Ecdysteroid Metabolism in *Spodoptera littoralis*

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by

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"Scientists are Peeping Toms
at the keyhole of eternity"

A. Koestler
(British Philosopher)

FOREWORD

The work described in this thesis was carried out in the Biochemistry Department of the University of Liverpool between 1985 and 1988. During this period, the research was funded by the Agricultural and Food Research Councils.

I am grateful to Professor B.E.H. Maden, Ph.D., F.R.S.E. for giving me the opportunity to work in this Department. Many thanks are due to all members of 'Lab 216' and my friends in Liverpool (past and present) for their friendship, assistance and helpful discussion. Also thanks to Jane for typing this manuscript so efficiently.

I would like to express my special thanks to Professor H.H. Rees for his enthusiastic guidance and encouragement he gave during my years of research and for all his help in completing this thesis.

Finally, I would like to thank my family for their love and encouragement throughout my education. It is with gratitude that I dedicate this thesis to my parents.

SUMMARY

The feasibility of the activation-inactivation of ecdysone 20-monooxygenase activity, from Spodoptera littoralis fat body, in a manner commensurate with possible modulation of the enzyme's activity, by reversible phosphorylation-dephosphorylation was investigated. Initially, the sub-cellular localization of the ecdysone 20-monooxygenase was determined in the fat body. It was found to be located primarily in the mitochondria with a small, but significant, proportion being apparently associated with the microsomal fraction.

Microsomal fractions showed higher ecdysone 20-monooxygenase activity after preincubation at 37°C in phosphate buffer rather than Hepes or Imidazole buffer systems. Similarly, activity of the monooxygenase was elevated following preincubation with fluoride, an inhibitor of phosphoprotein phosphatases. Preincubation with alkaline phosphatase, or with cyclic AMP-dependent protein kinase and appropriate cofactors, resulted in appreciable diminution or enhancement, respectively, in monooxygenase activity. Activation of ecdysone 20-monooxygenase activity could also be effected by incubation with a cytosolic fraction in the presence of cyclic AMP, ATP and fluoride; this activation was prevented by a cyclic AMP-dependent protein kinase inhibitor. Similarly, inactivation of the monooxygenase was achieved by preincubation with cytosol, the effect being enhanced by Ca^{2+}-calmodulin and/or by Mg^{2+} ions. Use of a mixture of proteinase inhibitors established that the results are not complicated by the effects of such enzymes. The combined results

provide indirect evidence that the microsomal ecdysone 20-monooxygenase exists in an active phosphorylated form and an inactive dephosphorylated form, interconvertible by a cyclic AMP-dependent protein kinase and a phosphoprotein phosphatase.

Mitochondrial fractions also showed higher ecdysone 20-monooxygenase activity after preincubation with fluoride. Preincubation of a broken mitochondrial preparation with alkaline phosphatase resulted in appreciable diminution of monooxygenase activity, whereas activity was retained when the phosphatase was inhibited by EDTA and inorganic phosphate. Monooxygenase activity was increased when a broken mitochondrial preparation was incubated with cyclic AMP-dependent protein kinase and appropriate cofactors. Similarly, when the activity of the mitochondrial ecdysone 20-monooxygenase was reduced by incubation with alkaline phosphatase followed by inactivation of the phosphatase by EDTA and phosphate, there was an appreciable recovery of activity upon rephosphorylation of the enzyme system by incubation with protein kinase and appropriate cofactors. The combined results provide indirect evidence that the mitochondrial ecdysone 20-monooxygenase may also exist in an active phosphorylated form and an inactive dephosphorylated form, which are interconvertible by appropriate phosphoprotein phosphatase(s) and protein kinase(s). Of course, in vivo a cyclic AMP-dependent protein kinase would not be expected to be involved in modulation of the mitochondrial monooxygenase.

The effect of various known cytochrome P-450 inhibitors was investigated on a microsomal plus mitochondrial preparation from fat body. The most potent inhibitors were ketoconazole and imazalil which are both azoles with I_{50} values of 0.26ppm and 0.32ppm, respectively.

The major ecdysteroids occurring during oogenesis and embryogenesis in Spodoptera littoralis were analysed. Initially, the metabolism of injected [$^3H_2$]cholesterol was investigated. Four major metabolites were identified; 2-deoxyecdysone, 2-deoxyecdysone 22-phosphate and unusually the corresponding 5α-epimers. It is possible that the latter isomers do not occur in vivo, but arise by keto-enol tautomerism during isolation. 2-Deoxyecdysone and 2-deoxyecdysone 22-phosphate were present throughout oogenesis and embryogenesis. The structures were ascertained by mass spectrometric and nuclear magnetic resonance spectrometric analysis. In addition, small amounts of ecdysone and 20-hydroxyecdysone were identified during embryogenesis by h.p.l.c./R.I.A.

The changes in ecdysteroid titre and composition during oogenesis and embryogenesis were determined. In the ovaries of Spodoptera, the total ecdysteroid concentration remains almost constant. Appreciable amounts of ecdysteroids appear to be passed into the eggs where the titre per batch of eggs decreases early in embryogenesis. Towards the end of embryogenesis the total ecdysteroid titre per batch of eggs rises again.

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CHAPTER ONE

INTRODUCTION

1. Insect Endocrinology

The Class Insecta can be broadly divided into two groups, the Hemimetabolous and the Holometabolous insects. Hemimetabolous insects hatch in a form closely resembling the adult, but have no wings. During successive moults, the wings gradually develop and the sex organs mature. In the Holometabolous insects, the larval stages are structurally dissimilar to the adult and a series of larval-larval moults is followed by a pupal stage. Hence, metamorphosis is said to be complete in Holometabolous insects and incomplete in Hemimetabolous insects.

For the development and growth of an insect to occur, it is necessary for the cuticle to be periodically removed and replaced. During the moulting cycle, the cuticle separates from the epidermis (apolysis), the epidermal cells divide, and deposit the new cuticle. The old cuticle is then shed (ecdysis) and the insect increases in size. It is only during this short period when the epidermis is detached from the cuticle that development can take place. The moulting cycle is controlled by ecdysteroids, the titre of which generally rises at the appropriate time.

During the life cycle of insects, ecdysteroids, often called the moulting hormones, act in concert with juvenile hormone to ensure the correct development of the insect. Increased levels of moulting hormone induces moulting, whereas juvenile hormone modulates this effect by determining the nature of the moult (Fig. 1). While

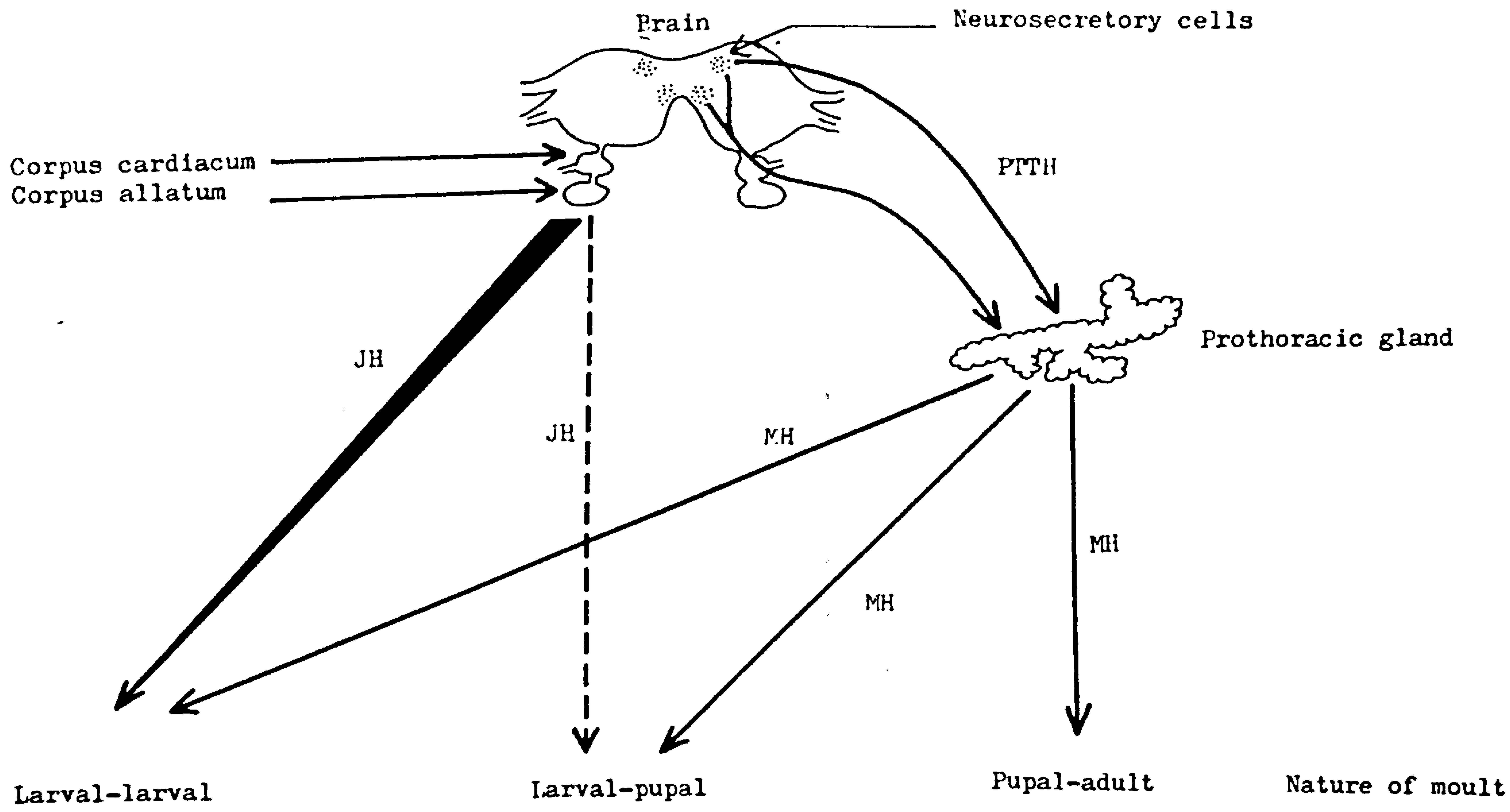


Fig 1. Diagrammatical representation of the classical scheme of hormonal control of insect post-embryonic development.

physiological mechanisms such as metabolism, excretion and sequestration all contribute to the regulation of a hormone titres, the initial regulation of the titres of ecdysteroids and juvenile hormones occurs at the level of synthesis. The secretion of both these hormones is controlled by an endocrine system.

Different groups of cerebral neurosecreatory cells, in response to appropriate enviromental stimuli synthesize a prothoracicotropic hormone (PTTH) (Bollenbacher and Granger, 1985). At present, it appears that PTTH exists as a series of molecular variants, but little is known about the function of the individual components (Kataoka et al., 1987). Following synthesis, PTTH is transferred to the retrocerebral complex (corpora cardiaca and corpora allata) via nerve axons. The retrocerebral complex appear to serve as storage organs for this secretion from where it can be released into the haemolymph as required. Once in the haemolymph, the PTTH stimulates the prothoracic glands or analogous structures to synthesize and secrete the moulting hormone, ecdysone. Other factors also appear to regulate the prothoracic glands. These include the competency of the glands to be stimulated by a trophic effector and the presence of one or more haemolymph protein factors (Watson et al., 1987). Ecdysone acts mainly as a precursor of the major active hormone 20-hydroxyecdysone, although it does have hormonal activity per se. It is the hormonally more active, 20-hydroxyecdysone, which initiates the 'moult'.

Although ecdysone is the main ecdysteroid synthesized by the prothoracic glands, several others occur in the haemolymph suggesting that these individual ecdysteroids or a critical ratio of the ecdysteroids may have discrete functions in the control of insect development.

The type of moult the insect undergoes is determined by the level of juvenile hormone which is synthesized by the corpora allata. In the presence of a high juvenile hormone titre, larval-larval moults occur, whereas in the presence of a low titre or in the absence of juvenile hormone, larval-pupal and pupal-adult moults occur.

In some Lepidoptera the corpora allata produce several juvenile hormone homologues [JH-I, JH-II, JH-III, JH-0 and 4 methyl JH-I], whereas others can secrete juvenile hormone acids as well (Granger et al., 1982). As a result, fluctuations occur not only in the haemolymph titres of juvenile hormones but in the titres of their acids as well (Baker et al., 1987). Regulation of synthesis of the juvenile hormones and their acids is a major mechanism by which their titres are controlled. The juvenile hormone titre has been shown to increase due to methylation of synthesized acids by peripheral tissues (eg. imaginal discs) in the Manduca sexta pupae (Sparagana et al., 1985). The synthesis of juvenile hormones and their acids is controlled by cerebral neuroendocrine and nervous mechanisms (Feyereisen, 1985) which themselves may be affected by other hormones, notably ecdysteroids (Granger et al., 1987). The juvenile hormone titre may, in turn, regulate the competency of the prothoracic glands to be stimulated by a trophic effector (Watson and Bollenbacher, 1988).

