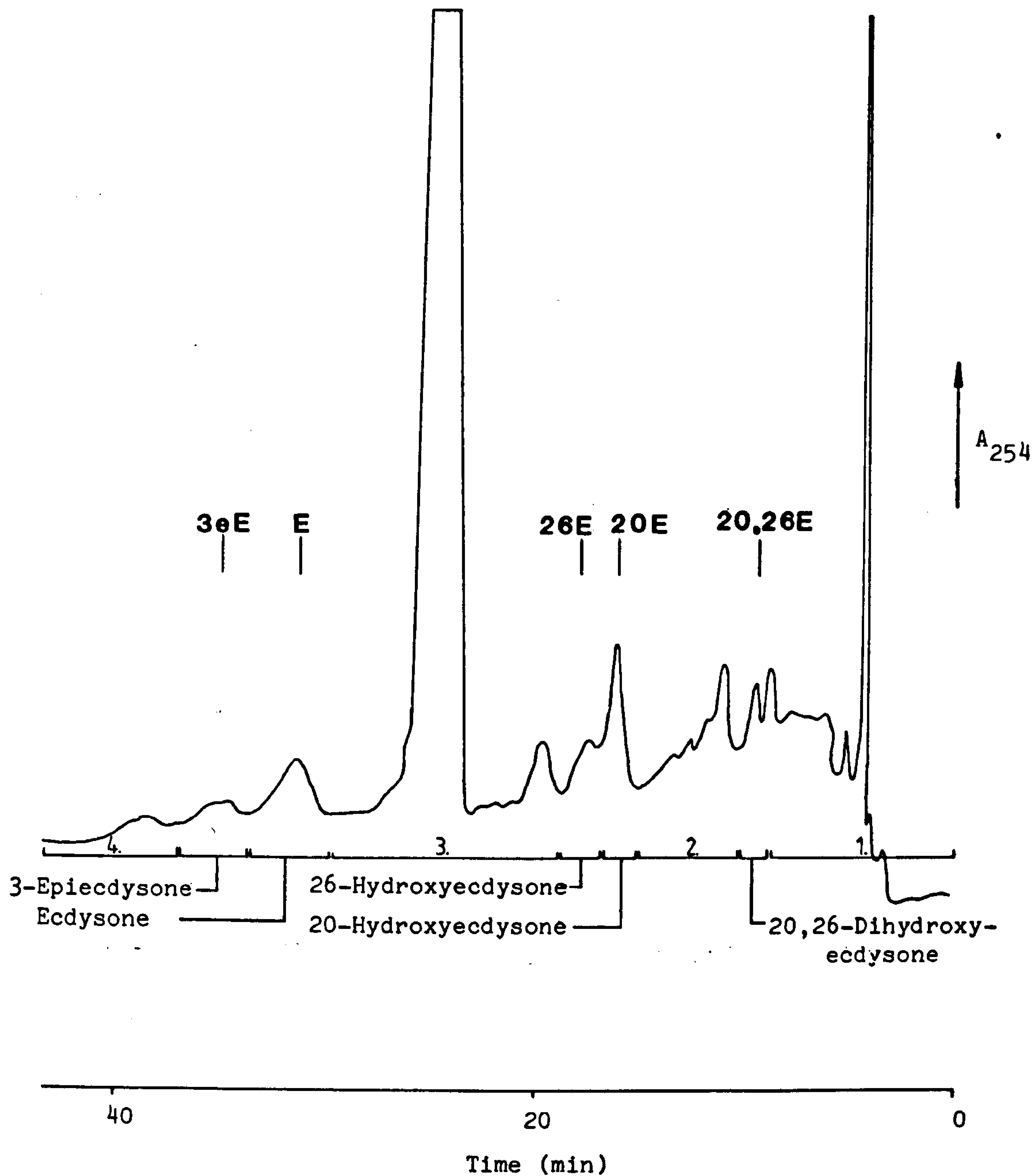


Figure 3C-11 : Reversed-phase h.p.l.c.-R.I.A. analysis of a pupal sample 168 h female). Areas corresponding to ecdysteroids were collected as were the intervening areas, and these fractions subjected to R.I.A. analysis. Authentic compounds were subsequently chromatographed on the same system. For results see Table 3C-2.

Table 3C-2 : Comparison of the amounts of ecdysteroids detected by h.p.l.c. (U.V.) and h.p.l.c. - RIA from the female pupal sample at 168 hr

		Nanograms detected by		Cross reaction ratio
Ecdysteroid		U.V. absorbance A_{254}	R.I.A. ecdysone equivalents	ng by R.I.A. / ng by A_{254}
Ecdysone		28	24	0.86
3-epiecdysone		10	1	0.10
20-hydroxyecdysone		51	19	0.37
26-hydroxyecdysone		10 †	38	3.80
20,26-dihydroxyecdysone		10 †	11	1.10
Intervening areas (see figure 3C-11)	1.	-	14.5	-
	2.	-	16.5	-
	3.	-	10.0	-
	4.	-	7.0 (1.–4. = 48)	-
Methanol wash		-	14	-
Total		109	155	-

The amount of sample injected onto h.p.l.c. corresponds to five insect equivalents, 192 ng ecdysone equivalents by RIA, 97.5 ng from h.p.l.c. with U.V. monitoring of peaks (figure 3C-11).

† Quantities calculated from the h.p.l.c. titre curves (figures 3C-7 and 3C-8) i.e. from h.p.l.c. (U.V.) using an APS-Hypersil column.

ecdysone nucleus (as in compounds such as; 2-deoxyecdysone, 2-deoxy-20-hydroxyecdysone, 3-dehydroecdysone and 3-dehydro-20-hydroxyecdysone) leads to a sharp diminution of steroid binding (Reum _et al_., 1979). This, therefore, accounts for the considerably reduced quantity of 3-epiecdysone detected by R.I.A. ($^{1}/10$th of that detected by h.p.l.c. with U.V. monitoring). The particular batch of antiserum used in this analysis had 2.5 times the affinity for ecdysone as for 20-hydroxy-ecdysone (K.P. Wigglesworth, unpublished), and, indeed, the lower affinity of the ICT-1 antiserum towards 20-hydroxyecdysone is observed in this experiment (19 ng ecdysone equivalents by RIA, 51 ng by h.p.l.c with U.V. monitoring). The effect of 26-hydroxylation of the side chain of ecdysteroids on the cross reactivity of the ICT-1 antiserum has not been reported. However, compounds such as Makisterone A, inokosterone, and cyasterone, which differ from the structure 20-hydroxyecdysone at the end of the side chain do not significantly affect the cross-reactivity of the antiserum (Reum _et al_., 1979). It would, therefore, have been reasonable to expect 26-hydroxyecdysone and 20,26-dihydroxyecdysone to have similar cross-reactivities as ecdysone and 20-hydroxyecdysone, respectively. However the results shown in Table 3C-2 (final column) suggest that addition of a hydroxy group at C-26 of ecdysone and 20-hydroxyecdysone apparently enhances the cross reactivity 4-fold and 3-fold, respectively. Whether this is really the case, or whether it is due to anomalous interference in the RIA or the presence of contaminating UV-absorbing material during the estimation of the compounds by h.p.l.c. is uncertain. Whatever the case,the material associated with these areas of the chromatogram contribute a significant proportion of the immunoreactive material detected by the ICT-1 antiserum.

A fairly high proportion of immunoreactive material was found in the areas of the chromatogram intervening the ecdysteroids and in the methanol wash which followed the h.p.l.c. run. However, this does not account totally for the difference between the quantity of ecdysteroid detected by h.p.l.c. (97.5 ng, see p. 78), and detected by RIA of the total sample (192 ng see p. 78). In fact, the sum of the ng ecdysone equivalents detected by h.p.l.c.-R.I.A. (155 ng) falls short of the R.I.A. analysis of the total sample (192 ng). Therefore, although the immunoreactivity of these areas of the chromatogram can be attributed to some of the increased ecdysteroid measurement by R.I A., it does not entirely account for the difference between measurement by h.p.l.c. analysis and R.I.A. So it must be concluded that some form of artificial enhancement of immunoreactive material is responsible for the remaining increased quantities detected by radioimmunoassay.

PART 3 SECTION D

PART 3 SECTION D

Ecdysteroids associated with the ovaries of *S. littoralis*

Page No.

Introduction 82

Experimental and results

1. Incorporation *in vivo* of [4-^{14}C] cholesterol into ecdysteroids in the ovaries of female pupae of *S. littoralis* 83

   [^{14}C] Ecdysteroids isolated from the ovaries of day 8½ female pupae 84

   [^{14}C] Ecdysteroids isolated from the carcass plus haemolymph of day 8½ female pupae 84

   Overall pattern of metabolites 84

2. Gas-liquid chromatography/mass spectrometry of the ecdysone metabolites associated with the ovaries of *S. littoralis* pupae 85

   Analysis of 204 hr female pupal ecdysteroid titre sample 85

   Analysis of the ecdysteroids in the ovaries of 204 hr female pupae 86

   Analysis of the highly polar ecdysteroids from 204 hr (day 8½) female pupae 87

PART 3 SECTION D

Ecdysteroids associated with the ovaries of *S. littoralis*

INTRODUCTION

Ecdysteroid titres in male and female pupae have been studied in *Galleria mellonella*, (Bollenbacher *et al*., 1978; Hsiao and Hsiao, 1977) *Bombyx mori* (Hanaoka and Ohnishi, 1974), *Manduca sexta* (Bollenbacher *et al*., 1981) and in *Spodoptera littoralis* (Clarke, 1981). All these titres demonstrate that an additional peak of ecdysteroid material is present in the female titre.

The character of the female peak in *S. littoralis* has been further investigated in this work (see Part 3, section C). Although additional purification by reversed-phase SEP-PAK cartridges reduces the relative size of this peak, a noticeable peak of ecdysteroid (assayed by R.I.A. or by h.p.l.c. analysis) is present in the female (see figure 3D-1) with no such peak occurring in the male.

The additional peak found in female pupae has been demonstrated to be associated with the ovaries in some species. Indeed, in ovariectomized female pupae of *B. mori* (Hanaoka and Ohnishi, 1974) and *G. mellonella* (Bollenbacher *et al*., 1978) the second peak observed in normal females is completely missing.

This section describes the investigation of the ecdysteroid content of ovaries in *S.littoralis* pupae by GC/MS. Although the gas chromatography column employed allows ready resolution of ecdysone, 20-hydroxyecdysone, 26-hydroxyecdysone and 20,26-dihydroxyecdysone, 3-epi-ecdysteroids are not separated from the parent ecdysteroids. In addition the incorporation of [^{14}C] cholesterol into ecdysteroids in the ovaries was attempted, and the ecdysteroids formed in this way identified by reversed-phase h.p.l.c.

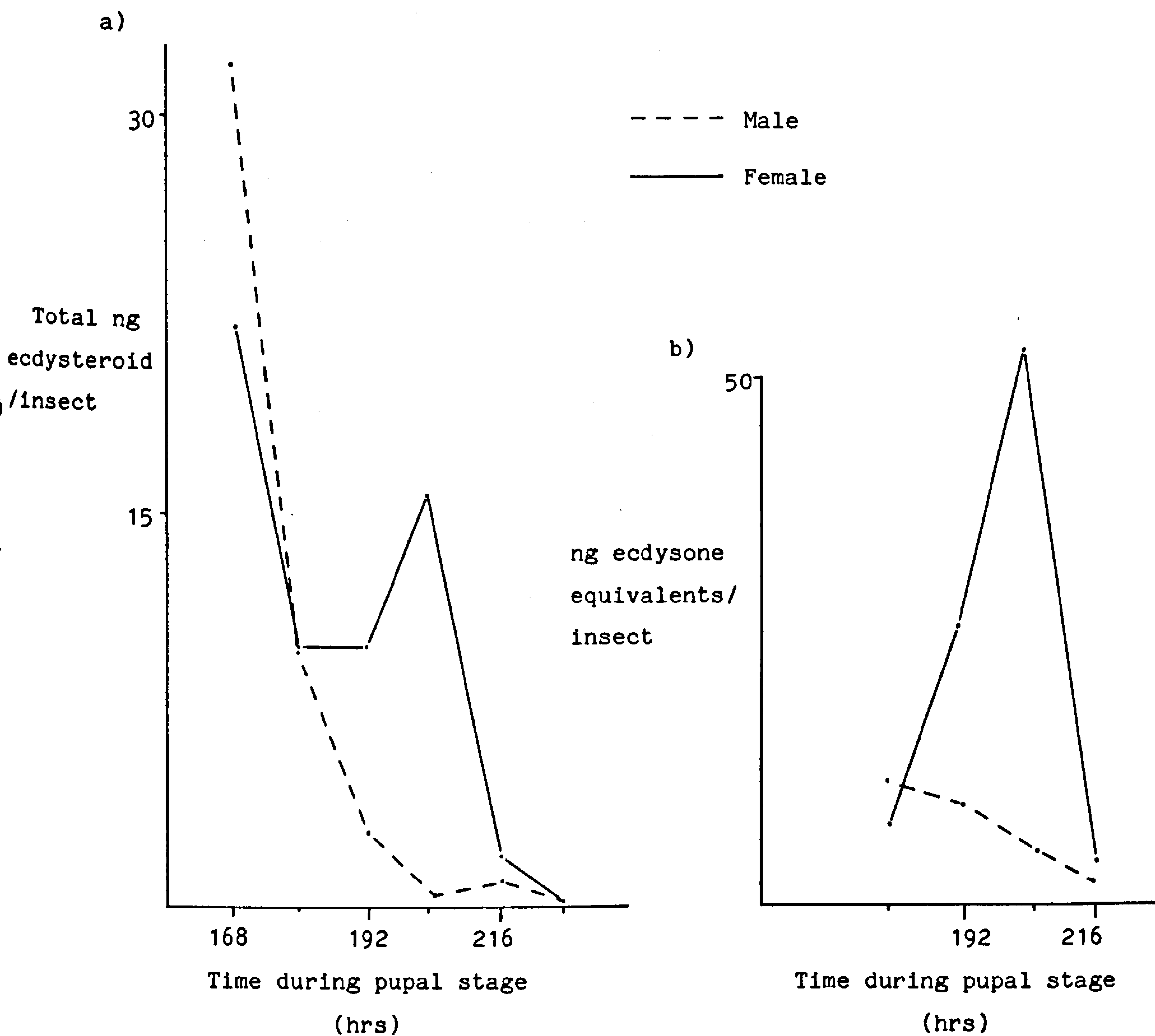


Figure 3D-1 : The amount of ecdysteroid detected a) by h.p.l.c., b) by radioimmunoassay (mean of single samples assayed in duplicate), during the later stages of pupal development in male and female pupae (samples, ex. G.S. Clarke (1981) assayed after SEP-PAK chromatography, see p. 74). The results demonstrate the presence of an additional ecdysteroid peak at day 8½ in the female pupal titre.

A high proportion of ovarian ecdysteroids in several insect species are known to occur as polar conjugates, hydrolysable by *Helix pomatia* hydrolases (Hoffmann *et al*., 1980). For example, at the ovarian peak titre of the female pupal stage of the lepidopteran *G. mellonella*, 85% of the total ecdysteroids (as assessed by bioassay) were present in the form of conjugates (Hsiao and Hsiao, 1979). In view of these considerations the ecdysteroid conjugate fraction from female *S. littoralis* pupae at the time of the second hormone peak was investigated by enzymic hydrolysis followed by GC/MS analysis of the ecdysteroid moieties thus released from conjugation.

EXPERIMENTAL AND RESULTS

1. Incorporation *in vivo* of [4-^{14}C] cholesterol into ecdysteroids in the ovaries of female pupae of *S. littoralis*

Fifteen *S. littoralis* pupae were synchronised at the final larval moult ($\pm$ 2 hours) and allowed to develop under normal conditions. At day 2 of pupal development the insects were injected with 0.2 µCi [4-^{14}C] cholesterol per insect (3 µCi in total). This time of injection was chosen since it allowed the injected [^{14}C] cholesterol maximum time to reach the proposed site of biosynthesis in the ovaries, and allowed time for the pupal cuticle to harden so that injection caused little damage to the insect.

At day 8½ of pupal development, the time corresponding to the ecdysteroid titre peak believed to be associated with ovarian synthesis (figure 3D-1), the insects were sacrificed, the ovaries (112 mg) dissected and rinsed twice in insect Ringer solution. The ovaries and the carcass plus haemolymph (4.632g) were then extracted and the ecdysteroids purified by the method described in Part 2. The details

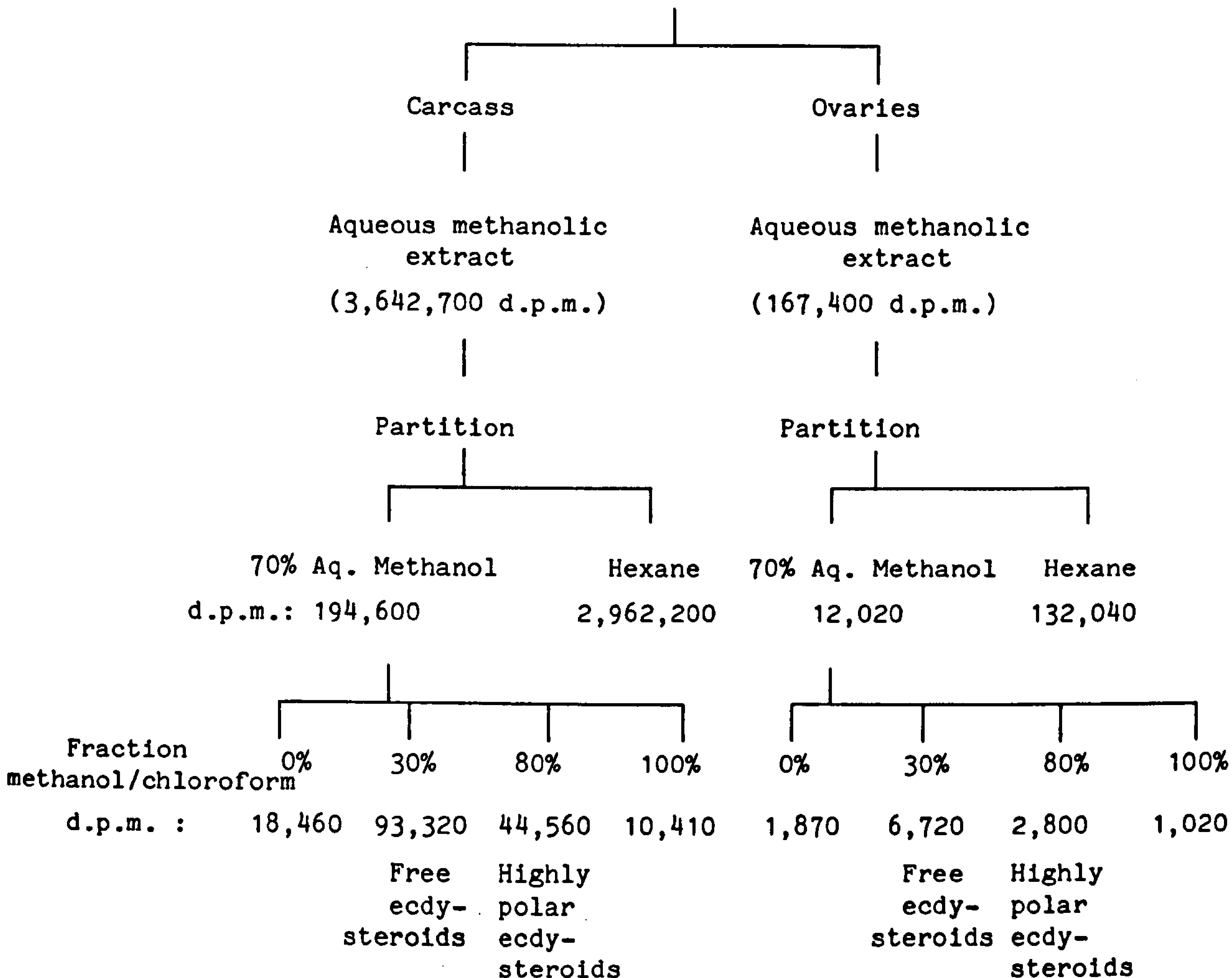


Figure 3D-2: Summary of the distribution of [^{14}C] radioactivity in day 8½ female pupae administered with [^{14}C] cholesterol at day 2.

of the distribution of radioactivity is illustrated in figure 3D-2.

[^{14}C] Ecdysteroids isolated from the ovaries of day 8½ female pupae

The analysis of the free ecdysteroids (30% methanol/chloroform column fraction) from the ovaries of female pupae was achieved by using h.p.l.c. system 1 [figure 3D-3 (a)]. The results show the presence of ecdysone and small amounts of [^{14}C] compounds co-chromatographing with 20-hydroxyecdysone and 2-deoxyecdysone.

The highly polar ecdysteroid fraction was analysed using h.p.l.c. [figure 3D-3 (b)] revealing the presence of radioactivity co-chromatographing with 20-hydroxyecdyson-26-oic acid as the principal component. However, the amount of radioactivity was insufficient for further characterisation of the putative acid.

[^{14}C] Ecdysteroids isolated from the carcass plus haemolymph of day 8½ female pupae

The free ecdysteroid fraction (30% methanol/chloroform column fraction) was analysed with h.p.l.c. system 1 [figure 3D-4(a)], the results demonstrating that the only free ecdysteroid present is [^{14}C] ecdysone.

The highly polar ecdysteroid fraction analysed by h.p.l.c. system 2 [figure 3D-4(b)] revealed only one [^{14}C] component, co-chromatographing with 20-hydroxyecdyson-26-oic acid.

Overall pattern of metabolites

A summary of the quantitative analysis of the metabolites formed from [^{14}C] cholesterol is shown in Table 3D-1. The results indicate that the ecdysteroids represent 0.44% of the radioactivity recovered

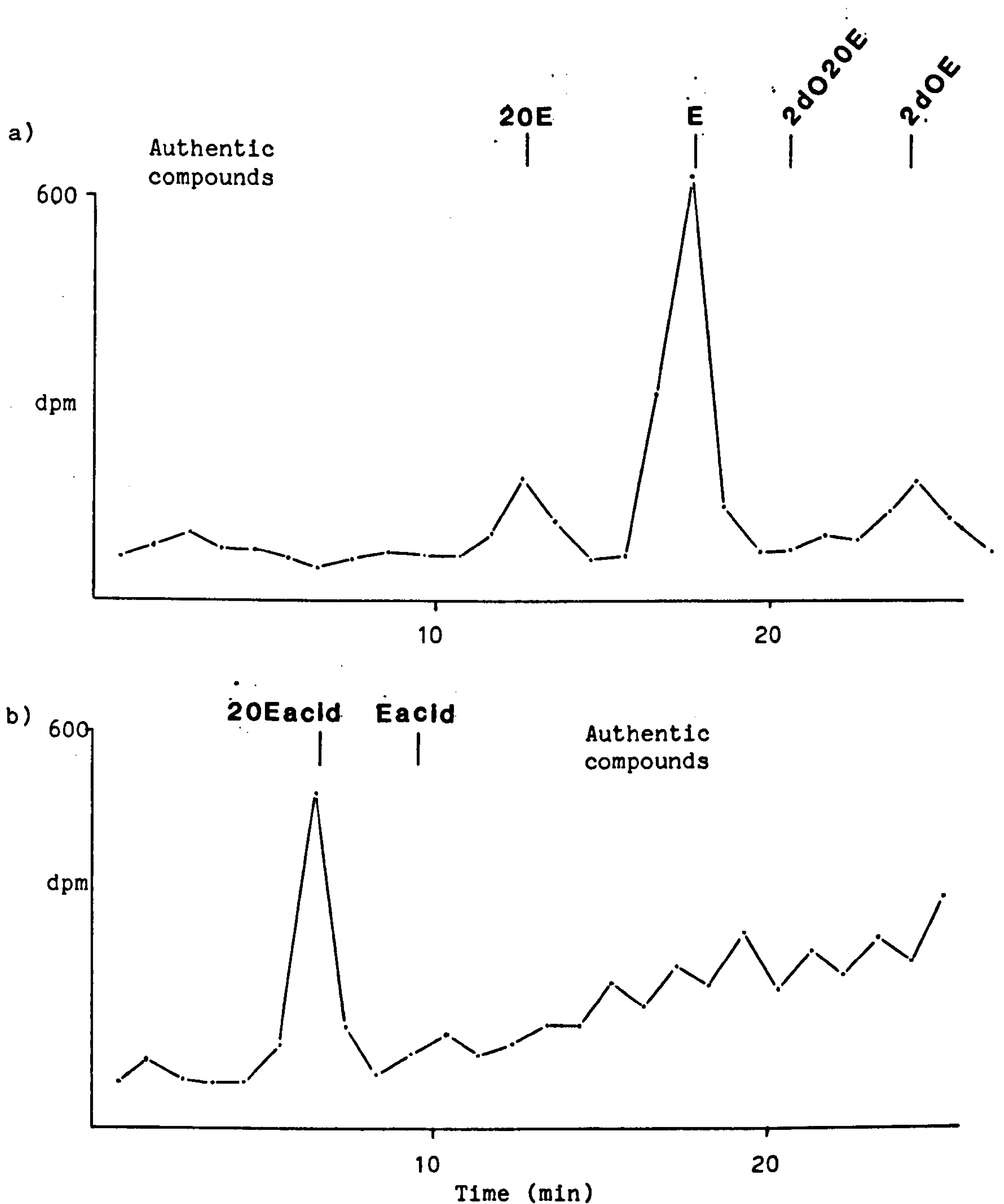


Figure 3D-3 : H.p.l.c. analysis of [^{14}C] ecdysteroids from the ovaries of day 8½ female pupae after administration of cholesterol at day 2.

a) [^{14}C] free ecdysteroids [an Ultrasphere-ODS reversed-phase column eluted at a flow rate of 1 ml/min with a linear gradient (30 mins.) of methanol in water changing from (2:3, $^{v}/v$) to (4:1, $^{v}/v$)]

b) The [^{14}C] highly polar ecdysteroids [a Partisil ODS-3 reversed-phase column eluted at a flow rate of 2 ml/min with a linear gradient (30 mins) of acetonitrile in 0.02M Tris-perchlorate buffer pH 7, (1:9 v/v) changing to (2:3, v/v)]

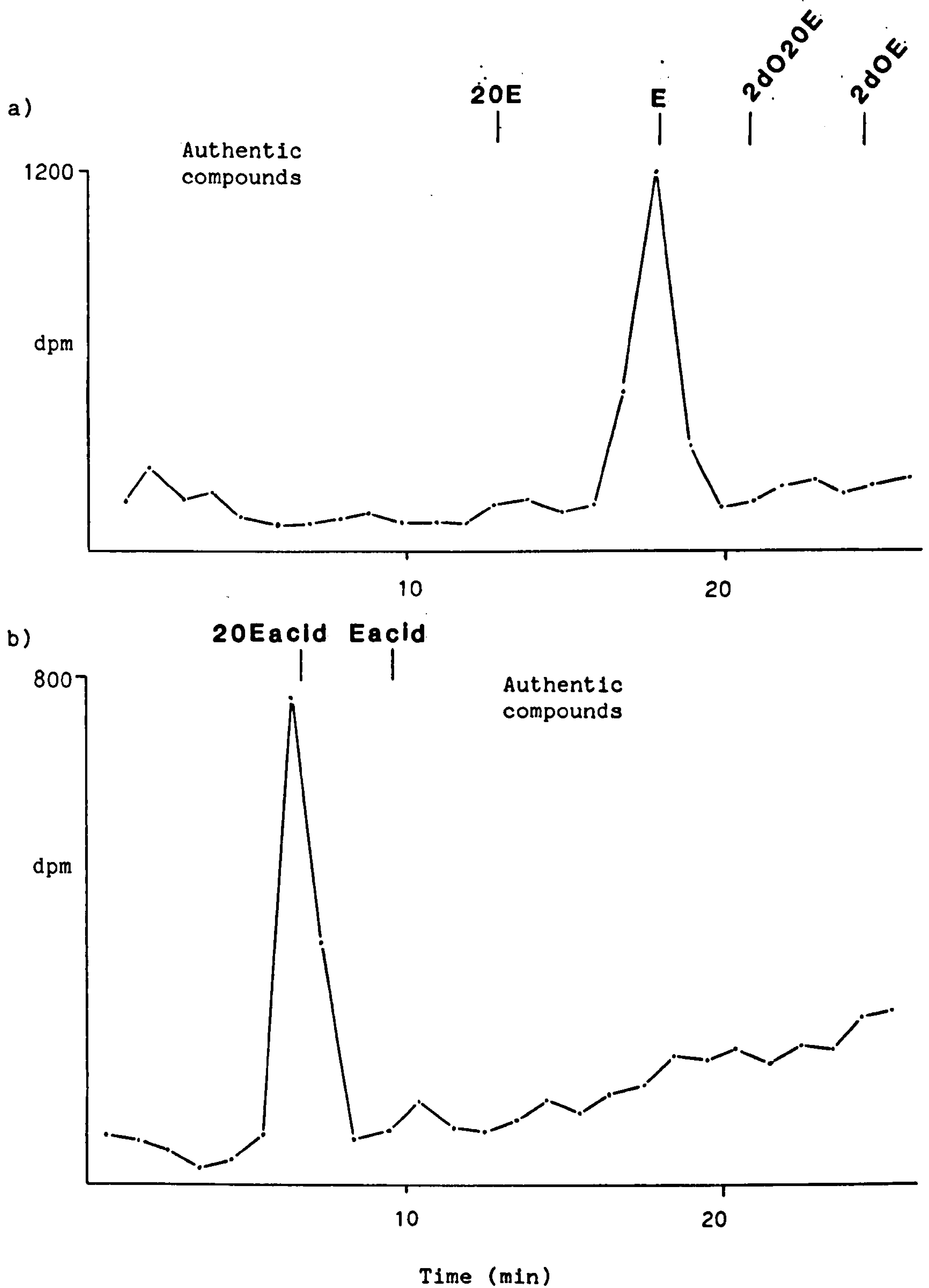


Figure 3D-4 : H.p.l.c. analysis of a) the [^{14}C] free ecdysteroids (h.p.l.c. system as Figure 3D-3a) and b) the [^{14}C] highly polar ecdysteroids (h.p.l.c. system as Figure 3D-3b) from the 'carcass plus haemolymph' of day 8½ female pupae after administration of [^{14}C] cholesterol at day 2.

Table 3D-1 : Summary of the [^{14}C] ecdysteroids found in day 8½ female pupae after metabolism of 3 μCi [^{14}C] cholesterol administered on day 2.

Ecdysteroid	d.p.m.	
	Carcass plus haemolymph	Ovaries
Ecdysone	8800	1500
20-hydroxyecdysone	-	350
2-deoxyecdysone	-	300
20-hydroxyecdyson-26-oic acid?	5200	450
Total	14,000	2,600

The total incorporation into ecdysteroids is 0.44% of the radioactivity recovered in the initial aqueous methanolic extract.

Total radioactivity recovered in the initial aqueous methanol extract : 3,810,100 d.p.m.

Total radioactivity recovered (by h.p.l.c.) in the form of ecdysteroids : 16,600 d.p.m.

in the initial aqueous methanolic extract. The results also demonstrate that the radioactive ecdysteroids recovered from both the 30% and 80% methanol/chloroform column fractions do not account for the total radioactivity in each of these fractions (c.f. figure 3D-2 and Table 3D-1). Indeed, this phenomenon was encountered from all [^{14}C] cholesterol injection experiments _in vivo_, and an investigation into this has been undertaken (see Part 3, section A).

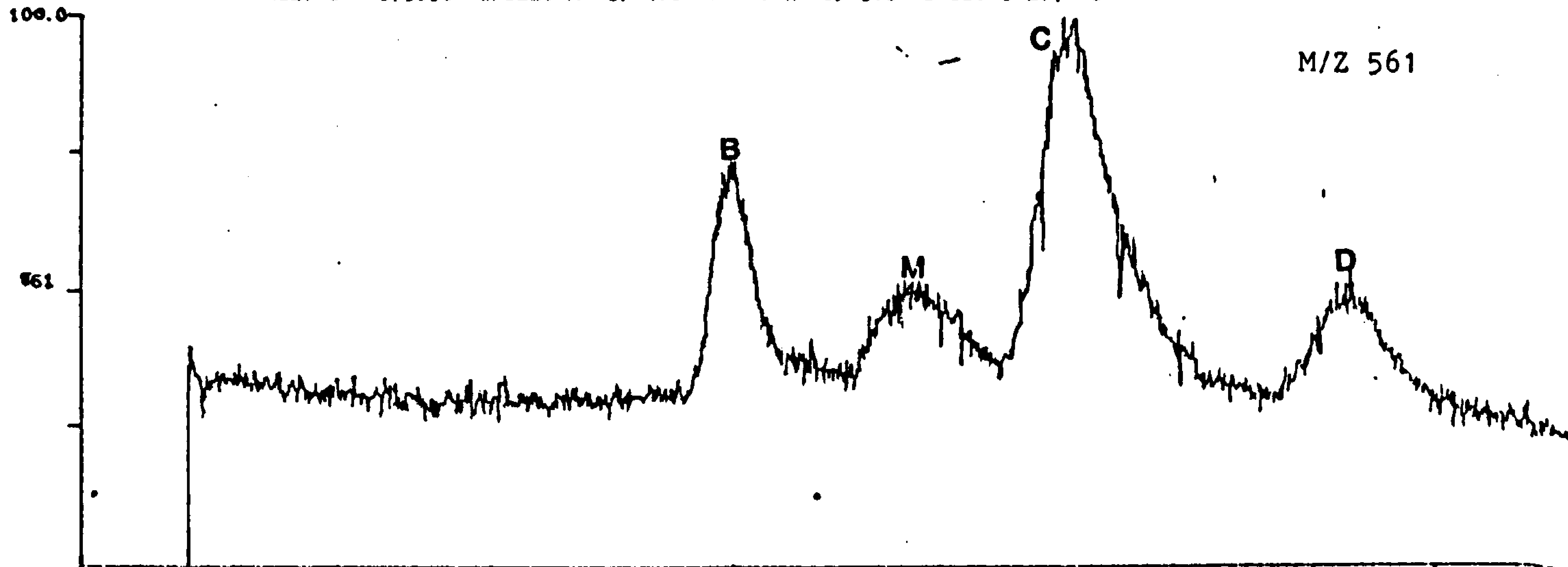
The free [^{14}C] ecdysteroids present at day 8½ after administration of [^{14}C] cholesterol at day 2, consist mainly of ecdysone in both the ovaries and in the rest of the body (carcass plus haemolymph). In fact, the free ecdysteroid complement from this experiment is similar to that present at day 9 of male pupal development after administration of [^{14}C] cholesterol at day 2 (i e. mainly consisting of ecdysone, see Table 3A-4). [^{14}C] Ecdysone was the sole free ecdysteroid present in the carcass plus haemolymph from females, and in the ovaries ecdysone was accompanied by only small amounts of 20-hydroxyecdysone and 2-deoxy-ecdysone. The highly polar fraction contains almost exclusively radio-activity co-chromatographing with 20-hydroxyecdyson-26-oic acid, once again very similar to [^{14}C] ecdysteroid formation in day 9 male pupae.

2. Gas-liquid chromatography/mass spectrometry of the ecdysone metabolites associated with the ovaries of _S.littoralis_ pupae

Analysis of 204 hr female pupal ecdysteroid titre sample

The sample from the female ecdysteroid titre curve corresponding to 204 hrs of pupal development was analysed by GC/MS (figure 3D-5) by the method described in Part 2. The ecdysteroid peaks were identified by their retention times relative to Makisterone A (internal standard)

MID MASS CHROMATOGRAMS DATA: AM184 #1 SCANS 1 TO 1200
11/26/82 12:53:00 CALI: AM180 #1
SAMPLE: NICKI
RANGE: G 1,1500 LABEL: N 0, 4.0 QUAN: A 0, 1.0 BASE: U 20, 3



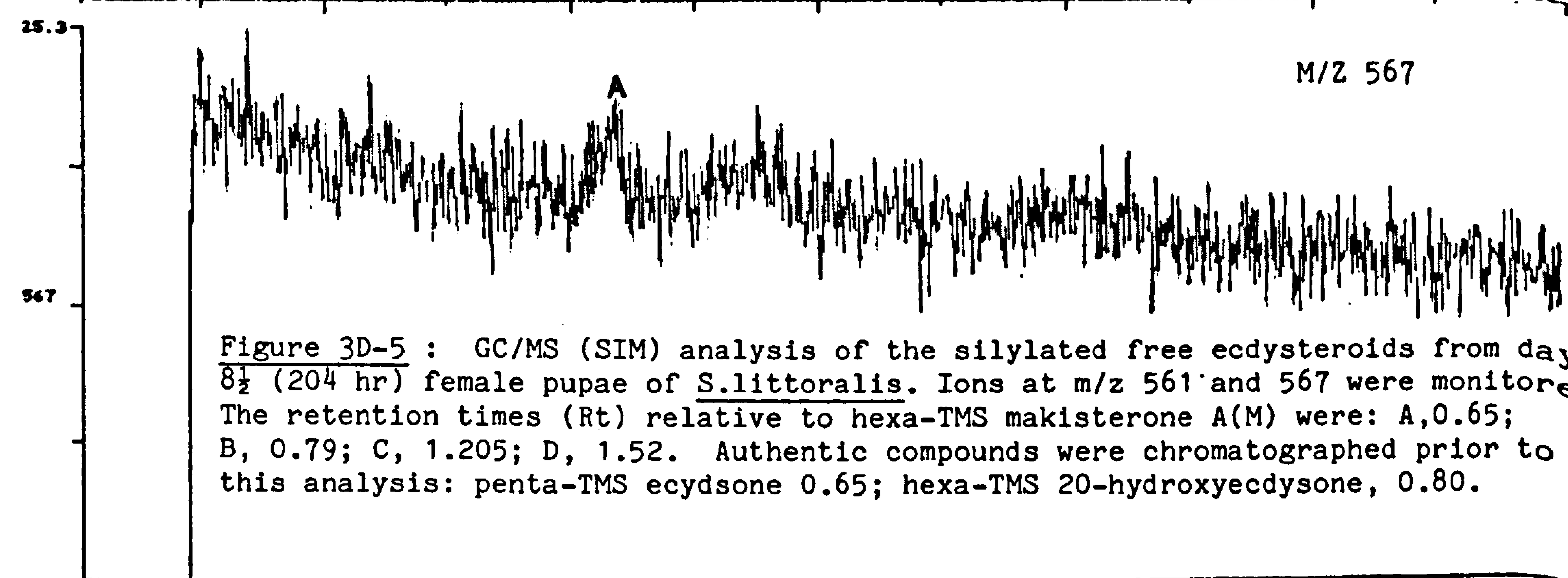


Figure 3D-5 : GC/MS (SIM) analysis of the silylated free ecdysteroids from day 8½ (204 hr) female pupae of S.littoralis. Ions at m/z 561 and 567 were monitored. The retention times (Rt) relative to hexa-TMS makisterone A(M) were: A,0.65; B, 0.79; C, 1.205; D, 1.52. Authentic compounds were chromatographed prior to this analysis: penta-TMS ecydsone 0.65; hexa-TMS 20-hydroxyecdysone, 0.80.

100.0
808
Total ion chromatogram
533
671
1021
RIC
84
214
345
439

scan 200 400 600 800 1000 1200
time 2:20 4:40 7:00 9:20 11:40 14:00

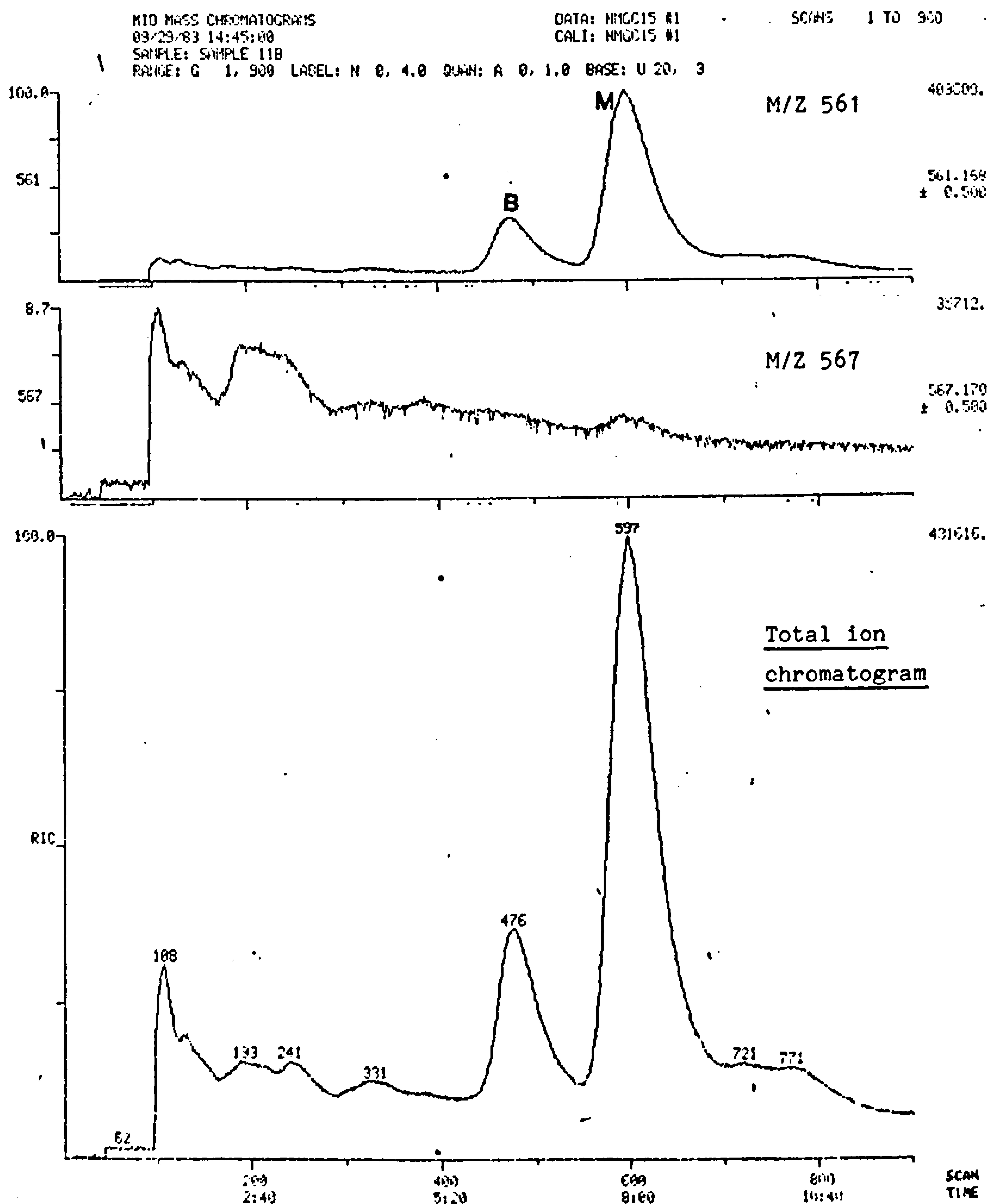


Figure 3D-7 : GC/MS (SIM) analysis of the silylated free ecdysteroid fraction from the "carcass plus haemolymph" of day 8½ female pupae of S.littoralis. Ions at M/Z 561 and 567 were monitored. The retention time (R_t) relative to hexa TMS makisterone A (M) was: B, 0.80. Authentic compounds were chromatographed prior to the present analysis, the retention times (R_t) relative to hexa-TMS makisterone A(M) were: penta-TMS ecdysone, 0.65; hexa-TMS 20-hydroxyecdysone, 0.80.

(Table 3D-2) and quantified by peak area as described in Part 2.

This analysis revealed the presence of peaks co-chromatographing with 20,26-dihydroxyecdysone (20 ng; 10 ng/insect), 20-hydroxyecdysone (8 ng; 4 ng/insect), ecdysone (6.5 ng; 3.25 ng/insect). However, it must be noted that the column employed did not resolve ecdysteroids from their 3-epimers. Therefore, since 3-epi-ecdysone and 3-epi-20-hydroxyecdysone have been detected by h.p.l.c. analysis of pupae at an early stage of development, these compounds might be included with the corresponding 3β-hydroxyecdysteroids.

<u>Analysis of the ecdysteroids in the ovaries of 204 hr female pupae</u>

Ten female pupae were synchronised at the pupal moult and allowed to develop until 204 hours (± 2 hrs) of pupal development. The ovaries (83 mg) were dissected, separated from the carcass plus haemolymph (3.140 g) and rinsed twice with insect Ringer solution. Each of these was then extracted and purified by silicic acid column chromatography by the method described in Part 2. The free ecdysteroid fractions, thus purified from both the ovaries and the carcass plus haemolymph, were then derivatised by silylation and analysed by GC/MS (SIM) (for details see Part 2).

The analysis of the ovarian ecdysteroids [ex 20 mg of ovarian tissue (2.5 insects)] (figure 3D-6) revealed the presence of two major ecdysteroids, 20,26-dihydroxyecdysone and/or the 3-epimer (13.5 ng, 5.4 ng/insect), 20-hydroxyecdysone and/or the 3-epimer (19.2 ng, 7.68 ng/insect, and smaller amounts of ecdysone and/or the 3-epimer (5.1 ng, 2.04 ng/insect).

The analysis of the ecdysteroids in the carcass and haemolymph [ex 314 mg tissue (1 insect)] (figure 3D-7) showed the presence of only 20-

Table 3D-2 : Relative retention times of fully silylated TMS ethers of some ecdysteroids by GC/MS (SIM), using a 1.5m x 2 mm glass column packed with 1% (w/w) OV-1 silicone phase on Gas Chrom. Q (100 - 200 mesh), (Mendis *et al.*, 1983)

Ecdysteroid	Retention time relative to Makisterone A
Ponasterone A	0.54
Ecdysone	0.63
3-Epiecdysone	0.63
20-Hydroxyecdysone	0.79
3-Epi 20-hydroxyecdysone	0.79
Inokosterone	0.85
26-Hydroxyecdysone	0.95
Makisterone A	1
20,26-Dihydroxyecdysone	1.21
Podecdysone A	1.17

hydroxyecdysone and/or its 3-epimer (1.05 ng, 1.05 ng/insect). The quantitative and qualitative results for the ecdysteroids detected by GC/MS are quite different from those obtained for the [^{14}C] cholesterol administration; the reasons for this are obscure.

Analysis of the highly polar ecdysteroids from 204 hour (day 8½) female pupae

In addition to the analysis of the free ecdysteroid fractions of the ovaries and carcass plus haemolymph, five day 8½ female pupae (ovaries not dissected) were also extracted by the method described in Part 2. The highly polar ecdysteroid fraction from silicic acid column chromatography was then subjected to hydrolysis by *H. pomatia* enzyme preparation. The ecdysteroids released from conjugation by this treatment were then further purified by a second silicic acid column separation (see Part 2 for details). The released ecdysteroids were then silylated and analysed by GC/MS (SIM) (figure 3D-8). The chromatogram demonstrates that only a small amount of ecdysteroid (less than 0.05 ng 20-hydroxyecdysone/insect) is present in the form of conjugates, whether it be in the ovaries or elsewhere in the pupae.

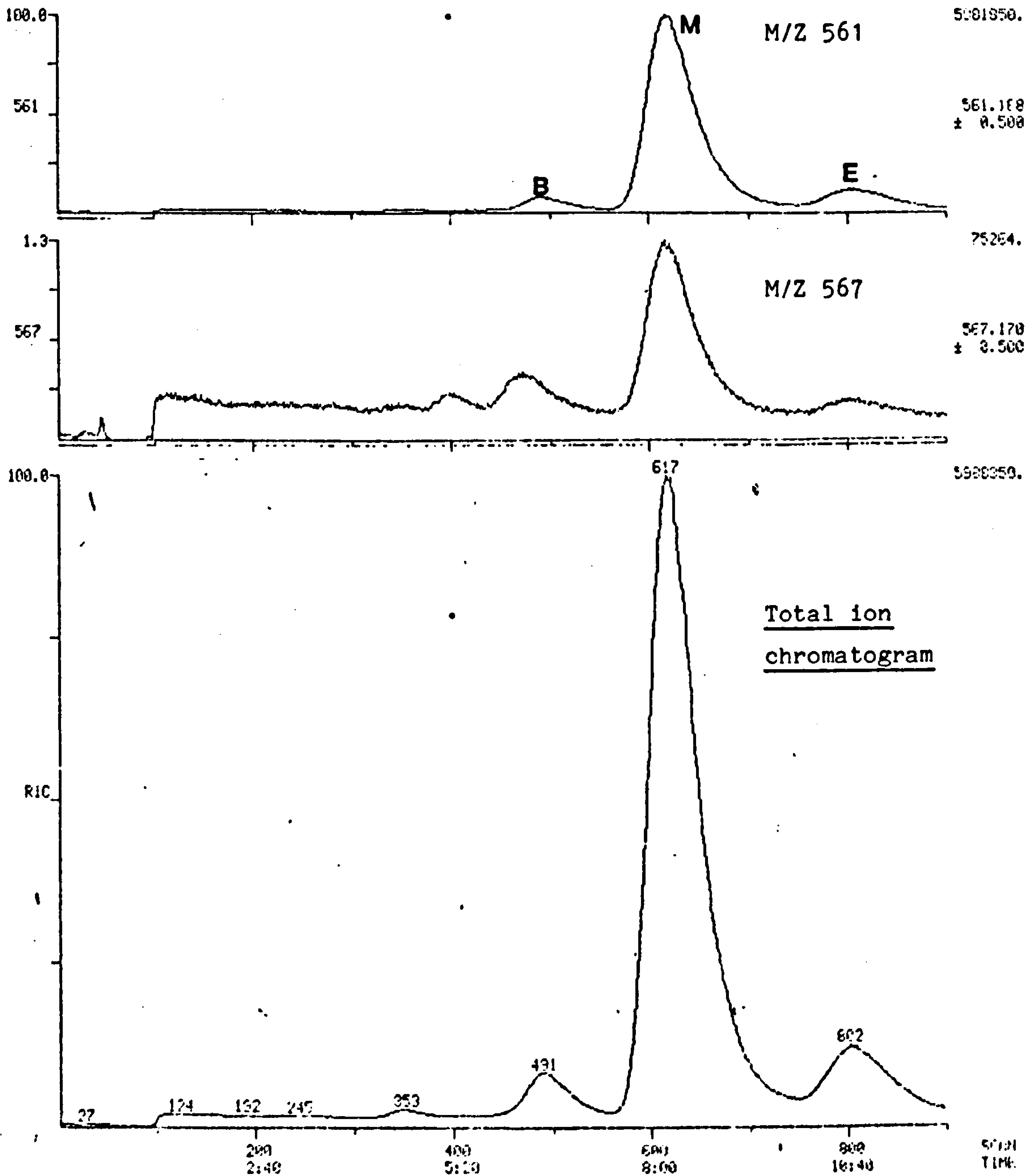


Figure 3D-8 : GC/MS (SIM) analysis of ecdysteroids (silylated) enzymically released from the corresponding polar conjuages isolated from day 8½ female pupae of S. littoralis. Ions at M/Z 561 and 567 were monitored. The retention times (T_t) relative to hexa-TMS makisterone A(M) were: B, 0.80; E, 1.30. Authentic compounds were chromatographed prior to this analysis, the retention times (R_t) relative to hexa-TMS makisterone A(M) were: penta-TMS ecdysone, 0.65; hexa-TMS 20-hydroxyecdysone, 0.80.

PART 3 SECTION E

PART 3 SECTION E

Discussion of the studies on *S. littoralis* pupae

		Page No.
1.	Ecdysteroid metabolites present in the pupal stage of *S. littoralis*	89
2.	The occurrence of ecdysteroid-26-oic acids in insects	97
3.	Titres of individual ecdysteroids during the pupal stage of *S. littoralis*	100
4.	Ecdysteroids in the ovaries of *S. littoralis*	108

PART 3 SECTION E

Discussion of the studies on *S. littoralis* pupae

1. Ecdysteroid metabolites present in the pupal stage of *S. littoralis*

The radioactive free ecdysteroids isolated from metabolism *in vivo* of [^{3}H] ecdysone injected into pupae (Table 3A-1) are principally in the form of 20- and 26-hydroxylated ecdysteroids. The formation of these compounds was confirmed by [^{14}C] cholesterol metabolism *in vivo*, which, after injection at day 2 of pupal development was converted into ecdysone and 20- and 26-hydroxylated ecdysteroids. In addition, such compounds have been found as endogenous ecdysteroids, although the 26-hydroxy ecdysteroids only appear as minor components (figures 3C-5,6,7 and 8).

Whereas metabolism of ecdysone by dehydroxylation has not been reported, side chain hydroxylation is widely observed during post-embryonic development of many arthropod species. 20-Hydroxyecdysone is widely accepted as the major active form of insect moulting hormone, and has been found in most insects. However, in this study, the further metabolism of ecdysone and 20-hydroxyecdysone, to less hormonally active ecdysteroids, was of most interest. Therefore, the discussion of the hydroxylating processes will be mainly limited to 26-hydroxylation. These compounds are usually present only as minor compounds (Koolman *et al.*, 1979; Feyereisen *et al.*, 1976; Russel *et al.*, 1977). In *Calliphora vicina*, the metabolism *in vivo* of [^{3}H] ecdysone was monitored (Karlson and Koolman, 1973) and shown to progressively proceed via 20-hydroxyecdysone and minor metabolites [later identified as 26-hydroxy-ecdysone, 20,26-dihydroxyecdysone, and inokosterone (Koolman *et al.*, 1979)] to polar ecdysteroids (possibly 26-oic acids).