2. Structure and Occurrence of Ecdysteroids

Ecdysteroids are polyhydroxylated ketosteroids possessing a 14α-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 (Fig. 2). This conformational arrangement is critical for moulting hormone activity, 5α-ecdysteroids being hormonally inactive.

The numbering and lettering of structures in this thesis are in accordance with the guidelines laid out in the IUPAC-IUB Definitive Rules for Steroid Nomenclature (1971; Fig. 3). This means that notation differentiates between nuclear substituents above the plane of the ring, termed 'β' (denoted by ————) and substituents below the plane, termed 'α' (denoted by ---------). The ecdysteroid side chain is linked to the tetracycline 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 such centres, using the R and S symbols to indicate the three dimensional positions of each substituent. A 22R hydroxyl group is essential for compounds to exhibit high moulting hormone activity and in 20-hydroxyecdysteroids a 20-hydroxy group is present producing a 20R stereochemical centre (Fig. 4).

The structures of a number of free ecdysteroids isolated from arthropods are given in Fig. 5. Polar and apolar conjugates of ecdysteroids have also been detected in arthropods, usually in the ovaries or eggs and a number of these are listed in Fig. 6.

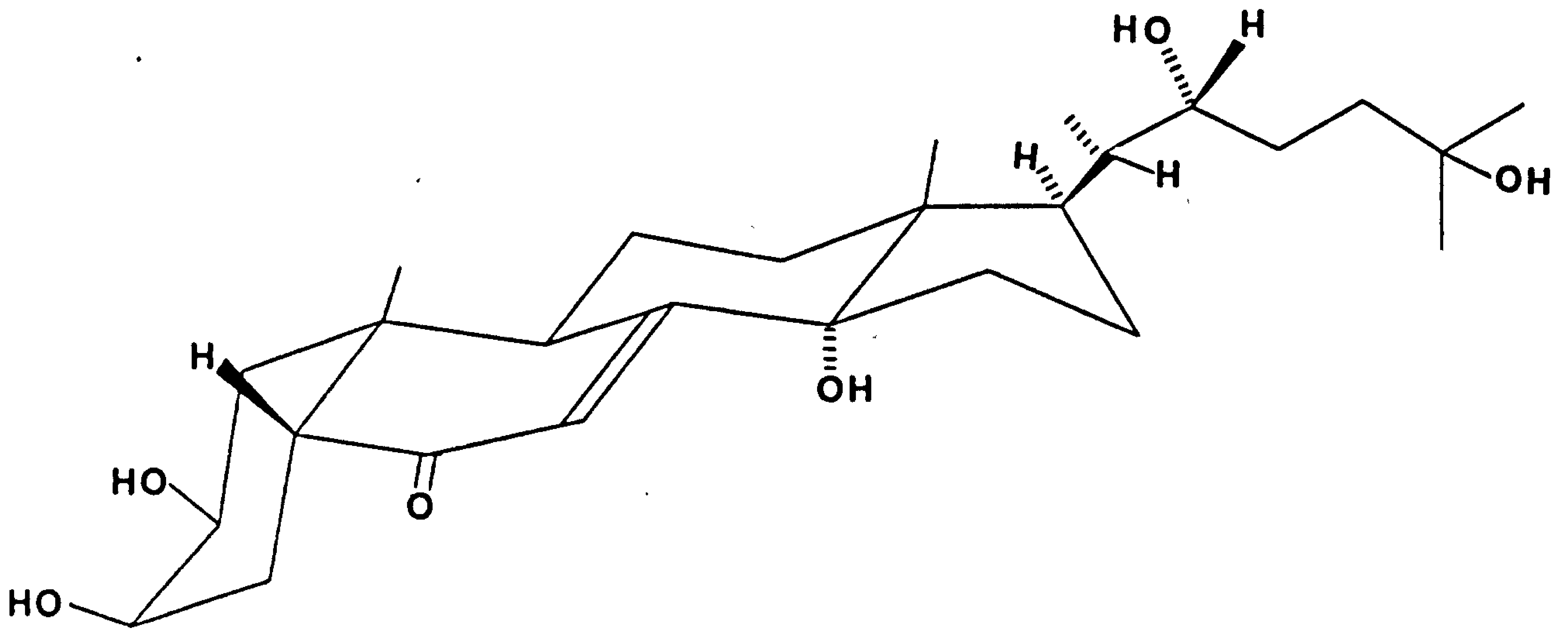


Fig 2. Structure of ecdysone, showing 5β configuration and asymmetric centres.

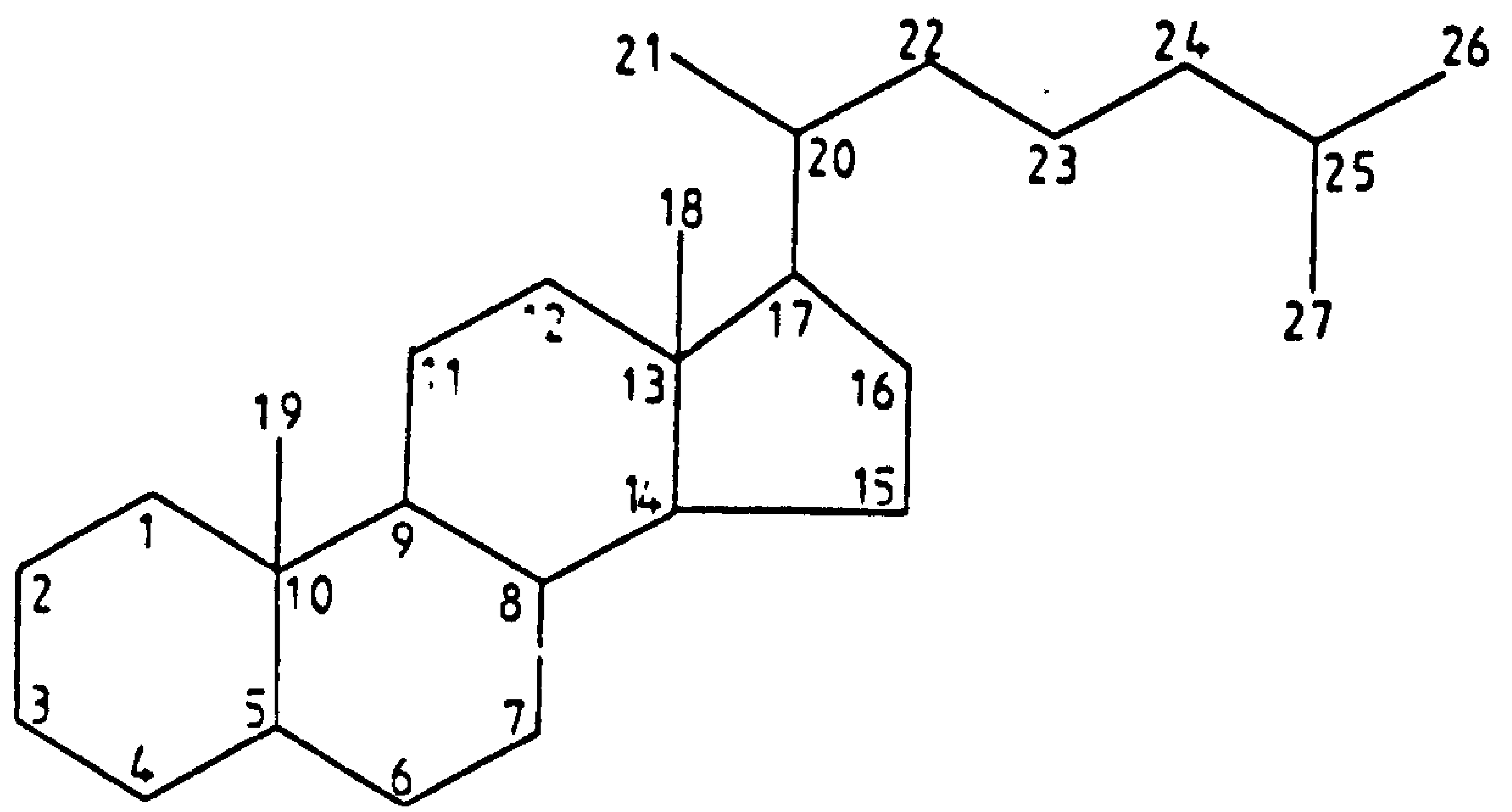


Fig 3. Basic steroid structure and numbering.

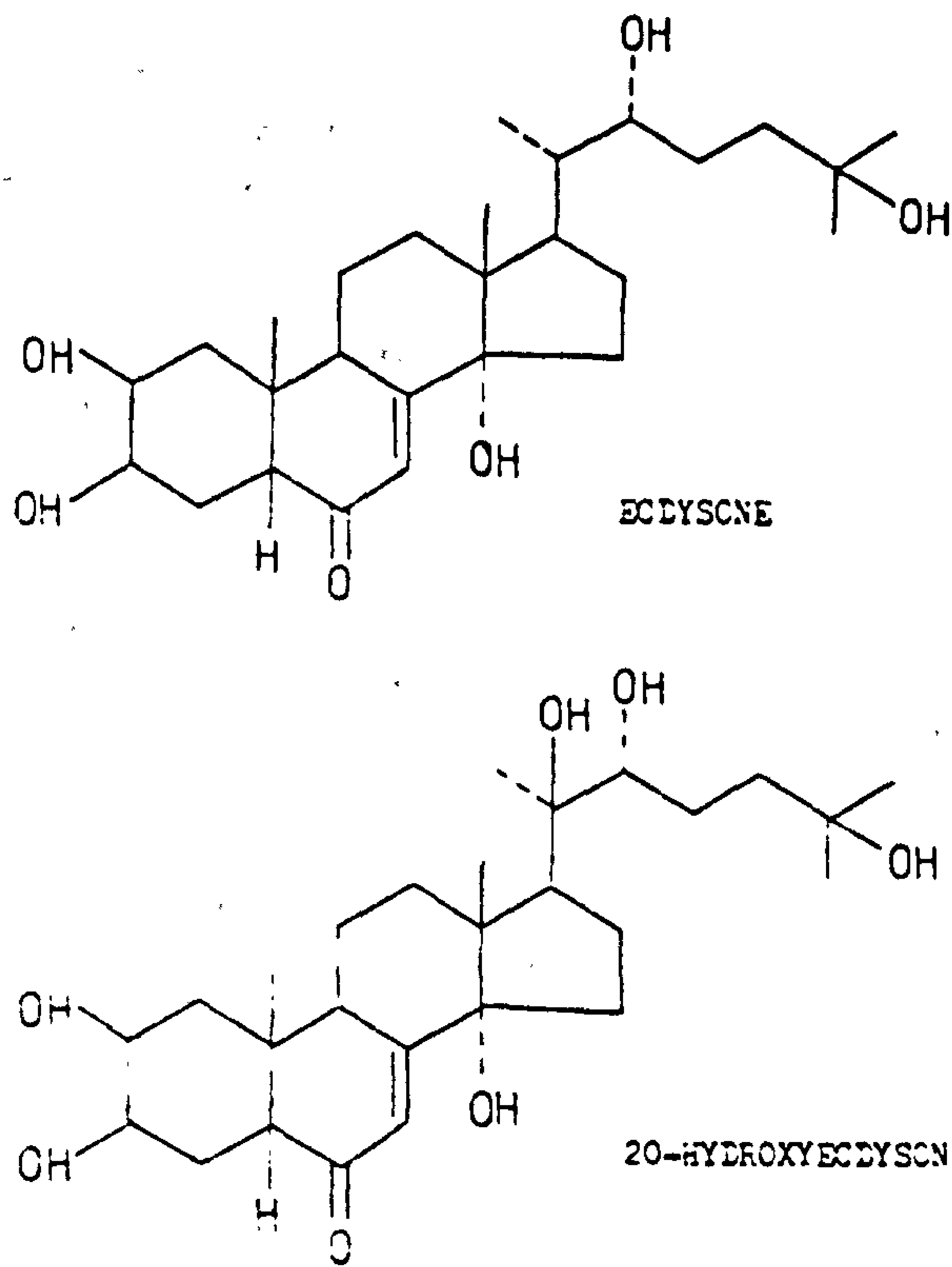


Fig 4. Chemical structures of ecdysone (2β,3β,14α,22R,25-pentahydroxy-5β-cholest-7-en-6-one) and 20-hydroxyecdysone (2β,3β,14α,20R,22R,25-hexahydroxy-5β-cholest-7-en-6-one)

Fig. 5 **Structures of a number of free ecdysteroids isolated from arthropods**

	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
Ecdysone	OH	OH	H	H	OH	H	OH	CH_3
20-Hydroxyecdysone	OH	OH	H	OH	OH	H	OH	CH_3
Ponasterone A	OH	OH	H	OH	OH	H	H	CH_3
2-Deoxyecdysone	H	OH	H	H	OH	H	OH	CH_3
2-Deoxy-20-hydroxyecdysone	H	OH	H	OH	OH	H	OH	CH_3
2,22-Dideoxyecdysone	H	OH	H	H	H	H	OH	CH_3
2,22-Dideoxy-20-hydroxyecdysone	H	OH	H	OH	H	H	OH	CH_3
2,22,25-Trideoxyecdysone	H	OH	H	H	H	H	H	CH_3
26-Hydroxyecdysone	OH	OH	H	H	OH	H	OH	CH_2OH
20,26-Dihydroxyecdysone	OH	OH	H	OH	OH	H	OH	CH_2OH
Inokosterone	OH	OH	H	OH	OH	H	H	CH_2OH
Ecdysonoic acid	OH	OH	H	H	OH	H	H	COOH
20-Hydroxyecdysonoic acid	OH	OH	H	OH	OH	H	H	COOH
3-Dehydro-2-deoxyecdysone	H	O	H	H	OH	H	OH	CH_3
3-Epiecdysone	OH	H	OH	H	OH	H	OH	CH_3
3-Epi-20-hydroxyecdysone	OH	H	OH	OH	OH	H	OH	CH_3
3-Epi-2-deoxyecdysone	H	H	H	H	OH	H	OH	CH_3
3-Epi-26-hydroxyecdysone	OH	H	H	H	OH	H	OH	CH_2OH
3-Epi-20,26-dihydroxyecdysone	OH	H	H	OH	OH	H	OH	CH_2OH
Makisterone A	OH	OH	H	OH	OH	CH_3	OH	CH_3

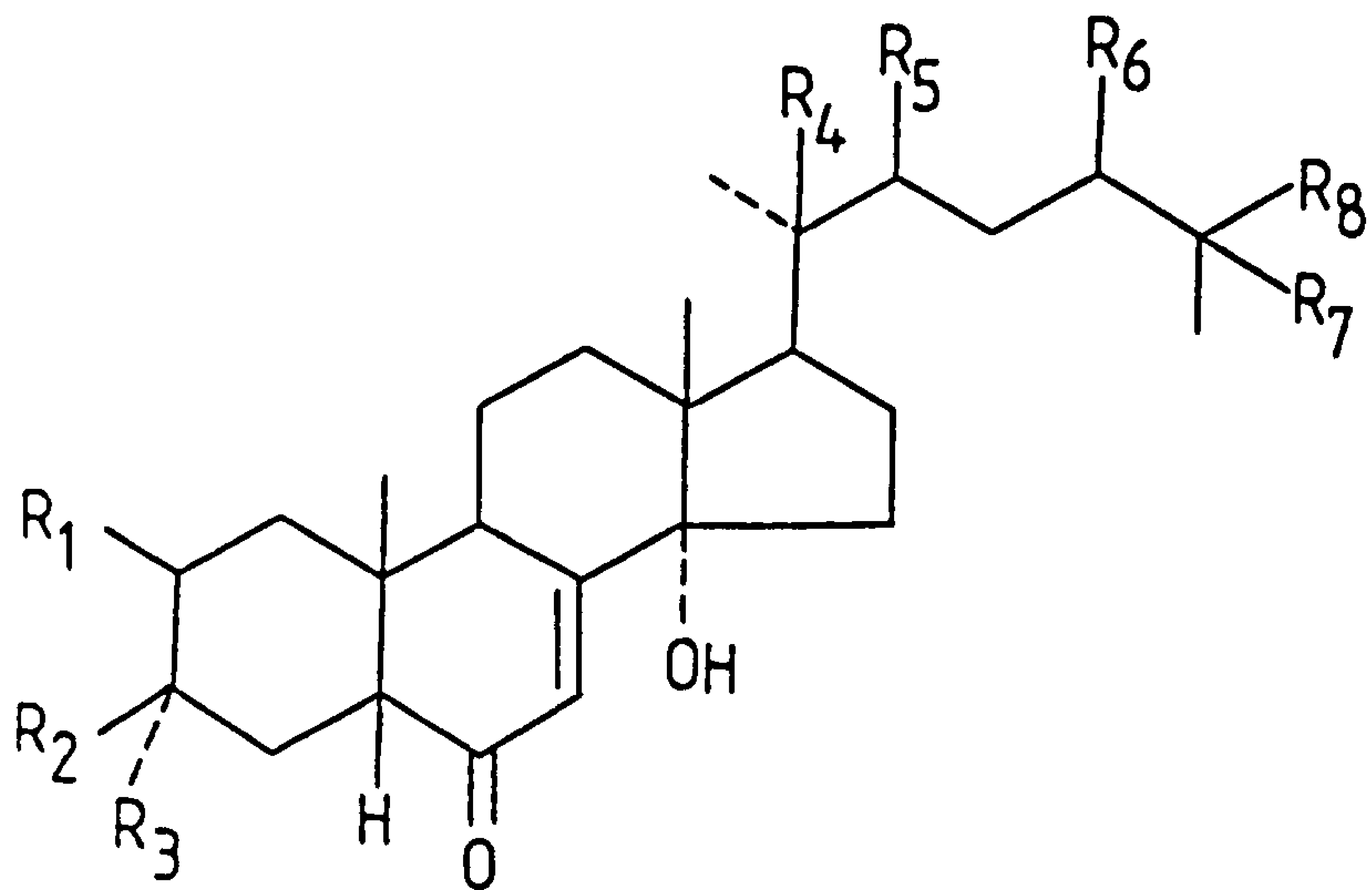


Fig. 6 **A list of a number of ecdysteroid conjugates isolated from arthropods**

Name	Reference
2-Deoxyecdysone 22-phosphate	Isaac *et al.*, 1982.
2-Deoxy-20-hydroxyecdysone 22-phosphate	Tsoupras *et al.*, 1982a; Isaac *et al.*, 1983.
Ecdysone 2-phosphate	Isaac *et al.*, 1984.
Ecdysone 22-phosphate	Isaac *et al.*, 1982.
20-Hydroxyecdysone 22-phosphate	Isaac *et al.*, 1983.
20-Hydroxyecdysone 2-(or 3)phosphate	Tsoupras *et al.*, 1983a.
3-Epi-2-deoxyecdysone 3-phosphate	Tsoupras *et al.*, 1982b; Isaac *et al.*, 1984.
3-Epiecdysone 3-phosphate	Tsoupras, 1982.
Ecdysone 3-acetate	Isaac *et al.*, 1981.
20-Hydroxyecdysone 3-acetate	Modde, 1983.
20-Hydroxyecdysone 22-acyl ester	Connat *et al.*, 1986.
Ecdysone 22-acyl ester	Crosby *et al.*, 1986.
2-Deoxyecydsone 22-adenosine-monophosphate	Tsoupras *et al.*, 1982b.
Ecdysone 22-adenosine-monophosphate	Tsoupras, 1982.
Ecdysone 22-N^6-(isopentenyl)-adenosine-monophosphate.	Tsoupras *et al.*, 1983a.
3-Acetylecdysone 2-phosphate	Isaac *et al.*, 1984.
3-(or 2) Acetylecdysone 22-phosphate	Isaac and Rees, 1984.
3-Acetyl-20-hydroxyecdysone 2-phosphate	Modde, 1983; Isaac and Rees, 1984.
3-Acetyl-20-hydroxyecdysone 22-phosphate	Tsoupras *et al.*, 1982a, 1983b.
26-Hydroxyecdysone 26-phosphate	Thompson *et al.*, 1985.
26-Hydroxyecdysone 22-glucoside	Thompson *et al.*, 1987.

Ecdysteroids are also detected in invertebrates besides insects. These include cestodes (Mendis _et_ _al_., 1984), crustacea (Rees, 1971), annelids (Porchet _et_ _al_., 1984), trematodes (Nirde _et_ _al_., 1983), nematodes (Mendis _et_ _al_., 1983), arachnids (Wigglesworth _et_ _al_., 1985), gastropods (Romer, 1979), myriapods (Juberthie-Jupeau _et_ _al_., 1979), and coelenterates (Hoffmann and Porchet, 1984).

In addition, ecdysteroids have been detected in a variety of plants: pteridophyta, gymnosperms and angiosperms (Hetru and Horn, 1980; Bergamasco and Horn, 1983). These ecdysteroids have been termed phytoecdysteroids, and although the exact significance of these metabolites is not fully understood, it has been proposed that they may protect the plant from predatory insects.

Ecdysone and 20-hydroxyecdysone are the most abundant ecdysteroids, found in virtually every arthropod species analysed and there are few species which possess neither metabolite. Makisterone A (24-methyl-20-hydroxyecdysone) is the major ecdysteroid found in certain phytophagous Heteroptera (eg. _Oncopeltus_ _fasciatus_) which cannot dealkylate phytosterols (Feldlaufer and Svoboda, 1986). Ponasterone A [25-deoxy-20-hydroxyecdysone] (Lachaise _et_ _al_., 1981) and inokosterone [25-deoxy-20, 26-dihydroxyecdysone] (Faux _et_ _al_., 1969) have, as yet, only been detected in crustacea. The prothoracic glands are the prime physiological source of moulting hormone during larval-larval, larval-pupal and pupal-adult transformations in insects. There is also much evidence for the physiological production of ecdysteroids in the abdomens of _Tenebrio_ _molitor_ during pupal-adult development (Delbecque _et_ _al_., 1978).

Isolated larval abdomens of other insect species have also been reported to be able to produce some ecdysteroids (Nakanishi *et al.*, 1972; Hsiao *et al.*, 1975). It is also well-documented that the ovaries of reproductively competent female insects synthesize ecdysone. This can occur during pupal development, as in *Bombyx mori* (Ohnishi *et al.*, 1977) or in adult insects such as *Aedes aegypti* (Hagedorn *et al.*, 1975) or *Locusta migratoria* (Lagueux *et al.*, 1977). The ovarian follicle cells have been established as the exact site of ecdysteroid synthesis in *Locusta* (Lagueux *et al.*, 1977; Zhu *et al.*, 1983).

In many species the bulk of the ovarian ecdysteroids are transferred to the ooplasm of eggs (Goltzene *et al.*, 1978) where they may have a function during embryonic development (Hoffmann, 1986). However, in others, an appreciable amount of the ovarian ecdysteroids are released into the maternal haemolymph where they might play a role in the female (Hoffmann, 1986). Ovarian ecdysteroids are thought in higher insect orders (eg. *Aedes*) to play a role in vitellogenin synthesis and in lower insect orders to play a role in the early events of embryogenesis (Hagedorn, 1985).

In insect oocytes, the presence of free ecdysone and 20-hydroxy-ecdysone is characteristic of the cockroaches, termites and moths, while in locusts ecdysone and 2-deoxyecdysone predominate (Lagueux *et al.*, 1981; Rees and Isaac, 1984). Most of the ecdysteroids found in ovaries and eggs of Lepidoptera and locusts are conjugated to more polar molecules, primarily phosphate (Hagedorn, 1985).

There exists as mentioned previously, a large amount of indirect evidence that one of the functions of egg ecdysteroids is in the control of moulting. In several insect species, a correlation between moulting events in the embryo and the concentration of moulting hormone activity, was clearly evident (Imboden and Lanzrein, 1982; Fournier and Radallah, 1988). The free ecdysteroid titre in Locusta eggs exhibits four peaks, which show a strict temporal correlation with the successive deposition of the serosal cuticle and the first, second and third embryonic cuticles (Lagueux et al., 1979). It has also been shown that the synthesis of RNA, proteins and polysaccharides, associated with the formation of the cuticle, are prematurely triggered and accelerated in vivo by the addition of an exogenous ecdysteroid to a culture of epidermal cells from the legs of Blaberus craniifer embryos (Bulliere et al., 1979).

In some insect species (eg. Locusta, Schistocerca and Bombyx) the peaks of free ecdysteroids at least during early embryogenesis may result from hydrolysis of ecdysteroid conjugates of maternal origin rather than de novo synthesis (Hagedorn, 1985). However, this does not exclude the possibility that during later stages of development, the embryos synthesize ecdysteroid de novo once the prothoracic glands (or other tissues capable of ecdysone biosynthesis) have differentiated. The biosynthetic origin of embryonic ecdysteroids in those insects where the presence of maternal ecdysteroids has either not been reported or is minimal (eg. Nauphoeta cinerea) suggests de novo synthesis, but this awaits demonstration (Hagedorn, 1985). Maternal conjugates are gradually metabolized within the Locusta and

Schistocerca embryos as development proceeds (Lagueux et al., 1981; Rees and Isaac, 1984). Schistocerca embryos contain a phosphatase enzyme which can hydrolyse ecdysteroid 22-phosphates to release mainly ecdysone (Isaac et al., 1983b).