Sequential hydroxylation of the ecdysone side chain is therefore an important metabolic pathway. However, the order of hydroxylation does not appear to be critical, as both 20-hydroxyecdysone and 26-hydroxyecdysone can be converted into 20,26-dihydroxyecdysone in Pieris (Lafont et al., 1980). In addition, it must be noted that 25- and 26-hydroxylated ecdysteroids can theoretically exist in the 25S and 25R form. However, the separation systems used during this study could not separate these isomers; a mixture of 25R and 25S 26-hydroxyecdysone has been indicated by h.p.l.c. analysis of this compound from Pieris (Lafont et al., 1983). Lafont et al. have performed a number of [^{3}H] ecdysone injection experiments in Pieris pharate pupae and pupae similar to those reported in this study. The principal metabolic differences observed in these 2 lepidopterans concerning the 26-hydroxy ecdysteroids, lies in the fact that both [^{3}H] 26-hydroxyecdysone and [^{3}H] 20,26-dihydroxyecdysone do not accumulate appreciably in Pieris. These occur in Pieris only as minor metabolites, whether it be in pharate pupae (Lafont et al., 1980) or during pupal development (Beydon et al., 1981), with the [^{3}H] 26-oic-acids (P_1 and P_2) constituting the major metabolites of ecdysone and 20-hydroxyecdysone. In Spodoptera, however, the 26-hydroxylated compounds represent a higher proportion of the metabolites. Whether this is merely due to different experimental procedures (e.g. different amounts of ecdysone injected per insect) or reflects a more active production of 26-oic acids in Pieris is unclear. However, one explanation could lie in the developmental time at which [^{3}H] ecdysone was injected and incubated. The [^{3}H] metabolites observed in Spodoptera were produced over the decrease in endogenous moulting hormone titre where, presumably, any [^{3}H] 26-hydroxyecdysteroids would be formed in the presence of endogenous 26-hydroxyecdysteroids. However, in Pieris such experiments were only performed during the first seven

days of pupal development when the ecdysteroid titre is low or is increasing to the peak 20-hydroxyecdysone titre at day 6 (Lafont _et al._, 1975). Indeed, ecdysteroid metabolism early in _S. littoralis_ pupae, as analysed by metabolism _in vivo_ of [^{14}C] cholesterol (figure 3A-7), produces effectively smaller relative amounts of the 26-hydroxylated ecdysteroids. It must be noted that the metabolism of [^{3}H] ecdysone during the decrease in the endogenous titre is probably most reflective of the physiological situation.

The presence of [^{3}H] 3-epi ecdysteroids from [^{3}H] ecdysone metabolism _in vivo_ in late pupae has not been observed. However, similar experiments in late 6th instar larvae (140 hrs) have yielded some [^{3}H] 3-epiecdysone, and the analysis of endogenous ecdysteroids (Part 3, section C) has revealed the presence of 3-epiecdysone and 3-epi 20-hydroxyecdysone, principally during the early stages of that instar. Once again, the reason for the absence of 3-epimers of ecdysteroids in the pupal experiments could be due to the developmental time at which [^{3}H] ecdysone was administered to the pupae. This seems likely, as 3-epiecdysteroids are no longer present endogenously after day 4 of pupal development (see Part 3, section C). Indeed, a similar phenomenon is observed in _Pieris_, where in pharate pupae (Lafont _et al._, 1980) and early pupae (up to 24 hours) (Beydon _et al._, 1981) considerable quantities of [^{3}H] 3-epiecdysteroids are produced after administration of [^{3}H] ecdysone. However, during the remainder of pupal development such 3-epimerisation is not encountered, all metabolites being in the form of 3β-hydroxyecdysteroids.

Conjugate formation during the pupal stage of _S. littoralis_ development appears to be a rather minor metabolic pathway of ecdysteroids. Conjugates have been identified mainly from eggs and ovaries of insects

(Isaac et al., 1983; Tsoupras et al., 1982), but through radiotracer experiments their presence has also been established in the larvae and faeces of insects (for review see Koolman, 1982). In pupae, however, reports of ecdysteroid conjugates are limited. Some [^{3}H] conjugate formation following administration of [^{3}H] ecdysone to the white pupae of C. vicina has been demonstrated (Karlson and Koolman, 1973), but conjugate formation in vivo is not apparently evident in Lepidopteran pupae. Indeed, the absence of conjugates in the pupae of P. brassicae has recently been remarked by Lafont et al. (1980), all the polar metabolites at this stage of the insects development occurring in the form of ecdysteroid-26-oic acids (P_1 and P_2).

This is essentially also the case in Spodoptera. Some conjugates are observed as metabolites of injected [^{3}H] ecdysone and [^{14}C] cholesterol, but conjugation is a relatively minor metabolic route in comparison to the formation of the unhydrolysable polar ecdysteroids, ecdyson-26-oic acid and 20-hydroxyecdyson-26-oic acid, (Table 3A-1). Indeed, the GC/MS (SIM) analysis of day 8½ female pupae (section D) confirms the low levels of hydrolysable conjugates present at this late stage of pupal development, when conjugates, if produced during the pupal instar,would have presumably accumulated as the end-products of ecdysteroid metabolism.

By far the most predominant [^{3}H] highly polar ecdysteroids produced during decreasing hormone titre from [^{3}H] ecdysone metabolism in vivo were the [^{3}H] ecdysteroid 26-oic acids (Table 3A-1), in agreement with similar results obtained in Pieris pupae (Lafont et al., 1980; Beydon et al., 1981). Both [^{3}H] 20-hydroxyecdyson-26-oic acid and lesser amounts of [^{3}H] ecdyson-26-oic acid were produced in these experiments, but in the endogenous situation it would appear that 20-hydroxyecdyson-26-oic acid is much the predominant form. Approximately 27 μg 20-

hydroxyecdyson-26-oic acid and only approximately 1 μg ecdyson-26-oic acid were isolated from day 9 pupae for characterisation of the ecdysteroid 26-oic acids (section B). Indeed, metabolism in vivo of [^{14}C] cholesterol produced [^{14}C] 20-hydroxyecdyson-26-oic acid as the predominant component of the highly polar ecdysteroid fraction (Tables 3A-3 and 4). This applies whether pupae were sacrificed at a late stage or at peak endogenous hormone titre. The [^{3}H] ecdysteroid 26-oic acids isolated from Pieris (Lafont et al., 1980) in early pupae, after injection of [^{3}H] ecdysone, included [^{3}H] 3-epiecdyson-26-oic acid and [^{3}H] 3-epi-20-hydroxyecdyson-26-oic acid. These compounds were not detected in the present study. In the case of the radioactive tracer experiments involving [^{3}H] ecdysone metabolism in vivo, this can be easily explained. These experiments were undertaken using late pupae (day 5), and at this stage of development it is apparent that 3-epiecdysteroids are no longer produced (see h.p.l.c. ecdysteroid titres, figures 3C-5,6,7 and 8); a similar situation is observed in Pieris pupae. In Pieris pupae more than 24 hrs after pupation, no 3-epiecdysteroids are produced from [^{3}H] ecdysone metabolism in vivo (Beydon et al., 1981). The extraction of endogenous 3-epiecdysteroid-26-oic acids was not undertaken in the present study, but it would be reasonable to expect that these do occur as end-products of 3-epiecdysteroid metabolism via a metabolic pathway parallel to that established for the ecdysteroids. This, implies the existence of 3-epi-26-hydroxylated ecdysteroids as intermediates in this pathway. The relative amounts of 3-epiecdysone and 3-epi 20-hydroxy-ecdysone as compared to ecdysone and 20-hydroxyecdysone present during the pupal instar of S. littoralis (see h.p.l.c. titres, figures 3C-5,6, 7 and 8) implies that 3-epiecdysteroids will probably be present in much lower levels than their 3β-hydroxyecdysteroid isomers.

There is evidence for the presence of 20-hydroxyecdyson-26-oic acid conjugate, hydrolysable with Helix hydrolases, in 6th instar larvae of S. littoralis (table 4A-2). Therefore, the existence of ecdysteroid-26-oic acid conjugates in the pupal instar cannot be discounted. However, this must be considered unlikely in view of the extremely low conjugation of ecdysone and 20-hydroxyecdysone encountered in the pupae (aforementioned in this discussion). It could be argued that, as end-products of oxidative metabolism of ecdysteroids, the 26-oic acids may be preferentially conjugated. However, the conjugation process has been found to be largely non-specific, the conjugated ecdysteroids often reflecting the pattern of free ecdysteroids. This indicates that the conjugate form of 26-oic acids probably does not occur at this stage of S. littoralis development.

The metabolism in vivo of [23,24 ^{3}H] ecdysone in S. littoralis has revealed the presence of a number of ecdysteroid metabolites. However, any ecdysone metabolites formed by side-chain cleavage would not be detected in these experiments. Indeed, the formation of post-sterone by C-20/C-22 scission of 20-hydroxyecdysone has been reported in the prepupae of Calliphora stygia (Galbraith et al., 1969), although it only occurred as a very minor metabolite. The administration of the nucleus-labelled [4-^{14}C] cholesterol in this study should have detected any poststerone produced. This was not the case, and it must be concluded that poststerone is not a significant metabolite of ecdysone, if indeed it is present at all.

Other notable compounds not detected in the present studies in vivo include 3-dehydroecdysteroids. The latter compounds which are potential intermediates in the 3-epimerisation reaction, have been apparently reported from a number of radioactive tracer experiments (review, see

Koolman, 1982) in a number of species, although significantly these have rarely been identified in Lepidopterans (Koolman, 1978). However, these compounds have frequently been identified solely on the strength of silica t.l.c. and it has recently been demonstrated that ecdysteroid acetates co-chromatograph with 3-dehydroecdysteroids on this system (Isaac et al., 1981). Thus, the presence in vivo of 3-dehydroecdysteroids in insects has perhaps to be re-assessed.

The formation of ecdysteroid 3-acetates was not encountered in S. littoralis at any time during either the pupal stage or the 6th instar larval stage of development. In fact, no ecdysone metabolite less polar than ecdysone, or 3-epiecdysone, was discovered in this study, although this was extensively investigated by h.p.l.c. methods using authentic marker compounds.

It is curious that in a number of metabolism experiments in vivo using radiolabelled cholesterol, an inordinate amount of ecdysone was produced. This phenomenon occurs in experiments that involve injection of [^{14}C] cholesterol at day 2 of pupal development with analysis of the products in late pupae (day 9) (table 3A-4 and table 3D-1), whereas if the products are analysed earlier in pupal development, such as at day 6, radioactive ecdysone is found at similar levels to other ecdysteroids (Table 3A-3). At the later stage of pupal development (day 9) ecdysone accounts for the majority of [^{14}C] ecdysteroids (table 3A-4), whereas the titres of individual ecdysteroids reveal similar proportions of each steroid (figures 3C-5,6,7 and 8). Interestingly, the proportions of individual [^{14}C] ecdysteroids isolated from day 6 females (table 3A-3), are in reasonable agreement with the proportions of ecdysteroids as determined by h.p.l.c. analysis (figures 3C-5,6,7 and 8).

It is normally assumed that the administration of [^{14}C] cholesterol should produce ecdysteroids reflecting the endogenous situation, but this seems not to hold true for the two aforementioned experiments. It certainly appears that after the peak of ecdysone secretion mid-way through the pupal stage, injected [^{14}C] cholesterol is subject to apparent unphysiological metabolism to ecdysone.

The reason for such an effect is uncertain. However, in prothoracic gland incubations in vitro in Locusta migratoria, it was found that the radioactivity from cholesterol could be incorporated more efficiently if the glands were cultured in a medium containing haemolymph derived from locusts which had been previously injected with radioactive cholesterol (Hoffmann et al., 1975). This suggests that cholesterol may not be the true hormone precursor used by the prothoracic glands, or that lipoprotein-bound sterol is a more effective precursor. Indeed, in Manduca sexta and Periplaneta americana only the prothoracic glands possess a large percentage of 7-dehydrocholesterol, implicating the prothoracic glands as a site of synthesis or storage of 7-dehydrocholesterol. In addition, incorporation of the radioactive label from [^{14}C] cholesterol into 7-dehydrocholesterol appears to vary depending on the developmental stage of the insect (Thompson et al., 1973).

Taking these findings into account it could be proposed that on injection into young pupae the radioactive cholesterol, before metabolism to ecdysteroids, needs to be converted into an intermediate of the ecdysone biosynthesis pathway (possibly 7-dehydrocholesterol) and to enter a pool of the intermediate compound in the prothoracic glands. Consequently, when the surge of ecdysone synthesis occurs midway through the pupal stage, the compartmentalized intermediate pool (containing some radioactive intermediate) would then be depleted, and non-radioactive

ecdysteroid plus radioactive ecdysteroids would be produced. Thus at the time of high ecdysteroid synthesis the radioactive ecdysteroids should reflect the endogenous situation, as was observed experimentally (see table 3A-3). However,the remaining radioactive cholesterol, still circulating in the haemolymph, can then enter the compartmentalized pool of the proposed intermediate compound, but now without having to enter a pool containing significant endogenous material as this has been largely depleted by the rapid synthesis of ecdysone at peak ecdysteroid titre. It, therefore, follows that even low level background synthesis of ecdysone would produce a high proportion of radioactive ecdysone, as the radioactive precursors of ecdysone are no longer diluted by endogenous compounds. Therefore, by this interpretation, the ecdysteroids detected at day 9 or day 8½ of pupal development (table 3A-4 and table 3D-1) can be divided into two groups (i) the [^{14}C] ecdysteroids produced via a pathway diluted by endogenous precursors and ecdysteroids and (ii) the [^{14}C] ecdysteroids (mainly ecdysone) produced in the presence of only a small amount of endogenous intermediates (i.e. after the main ecdysone synthesis period).

2. The occurrence of ecdysteroid-26-oic acids in Insects

In a number of radioactive tracer experiments, highly polar ecdysteroid metabolites, resistant to enzymic hydrolysis, have been observed: compound Ic in *Locusta* (Koolman *et al.*, 1973); compound OB in *Sarcophaga* (Moribayashi and Ohtaki, 1978); *Calliphora* (Sannasi and Karlson, 1974); *Bombyx* (Moriyama *et al.*, 1970). That this compound was a carboxylic acid derivative of the ecdysteroid molecule was first suggested in 1973 (Koolman *et al.*). Only relatively recently has this suggestion been

confirmed. The unequivocal characterisation by physico-chemical means of ecdyson-26-oic acid and 20-hydroxyecdyson-26-oic acid from insect material has been achieved concurrently in two laboratories (Schistocerca gregaria and Spodoptera littoralis, Isaac et al., 1983; Pieris brassicae, Lafont et al., 1983). In addition, these compounds have been identified as metabolites of administered radioactive ecdysone in a number of species; Pieris brassicae pupae (Lafont et al., 1980), Locusta migratoria larvae (Lafont et al., 1983), and Spodoptera littoralis pupae (this study). [^{3}H] 20-Hydroxyecdyson-26-oic acid has also been produced from incubations in vitro of tissue extracts from Calliphora vicina (Lafont et al., 1983). It would, therefore, seem that the formation of these compounds probably occurs in most insects.

These compounds have been shown to be produced from ecdysone via 26-hydroxylated intermediates in P.brassicae (Beydon et al., 1981), and in this study the conversion of 20,26-dihydroxyecdysone into 20-hydroxyecdyson-26-oic acid has been demonstrated. The accumulation of ecdyson-26-oic acid and 20-hydroxyecdyson-26-oic acid observed in Spodoptera and Pieris (Lafont et al., 1983) indicates that these compounds probably represent the end-product of hydroxylation and oxidation reactions of ecdysone and, at least in the absence of conjugation processes, represent the end-products of the inactivation pathway of the moulting hormones. Although the moulting hormone activity of these compounds has not been tested, it has been shown that a closely related structure, 20-hydroxy-25-deoxyecdyson-26-oic acid, is inactive in the Calliphora bioassay (Lafont et al., 1983) and in the Drosophila salivary gland puffing assay (Spindler et al., 1976). Although the 26-oic acids could possess an alternative physiological function in Arthropods, it is most probable that the conversion of ecdysone and 20-hydroxyecdysone into

the more polar acidic compounds facilitates the eventual excretion of the moulting hormones.

A C-25 hydroxyl group is not necessary for the formation of the carboxylic acid group at C-26 of the ecdysteroid molecule in insects, as both inokosterone (Spindler et al., 1976) and ponasterone A (Lafont et al., 1983) can be converted into their respective 26-oic acids. As with the 26-hydroxy ecdysteroids it is most probable that the 26-oic acids exist in two isomeric forms (25R and 25S). Indeed, the 25-deoxy-compound ponasterone A, was converted into two isomers of 25-deoxy-20-hydroxyecdyson-26-oic acid by Pieris wing discs (Lafont et al., 1983).

In addition to being inactivation products, conjugates of ecdysteroids are possibly storage forms of moulting hormone (Willig et al., 1971) and may be hydrolysed during insect development to release active moulting hormone. However, in contrast, ecdysteroid-26-oic acids seem to be terminal end-products and not recycled. Their formation is irreversible, injected 20-hydroxyecdyson-26-oic acid being insignificantly metabolised in Calliphora erythrocephala larvae (Young, 1976) and Pieris pupae (Beydon et al., 1981).

Comparison of the occurrence of the ecdysteroid-26-oic acids at different stages of insect development has not been thoroughly investigated. However, the principal polar metabolites found in both Pieris (Lafont et al., 1980) and Spodoptera pupae are in the form of ecdysteroid acids, indicating that during this stage of development they have an important role in the inactivation of moulting hormones.

The accumulation of these compounds at this stage is most probably due to the absence of conjugation of moulting hormones, which leaves ecdysone and 20-hydroxyecdysone to be metabolised in the closed pupal system to polar metabolites via side-chain hydroxylation and oxidation rather than via conjugation.

3. Titres of individual ecdysteroids during the pupal stage of S. littoralis

Ecdysteroid titres have been compiled for the pupal stages of a number of insects. Radioimmunoassay has been most frequently used for these studies of the ecdysteroid content of either haemolymph or whole pupae. Such studies in Calpodes ethlius (Dean et al., 1980), Manduca sexta (Bollenbacher et al., 1981), Galleria mellonella male and female (Bollenbacher et al., 1978), Tenebrio molitor (Delbecque et al., 1978), Bombyx mori male (Calvez et al., 1976), have produced moulting hormone titres characterised by a single large peak occurring at between one quarter and half way through the pupal instar. This moulting hormone profile is confirmed in the RIA titre of male S.littoralis pupae (Clarke, 1981; figure 3C-1), although in the female titre a second peak occurring at a later stage of pupal development is present (Clarke, 1981). However, in the female titre compiled by h.p.l.c. (figures 3C-7 and 8) this peak is not apparent; the reason for this is unclear, although artificial enhancement of the RIA response is thought to be responsible for the later peak in the female pupae (see Part 3, section C; p. 80). The presence of a genuine ecdysteroid peak at the later stage of female pupal development should not, however, be totally discounted, as it is possible that ecdysteroid molecules other than those detected by either h.p.l.c. or GC/MS (Part 3, section 4), may be responsible for such a peak. Indeed, the occurrence of an extra peak in the female pupal moulting hormone titre has been found in other insects. Notably, studies of the moulting hormone titres in Bombyx mori using bioassay techniques (Hanaoka and Ohnishi, 1974) revealed a second moulting hormone peak exclusive to the female pupae occurring late in the instar, and indeed showed that the extra peak was associated with the ovaries. Similar studies, analysing male and female pupae

separately, have been performed on Galleria mellonella (Bollenbacher et al., 1978) and Manduca sexta (Bollenbacher et al., 1981). In Galleria these studies have revealed an extra moulting hormone peak in the female pupae, although in this case the peak occurs around adult eclosion; in the case of Manduca the peak is very small (no more than one tenth of the early pupal peak) and occurs early in the adult. Such studies, analysing the two sexes separately, are quite limited. However, a number of studies have involved the analysis of the moulting hormone titre of whole pupae from mixed sex populations. It is therefore reasonable to expect that these studies will also reveal any supplementary moulting hormone peak associated with the female pupae, although of course, the quantitative nature of such a peak cannot be appreciated. In such studies in Tenebrio pupae (Delbecque et al., 1978) and Manduca pupae (Bollenbacher et al., 1981), titre analysis does not reveal a second peak of moulting hormone late in the pupal instar, even one of minor significance. Therefore, it would appear, that the authenticity of the female RIA peak obtained in S. littoralis pupae can neither be confirmed nor discounted by analogy to the aforementioned studies in other insect pupae. Indeed, the timing and relative size of such female peaks in the pupal moulting hormone titres, seems to vary considerably from species to species.

Consistent in all RIA pupal moulting hormone titres is the appearance of a single large peak occurring in the first halfof the titre or midway through the titre, which presumably triggers adult development. From these results it might appear that a simple process of ecdysteroid synthesis followed by further metabolism of the active moulting hormone is taking place. However, the analysis of the individual ecdysteroids by h.p.l.c. in S. littoralis reveals a more complex pattern (figures

3C-5,6,7 and 8). Similar studies, involving the compilation of pupal titres of ecdysone and 20-hydroxyecdysone by chromatographic methods, have been undertaken in *Pieris brassicae* (Lafont *et al.*, 1975) and in *Heliothis zea* (Holman and Meola, 1978). In *Heliothis* the pattern is simple, a single large peak of ecdysone is rapidly followed by a 20-hydroxyecdysone peak of similar size (figure 3E-1(A)). However, in *Pieris* a more complex picture is encountered, consisting of two peaks of ecdysone in addition to a large peak of 20-hydroxyecdysone [figure 3E-1(B)]. The ecdysone and 20-hydroxyecdysone titres in *S.littoralis* male and female pupae are different again from the titres previously described [for the male titre see figure 3E-1(C)]. The titres from *S. littoralis* do, however, resemble more closely the *Pieris* titres, with a major and a smaller peak of 20-hydroxyecdysone accompanied by two smaller ecdysone peaks, although the timing of the peaks is different. From the pupal ecdysteroid titre profiles determined in the aforementioned studies, it is apparent that the simple moulting hormone titres obtained by RIA in many insects may, in fact, hide a more complex pattern of individual ecdysteroid titres. Also apparent from the individual ecdysteroid titres compiled in *Pieris* and *Spodoptera* is the sharpness of some of the peaks, therefore emphasising the necessity of analysing insects at frequent intervals during the instar if a complete ecdysteroid titre is to be compiled. Indeed, this is confirmed by the analysis of the pupal RIA moulting hormone titre in *Galleria mellonella* (Sehnal *et al.*, 1981), which by taking data at 12hr intervals reveals the presence of an additional peak that was not identified by taking data at 24hr intervals, (Bollenbacher *et al.*, 1978).

The titres of the 26-hydroxylated ecdysteroids reveal that these compounds only occur in relatively small amounts (figure 3E-2),

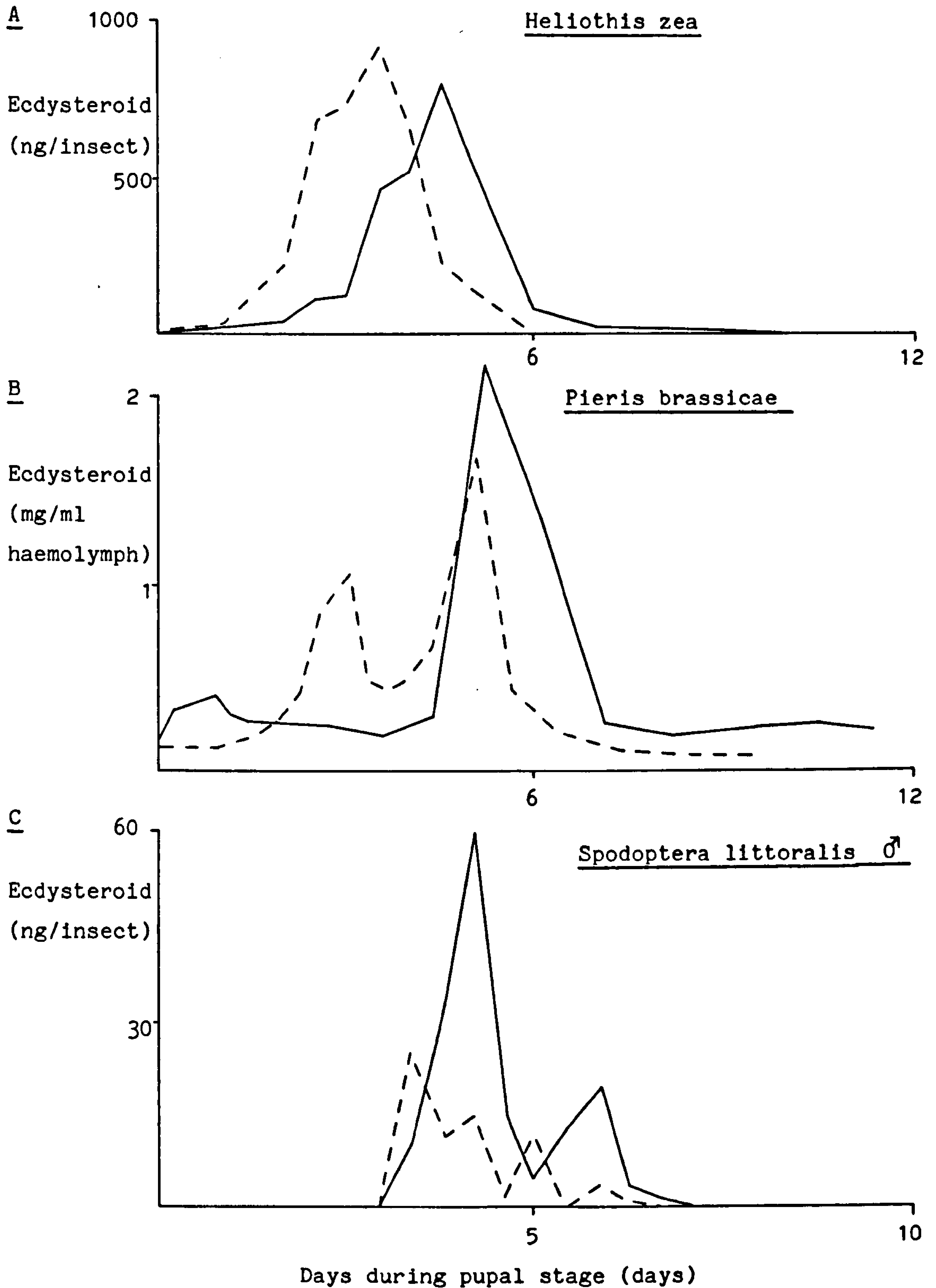


Figure 3E-1 : Ecdysone (dotted line) and 20-hydroxyecdysone (continuous line) titres determined during the pupal stages of Heliothis zea (Holman and Meola, 1978), Pieris brassicae (Lafont et al., 1975), and Spodoptera littoralis (male).

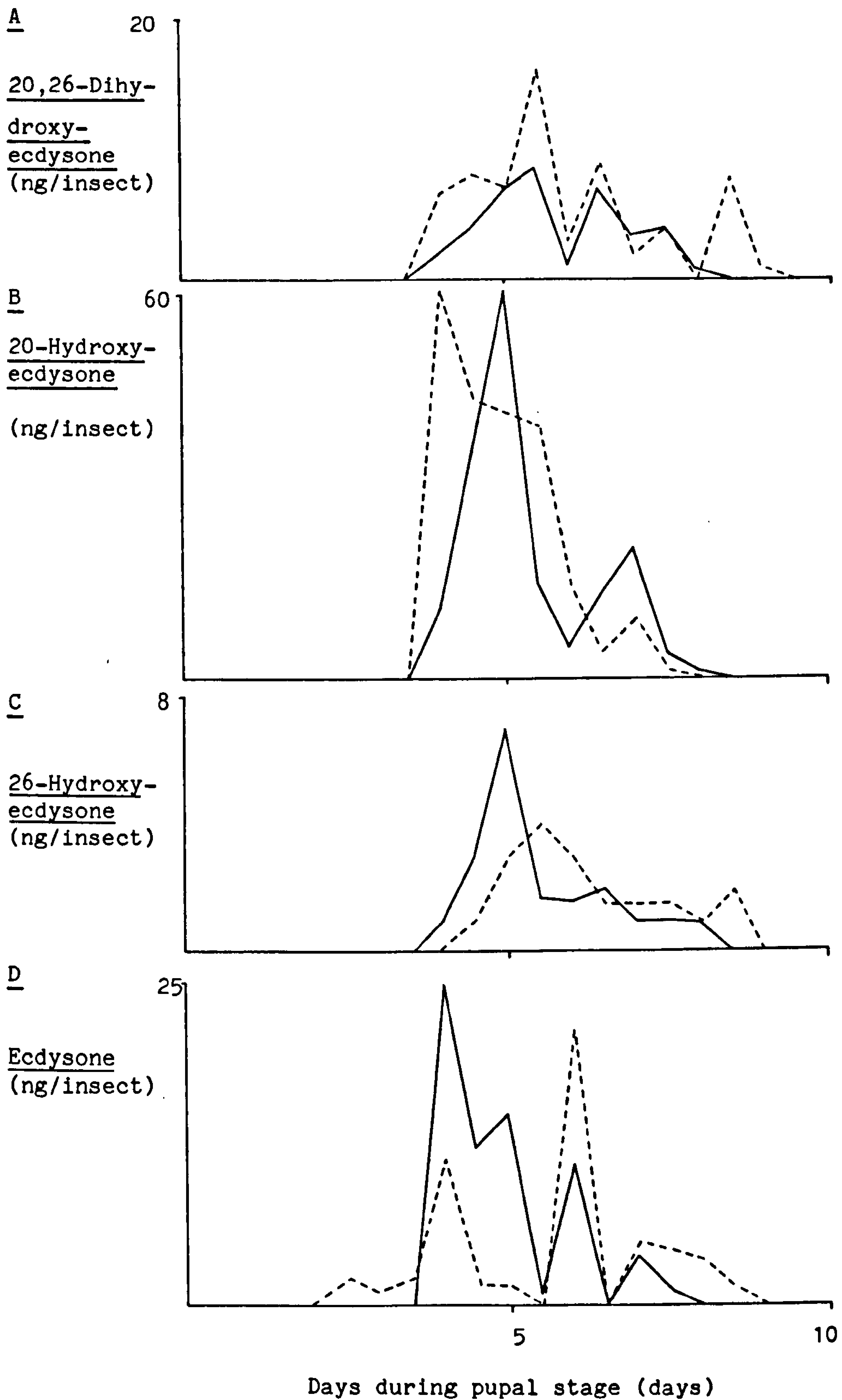


Figure 3E-2 : Ecdysteroid titres determined by h.p.l.c. in male (continuous line) and female (dotted line) pupae: A, 20,26-Dihydroxyecdysone; B, 20-Hydroxyecdysone; C, 26-Hydroxyecdysone; D,Ecdysone. The various titres are not all drawn to the same scale; 26-hydroxyecdysteroids occur in much lower amounts than ecdysone or 20-hydroxyecdysone.

indicating that these compounds do not accumulate appreciably and that they probably constitute intermediates in the formation of the polar 26-oic acids of ecdysone and 20-hydroxyecdysone. This seems to be the case in most insect species, after metabolism in vivo of administered [^{3}H] ecdysone in Pieris pupae (Lafont et al., 1980) only small amounts of [^{3}H] 26-hydroxylated ecdysteroids were produced, the main metabolites being ecdyson-26-oic acid and 20-hydroxyecdyson-26-oic acid. Also in kinetic experiments in Calliphora vicina, 26-hydroxy-ecdysteroids only occur as intermediates in the formation of more polar compounds (Karlson and Koolman, 1973).

In the h.p.l.c. titre in S.littoralis the major peaks of 20,26-dihydroxyecdysone accompany corresponding peaks of 20-hydroxyecdysone (figure 3E-2) indicating the active metabolism of 20-hydroxyecdysone via 26-hydroxylation to, most probably, the 20-hydroxyecdyson-26-oic acid. A corresponding association between the ecdysone and 26-hydroxyecdysone peaks is less clear, although the largest ecdysone peak in male pupae does have a significant underlying 26-hydroxyecdysone peak, thus suggesting a similar active metabolism of ecdysone via 26-hydroxyecdysone to presumably ecdyson-26-oic acid. Ecdysone is principally metabolised via two competing reactions, 20-hydroxylation and 26-hydroxylation. From the titres it would seem that when ecdysone appears in the insect (at day 4), it is predominantly metabolised to 20-hydroxyecdysone. However, as 20-hydroxylation decreases, 26-hydroxylation becomes more prominent (at approximately day 5).

The other group of ecdysteroids determined in the h.p.l.c. titres of S.littoralis pupae were the 3-epi derivatives of ecdysteroids; these are presented in figure 3E-3. 3-Epiecdysteroids show considerably less moulting hormone activity than the corresponding 3β-hydroxy-

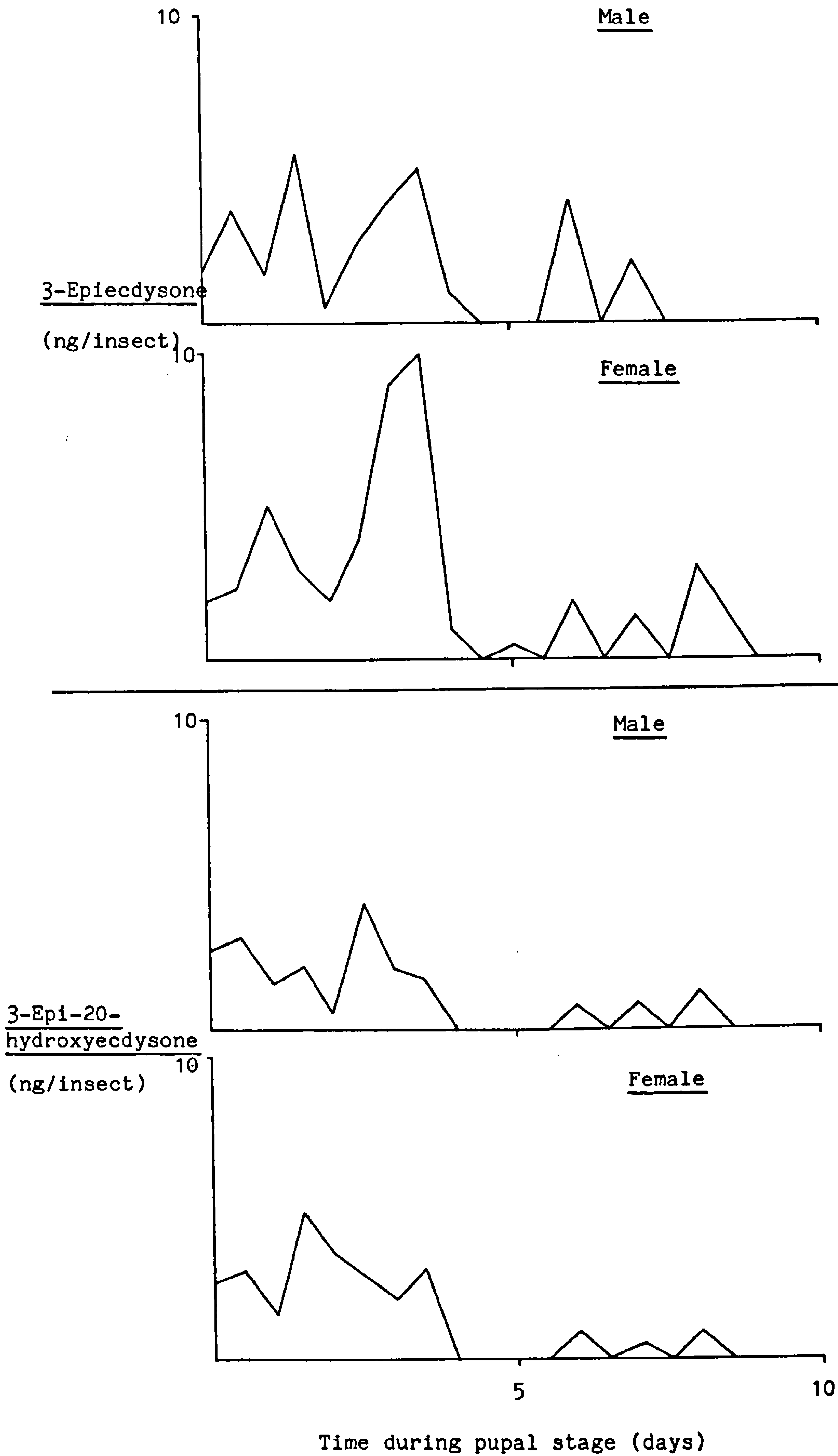


Figure 3E-3 : 3-Epiecdysteroid titres determined by h.p.l.c. in male and female pupae.

ecdysteroid equivalents (Koolman, 1982), and have been proposed as possible inactivation products of the moulting hormones (Kaplanis et al., 1979). It would be reasonable to expect 3-epiecdysone and 3-epi 20-hydroxyecdysone, as inactivation products of ecdysone and 20-hydroxyecdysone, to accumulate after the peaks of these two compounds. Surprisingly, this is not the case and, in fact, the 3-epiecdysteroids occur early in the instar well before the large increase in ecdysone and 20-hydroxyecdysone (figures 3C-5,6,7,8). A similar situation is encountered in Pieris, injected [^{3}H] ecdysone is converted into [^{3}H] 3-epiecdysteroids in pharate pupae and early pupae (Lafont et al., 1980), whereas during the rest of the pupal stage [^{3}H] ecdysone can only be converted into other 3β-hydroxyecdysteroids (Beydon et al., 1981). Indeed, it would seem that 3-epimer formation is characteristic of ecdysone metabolism at this stage of insect development (i.e. pharate and early pupae). 3-Epiecdysone is formed from [^{3}H] ecdysone if administered late in the sixth instar S. littoralis larvae (figure 4A-8), but if [^{3}H] ecdysone is administered either earlier in the 6th instar larvae or midway through the pupal stage, no [^{3}H] 3-epiecdysteroids are produced (figure 4A-3 and figure 3A-3). This would also seem to be the case in the Dipteran, Sarcophaga peregrina, where [^{3}H] 3-epi-20-hydroxyecdysone is the major metabolite of injected [^{3}H] 20-hydroxyecdysone in pharate pupae whereas mature larvae exhibited very little metabolism of injected [^{3}H] 20-hydroxyecdysone (Moribayashi and Ohtaki, 1978).

In both male and female titres, 3-epiecdysone occurs more or less as two peaks at approximately 24 hrs and 84 hrs with lesser amounts of 3-epi-20-hydroxyecdysone also occurring during this period of pupal development (see figure 3E-3). The disappearance of these compounds at 96 hrs of development is virtually concurrent with the appearance of

the 3β hydroxyecdysteroids, ecdysone and 20-hydroxyecdysone. This could possibly indicate that the 3-epiecdysteroids are converted into their corresponding 3β-hydroxyecdysteroids at this stage of pupal development. This, however, is highly unlikely, as 3-epimers of ecdysone and 20-hydroxyecdysone have been shown not to be converted into 3β-hydroxyecdysteroids in Pieris pharate pupae (Lafont et al., 1980). Indeed, the 3-epimerisation reaction has been shown to be irreversible in vitro in this study (Part 4, section D). The 3-epi-ecdysteroids are more likely to be further metabolised by a pathway parallel to that of the 3β-hydroxyecdysteroids, viz. via 26-hydroxylation and further oxidation to 3-epiecdysteroid-26-oic acids.

Small amounts of 3-epiecdysteroids occur later on in the pupal instar (figure 3E-3). However, there is no set pattern to be observed at this later stage of development. As the amounts of 3-epiecdysteroids present after the main peaks of 20-hydroxyecdysone and ecdysone are relatively small, and as [^{3}H] ecdysone administered at later stages of pupal development produces no detectable 3-epimers, 3-epimerisation appears not to play a significant part in the inactivation of ecdysone, at least at this stage of development. In contrast, in Manduca sexta, epimerisation was implicated as a means of inactivation of ecdysteroids at five days after pupal peak titre (Kaplanis et al., 1979). However, one must be careful when interpreting the results obtained in that study. Although 3-epiecdysteroids were, indeed, found to occur in larger amounts than 3β-hydroxyecdysteroids, the existence of polar ecdysteroids, such as ecdysteroid-26-oic acids, was not investigated. Therefore, a more complete study is required in Manduca before the principal inactivation compounds are confirmed. Indeed, it is interesting to note that 20,26-dihydroxyecdysone, an intermediate in 20-hydroxyecdyson-26-oic acid

formation, was isolated in higher quantities than any of the 3-epi-ecdysteroids in that study.

Certainly, in Pieris and Spodoptera, and perhaps Sarcophaga, the epimerisation process appears to be most active during pharate and early pupal development. This period of development in the insects life cycle corresponds to the time when much metamorphic activity is taking place. During this period, larval tissues are quite extensively broken down by autophagy and morphological changes occur in certain tissues (Dean et al., 1980). The more cytosolic environment, thus created, would favour the activity of soluble enzymes such as 3-epimerase, and reduce the effectiveness of membrane bound enzymes such as hydroxylases. This could, therefore, explain the predominance of 3-epiecdysteroids at this stage of development and the reduced level of 20-hydroxylase activity and apparent absence of 26-hydroxylase activity. In addition, the breakdown of membranes could also have the effect of releasing any compartmentalized 3-epimerase enzymes, thus bringing them into contact with ecdysteroids present in the haemolymph. As remodelling for adult morphogenesis occurs as pupal development progresses, presumably a more normal physiological environment is regained, leading to a return to metabolism via side chain hydroxylation and further oxidation rather than 3-epimerisation.

The anomalously high levels of ecdysteroids detected in the RIA moulting hormone titres in S. littoralis pupae (Clarke, 1981; figure 3C-1) have been discussed earlier (Part 3, section C). The difference between measurements by RIA and h.p.l.c. in the pupal moulting hormone titres are of considerable magnitude. For example, at male peak titre 500 ng 20-hydroxyecdysone equivalents/insect were detected by RIA, whereas by h.p.l.c. of the same sample, 60ng of 20-hydroxyecdysone,

15 ng of ecdysone, 7 ng of 20,26-dihydroxyecdysone and 7 ng of 26-hydroxyecdysone were detected per insect (89 ng total ecdysteroid/insect). As demonstrated in Part 3, section C the difference can, to some extent, be explained by the cross-reactivity of the ICT-1 antiserum to the individual ecdysteroids. However, it was concluded that a degree of anomalous artificial enhancement of the binding of the antiserum in the biological samples is responsible for this effect. Indeed, the effect of biological material on the results obtained by radioimmunoassay can be quite considerable. In a study investigating these effects, an unpurified haemolymph serum sample was assayed for ecdysone and compared to the same sample which had been purified by partition, T.l.c., and h.p.l.c. (Soumoff et al., 1981). This demonstrated that the unpurified sample enhanced the radioimmunoassay results by up to 3-fold.

It would appear that the enhancement effect is limited to the pupal stage of development. In the larvae, results obtained by RIA correlate reasonably well with results obtained by chromatographic methods (GC/MS) (see Part 4, section B). Indeed, the peak titre by RIA in larvae is approximately 100 times lower than that in pupae (20 ng 20-hydroxyecdysone equivalents/g insect in larvae compared to 1900 ng 20-hydroxyecdysone equivalents/g insect in pupae). A similar comparison using results from h.p.l.c. and GC/MS (SIM) analysis of 20-hydroxyecdysone reveals a six-fold larval to pupal difference. The latter results are, as would be expected, more in agreement with results obtained by other workers. The difference between moulting hormone peak titre in larval and pupal forms is usually less than ten-fold, varying from 2.5-fold in Calpodes ethlius (Dean et al., 1980) to 8-fold in Galleria mellonella (Bollenbacher et al., 1978).

From the analyses described in this study, and from the comparison to results obtained by other workers, it can therefore be concluded with some certainty that the RIA titre compiled for the pupal stage of S. littoralis development is subject to a great deal of error, if only in amplitude. This emphasises the reservation with which one should treat results obtained via radioimmunoassay.

4. Ecdysteroids in the ovaries of S. littoralis

In section D the identity of the ecdysteroids in the ovaries at day $8\frac{1}{2}$ of pupal development was investigated, and revealed the presence of 20-hydroxyecdysone (7 ng/insect) and 20,26-dihydroxyecdysone (5.4 ng/insect), with the notable absence of significant levels of ecdysone and ecdysteroid conjugates.

This ecdysteroid composition is somewhat surprising, particularly the presence of 20,26-dihydroxyecdysone. Although 26-hydroxyecdysone has been found to be the principal free ecdysteroid in the eggs of Manduca sexta (Kaplanis et al., 1980), no 26-hydroxylated ecdysteroids have been isolated as major components of ovarian ecdysteroids. Indeed, in the ovarian systems of Galleria mellonella (Hsiao and Hsiao, 1979) and Schistocerca gregaria (Gande et al., 1979), ecdysone and smaller amounts of 20-hydroxyecdysone were detected, whereas in Spodoptera ovaries, ecdysone is notably absent. In Bombyx mori and Schistocerca gregaria ovaries, 2-deoxyecdysteroids were identified as major ecdysteroids (Ohnishi et al., 1981; Dinan and Rees, 1981). In particular, in Bombyx ovaries a whole series of 2-deoxyecdysteroids were detected. These compounds, of course, would not be detected from S. littoralis ovaries with the analysis systems used in this study. GC/MS was used with selected ion monitoring, chosen only for the detection

of ecdysone, 20-hydroxyecdysone and their 26-hydroxylated derivatives. The presence of 2-deoxyecdysteroids, therefore, cannot be discounted. Indeed, a small amount of radiolabelled 2-deoxyecdysone was possibly present in the ovaries from the metabolism in vivo of [^{14}C] cholesterol (table 3D-1). 2-Deoxyecdysone, however, would not be detected by ICT-1 antiserum in the radioimmunoassay, as the cross-reaction of this ecdysteroid to the antiserum is one fiftieth of that of ecdysone (Reum et al., 1979). As previously explained, the RIA of pupal samples are perhaps not altogether reliable due, most probably, to enhancement effects by the biological sample. However, it is interesting to note, that whereas SEP-PAK purification of the samples did not reduce the enhancement effect in most samples, considerable reduction of the RIA response was achieved in samples associated with the second (ovarian) peak of ecdysteroids in the female pupae (figure 3C-10). Very low RIA activity was detected in the 'non-ecdysteroid' SEP-PAK fractions, so it was assumed that the SEP-PAK purification had removed some enhancing material of ovarian origin. However, this apparent purification effect could indicate that the ecdysteroids present at this stage were significantly different from those encountered at other stages of pupal development. By analogy to the ovarian ecdysteroids found in other Lepidopterans, especially Bombyx (Ohnishi et al., 1981), the most probable candidates would be 2-deoxyecdysteroids or possibly 22-deoxyecdysteroids.

It was also surprising to detect very little conjugated ecdysteroids in the ovaries at this stage of development (less than 0.05 ng 20-hydroxyecdysone conjugate/insect compared to 7 ng free 20-hydroxyecdysone/insect). In a number of studies in various insects; Schistocerca gregaria (Gande et al., 1979), Galleria mellonella (Hsiao and Hsiao, 1979),

<u>Bombyx mori</u> (Ohnishi <u>et al</u>., 1981), the vast majority of the ovarian ecdysteroids were in the conjugated form. However, as 2-deoxyecdysteroids were not analysed by GC/MS (SIM) in this study, the quantity of 2-deoxyecdysone conjugate remains unknown, although conjugation is not usually confined to one ecdysteroid species. It is quite likely that conjugation occurs later in ovarian development so that at another stage of development considerable amounts of conjugates might be present.

The work completed on the ovarian ecdysteroids in this study does not demonstrate the actual origin of the ovarian ecdysteroids. Although the ecdysteroids could be synthesized in the ovaries, it is also possible that they are synthesized outside the ovaries and then sequestered by the ovaries. Indeed, the radiolabelled ecdysteroids isolated at day 8½ of female pupae after administration of [^{14}C] cholesterol at day 2 will presumably contain ecdysteroids synthesized both at the time of the first RIA peak and at the time of the second RIA peak (present only in the female) (see figure 3C-1 for RIA tire curve). However, by comparing the ecdysteroids obtained in a similar experiment performed in male pupae (i.e. injection of [^{14}C] cholesterol at day 2 and analysis of the [^{14}C] ecdysteroids at day 9 of pupal development), the ecdysteroids produced at the second peak of the RIA titre can be assessed. In fact, the only difference seems to be the presence of small amounts of [^{14}C] 20-hydroxyecdysone and [^{14}C] 2-deoxyecdysone in the ovaries of the female pupae (cf. tables 3D-1 and 3A-4). It would appear also that the [^{14}C] ecdysteroid pattern outside the ovaries is similar to that obtained in the intact male pupae, indicating that the large amount of [^{14}C] 20-hydroxyecdyson-26-oic acid found at this time in females probably originates from metabolism of the ecdysteroids synthesized in the first RIA titre peak. Indeed, because of this it would appear that ecdysteroids

probably do not pass out of the ovaries. This view is also supported by the higher concentration of ecdysteroids found inside the ovaries compared to the rest of the insect (see Part 3, section D, p. 86). It is, however, unclear why 20,26-dihydroxyecdysone is apparently found in the ovaries by GC/MS analysis whereas in the [^{14}C] cholesterol incorporation experiment this ecdysteroid was not present.

PART 4 INVESTIGATION OF THE ECDYSTEROIDS IN THE SIXTH INSTAR LARVAE OF *SPODOPTERA LITTORALIS*

PART 4

Investigation of the ecdysteroids in the sixth instar larvae of *Spodoptera littoralis*

Page No.

Section A	The metabolism *in vivo* of [23,24 $^3H_2$] ecdysone in the sixth instar larvae of *S. littoralis*	113
Section B	Titre and identification of ecdysteroids present in sixth instar *S. littoralis* larvae	123
Section C	Developmental variation of the ecdysone metabolising enzymes *in vitro* during the sixth instar larval stage of *S. littoralis*	140
Section D	Investigation of the ecdysone 3-epimerisation reaction in *S. littoralis*	152
Section E	Discussion of the studies on *S.littoralis* larvae	166

PART 4 SECTION A

PART 4 SECTION A

The metabolism *in vivo* of [23,24 $^3H_2$] ecdysone in the sixth instar larvae of *S. littoralis*

Page No.

Introduction 114

Experimental and results

High-performance liquid chromatography systems used in this section 116

1. The metabolism *in vivo* of [23,24 $^3H_2$] ecdysone in early final instar larvae 116

   Injection of [23,24 $^3H_2$] ecdysone and extraction of insects and faeces 116

   Analysis of the free ecdysteroids 117

   Analysis of the highly polar ecdysteroids 117

2. The metabolism *in vivo* of [23,24 $^3H_2$] ecdysone in late final instar larvae (prepupae) 120

   Injection of [23,24 $^3H_2$] ecdysone and extraction of insects 120

3. Comparison of [^3H] ecdysone metabolism in early and late 6th instar larvae 121

PART 4 SECTION A

The metabolism *in vivo* of [23,24 $^3H_2$] ecdysone in the sixth instar larvae of *S. littoralis*

INTRODUCTION

Investigation of the nature of the ecdysteroid inactivation products formed during the decrease in hormone titre in 6th instar larvae is described in this section. For this, [^{3}H] ecdysone metabolism was investigated *in vivo*. A wide variety of ecdysone metabolites have been identified using this technique in the pupae of *S. littoralis* (see Part 3) and in many other insects (see Part 3, Section A).

However, compared to the studies with pupae (Part 3) additional factors require consideration. Firstly, the quantities of ecdysone (determined by R.I.A.) in the larvae (22 ng/g insect at peak titre, see figure 4A-1) are considerably smaller than those in the pupae (1900 ng/g insect at peak titre, see figure 3A-1, Part 3 Section A). Therefore, the amounts of ecdysone administered must be carefully calculated so that disruption of the physiological levels of ecdysteroids is kept to a minimum, and so that the metabolism of ecdysone reflects, as closely as possible,the endogenous ecdysteroid pathways. The larvae also differ from pupae in that during the first four days of larval development the insect is actively eating and excreting. So the study of ecdysone metabolites in the larvae must extend to the analysis of the faeces.