Another function of embryonic ecdysteroids in insects may be the control of diapause. It has been observed in eggs of Bombyx mori that the level of moulting hormone activity was diminished during diapause and re-elevated upon resumption of embryonic development (Ohnishi et al., 1971; Coulon, 1988). There is also a correlation between the concentration of moulting hormone activity and mitotic activity in the late stages of embryogenesis in Schistocerca gregaria (Scalia et al., 1987).

3. Biosynthesis of Ecdysteroids

Cholesterol is the distal precursor of ecdysone. Insects cannot synthesize sterols de novo from small molecules such as acetate and rely upon a dietary source of these compounds (Clayton, 1964). Most insect species require some cholesterol or a sterol which is convertible into cholesterol, for satisfactory growth, development and reproduction. Carnivorous insects derive their cholesterol directly from their diet, whereas phytophagus insects, such as Spodoptera littoralis, obtain such a sterol primarily by dealkylation of C_{28} and C_{29} phytosterols (eg. sitosterol, stigmasterol and campesterol). This involves removal of the alkane or alkene substituents from C-24 on the sterol side chain. However, the ability to dealkylate plant sterols

is not universal amongst phytophagus insect species. Several Hemipteran species, such as Oncopeltus fasciatus (Svoboda et al., 1977), Dysdercus fasciatus (Gibson et al., 1983) and Trogoderma granarium (Svoboda et al., 1980), lack this ability. In Oncopeltus fasciatus, the methylated sterol, campesterol is utilized to produce the C_{28} compound, makisterone A, as the principal ecdysteroid (Feldlaufer and Svoboda, 1986).

During ecdysone biosynthesis from cholesterol, extensive modification of the sterol nucleus occurs before side-chain hydroxylation (Rees, 1985). Both cholesterol and 7-dehydrocholesterol are incorporated into ecdysteroids (Johnson and Rees, 1977a). Owing to the reversible interconversion of the two sterols in vivo, it has only recently been demonstrated that 7-dehydrocholesterol is an obligatory intermediate and not incorporated indirectly via cholesterol (Milner et al., 1986; Warren et al., 1988).

There is appreciable evidence to support the intermediacy of 3β, 14α-dihydroxy-5β-cholest-7-en-6-one (2,22,25-trideoxyecdysone; often referred to as 5β-ketodiol) in ecdysone biosynthesis (Haag et al., 1985). However, the nature of the preceding steps is unclear, such as the introduction of the 6-oxo group, the Δ^7 double bond, the proton at C-5 and the hydroxylation at C-14.

The formation of the A/B cis ring junction also appears to occur during the early stages of ecdysteroid biosynthesis. This was supported by experiments involving excised prothoracic glands from

Manduca sexta in which only the 5β isomer of ketodiol was converted into ecdysone. The 5α isomer of ketodiol was converted into 5α-2-deoxyecdysone (Bollenbacher et al., 1977a). Thus, introduction of the A/B cis ring junction must occur before formation of ketodiol. Experiments involving the incorporation of stereospecifically labelled cholesterols in S. gregaria (Davies et al., 1981) showed that the 3α- and 4β-hydrogens of cholesterol are eliminated during ecdysteroid formation. These results were interpreted in terms of the possible intermediacy of a 3-oxo-Δ^4-steroid in formation of the A/B cis ring junction. Although none of the 3-oxo-Δ^4-steroids, which have been investigated as putative ecdysteroid precursors, were incorporated, there may be an obligatory requirement for other structural features in the molecule (Rees, 1985).

A potential precursor of 5β-ketodiol, 3β-hydroxy-5β-cholest-7-en-6-one (2,14,22,25-tetradeoxyecdysone, often referred to as 5β-ketol) is not significantly incorporated into 20-hydroxyecdysone in pupariating Calliphora stygia (Faux et al., 1979) and yielded 14-deoxyecdysone when prothoracic glands from Manduca sexta (Bollenbacher et al., 1977a) or Locusta migratoria (Haag et al., 1987) were cultured in vivo. This implies that 5β-ketol is not the immediate precursor of 5β-ketodiol and, thus, C-14 hydroxylation must occur on a hitherto unknown substrate other than 5β-ketol.

The favoured hydroxylation sequence following the formation of 5β-ketodiol is: C-25, C-22 and C-2 in the prothoracic glands and ovarian follicle cells of Locusta migratoria (Meister et al., 1985).

In the prothoracic glands of Locusta migratoria, the C-2 hydroxylase appears to be a mitochondrial oxygenase which differs from conventional cytochrome P-450 dependent monooxygenases by its relative insensitivity to CO. In contrast, the C-22 and C-25 hydroxylases appear to be classical cytochrome P-450 monooxygenases (Kappler et al., 1988). The C-22 hydroxylase is a mitochondrial enzyme and current evidence suggests that the C-25 hydroxylase is probably a microsomal enzyme. The C-2 hydroxylase found in Malpighian tubules and in the prothoracic glands differ only in their preference for cofactors. NADPH is a more efficient cofactor for the prothoracic gland C-2 hydroxylase when compared with isocitrate, whereas isocitrate is a more efficient source of reducing power for the Malpighian tubule enzyme. The C-2 hydroxylase is also interestingly, competitively inhibited by 2-deoxyecdysone and by 2,22,25-trideoxy-ecdysone. The end-product ecdysone appears to be a noncompetitive inhibitor (Kappler et al., 1988).

The hydroxylation of ecdysone by ecdysone 20-monooxygenase [E.C. 1.4.99.22] at position C-20 to yeild 20-hydroxyecdysone has been well documented. Ecdysone 20-monooxygenase is a cytochrome P-450-dependent mixed function oxidase (Rees et al., 1981). It has been reported that the enzyme system in various species and tissues is either largely mitochondrial or microsomal or has dual location in both sub-cellular fractions (Weirich et al., 1984). The activity of the Locusta microsomal monooxygenase system is enhanced following injection of ecdysone and reduced by extirpation of prothoracic glands (Feyereisen

and Durst, 1980), suggesting that the enzymic activity may be controlled by the concentrations of its substrate. In addition, it has been demonstrated that ecdysone 20-monooxygenase shows competitive inhibition by its product, 20-hydroxyecdysone (Feyereisen and Durst, 1978; Smith _et al_., 1979; Greenwood and Rees, 1984). A summary of our present understanding of the ecdysteroid biosynthetic pathway is given in Figure 7.

4. Vertebrate Steroid Hydroxylations

Since there is much information on steroid hydroxylation mechanisms in vertebrates, it is appropriate to consider these as background for the ecdysteroid hydroxylations in insects.

The biosynthesis of sterols involves a series of cytochrome P-450-catalysed monooxygenase reactions. Cytochrome P-450 is now known to encompass a family of haemoproteins, each of which exhibits distinct but overlapping substrate specificity. The distinct requirement of steroid synthesis in specific cell types may involve discrete P-450 isoenzymes adapted to a particular function (Lieberman _et al_., 1984).

A characteristic of many steroidogenic pathways is that reactions occur both in the mitochondria and the endoplasmic reticulum. The distribution of steroidogenic cytochromes P-450 between the mitochondria and microsomes is summarized in Figure 8. The reactions in the mitochondria and in the endoplasmic reticulum require very different electron transport chains.

Fig. 7 **Ecdysteroid biosynthesis (from Hoffmann, 1986)**

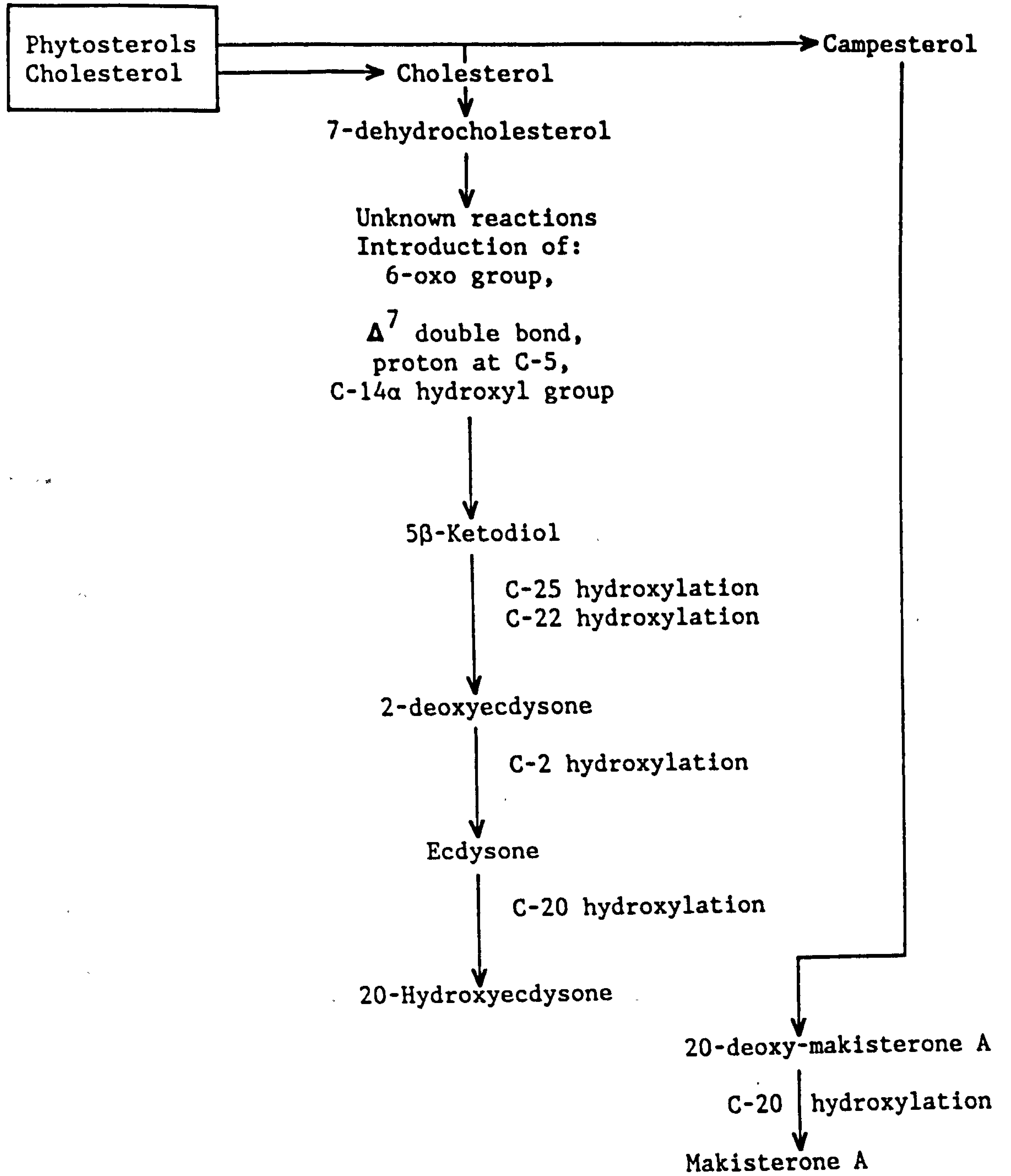


Fig. 8 Distribution of Steroid Hydroxylases between Mitochondria and Microsomes (Jefcoate, 1986)

Reaction	Substrate	Tissues
Mitochondria		
20,22-Cleavage[a]	Cholesterol	Adrenal, testis, ovary, placenta.
11β-, 18-HO[b]	Deoxycorticosterone	Adrenal
18-HO[a]	Corticosterone	Adrenal
	18-Hydroxycorticosterone	Glomerulosa
1α-HO	25-Hydroxyvitamin D_3	Kidney
24-HO		
26-HO	Cholesterol	Liver
Microsomes		
21-HO	17α-Hydroxyprogesterone[c]	Adrenal
17α-HO[a]	Progesterone[d]	Adrenal, testis.
17-20-lyase		
Aromatase[a]	Androgens	Ovary, placenta.
25-HO	Vitamin D	Liver
7α-HO	Cholesterol	Liver
12α-HO	3α,7α dihydroxy-cholestane	Liver
25-HO	3α,7α,12α Trihydroxy-cholestane	Liver
14α-HO	Dihydrolanosterol	Liver

[a]Multiple hydroxylases
[b]HO, hydroxylation
[c]Progesterone is also a substrate
[d]Lyase activity measured with 17α-hydroxyprogesterone

Four different mechanisms can potentially be used to rapidly affect the activity of cytochrome P-450: control of substrate, control of electron transfer, allosteric activation, or protein modification. In addition, cytochromes P-450 are all membrane bound enzymes and all the potential control elements listed above may be changed by the membrane environment.

Reactions involved in steroidogenesis include the following: the conversion of cholesterol into pregnenolone known as cholesterol side-chain cleavage catalysed by side-chain cleavage P-450 (P-450scc); 11β-hydroxylation catalysed by cytochrome $P\text{-}450_{11\beta}$; 21-hydroxylation catalysed by $P\text{-}450_{C21}$ and 17α-hydroxylation catalysed by cytochrome $P\text{-}450_{17\alpha}$.

Such reactions require, the reducing equivalent in the form of NADPH, molecular oxygen and a substrate to be monooxygenated (Simpson and Estabrook, 1969). The stoichiometry of these monooxygenase reactions is generally expressed by the following equation:

$$NADPH + H^+ + S + O_2 \longrightarrow NADP^+ + SO + H_2O$$

Where S denotes a substrate to be monooxygenated. During the reaction, one atom of molecular oxygen is incorporated into a substrate while the other atom is reduced to water and, therefore, the reaction is also referred to as a mixed function oxidase reaction. In the mitochondrial cytochrome P-450 system, besides cytochrome P-450, a specific electron-transfer system composed of adrenodoxin, an iron-

sulphur protein, and adrenodoxin reductase, a flavoprotein, is necessary to convey electrons from NADPH to cytochrome P-450. In the microsomal cytochrome P-450 system it consists only of a flavoprotein, cytochrome P-450 reductase (Omura _et al._, 1966; Kimura, 1968; Cohen and Estabrook, 1971).

Figure 9 shows the reaction cycle of steroid monooxygenations catalyzed by mitochondrial cytochrome P-450. This cycle has been considered to be common to both 11β-hydroxylation and cholesterol side chain cleavage reactions (Kapke and Baron, 1976). The initial step of the process is postulated to be the binding of a substrate to the oxidized form of cytochrome P-450 ('a' in Fig. 9). This is followed by the reduction of cytochrome P-450 by a reduced adrenodoxin. The reduced cytochrome P-450-substrate complex ('b' in Fig. 9) is then oxygenated by molecular oxygen to form a ternary complex with the substrate, oxygen and reduced cytochrome P-450 ('c' in Fig. 9). Subsequently, the second electron is given to the ternary complex resulting in the release of a hydroxylated product and H_2O (Ando and Horie, 1971; Schleyer _et al._, 1973). The three states of cytochrome P-450 described above ('a-c' in Fig. 9) are recognizable as spectral shifts in the Soret region as well as in the longer wavelength region of the substrate-free cytochrome P-450 (Schleyer _et al._, 1973).

Since NADPH does not freely penetrate into intact mitochondria, control of the activity of steroid hydroxylases also depends on the generation of NADPH within the mitochondria. Three principal routes are available: (1) $NADP^+$-linked malic enzyme, (2) $NADP^+$-linked

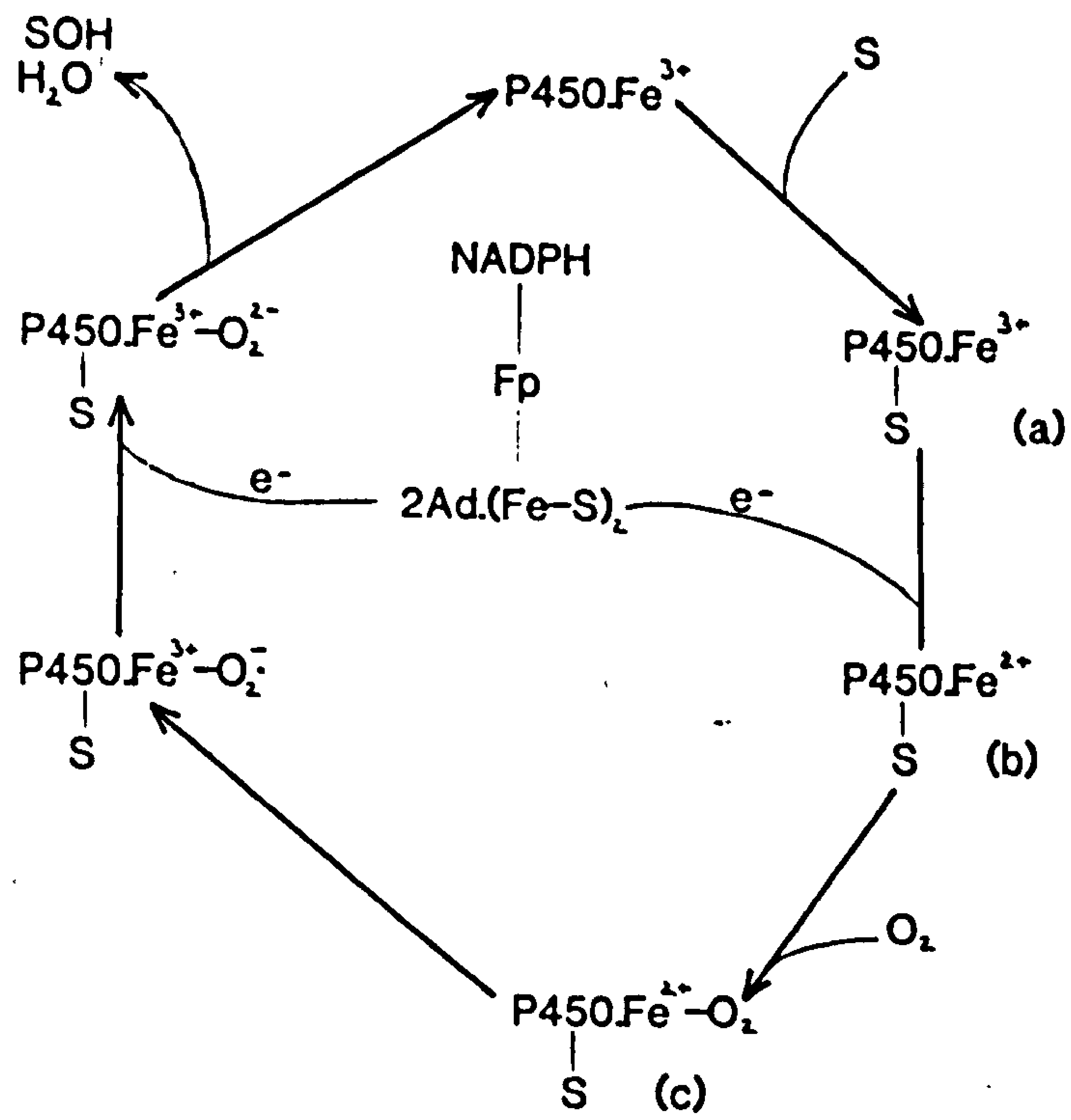


Fig 9. Reaction mechanism of the mitochondrial cytochrome P-450 dependent hydroxylation system (Huang and Kimura, 1971)

S:substate, Fp:adrenodoxin reductase, Ad.(Fe-S) :adrenodoxin

isocitrate dehydrogenase and (3) energy linked transhydrogenation of $NADP^+$ by NADH (from α ketoglutarate dehydrogenase, malic dehydrogenase, or reverse electron flow from succinate) [Kimura, 1981; Tuckey and Kamin, 1982].

The mechanism of microsomal cytochrome P-450 function is complex and has not been totally established (Fig. 10). The initial step consists of the binding of substrate to oxidized cytochrome P-450 followed by a one electron reduction catalysed by the NADPH-cytochrome P-450 reductase to form a reduced cytochrome-substrate complex. This complex can interact with carbon monoxide to form the CO complex. The next several steps are less well understood. They involve an initial reaction with oxygen to form a ternary oxygenated complex. This ternary complex accepts a second electron resulting in the further formation of one or more poorly understood complexes. One of these however is probably the equivalent of the peroxide anion derivative of the substrate-bound haemoprotein. After the transfer of one atom of oxygen to the substrate and the reduction of the other to form water, dismutation reactions occur that lead to the formation of the hydroxylated product, water and oxidized cytochrome (Cooper _et al._, 1979). The possibility that the second electron is derived from NADH via cytochrome b_5 has been the subject of argument for some time and is still to be completely resolved. It is clear, that this pathway is not essential for all cytochrome P-450 dependent monooxygenases, since many occur in systems reconstituted from NADPH, O_2, phosphatidylcholine, NADPH-cytochrome P-450 reductase and cytochrome P-450. Nevertheless, evidence is available that this pathway can

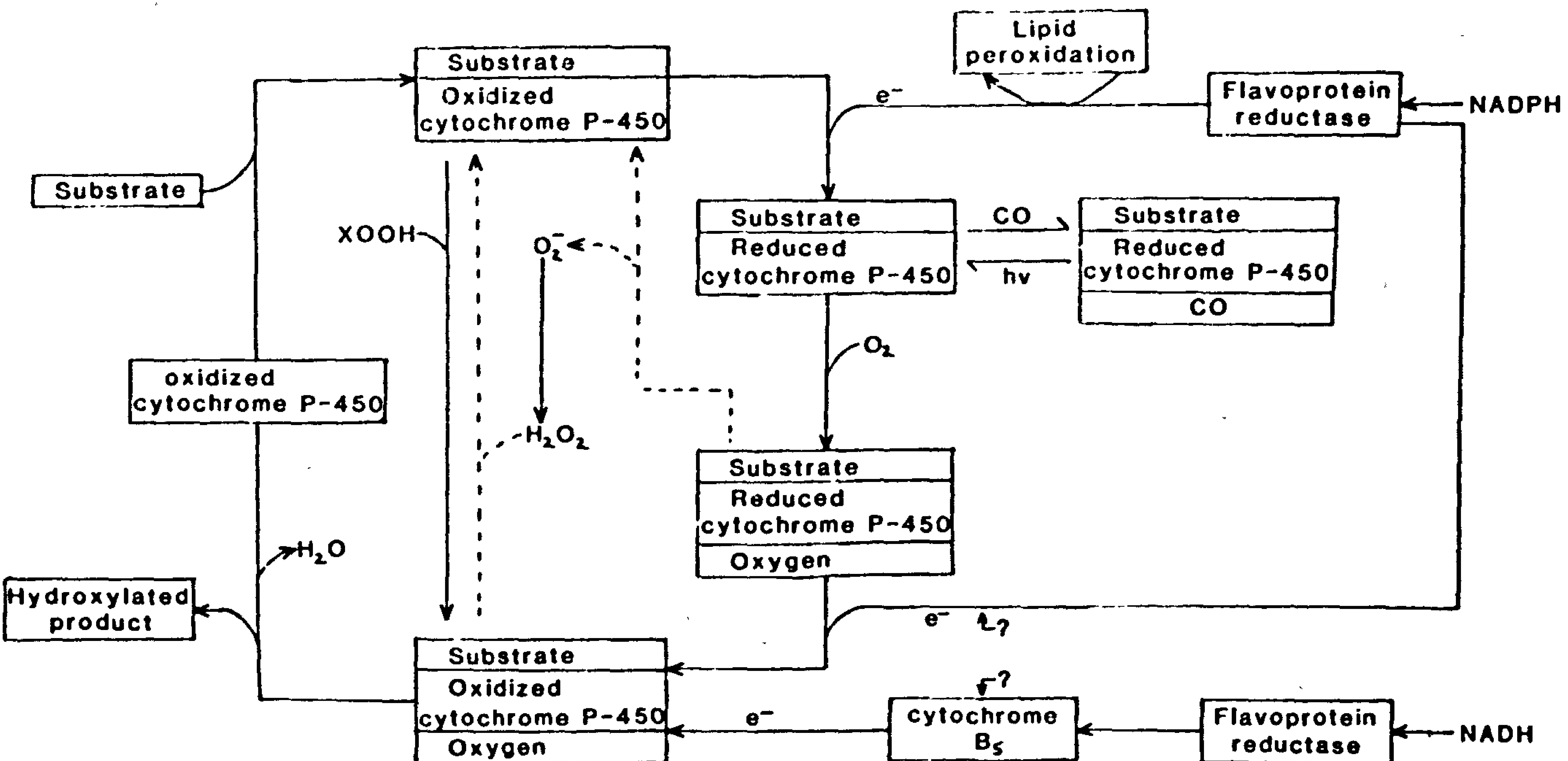


Fig 10. Reaction mechanism of the microsomal cytochrome P-450-dependent monooxygenase system

(Hodgson and Dauterman, 1980)

occur under some circumstances, and it may facilitate oxidative activity in the intact endoplasmic reticulum (Cohen and Estabrook, 1971; Hildebrant and Estabrook, 1971).