As shall be evident in this chapter, analysis of the faeces reveals the production of comparatively large amounts of conjugated ecdysteroids, which are only present in small amounts in the pupal stage of *S.littoralis* (see Part 3). Indeed, this low level of ecdysteroid conjugation in the pupal stage has also been found in another Lepidopteran, *P. brassicae* (Lafont, 1980). Ecdysteroid conjugates detected in experiments

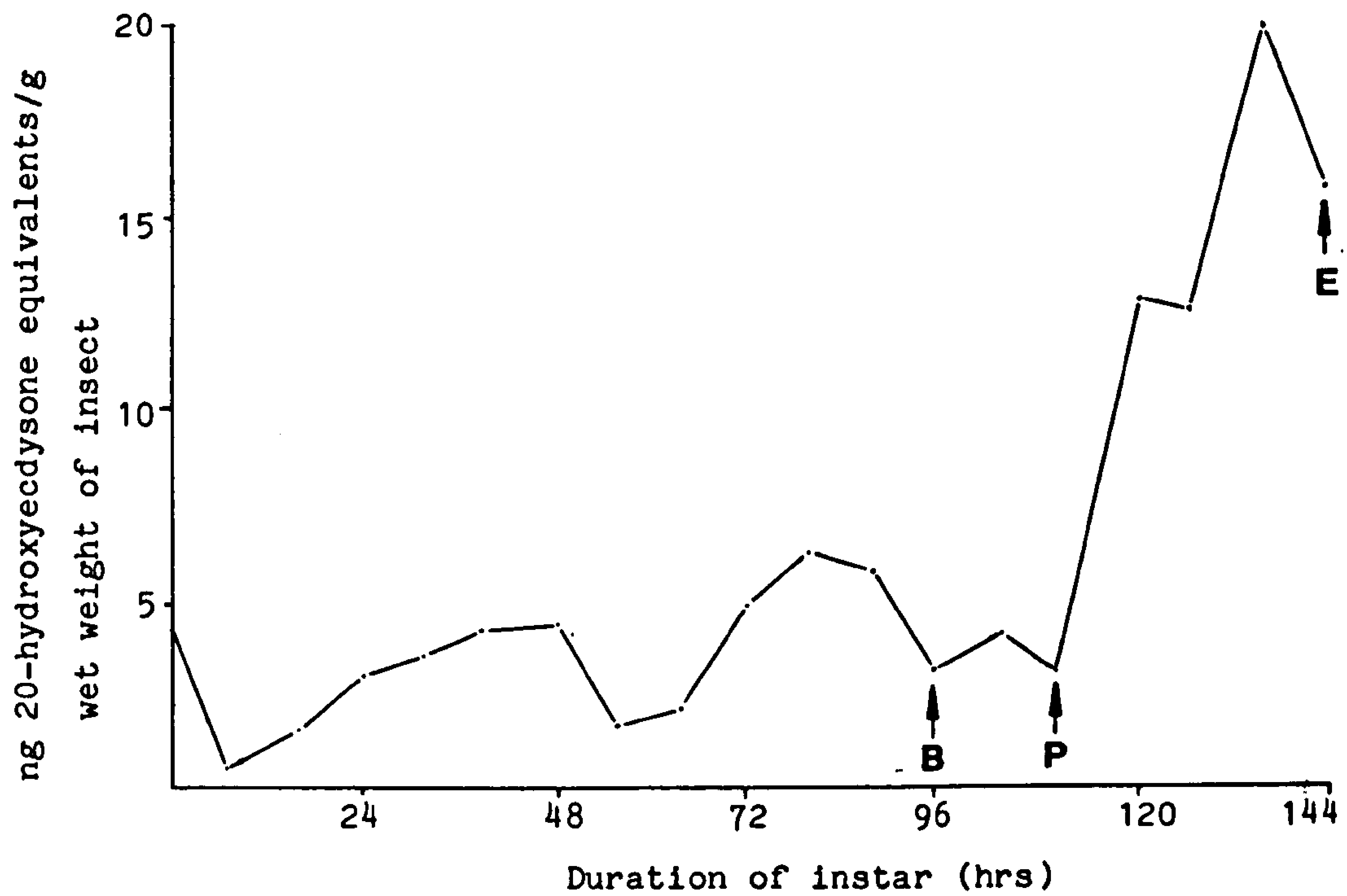


Figure 4A-1 : R.I.A. response to ICT-1 antiserum during the sixth instar of S. littoralis (Clarke, 1981) B : Burrowing, P : Onset of pre-pupal stage, E = Ecdysis

utilising [^{3}H] ecdysone have largely been identified on the strength of enzymic hydrolysis, which liberates free ecdysteroids from the highly polar fraction. However, the enzyme preparations used in these experiments have been found to be highly contaminated with a number of enzymes. For example, the frequently used, so called aryl sulphatase preparation from Helix pomatia is capable of hydrolysing ecdysteroid phosphates (Isaac et al., 1982). Indeed, only phosphate conjugates of ecdysteroids have been identified, as yet, by physico-chemical means. Ecdysone 22-phosphate, 2-deoxyecdysone - 22-phosphate, 20-hydroxyecdysone 22-phosphate and 2-deoxy-20-hydroxyecdysone 22-phosphate have been identified in the eggs of S.gregaria (Isaac et al., 1983), and the 3-phosphate of 3-epi-2-deoxyecdysone and 22-adenosinemonophosphoric ester of 2-deoxyecdysone (Tsoupras et al., 1982),have been characterised in the eggs of Locusta migratoria. However, the occurrence of ecdysteroid sulphates (Koolman et al., 1973) and ecdysteroid glucosides (Kaplanis et al., 1974) has also been suggested on the basis of preliminary evidence.

In this study [^{3}H] ecdysone metabolism was studied at two stages of development, one at the stage when the larvae are actively eating and excreting, the other at the prepupal stage when feeding and excretion have ceased. The ecdysteroid titre determined by R.I.A. during the final larval instar (figure 4A-1) was used as a basis for choosing the times at which the insects were injected and incubated with [^{3}H] ecdysone. For the first experiment, larvae were injected at the first titre peak (48 hours, see figure 4A-1) and incubated for a further 10 hours, whereas for the second, the larvae were injected after 136 hours into the 6th instar and incubated for a further 6 hours.

EXPERIMENTAL AND RESULTS

High-performance liquid chromatography systems used in this section

System 1 : Two Partisil ODS-3 reversed-phase columns in series eluted with methanol/water (9:11, v/v) at 1.5 ml/min.

System 2 : An Altex Ultrasphere ODS-reversed-phase column eluted at 1 ml/min with a linear gradient (30 minutes) of methanol in 0.02M sodium citrate buffer pH 6.5, (3:7, v/v) changing to methanol in sodium citrate buffer (7:3, v/v).

System 3 : An aminopropyl silica (APS-Hypersil) column eluted with either 6 or 8% methanol in dichloroethane at 2 ml/min.

The chromatographic positions of authentic compounds are shown on the chromatograms.

1. The metabolism in vivo of [23, 24 $^3H_2$] ecdysone in early final instar larvae

Injection of [23,24 $^3H_2$] ecdysone and extraction of insects and faeces

Eighteen final instar larvae (day 2 ± 2 hr, 13.38g) were injected (see Methods) with a total of 0.72 µCi [^{3}H] ecdysone (specific radioactivity 4 Ci/mmol). Thus each insect was injected with 0.04 µCi (4.6 ng) ecdysone. This level of ecdysteroid is similar to the amount of endogenous hormone present at this stage of the insect's development, vis. 1.9 ng 20-hydroxyecdysone equivalent/insect (see figure 4A-1) or approximately 7.1 ng ecdysone equivalent/insect [calculated from the cross reaction factors found by Reum et al. (1979)]. So, these levels of exogenous hormone should be metabolised at a similar rate to the endogenous hormone, and therefore reflect, as closely as possible, the physiological situation.

The insects were then reared for 10 hours under normal conditions together with food, and then sacrificed. The gut contents were removed and combined with the faeces produced during the 10 hours. The larvae and faeces plus gut contents were then extracted separately by the method described in Part 2. The distribution of radioactivity amongst various fractions after silicic acid column chromatography is represented in figure 4A-2. The metabolites contained in the 30% (free ecdysteroids) and the 80% (highly polar ecdysteroid metabolites) methanol/chloroform fractions were then analysed by h.p.l.c.

Analysis of the free ecdysteroids (30% Methanol/Chloroform fraction)

The [^{3}H] metabolites found in this fraction in the faeces and in larvae were analysed by h.p.l.c., using System 1. Authentic compounds were used to identify the metabolites and radioactivity was monitored by collecting fractions every minute for radioassay (see Methods). The chromatograms for both fractions (faeces and larvae) are shown in figure 4A-3. By co-chromatography of these compounds with authentic material on an A.P.S.-Hypersil column (System 3) their identities were confirmed as; 20,26-dihydroxyecdysone, 20-hydroxyecdysone, 26-hydroxyecdysone, and the unmetabolised ecdysone, although only 20,26-dihydroxyecdysone and 20-hydroxyecdysone were present in the larval extract. There was no evidence for the presence of 3-epiecdysteroids in this fraction.

Analysis of the highly polar ecdysteroids (80% Methanol/Chloroform)

These fractions were also analysed by reversed-phase h.p.l.c. using System 2. The chromatograms, represented in figure 4A-4, show the presence of a major peak chromatographing just after 20-hydroxyecdysone 22-phosphate in the faeces and larval extracts. In addition, the

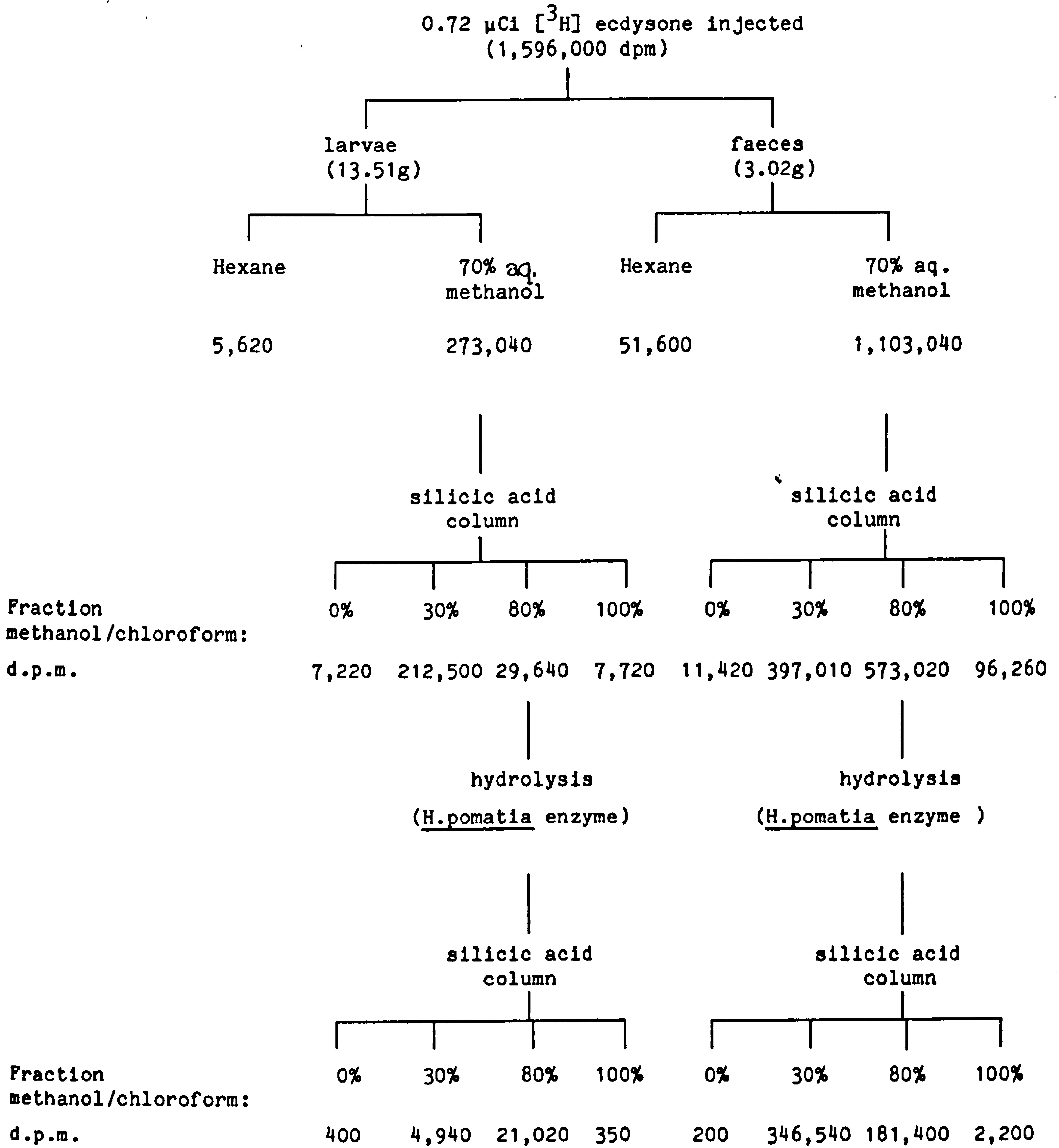


Figure 4A-2 : Summary of the distribution amongst various fractions of metabolites formed from $[^3H]$ ecdysone in early sixth instar larvae (numbers are in d.p.m.)

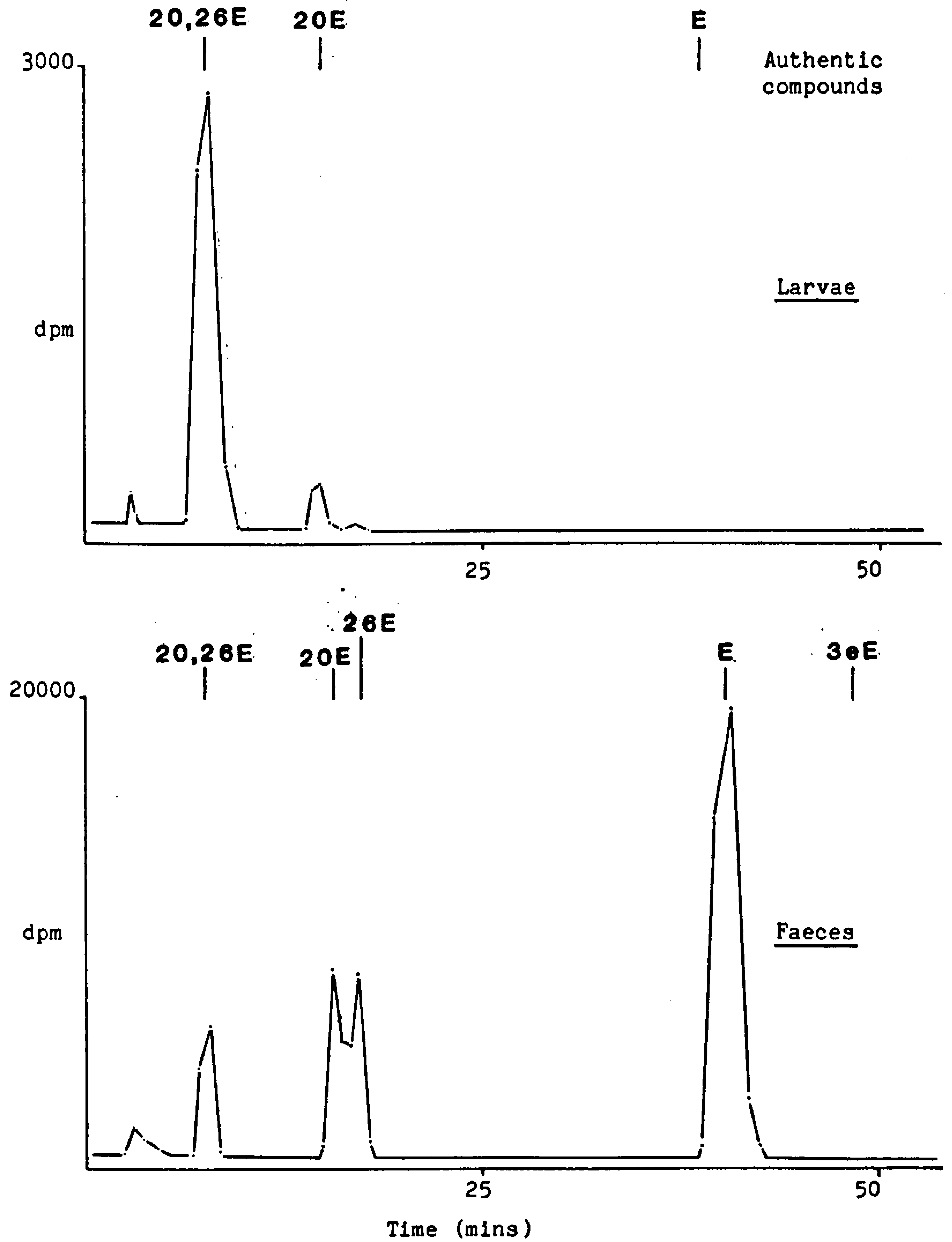


Figure 4A-3 : Reversed-phase h.p.l.c. analysis (system 1) of the free ecdysteroid fractions in the faeces and larvae of early 6th instar S. littoralis

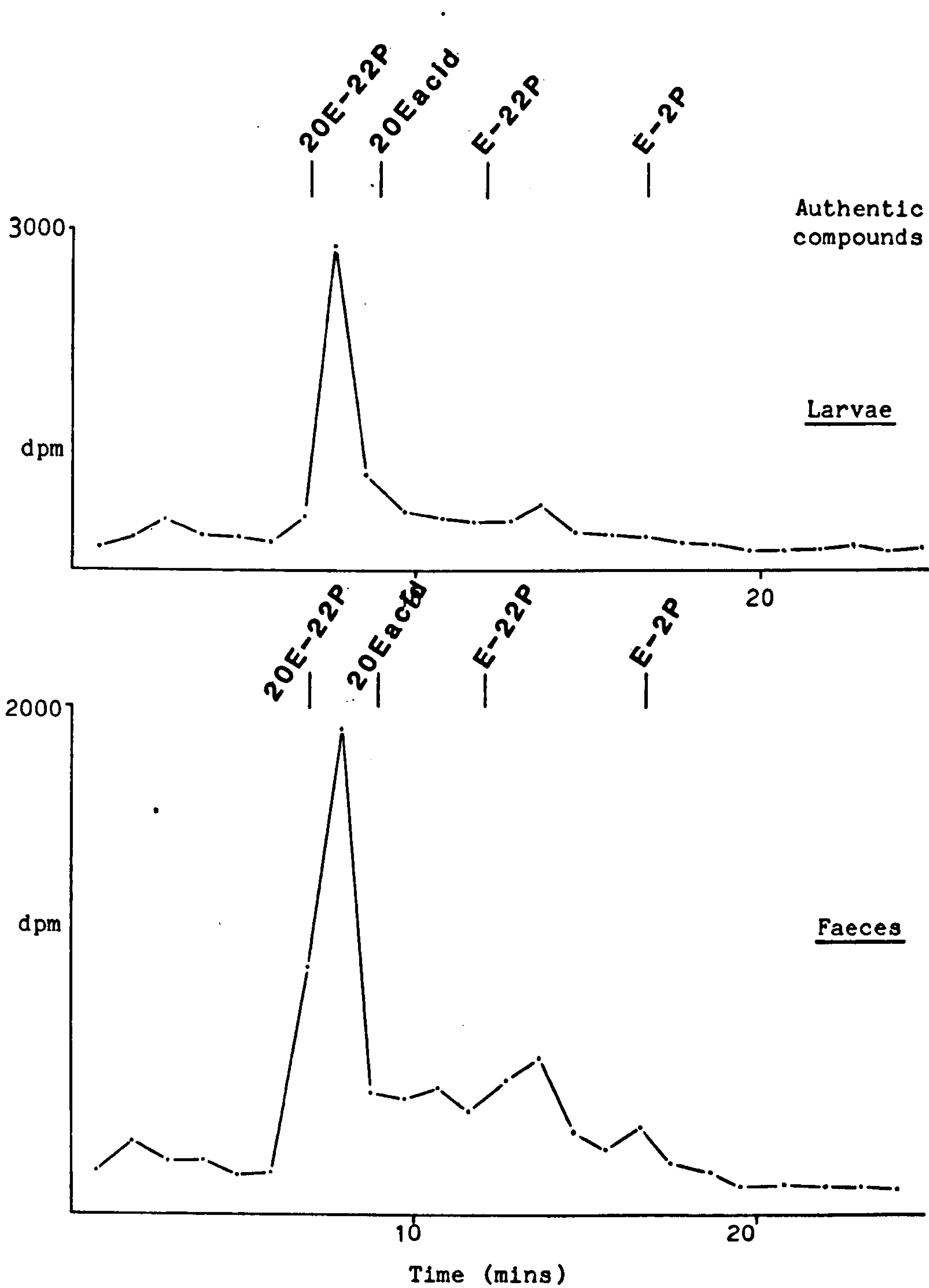


Figure 4A-4 : Reversed-phase h.p.l.c. analysis (system 2) of the highly polar ecdysteroid fraction in the faeces and larvae of early 6th instar S. littoralis.

faeces extract also contains significant radioactivity associated with other areas of the chromatogram which represent 55% of the radioactivity eluted from the column.

As explained earlier (Part 3(A)) the highly polar fraction from silicic acid column chromatography of S. littoralis pupal in vivo incubations has been found to contain small amounts of ecdysteroid conjugates together with greater amounts of unhydrolysable metabolites. To quantify the amounts of conjugates formed in early 6th instar larvae and to analyse the ecdysteroid moieties of these compounds the highly polar fractions were subjected to enzymic hydrolysis by the Helix pomatia enzyme preparation (see Methods). The products were then separated by silicic acid column chromatography (see Methods) and the fractions analysed by h.p.l.c. The distribution of the radioactivity after this separation are shown in figure 4A-2.

The ecdysteroids released from conjugation (contained in the 30% methanol/chloroform fraction) were analysed by reversed-phase chromatography (System 1, figure 4A-5), and the peaks of radioactivity from System 1 re-chromatographed on System 3 to confirm the identities of the ecdysteroids. The results demonstrated that conjugated ecdysteroids occurring in both the faeces and larvae consist of ecdysone, 20-hydroxyecdysone, and 20,26-dihydroxyecdysone, no 3-epi ecdysone was detected.

The 80% methanol/chloroform fraction after hydrolysis (containing unhydrolysable highly polar ecdysteroids) was analysed using h.p.l.c. system 2. The elution profiles (figure 4A-6) for both larvae and faeces show single peaks co-chromatographing with 20-hydroxyecdyson-26-oic acid. For further characterisation these two compounds were methylated by diazomethane (see Methods) and co-chromatographed (System 2) with the methyl ester of 20-hydroxyecdyson-26-oic acid isolated and characterised from

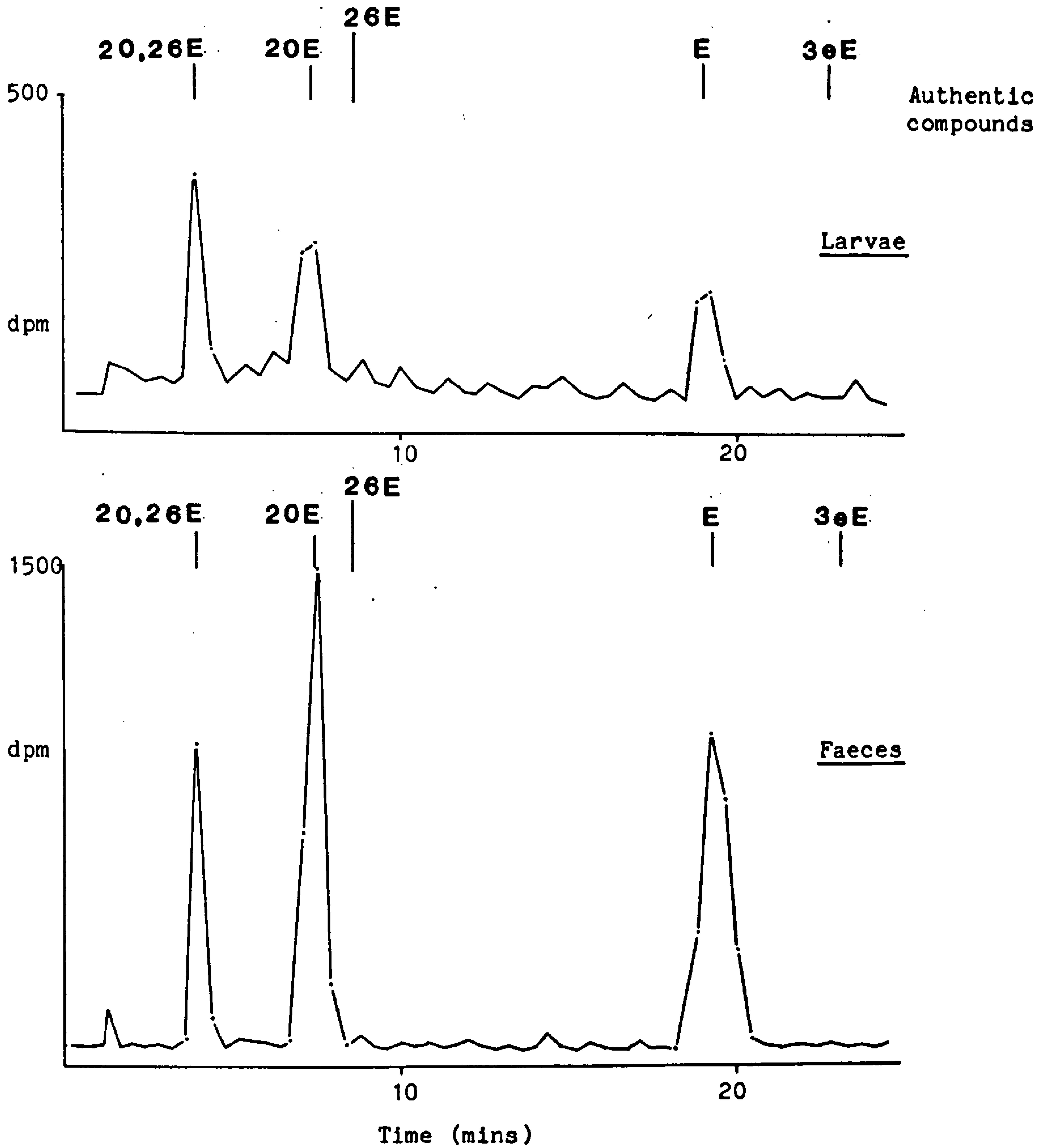


Figure 4A-5 : Reversed-phase h.p.l.c. analysis (system 1) of the [^{3}H] ecdysteroids released by H. pomatia enzyme from conjugated ecdysteroids derived from the faeces and larvae of early 6th instar S. littoralis

S. littoralis pupae and S. gregaria eggs (see Part 3 section B).

This detailed analysis of the highly polar ecdysteroids in the faeces and larvae of early 6th instar larvae has demonstrated that, in addition to the faeces containing most of the [^{3}H] ecdysteroids (Table 4A-2), it also contains a high proportion of conjugated ecdysteroids. In contrast to the faeces, most of the highly polar ecdysteroids in the larval extract can be accounted for by polar ecdysteroids of an unhydrolysable nature (see Table 4A-2).

Broadly speaking, the faeces highly polar ecdysteroid fraction as analysed by h.p.l.c. (figure 4A-4) contains one major peak consisting of 45% of the eluted radioactivity, and an area from Rt=7 min to Rt=18 min containing a broad spread of radioactivity, including two small peaks. By enzymic hydrolysis of this fraction, it was shown that not only free ecdysteroids resulted [ecdysone, 20-hydroxyecdysone and 20-26-dihydroxyecdysone; see figure 4A-5 (identities also confirmed by h.p.l.c. analysis using System 3)] but also an unhydrolysable highly polar ecdysteroid, 20-hydroxyecdyson-26-oic acid (figure 4A-6). Therefore, it must be assumed that the major peak in the highly polar ecdysteroid fraction of the faeces extract (figure 4A-4), gave rise to 20-hydroxyecdyson-26-oic acid after enzymic hydrolysis, and was, therefore, a 20-hydroxyecdyson-26-oic acid conjugate. Similarly, the remaining radioactivity not in the major peak must have given rise to the free ecdysteroids after enzymic hydrolysis (figure 4A-5), and so the spread of radioactivity shown by h.p.l.c. in figure 4A-4, represent conjugates of ecdysone, 20-hydroxyecdysone and 20,26-dihydroxyecdysone. Indeed, to confirm this interpretation, the quantitative analysis of the distribution of [^{3}H] ecdysteroids, as detected before (figure 4A-4) and after (figures 4A-5 4A-6) enzymic hydrolysis, were compared (Table 4A-1).

Table 4A-1 : Distribution of the [^{3}H] highly polar ecdysteroids in the faeces of early 6th instar larvae, as calculated from h.p.l.c. analysis (a) before enzymic hydrolysis (b) after enzymic hydrolysis.

Numbers in d.p.m. represent the distribution of the total amount of [^{3}H] highly polar ecdysteroids produced from the incubations *in vivo*.

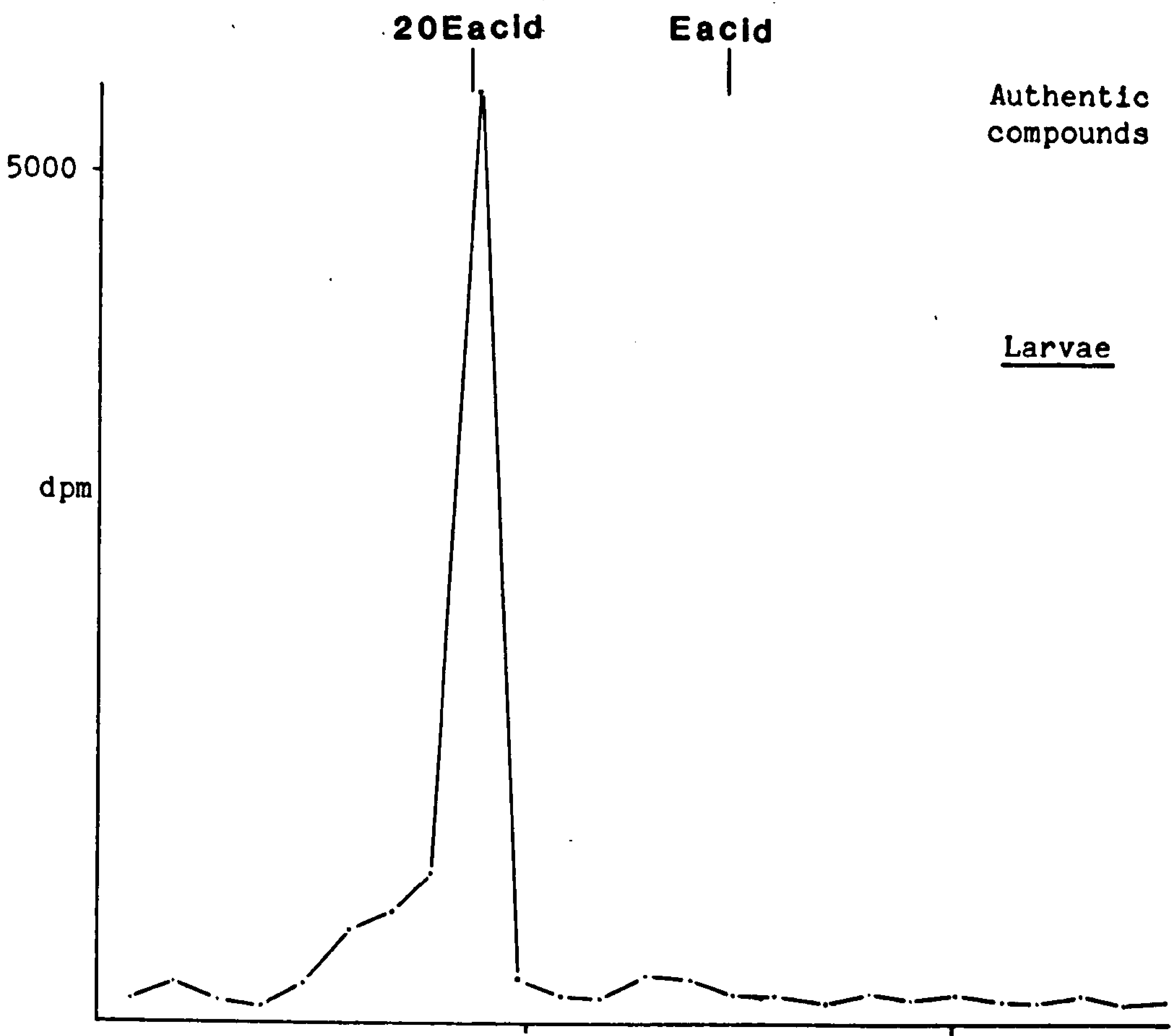
	Conjugates of	(a) Before hydrolysis (Fig.4A-4)	(b) After hydrolysis (Figs. 4A-5, 4A-6)	
Unhydrolysable polar ecdysteroids	20-hydroxyecdyson-26-oic acid	220,500 (peak)	165,900	
Free ecdysteroids	20-hydroxyecdysone	271,500 (remaining chromatogram)	142,300	344,100
	ecdysone		141,100	
	20,26-dihydroxy-ecdysone		60,700	
Total		492,000	510,000	

Table 4A-2 : Summary of the metabolites formed after administration of [^{3}H] ecdysone to early and late 6th instar *S. littoralis* larvae.

	Early sixth instar		Late
	larvae	faeces	sixth instar
FREE ECDYSTEROID FRACTION	212,500	397,010	574,330
(30% methanol/chloroform silicic acid column fraction)			
ecdysone	-	45.9%	6.9%
20-hydroxyecdysone	7.8%	18.8%	18.4%
26-hydroxyecdysone	-	18.3%	30.2%
20,26-dihydroxyecdysone	82.5%	11.4%	32.2%
3-epiecdysone	-	-	4.5%
POLAR ECDYSTEROID FRACTION	29,640	573,020	155,320
(80% methanol/chloroform silicic acid column fraction)			
Ecdysteroids released from conjugates			
ecdysone	4.7%	24.6%	-
20-hydroxyecdysone	4.0%	24.8%	-
20,26-dihydroxyecdysone	4.0%	10.6%	7.8%
ecdyson-26-oic acid	-	-	-
20-hydroxyecdyson-26-oic acid	68.8%	28.9%	-
Unhydrolysable polar ecdysteroids (not conjugated)			
ecdyson-26-oic acid	-	-	15.6%
20-hydroxyecdyson-26-oic acid	-	-	64.9%

Percentages represent the proportion of the total radioactivity recovered from reversed-phase h.p.l.c. in various compounds.

-, denotes not detected.



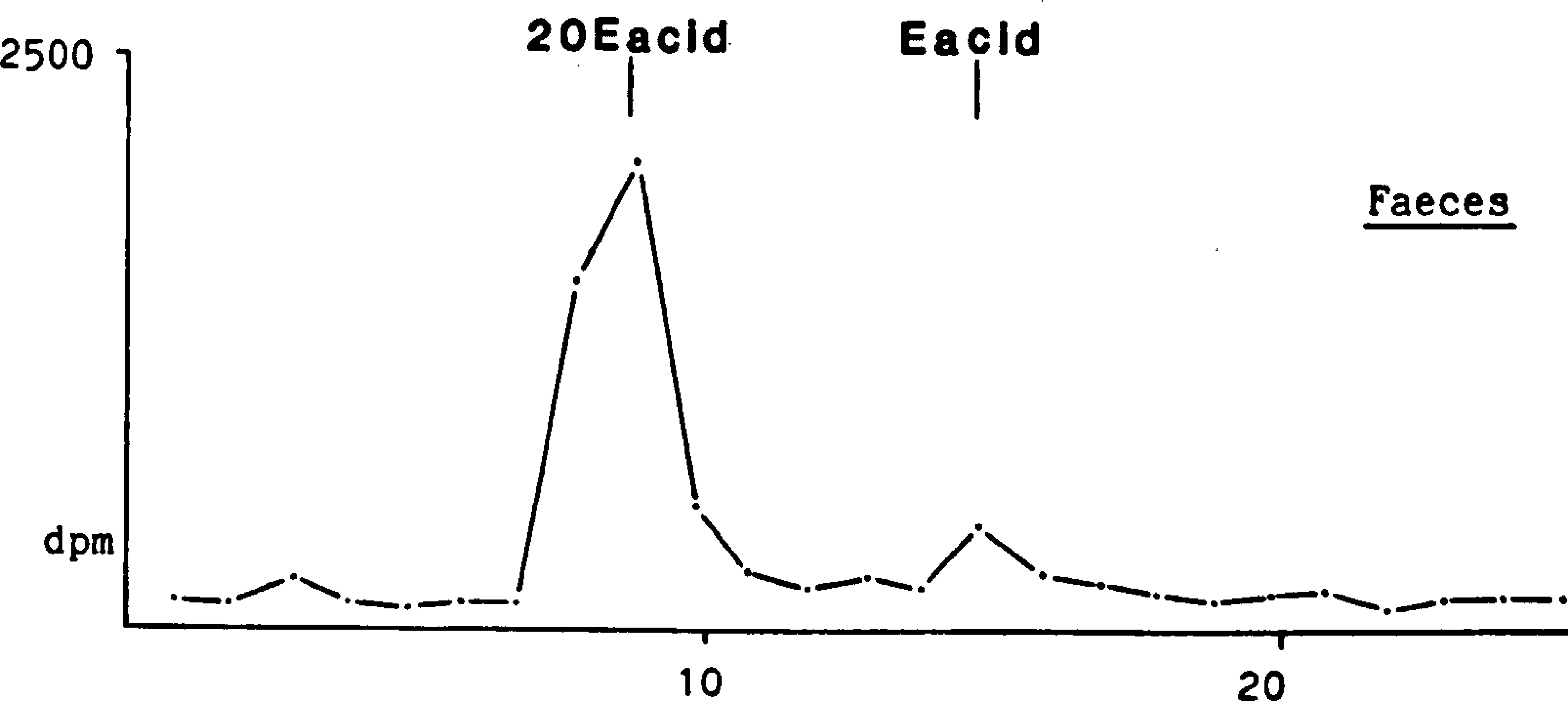


Figure 4A-6 : Reversed-phase h.p.l.c. analysis (system 2) of the unhydrolysable polar ecdysteroid fraction in the faeces and larvae of early sixth instar *S. littoralis*

These results show that this is indeed the most feasible interpretation, although some discrepancies are evident. Indeed, 54,600 dpm of the major peak before hydrolysis (figure 4A-4) cannot be accounted for by the 20-hydroxyecdyson-26-oic acid produced after hydrolysis (figure 4A-6). However, the free ecdysteroids produced from enzymic hydrolysis (figure 4A-5) represent 72,600 dpm more than the presumed free-ecdysteroid conjugates region as detected before hydrolysis (figure 4A-4). Therefore, it is likely that the 54,600 dpm left unaccounted from the major peak in figure 4A-4, in fact, consisted of underlying free ecdysteroid conjugate.

2. The metabolism *in vivo* of [23, 24 $^3H_2$] ecdysone in late final instar larvae (prepupae)

Injection of [23,24 $^3H_2$] ecdysone and extraction of insects

Ten final instar larvae (136 ± 2 hr; 3.52 g) were injected with 0.5 μCi [^{3}H] ecdysone (specific radioactivity 4 Ci/mmol). Thus, each insect was injected with 0.05 μCi (7.75 ng) ecdysone.

The insects were then reared for a further 6 hours under normal conditions and then sacrificed. At this stage of development the larvae do not produce any faeces, so only a larvae extraction was required.

The larvae were then extracted in the normal way (see Methods) and silicic acid column chromatography performed to separate the ecdysteroids into a free ecdysteroid fraction and a highly polar fraction (figure 4A-7). These fractions were then analysed by h.p.l.c. systems 1 and 2, respectively (figures 4A-8, 4A-9). The results demonstrate that in the free ecdysteroid fraction (figure 4A-8) not only were 20 and 26 hydroxylated ecdysteroids produced as in early 6th instar, but 3-epiecdysone was also present.

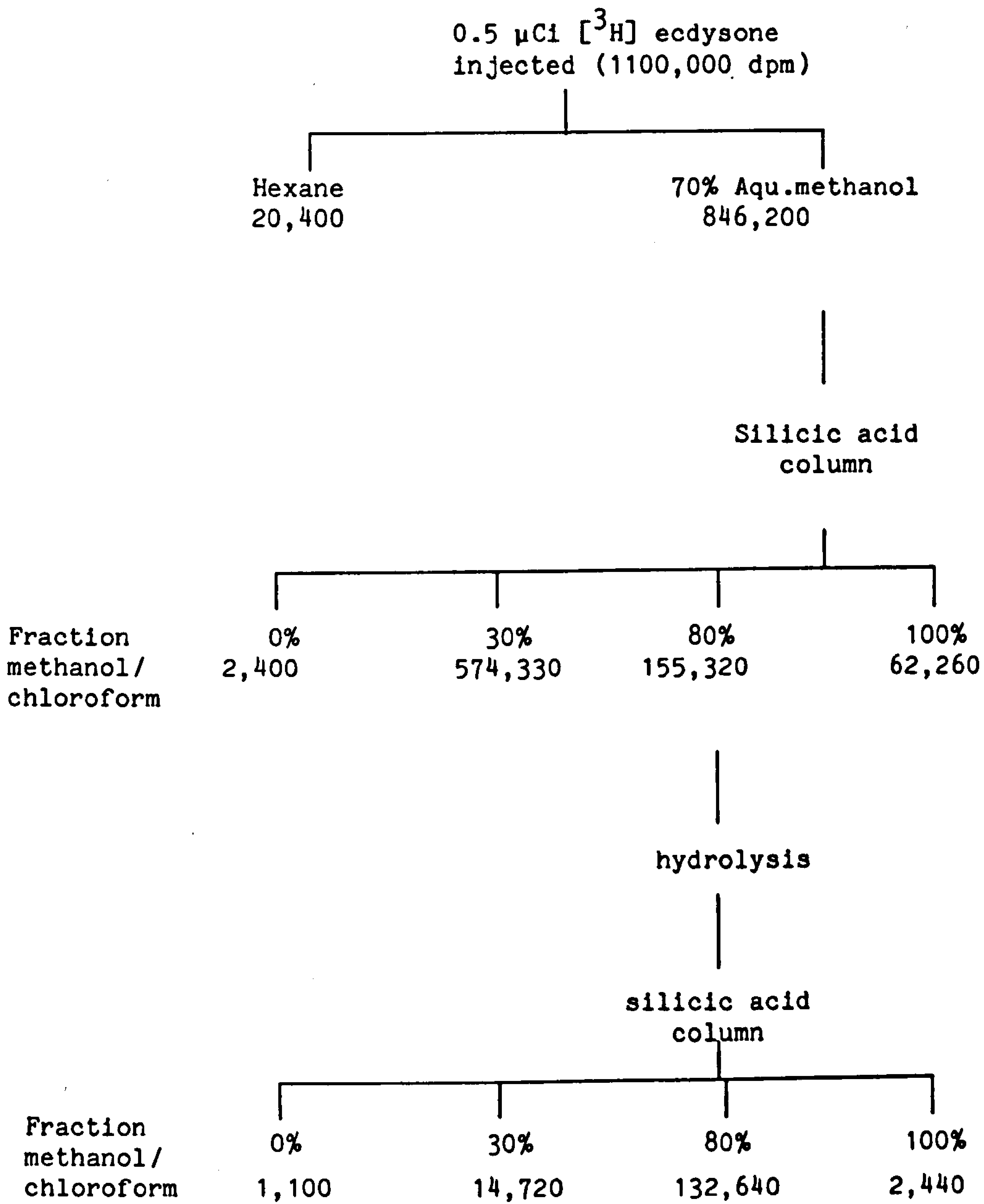


Figure 4A-7 : Summary of the distribution amongst various fractions of metabolites formed from [^3H] ecdysone in late sixth instar larvae (numbers in d.p.m.)

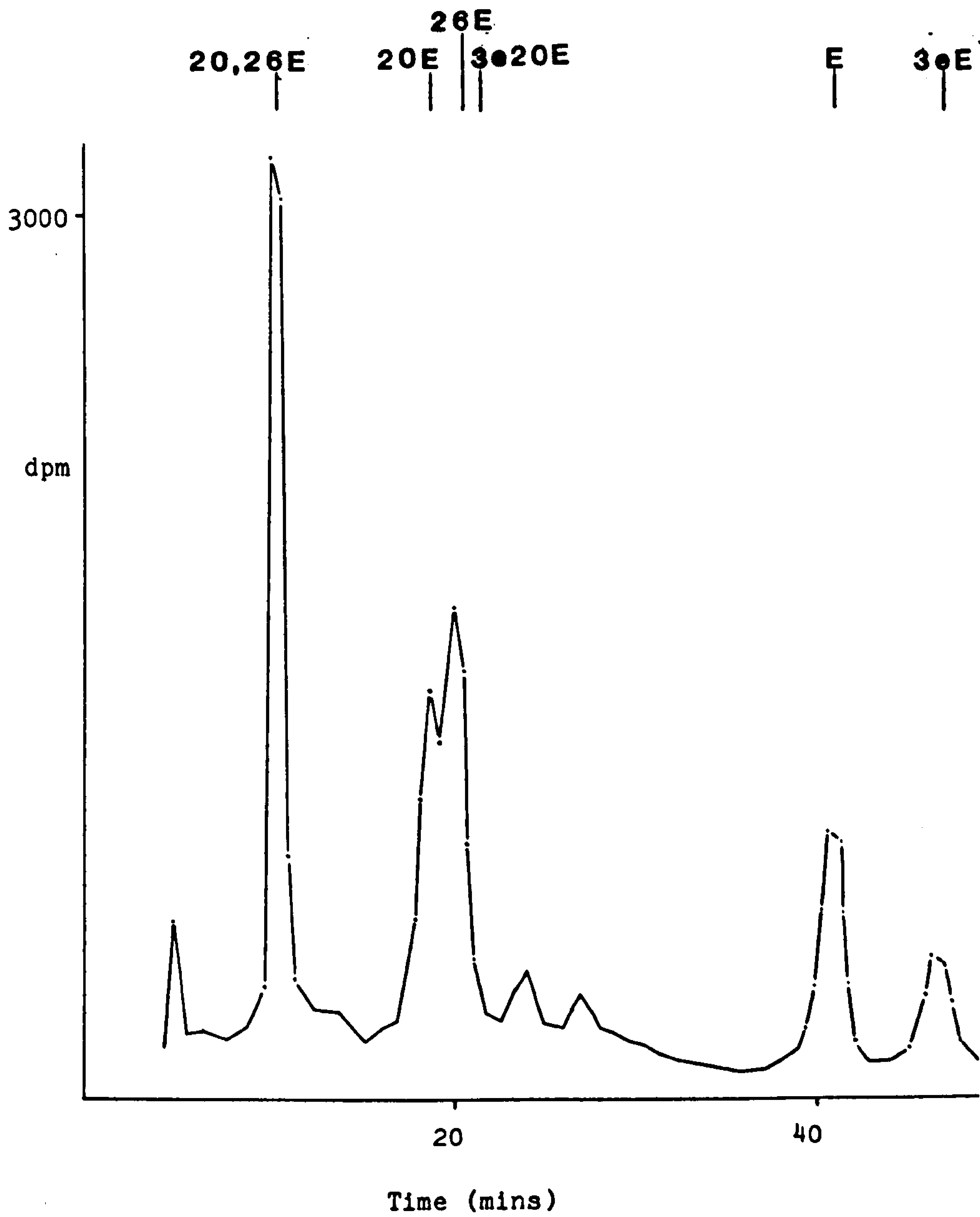


Figure 4A-8 : Reversed-phase h.p.l.c. analysis (system 1) of the free ecdysteroid fraction derived from late sixth instar larvae

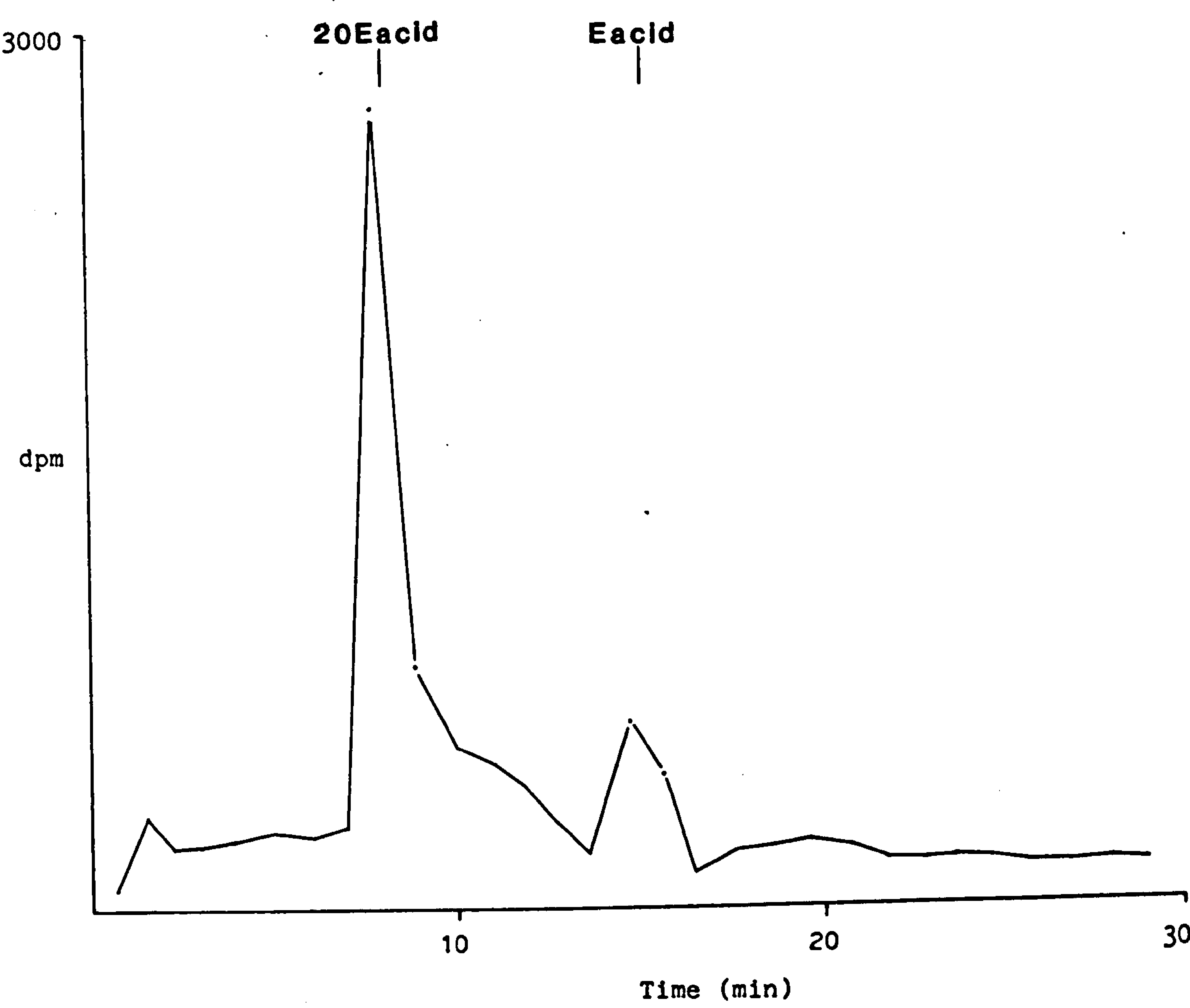


Figure 4A-9 : Reversed-phase h.p.l.c. analysis (system 2) of the [^{3}H] polar ecdysteroid metabolites in late sixth instar larvae.

The further characterisation of [^{3}H] 3-epiecdysone is shown in Part 4 section C. The h.p.l.c. analysis of the highly polar ecdysteroid fraction (figure 4A-9) indicates that some ecdyson-26-oic acid is present in addition to 20-hydroxyecdyson-26-oic acid which was also previously detected in early 6th instar larvae (figure 4A-6). The highly polar fraction was then hydrolysed by the Helix pomatia enzyme preparation (see Methods) and the released ecdysteroids separated from the unhydrolysable highly polar ecdysteroids by silicic acid column chromatography. The released ecdysteroids were analysed by h.p.l.c. system 1 (figure 4A-10) and the unhydrolysable fraction analysed by system 2 (figure 4A-11). The results show that the unhydrolysable polar ecdysteroids, 20-hydroxyecdyson-26-oic acid and ecdyson-26-oic acid (figure 4A-11), constitute the majority of the highly polar ecdysteroid metabolites, with the remainder being made up of 20,26-dihydroxyecdysone conjugate (figure 4A-10). Conjugation was however very small, only accounting for 1.8% of the [^{3}H] ecdysteroids recovered (Table 4A-2).

3. Comparison of [^{3}H] ecdysone metabolism in early and late 6th instar larvae

The quantitative distribution of radioactivity amongst various ecdysteroid metabolites in early and late 6th instar larvae is summarised in Table 4A-2. The composition of the free ecdysteroid fraction is broadly similar in both experiments. In the early 6th instar, ecdysone, 20-hydroxy and 26-hydroxy ecdysteroids are all present in the faeces, although in the larvae 20- and 26-hydroxylation was more active, producing 20-hydroxyecdysone and 20,26-dihydroxyecdysone without any trace of intermediate 26 hydroxyecdysone nor of the substrate, ecdysone. During late 6th instar development, in addition to 20- and 26-hydroxy-ecdysteroids, some 3-epi-ecdysone has been produced.