As observed in Figure 11 all four classical steroidogenic tissues, namely the adrenals, testes, ovaries and placenta, contain the enzymic system necessary for cholesterol side-chain cleavage, but the presence or absence of the other enzymes depends on the tissue and species in question. This diversity of gene expression of other steroid hydroxylases results in tissue- and species-specific steroid hormone production.

The steroid hydroxylases of the adrenal cortex have been examined in greater detail than those in other steroidogenic tissues (Pfeiffer et al., 1972; Purvis et al., 1973). Adrenocorticotrophic hormone (ACTH), the peptide hormone whose target tissue is the adrenal cortex, plays a key role in regulating the synthesis of adrenocortical steroid hydroxylases. The synthesis of cholesterol side-chain cleavage cytochrome P-450 (P-450scc) increases with ACTH treatment of bovine adrenocortical cells (DuBois et al., 1981). The temporal pattern of this response shows that little change in synthesis occurs during the first 12 hr. of ACTH treatment. At longer times, an increase in synthesis of cytochrome P-450scc is observed which peaks at 36hr. following initiation of treatment. The presence of ACTH beyond 36hr. results in a decrease in synthesis. A similar profile is observed for other mitochondrial components of the steroidogenic pathway, synthesis

Fig. 11 Significant levels of enzymic activities in steroidogenic tissues (Waterman and Simpson, 1985)

	Adrenal cortex (Fasciculata-reticularis)	Testis (Leydig)	Ovary (Granulosa luteal)	Placenta (Trophoblast)
Mitochondria	P-450scc $P\text{-}450_{11\beta}$	P-450scc -	P-450scc -	P-450scc -
Endoplasmic reticulum	$P\text{-}450_{17\alpha}$ [a] $P\text{-}450_{C21}$ -	$P\text{-}450_{17\alpha}$ - P-450aromatase	$P\text{-}450_{17\alpha}$ - P-450aromatase	$P\text{-}450_{17\alpha}$ [b] - P-450aromatase

a Activity found to be at extremely low levels or absent in most rodents leading to production of corticosterone as major glucocorticoid.

b Significant activity found in certain species including sheep, but in man only P-450scc and P-450aromatase activities are expressed.

of 11β-hydroxylase (cytochrome P-$450_{11\beta}$) adrenodoxin and adrenodoxin reductase. (Kramer et al., 1983a). Therefore, ACTH regulates the synthesis of mitochondrial steroid hydroxylases in a coordinate fashion (Waterman, 1982; Simpson and Waterman, 1983). It has been clearly established in several laboratories that ACTH treatment of adrenocortical cells leads to increased levels of cyclic AMP and that the acute effects of ACTH to stimulate steroidogenesis can be mimicked by analogues of cyclic AMP (Hayashi et al., 1979; Kramer et al., 1982; Rani et al., 1983). It has been shown that analogues of cyclic AMP can also mimic the effects of ACTH on the synthesis of mitochondrial components of the steroid hydroxylase pathway (Kramer et al., 1984). The synthesis of cytochromes P-450scc and P-$450_{11\beta}$ and adrenodoxin are all increased in response to treatment of bovine adrenocortical cells with dibutyryl cyclic AMP, the temporal pattern being similar to that observed following ACTH treatment (Boggaram et al., 1984a). It is suggested that while the synthesis of these mitochondrial components in response to ACTH appears to be coordinate, each enzyme may respond to different levels of cyclic AMP.

The increases in amount of cytochromes P-450scc and P-$450_{11\beta}$ and adrenodoxin in adrenocortical cells in response to ACTH are clearly due in part to an increased rate of synthesis. However, it is also possible that ACTH may decrease the turnover rates of these enzymes and in this way also contribute to their elevated levels. This has been found not to be the case for cytochrome P-450scc and adrenodoxin, but in the case of cytochrome P-$450_{11\beta}$, there appears to be a significant effect of ACTH to increase the half-life (Boggaram et al., 1984b).

ACTH also influences the rate of synthesis of microsomal steroid hydroxylases. Treatment of bovine adrenocortical cells with ACTH leads to increased synthesis of steroid 21-hydroxylase (cytochrome P-450_{C21}) [Funkenstein et al, 1983]. In this case, optimal synthesis is observed 24hr. following initiation of ACTH treatment and beyond 24hr. the synthesis of cytochrome P-450_{C21} decreases. The synthesis of cytochrome P-450_{C21} can also be induced by dibutyryl cyclic AMP (Boggaram et al., 1984a). Despite the fact that increased synthesis of cytochrome P-450_{C21} occurs, no concomitant increase in steroid 21-hydroxylase activity is observed; this has yet to be explained.

The other microsomal steroid hydroxylase, 17α-hydroxylase (cytochrome P-$450_{17\alpha}$) is also increased in bovine adrenocortical cells in response to ACTH treatment (Kramer et al., 1983b; McCarthy et al., 1983). Microsomes derived from ACTH-stimulated cells had much greater capacity to convert progesterone to 17α-hydroxyprogesterone than microsomes from untreated cells (McCarthy et al., 1983). Analogues of cyclic AMP also stimulate synthesis of cytochrome P-$450_{17\alpha}$ (Waterman and Simpson, 1985). The effect of ACTH on 17α-hydroxylase is much more dramatic than that on any other steroidogenic enzyme. The temporal pattern for this increase in synthesis resembles that described for cytochrome P-450_{C21}. Thus, the synthesis of the microsomal components of the steroidogenic pathway responds to the same stimuli as the mitochondrial components but with a different temporal response.

The decrease in synthesis observed at longer times of continued ACTH treatment has been attributed to desensitization of the cells,

perhaps in part at the level of the ACTH receptor, but also at a site distal to the production of cyclic AMP (Kramer et al., 1984).

ACTH serves two crucial regulatory roles in its target tissue, the adrenal cortex. The first is an acute role which is well established as regulation of the availability of substrate (cholesterol) for the pathway (Simpson and Waterman, 1983; Privalle et al., 1983). The second regulatory role is a chronic one, resulting in maintenance of optimal levels of steroid hydroxylases in the adrenal cortex. Under normal physiological conditions, the adrenal cortex experiences a rather constant pattern of ACTH stimulation leading to a regular diurnal pattern of production of glucocorticoids and adrenal androgens. Fluctuations in the level of ACTH lead to fluctuations in the levels of these products due to changes in the amount of cholesterol entering the pathway. In addition, the constant stimulation by ACTH leads to optimal levels of the enzymes in this pathway always being present (Waterman and Simpson, 1985). Thus, it is presumed that these forms of cytochrome P-450 in the adrenal cortex are constitutive and their levels do not change dramatically, unlike certain hepatic forms of cytochrome P-450 whose synthesis is altered in vivo as the organism encounters varying levels of xenobiotics. This suggests that the synthesis of the adrenocortical steroid hydroxylases is mediated by cyclic AMP produced by activation of adenylate cyclases by ACTH. The long time period required for increased synthesis, as well as the variability of synthesis of mitochondrial components, depending on the level of cyclic AMP suggests that cyclic AMP mediates the effect via a series of as yet undescribed steps. This has yet to be proved conclusively (Waterman and Simpson, 1985).

Adrenal cortex cholesterol ester hydrolase has been shown to be activated after cyclic AMP-dependent phosphorylation (Trzeciak and Boyd, 1974; Naghshineth et al., 1978). The 11β-hydroxylation cytochrome P-450 ($P\text{-}450_{11\beta}$) has been demonstrated to be a good substrate for cyclic AMP-dependent protein kinase (Defaye et al., 1982). In addition, the adrenal cortex contains several cyclic nucleotide-independent protein kinase activities which could participate in phosphorylation of substrates of key metabolic significance (Cochet et al., 1977; Cochet et al., 1980).

Cholesterol 7α-hydroxylase is a microsomal mixed function oxidase that includes cytochrome P-450 as one of its subunits and catalyses the NADPH-dependent hydroxylation of cholesterol. It is the rate-limiting enzyme in the conversion of cholesterol into bile acids. It is interesting to note that evidence has been provided for the modulation of cholesterol 7α-hydroxylase by changes in its phosphorylation state (Sanghvi et al., 1981; Goodwin et al., 1982). Phosphorylation of the phenobarbital-inducible form of cytochrome P-450 isoenzyme LM2 (purified from rabbit liver microsomes) in solution (Pyerin et al., 1983) and reconstituted membranes (Pyerin et al., 1984) by cyclic AMP-dependent protein kinase was shown to decrease its enzymic activity by conversion into cytochrome P-420 with consequent loss of haeme (Taniguchi et al., 1985). Thus, breakdown of this cytochrome P-450 and, therefore, its turnover may be under the control of adrenergic transmitters mediated by the second messenger cyclic AMP (Taniguchi et al., 1985; Pyerin et al., 1986). The cytochrome P-450 isoenzyme LM2 from rabbit liver microsomes is

phosphorylated at a single site, the serine residue in position 128 of the amino acid sequence. This serine is located in a partial sequence which is specific for substrates of cyclic AMP-dependent protein kinase (Muller et al., 1985). Phosphorylation of cytochrome P-450 is not restricted to the cytochrome P-450 mentioned above (Pyerin et al., 1987). However, the phosphorylation is very specific and restricted to certain combinations of cytochrome P-450 isoenzymes and protein kinases. Other enzymes involved in drug metabolism and chemical carcinogenesis have been found to be poor kinase substrates, except several isoenzymes of glutathione transferase (Pyerin et al., 1987).

5. Metabolism of Ecdysteroids

Isolation of ecdysteroids alone does not indicate their metabolic relationship, which can only be achieved by studying the interconversions of ecdysteroids. The main reactions in ecdysteroid metabolism can be summarized as follows (Fig. 12):

(a) hydroxylation of ecdysone to 20-hydroxyecdysone.

(b) epimerization at C-3 to yeild a stereoisomer.

(c) hydroxylation at C-26, eventually followed by oxidation to a carboxylic acid.

and (d) conjugation.

The titre of any hormone is the result of two main processes: biosynthesis and secretion on the one hand and metabolic inactivation and excretion on the other. In most hormonal systems, the amount of hormone assimilated by the target tissue is rather small compared with

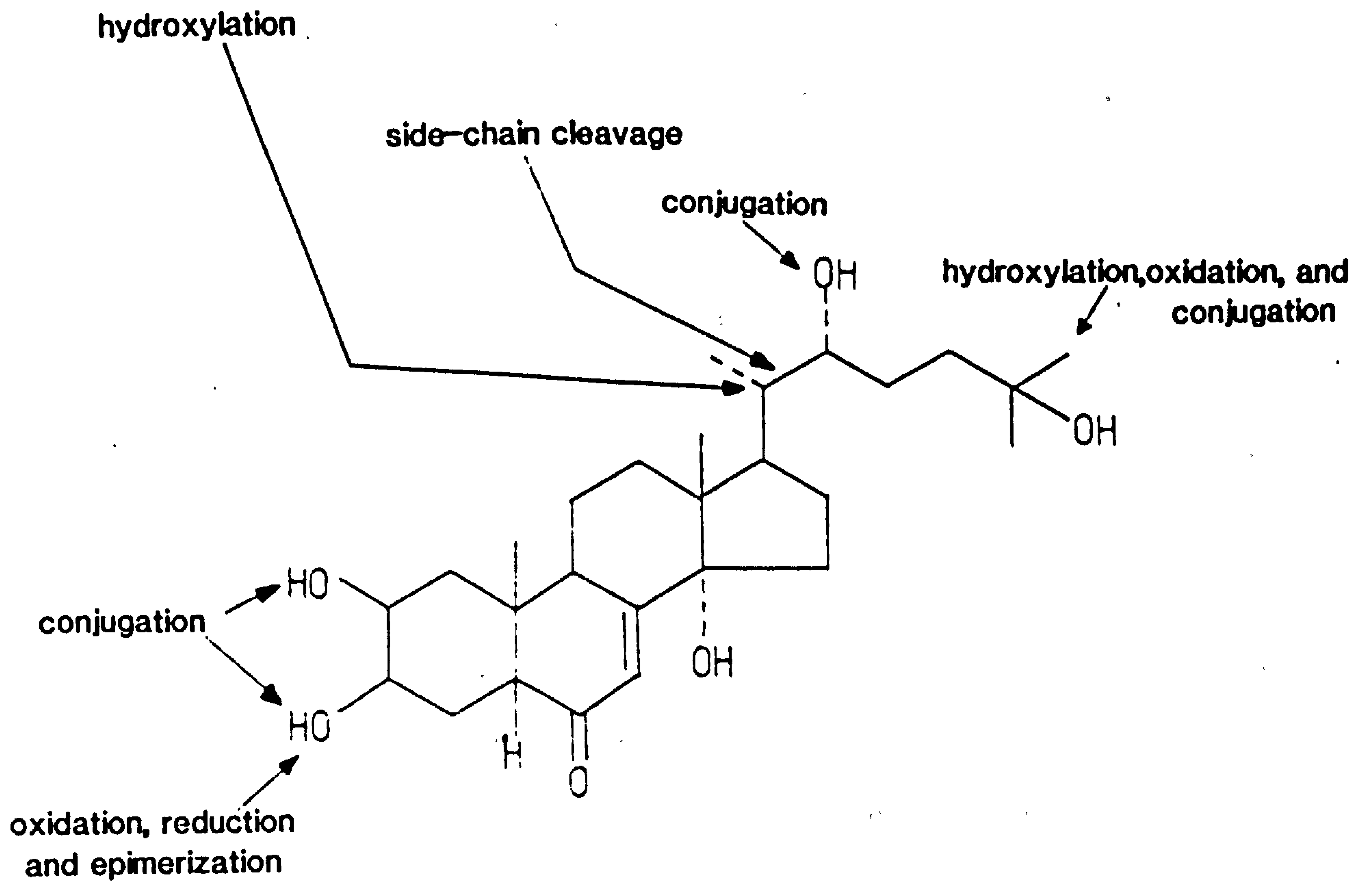


Fig 12 General reactions comprising ecdysone metabolism

the amount that is metabolically inactivated. This is borne out by the short half-lives of many hormones, which are in the order of a few minutes to one hour. Steroid hormones circulating in the blood are often protected from metabolic degradation by binding to blood protein(s). This phenomenon may be important in the regulation of ecdysteroid titres, as ecdysteroid-binding proteins have been demonstrated in the haemolymph of Locusta migratoria (Feyeresisen, 1980) as well as a number of other species (Yund and Osterbur, 1985).

The most important hydroxylation reaction is that of ecdysone at the C-20 position to yield 20-hydroxyecdysone. This reaction has been demonstrated in all insect species investigated (Koolman, 1982). In most insect species studied, the conversion of ecdysone to 20-hydroxyecdysone occurs rather rapidly and the circulating hormone is generally, but not exclusively 20-hydroxyecdysone which is mainly responsible for eliciting the moulting process.

Further hydroxylation of ecdysone and 20-hydroxyecdysone usually occurs at position C-26, producing 26-hydroxyecdysone and 20,26-dihydroxyecdysone, both of which have much reduced hormonal activity (Thompson et al., 1967; Kaplanis et al., 1973). These metabolites have been identified and characterized in a number of insects including Manduca sexta (Thompson et al., 1967; Kaplanis et al., 1973) and Calliphora vicina (Greenwood and Russel, 1978). 26-Hydroxyecdysone was identified as a metabolite of ecdysone in Pieris brassicae (Lafont et al., 1980) demonstrating that the metabolism of ecdysone can also begin with hydroxylation at C-26.

Removal of hydroxyl groups, has as yet, only been observed in micro-organisms (Hayakawa, 1973). However, inokosterone (25-deoxy-20,26-dihydroxyecdysone) has been identified as a metabolite of labelled ecdysone in Calliphora (Koolman et al., 1979). It is conceivable that dehydroxylation through the elimination of sulphuric acid from a 25-sulphate of either 20-hydroxyecdysone or 20,26-dihydroxyecdysone occurs and that the product is then converted into inokosterone.

Ecdysone and other ecdysteroids are readily oxidized at C-3 to yield 3-dehydroecdysone. The reaction is catalysed by ecdysone oxidase (E.C. 1.1.3.16) (Milner and Rees, 1985). This enzyme will not oxidize cholesterol, 20-hydroxycholesterol or 22,25-dideoxyecdysone.

Ecdysone and most of its metabolites can undergo irreversible epimerization to yield 3α-stereoisomers of ecdysteroids (3-epiecdysteroids), in some insect species. The 3-epiecdysteroids differ from the ecdysteroids only in their configuration at C-3. The reaction involves the intermediacy of 3-dehydro compounds, which are reduced to the 3-epi compounds by 3-dehydroecdysone reductase (Koolman, 1976; Milner and Rees, 1985).

A further oxidation of 26-hydroxyecdysteroids yields the corresponding carboxylic acid. Such oxidation products may be expected from ecdysone, 20-hydroxyecdysone and possibly also 3-epiecdysone and 3-epi-20-hydroxyecdysone (Lafont et al., 1980). The major metabolite to accumulate is 20-hydroxyecdysonoic acid, which is the end-product of an ecdysteroid inactivation pathway in a number of Lepidoptera species (Lafont et al., 1983).

In many insects, the major metabolic route of ecdysteroids appears to be the formation of conjugates. Numerous ecdysteroids can exist in conjugate form, most notably ecdysone, 20-hydroxyecdysone, 20,26-dihydroxyecdysone and several 3-dehydro- and 3-epiecdysteroids. Thus, the conjugating enzymes are apparently very efficient and not specific for a particular metabolite. The presence of sulphate esters of some ecdysteroids has been reported (Koolman et al., 1973).

The existence of ecdysteroid phosphate esters has only been recognized comparatively recently. These include ecdysone 22-phosphate, 2-deoxyecdysone 22-phosphate and the corresponding 20-hydroxylated compounds in the eggs of Schistocerca gregaria (Isaac and Rees, 1984). The 2- and 3-acetate of ecdysone 22-phosphate along with the 3-acetyl 2-phosphate of ecdysone and 20-hydroxyecdysone, and 3-epi-2-deoxyecdysone 3-phosphate appear in Schistocerca eggs just prior to hatching (Isaac and Rees, 1984). 3-Epi-2-deoxyecdysone 3-phosphate has also been identified in embryos from Locusta migratoria (Tsoupras et al., 1982b).

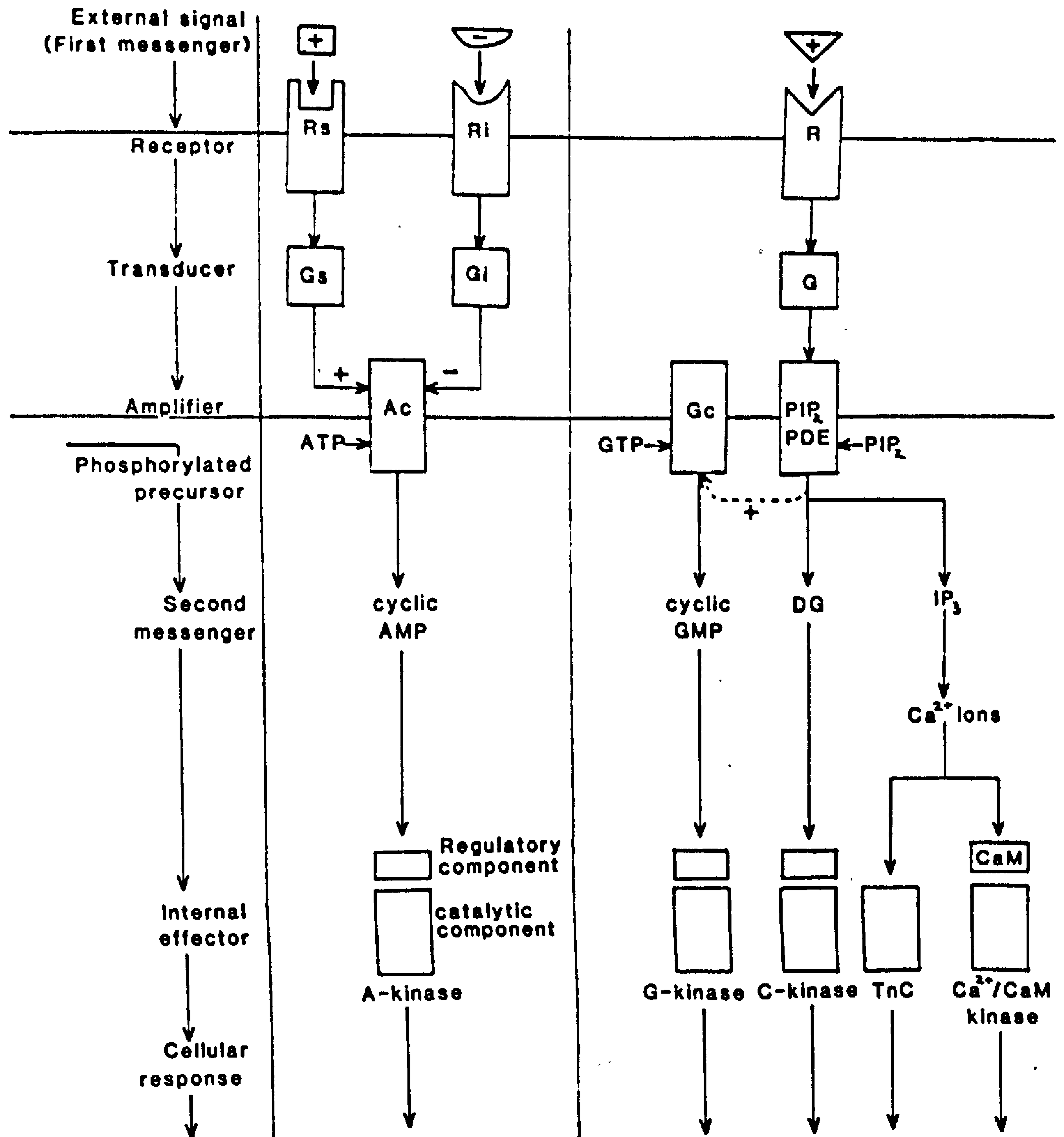
More recently, 26-hydroxyecdysone 22-glucoside has been characterised fully from the developing eggs of Manduca sexta (Thompson et al., 1987). Ecdysteroid 22-fatty acyl esters have been isolated from Arachida (ticks; Connat et al., 1984; Crosby et al., 1986). More recently, fatty acyl ester derivatives of ecdysteroids have also been detected in insects (Hoffmann et al., 1985; Whiting and Dinan, 1988; Slinger and Isaac, 1988).

6. Covalent Modification

In a multicellular organism, the major barrier to the flow of information is the cells plasma membrane. Dispersed on the outer surface of the cell are protein receptors which detect an incoming messenger and transmit this information by inducing a conformational change in the protein next in line. At some point, the information is assigned to small molecules or even to ions within the cells cytoplasm. These are the second messengers, whose diffusion enables a signal to propagate rapidly throughout the cell. Although the internal signal pathways in cells are very universal, the small number of known second messengers are capable of regulating a vast variety of physiological and biochemical processes (Krebs and Beavo, 1979).

Three major signal pathways are known to exist. The first and second employ the second messengers, cyclic adenosine monophosphate (cyclic AMP) and cyclic guanosine monophosphate (cyclic GMP). The other employs a combination of second messengers that includes calcium ions, inositol trisphosphate (IP_3) and diacyl glycerol (DG) (Fig. 13). (Berridge, 1985). Of all the steps of the known second-messenger pathways, the ones best understood are the ones of transduction and amplification that activate cyclic AMP (Fig. 14). For information to flow across the cells plasma membrane two events must first occur. At the surface of the cell an external signal molecule must bind to its receptor and a GTP (guanosine triphosphate) molecule must then act on its G protein in the cell (Krebs and Beavo, 1979). Two types of G proteins are involved, one of them stimulatory and other inhibitory.