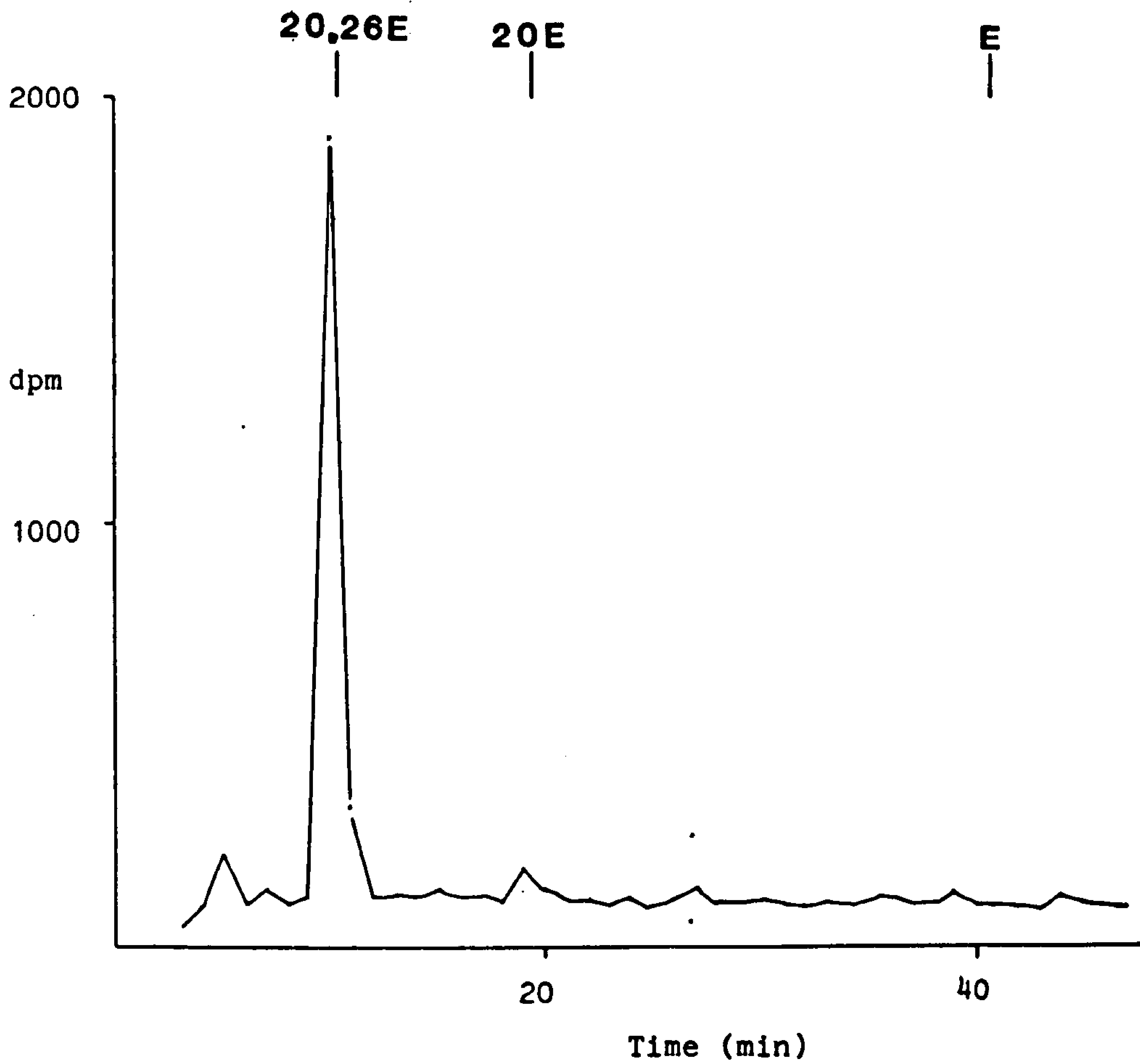


Figure 4A-10 : Reversed-phase h.p.l.c. analysis (system 1) of the ecdysteroids released by *H. pomatia* enzyme from conjugated ecdysteroids derived from late 6th instar larvae of *S. littoralis*.

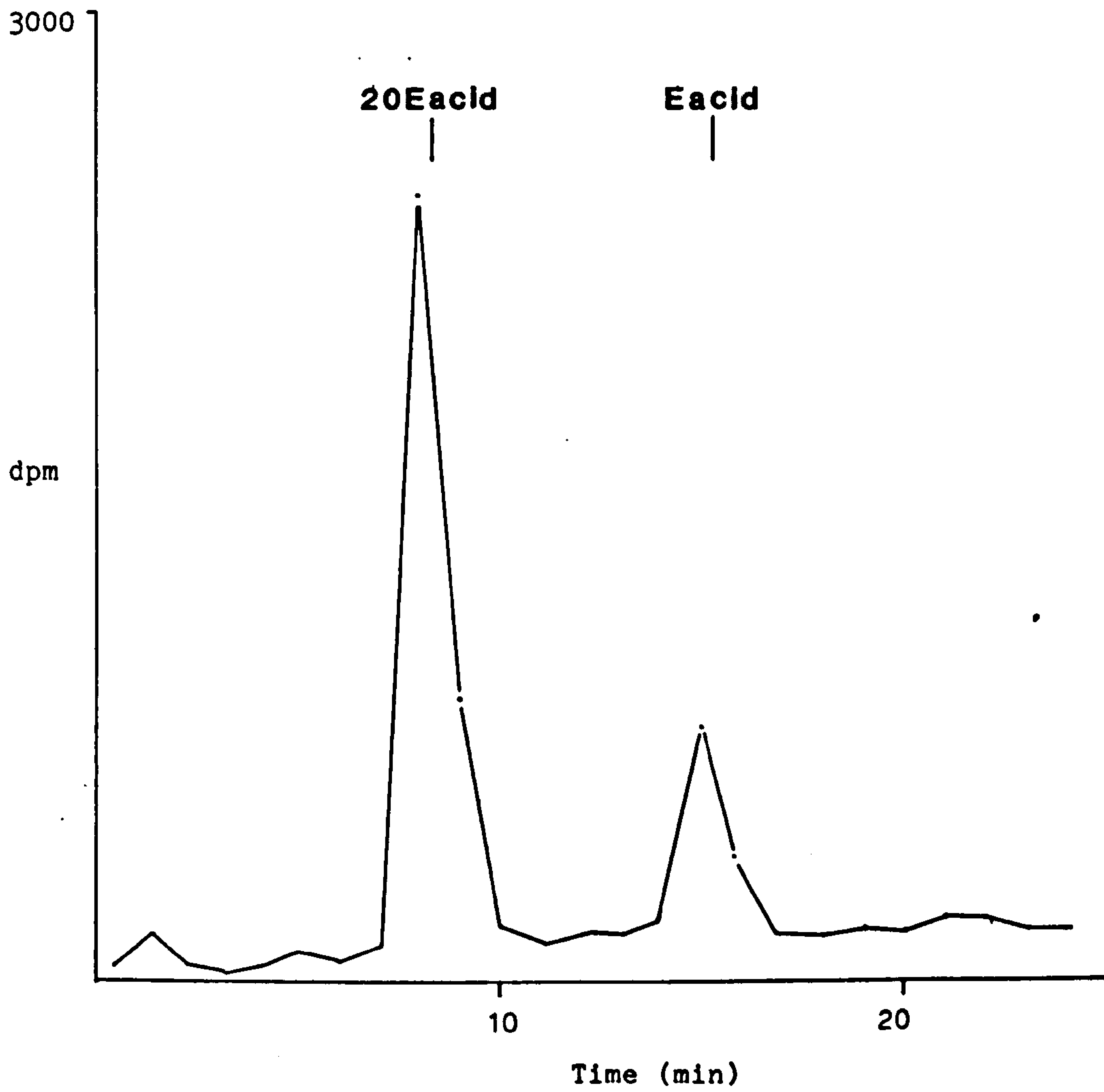


Figure 4A-11 : Reversed-phase h.p.l.c. analysis (system 2) of the unhydrolysable polar ecdysteroid fraction from late 6th instar larvae.

Over the first half of the final larval instar the insect is at its most active, rapidly eating and excreting and increasing in size and weight (from 250 mg to 770 mg in 48 hours). Therefore, it is not surprising to find that at this stage the majority of radiolabelled ecdysteroids have been excreted (81%). The faeces extract, as well as containing more of the metabolites, has a high proportion (58%) of its ecdysteroids in the form of conjugates. These include not only conjugates of 20- and 26-hydroxylated ecdysteroids (figure 4A-5) but also probably a conjugate of 20-hydroxyecdyson-26-oic acid (viz. the major peak in figure 4A-4) which is hydrolysed to release 20-hydroxyecdyson-26-oic acid (see figure 4A-6). This is in contrast to the ecdysone metabolism in the late 6th instar larval system, where very little conjugation has occurred. At this stage of development 26-oic acid derivatives of ecdysone and 20-hydroxyecdysone accounted for the majority of the polar ecdysteroids.

PART 4 SECTION B

PART 4 SECTION B

Titre and identification of ecdysteroids present in sixth instar *S. littoralis* larvae

	Page No.
Introduction	125
Experimental and results	
1. Determination of the ecdysteroid titre curve in the haemolymph of sixth instar *S.littoralis* larvae by radioimmunoassay	128
Preparation of samples for radioimmunoassay	128
Examination of the reversed-phase SEP-PAK method as a purification step for ecdysteroids	128
Radioimmunoassay of the haemolymph samples	129
Validity tests for assay of samples from larval haemolymph with both antisera (ICT-1 and DHS 1-15)	131
Radioimmunoassay of the carcass samples from the later stages of 6th instar larvae	131
2. Characterisation of the moulting hormone in the haemolymph of sixth instar *S. littoralis*	132
Analysis of the free ecdysteroids in the haemolymph of sixth instar *S. littoralis* larvae	132
Analysis of the conjugated ecdysteroids present in the haemolymph of sixth instar *S. littoralis* larvae	134
Summary of the ecdysteroid composition in haemolymph of 6th instar larvae as detected by GC/MS	136

Page No.

3. Ecdysteroid analysis of the faeces of sixth instar *S. littoralis* larvae 137

Analysis by radioimmunoassay of the free and conjugated ecdysteroids present in the faeces of sixth instar *S. littoralis* larvae 137

The ecdysteroid composition of the faeces of 6th instar larvae of *S. littoralis* 138

PART 4 SECTION B

Titre and identification of ecdysteroids present in 6th instar *S. littoralis* larvae

INTRODUCTION

To study the metabolites of ecdysone during the 6th instar stage of *S. littoralis*, it is necessary to establish the levels of ecdysteroids throughout the instar. The compilation of such a moulting hormone titre curve can then be used as a basis for further experiments so that the metabolic fate of ecdysone can be uncovered.

For the purpose of such determinations a suitable assay must be chosen, combining specificity, sensitivity, and, because a large number of samples are to be assayed, ease of application.

The first assay for ecdysone, as used by Butendandt and Karlson (1954), used ligated abdomens of *Calliphora erythrocephala* for the bioassay of moulting hormone. Since then, a number of more sensitive and reliable detection methods have been made available for the determination of moulting hormone levels in arthropods and crustacea. Of these methods, the most sensitive, with the exception of gas-liquid chromatography linked to electron capture detection (Poole and Morgan, 1975), has proved to be radioimmunoassay detecting levels of moulting hormone of less than 100 pg (Borst and O'Connor, 1972; Lauer *et al.*, 1974).

Due to the nature of the radioimmunoassay technique, relying on recognition of an ecdysteroid by the antibody, it is not totally specific for any single ecdysteroid because a number of closely related structures will also be recognised, albeit to a lesser extent. However, as this method can detect low levels of ecdysteroid (100 pg), and many samples can be assayed simultaneously, the method provides an ideal blend of sensitivity, specificity, and ease of application, for the compilation

of the moulting hormone titre curve in sixth instar S. littoralis larvae.

Several antibodies have been produced for the moulting hormone assay. Their specificity towards individual ecdysteroids vary quite considerably, depending on the ecdysteroid-hapten used to produce the antibody. Using these antisera a number of radioimmunoassays have been developed for ecdysteroids. Two different antisera were chosen for the analysis of S. littoralis larval ecdysteroids; ICT-1 (Spindler et al., 1978) and DHS 1-15 (Soumoff et al., 1981). ICT-1 antiserum, raised using inokosterone-26-acid bound to thyroglobulin, is primarily specific for the nucleus, whereas DHS 1-15, raised using 20-hydroxyecdysone 2-hemisuccinate conjugated to thyroglobin, exhibits greater specificity for the ecdysteroid side chain than for the nucleus. Therefore, using both antisera, some knowledge of the specific structures of the ecdysteroids present may be obtained, thus rendering the RIA assay somewhat more specific.

Moulting hormone titre curves determined by radioimmunoassay of the haemolymph of the final larval instar in a number of other lepidopteran species have been compiled; Galleria mellonella (Bollenbacher et al., 1978; Hsiao and Hsiao, 1977), Manduca sexta (Bollenbacher et al., 1975), Bombyx mori (Calvez et al., 1976), Calpodes ethlius (Dean et al., 1980). The moulting hormone titre for Spodoptera littoralis 6th instar larvae has been determined using whole insects (figure 4A-1; Clarke, 1980), a major peak being detected near the end of the instar, which is preceded by two smaller peaks. To confirm this multiplicity of peaks, in the present study the haemolymph moulting hormone titre will be compiled, and the ecdysteroid complement determined at peak moulting hormone titres by GC/MS. As haemolymph provides samples containing less extraneous material than whole insect extracts, this lends itself well to

both analysis by RIA and by GC/MS, by facilitating extraction and requiring only limited purification.

Similar investigations, involving the determination of the RIA moulting hormone titre followed by analysis of the nature of the ecdysteroids at selected points during the titre curve, have been undertaken in some insects. In the locust, Locusta migratoria an RIA moulting hormone titre in the blood of last instar larvae was compiled (Hirn et al., 1979). In addition to this the relative importance of ecdysone and 20-hydroxyecdysone was assessed by t.l.c.-RIA, demonstrating that depending on the age within the instar, the predominant moulting hormone could be either ecdysone or 20-hydroxyecdysone. In the lepidopteran, M. sexta, the moulting hormone titre was determined in the 5th instar larvae, in both haemolymph samples and whole animal extracts. Further qualitative and quantitative analysis of the ecdysteroids, determined by RIA, t.l.c. and g.l.c., revealed that in both haemolymph and whole-animal samples, early in the instar (1-4 days) the ecdysone to 20-hydroxyecdysone ratio was approximately 1, and late in the instar the ratio was approximately 0.2 (Bollenbacher et al., 1975).

For the present study, GC/MS was employed to provide a detailed analysis of fractions at peak moulting hormone titre during the 6th instar of S.littoralis. This method permits the sub-nanogram quantitative detection of the ecdysteroids, ecdysone, 20-hydroxyecdysone and 20,26-dihydroxyecdysone. The metabolism in vivo of [^{3}H] ecdysone (Part 4, section A) has demonstrated that a high proportion of the metabolites in the larvae of S. littoralis exist in the form of conjugates present in the faeces. Therefore, this study was extended to include the characterisation of conjugated ecdysteroids and to include the analysis (by RIA and GC/MS) of the faeces.

EXPERIMENTAL AND RESULTS

1. Determination of the ecdysteroid titre curve in the haemolymph of sixth instar *S. littoralis* larvae by radioimmunoassay

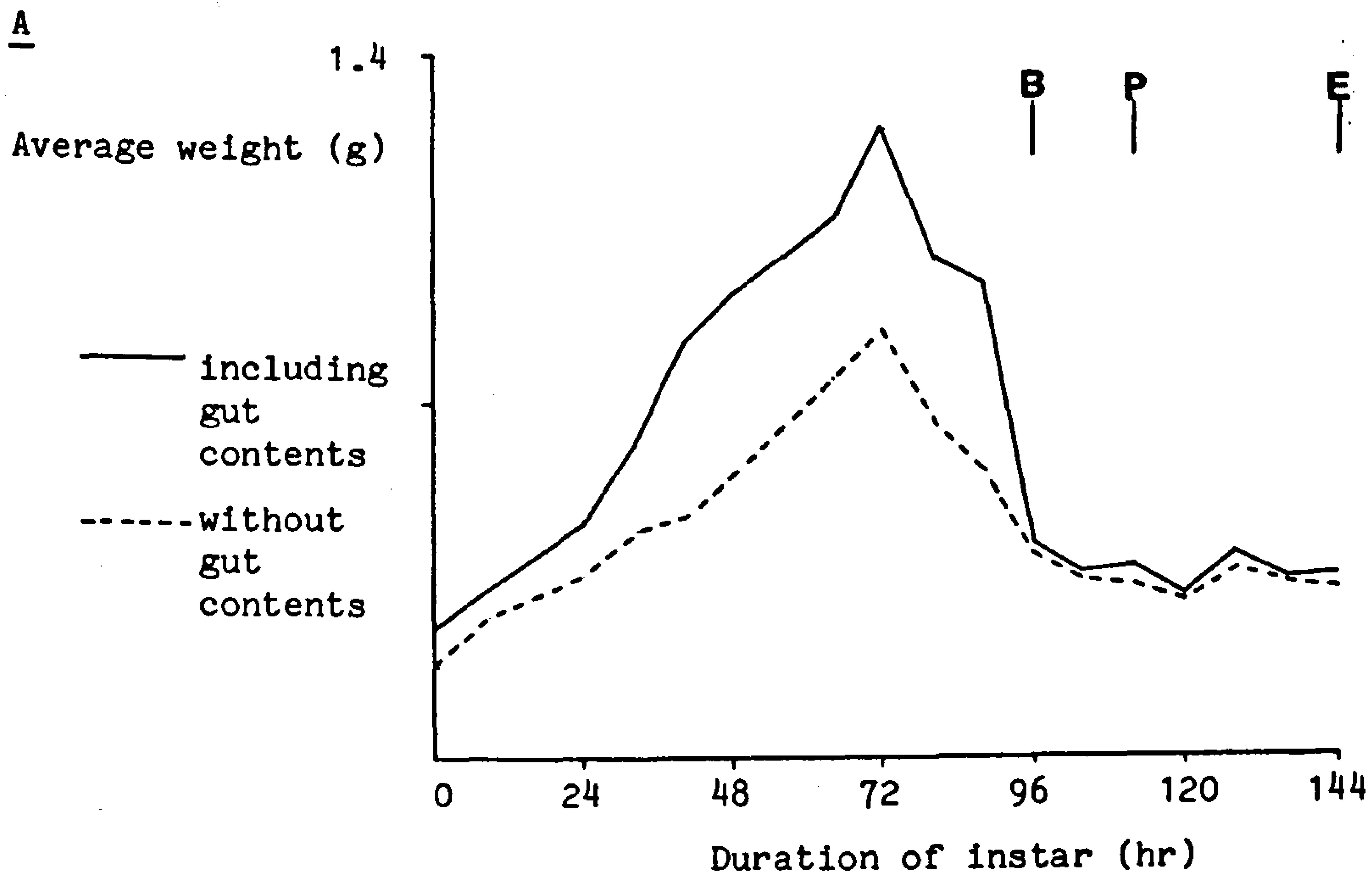
Preparation of samples for radioimmunoassay

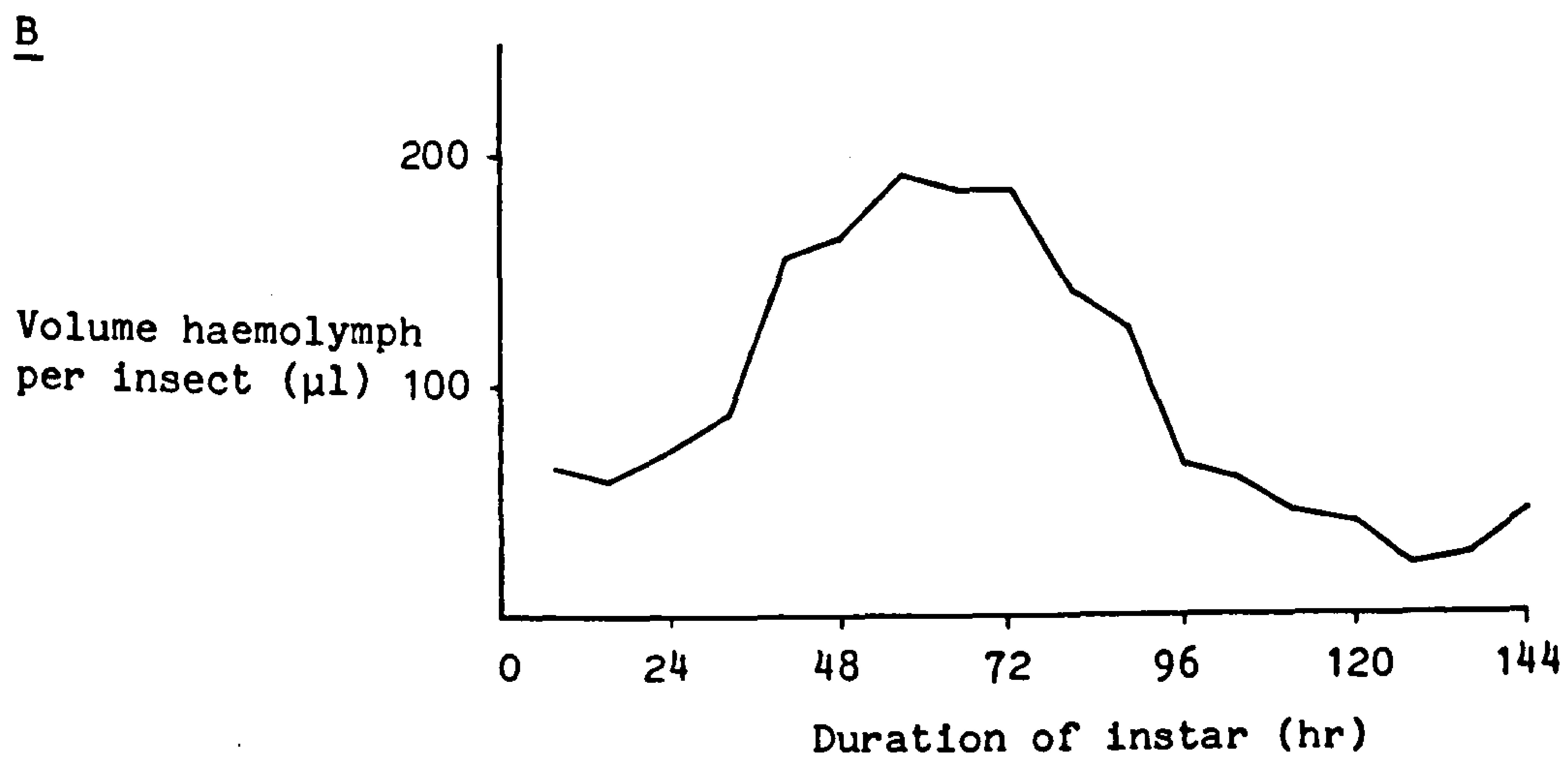
Groups of three larvae were synchronised at the 5th/6th instar moult and collected at intervals (every 8 hours) throughout the instar (until pupation). The collected insects were then weighed, and as much as possible of the haemolymph drained from the larvae through a small dorsal incision made at the rear of the larvae. The volume of haemolymph was then measured; these measurements and the weights of the insects throughout the instar are presented in figure 4B-1.

The haemolymph samples thus collected were then extracted. 3ml of chilled ethanol was added to the haemolymph to precipitate proteins and extract ecdysteroids, this was then centrifuged for 5 minutes, the supernatant collected and the pellet re-extracted twice with 3 ml methanol. The combined methanol extracts were then evaporated to dryness under vacuum and transferred to a small vial. The sample was dissolved in 200 µl methanol and then made up to a 10% ($^{v}/v$) methanol/water solution by the addition of 1.8 ml of water. The samples were then ready for reversed-phase SEP-PAK purification by the method explained in Part 2. [^{3}H] ecdysteroids were subjected to chromatography on reversed-phase SEP-PAK to establish the recovery of material during this method of purification.

Examination of the reversed-phase SEP-PAK method as a purification step for ecdysteroids

[23,24 $^{3}H_{2}$] Ecdysone was purified by silicic acid column chromatography (see Part 2), and 496,000 dpm [^{3}H] ecdysone added to 500 µl of





B : burrowing

P : onset of pre-pupal stage

E : ecdysis

Figure 4B-1 : A) Growth curve of *S. littoralis* sixth instar larvae.

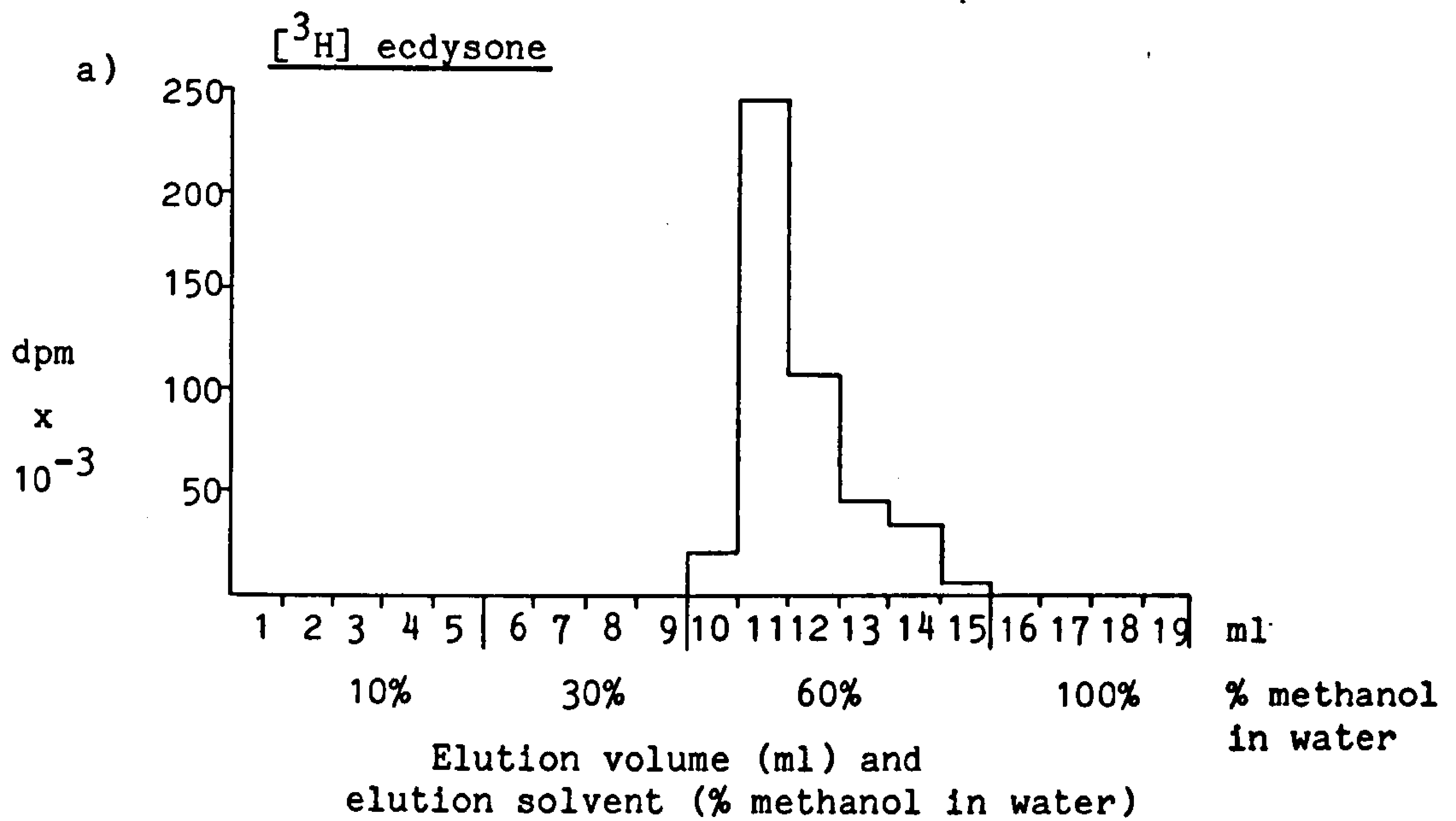
B) The volume of haemolymph recovered throughout the instar.

larval haemolymph. The haemolymph sample was then purified by the aforementioned procedure (extraction with 3 ml ethanol and 2 x 3 ml methanol) and then prepared in a 2 ml 10% methanol/water solution as previously explained. The sample was then loaded onto the reversed-phase SEP/PAK cartridge, the vial washed twice with 10% ($^{v}/v$) methanol/water, and the washings loaded onto the column. This was followed by elution with 4 ml of 30% methanol/water, 6 ml of 60% methanol/water (the free ecdysteroid fraction) and 4 ml of methanol. The eluate was collected in 1 ml fractions and assayed for radioactivity.

The free ecdysteroids in S. littoralis have been shown to contain ecdysone metabolites of varying polarity (Part 3, section A; Part 4, section A) the most polar of these being 20,26-dihydroxyecdysone. To ensure that this increase in polarity does not cause such an ecdysteroid to elute before the 'free ecdysteroid' fraction collected on this reversed-phase SEP-PAK system, [^{3}H] 20,26-dihydroxyecdysone [as isolated from pupae (Part 3, section A)] was also subjected to the same examination as described for [^{3}H] ecdysone. The results of these experiments are illustrated in figure 4B-2 and demonstrate that the ecdysteroids, ecdysone and 20,26-dihydroxyecdysone, can be purified on reversed-phase SEP-PAK cartridges with recoveries of 95% and 90.5%, respectively.

Radioimmunoassay of the haemolymph samples

Radioimmunoassay was performed using different antisera (ICT-1 and DHS 1-15: see Part 2 for details). Each sample was assayed in duplicate with both antisera and the results expressed in nanogram ecdysone equivalents per insect. The RIA moulting hormone titre curves are illustrated in figure 4B-3. The moulting hormone titre curve shows the presence of three distinct peaks at 40, 72 and 120 hrs. The



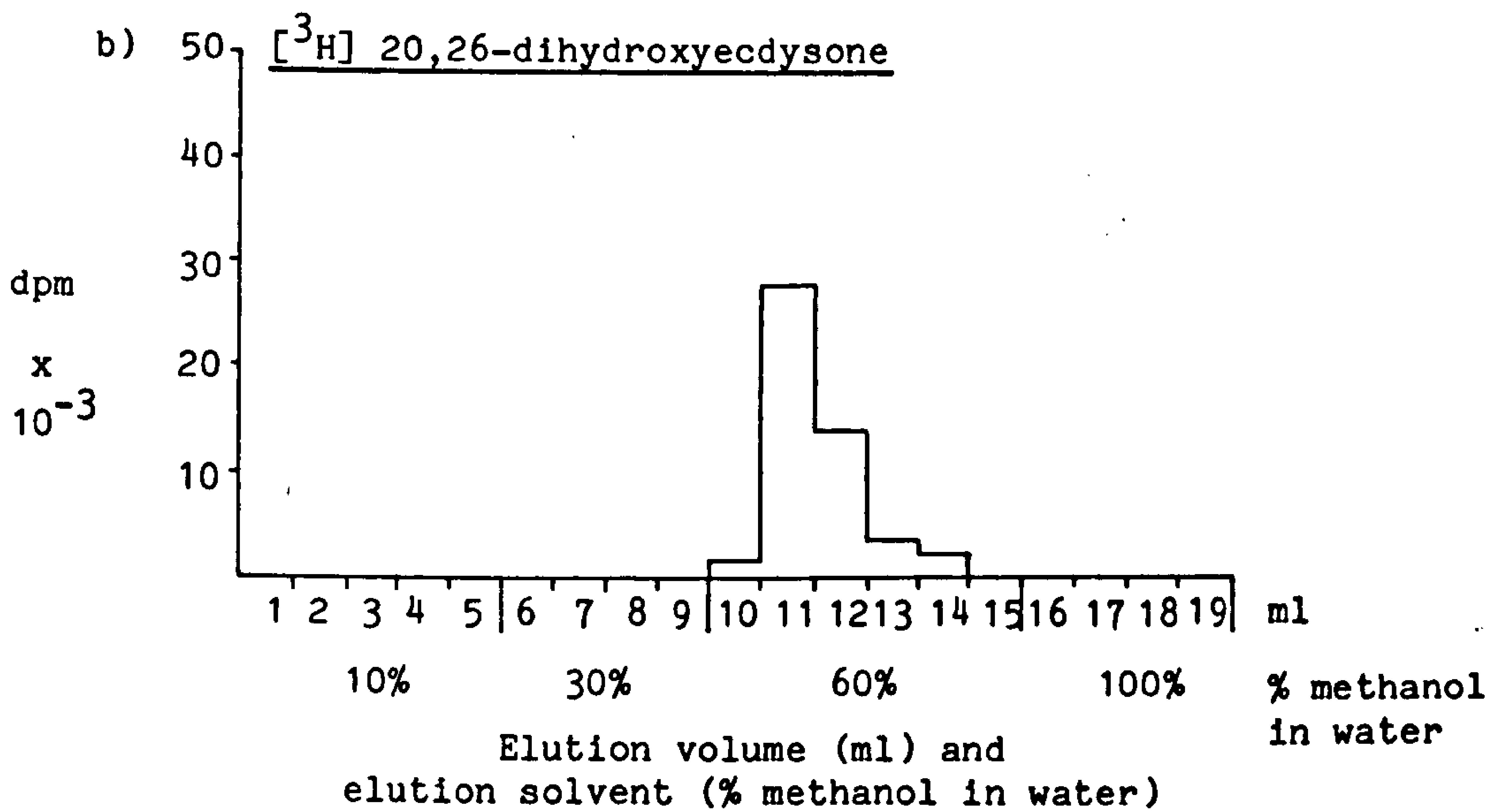


Figure 4B-2 : Elution profiles of [^{3}H] ecdysone (a) and [^{3}H] 20,26-dihydroxyecdysone (b) from reversed-phase SEP-PAK cartridges

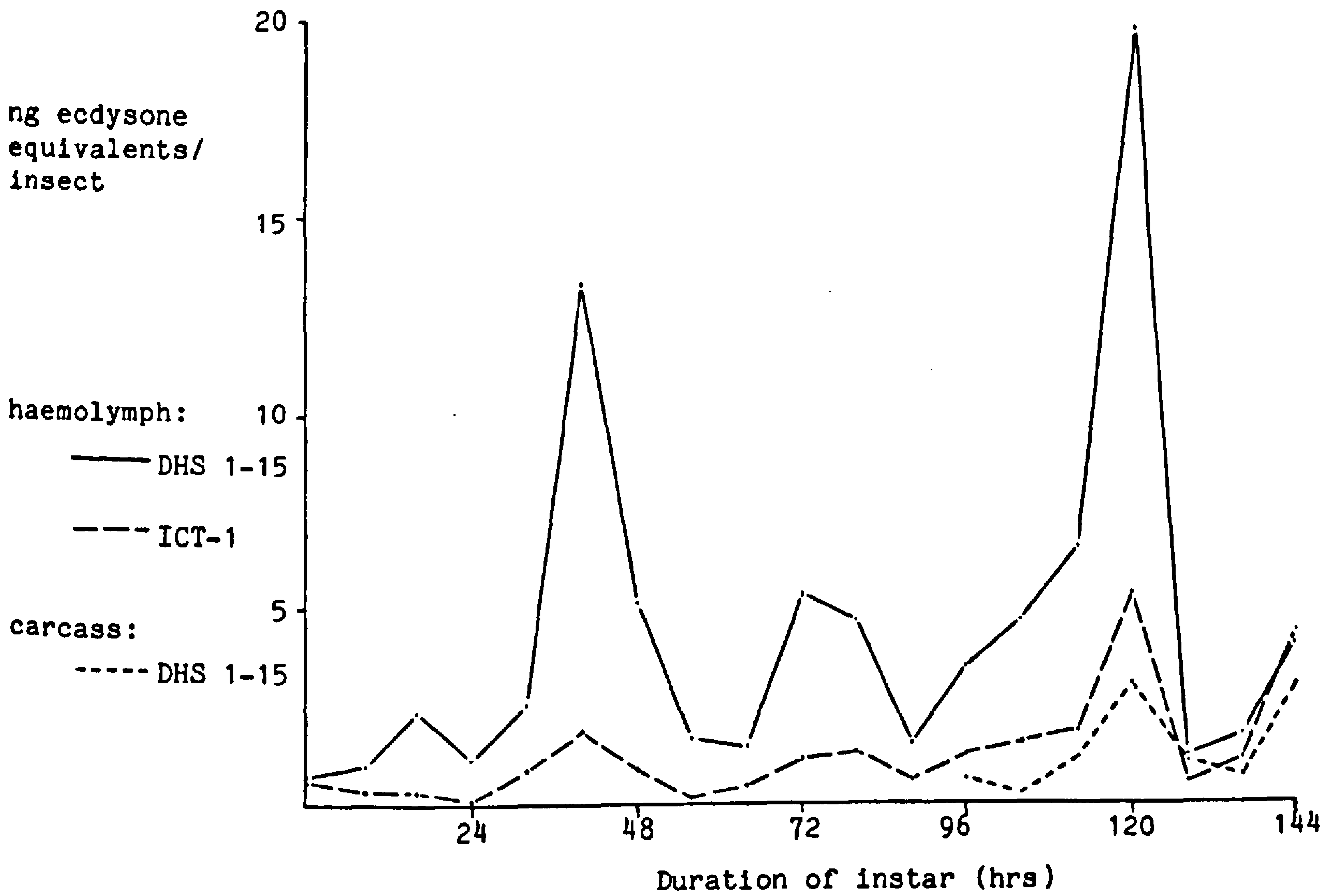


Figure 4B-3 : RIA response to ICT-1 and DHS1-15 antisera in the haemolymph of 6th instar S. littoralis larvae, and RIA response to DHS1-15 antiserum in the carcass of the same larvae. Each point represents the average of two measurements.

appearance of three distinct peaks is similar to the pattern obtained for the moulting hormone titre from whole-animal extracts (figure 4A-1; Clarke 1980). The timing of the first two peaks coincides with those observed in the whole-animal titre, however, the final peak in the haemolymph occurred at 120 hrs while in the whole-animal titre this peak occurred at 136 hrs. A possible reason for this could be attributed to different rearing conditions for the insects used in the two studies, which would effectively shorten or lengthen the developmental stages. However, this is apparently not the case, rearing conditions were the same in both studies and consequently the duration of the 6th larval instar was the same in both studies (144 hrs). An alternative explanation would, therefore, be the possible sequestering of moulting hormone at around 136 hrs of 6th instar development from the haemolymph into one or more of the insect's tissue (this possibility is investigated p. 131).

Comparison of the haemolymph titres as compiled by antisera ICT-1 and DHS 1-15 shows principally a quantitative difference. DHS 1-15 antiserum consistently detected higher levels of ecdysteroid (3-6 times higher). This is presumably due to the different cross-reactivities of the two antisera to the ecdysteroid species in the haemolymph samples. Any change of a ligand in ring A of the ecdysone nucleus leads to a drastic diminution of steroid binding to the ICT-1 antiserum (Reum and Koolman, 1979). So, if some of the ecdysteroid present in the haemolymph was in the form of, for example, 3-epi-ecdysone only the DHS 1-15 antiserum would detect it, as the binding of this antiserum is less affected by ring A ligand modifications (Soumnoff _et al._, 1981). In fact, compounds such as 2- and 3-acetates of ecdysone have been found to exhibit increased binding to the DHS 1-15 antiserum by at least four times

that achieved by ecdysone (K.P. Wigglesworth, unpublished). If the same is true for the binding of 3-epiecdysteroids this could well account for the increased quantities of moulting hormone detected by DHS 1-15 antiserum.

Validity tests for assay of samples from larval haemolymph with both antisera (ICT-1 and DHS 1-15).

It is often the case that in an insufficiently purified sample, results obtained by RIA are affected by impurities in the sample, either causing interference of the ecdysteroid-antiserum interaction or causing artificial enhancement of the antiserum binding. To verify that these types of interactions are not occurring, validity tests for the assays were undertaken at points during the moulting hormone titre. This was achieved by repeating RIA of the peak titre samples (40 hrs and 120 hrs) in the presence and absence of known amounts of added exogenous ecdysone. The results (Table 4B-1) demonstrate that there is evidence of some interference, especially in the 40 hours sample. However, the difference between the amounts of exogenous ecdysone detected and actually added are within acceptable limits.

Radioimmunoassay of the carcass samples from the later stages of 6th instar larvae

As previously indicated, the final peak of moulting hormone in the whole-animal titre of 6th instar larvae occurring at 136 hours does not correspond to an equivalent peak in the haemolymph titre. This suggests that there is a possibility that some ecdysteroid is sequestered into the insect's tissue. To verify this, the carcasses (larvae minus haemolymph) of the later stages of 6th instar larval development were also analysed by RIA.

Table 4B-1 : Validity test for the radioimmunoassays: the effect of adding exogenous ecdysone to aliquots of peak fractions from the ecdysteroid titre curve for sixth instar *S. littoralis* larvae.

Antiserum	Age within instar	RIA value (pg ecdysone equivalents)	RIA value obtained after addition of 85 pg ecdysone (pg ecdysone equivalents)	Difference
ICT-1	40	116	208	92
		118	220	102
	120	124	201	77
		118	196	78
DHS 1-15	40	102	194	92
		110	197	87
	120	106	189	83
		100	183	83

Carcasses (at 96, 104, 112, 120, 128, 136 and 144 hrs of 6th instar larval development) were extracted in aqueous methanol and methanol and the free ecdysteroids separated by silicic acid column chromatography. RIA were performed using DHS 1-15 antiserum. The resulting titre curve is shown in figure 4B-3, and reveals the presence of ecdysteroids in these samples at a much lower level than in the haemolymph. However, the titre profile is similar to that obtained for the haemolymph titre, implying that the ecdysteroid content of the tissues reflects that of the haemolymph; this is also the case in _Manduca sexta_ 5th instar larvae (Bollenbacher _et al_., 1975). These results, therefore, exclude the possibility that major sequestration of ecdysteroids by the insect tissues is responsible for the discrepancy between the haemolymph and whole larval titres of ecdysteroids

2. Characterisation of the moulting hormone in the haemolymph of sixth instar S. littoralis

Analysis of the free ecdysteroids in the haemolymph of sixth instar S. littoralis larvae

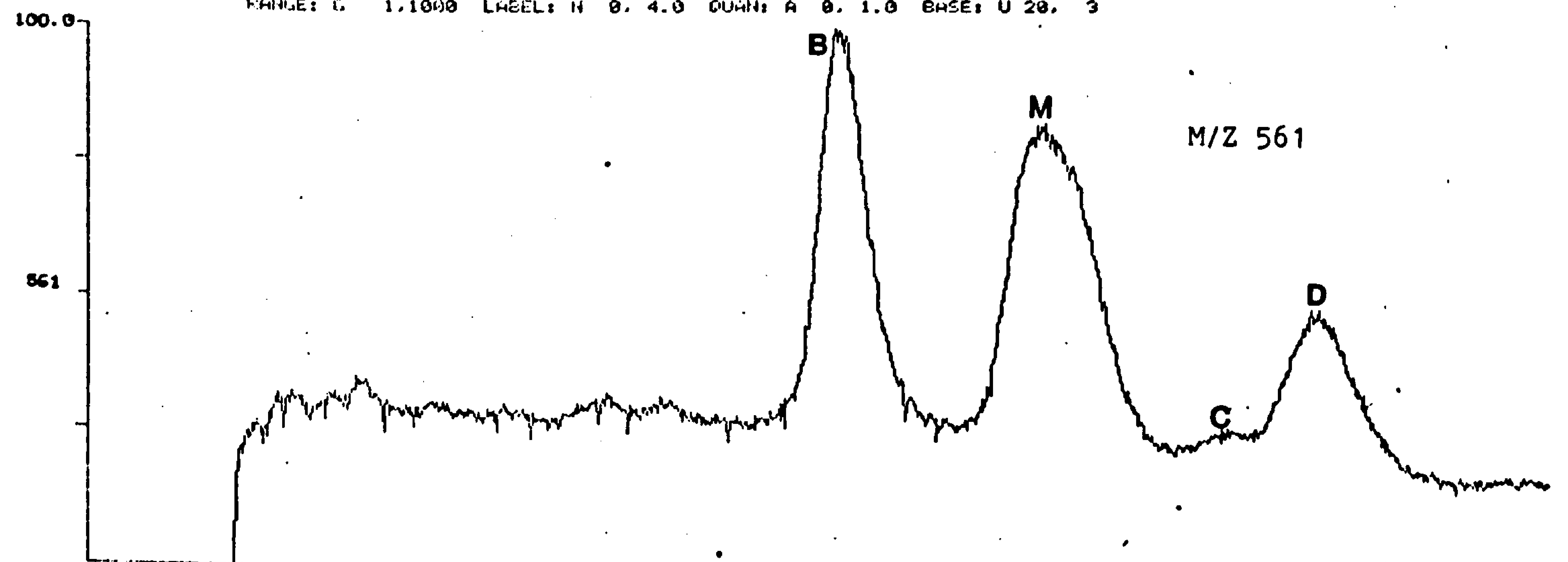
Moulting hormone titre points representing the peak titre points (40 hrs plus 48 hrs, 72 hrs plus 80 hrs, and 120 hrs) were chosen for further analysis by Gas-liquid chromatography/Mass spectrometry (selected ion monitoring) [GC/MS (SIM)]. The three samples were first re-analysed by RIA to confirm the quantities of ecdysteroid remaining in each sample. Insufficient material was available for GC/MS(SIM) analysis from the 72 hr plus 80 hr peak titre samples. However, the quantities detected for the 40 hr plus 48 hr peak titre (4760 pg ecdysone equivalents by DHS 1-15) and the 120 hr peak titre (4700 pg ecdysone equivalents by

DHS 1-15) were adequate for such an analysis.

These two samples were, therefore, derivatised by silylation and subjected to GC/MS(SIM) analysis (for details see Part 2). Fragment ions were monitored for the detection of 20-hydroxyecdysone related structures (m/z 561) and ecdysone related structures (m/z 567) (figures 4B-4, 4B-5), and ecdysone and 20-hydroxyecdysone quantified by reference to appropriate standard curves (see Part 2), and identified by their relative retention times to Makisterone A (table 4B-2).

20-Hydroxyecdysone (labelled B in figures 4B-4 and 4B-5) is the principal identifiable ecdysteroid in both the samples with smaller amounts of ecdysone (A) and 20,26-dihydroxyecdysone (C) present in the 40 plus 48 hrs haemolymph sample. Also present in the 40 plus 48 hour chromatogram is a peak (m/z 561) at RRt=1.3 (labelled D, figure 4B-4). Although this peak shows the ion m/z 561 it could be non-ecdysteroidal. The chromatographic position of hepta-TMS 20-hydroxyecdyson-26-oic acid is not known. The total ion chromatogram of the silylated ecdysteroids from the 128 hr haemolymph sample displays two major peaks (E,RRt=0.51; F,RRt=0.62) chromatographing earlier than 20-hydroxyecdysone. These are most likely to be non-ecdysteroidal; none of the investigated authentic ecdysteroids detectable with selected ion monitoring at m/z 561 or 567 is known to chromatograph at these retention times.

The aforementioned GC/MS analyses were originally designed merely as a preliminary to further experiments for the full characterisation of ecdysteroids at peak hormone titres. A fresh set of haemolymph samples of larvae at all peak titres were extracted and prepared in the same manner as before. Silylation of the samples, however, was not achieved, although standard ecdysteroids in separate samples were silylated. Even silylation of the internal standard, Makisterone A did not occur in the biological samples. The reason for this is unknown, and as insufficient



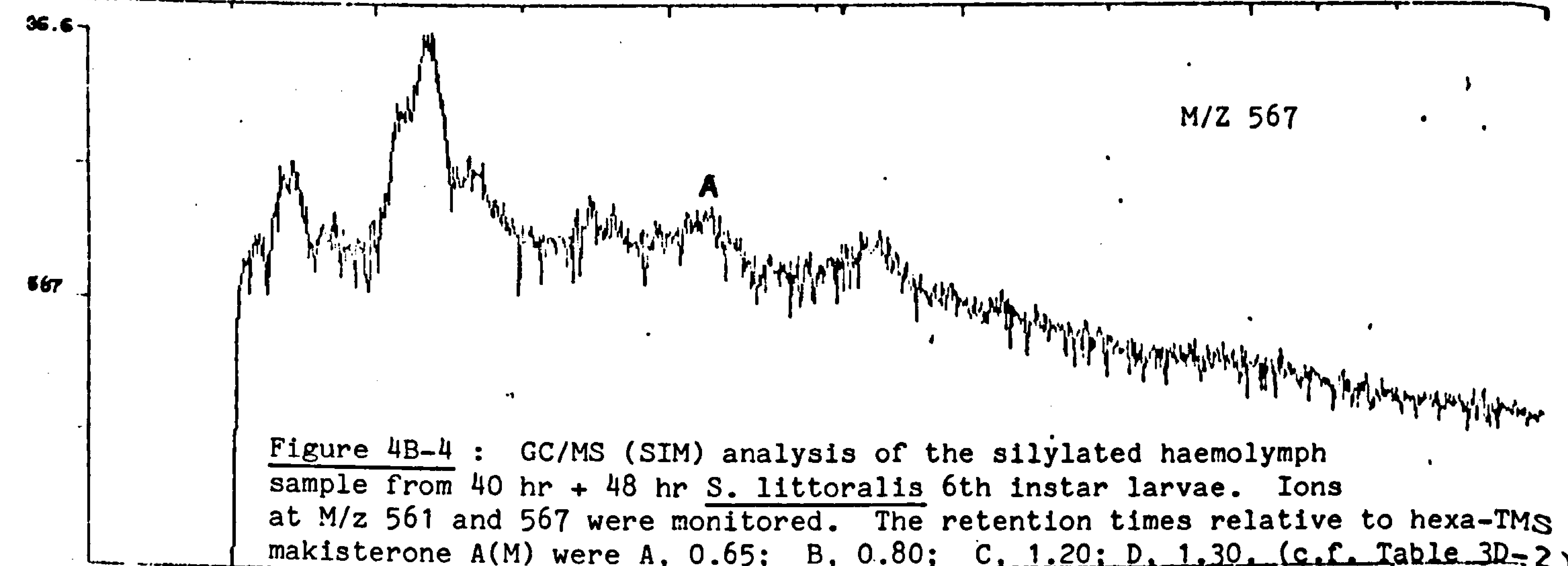
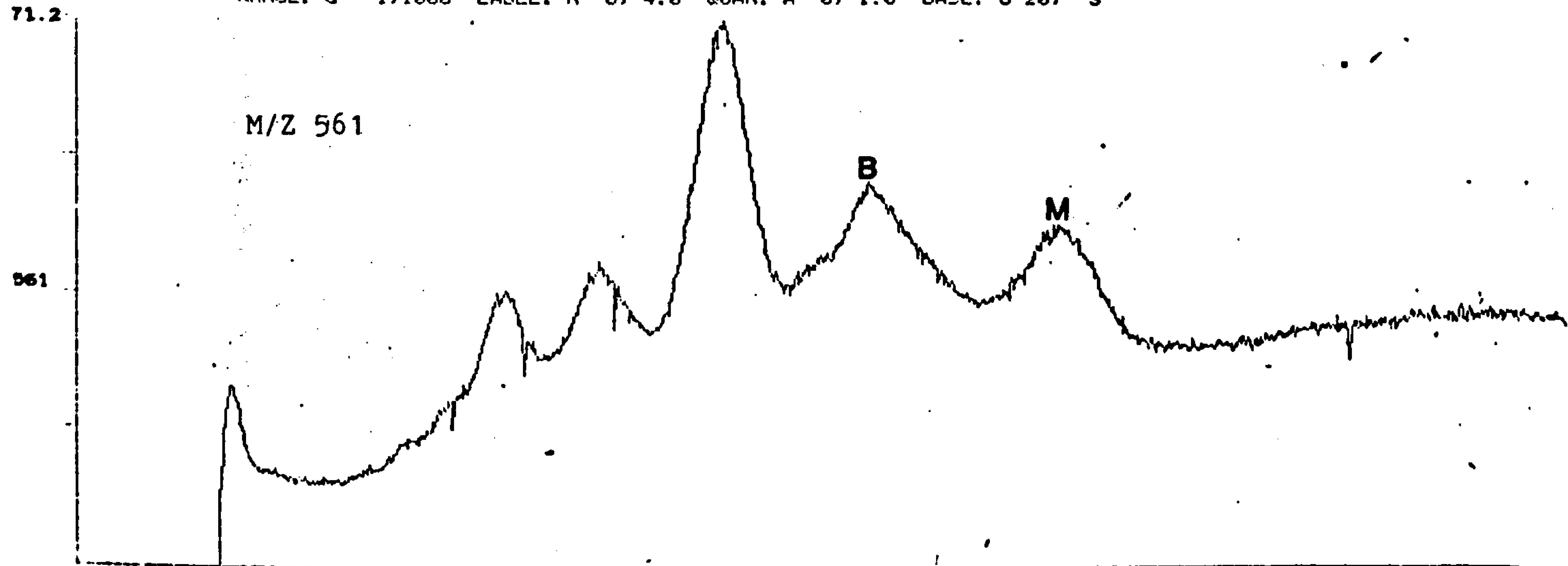


Figure 4B-4 : GC/MS (SIM) analysis of the silylated haemolymph sample from 40 hr + 48 hr S. littoralis 6th instar larvae. Ions at M/z 561 and 567 were monitored. The retention times relative to hexa-TMS makisterone A(M) were A, 0.65; B, 0.80; C, 1.20; D, 1.30, (c.f. Table 3D-2)

100.0
RIC
521
651
645
239
142
357
Total ion chromatogram
scan time
200 2:20
400 4:40
600 7:00
800 9:20



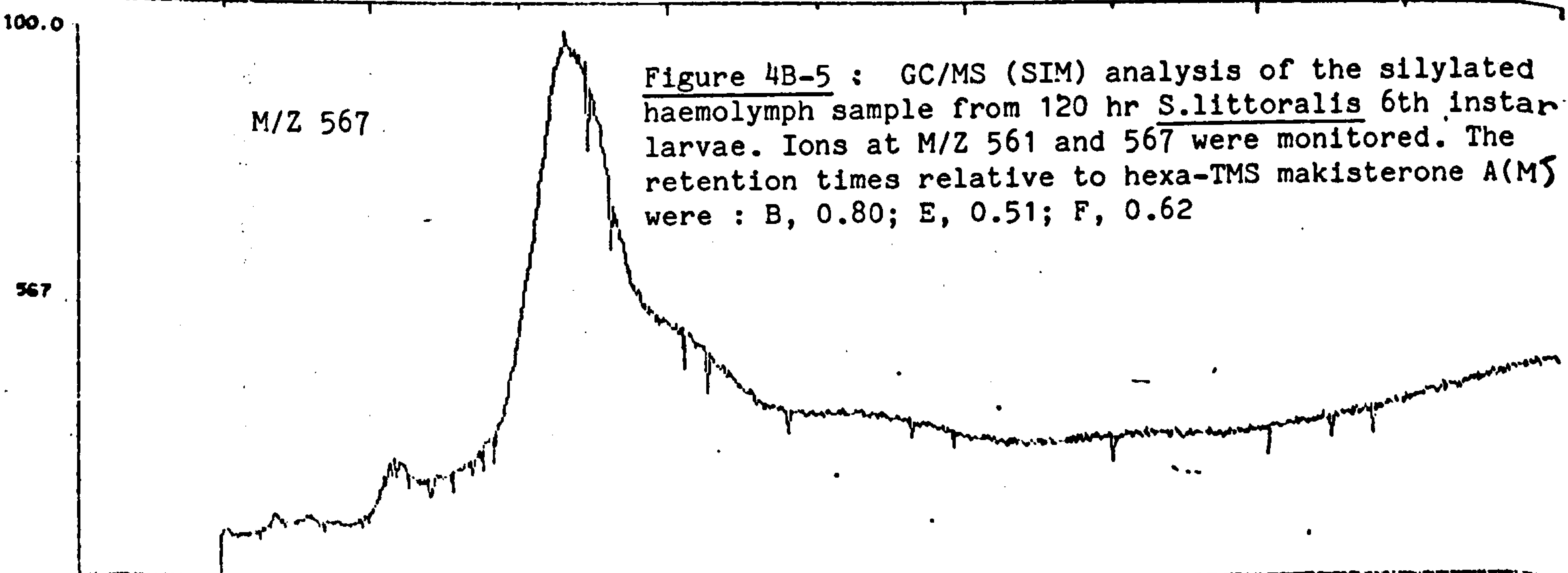


Figure 4B-5 : GC/MS (SIM) analysis of the silylated haemolymph sample from 120 hr S.littoralis 6th instar larvae. Ions at M/Z 561 and 567 were monitored. The retention times relative to hexa-TMS makisterone A(M) were : B, 0.80; E, 0.51; F, 0.62

100.0

Total ion chromatogram

E 344

F 438

538

670

745

218

106

RIC

scan time

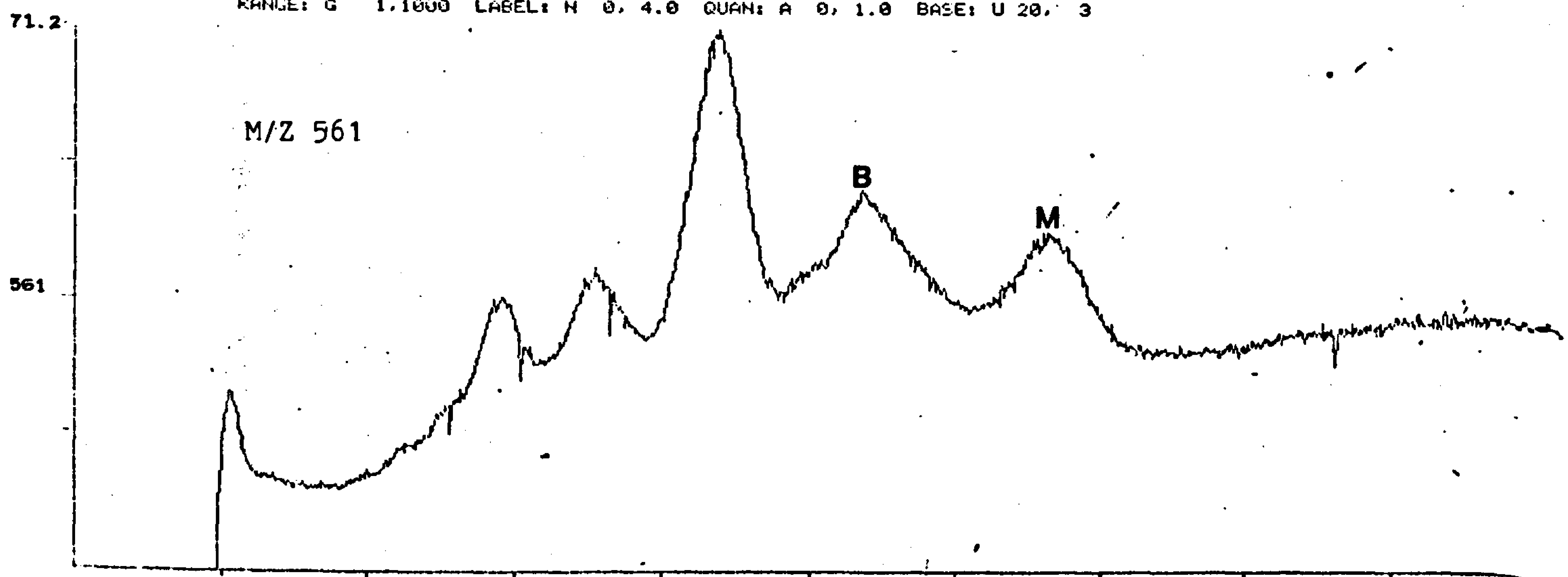
200 2:20

400 4:40

600 7:00

800 9:20

1000



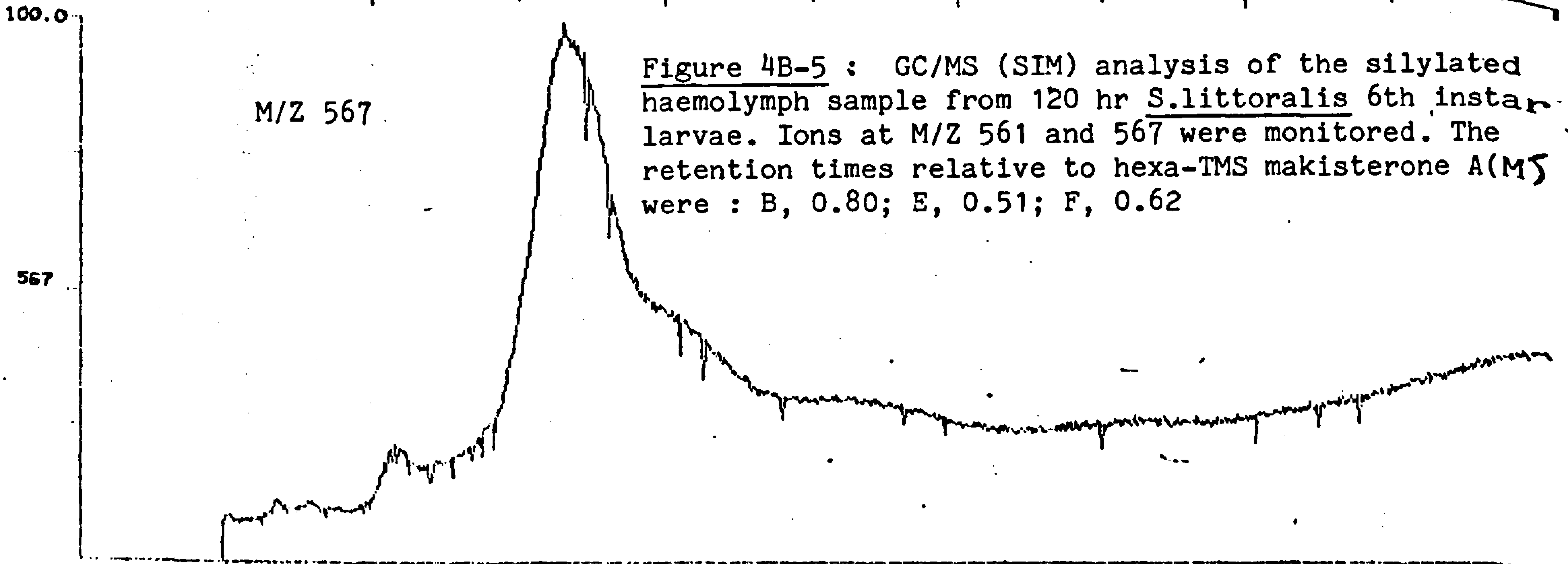


Figure 4B-5 : GC/MS (SIM) analysis of the silylated haemolymph sample from 120 hr S.littoralis 6th instar larvae. Ions at M/Z 561 and 567 were monitored. The retention times relative to hexa-TMS makisterone A(M) were : B, 0.80; E, 0.51; F, 0.62

100.0

E 344

Total ion chromatogram

F 438

538

RIC

670

745

218

106

scan time

200 2:20

400 4:40

600 7:00

800 9:20

1000

Table 4B-2: Relative retention times of fully silylated TMS ethers of some ecdysteroids by GC/MS (SIM) using a 1.5 m x 2 mm glass column packed with 1% (w/w) OV-1 silicone phase on Gas Chrom. Q (100-200 mesh) (Mendis *et al.*, 1983).

Ecdysteroid	Retention time relative to Makisterone A
Ponasterone A	0.54
Ecdysone	0.63
3-Epiecdysone	0.63
20-Hydroxyecdysone	0.79
3-Epi-20-hydroxyecdysone	0.79
Inokosterone	0.85
26-Hydroxyecdysone	0.95
Makisterone A	1.00
20,26-Dihydroxyecdysone	1.21
Podecdysone A	1.17

time was available for the completion of this aspect of the research, a thorough characterisation of ecdysteroids at all stages of the 6th instar larvae was unfortunately not achieved.

However, the GC/MS analyses of the two samples successfully completed provide much information on the composition of the ecdysteroids, during this stage of S. littoralis development. The quantities of ecdysteroids identified by this method for both haemolymph samples (40 plus 48 hrs, 120 hrs) are presented in table 4B-4. The results demonstrate that 20-hydroxyecdysone (and/or 3-epi-20-hydroxyecdysone) is the main ecdysteroid at the first and the last peak of the haemolymph moulting hormone titre. Smaller amounts of ecdysone and 20,26-dihydroxyecdysone were detectable in the 40 plus 48 hr sample. Although ecdysone was not detected in the 120 hr sample it would be reasonable to expect that some is present but was not detectable because the sensitivity of selected ion monitoring at m/z 567 for ecdysone is only one tenth of that for 20-hydroxyecdysone detected at m/z 561.

It is interesting to note that the total ecdysteroids detected by GC/MS in both samples are considerably higher than the values obtained by RIA with the ICT-1 antiserum, indicating that, as previously suggested, many of the ecdysteroids could be in the 3α-hydroxy isomeric form.

Analysis of the conjugated ecdysteroids present in the haemolymph of sixth instar S. littoralis larvae

As was indicated in Part 4 section A, ecdysteroids during this instar are also present in the form of conjugates. It was, therefore, decided to analyse the ecdysteroid conjugates at the moulting hormone peak titres (viz. 40 hours, 76 hours, 120 hours, 144 hours).

Groups of sixth instar larvae representing the peak ecdysteroid titre points at 40 hours, 76 hours, 120 hours, and 144 hours of 6th instar development were collected and the haemolymph drained from the larvae as previously explained.

The haemolymph samples were then purified by silica SEP-PAK chromatography to separate free ecdysteroids from the highly polar ecdysteroid fraction (containing conjugated ecdysteroids) (for details see Part 2). The highly polar ecdysteroid fraction was then subjected to hydrolysis by a H. pomatia enzyme preparation to release ecdysteroids from conjugation. The ecdysteroids thus produced were then further purified by a second silica SEP-PAK purification, the ecdysteroids eluting in the 30% methanol/chloroform fraction (for details see Part 2).

The quantities of ecdysteroids released from conjugation in this way were then analysed by radioimmunoassay, as were the quantities of free ecdysteroids isolated from the haemolymph (Table 4B-3). The quantities of moulting hormone equivalents detected in the free ecdysteroid fraction are, as expected, very similar to those obtained for the RIA moulting hormone titre (using ICT-1 antiserum) (c.f. figure 4B-3).

The ecdysteroids released from conjugation were also analysed by GC/MS by the method previously explained. Because of the low levels of conjugated ecdysteroids (see Table 4B-3), the four haemolymph samples (40, 76, 120 and 140 hrs) were combined to provide sufficient material for GC-MS. A quarter of the sample of the released ecdysteroids (8ng ecdysone equivalents) were silylated by the method described in Part 2, and subjected to GC/MS. Fragment ions were monitored for the detection of 20-hydroxyecdysone related structures (m/z 561) and ecdysone-related structures (m/z 567) (figure 4B-6). Ecdysone and 20-hydroxyecdysone were quantified by the method described in Part 2. The predominant

Table 4B-3: The quantities of ecdysteroids present in (1) the free ecdysteroid fraction and (2) the fraction containing ecdysteroids released from conjugation by *H. Pomatia* enzyme, for the peak R.I.A. ecdysteroid titre points of the haemolymph of sixth instar *S.littoralis* larvae.

Age within instar (hours)	RIA value (ICT-1) (pg ecdysone equivalents/insect)	
	(1) Free ecdysteroids	(2) Conjugated ecdysteroids (ecdysteroids released from conjugation by *H. pomatia* enzyme)
40	2420	38
76	1240	94
120	5740	454
144	3940	1014

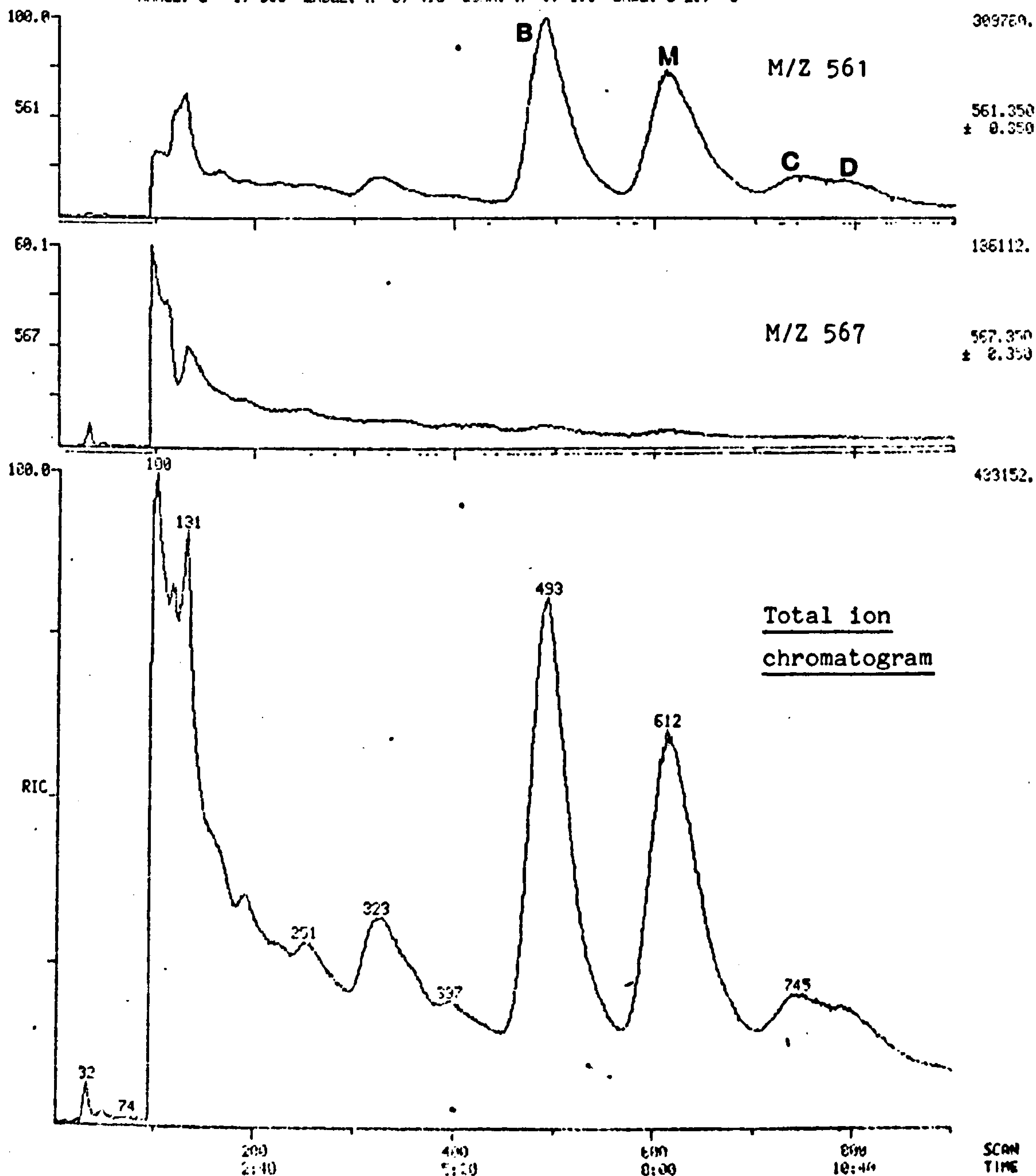


Figure 4B-6 : GC/MS (SIM) analysis of the silylated ecdysteroids released from conjugation by hydrolysis with H. pomatia enzyme, as isolated from the haemolymph of 6th instar of S. littoralis larvae. The retention times (R_t) relative to hexa-TMS makisterone A(M) were: B, 0.80; C, 1.22; D, 1.29. Authentic compounds were chromatographed prior to this analysis, the retention times (R_t) relative to hexa-TMS makisterone A(M) were: penta-TMS ecdysone, 0.65; hexa-TMS 20 hydroxyecdysone, 0.80.

ecdysteroid identified in this fraction was 20-hydroxyecdysone (B; 0.77 ng/insect) with a much smaller amount of 20,26-dihydroxy-ecdysone being present (C; 0.05 ng/insect). The composition of the ecdysteroids in the conjugated form thus reflects the composition of the free ecdysteroids, the predominant ecdysteroid being 20-hydroxyecdysone.

Summary of the ecdysteroid composition in the haemolymph of 6th instar larvae as detected by GC/MS

The quantitative results obtained from the three GC/MS analyses are shown in Table 4B-4. The peaks on GC-MS were identified by comparison of their retention times relative to Makisterone A with those of authentic standards (Table 4B-2), and by the detection of selected ions.

The peaks corresponding to 20,26-dihydroxyecdysone were quantified from GC-MS analysis of a standard solution of 20-hydroxyecdysone. Therefore, the results obtained for 20,26-dihydroxyecdysone must only be considered as approximations.

The results expressed in Table 4B-4 establish that the principal ecdysteroid present in the haemolymph of the 6th instar larvae is 20-hydroxyecdysone (and/or 3-epi-20-hydroxyecdysone). Smaller amounts of ecdysone, and 20,26-dihydroxyecdysone were also detected at the 40 hrs plus 48 hrs peak RIA titre. Although ecdysone is not detected at 120 hrs, it would be reasonable to assume that a small quantity of ecdysone is present, and that the failure to detect this compound is probably due to the reduced sensitivity of the GC/MS method for this molecule (ten times less sensitive than for 20-hydroxyecdysone).

The ecdysteroid composition of the ecdysteroids released from conjugation is, as expected, similar to the free ecdysteroids.

Table 4B-4: Quantitative analysis by GC/MS of the ecdysteroids in the peak titre fractions 40 hours plus 48 hours and 120 hours of the haemolymph of sixth instar S.littoralis larvae and of the ecdysteroids released from conjugation by H.pomatia enzyme from the haemolymph of 6th instar S.littoralis larvae.

Ecdysteroids	Free ecdysteroids		Ecdysteroids present in the form of conjugates
	Peak titre fraction 40 + 48 hrs ng/insect	Peak titre fraction 120 hrs ng/insect	ng/insect
Ecdysone (3-epiecdysone)	2.83	-	-
20-Hydroxyecdysone (3-epi-20-hydroxy-ecdysone)	11.46	9.57	0.77
20,26-Dihydroxy-ecdysone	0.40	-	0.05

- Denotes not detected

20-hydroxyecdysone is the major component with a small quantity of 20,26-dihydroxyecdysone present.

3. Ecdysteroid analysis of the faeces of sixth instar S. littoralis larvae

Analysis by radioimmunoassay of the free and conjugated ecdysteroids present in the faeces of sixth instar S. littoralis larvae

During early 6th instar development, S. littoralis larvae undergo a rapid increase in size and weight (figure 4B-1). This is, of course, accompanied by a particularly active period of feeding and excreting, and so the faeces must be analysed to provide a complete assessment of larval ecdysteroids. Indeed, the faeces of larvae have been found to contain relatively large amounts of ecdysteroid inactivation products after the incubation in vivo of [^{3}H] ecdysone (see Part 4, section A). Therefore, the examination of the endogenous ecdysteroids present in the faeces was undertaken.

Eighteen S. littoralis larvae were synchronised at the 5th/6th instar larval moult, and reared under normal conditions until 60 hours of 6th instar development. The faeces produced during that time were collected and combined with the gut contents of the larvae.

The faeces plus gut contents (14.1g), thus collected, were then extracted by the procedure described in Part 2 and further purified by silicic acid column chromatography, which separates free ecdysteroids (30% methanol/chloroform column fraction), and highly polar ecdysteroids, including ecdysteroid conjugates (80% methanol/chloroform column fraction). The ecdysteroid conjugate fraction was then subjected to enzymic hydrolysis with the H.pomatia enzyme preparation. The ecdysteroids released from conjugation were then separated by the use

of a second silicic acid column, the released ecdysteroids being eluted in the 30% methanol/chloroform column fraction (for details of this method see Part 2).

The ecdysteroid content of the free ecdysteroid fraction, the fraction containing ecdysteroids released from conjugation by H.pomatia enzyme preparation, and a sample of the diet used for feeding the larvae during this experiment, were all analysed by radioimmunoassay using the ICT-1 antiserum (see Part 2 for details). The results of this analysis are shown in Table 4B-5, demonstrating that the majority of the ecdysteroids are in the form of conjugates (84%).

The analysis of the faeces by RIA has demonstrated that a large proportion of the ecdysteroids are in the conjugated form. For further analysis of the fraction containing the ecdysteroids released from conjugation, GC/MS (SIM) was employed.

An aliquot of the sample (equivalent to 0.778g; 1 insect) was derivatised by silylation and subjected for GC/MS analysis (for method see Part 2). Ions were monitored at m/z 561, and m/z 567 (figure 4B-7).

The ecdysteroid composition of the faeces of 6th instar larvae of S. littoralis

Radioimmunoassay of the free and the conjugated ecdysteroids in the faeces, has demonstrated that, as excretory products, ecdysteroids occur predominantly in the form of conjugates (14.714 ng ecdysone equivalents/g faeces) with much smaller quantities of free ecdysteroids (2.786 ng ecdysone equivalents/g faeces).

The composition of the ecdysteroid moieties of the conjugates has been investigated by GC/MS (figure 4B-7). Once again, 20-hydroxy-ecdysone (B; relative Rt=0.80) proved to be the major ecdysteroid

Table 4B-5: Radioimmunoassay (using ICT-1 antiserum) of the ecdysteroids from the free ecdysteroid fraction and the fraction containing ecdysteroids released from conjugation by *H. pomatia* enzyme, as isolated from the faeces of *S.littoralis* larvae.

	ng Ecdysone equivalent/g faeces (or diet)	ng Ecdysone equivalent/ insect
Diet	0.305	-
Free ecdysteroids	2.786	2.182
Ecdysteroids released from conjugation by *H.pomatia* enzyme	14.714	11.526

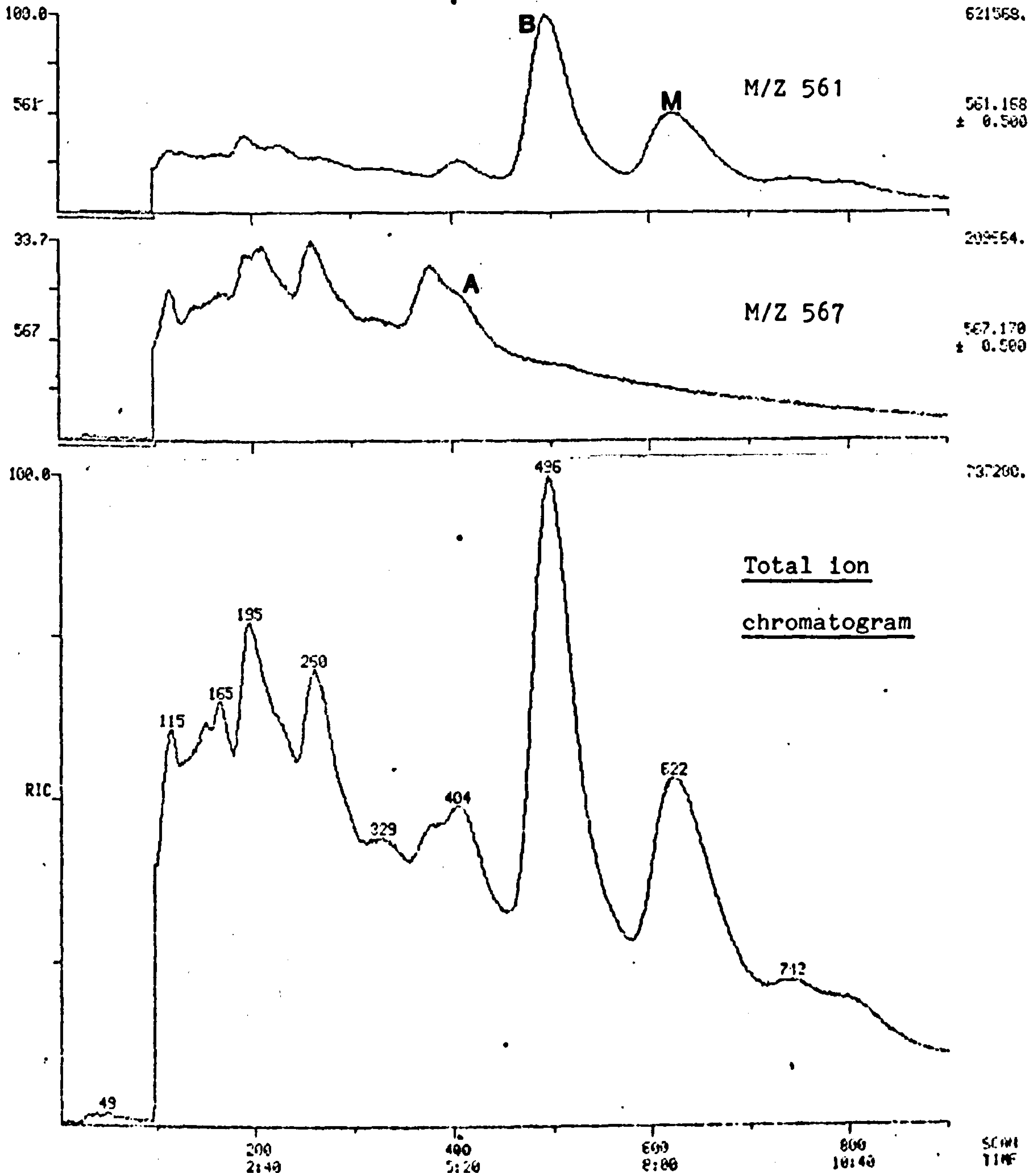


Figure 4B-7 : GC/MS (SIM) analysis of the silylated ecdysteroids released from conjugation by hydrolysis with H. pomatia enzyme, as isolated from the faeces of 6th instar S.littoralis larvae. The retention times relative to hexa-TMS makisterone A(M) were: A,0.65; B, 0.80. Authentic compounds were chromatographed prior to this analysis, the retention times (Rt) relative to hexa-TMS makisterone A(M) were : penta-TMS ecdysone, 0.69; hexa-TMS 20-hydroxyecdysone, 0.80.

(6.08 ng/insect) in the conjugated fraction. Ecdysone was also detected, and although the GC/MS peak corresponding to ecdysone (A; relative Rt=0.65) occurs as a shoulder on a contaminant peak, the quantity was calculated as 2.01 ng/insect.

PART 4 SECTION C

PART 4 SECTION C

Developmental variation of the ecdysone metabolising enzymes *in vitro* during the 6th instar larval stage of *S. littoralis*

Page No.

Introduction 141

Experimental and results

1. Tissue localisation of the ecdysone hydroxylase and 3-epimerase enzymes in 6th instar larvae of *S. littoralis* 144

    Tissue localisation of 20-hydroxylase, 26-hydroxylase and 3-epimerase enzymes in 116 hour 6th instar larvae of *S. littoralis* 144

    Extraction and assay procedure 145

    Analysis of the enzymic activities in the various tissues 146

    Additional tissue localisation studies of the enzymic activity at 72 hrs and 126 hrs of development 147

2. Determination of 20-hydroxylase titre curves in the homogenates of fat body and Malpighian tubules of developing 6th instar larvae of *S. littoralis* 148

3. Determination of 3-epimerase titre curves in the homogenates of midgut tissue of developing 6th instar larvae of *S. littoralis* 149

PART 4 SECTION C

Developmental variation of the ecdysone metabolising enzymes *in vitro* during the 6th instar larval stage of *S. littoralis*

INTRODUCTION

The metabolism *in vivo* of ecdysone in *Spodoptera* 6th instar larvae has demonstrated the presence of 20- and 26-hydroxylating processes and some 3-epimerisation. The developmental variations of the enzymes responsible for these reactions was investigated in tissue homogenates of the 6th instar larvae to endeavour to assess the contribution of each enzyme to the control of the ecdysteroid titre.

Both the 20- and 26-hydroxylase enzyme systems have been shown to be present in intact fat body and Malpighian tubules of the related Lepidopteran *Manduca* prepupae (King, 1972). However, during the present study, no 26-hydroxylation was observed in any tissue homogenates of *S.littoralis* during the entire development of 6th instar larvae. Therefore it appears that homogenization disrupted the 26-hydroxylase enzyme system, and so an intact or sliced tissue incubation would probably be required to detect 26-hydroxylase activity. However, this was not undertaken and research was concentrated on the enzymic activities displayed by the homogenate preparation (i.e. 20-hydroxylation and 3-epimerisation).

The ecdysone C-20 hydroxylase enzyme has been investigated in a number of insect tissues: *Manduca sexta* midgut (Nigg *et al.*, 1976; Mayer *et al.*, 1978; Kaplanis et al., 1980), *M. sexta* fat body (Bollenbacher *et al.*, 1977; Smith et al., 1980), *Locusta migratoria* fat body, Malpighian tubules and midgut (Feyereisen and Durst, 1978), *Musca domestica* (Yu and Terriere, 1974), and *Schistocerca gregaria* Malpighian

tubules and fat body (Johnson, 1976; Greenwood, 1981). Indeed, the activity of this enzyme is principally found in these tissues. In the lepidopteran, M. sexta, activity was highest in the fat body (Smith et al., 1980), but in the Orthopterans, S. gregaria and L.migratoria, the highest specific activity was found in the Malpighian tubules (Greenwood, 1981; Feyereisen and Durst, 1978).

The specific activity of 20-hydroxylase in the Malpighian tubules has been shown to undergo developmental variation during the final larval instar of S. gregaria (Johnson and Rees, 1977) and L.migratoria (Feyereisen and Durst, 1980). In these cases the changes in hydroxylase activity correlates closely to the total ecdysteroid titre. However, the hydroxylase activity observed in fat body preparations during the final larval instar of the holometabolous insects, M. sexta (Smith et al., 1980) and Calliphora erythrocephala (Koolman, 1980), shows that a decrease in 20-hydroxylase occurs before the major ecdysteroid peak. The control mechanism of the ecdysteroid titre may therefore vary from species to species, although the tissues chosen for such an investigation and the preparation employed may have considerable bearing upon the developmental variations of enzyme activity observed. Indeed, in this study, 20-hydroxylase activity was monitored in both Malpighian tubules and fat body in order to assess their relative importance and to correlate these to the haemolymph ecdysteroid titre of 6th instar larvae (figure 4B-3).

The incubation procedure for the present study was based on that employed for determination of 20-hydroxylase activity in the fat body of the Lepidopteran, M. sexta (Smith et al., 1979). In that study, pH and incubation temperature were optimised at 7.5 and 30°C, respectively, and hydroxylation of ecdysone by the fat body mitochrondrial fractions was linear up to 30 min at enzyme concentrations of 2.5 and 7.5 mg tissue

equivalent. 20-Hydroxylase activity in fat body homogenates was NADPH dependant, with the addition of an NADPH-generating system being slightly more effective than the addition of exogenous NADPH (Smith _et al._, 1979).

In addition to the presence of 20-hydroxylase activity, ecdysone 3-epimerase activity has been observed in the midgut tissue of 6th instar _Spodoptera littoralis_ larvae. Therefore, it was possible to monitor the activity of this enzyme throughout this stage of _S.littoralis_ development by using midgut tissue homogenates.

The ecdysone 3-epimerase reaction has been studied in _Manduca sexta_ (Nigg _et al._, 1974; Meyer _et al._, 1979). However, very little data is available on the variation of its activity during insect development. Therefore, it would be interesting to establish any correlation between the ecdysteroid titre and 3-epimerase activity.

Ecdysone 3-oxidase, originally studied in _Calliphora erythrocephala_, resembles ecdysone 3-epimerase in its requirement for molecular oxygen. Indeed, it has been suggested that the ecdysone 3-epimerase system consists of two enzymes, ecdysone 3-oxidase and 3-dehydroecdysone reductase (Koolman, 1976) which convert ecdysone via 3-dehydroecdysone into 3-epiecdysone.

The developmental variation of the ecdysone oxidase enzyme has been investigated in _Calliphora_ (Koolman, 1978). In that study it was shown that the ecdysteroid titre of the insect and the ecdysone 3-oxidase activity varied in parallel. Where the ecdysteroid titre was high, in the eggs and pupae, ecdysone oxidase was also high, whereas during the larval stages the ecdysteroid levels were low as was the ecdysone oxidase activity. As 3-dehydroecdysone is much less active than ecdysone in biological assays (Koolman and Spindler, 1977), this would therefore imply, that metabolism via ecdysone oxidase is an important part of the

inactivation of moulting hormones in Calliphora. In the present study the possibility of an equivalent role for the 3-epimerisation process in Spodoptera will be investigated.

EXPERIMENTAL AND RESULTS

1. Tissue localisation of the ecdysone hydroxylase and 3-epimerase enzymes in 6th instar larvae of S. littoralis

The ecdysteroid titre (by RIA) of the haemolymph of 6th instar larvae (see figure 4B-3) indicates a large peak of ecdysteroid at 120 hrs of development. The GC/MS analysis of the ecdysteroids at this stage of development (120 hrs) identified 20-hydroxyecdysone as the principal ecdysteroid present (Table 4B-4). Therefore it can be assumed that during increase in the titre of this R.I.A. peak the 20-hydroxylase enzyme will be active. For this reason, larvae at 116 hours of 6th instar development were chosen for the initial tissue localisation of 20-hydroxylase, 26- hydroxylase and 3-epimerase enzymes.

Tissue localisation of 20-hydroxylase, 26-hydroxylase and 3-epimerase enzymes in 116 hour 6th instar larvae of S. littoralis

Larvae (animals at 116 hr within the 6th instar) were dissected in chilled insect Ringer solution, under a binocular microscope. The tissues dissected were as follows: Malpighian tubules, fat body, testes, salivary glands, midgut (gut contents and peritrophic membrane removed), hindgut, head plus gizzard, and nervous tissue. These tissues plus the carcass and the haemolymph were separated for individual analysis.

Each tissue was rinsed with homogenisation buffer (0.05M potassium phosphate buffer pH 7.5 containing 0.25M sucrose) most of the buffer was

blotted and the tissue was then weighed and kept on ice. The tissues were then homogenised in a known volume of homogenisation buffer to achieve a final tissue concentration of 80 mg tissue/ml buffer. Homogenisation was performed using a 2 ml Potter Elvehjem homogeniser, with 4 gentle passes of the pestle for each homogenate. All apparatus and solutions were kept on ice during the procedure.

The homogenates were incubated and assayed for a number of reactions: ecdysone 3-epimerisation, 20-hydroxylation and 26-hydroxylation. The amount of homogenate was derived from approximately the same weight of tissue in each case. The incubation mixtures were as follows:

- 50 µl homogenate (80 mg/ml)
- 50 µl NADPH Generating system (2×10^{-4}M $NADP^+$, 2×10^{-3}M Glucose-6-phosphate, 0.6 i.u. Glucose-6-phosphate dehydrogenase) in 0.05M Potassium phosphate buffer pH 7.5.
- 50 µl [23,24 $^3H_2$] ecdysone solution (615,000 dpm, 53.6 Ci/mmol)

The tissues were incubated for 30 minutes at 30°C with moderate agitation in a reciprocating water bath. The incubations were then extracted and assayed using h.p.l.c. analysis.

Extraction and assay procedure

Each reaction was stopped by the addition of 0.8 ml of chilled ethanol and the mixture was then centrifuged to separate the precipitated protein from the supernatant. The pellet was re-extracted with ethanol, the supernatants were combined and the supernatants evaporated to dryness. The residue was dissolved in 10% methanol/water and the potassium phosphate salts were removed by reversed-phase SEP-PAK, as explained in Part 2. The de-salted sample was then ready for h.p.l.c. analysis.

The h.p.l.c. analysis was used to monitor either 20-hydroxylation of ecdysone to 20-hydroxyecdysone or 3-epimerisation of ecdysone to 3-epiecdysone. For these assays, two different h.p.l.c. systems were required:

(i) 20-hydroxylase assay: For separation of [^{3}H] ecdysone (substrate) from [^{3}H] 20-hydroxyecdysone (the product) a Waters Associates Z module ODS reversed-phase column eluted with a 40% to 80% (^{v}/v) methanol/water gradient over 20 minutes (1.5 ml/min) was utilised (see figure 4C-1). Fractions were collected every minute and radioassayed in scintillation cocktail.

(ii) 3-epimerase assay: An APS-Hypersil column developed isocratically with 5.5% (^{v}/v) methanol/dichloroethane (2 ml/min) provided good separation of ecdysone and 3-epiecdysone (see figure 4C-2). Fractions were collected every minute and radioassayed in scintillation cocktail.

Analysis of the enzymic activities in the various tissues

In all the tissues assayed formation from [^{3}H] ecdysone of 26-hydroxylated ecdysteroids, either in the form of 26-hydroxyecdysone or 20,26-dihydroxyecdysone could not be detected. However, in a number of tissues, considerable 20-hydroxylase activity was detected. The conversion of ecdysone into 20-hydroxyecdysone by the different tissues is presented in Table 4C-1. It is apparent that, at least at this stage of development (116 hr within the 6th instar) the Malpighian tubules contain the highest 20-hydroxylase activity per unit weight of tissue. It appears also that the fat body contains most of the 20-hydroxylase activity per insect.

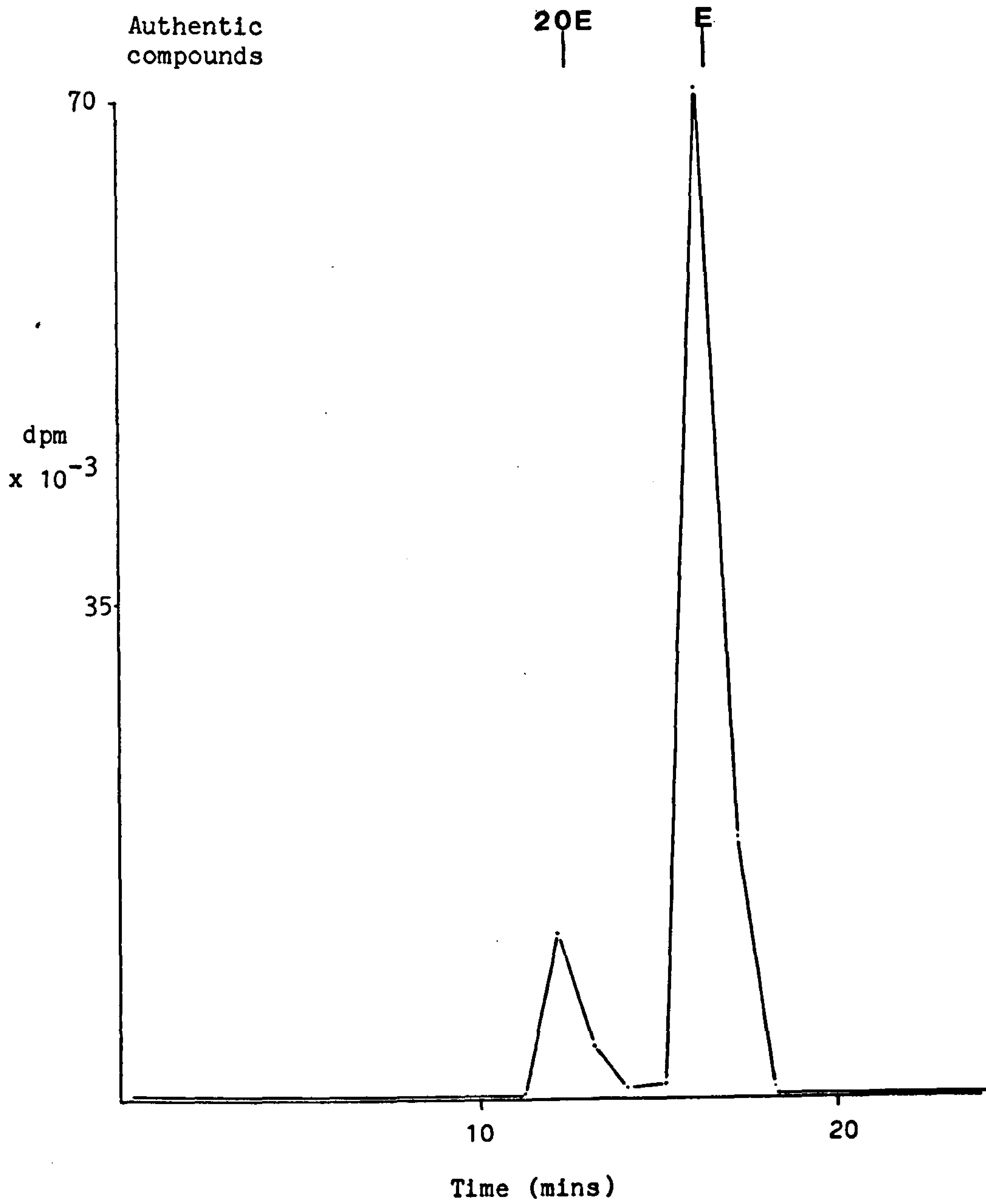


Figure 4C-1 : Reversed-phase h.p.l.c. analysis of the products of metabolism *in vitro* of [^{3}H] ecdysone by ecdysone 20-hydroxylase (for conditions see text)

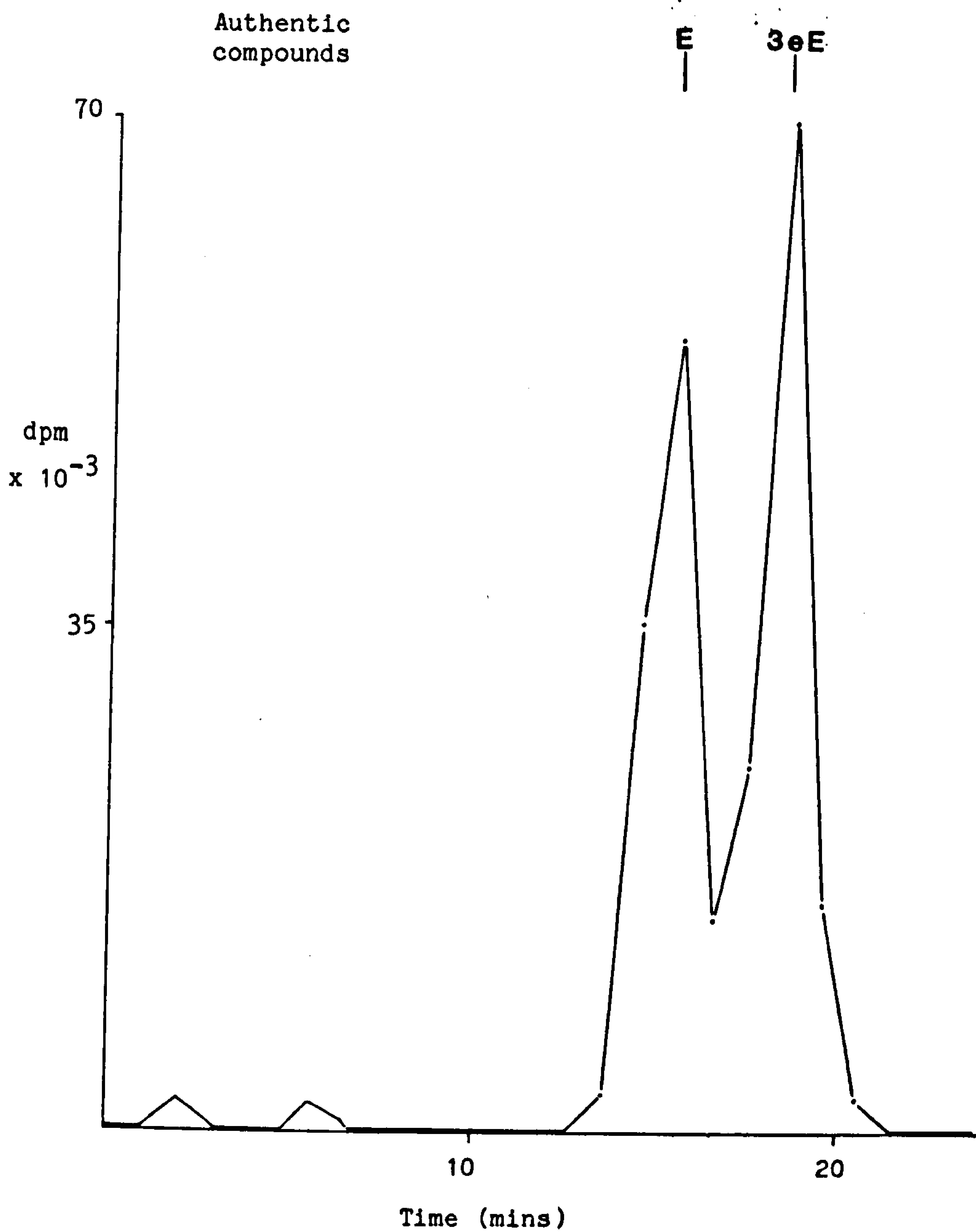


Figure 4C-2 : H.p.l.c. analysis on an APS-Hypersil column (adsorption) of the products of the metabolism *in vitro* of [^{3}H] ecdysone by ecdysone 3-epimerase (for conditions see text).

Table 4C - 1 : The percentage conversion of [^{3}H] ecdysone by 3-epimerase and 20-hydroxylase enzymes into [^{3}H] 3-epiecdysone and [^{3}H] 20-hydroxyecdysone, respectively, in *S. littoralis* larval tissue homogenates taken at various times during 6th instar development. -, denotes not detected.

Tissue	Number of insects the tissue was derived from [c]			% Conversion [a,b] of [^{3}H] ecdysone to					
				20-hydroxyecdysone			3-epiecdysone		
Age(hr)	72	116	126	72	116	126	72	116	126
Fat body	0.12	0.08	0.07	86	6	5	-	-	-
Malpighian tubules	1.44	3.3	3.8	7	46	-	-	-	-
Midgut	0.24	0.86	1.14	-	16	-	94	67	-
Testes	3	3	3.6	14	10	-	-	-	-
Head and gizzard		1.07			5			5	
Salivary glands		1.33			-			-	
Hind gut	016[d]	2.93	0.08[d]	7[d]	5	5[d]	-[d]	-	-[d]
Nervous tissue		1.62			-			-	
Haemolymph		0.26			-			-	
Remaining carcass		0.04			-			-	

a. Based on the total [^{3}H] recovered from the h.p.l.c. in ecdysone, 3-epiecdysone, 20-hydroxyecdysone

b. A boiled fat body incubation was used as control for all three developmental stages (72, 116, 126 hr). No conversion of ecdysone was detected.

c. The amount of homogenate incubated was derived from approximately 12 mg of tissue in each case. The number of insects from which this was derived is presented in this column.

d. Conversion obtained from homogenates of carcass minus fat body, Malpighian tubules, midgut and testes.

3-Epimerase activity was virtually limited to the midgut, 67% (1815 pg ecdysone converted) of the radioactivity recovered being in the form of 3-epi ecdysone (66%) and 3-epi 20-hydroxyecdysone (1%; Table 4C-1).

<u>Additional tissue localisation studies of enzymic activity at 72 hrs and 126 hrs of development</u>

Tissue localisation of ecdysteroid metabolising enzymes were also carried out at other times during 6th instar development, early in the instar at 72 hrs of 6th instar development, and also at 126 hrs of development at which time the ecdysteroid titre (by RIA) undergoes a sharp decrease (see figure 4B-3). At the latter time, rapid metabolism of 20-hydroxyecdysone would be expected and, therefore, 26-hydroxylating enzymes should be active.

The principal ecdysteroid metabolising tissues, Malpighian tubules, fat body, midgut and testes, were dissected from the larvae. These tissues plus the remaining carcass were then all analysed for enzyme activity by the procedure previously employed.

The conversion of [^{3}H] ecdysone into 20-hydroxyecdysone and 3-epi ecdysone is represented in Table 4C-1 along with the results obtained from the tissue localisation of 116 hrs of 6th instar development. Once again no 26-hydroxylase activity was detected in these tissues. 3- Epimerase was highly active early in the instar converting 94% of the incubated [^{3}H] ecdysone into 3-epiecdysone, although later in the instar at 126 hr no such activity was detected. In fact, metabolism of [^{3}H] ecdysone was almost non-existant at 126 hrs of development, only the fat body displayed any 20-hydroxylase activity at this stage, converting 5% of the incubated

$[^3H]$ ecdysone into 20-hydroxyecdysone. However, 20-hydroxylation was very active at 72 hrs of development, $[^3H]$ ecdysone being 20-hydroxylated in the fat body (86%), Malpighian tubules (7%) and testes (14%).

2. Determination of 20-hydroxylase titre curves in the homogenates of fat body and Malpighian tubules of developing 6th instar larvae of *S. littoralis*

The fat body and Malpighian tubule tissues have both demonstrated considerable ecdysone 20-hydroxylase activity. Therefore, these tissues were chosen for a study of the 20-hydroxylase activity throughout the 6th instar larval development of *S. littoralis*.

Groups of insects were synchronized ($\pm$ 2 hr) at the 5th/6th larval instar moult. The larvae were sacrificed at 8 hour intervals during 6th instar development and the fat body and Malpighian tubules dissected.

The homogenisation and assay procedure for these tissues was the same as previously described. However, for each tissue homogenate, three different incubations were performed using different substrate (ecdysone) concentrations,to permit the detection of low level 20-hydroxylase activity,and to ensure that the reaction could proceed under conditions where the substrate concentration was not limiting in at least one incubation. Three different quantities (1.1 ng, 20 ng, 200 ng) of ecdysone [labelled with 275,000 dpm $[^3H]$ ecdysone (1.1 ng)] were incubated for 30 mins at 30°C with each homogenate,and all incubations were performed in duplicate. The conversion of ecdysone into 20-hydroxyecdysone was monitored by the same h.p.l.c. procedure as previously described. However, results were only used from the incubations showing less than 20% conversion of substrate (i.e. assumed not to be substrate-limited).

Incubations with different substrate concentrations are not comparable. Therefore, in figures 4C-3 and 4C-4 the regions of the titres calculated from the differing substrate concentrations employed are represented differently. However, incubations of high, intermediate and low activity can be distinguished by this method.

The resulting 20-hydroxylase activity titres (expressed in ng 20-hydroxyecdysone/min/insect) for the fat body and Malpighian tubules are presented in figure 4C-3. The results show that the majority of 20-hydroxylase activity in the insect is present in the fat body tissue. Three peaks of activity are present, approximately corresponding to peaks of ecdysteroid shown in the RIA moulting hormone titre in the haemolymph (figure 4B-3). The results were also expressed in pg/min/mg tissue (figure 4C-4) and demonstrate that although the Malpighian tubules contribute only a limited amount of the total 20-hydroxylase activity in the whole insect, it does nonetheless have a high specific activity (i.e. activity per mg tissue). In addition, the results from figures 4C-3 and 4C-4 suggest that the hydroxylase activity in the Malpighian tubules both increases and decreases later than that of the fat body. For further discussion see Part 4, section E (p.173).

3. Determination of 3-epimerase titre curves in the homogenates of midgut tissue of developing 6th instar larvae of *S. littoralis*

The midgut tissue has shown considerable ecdysone 3-epimerase activity. Therefore, this tissue was chosen to monitor the 3-epimerase activity throughout the 6th instar larval development of *S. littoralis*. For this the midgut from larvae used for the 20-hydroxylase titre study was used. The gut contents and peritrophic membrane were removed from the midgut.

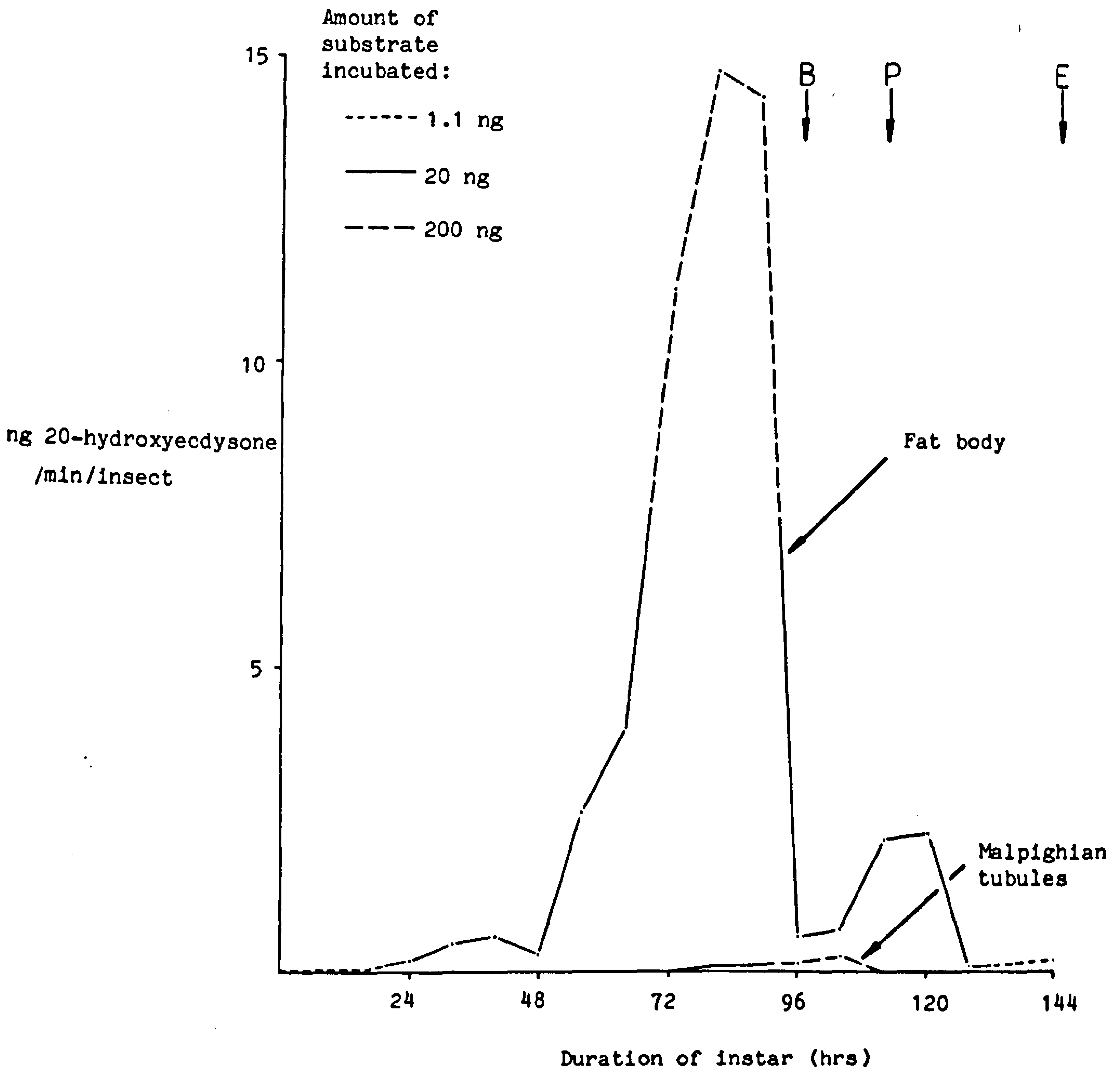


Figure 4C-3: Ecdysone 20-hydroxylase activity titre of fat body and Malpighian tubules homogenates in the 6th larval instar of *S. littoralis*. The regions of the titres calculated from the differing amounts of substrate employed (1.1 ng, 20ng, 200 ng) are represented differently. Results are expressed in ng 20-hydroxyecdysone produced/min/insect. Each point represents the mean of two points, the standard error of the mean varied from 2 to 18%

B : Burrowing E : Ecdysis P : Onset of prepupal stage

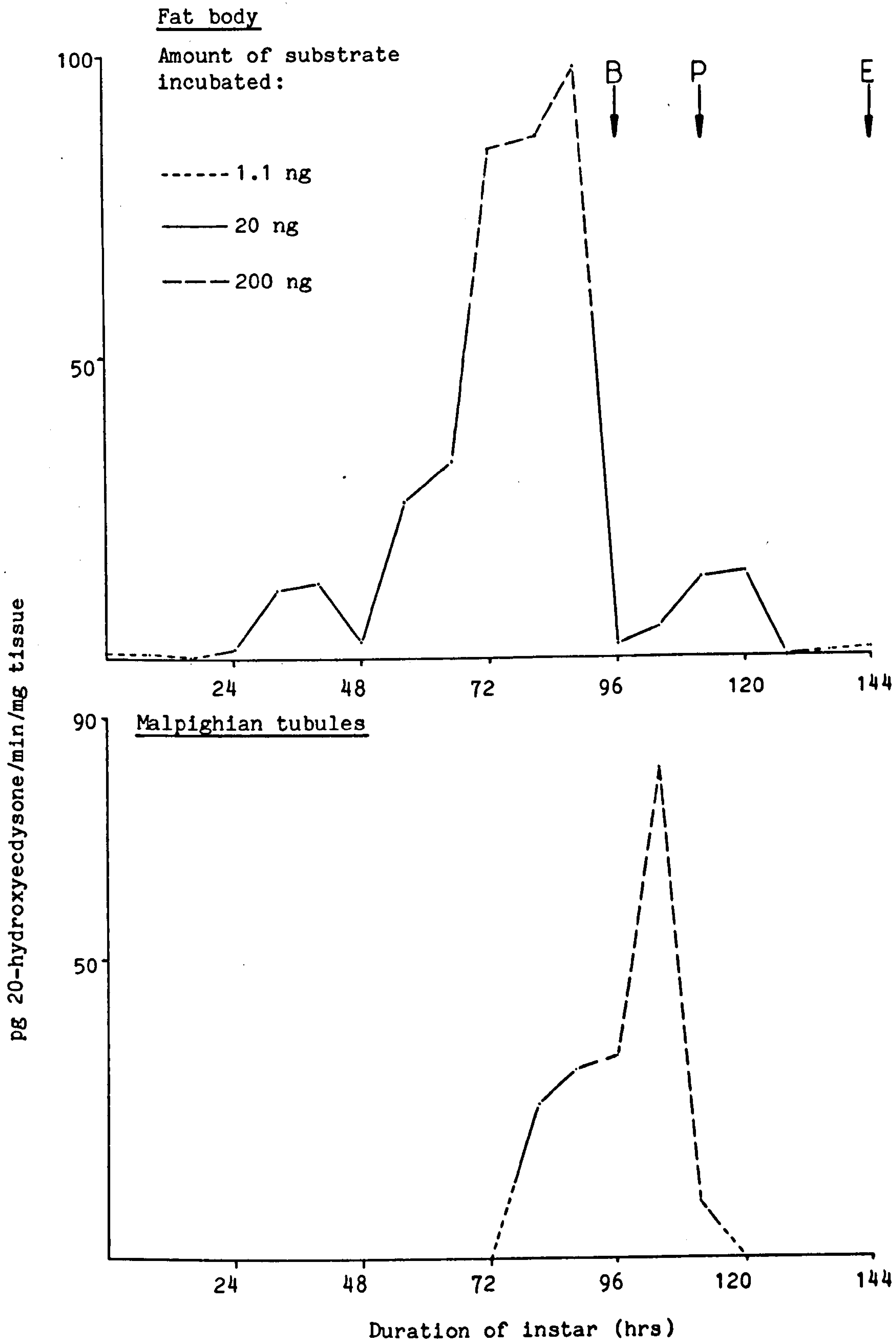


Figure 4C-4 : Ecdysone 20-hydroxylase activity titre of fat body and Malpighian tubule homogenates in the 6th larval instar of S. littoralis. The regions of the titres calculated from the differing amounts of substrate employed (1.1 ng, 20ng, 200 ng) are represented differently. Results are expressed in pg 20-hydroxyecdysone produced/min/mg tissue. Each point represents the mean of two points, the standard error of the mean varied from 2 to 18%. B : Burrowing, P : Onset of prepupal stage, E : Ecdysis.

The homogenisation and assay procedures used in this experiment were essentially the same as previously described. However, due to the high activity shown by the ecdysone 3-epimerase enzyme system, higher substrate concentrations were used. Three different quantities (20 ng, 200 ng, 1000 ng) of ecdysone [labelled with 275,000 dpm [^{3}H] ecdysone (1.1 ng)] were incubated with each homogenate for 30 mins at 30°C and all incubations were performed in duplicate. The ecdysteroids were extracted from the incubation medium as described earlier, and chromatographic analysis of the products was undertaken using an APS-Hypersil h.p.l.c. column (for conditions see p. 146). As with 20-hydroxylase assays, results were only used from the incubations showing less than 20% conversion (i.e. assumed not to be substrate-limited). As in the case of the 20-hydroxylase assays, incubations with different substrate concentrations are not comparable. Therefore in figure 4C-5 the regions of the titre calculated from the differing substrate concentrations employed are represented differently. However incubations of high, intermediate and low activity can be distinguished by this method.

The resulting ecdysone 3-epimerase activity titres for midgut homogenates are presented in figure 4C-5. The overall titre profile shows somewhat less of an ordered pattern than that observed in the case of the 20-hydroxylase titres. 3-Epimerase shows much increased activity from 56 hrs to 128 hrs of 6th instar development, reaching a maximum at 64 hrs (6000 pg/min/mg tissue). Indeed, the activity of this enzyme far exceeds the maximum 20-hydroxylase activity observed in the fat body; accurate comparisons between the maximal activities of the two enzymes are not possible since they were determined at different substrate concentrations.

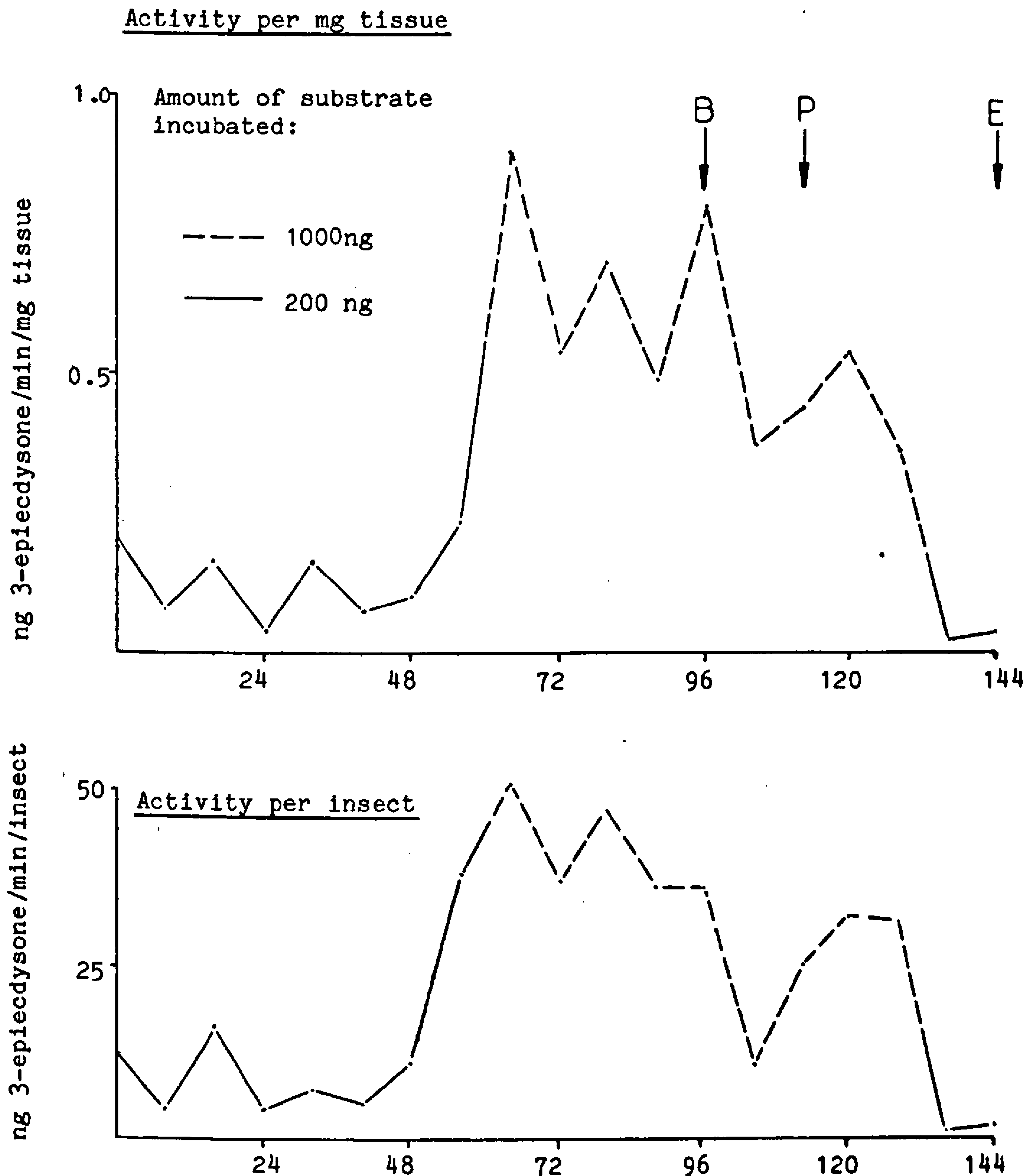


Figure 4C-5 : Ecdysone 3-epimerase activity titre of midgut homogenates in the 6th larval instar of S. littoralis. The regions of the titres calculated from the differing amounts of substrate employed (200 ng, 1,000ng) are represented differently. Activity is expressed per mg tissue and per insect. Each point represents the mean of two points, the standard error of the mean varied from 6 to 28%. B : Burrowing, P : Onset of prepupal stage, E : Ecdysis.

It certainly appears that there is no obvious correlation between the endogenous ecdysteroid titre (figure 4B-3) and the activity of this enzyme during the 6th larval instar. However, a more detailed discussion is presented in Part 4 section E (p. 180).

PART 4 SECTION D

PART 4 SECTION D

Investigation of the ecdysone 3-epimerisation reaction in *S. littoralis*

Page No.

Introduction 153

Experimental and results

High-performance liquid chromatography systems used 155

1. Epimerisation *in vitro* of [23,24-^{3}H] ecdysone by a midgut cytosol preparation from *S. littoralis* : the involvement of 3-dehydroecdysone in the reaction 155

    Preparation of the midgut cytosol 155

    An attempt to demonstrate 3-dehydroecdysone as an intermediate in the 3-epimerisation of ecdysone using the midgut cytosol preparation 156

    The isolation of 3-dehydroecdysone as an intermediate in the 3-epimerisation of ecdysone by using dialysed midgut cytosol preparation 158

    Further characterisation of the radioactivity corresponding to 3-dehydroecdysone formed from [^{3}H] ecdysone 159

    Reversed-phase h.p.l.c. analysis of the unidentified [^{3}H] ecdysone metabolite (U) 160

    Incubation of [^{3}H] 3-epiecdysone with the midgut cytosol preparation, to establish the irreversibility of the 3-epimerisation reaction 161

2. Investigation of the nature of the enzyme involved in the conversion of ecdysone into 3-dehydroecdysone 162

3. Proposed metabolic scheme for the conversion of ecdysone into 3-epiecdysone 164

PART 4 SECTION D

Investigation of the ecdysone 3-epimerisation reaction in S. littoralis

INTRODUCTION

3-Epiecdysone, and indeed 3-epi-20-hydroxyecdysone, have been detected during this study of ecdysone metabolites in S. littoralis. Metabolism in vivo of [^{3}H] ecdysone in 6th instar larvae yielded a small amount of 3-epiecdysone (figure 4A-8), and the majority of the ecdysteroid present in early pupal development consisted of 3-epiecdysone and 3-epi-20-hydroxyecdysone (figures 3C-5 to 8). In addition, high ecdysone 3-epimerase activity has been observed during metabolism in vitro of [^{3}H] ecdysone by homogenates of 6th instar larvae midgut tissue (figure 4C-5).

3-Epiecdysone and 3-epi-20-hydroxyecdysone have been characterised from insect material by physico-chemical means only relatively recently; 3-epi-20-hydroxyecdysone from Manduca sexta meconium (Thompson et al., 1974) and 3-epiecdysone from Manduca sexta pupae (Kaplanis et al., 1979). A number of 3α-ecdysteroids, formed in vivo from radiolabelled ecdysone injected into Pieris pharate pupae (Lafont et al., 1980) were identified as 3-epimers of ecdysone, 20-hydroxyecdysone, 26-hydroxyecdysone 20,26-dihydroxyecdysone, and possibly 3-epimers of 26-oic acids of ecdysteroids. The reaction was also demonstrated to be irreversible and proved to be very active at that stage of development. This reaction appears to constitute an important inactivation process by converting 3β-ecdysteroids to their hormonally less active 3α-epimer.

Enzyme activity converting ecdysone into 3-epiecdysone has been detected in vitro in Manduca sexta (Nigg et al., 1974). Homogenates of midgut tissue were shown to convert a number of 3β-ecdysteroids to

their corresponding 3α-ecdysteroids and the reaction was found to be dependent on NADH or NADPH and oxygen. Subcellular localisation of the ecdysone 3-epimerase activity in the midgut tissue revealed that the majority of the activity occurs in the cytosol fraction (85,000g for 90 mins.). It was postulated that the epimerisation of ecdysone involved the formation of 3-dehydroecdysone as a transient intermediate (Nigg *et al.*, 1974) implicating an ecdysone oxidase and a 3-dehydroecdysone reductase in the 3-epimerisation reaction (figure 4D-1).

3-Dehydroecdysone has not been isolated as an ecdysone metabolite in *Manduca* nor indeed in the present study in *Spodoptera*. However, this compound has been reported in radiotracer experiments *in vivo* in a number of species of insects (for review see Koolman, 1982). Most of these observations, however, were made largely on the strength of t.l.c. analysis, and so the identity of the compound could not be confirmed. In particular it must be noted that the recently discovered ecdysteroid 3-acetates co-chromatograph with 3-dehydroecdysteroids on silica t.l.c. (Gibson, 1982). Although the occurrence of 3-dehydroecdysone *in vivo* is perhaps unclear, the conversion of ecdysone into 3-dehydroecdysone *in vitro* by tissue homogenates of *C. erythrocephala* has been well characterised (Koolman, 1978). This demonstrates that the metabolism of ecdysteroids in Diptera could be different from that of Lepidopterans, such as *Manduca*, where 3-epiecdysteroids are formed with no trace of 3-dehydroecdysone. However, in *Calliphora vicina* prepupae, 3-epi-ecdysteroids are probably formed after the injection of radiolabelled 3-dehydroecdysone (Koolman, 1980), implicating the latter structure in the formation of the 3-epimer of ecdysone. Indeed, the conversion of 3-dehydroecdysone into 3-epiecdysone has also been observed in the midgut tissue of *S.gregaria* (Dinan, 1980).

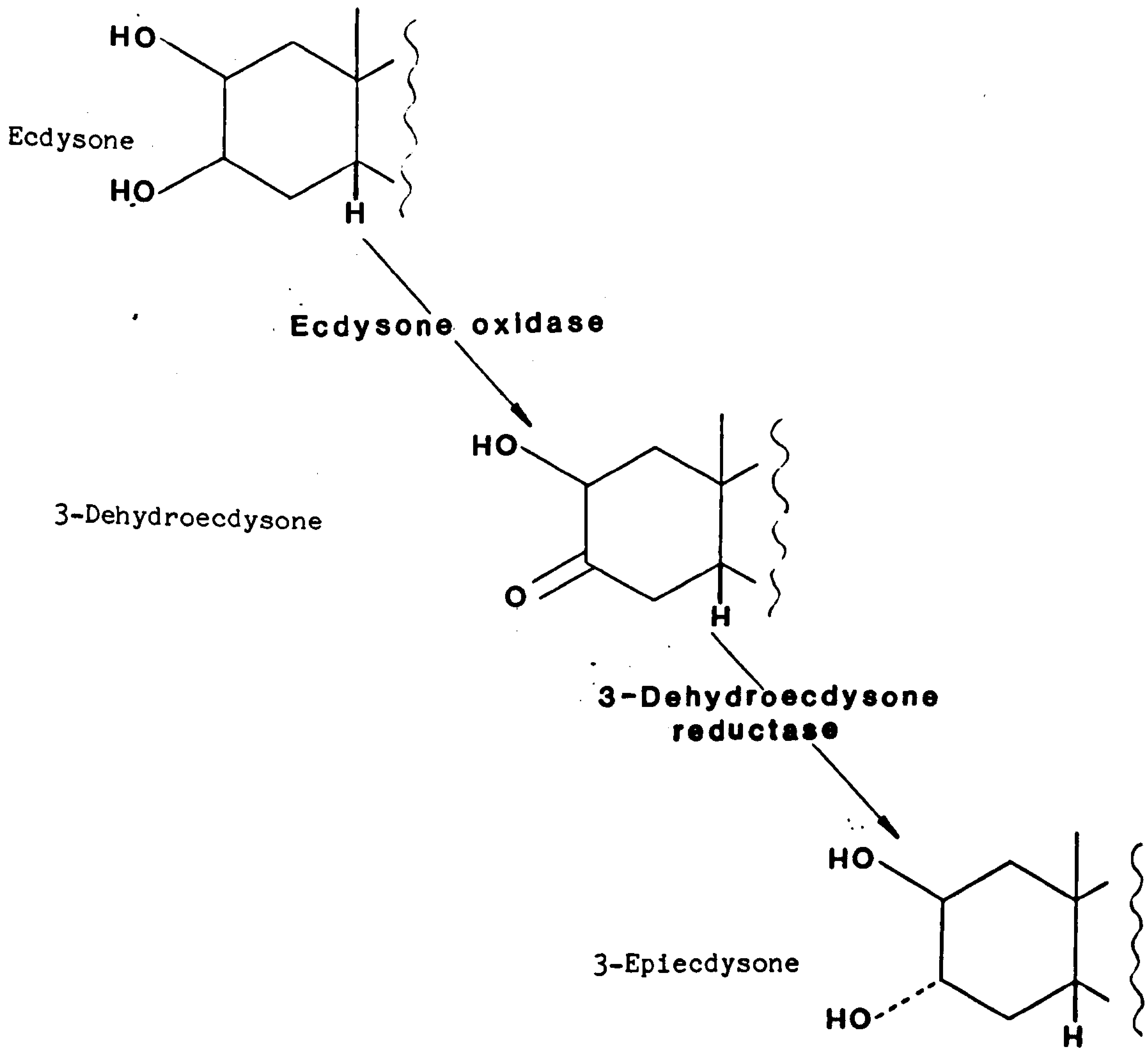


Figure 4D-1 : The proposed intermediacy of 3-dehydroecdysone in the 3-epimerisation of ecdysone (Nigg *et al.*, 1974)

Since the 3-epimerisation process in the midgut of Spodoptera was highly active, this provided an opportunity to investigate the reaction in more detail. The involvement of 3-dehydroecdysone, the cofactor and oxygen requirements, and the reversibility of the reaction were all investigated in the light of the above mentioned investigations in Manduca (Nigg et al., 1974; Meyer et al., 1979) and Diptera (Koolman, 1978).

EXPERIMENTAL AND RESULTS

High-performance liquid chromatography systems used

System 1 : A Partisil ODS-3 reversed-phase column eluted with methanol/water (48%, v/v) at 2 ml/min.

System 2 : An aminopropyl silica column (APS-Hypersil) eluted with 6% methanol in dichloroethane at 2 ml/min.

System 3 : An Altex Ultrasphere ODS reversed-phase column eluted at 1 ml/min with a linear gradient (20 minutes) of methanol in water (2:3, v/v) changing to methanol in water (4:5, v/v).

1. Epimerisation in vitro of [23,24-^{3}H] ecdysone by a midgut cytosol preparation from S. littoralis: the involvement of 3-dehydroecdysone in the reaction

Preparation of the midgut cytosol

The midguts of twenty 3 day old 6th instar larvae were dissected in ice-cold insect Ringer solution (7.5g NaCl, 0.35g KCl and 0.21g CaCl. $2H_2O$ per litre distilled water). The Malpighian tubules, peritrophic membrane and hindgut, together with the gut contents, were carefully removed from each midgut. The midguts were then thoroughly rinsed in

Ringer solution and homogenized in 0.15M KCl, by 25 passes of a Potter-Elvehjem homogeniser, to give a 20% (w/v) homogenate. The homogenate was centrifuged for 5 minutes in a microcentrifuge; the pellet was rehomogenised in 0.15M KCl, and then centrifuged as before. The supernatants were combined and centrifuged in a Beckman 40.2 Rotor of a Beckmann Spinco ultracentrifuge at 100,000g for 1 hour, to yield the gut cytosol fraction. All experiments in this section utilized freshly prepared cytosol preparation, although it could be stored frozen (-20°C) without appreciable loss of epimerase activity for at least two weeks.

<u>An attempt to demonstrate 3-dehydroecdysone as an intermediate in the 3-epimerisation of ecdysone using the midgut cytosol preparation</u>

To demonstrate the intermediacy of 3-dehydroecdysone in the 3-epimerisation of ecdysone, [^{3}H] ecdysone was incubated with midgut cytosol in the presence of a non-radioactive trap of 3-dehydroecdysone. In addition, [^{3}H] 3-dehydroecdysone was incubated to confirm the intermediacy of this compound in the epimerisation reaction and to assess the reversibility of the oxidation reaction.

The following solutions were prepared for the incubations:

1. 0.2M Tris/HCl Buffer pH 7.5
2. NADH solution (6 mg/ml buffer)
3. [23,24-^{3}H] ecdysone solution (4 mCi/µmol)
4. [23,24-^{3}H] 3-dehydroecdysone prepared from [23,24 ^{3}H] ecdysone (3 mCi/µmol).

Four incubation mixtures were then made up as shown in Table 4D-1. 100 µl of [^{3}H] ecdysone (1 µCi) was added to incubation vials, 1, 2 and 3; 100 µl [^{3}H] 3-dehydroecdysone (.75 µCi) was added to vial 4; in addition,20 µg of unlabelled 3-dehydroecdysone was added to vial 3. All four solutions were then reduced to dryness under a gentle stream of nitrogen.

Table 4D-1 : Incubation mixtures used in an attempt to demonstrate 3-dehydroecdysone as an intermediate in the 3-epimerisation reaction.

Incubation			
1	2	3	4
[^{3}H] ecdysone (1μCi)	[^{3}H] ecdysone (1μCi)	[^{3}H] ecdysone (1μCi)	[^{3}H] 3-dehydro-ecdysone (0.75μCi)
-	-	20μg 3-dehydro-ecdysone	-
NADH (50 μl)	NADH (50 μl)	NADH (50 μl)	NADH (50 μl)
Buffer (50 μl)	Buffer (50 μl)	Buffer (50 μl)	Buffer (50 μl)
Cytosol preparation (Boiled) (140 μl)	Cytosol preparation (140 μl)	Cytosol preparation (140 μl)	Cytosol preparation (140 μl)

The NADH solution and buffer solution were then added and mixed and the reaction started by the addition of 140 µl cytosol preparation. In the case of the control incubation (vial 1) the enzyme preparation was boiled prior to addition. All four tubes were incubated at 37°C for 90 minutes. When the incubation was complete 750 µl of chilled ethanol was added to precipitate the protein. After centrifugation in the bench centrifuge the supernatant was collected and the pellet re-extracted twice with methanol. The supernatants were combined and each sample reduced to dryness under a gentle stream of nitrogen ready for analysis by h.p.l.c.

Adsorption h.p.l.c. (aminopropyl column) provided the most useful analysis as it clearly separates ecdysone, 3-epiecdysone, and the putative intermediate 3-dehydroecdysone. However, the samples were also analysed by reversed-phase h.p.l.c. to confirm the identity of the compounds and to detect the possible formation of highly polar ecdysteroids.

The h.p.l.c. analysis (aminopropyl column, system 2) of the products of all four incubations are presented in figure 4D-2. In all the incubations no significant amount of 3-dehydroecdysone was observed. However, incubation of the cytosol with [^{3}H] ecdysone in the presence of the non-radioactive trap 3-dehydroecdysone (incubation 3) effectively lowered the conversion of ecdysone into 3-epiecdysone and yielded a small trace of possible [^{3}H] 3-dehydroecdysone [figure 4D-2 (3) c.f. 4D-2 (2)]. The incubation of [^{3}H] 3-dehydroecdysone [figure 4D-2 (4)] yielded [^{3}H] 3-epiecdysone as virtually the only product, with no 3-dehydroecdysone remaining. Indeed, from the foregoing incubations it would appear that 3-dehydroecdysone is very rapidly reduced to 3-epiecdysone. Therefore, in an attempt to isolate the intermediate, [^{3}H] ecdysone was incubated without cofactor. However, once again, the only product was [^{3}H] 3-epiecdysone.

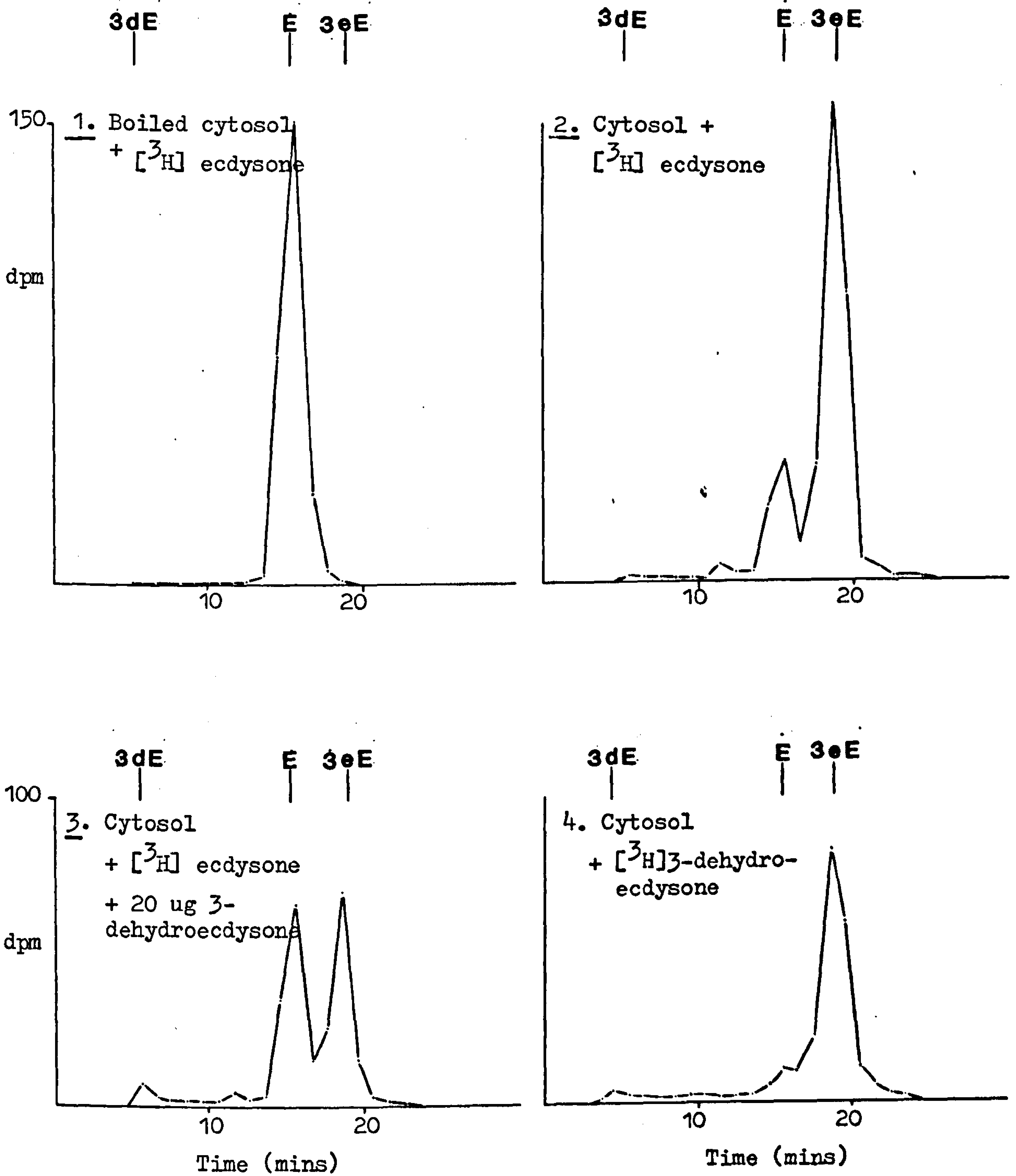


Figure 4D-2 : H.p.l.c. analysis on APS-Hypersil (system 2) of the products of incubations used in an attempt to demonstrate [^{3}H] 3-dehydroecdysone as an intermediate in the 3-epimerisation reaction. Authentic compounds were chromatographed prior to chromatography of the radioactive samples.

The isolation of 3-dehydroecdysone as an intermediate in the 3-epimerisation of ecdysone by using dialysed midgut cytosol preparation

The foregoing incubations have indicated that 3-dehydroecdysone could possibly be an intermediate in the 3-epimerisation reaction, but due to the active reduction of this compound to 3-epiecdysone, this could not be confirmed. To overcome this, the midgut cytosol preparation was subject to dialysis to remove endogenous cofactors.

A series of incubations using this preparation were undertaken. The experiment was designed to isolate 3-dehydroecdysone (in the absence of reducing cofactor) and to test the involvement of the cofactors, NADH and NADPH, in the epimerisation reaction.

The following solutionswere prepared for the incubations:

1. 0.2M Tris/HCl Buffer pH 7.5
2. NADH solution (6 mg/ml buffer)
3. NADPH solution (6 mg/ml buffer)
4. [23,24-^{3}H] ecdysone solution (0.11 mCi/µmol)
5. [23,24-^{3}H] 3-dehydroecdysone solution synthesised from [23,24-^{3}H] ecdysone (3 mCi/µmol)

Five incubations were then made up as shown in table 4D-2. 100 µl [^{3}H] ecdysone (0.25 µCi) was added to incubation vials A,B,C and D; 100 µl [^{3}H] 3-dehydroecdysone (0.16 µCi) was added to incubation vial E. All five solutions were then reduced to dryness under a gentle stream of nitrogen. The cofactors and buffer solutions were then added and mixed, and the reaction started by the addition of 150 µl of the dialysed midgut cytosol preparation. In the case of the control incubation (vial A) the enzyme preparation was boiled prior to addition. All five tubes were then incubated at 37°C for 90 minutes, and the reaction mixtures extracted

Table 4D-2 : The incubation mixtures used with a dialysed enzyme preparation to demonstrate 3-dehydroecdysone as an intermediate in the 3-epimerisation reaction.

	Incubation				
	A	B	C	D	E
[^{3}H] ecdysone	0.25μCi	0.25μCi	0.25μCi	0.25μCi	-
[^{3}H] 3-dehydro-ecdysone	-	-	-	-	0.16μCi
NADPH	50 μl	50 μl	-	-	-
NADH	-	-	50 μl	-	100 μl
Buffer	50 μl	50 μl	50 μl	100 μl	100 μl
Cytosol enzyme preparation	150 μl (Boiled)	150 μl	150 μl	150 μl	150 μl

and analysed by h.p.l.c. (systems 1 and 2) as previously described (p. 155).

The adsorption h.p.l.c. analysis (system 2) of all five samples is presented in figure 4D-3. In the presence of NADPH, 3-epiecdysone was formed [figure 4D-3 (B)] whereas without cofactor 3-epiecdysone was not formed but a trace of possible 3-dehydroecdysone was detectable [figure 4D-3 (D)]. Incubation in the presence of NADH produced no 3-epiecdysone, but a peak corresponding to 3-dehydroecdysone and an unknown metabolite (U) were formed [figure 4D-3 (C)].

In the presence of cofactor (undialysed preparation; figure 4D-2 (4)] [^{3}H] 3-dehydroecdysone was reduced almost entirely to 3-epiecdysone, whereas in the present case, with the concentration of cofactor lowered by dialysis [dialysed preparation, figure 4D-3 (E)] less 3-dehydroecdysone was reduced, both ecdysone and 3-epiecdysone being produced.

Further characterisation of the radioactivity corresponding to 3-dehydroecdysone formed from [^{3}H] ecdysone

The radioactivity corresponding to 3-dehydroecdysone from incubations C and D (figures 4D-3 (C) and (D)) was collected, combined, and further characterised by reversed-phase h.p.l.c. (system 3) (figure 4D-4). The majority of the radioactivity did indeed co-chromatograph with 3-dehydroecdysone. However, some radioactivity co-chromatographed with ecdysone and a 'shoulder' of radioactivity is apparent on the trailing edge of the large peak. This indicated that possibly 3-dehydro-ecdysone was not the only metabolite of [^{3}H] ecdysone contained in the peak co-chromatographing with 3-dehydroecdysone on adsorption h.p.l.c. (system 2).

A. Boiled enzyme control

B. Dialysed cytosol + [^{3}H] ecdysone + NADPH

C. Dialysed cytosol + [^{3}H] ecdysone + NADH

D. Dialysed cytosol + [^{3}H] ecdysone

E. Dialysed cytosol + [^{3}H] 3-dehydroecdysone

3dE E 3eE

U

dpm (10^{-3})

dpm ($\times 10^{-3}$)

Time (mins)

Figure 4D-3 : H.p.l.c. analysis (system 2) of the incubations of [^{3}H] ecdysteroids with dialysed cytosol enzyme preparation. For details of incubations see Table 4D-2. Authentic compounds were chromatographed prior to chromatography of the radioactive samples.

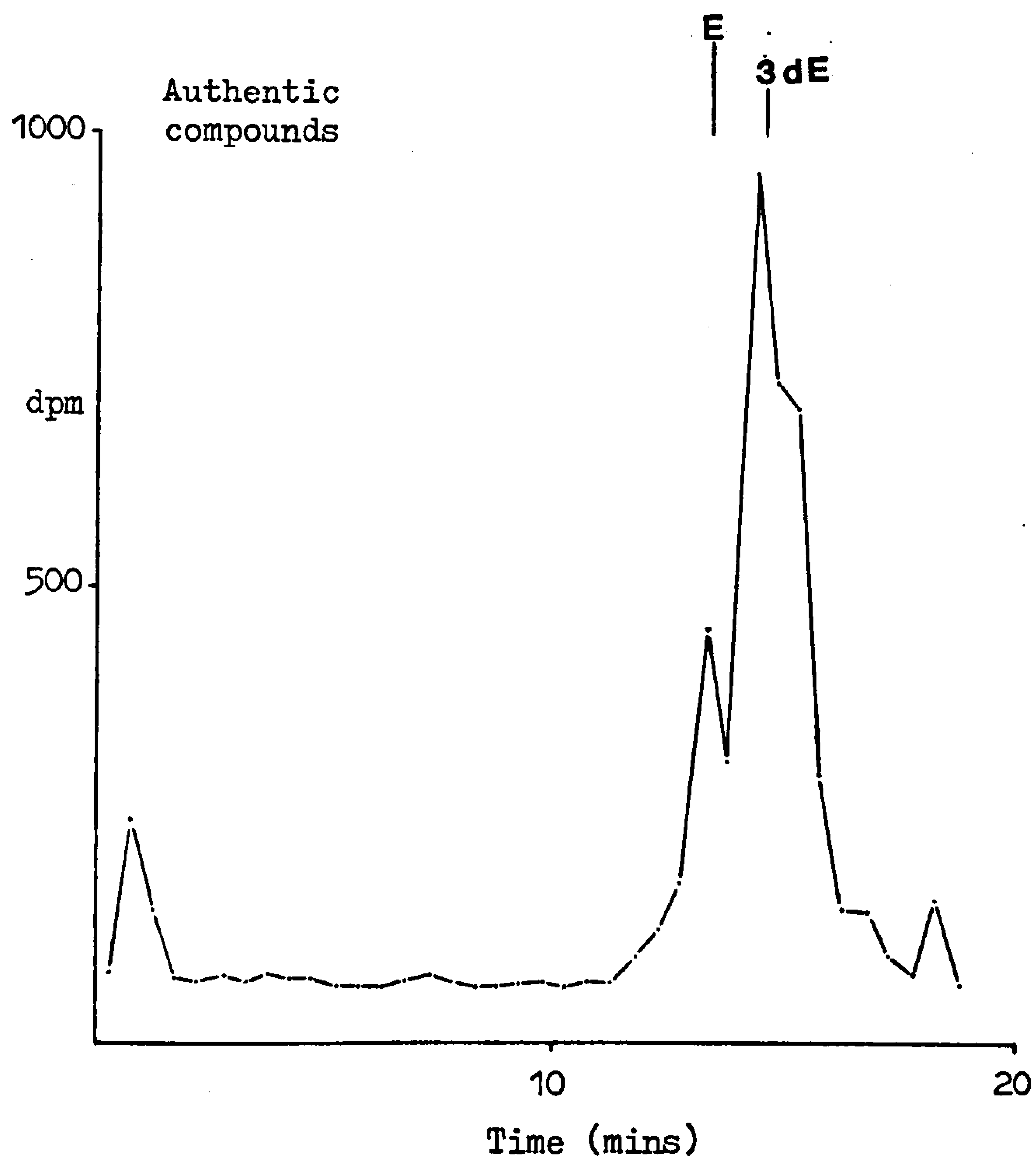


Figure 4D-4 : Reversed-phase h.p.l.c. analysis (system 3) of the putative [^{3}H] 3-dehydroecdysone collected from adsorption h.p.l.c. (APS-Hypersil column) analysis of incubations C and D. Authentic compounds were co-chromatographed with the radioactive sample.

For additional characterisation, the putative [^{3}H] 3-dehydro-ecdysone was also subjected to reduction with $NaBH_4$ (for method see Part 2). The products of the reaction were analysed by adsorption h.p.l.c. (system 2; figure 4D-5). Both ecdysone and 3-epiecdysone are produced as expected, with a small amount of radioactivity chromatographing at the same position as the unknown metabolite (U) [c.f. figure 4D-3 (C) and 4D-5].

The occurrence of the metabolite 'U' after chemical reduction by sodium borohydride implies that the putative 3-dehydroecdysone peak from h.p.l.c. on APS-Hypersil (system 2) in fact contains an additional compound which was reduced by sodium borohydride to the unknown compound 'U'. Indeed, it would tend to suggest that the '3-dehydroecdysone' peak from incubation C [figure 4D-3 (C)] contains a dehydroecdysone with a keto group at a position other than C-3 (i.e. at positions C-2 or C-22). The reduction of this compound would therefore produce, in addition to ecdysone, either 2-epiecdysone or 22-isoecdysone as the unknown compound 'U'. The chromatographic properties of 22-isoecdysone on h.p.l.c. on an aminopropyl column are known (retention time relative to ecdysone = 1.52). 22-Isoecdysone shows characteristics more polar than ecdysone and the unknown metabolite 'U'. This leaves 2-epiecdysone as the possible identity of 'U' with 2-dehydroecdysone as its intermediate which would presumably co-chromatograph with 3-dehydroecdysone on aminopropyl h.p.l.c. For further characterisation of this compound, it was also analysed by reversed-phase h.p.l.c.

<u>Reversed-phase h.p.l.c. analysis of the unidentified [^{3}H] ecdysone metabolite (U)</u>

The peak of radioactivity corresponding to the unidentified [^{3}H] metabolite (U) from incubation C [figure 4D-3 (C)] was analysed using

3dE E 3eE Authentic compounds

1000

dpm

500

U

10 20 30

Time (mins)

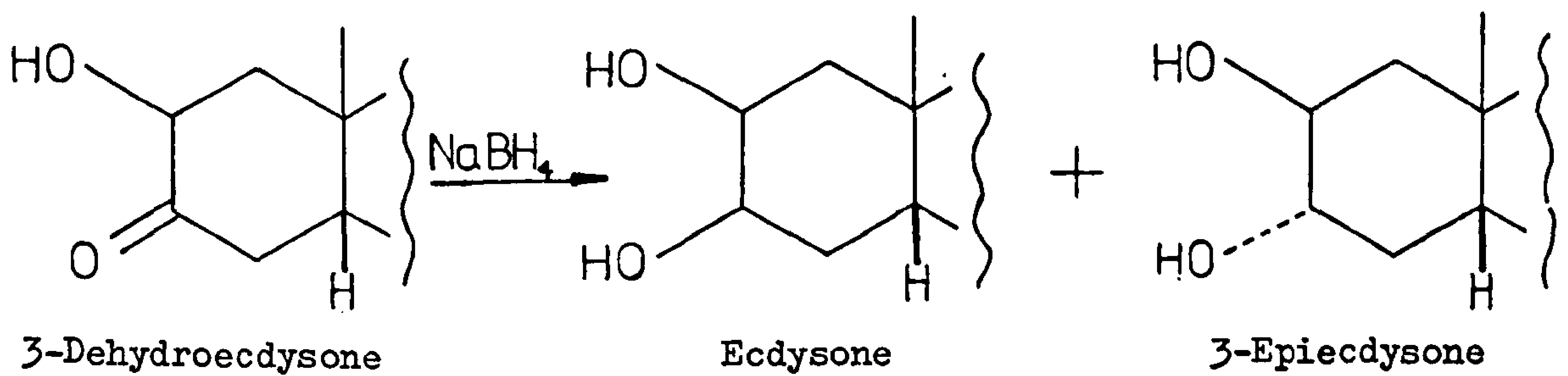


Figure 4D-5 : H.p.l.c. analysis (APS-Hypersil; system 2) of the products of the reduction of putative [^{3}H] 3-dehydroecdysone by sodium borohydride. Authentic compounds were co-chromatographed with the radioactive sample.

reversed-phase h.p.l.c. (system 3; figure 4D-6). A single peak was observed chromatographing at a similar retention time to 3-epiecdysone. The behaviour indicates that the compound 'U' has a structure similar to 3-epiecdysone and could indeed by an epimer of ecdysone with the α hydroxy group occurring on the nucleus (i.e. 2-epiecdysone). Figure 4D-7 represents the possible involvement of the putative 2-epiecdysone as a minor metabolite in the metabolism _in vitro_ of [^{3}H] ecdysone by the midgut cytsol preparation. However, more definitive characterisation of the structure of 'U' is necessary.

<u>Incubation of [^{3}H] 3-epiecdysone with the midgut cytosol preparation, to establish the irreversibility of the 3-epimerisation reaction</u>

[^{3}H] 3-Epiecdysone used in this experiment was obtained from the metabolism of [^{3}H] ecdysone by the incubations previously described in this section. The radioactivity corresponding to [^{3}H] 3-epiecdysone was purified by reversed-phase h.p.l.c. (system 1) followed by adsorption h.p.l.c. (system 2).

[^{3}H] 3-Epiecdysone (0.05 μCi) was incubated with; (i) undialysed midgut cytosol preparation, (ii) dialysed midgut cytosol preparation with NADPH as cofactor, (iii) dialysed midgut cytosol preparation with NADH as cofactor, and (iv) dialysed midgut cytosol preparation without cofactor. All the incubations were carried out using the same method as previously described .

Incubation of [^{3}H] 3-epiecdysone with either undialysed (figure 4D-8) or dialysed cytosol produced no other detectable compounds, indicating that under the conditions used the epimerisation reaction is irreversible.

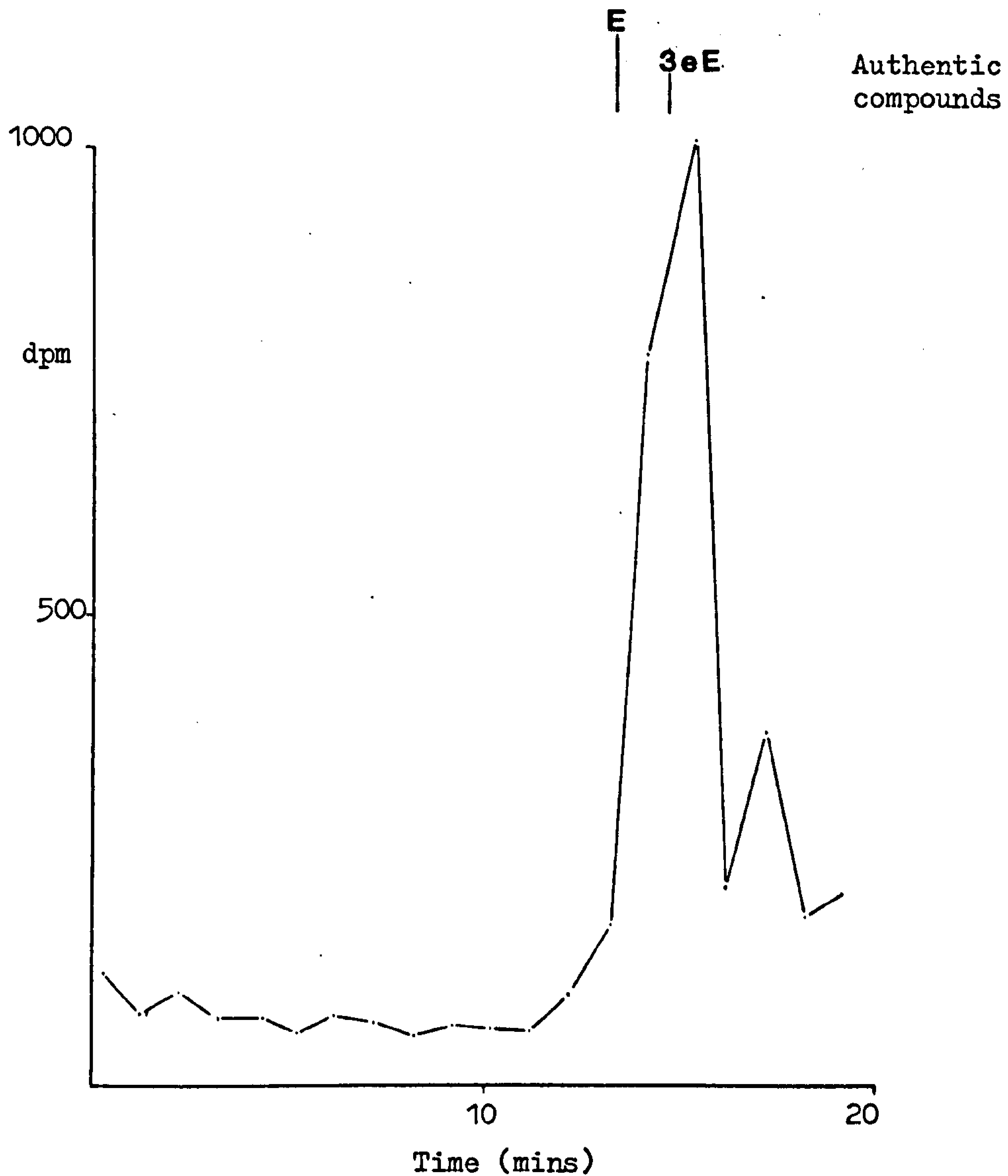


Figure 4D-6 : Reversed-phase analysis of the unidentified compound (U) collected from adsorption h.p.l.c. (APS-Hypersil column) [figure 4D-3(c)]. Authentic compounds were co-chromatographed with the radioactive sample.

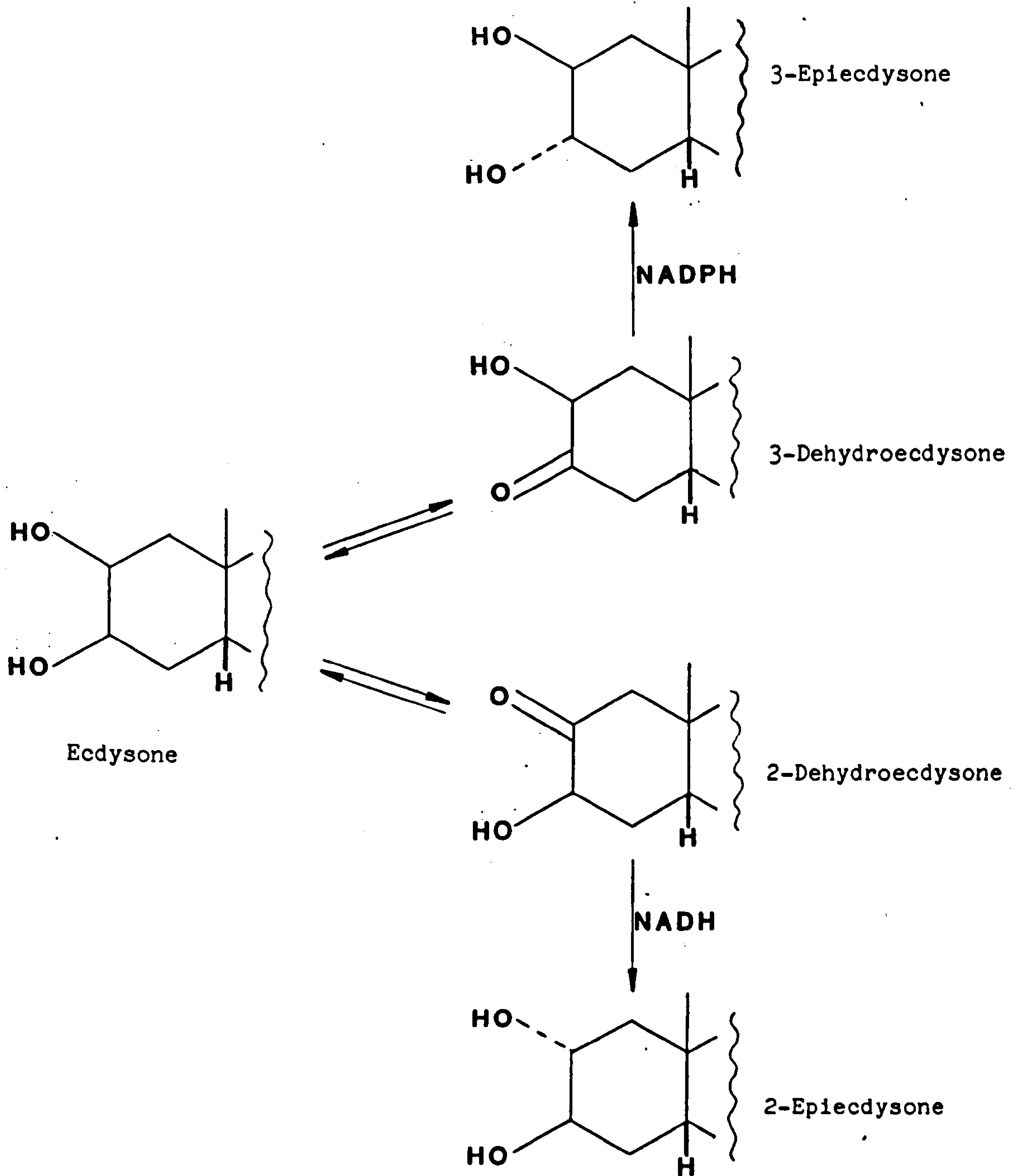


Figure 4D-7 : The possible metabolic pathway of ecdysone *in vitro* (using the midgut cytosol preparation) showing the involvement of the proposed metabolite, 2-epiecdysone.

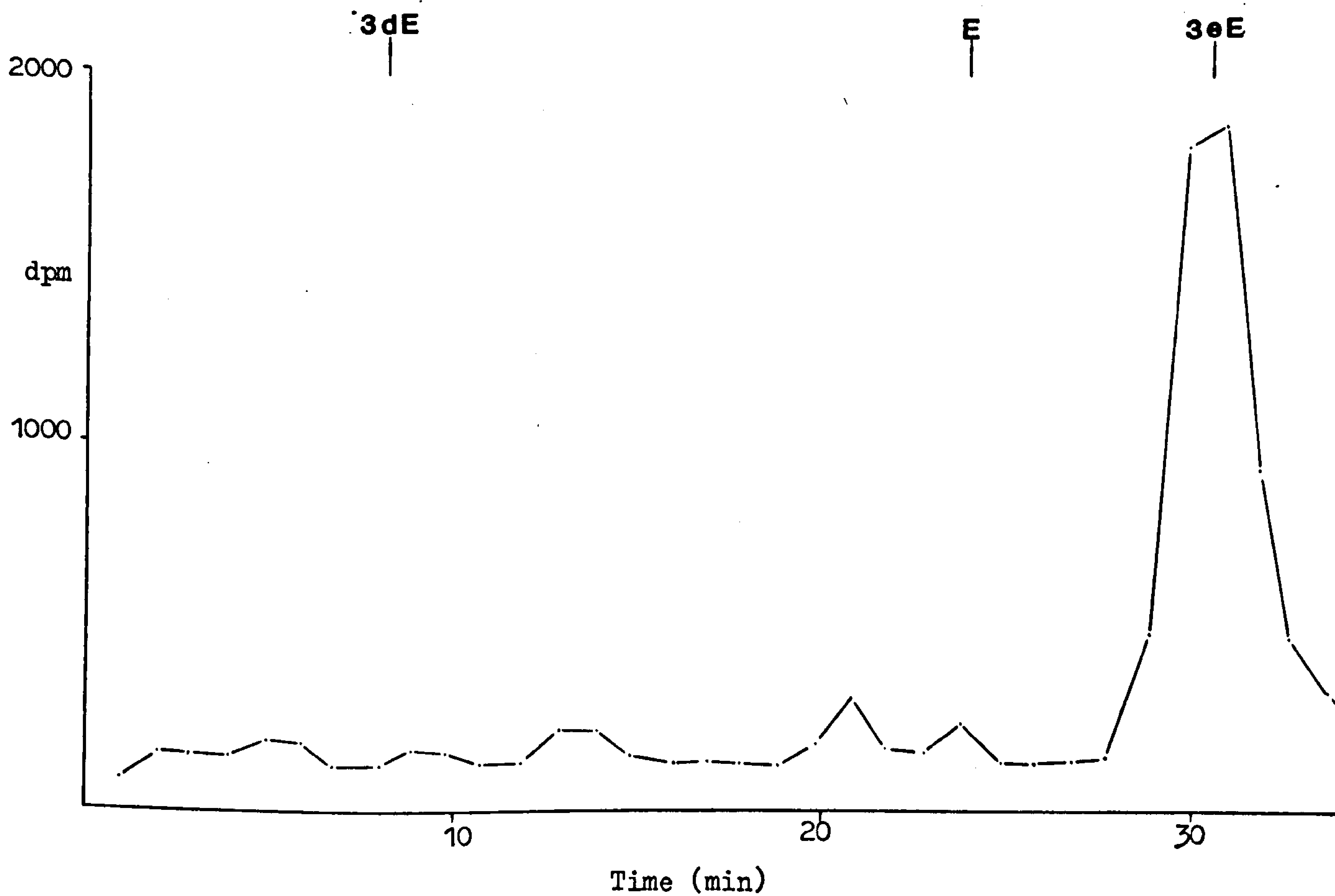


Figure 4D-8 : Adsorption h.p.l.c. analysis of the products of the incubation of [^{3}H] 3-epiecdysone in undialysed midgut cytosol preparation. Authentic compounds were co-chromatographed with the radioactive sample.

2. Investigation of the nature of the enzyme involved in the conversion of ecdysone into 3-dehydroecdysone

The foregoing results have implicated 3-dehydroecdysone as an intermediate in the 3-epimerisation reaction of ecdysone. The formation of 3-dehydroecdysone is presumably due to the involvement of a dehydrogenase or oxidase enzyme. In an attempt to identify which of these two types of enzymes is involved, the epimerisation reactions requirements for molecular oxygen and the cofactors, NAD^+ and $NADP^+$, was assessed by a series of incubations with dialysed midgut cytosol preparation.

A dialysed midgut cytosol preparation from 18 insects was prepared by the method previously described.

The following solutions were prepared for the incubations:

1. 0.2M Tris/HCl Buffer pH 7.5
2. NADPH solution (6 mg/ml buffer)
3. NADH solution (6 mg/ml buffer)
4. $NADP^+$ solution (6 mg/ml buffer)
5. NAD^+ solution (6 mg/ml buffer)
6. EDTA solution (0.02M in buffer)
7. [23,24 $^3H_2$] ecdysone (0.11 mCi/μmol)

Seven incubation mixtures were then made up as shown in Table 4D-3 100 μl [^{3}H] ecdysone (0.25 μCi) was added to all incubation test tubes. All solutions were then reduced to dryness under a gentle stream of nitrogen. The cofactors and buffer solutions were then added and mixed, and the reaction started by the addition of 150 μl of dialysed midgut cytosol preparation. Incubations II to VII proceeded under atmospheric conditions while incubation I was conducted under nitrogen (i.e. in the

Table 4D-3 : The incubation mixtures used to investigate the nature of the enzyme involved in the conversion of ecdysone into 3-dehydroecdysone.

	Incubation						
	I	II	III	IV	V	VI	VII
Under air or nitrogen	N_2	air	air	air	air	air	air
[^{3}H] Ecdysone	0.25μCi	0.25μCi	0.25μCi	0.25μCi	0.25μCi	0.25μCi	0.25μCi
NADPH	50μl	50μl	50μl	50μl	50μl	50μl	-
NADH	-	-	-	-	-	-	50μl
NAD^+	-	-	-	50μl	-	-	-
$NADP^+$	-	-	-	-	50μl	-	-
EDTA	-	-	-	-	-	50μl	-
Buffer	50μl	50μl	50μl	-	-	-	50μl
Cytosol enzyme preparation	150μl	150μl (Boiled)	150μl	150μl	150μl	150μl	150μl

absence of molecular oxygen). In the latter case, just prior to the addition of the cytosol preparation, air was flushed from the tube by alternately applying vacuum and then refilling the test tube with nitrogen. The test tube was purged in this way six times to ensure all oxygen was flushed from the system. In addition, this process was repeated just after the addition of the cytosol preparation and repeated at 10 minute intervals during incubation. All the tubes were incubated at 37°C for 30 minutes, then 750 µl of chilled ethanol was added to precipitate the protein, and the reaction mixture extracted and analysed by reversed-phase and adsorption h.p.l.c. as previously described .

Analysis by adsorption h.p.l.c. (APS-Hypersil column) provides good separation of the three components of the 3-epimerisation reaction; ecdysone, the intermediate 3-dehydroecdysone, and 3-epiecdysone. Radioactivity corresponding to ecdysone, 3-dehydroecdysone and 3-epiecdysone in each incubation mixture is given in Table 4D-4.

The conversion of ecdysone into its 3-epimer is considerably reduced in the absence of oxygen (c.f. incubations I and III), although some conversion has occurred possibly due to the presence of residual oxygen dissolved in the incubation mixture. This reduction could possibly imply the involvement of an oxidase enzyme in the 3-epimerisation reaction. However, in the presence of oxidised cofactors, NAD^+ and $NADP^+$ (incubations IV and V), 3-epimerisation is considerably increased. Although the exact significance of this is uncertain, it could be an indirect effect.

Incubation with NADH (Incubation VII) produces a small amount of 3-epiecdysone, and also a small quantity of the unidentified compound (U) thus confirming earlier results [figure 4D-3 (C)]. The addition of the

Table 4D-4 : The radioactivity corresponding to various [^3H] ecdysteroids separated by adsorption h.p.l.c. (system 2). Results are expressed as d.p.m. per incubation mixture.

Incubation	Cofactors and EDTA	d.p.m. in various [^3H] ecdysteroids/incubation				% of recovered radioactivity converted into 3-epiecdysone
		Unidentif. comp. (U)	Ecdysone	3-Dehydro-ecdysone	3-Epi-ecdysone	
I (under N_2)	NADPH	-	500,100	15,300	25,200	4.7%
II (Boiled enzyme preparation	NADPH	-	535,200	-	-	0%
III	NADPH	-	440,700	8,000	101,200	18.4%
IV	NADPH+NAD^+	-	356,400	21,600	141,600	27.3%
V	NADPH+$NADP^+$	-	324,100	16,200	202,300	37.3%
VI	NADPH+EDTA	-	435,800	24,000	120,300	20.7%
VII	NADH	24,600	476,600	22,400	18,900	3.5%

metalloenzyme inhibitor, EDTA (Incubation VI), apparently causes no loss of activity. Thus implying the absence of any metalloflavoprotein in the enzyme catalysing the oxidation of ecdysone to 3-dehydroecdysone.

3. Proposed metabolic scheme for the conversion of ecdysone into 3-epiecdysone

The intermediacy of 3-dehydroecdysone in this reaction was indicated by the effect of adding mass of 3-dehydroecdysone to the incubation mixture, which considerably reduced the conversion of [^{3}H] ecdysone into [^{3}H] 3-epiecdysone [c.f. figures 4D-2 (2) and (3)]. Confirmation of 3-dehydroecdysone as an intermediate in the reaction was achieved by the incubation of [^{3}H] ecdysone with dialysed midgut cytosol preparations, 3-dehydroecdysone being accumulated in incubations without NADPH [figure 4D-3 (C) and (D)]. 3-Dehydroecdysone was characterised by co-chromatography with authentic material on two h.p.l.c. systems (reversed-phase and adsorption), and by chemical reduction to ecdysone and 3-epiecdysone (figure 4D-5).

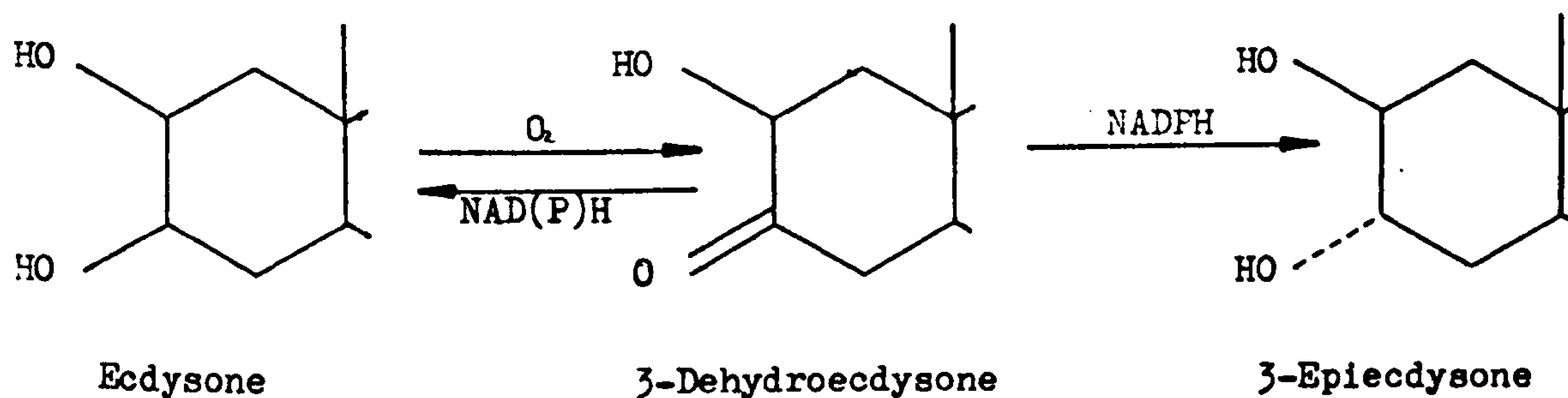
3-Dehydroecdysone can be converted into either ecdysone or 3-epiecdysone and in the presence of NADPH 3-dehydroecdysone is virtually all converted into 3-epiecdysone [figure 4D-3(B)]. Indeed, the reduction to 3-epiecdysone is apparently irreversible as demonstrated by the incubation of [^{3}H] 3-epiecdysone (figure 4D-8).

It is apparent that NADPH is required for the reduction of 3-dehydroecdysone to 3-epiecdysone whereas NADH is not [c.f. figures 4D-3 (B) and (C)].

The results of the final experiment (Table 4D-4) implicate oxygen in the reaction, leading to the assumption that an oxidase enzyme is involved in the formation of the intermediate 3-dehydroecdysone.

If, indeed, an oxidase is involved it appears that it is probably non-metal linked, as the reaction is apparently unaffected by EDTA. Indeed, this is in agreement with studies of the ecdysone oxidase enzyme from _Calliphora vicina_, where the addition of EDTA to the enzyme preparation has no effect on oxidase activity (Koolman and Karlson, 1978).

The combined results obtained in this study indicate the intermediacy of 3-dehydroecdysone in the 3-epimerisation of ecdysone, and implicate the involvement of NADPH and molecular oxygen in this reaction:



PART 4 SECTION E

PART 4 SECTION E

Discussion of the studies on *S. littoralis* larvae

Page No.

1. Ecdysone metabolites during the 6th larval instar of *S. littoralis* — 167

2. Ecdysteroid titre in the 6th instar larvae of *S. littoralis* — 169

3. The conversion of ecdysone into 20-hydroxyecdysone in the tissues of 6th instar larvae of *S. littoralis* — 173

4. The conversion of ecdysone into 3-epiecdysone in the midgut of 6th instar larvae of *S. littoralis* — 180

5. The ecdysteroids contained in the faeces of *S. littoralis* 6th instar larvae — 184

PART 4 SECTION E

Discussion of the studies on *S. littoralis* larvae

1. Ecdysone metabolites during the 6th larval instar of *S. littoralis*

The ecdysone metabolites observed at this stage of development are similar to those in the pupal stage. Both 20- and 26-hydroxylation processes are active and the presence of ecdysteroid-26-oic acids is observed. However, in addition to the ecdysteroids characterised by metabolism *in vivo* of [^{3}H] ecdysone in pupae, similar experiments in the 6th instar larvae yielded considerable amounts of ecdysteroid conjugates and some 3-epiecdysone (Table 4A-2).

20-Hydroxyecdysone appears to be the major free ecdysteroid found by analysis of the endogenous hormone at the peaks of the RIA moulting hormone titres, with smaller amounts of 20,26-dihydroxyecdysone and ecdysone also being detected (Table 4B-4). Indeed, the metabolism *in vivo* of [^{3}H] ecdysone also demonstrates that considerable 20-hydroxylation occurs with the production of radiolabelled 20-hydroxyecdysone, 20,26-dihydroxyecdysone and 20-hydroxyecdysonoic acid (Table 4A-2).

Although 26-hydroxyecdysone was present as a radiolabelled metabolite of injected [^{3}H] ecdysone (Table 4A-2), it was not actually detected by GC/MS (SIM) analysis of the endogenous moulting hormones. Its presence in the radiolabelled experiments is perhaps slightly unphysiological, probably due to the fact that the injection of exogenous [^{3}H] ecdysone does not reflect the endogenous moulting hormone complement, which is mainly in the form of 20-hydroxyecdysone [see GC/MS (SIM) analyses, Table 4B-4]. Furthermore, the metabolism of an exogenous ecdysteroid may not accurately reflect that of the same hormone *in vivo*, where factors such as protein binding and compartmentation may be important.

In the pupal stage, 3-epiecdysteroids were found in the young pupae (figures 3C-4, 5, 6 and 7), although metabolism *in vivo* of [^{3}H] ecdysone at a later pupal stage did not yield any 3-epimers of ecdysteroids (figure 3A-3). However, such experiments in late (140 hr) 6th instar larvae did yield some 3-epiecdysone (Table 4A-2). This situation is similar to that found in *Pieris* where 3-epimerisation is principally active during the pharate pupae and early pupal stage (Beydon *et al.*, 1981).

The major difference observed when comparing the metabolism of ecdysone in the larvae and pupae is the occurrence of ecdysteroid conjugates in the 6th instar larvae. Whereas the polar ecdysteroids in the pupal stage and late 6th instar larvae consist mainly of ecdysteroid-26-oic acids (Table 3A- 1 and 4A-2), the polar ecdysteroid fraction in the 6th instar larvae (excluding late larvae) are principally in the form of conjugates. However, these compounds are largely excreted and are to be found in large quantities in the faeces (Table 4A-2).

Although the analysis of the faeces collected between 48 and 58 hr after administration of [^{3}H] ecdysone at 48 hr of development revealed similar amounts of radiolabelled free and conjugated ecdysteroids, the analysis by mass of the moulting hormones in faeces collected between 0 and 60 hr indicates that most ecdysteroids are in the conjugated form (table 4B-5). Further analysis by GC/MS (SIM) revealed that 20-hydroxyecdysone was the major conjugated ecdysteroid (figure 4B-7) thus reflecting the presence of 20-hydroxyecdysone in the haemolymph as the major free ecdysteroid. However, it must be noted that ecdysteroid-26-oic acids could not be detected by the GC/MS (SIM) procedure employed in this study. Therefore the radiolabelled 20-hydroxyecdyson-26-oic acid conjugate detected in faeces after administration of [^{3}H] ecdysone (Table 4A-2) cannot be confirmed as an endogenous product. It is

interesting that considerable amounts of radiolabelled ecdysone conjugate were isolated from the faeces after [^{3}H] ecdysone injections into the larvae (58 hr), whereas the analysis of the moulting hormones by mass revealed only a small quantity. The reason for this is, once again, probably due to the injection of [^{3}H] ecdysone at a time when the predominant endogenous moulting hormone is 20-hydroxyecdysone. This emphasises that results obtained from such experiments must be treated with caution, and considered as a qualitative rather than quantitative analysis.

The regulation of the moulting hormone titre during this instar is governed mainly by the synthesis of the moulting hormone, its further metabolism, and excretion. In this study, a number of aspects of these processes have been investigated: the identification of mass of the ecdysteroids and their levels in the haemolymph, the analysis of the activities of enzymes responsible for ecdysone metabolism, and an examination of the moulting hormone excretion products. All these give some indication of the means by which the moulting hormone titre is regulated, and shall be examined in the rest of this section.

2. Ecdysteroid titre in the 6th instar larvae of *S. littoralis*

The RIA titre curves compiled in this study reveal the presence of three peaks of immunoreactive ecdysteroids in the haemolymph with either ICT-1 or DHS1-15 antiserum (figure 4B-3). Many such ecdysteroid RIA titres have been compiled in the final larval instar of holometabolous insects, either by assaying the moulting hormone content of haemolymph or whole animal samples.

Most immunoreactive ecdysteroid titres reported for Lepidopterans reveal the presence of a major peak just prior to the shedding of the larval skin (Manduca sexta, Bollenbacher et al., 1975; Calpodes ethlins, Dean et al., 1980; Bombyx mori and Philosamia cynthia, Calvez et al., 1976). This is probably analogous to the major peak in the S.littoralis moulting hormone occurring at 120 hours.

In addition to this peak, a smaller but nonetheless significant ecdysteroid peak is also observed in some Lepidopterans occurring in the second half of last larval instar development just prior to the major peak; Galleria mellonella (Sehnal et al., 1981), Tenebrio molitor (Delbecque et al., 1975), Pieris brassicae (Lafont et al., 1975). In Galleria a peak occurring 24 hrs before the major peak, is associated with the wandering stage (figure 4E-1). The equivalent peak in Spodoptera (i.e. associated with a behavioural change to the burrowing stage) would, therefore, be represented by the increase in the titre observed at 96 to 104 hr (see figure 4E-1). Although a clear peak is not resolved at this time, possibly because insufficient titre points were taken, or due to asynchrony of the insect population, it would seem that this 'shoulder' at 96 to 104 hr represents the peak associated with behavioural change. The profile of the moulting hormone titre in late last larval instar Calpodes (Dean et al., 1980) closely resembles that of S. littoralis (i.e. with a shoulder on the major peak; see figure 4E-1). A study of the ecdysone and 20-hydroxyecdysone titres in the final larval instar of the Lepidopteran Pieris brassicae (Lafont et al., 1975) revealed a peak of ecdysone closely preceding and slightly overlapping the major 20-hydroxyecdysone peak. By analogy with that study, it would appear that in Spodoptera littoralis the shoulder could be due to the presence of a distinct peak of ecdysone preceding the major peak of 20 hydroxy-ecdysone.

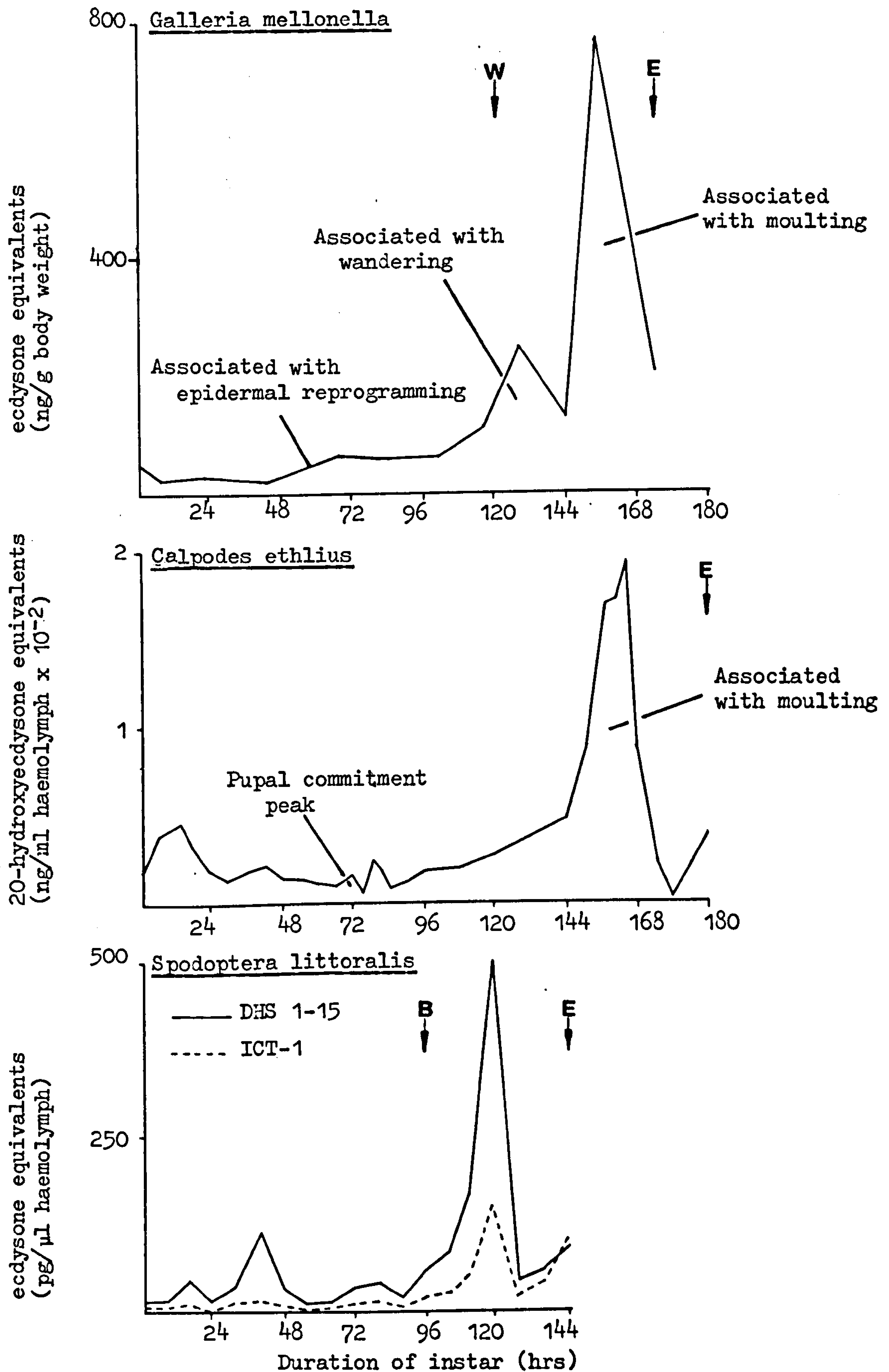


Figure 4E-1 : Ecdysteroid titres in Spodoptera 6th instar larvae compared to equivalent titre in Galleria (Sehnal et al., 1981) and Calpodes (Dean et al., 1980). In the latter two studies, the various increases in ecdysteroid titre were related to developmental events. E : Ecdysis, W : Wandering, B: Burrowing

The significance of two smaller peaks occurring earlier in the haemolymph moulting hormone titre of S. littoralis 6th instar larvae at 40 and 72 hrs of development is perhaps less clear. However, the peak at 72 hrs in S. littoralis is probably equivalent to the tire increase observed in Galleria at 60 hr (see figure 4E-1) which has been suggested to be responsible for epidermal reprogramming from larval to pupal activities (Sehnal et al., 1981). Furthermore, similar peaks in Calpodes at 72 hr (see figure 4E-1) and in Manduca sexta at 108 hr of final larval stage development have been convincingly shown as the 'pupal commitment peak' (Dean et al., 1980; Riddiford, 1976). In the two latter species this peak, occurring approximately midway through the instar, has been demonstrated to be responsible for the change in epidermal commitment from larval to pupal activities. By analogy it would be reasonable to assign the peak at 72 hr in S. littoralis to this function. However, it must be noted that the peak at 108 hr in M. sexta was also associated with a behavioural change to the wandering stage (Riddiford, 1976). This is in contrast to the observations in Galleria where pupal commitment and behavioural change are assigned to different peaks (Sehnal et al., 1981).

The ecdysteroid titre in Calpodes revealed a number of minor peaks occurring early in the final larval instar, which are not so much in evidence in other ecdysteroid RIA titres that have been reported. This is probably due to the fact that very short time intervals were used for the study (6 hr). For the present study, larvae were also assayed at frequent time intervals (8 hrs) and this could explain why, in a number of RIA moulting hormone titre curves in other insects subtle ecdysteroid peaks early in the titre are not observed, (Philosamia and Bombyx, Calvez et al., 1976; Galleria, Maroy and Tarnoy, 1978).

In this study, the predominant ecdysteroid present has been shown by GC/MS (SIM) to be 20-hydroxyecdysone at least at the peak moulting hormone titres at 40 hrs and 120 hrs (Table 4B-4). A number of similar studies in other insects aimed principally at determining the ecdysone to 20-hydroxyecdysone ratio, have been undertaken. In Manduca (Bollenbacher et al., 1981) and Pieris (Lafont et al., 1975) the final larval peak was shown to contain mainly 20-hydroxyecdysone as is the case in Spodoptera, whereas in Galleria ecdysone and 20-hydroxyecdysone are present in similar amounts (Maroy and Tarnoy, 1978). However, these differences may only reflect the time of sampling. Indeed, a similar analysis of the last peak in the final larval instar of Bombyx (Calvez et al., 1976) showed that the ratio of ecdysone to 20-hydroecdysone varies quite considerably (from 1:2 to 9:1) depending on which time during the final peak the analysis was taken. This demonstrates as does the ecdysone and 20-hydroxyecdysone titres in Pieris (Lafont et al., 1975), and indeed the individual ecdysteroid titres performed in the S. littoralis pupae (see Part 3, section C), that what may appear to be a single peak by RIA analysis may, in fact, consist of a number of sharp ecdysteroid peaks.

It is perhaps surprising to find that the RIA analysis using the DHS 1-15 antiserum produces much higher values than the same analysis using ICT-1 antiserum. This could possibly be explained by the presence of ecdysteroids modified in the nucleus (e.g. 3-epiecdysteroids, ecdysteroid-3-acetates, 2-deoxyecdysteroids) in the haemolymph. These compounds would not be recognised by the ICT-1 antiserum, whereas the DHS 1-15 antserum, for which the binding to ecdysteroids is more affected by side chain modifications, would detect such compounds. In fact, it has been shown that some A-ring modified ecdysteroids, viz. ecdysone 2- and 3-acetates, actually improve binding to the DHS 1-15 antiserum by some 4-fold

(K.P. Wigglesworth, unpublished results). If this type of compound were indeed present in the haemolymph, even in relatively small amounts, the greater quantity of moulting hormone detected by DHS 1-15 antiserum could be explained. However, the pattern of radiolabelled ecdysteroids produced from injected [^{3}H] ecdysone did not support this notion; only late in the instar was an A-ring modified ecdysteroid produced (3-epi-ecdysone). This does not preclude the possible presence of an ecdysone precursor, such as 2-deoxyecdysone.

3. The conversion of ecdysone into 20-hydroxyecdysone in the tissues of 6th instar larvae of *S. littoralis*

The metabolism of ecydsone in various tissues of *S. littoralis* 6th instar larvae revealed two major products, 3-epiecdysone and 20-hydroxy-ecdysone. As 26-hydroxyecdysteroids were formed by the metabolism *in vivo* of [^{3}H] ecdysone, it was hoped that some conversion *in vitro* into 26-hydroxyecdysteroids might also occur. Such a conversion *in vitro* has been previously reported in the fat body and Malpighian tubules of *Manduca* (King, 1972) and *Pieris* imaginal wing discs (Blais and Lafont, 1980), but no formation *in vitro* of 26-hydroxyecdysteroids was observed in this study.

However, the exclusive conversion *in vitro* of ecdysone into 20-hydroxyecdysone in certain tissues of *S. littoralis* larvae enables a thorough analysis of the developmental variation of the 20-hydroxylation reaction throughout the 6th larval instar and its possible correlation with the endogenous ecdysteroid titre. 20-Hydroxylase activity was principally found in the homogenates of fat body and Malpighian tubules with some activity also in the midgut. Indeed, this is in agreement with reports in other insect species. The importance of the fat body

and Malpighian tubules as sources of 20-hydroxylase activity has been demonstrated in a similar study in Manduca sexta (Smith et al., 1980). In that case, the homogenates of fat body and Malpighian tubules demonstrated the highest specific activity, with the integument and midgut also displaying considerable activity. In the orthopterans, Locusta migratoria (Feyereisen and Durst, 1978) and Schistocerca gregaria (Johnson and Rees, 1977) the Malpighian tubules contained the highest specific activity with some 20-hydroxylase activity also present in the fat body. Therefore, it is apparent that one or both of these tissues probably plays an important role in the regulation of the ecdysone to 20-hydroxyecdysone ratio present in the insect.

In the present study, 20-hydroxylase activity in both tissues appears to reach a similar maximum specific activity (approximately 90 pg. 20-hydroxyecdysone/min/mg tissue; see figure 4C-4). However, in terms of total activity per insect (or tissues), the activity shown by the fat body far exceeds that shown by Malpighian tubules 20-hydroxylase activity. In addition, the fat body shows activity at many times during the instar, whereas the Malpighian tubule enzyme seemingly shows activity only over a short period towards, the end of the final larval instar. It would therefore appear that the 20-hydroxylase activity in the fat body has most influence on the ecdysteroid composition of the haemolymph. Indeed, the fat body's role as a major metabolic organ in insects, its close proximity to the haemolymph, and its distribution throughout the whole of the insect, suggests that this tissue is the most obvious for controlling the conversion of ecdysone into 20-hydroxyecdysone. The Malpighian tubules, being closely associated with the alimentary tract, are more likely to be involved in the catabolism of ecdysone for eventual excretion via the gut. These possible functions of the 20-hydroxylase enzyme in the fat body and the Malpighian tubules

have also been suggested on the basis of the kinetic properties of the 20-hydroxylase from fat body and Malpighian tubules (Koolman, 1982). The 20-hydroxylase of the fat body is characterised by a low Km and limited capacity (Bollenbacher et al., 1977), while in the tissues associated with the alimentary tract, the midgut and Malpighian tubules, the enzyme displays a high Km and high capacity (Kaplanis et al., 1980; Johnson and Rees, 1977). This indicates that the fat body enzyme may function at relatively low ecdysone concentrations and so modulate the ecdysone to 20-hydroxyecdysone ratio in the haemolymph. Whereas the enzyme associated with the alimentary tract may hydroxylate high concentrations of ecdysone destined for excretion. However, it must be noted that in *Locusta migratoria* the apparent Km for ecdysone was not significantly different when using fat body or Malpighian tubule microsomes (Feyereisen and Durst, 1978).

The activity of the fat body ecdysone 20-hydroxylase system during the development of sixth instar larvae is characterised by three periods of increased activity [figure 4E-2(C)]. By far the most significant increase is that observed mid way through the instar with maximum activity at 80 h. Significantly, the gradual increase of this peak accompanies a period of considerable growth and increase in body weight. The increase in body weight is not only due to increased haemolymph in the insect [figure 4E-2(A)] but also due to actual increase in weight of the tissues. For example, the fat body weight increases from 57mg to 162 mg from 48hr to 88 hr[figure 4E-2(B)]. Also, when larvae undergo a behavioural change at 96hr by entering a burrowing stage, ceasing excretion via faeces and apparently reducing growth rate, the 20-hydroxylase activity drops dramatically from 14300 pg/min/insect to 600 pg/min/insect in 8 hours. This suggests perhaps a temporal relationship between 20-hydroxylase activity and the excretory and/or growth of the insect.

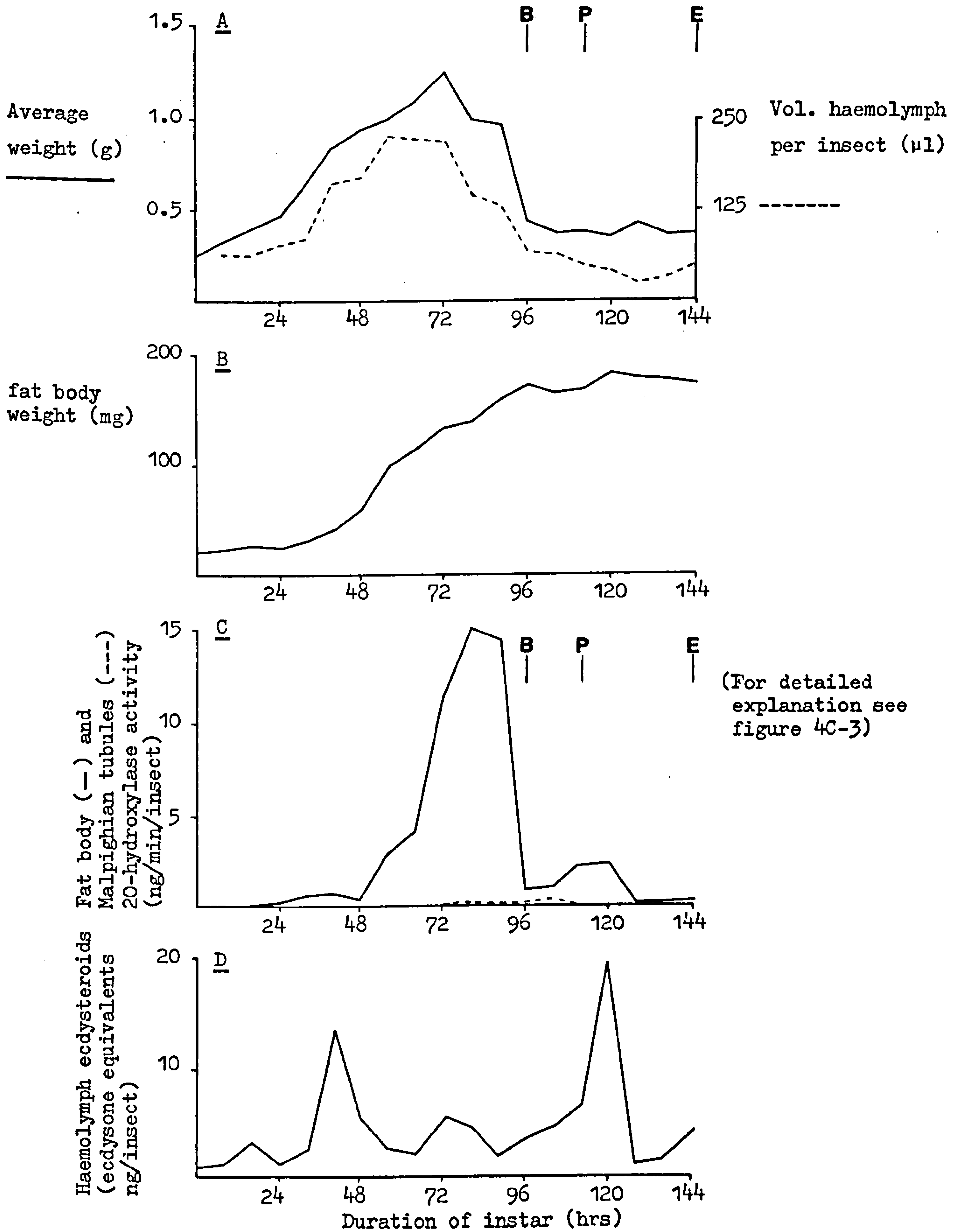


Figure 4E-2 : Comparison of aspects of 6th instar S. littoralis development. (A) The growth curve and volume of haemolymph during the instar, (B) the weight of the fat body, (C) 20-hydroxylase activity titre in fat body, and Malpighian tubules, (D) endogenous ecdysteroid titre determined by RIA (DHS 1-15 antiserum). B : Burrowing, P : Onset of prepupal stage, E = Ecdysis

A similar study of the 20-hydroxylase activity in fat body homogenates during the final larval instar of Manduca (Smith et al., 1983) has been undertaken. This showed that 20-hydroxylase activity also increases dramatically during the growth stage (days 0 to 4) of the last larval instar, but decreases sharply at day 6, prior to the maximum haemolymph ecdysteroid titre at day 7. As in Spodoptera the decrease in activity in Manduca occurs around the time of a behavioural change (i.e. wandering stage).

In both studies, the drop in 20-hydroxylase activity corresponds closely to the cessation of faeces production. In the present study considerable amounts of ecdysteroid conjugates have been found in the faeces of early 6th instar larvae. This is in contrast to the metabolism of [^{3}H] ecdysone in late 6th instar larvae (i.e. in the absence of faeces production) where only small amounts of conjugates are produced. Therefore, this indicates that there is, perhaps, a temporal correlation between 20-hydroxylation activity and the conjugation and excretion processes. It could be postulated that during the growth stage (day 0 to 4), because the conjugation and excretion processes are active and only small amounts of ecdysone are being secreted by the prothoracic glands, high 20-hydroxylase activity is required to maintain the concentration of 20-hydroxyecdysone at a sufficient level for normal development to continue. Indeed Smith et al. (1983) suggested a similar explanation for the elevated 20-hydroxylase activity observed in Manduca over the growth stage of fifth instar larvae development. They suggested that the high 20-hydroxylase activity (with its low K_m) may be necessary to ensure that even the small amount of ecdysone produced by the prothoracic glands could be hydroxylated to 20-hydroxyecdysone in order to maintain critical levels of 20-hydroxyecdysone so that development can continue.

These findings in Manduca and Spodoptera, therefore, suggest that there is no precise quantitative correlation between the change in 20-hydroxylase activity and changes in the ecdysteroid titre. The major peak of 20-hydroxylase activity decreases well before the appearance of the major peak of ecdysteroid at 120 hrs (figure 4E-2). Similarly, in Schistocerca gregaria (Greenwood, 1981) peak 20-hydroxylase activity precedes the peak ecdysteroid titre. Furthermore, in Calliphora erythrocephala (Young and Young, 1976) data suggest that elevated 20-hydroxylase activity corresponds to a low ecdysteroid titre.

This is in contrast to the findings in larvae of other species where some correlation has apparently been established [Locusta migratoria (Koolman et al.,1975); Tenebrio molitor (Weinheimer and Romer, 1977); Cholistoneura fumiferana (Lagueux et al., 1976); Gryllus bimaculatus (Wildman and Romer,1977)].

The present work essentially agrees with the findings in Manduca and Calliphora. However, because in the present study 20-hydroxylase activity was monitored at frequent intervals (8 hr) during the instar, two relatively minor peaks of activity were also distinguished early (40 hrs) and late (120 hrs) in the instar [figure 4E-2(C)]. Such subtle, yet possibly significant fluctuations in the 20-hydroxylase titre, could not have been observed in Manduca or Calliphora as 20-hydroxylase activity was assayed at only a few selected points during Calliphora development, and at 24 hr intervals in Manduca.

In contrast to the peak of high activity, the two minor peaks do correlate well with corresponding peaks of endogenous ecdysteroid observed in the RIA moulting hormone titre (see figure 4E-2). This is particularly true of activity corresponding to the major endogenous ecdysteroid peak at 120 hrs. The sole ecdysteroid detected by GC/MS

(SIM) at this time (120 hrs) was found to be 20-hydroxyecydsone (table 4B-4), this correlates well with the increased 20-hydroxylase activity observed at 112 and 120 hrs (figure 4E-2). The increase in activity observed is possibly a result of induction of the 20-hydroxylase enzyme by an increased level of ecdysone in the haemolymph. Indeed, such an inductive effect of the 20-hydroxylase system by ecdysone and 20-hydroxyecdysone has been indicated in Locusta (Feyereisen and Durst, 1980).

The activity monitored at 120 hrs is much lower than the activity observed earlier in the instar at 72-80 hrs. As explained earlier, excretion via the faeces ceases at approximately 96 hr. Therefore, at the later stages of 6th instar larvae development removal of ecdysteroids via conjugation is reduced in the absence of excretion, therefore levels of 20-hydroxyecdysone can be maintained with lower 20-hydroxylase activity.

The sharp decrease of the endogenous ecdysteroid titre between 120 and 128 hrs is concurrent with an equally sharp decrease in 20-hydroxylase activity (figure 4E-2). This, provides further evidence for the direct involvement of the 20-hydroxylase enzyme system in the control of the titre at the prepupal stage. It has been shown that 20-hydroxyecdysone effectively inhibits 20-hydroxylase activity (Feyereisen and Durst, 1977, in Locusta; Beydon and Lafont, 1983, in Pieris), and in addition, 20-hydroxyecdysone has been shown to be a very effective inhibitor of ecdysone biosynthesis (Beydon and Lafont, 1983). These effects and, of course, further metabolism of 20-hydroxyecdysone to 3-epiecdysteroids and/or ecdysteroid-26-oic acids probably all contribute to the decrease in the moulting hormone titre. This implies that during late 6th instar larvae development (i.e. later than 96 hrs) 20-hydroxylase may play a central role in the control of the ecdysteroid titre.

The increase and decrease in the 20-hydroxylase activity in the Malpighian tubules occurs later than that of the fat body (see figure 4C-4). However, the enzymic activity still decreases prior to the endogenous ecdysteroid peak titre at 120 hrs. This suggests that this tissue does not play a major part in the control of the haemolymph ecdysteroid titre. The Malpighian tubules 20-hydroxylase activity could perhaps be implicated in the control of catabolic and excretory processes.

The 20-hydroxylase activity of Manduca sexta midgut during the final larval stage has been investigated (Smith et al., 1983). As the midgut and Malpighian tubules are both associated with the alimentary tract, the Manduca midgut and Spodoptera Malpighian tubules 20-hydroxylase titres are probably worthy of comparison. As in the present study with Malpighian tubules, Manduca midgut homogenates showed a peak of activity approximately 24 hrs later than that observed in the fat body. Also, a dramatic decrease in the Manduca midgut 20-hydroxylase activity is concurrent with the increase in haemolymph ecdysteroid titre, which is also observed in this study.

Due to the lower mass of midgut compared with fat body, it would also appear that the midgut 20-hydroxylase does not contribute significantly to the ecdysteroid titre in Manduca. Therefore, most 20-hydroxylase activity per insect appears to be present in the fat body, both in Manduca and Spodeptera.

In both these studies, the 20-hydroxylase activity in these two tissues (midgut and Malpighian tubules) is coincident with quite dramatic changes in the insect: a behavioural change, cessation of feeding and the purging of the gut contents. As suggested by Smith et al. the sharp peak of hydroxylase activity observed in these tissues

may be part of a general increase in the metabolic activity of the alimentary tissues as they undergo dramatic physiological changes associated with these events. Thus, the fluctuation of the 20-hydroxylase enzyme activity observed in the Malpighian tubules may not entirely be a result of any specific increase in enzyme activity. However, it is always possible that the activities of enzymes *in vitro* may not reflect their true pattern of activities *in vivo*.

4. The conversion of ecdysone into 3-epiecdysone in the midgut of 6th instar larvae of *S. littoralis*

The properties of the 3-epimerisation reaction as demonstrated in this study (oxygen dependence, reduced nicotinamide nucleotide cofactor requirement, and the irreversibility of the reaction) are in agreement with those established in previous investigations in *Manduca sexta* midgut (Nigg *et al.*, 1974; Meyer *et al.*, 1979). However, in *Manduca* midgut it was demonstrated that either NADPH or NADH could be used as cofactor, whereas in this study the addition of NADH to the incubation did not produce 3-epiecdysone. In fact, the presence of NADH lead to the production of another metabolite, which could possibly be 2-epiecdysone.

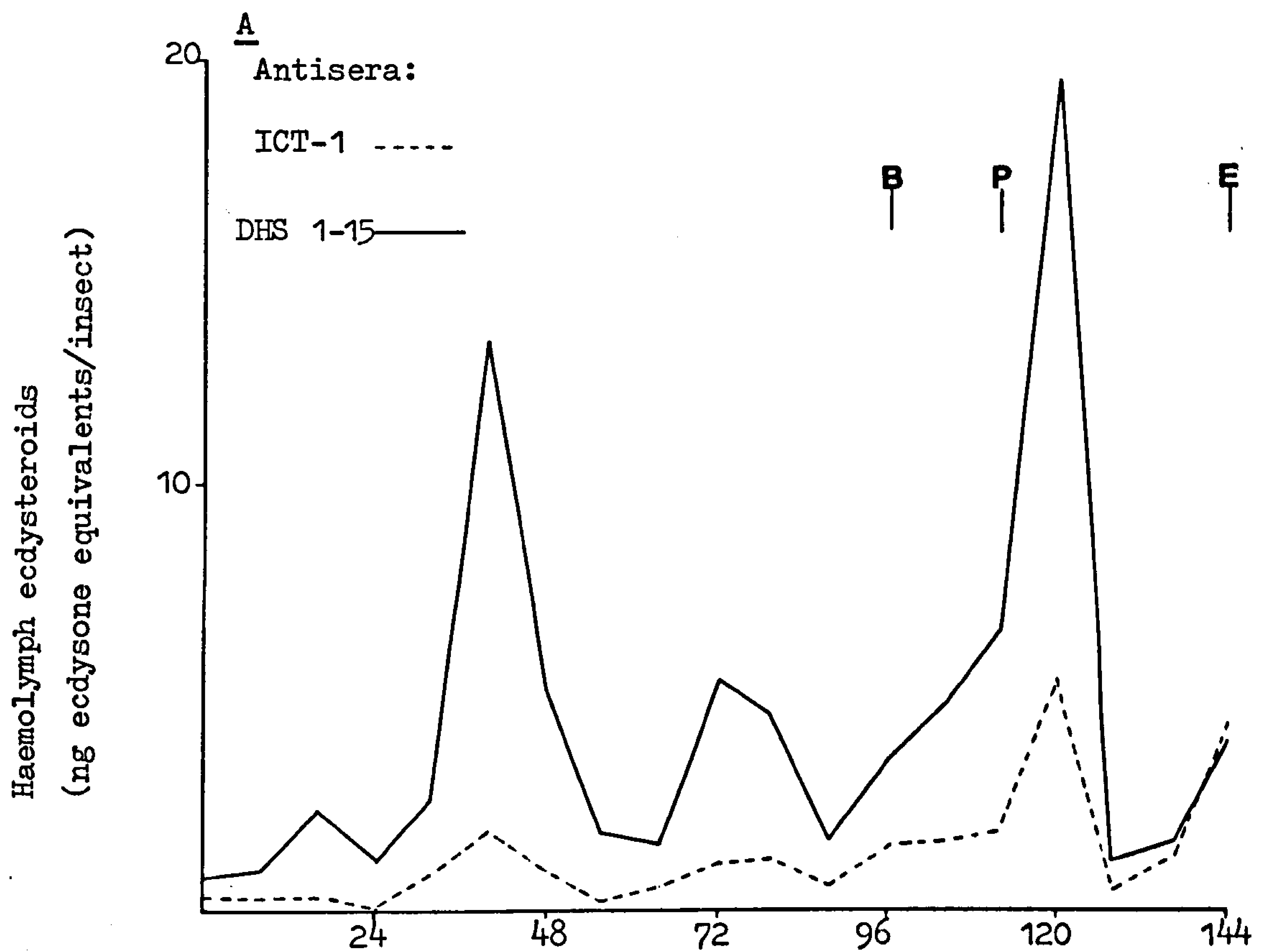
Ecdysone 3-oxidase activity, which converts ecdysone into 3-dehydroecdysone, has been detected in the midgut of *Calliphora erythrocephala* (Koolman and Karlson, 1975). Although the reaction was shown to be irreversible *in vitro* (Koolman and Karlson, 1978), 3-dehydroecdysone injected into the insect was converted into 3-epiecdysone (Koolman, 1982). This suggests the possible intermediacy of 3-dehydroecdysone in the 3-epimerisation reaction, with the 3-epimerase system consisting of two enzymes, ecdysone oxidase and 3-dehydroecdysone reductase (Koolman,

1976). In the present study, the formation and metabolism of 3-dehydro-ecdysone in *in vitro* incubations indicated the presence of these two enzymes in *S. littoralis*. However, the actual separation of the two enzymes has yet to be performed.

In *Manduca* and *Spodoptera* midgut tissue, ecdysone is metabolised to 3-epiecdysone whereas in *Calliphora* midgut ecdysone is metabolised to 3-dehydroecdysone. The reason for the different metabolism is unclear. It could, of course, possibly be due to the different experimental procedures employed. In studies utilising *Calliphora* midgut preparations, the addition of reduced cofactor was not reported (Koolman and Karlson, 1975; Koolman and Karlson, 1978; Koolman, 1978). A possible absence of appropriate reducing cofactors in the enzyme incubation would, at the least, lead to a less active conversion of 3-dehydroecdysone into 3-epiecdysone and would perhaps cause accumulation of 3-dehydroecdysone in the *Calliphora* preparation. Indeed, this would also explain the reported irreversible nature of the ecdysone 3-oxidase reaction in *Calliphora*, where 3-dehydroecdysone is not converted back to ecdysone (Koolman and Karlson, 1978). However, in *Spodoptera* midgut cytosol preparation, 3-dehydroecdysone is converted into both ecdysone and 3-epiecdysone. Significantly, this conversion is considerably lowered in dialysed cytosol preparation [cf. figures 4D-2(4) and 4D-3 (E)].

An enzyme capable of reducing 3-dehydroecdysone to 3-epiecdysone does seem to be present in *Calliphora*, as injected radiolabelled 3-dehydroecdysone is apparently metabolised rapidly to 3-epiecdysone (Koolman, 1982). In addition to any differences caused by the experimental procedures employed, the inter-species variations observed *in vitro* could be attributed to the localization of the 3-dehydroecdysone reductase enzyme. The titre of 3-epimerase activity in midgut (figure 4C-5)

shows that the activity of this enzyme can be as high as 1400 pg 3-epiecdysone produced/min/mg tissue, far exceeding the rate of 20-hydroxylation observed in fat body homogenates (98 pg/min/mg tissue). A similar situation is observed in Calliphora where the ecdysone oxidase activity in the midgut shows higher activity than the 20-hydroxylase system of any tissue. However, the ecdysone oxidase titre in Calliphora seems to correlate well with the endogenous ecdysteroid titre, whereas the 3-epimerase titre in midgut homogenates of Spodoptera 6th instar larvae bears no apparent relationship to the endogenous ecdysteroid titre (figure 4E-3). By investigation of the endogenous ecdysteroids during the pupal stage and from the metabolism *in vivo* of [^{3}H] ecdysone in late 6th instar larvae, it is apparent that 3-epi-ecdysteroids occur during the prepupal stage and in early pupal development. However, the 3-epimerase titre shows a decrease in activity towards the end of the instar. This suggests that factors other than the actual 3-epimerase activity control the level of 3-epi-ecdysteroids in the insect. The actual 3-epimerase titre curve in fact shows no distinct pattern, save a much lower activity over the first 48 hrs of the instar and 8 hrs prior to ecdysis to the pupal stage. It has previously been suggested, on the basis of RIA analysis with two different antisera (ICT-1 and DHS 1-15), that during 6th instar peak ecdysteroid titre some 3-epiecdysteroids may be present (p.131). On that basis it would be reasonable to expect peaks of 3-epimerase activity corresponding to peaks of endogenous ecdysteroid. There is, in fact, a discernible increase in epimerase activity from 104 hrs to 120 hrs followed by a sharp decrease towards 136 hrs, which could possibly be associated with the major peak of endogenous ecdysteroid at 120 hrs (figure 4E-3). However, the overall pattern of the titre,



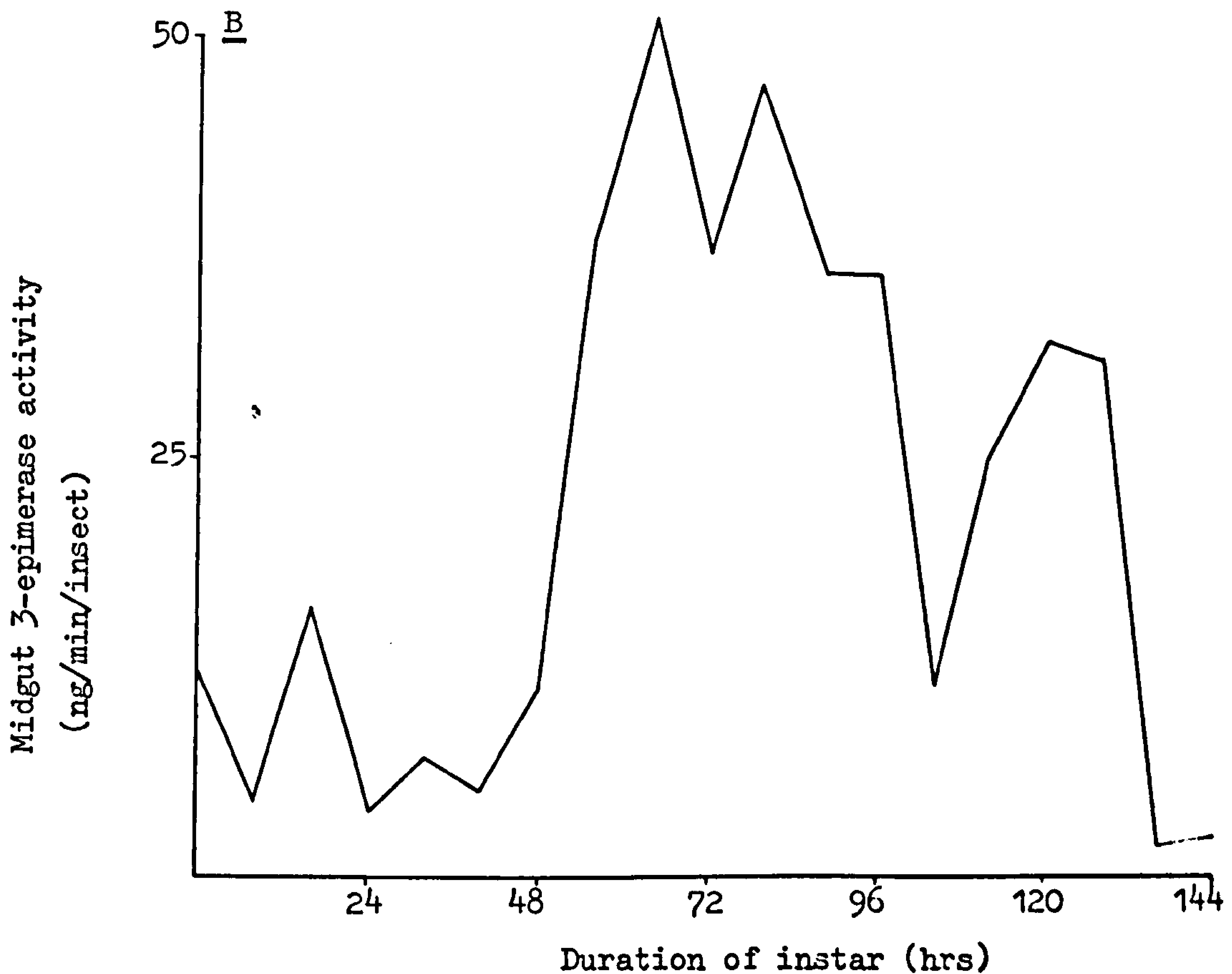


Figure 4E-3 : Comparison of (A) the endogenous haemolymph moulting hormone titre by RIA, and (B) midgut 3-epimerase activity titre, P : Onset of prepupal stage, E : Ecdysis, B : Burrowing.

and indeed the very high activity of this enzyme *in vitro*, suggest that other factors control the metabolism of ecdysone to 3-epiecdysone.

It is possible that the activity indicated by *in vitro* experiments does not reflect the *in vivo* situation. Other enzymes may, in fact, have higher affinity for the substrate ecdysteroids. Indeed, the K_m values of ecdysone 20-hydroxylase and ecdysone 3-epimerase (1.6×10^{-7}M and 5.13×10^{-5}M respectively) as calculated in *Manduca sexta* tissues (Bollenbacher *et al.*, 1977; Mayer *et al.*, 1979) suggest that 20-hydroxylase has a higher affinity for ecdysone. Alternatively, ecdysone 3-epimerase may be compartmentalized and, therefore, separated from the endogenous pool of ecdysteroids. Perhaps the 3-epimerase system of the midgut may be directed at the inactivation of moulting hormones present in the food rather than controlling the ecdysteroid concentrations within the insect.

Even if the ecdysone 3-epimerase system is active the actual concentration of 3-epiecdysteroids in the insect may, in fact, be controlled by the further metabolism of 3-epiecdysone and 3-epi 20-hydroxyecdysone, via conjugation or possibly via ecdysteroid-26-oic acid formation. It is interesting to note that at the times at which conjugation is considerably reduced (prepupal and early pupal stage) the presence of 3-epiecdysteroids has been observed (Table 4A-2, figures 3C- 4,5,6 and 7), whereas during the times of high conjugation these ecdysteroids are not detectable. This phenomenon is also indicated in *Pieris* (Lafont *et al.*, 1980; Beydon *et al.*, 1981).

5. The ecdysteroids contained in the faeces of *S. littoralis* 6th instar larvae

In the haemolymph of 6th instar larvae, conjugated ecdysteroids constitute only a small proportion of the total ecdysteroid complement (Table 4B-3). In the faeces, however, the endogenous ecdysteroids and, indeed, ecdysteroids produced from the metabolism *in vivo* of injected ecdysone consist of a large proportion of conjugated ecdysteroids (Table 4B-5 and Table 4A-2). In addition, the analysis of the metabolites from the metabolism *in vivo* of [^{3}H] ecdysone in early larvae revealed that the majority of the ecdysteroid metabolites had been excreted into the faeces (81%). It would therefore appear that during the times at which the larvae produce faeces, until approximately 88 hrs of 6th instar development, conjugation and excretion could be important in maintaining the moulting hormone titre at a low level.

Indeed, this appears to be true in *Locusta migratoria*. The low moulting hormone titre observed up to day 6 of 5th instar development of *Locusta* is, at least in part, controlled by the very active excretion of ecdysteroids (Koolman *et al.*, 1975). In *Calliphora erythrocephala* (Young, 1976) the increase in the endogenous 20-hydroxyecdysone titre at puparium formation is accompanied by a decrease in the rate of metabolism of moulting hormones. This could also be the case in 6th instar *S.littoralis*, where at the late stage of the instar, conjugation and excretion are necessarily reduced because of the lack of faeces production. Thus contributing to the increase in moulting hormone titre at 120 hrs. Even in the haemolymph the proportion of ecdysteroids in the form of conjugates does not rise significantly at peak titre (Table 4B-3).

In the absence of faeces production, the decrease in the peak titre at 120 hrs is probably affected by the metabolism to ecdysteroid-26-oic acids and possibly 3-epimerisation (Table 4A-2). However, a small amount of conjugation is still present at this stage, and, indeed, an increase in the amount of ecdysteroid conjugate is observed at 144 hrs (Table 4B-3). This process may therefore still contribute to the control of the moulting hormone titre at the prepupal and pupal stage, albeit to a lesser degree.

As in Tenebrio molitor larvae (Weinheimer and Romer, 1977) the principal conjugates in the faeces contained ecdysteroid moieties of 20-hydroxyecdysone and ecdysone. In faeces collected over the first 60 hours of 6th instar development, 20-hydroxyecdysone (6.08 ng/insect) was the major ecdysteroid with a smaller amount of ecdysone (2.0/ng/ insect). As the conjugation process has not been found to be specific for any single ecdysteroid, the quantities observed probably reflect the concentration of 20-hydroxyecdysone and ecdysone in the haemolymph. Indeed the GC/MS (SIM) analysis of the free ecdysteroids at 40 + 48 hrs of development shows 20-hydroxyecdysone to be the principal ecdysteroid (11.46 ng/insect) with ecdysone (2.83 ng/insect) also present but at lower concentration.

PART 5 GENERAL DISCUSSION

PART 5

General Discussion

Insects have many advantages as experimental animals. Their short life cycle makes them ideal for developmental studies, and because of characteristic modifications in morphology throughout development, large groups of animals can be synchronised with considerable accuracy. This is of particular importance in the study of hormonal systems. The ability to obtain large groups of synchronous insects with confidence allows the problem of low endogenous hormone level to be largely overcome. However, the presence of relatively small amounts of ecdysteroids in insects and the large pool of cholesterol, the distal precursor of ecdysone, still makes the incorporation of label via administered radioactive cholesterol extremely low. Therefore much care is required in the purification of radioactive cholesterol, which must be administered to the insect at relatively high levels of radioactivity. As the present study was principally concerned with the further metabolism of ecdysone, the administration of radioactive ecdysone provided a great deal of information for this study. Although such studies provided good incorporation of the radioactivity into numerous ecdysone metabolites, the results need to be treated with some caution. The quantity of exogenous ecdysone administered may not reflect the endogenous situation nor may it be subject to the same possible protein binding or compartmentalization as the endogenous hormone.

The present study has provided an insight into the processes affecting the modulation of the moulting hormone activity circulating in the insect. At most times of development that were studied in <u>S. littoralis</u>, 20-hydroxyecdysone and 20-hydroxylated metabolites were

consistently more prominent than ecdysone and its corresponding metabolites. However, the quantitative importance of different pathways of inactivation of these hormones appeared to differ depending on the developmental stage of the insect. Throughout the developmental stages studied, metabolism of ecdysone and 20-hydroxyecdysone via 26-hydroxylation and oxidation to the corresponding acids appears to be prominent (figure 5-1). However, the overall metabolite pattern is also dependent upon the activity of two other routes of inactivation, viz. conjugation and 3-epimerisation of ecdysteroids. The 6th larval stage and pupal stage, encompassing the metamorphic transformation of the insect, is characterised by three distinct periods of different metabolism of ecdysone and 20-hydroxyecdysone. During the first four days of 6th instar larval development, when the endogenous ecdysteroid titre is low, the production of faeces was accompanied by the metabolism of ecdysteroids via conjugation. With the cessation of faeces production, conjugation was considerably reduced and did not constitute a major metabolic route throughout the pupal stage. The early pupal stage and possibly the prepupal stage were characterised by metabolism of ecdysone to 3-epiecdysone and 3-epi-20-hydroxyecdysone. At the later stages of pupal development (from day 4 of pupal development to adult emergence), where ecdysteroid levels are at their highest, the absence of 3-epimerisation and conjugation leave hydroxylation and oxidation of the side chain as the main metabolic route of ecdysone and 20-hydroxyecdysone. These pathways are shown in figure 5-1 and demonstrate the influence of the two development-dependant reactions, conjugation and epimerisation, on the metabolites present in the insect at any given time during the 6th larval and pupal stages. The metabolism to the ecdysteroid-26-oic acids is active throughout development but may vary in amplitude.

Figure 5-1 : Major pathways of ecdysone metabolism in *Spodoptera littoralis* as established in the present study

The precise nature of the conjugates was not investigated in this study. Although a number of polar ecdysteroid conjugates have been characterised from ovaries or eggs of insects, the identification of post-embryonic ecdysteroid conjugates has been limited. Recently, 3-(or 2-)acetate 22-phosphate and 3-(or 2-) phosphate derivatives of 20-hydroxyecdysone have been identified as metabolites of 20-hydroxy-ecdysone incubated in vitro with gut-Malpighian tubule complexes from Locusta larvae (Tsoupras et al., 1983). Similar ecdysteroid conjugates may well be present in Spodoptera larvae.

As 3-epiecdysteroids have been identified during periods when no excretion is possible (i.e. in a closed system) epimerisation may represent an alternative means of inactivation in these situations. However, as 3-epiecdysteroids are not observed in significant amounts at the later stages of pupal development this cannot be strictly true. The occurrence of 3-epiecdysteroids corresponds more closely to the period when larval tissues are being degraded and adult tissue synthesized. Therefore, 3-epimerisation could occur as a result of a different physiological environment present at that time. However, it is possible that 3-epiecdysteroids may have a specific hormonal function during the process of metamorphosis.

Whether any or all of the ecdysteroid metabolites identified in this study have distinct hormonal functions is unclear. It has been indicated that in isolated nuclei from crayfish integument ecdysone and 20-hydroxyecdysone have different binding sites (Kuppert and Spindler, 1982) suggesting different functions for these two ecdysteroids. As far as moulting hormone activity is concerned, the metabolism of ecdysone and 20-hydroxyecdysone either via 26-hydroxylation or 3-epimerisation causes considerable inactivation, reducing the biological activity by some 5 to 10-fold (house fly assay).

The modulation of moulting hormone activity through metabolism of the ecdysteroids is probably most critically effected at the target cell and more particularly by the interaction of the ecdysteroid and the receptor.

In a study of the biological activities of ecdysteroids and ecdysteroid analogues the essential groups required for moulting hormone activity were assessed (Bergamasco and Horn, 1980). A 22R-hydroxy group was essential for high activity, and in most biological activity assays, analogues containing a 20,22 diol function were always the most active. This supports the suggestion that ecdysone acts primarily as a prohormone, and is activated by 20-hydroxylation to 20-hydroxyecdysone. Therefore, the C-22 hydroxy group is probably involved in a critical interaction with the receptor. In the presence of the 20-hydroxy group, this interaction is enhanced, possibly because of increased local polarity on the side-chain or by restricting the orientation of the C-22 hydroxy group into a position favourable for interaction with the receptor. Indeed, it would appear that this could occur. In the ecdysone molecule the side-chain is by no means a rigid structure, rotation around the C-17 to C-20 axis and around the C-20 to C-22 axis (see figure 5-2) leads to a side-chain continuously changing its orientation, obviously reducing the chances of a favourable spacial position of the 22 hydroxy group for interaction with the receptor. However, with the addition of the hydroxyl group at the chiral C-20 position this random movement of the side-chain will be considerably reduced. With the proximity of the C-18 methyl group and the presence of the C-21 methyl group, the addition of a bulky group at C-20 causes considerable steric hinderance between the C-21 methyl and C-20 hydroxy groups c the side-chain and the C-18 methyl group on the ecdysteroid nucleus

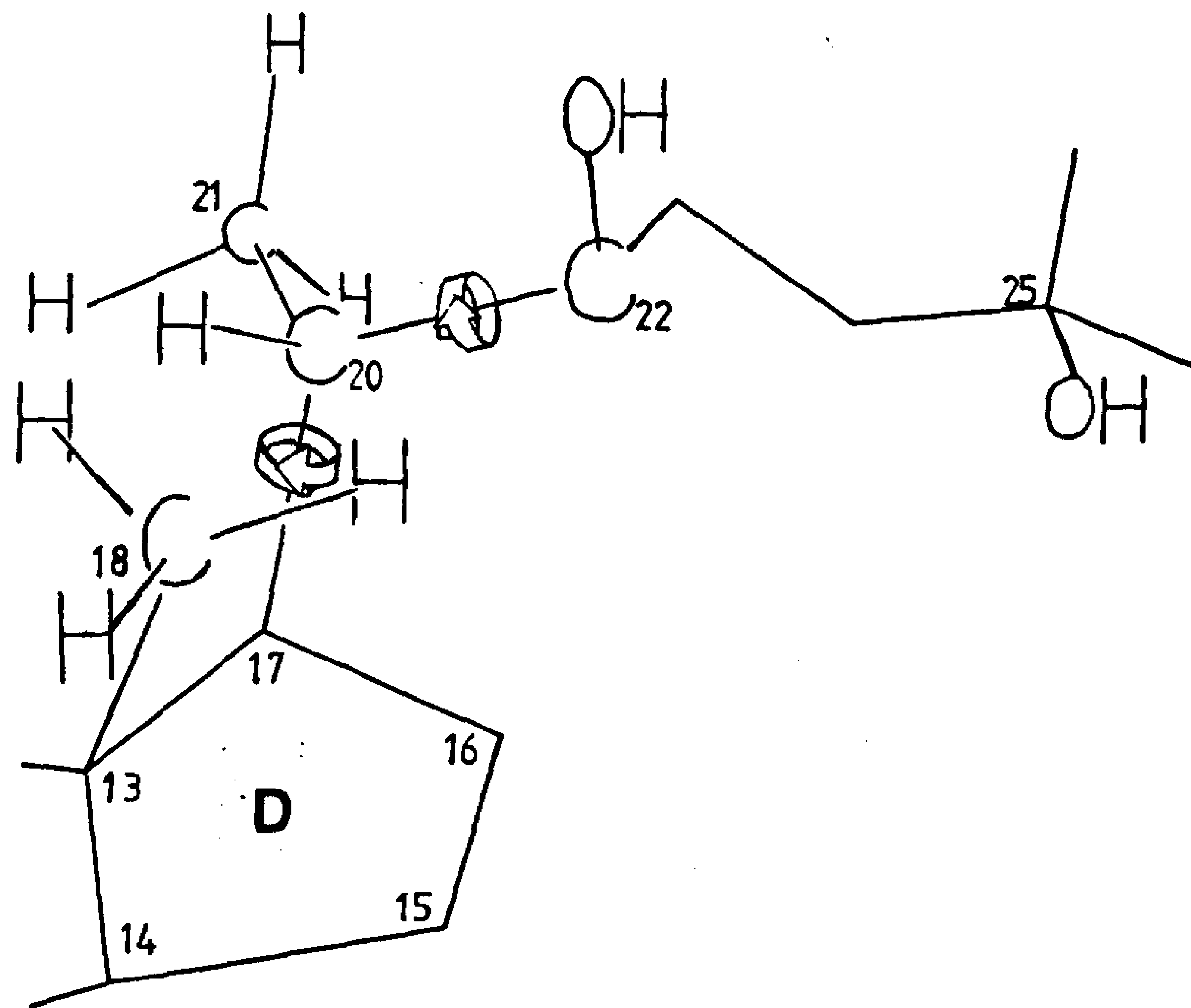


Figure 5-2 : The structure of ecdysone at ring D and the side chain, showing the rotation that can occur around the C-17/C-20 and C-20/C-22 axes.

(figure 5-3). Thus, movement around the C-17 to C-20 axis is restricted. In addition, due to the presence of a hydroxy group at C-20, steric hinderance between the C-20 hydroxy and C-22 hydroxy groups will tend to reduce rotation around the C-20 to C-22 axis, thus, largely confining the essential C-22 hydroxy group to the α side of the molecule. These conformational restrictions in 20-hydroxyecdysone, while not rendering the side chain completely rigid, would therefore increase the probability of the C-22 hydroxy group being in a favourable position for ecdysteroid-receptor interaction.

In the same study (Bergamasco and Horn, 1980) it was found that the most active ecdysteroids contain a 3β-hydroxy group. It is not surprising, therefore, that 3-epi-20-hydroxyecdysone and 3-epiecdysone show only 7-10% and 10-20%, respectively, of the 20-hydroxyecdysone activity in the *Musca* assay. The survey showed that binding to the β-face of the A-ring is required and also that the presence of a small 3β-group is necessary for activity. It is clear that the conversion of ecdysone and 20-hydroxyecdysone into their 3-epimers has a profound effect on the activity of the ecdysteroid. The orientation of the 3α-hydroxy group is very different from that of the 3β-hydroxy (figure 5-4). The different orientation will presumably also affect the conformation of the 'A' ring and, therefore, the shape of the β-face. The configuration of the A ring is constantly changing, continuously interconverting between chair and boat shape although it will be predominantly in the chair configuration. In the case of 3β-ecdysteroids, there is considerable diaxial interaction between the axial hydrogens at C-1 and C-5 and the axial hydroxy at C-3 (figure 5-5). However, in the case of 3-epi-ecdysteroids the C-3 hydroxy is in the equatorial position creating much less diaxial interaction. This produces a structure at the 'A'

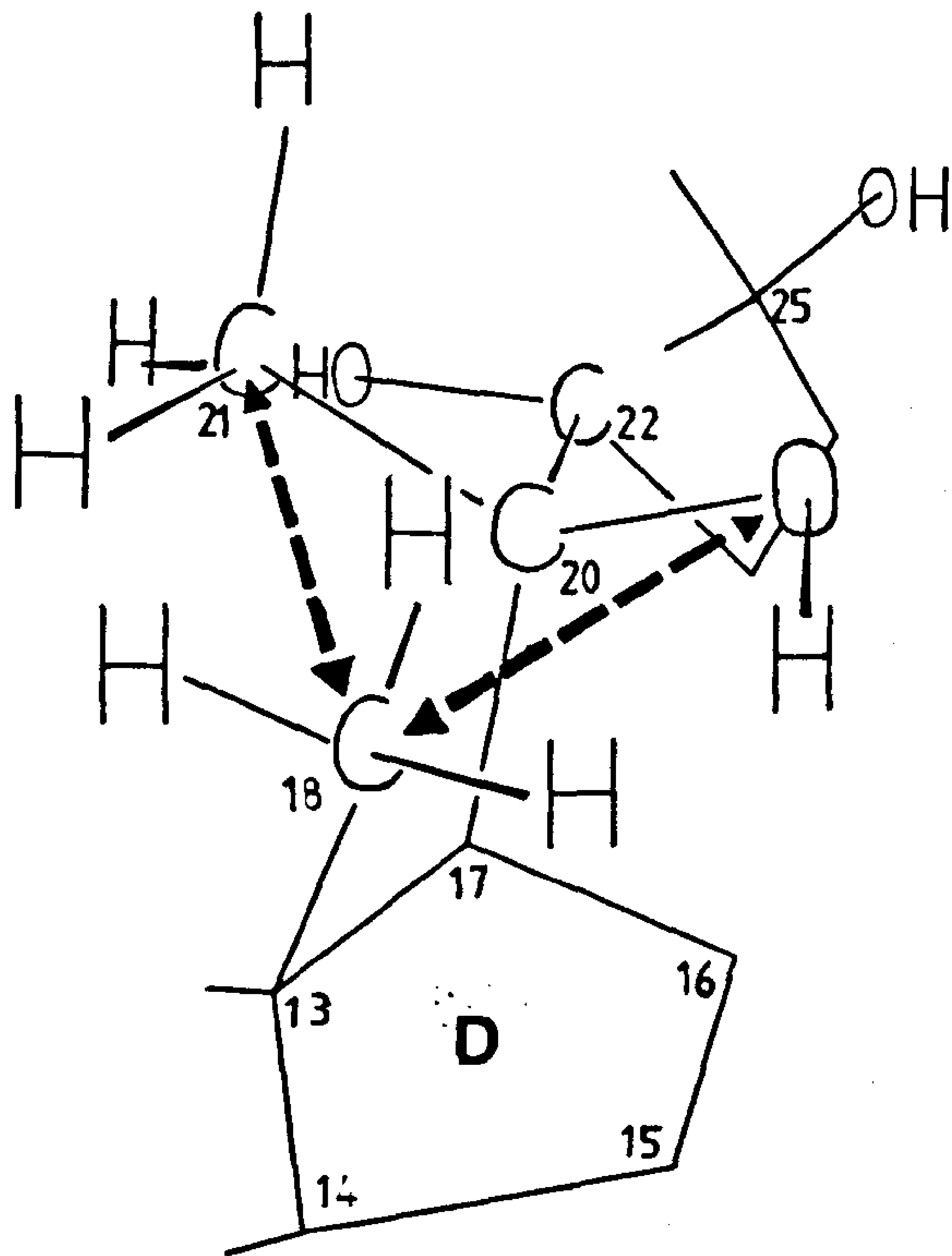


Figure 5-3 : The structure of 20-hydroxyedysone at ring D and the side chain, showing the interaction (dotted arrows) between the C-18 methyl of the nucleus and the 20 hydroxy and C-21 methyl groups on the side chain. Also demonstrating the most likely spacial configuration of the 20,22 diol.

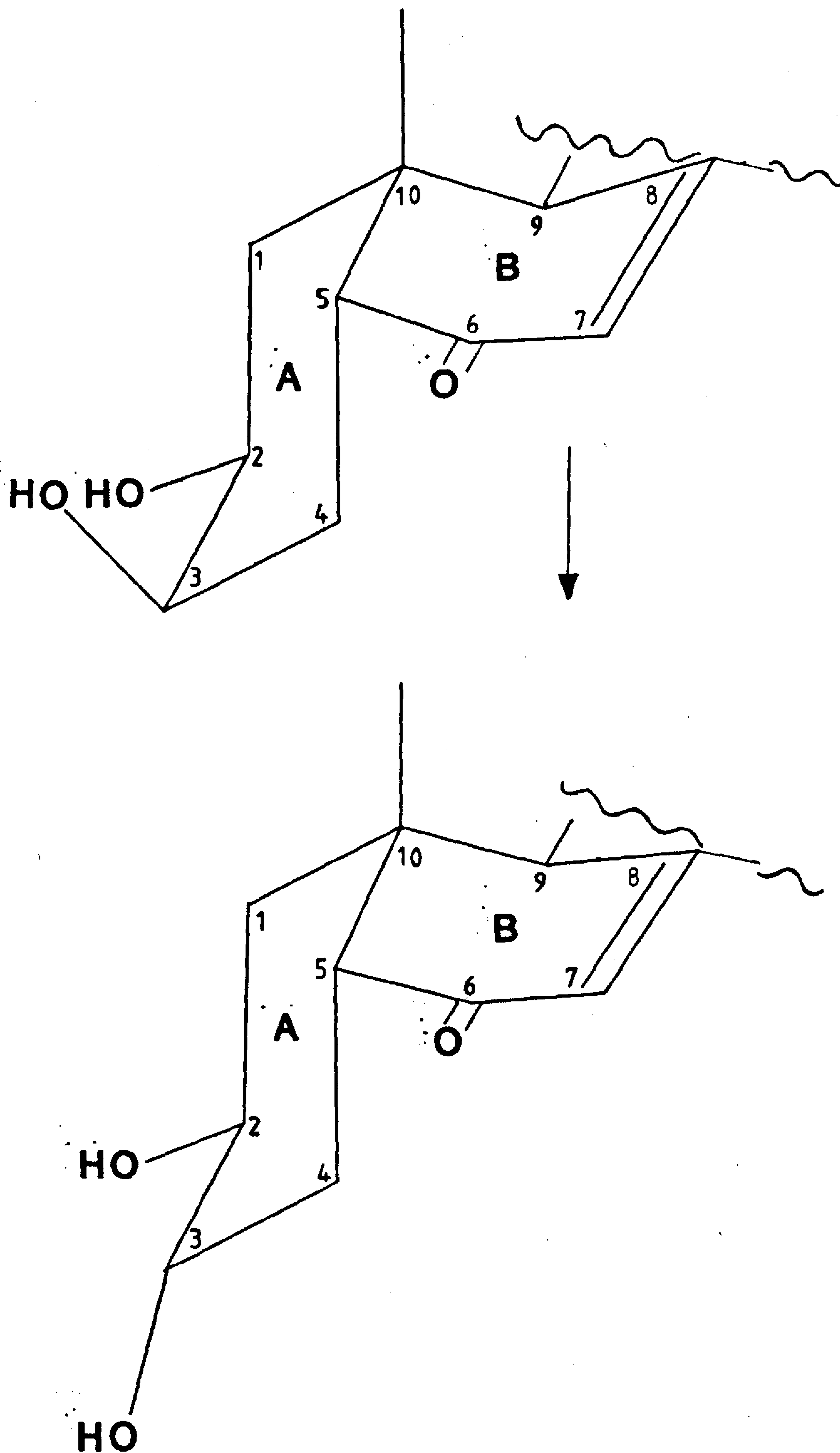
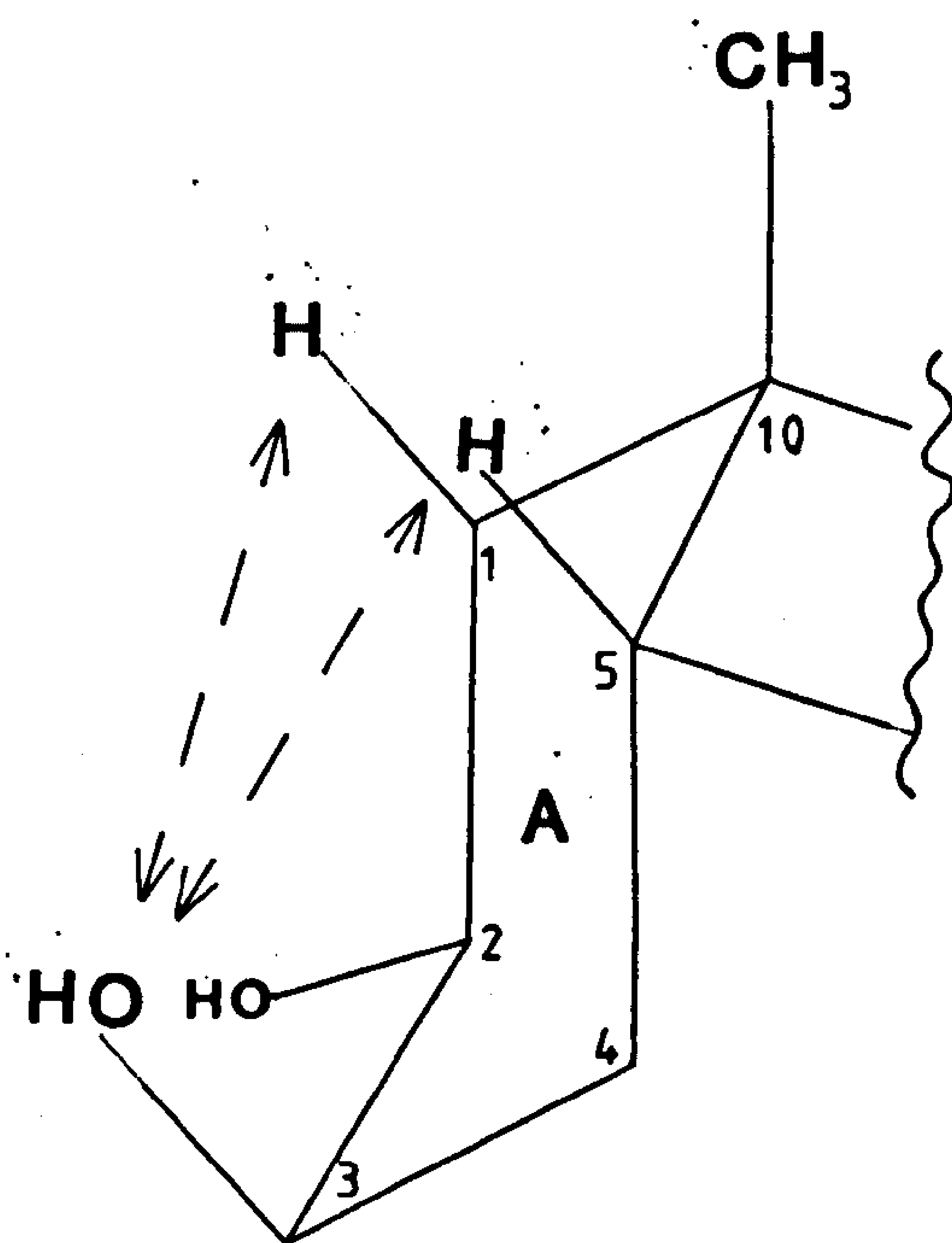


Figure 5-4 : Conversion of a 3β-ecdysteroid to a 3-epiecdysteroid demonstrating the change of conformation of the C-3 hydroxy group from axial to equatorial.



<u>Figure 5-5</u> : 1,3-Diaxial interactions between the two axial hydrogens at C-1 and C-5 with the axial C-3 hydroxy group in the A-ring of the ecdysteroid molecule.

ring much favouring the chair configuration, and perhaps contributing to a decrease in moulting hormone activity by effectively changing the overall shape of the β-face of the A ring.

The 26-hydroxyecdysteroids have considerably less activity than ecdysone and 20-hydroxyecdysone by both Musca and Calliphora moulting hormone assays. It is difficult to explain the significance of this inactivation step in terms of definite structural modification at the molecular level. However, it is interesting that 25-deoxy-20-hydroxyecdysone (Ponasterone A) is more active than 20-hydroxyecdysone in some assays. Therefore, it appears that the 25-hydroxy group is not required for activity and in fact the presence of the polar group at the end of the side chain causes some loss of activity. It would, therefore, follow that the presence of both 25 and 26 hydroxy groups on the ecdysteroid side chain would further decrease the moulting hormone activity by increasing the local polarity at the end of the side chain. The biological activity of the ecdysteroid-26-oic acid has not been assayed. However, the closely related ecdysteroid, inokosteron-26-oic acid, has been shown to be inactive (Spindler et al., 1976; Lafont et al., 1983). This indicates that the same is probably true for the ecdyson and 20-hydroxyecdyson-26-oic acids.

In addition to the effects of the various molecular transformations on receptor binding capability, the activity of ecdysteroids will also be governed by the solubility of these compounds and their ability to bind to hydrophilic carrier proteins. The water solubility of compounds such as 26-hydroxyecdysteroids, ecdysteroid-26-oic acids and ecdysteroid conjugates will enhance their excretion and make binding to the haemolymph proteins less likely, thus, effectively reducing their in vivo moulting hormone activity.

The enzymes involved in the interconversion of the ecdysteroids are obviously central to the control of moulting hormone activity present in the insect. The 20-hydroxylase activity shows considerable developmental variation, and would imply that 20-hydroxylation activity may have a role in the control of the ecdysone to 20-hydroxyecdysone ratio in the haemolymph. Furthermore, at certain times during 6th instar development there is an apparent temporal correlation between the 20-hydroxylase activity *in vitro* and the endogenous ecdysteroid titre. This suggests that 20-hydroxylation may regulate the levels of ecdysteroid in the insect, at least at some periods during development. However, it must be concluded that no single reaction controls the moulting hormone titre, but that synthesis of ecdysone, its 20-hydroxylation, and its further metabolism are all important factors in the regulation of moulting hormone activity in the insect.

The ecdysteroid 3-epimerase enzyme activity *in vitro* appears to have little bearing on the regulation of the moulting hormone titre, its activity during the 6th larval instar showing no correlation to variations in the endogenous ecdysteroid titre. It could be said that the epimerase activity *in vitro* may not reflect the physiological activity of the enzyme. However, as the methods used for detecting endogenous ecdysteroids during the 6th larval instar could not distinguish between ecdysteroids and 3-epiecdysteroids, there is still a possibility of the existence of 3-epiecdysteroids in the free or conjugated form being present at the time of apparent high 3-epimerase activity. Nonetheless, 3-epiecdysteroids were not detected as metabolites of injected radiolabelled ecdysone.

It is therefore evident that for a complete understanding of the regulatory role of metabolizing enzymes, some further study is required. This is particularly true for the developmental aspects of the 26-hydroxylation and conjugation processes which would appear to control the moulting hormone titre at some stages of development. As this investigation was primarily concerned with ecdysone metabolism, the production of ecdysone from the prothoracic glands was not specifically monitored. To obtain a complete picture of the factors controlling moulting hormone concentrations in the insect this aspect requires to be investigated. The results from such a study could then be considered alongside the information amassed on the metabolism and excretion of ecdysteroids in the present investigation in *Spodoptera littoralis*.

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APPENDICES

Appendix 1

Trivial and I.U.P.A.C. equivalent names

ecdysone = 2β, 3β, 14α, 22R, 25-pentahydroxy-5β- cholest-7-en-6-one

20-hydroxyecdysone = 2β, 3β, 14α, 20R, 22R, 25-hexahydroxy-5β- cholest-7-en-6-one

26-hydroxyecdysone = 2β, 3β, 14α, 22R, 25,26-hexahydroxy-5β-cholest-7-en-6-one

20,26-dihydroxyecdysone = 2β, 3β, 14α, 20R, 22R, 25,26-hepta-hydroxy-5β-cholest-7-en-6-one

3-epi-ecdysone = 2β, 3β, 14α, 22R, 25-pentahydroxy-5β-cholest-7-en-6-one

3-epi-20-hydroxyecdysone = 2β, 3β, 14α, 20R, 22R, 25-hexahydroxy-5β- cholest-7-en-6-one

3-epi-20,26-dihydroxyecdysone = 2β, 3α, 14α, 20R, 22R, 25,26-heptahydroxy-5β-cholest-7-en-6-one

3-dehydroecdysone = 2β, 14α, 22R, 25-tetrahydroxy-5β-cholest-7-en-3,6-dione

3-dehydro-20-hydroxyecdysone = 2β, 14α, 20R, 22R, 25-pentahydroxy-5β-cholest-7-en-3,6-dione

2-deoxyecdysone = 3β, 14α, 22R, 25-tetrahydroxy-5β-cholest-7-en-6-one

Appendix 2 : Mass spectra

Spectrum number

1. 3-Dehydroecdysone
2. 3-Epiecdysone (deuterated)
3. 3-Dehydro-20-hydroxyecdysone
4. 3-Epi-20-hydroxyecdysone (deuterated)
5. Cholic acid
6. Cholic acid methyl ester
7. 20-Hydroxyecdyson-26-oic acid
8. 20-Hydroxyecdyson-26-methyl ester

MASS SPECTRUM
01/28/81 15:23:00 + 1:40
SAMPLE: 3-DEHYDROECDYSONE

DATA: ECI2 #25
CALI: CICAL #3

BASE M/E: 99
RIC: 1048570.

Spectrum 1

100.0 — 50.0 — 135424.

81.1 99.1 109.1 126.1 145.1 165.1 179.1 199.1 211.1 225.1 241.1 255.1 267.1 283.1

M/E 100 150 200 250

ISOBUTANE CI 5X10^{-4} TORR,

250C, 100EV, 4KV, 500UA.

100.0 — 50.0 — 135424.

299.1 313.2 329.2 355.2 381.2 393.2 411.2 427.2 445.2 471.3 485.3 501.3

M/E 300 350 400 450 500

MASS SPECTRUM
01/28/81 14:57:00 + 1:44
SAMPLE: 3EPI-ECDYSONE EX. N.P.MILNER
DATA: ECI1 #26
CALI: CICAL #3
BASE M/E: 99
RIC: 1853430.
Spectrum 2
100.0
50.0
168192.
99.1
81.1
109.1
126.1
135.1
159.1
173.1
197.1
211.1
225.1
241.1
255.2
267.2
M/E
100
150
200
250
250C, 100EV, 4KV, 500UA.
ISOBUTANE CI35x10⁻⁴ TORR
301.2
313.2
331.2
355.2
377.2
393.2
412.2
430.3
448.3
470.3
488.3
350
400
450
500

MASS SPECTRUM
01/28/81 15:35:00 + 1:20
SAMPLE: 3-DEHYDRO-20-HYDROXYECDYSONE

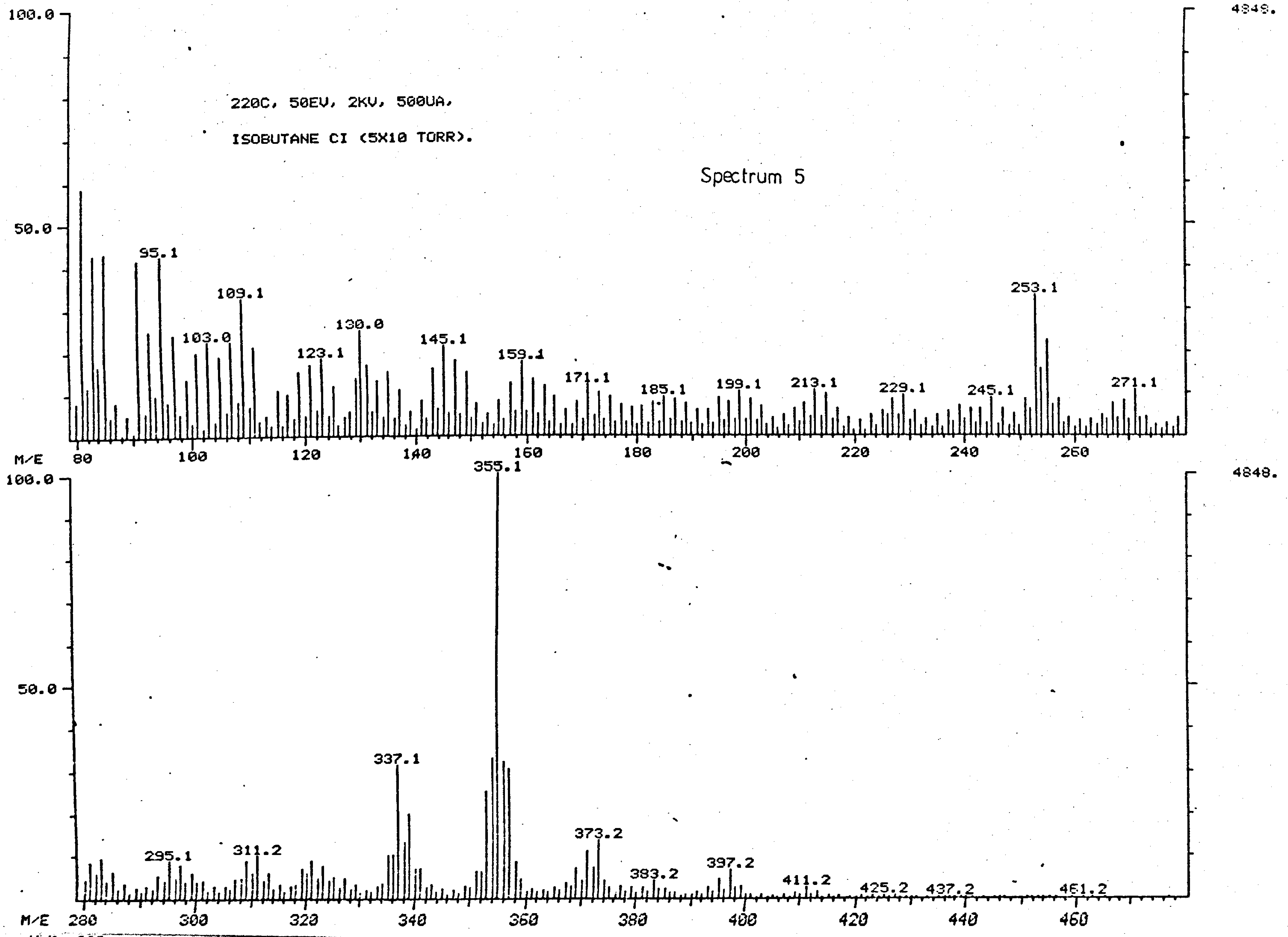
DATA: ECI3 #20
CALI: CICAL #3

BASE M/E: 99
RIC: 231936.

ISOBUTANE CI 3.5×10^{-4} TORR,

250C, 100EV, 4KV, 500UA.

Spectrum 3



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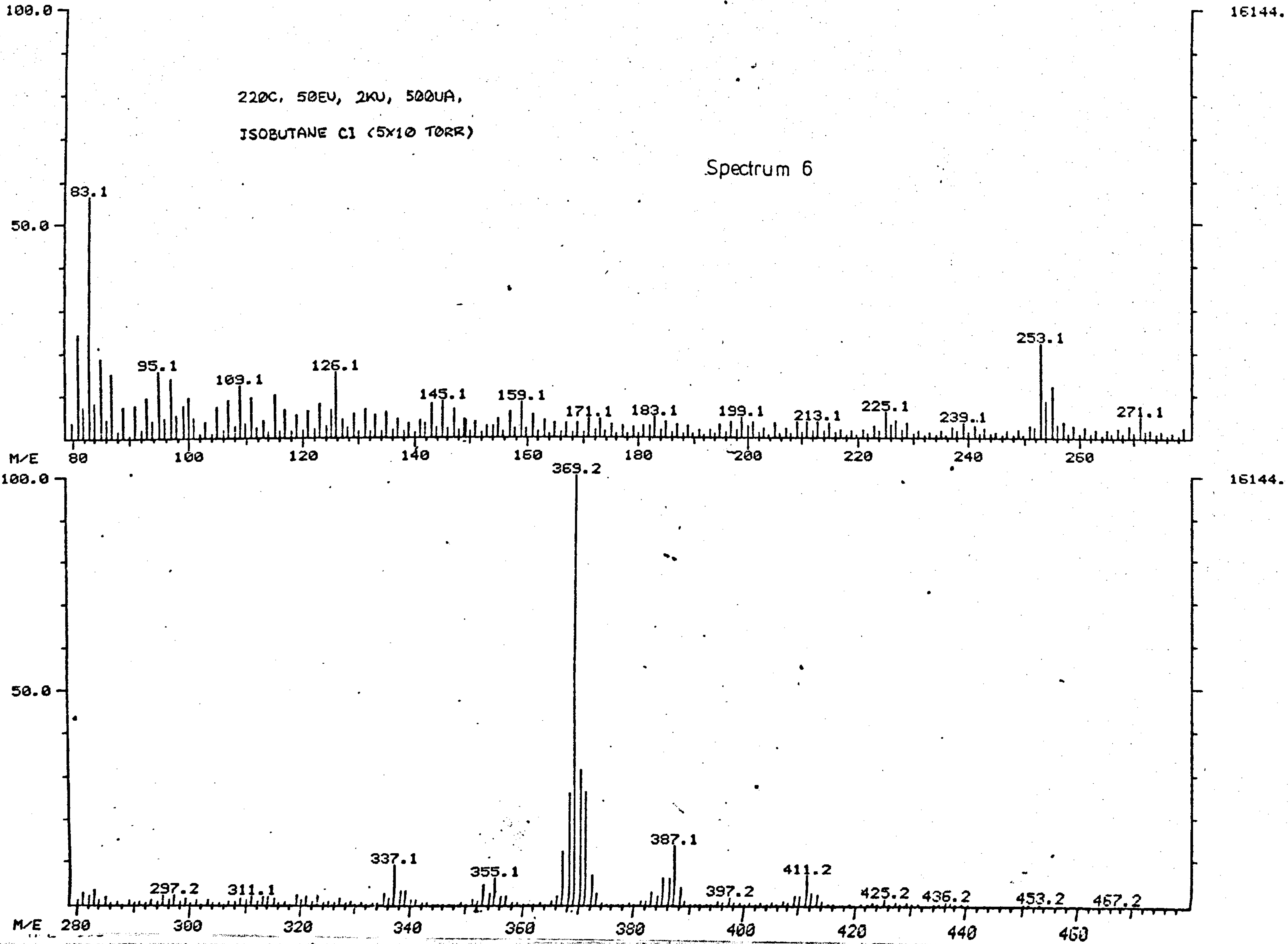
06/19/81 12:10:00 + 1:24
SAMPLE: CHOLIC ACID
DATA: NMCI1 #12
CALI: CICAL1 #2
BASE M/E: 355
RIC: 117632.
220C, 50EV, 2KV, 500UA,
ISOBUTANE CI (5X10 TORR).
Spectrum 5
4848.
100.0
50.0
M/E
95.1
103.0
109.1
123.1
130.0
145.1
159.1
171.1
185.1
199.1
213.1
229.1
245.1
253.1
271.1
80
100
120
140
160
180
200
220
240
260
4848.
100.0
50.0
M/E
295.1
311.2
337.1
355.1
373.2
383.2
397.2
411.2
425.2
437.2
461.2
280
300
320
340
360
380
400
420
440
460


MASS SPECTRUM
06/19/81 12:22:00 + 0:56
SAMPLE: CHOLIC ACID METHYL ESTER
DATA: NMCI2 #8
CALI: CICAL1 #2
BASE M/E: 369
RIC: 189184.
220C, 50EV, 2KV, 500UA,
ISOBUTANE CI (5X10 TORR)
Spectrum 6
100.0
50.0
16144.
M/E
83.1
95.1
109.1
126.1
145.1
159.1
171.1
183.1
199.1
213.1
225.1
239.1
253.1
271.1
80
100
120
140
160
180
200
220
240
260
369.2
297.2
311.1
337.1
355.1
387.1
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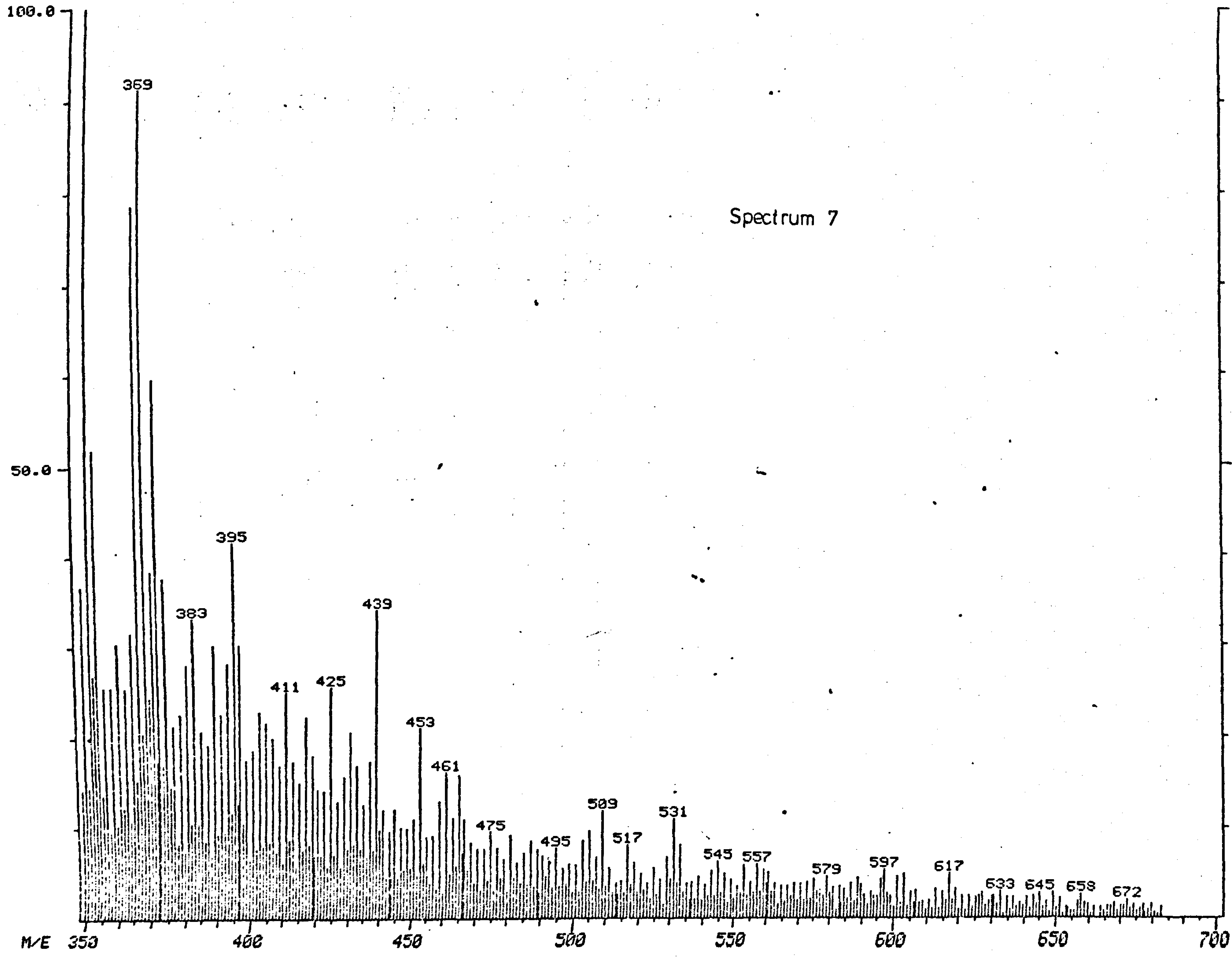
MASS SPECTRUM
11/03/82 15:21:00 + 0:50
SAMPLE: 20-HYDROXYECDYSONE-26-ACID.
DATA: NMNEG2 #5
-ve F.A.B.
BASE M/E: 58
RIC: 12402600.
49408.
100% FA
M/E 353
Spectrum 7
100.0
50.0
369
395
383
439
411
425
453
461
475
495
509
517
531
545
557
579
597
617
633
645
658
672
M/E
350
400
450
500
550
600
650
700