Fig 13. Known signal pathways in cells (Berridge, 1985)



Rs/i-Stimulatory /inhibitory receptor
Gs/i-Simulatory /inhibitory G protein
Ac-Adenylate cyclase
Gc-Guanylate cyclase
PDE-Phophodiesterase
PIP_2 -Phosphatidylinositol 4,5-bisphosphate
TnC-Troponin C

ATP-Adenosine triphosphate
GTP-Guanosine triphosphate
cAMP-cyclic adenosine monophosphate
cGMP-cyclic guanosine monophosphate
IP_3 -Inositol trisphosphate
DG-Diacyl glycerol
CaM-calmodulin

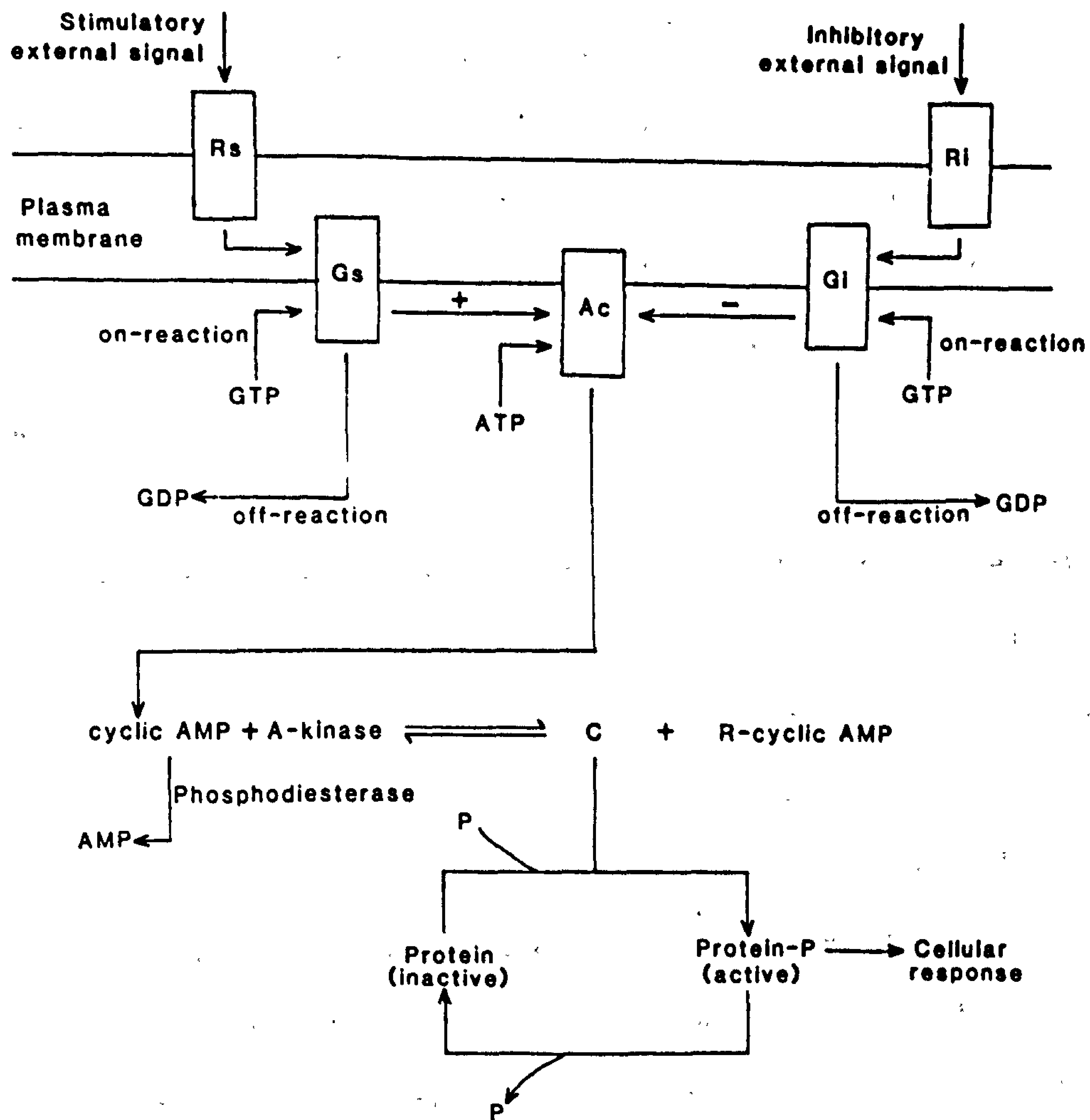


Fig 14. Cyclic AMP signal pathway. (Berridge, 1985)

Rs-stimulatory receptors

Ri-inhibitory receptors

Gs-Stimulatory G protein

Gi-inhibitory G protein

P-phosphate

Ac-adenylate cyclase

C-catalytic subunit of cyclic AMP

R-regulatory subunit of cyclic AMP

The stimulating protein called 'Gs' links itself to external receptors called 'Rs'. The binding of a stimulatory receptor signal to an external receptor induces a conformation change in that receptor. The change is transmitted through the cell membrane to 'Gs' making it susceptible to GTP, which is present within the cell. The binding of GTP to 'Gs' in the presence of adequate Mg^{2+} or Mn^{2+} ions forces 'Gs' into still another conformational change which enables it to activate adenylate cyclase (AC) and thereby instigate the formation of cyclic AMP (Fig. 14). The activity of the Gs-GTP complex is ended by the hydrolysis of GTP to GDP (guanosine diphosphate) catalysed by the enzyme GTPase.

The other type of G protein in the cyclic AMP pathway mediates an inhibitory transduction. The arrival of an external inhibitory signal at the receptor designated 'Ri' results in a conformational change in the G protein called 'Gi' (a change again dependent on the binding of GTP in the presence of adequate Mg^{2+} or Mn^{2+} ions). The 'Gi' protein, in turn, inhibits adenylate cyclase (Fig. 14) [Berridge, 1985]. The final chemical steps in the cyclic AMP pathway are mediated by an A-protein kinase which phosphorylates a particular protein when it is activated specifically by cyclic AMP. Each A-protein kinase consists of two components, a catalytic subunit and a regulatory subunit. Cyclic AMP binds to the regulatory subunit, thereby liberating the catalytic one, which is then free to phosphorylate proteins (Berridge, 1985).

Two isoenzyme forms of A-protein kinase designated types I and II have been identified and are present in most tissues, although the relative amounts of each form vary among tissues, species and developmental or physiological conditions (Corbin et al., 1975). Cyclic AMP is degraded by the enzyme phosphodiesterase. Sodium fluoride also activates adenylate cyclase through the 'Gs' protein, although the precise mechanism of action is unknown (Smith and Combest, 1985).

Cyclic GMP has all the hallmarks of a second messenger although its precise role in the cell is not well understood. The enzyme guanylate cyclase which converts GTP to cyclic GMP in the presence of Mn^{2+} or Mg^{2+}, is usually not connected to a receptor. Nevertheless, the formation of cyclic GMP often occurs together with the activation of the inositol-lipid pathway. The end of the signal pathway is equally obscure. Cyclic GMP is known to activate a protein kinase (in particular G-kinase) which in turn phosphorylates certain proteins, the functions of which are not clearly understood (Fig. 13).

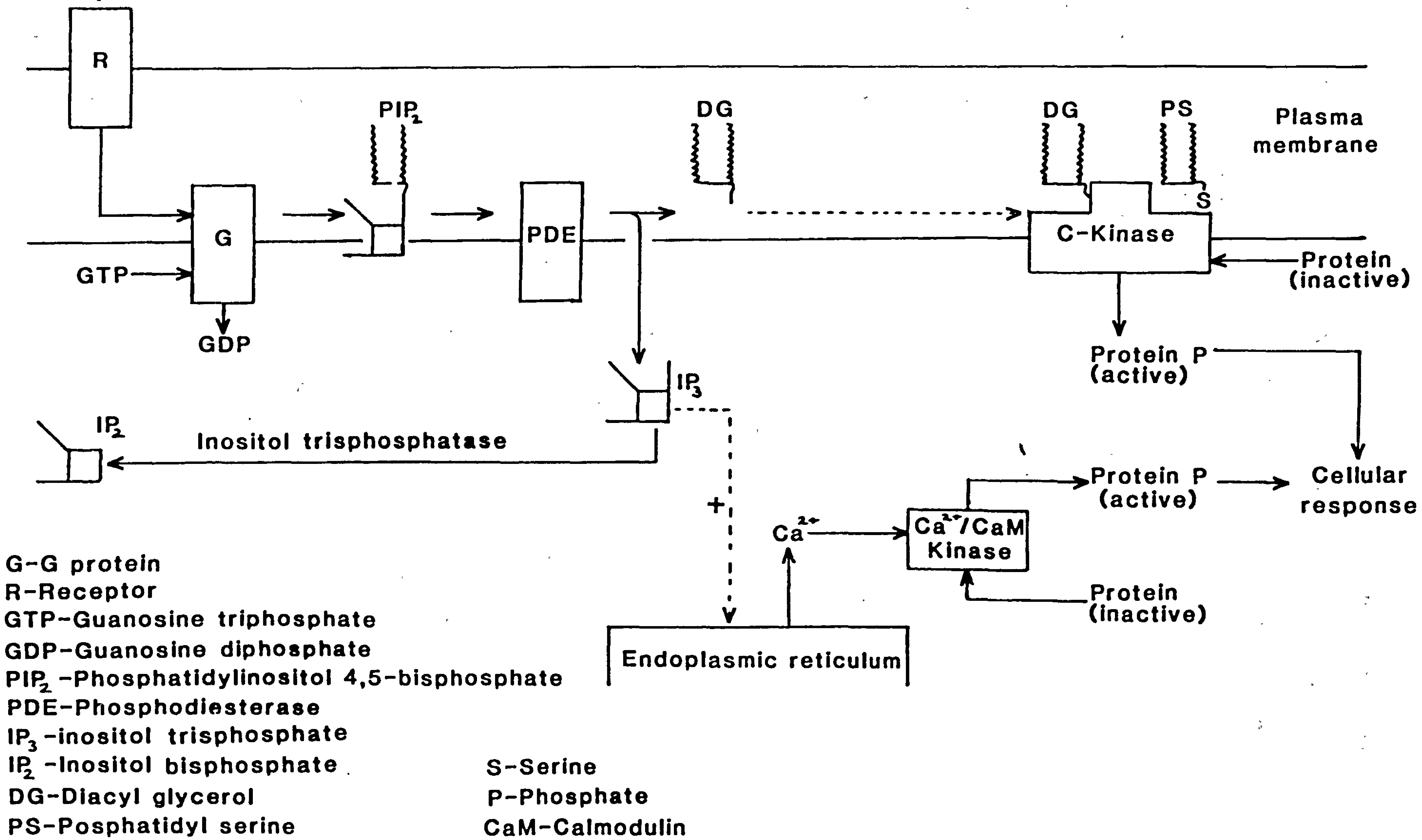
Intracellular levels of cyclic nucleotides are determined by a balance between synthesis (via cyclase) and degradation to 5'-mononucleotides (via cyclic nucleotide phosphodiesterase or PDE). At least three distinct forms of PDE have been characterized (Wells and Kramer, 1981). These three forms differ in their substrate specificity, ie. cyclic AMP vs cyclic GMP, and are present in different relative amounts from tissue to tissue (Wells and Kramer, 1981).

The third pathway, the inositol-lipid path, which employs a combination of messengers that includes calcium, inositol trisphosphate and diacyl glycerol is also complex and not fully understood (Fig. 15). The external signal binds to the receptors which transmit information through a G protein to activate phosphatidylinositol 4,5-bisphosphate phosphodiesterase. In turn phosphodiesterase cleaves phosphatidylinositol 4,5-bisphosphate into the second messengers inositol trisphosphate and diacyl glycerol. The inositol trisphosphate is water soluble, and so diffuses into the cytoplasm. There it releases calcium from storage in the membranous intracellular endoplasmic reticulum. In turn, the calcium stimulates a protein kinase. The diacyl glycerol remains in the membrane, where it activates the enzyme C-kinase. The membrane phospholipid, phosphatidyl serine, is a cofactor, or necessary adjunct, for the activation. The two limbs of the pathway lead to phosphorylation of distinct sets of proteins. This pathway is covered more fully in the review article by Berridge (1985).

There are still many enzymes, particularly those involved in biosynthetic pathways, that are controlled by protein kinases whose mechanism of regulation are not yet understood (Cohen, 1980).

To modulate cell function via protein phosphorylation there must also exist a phosphoprotein phosphatase to reverse the reaction catalysed by protein kinase (Krebs and Beavo, 1979). The protein phosphatase activities involved in regulating the major pathways of intermediary metabolism in vertebrates can be explained to-date by

Fig 15. The inositol-lipid pathway. (Berridge, 1985)



G-G protein
R-Receptor
GTP-Guanosine triphosphate
GDP-Guanosine diphosphate
PIP_2-Phosphatidylinositol 4,5-bisphosphate
PDE-Phosphodiesterase
IP_3-inositol trisphosphate
IP_2-Inositol bisphosphate
DG-Diacyl glycerol
PS-Posphatidyl serine

S-Serine
P-Phosphate
CaM-Calmodulin

only four enzymes which are divided into two classes, Type-1 and Type-2. Type-1 protein phosphatases dephosphorylate the β-subunit of phosphorylase kinase in preference to the α-subunit and are potently inhibited by two thermostable proteins termed inhibitor-1 and inhibitor-2, whereas Type-2 protein phosphatases preferentially dephosphorylate the α-subunit of phosphorylase kinase and are insensitive to inhibitor-1 and inhibitor-2.

The Type-2 protein phosphatases can be further divided into 2A, 2B and 2C protein phosphatases according to their substrate specificity. Protein phosphatase-1 (Type 1) and -2A have very broad substrate specifities. The major differences between these, apart from the site specificity for phosphorylase kinase, are the much higher myosin light chain phosphatase and ATP-citrate lyase phosphatase activities of protein phosphatase 2A. Protein phosphatase-2C (a Mg^{2+}-dependent enzyme) also has a broad specificity, but can be distinguished from protein phosphatase-2A by its extremely low phosphorylase phosphatase and histone H1 phosphatase activities, and its slow dephosphorylation of sites (3a + 3b + 3c) relative to site-2 of glycogen synthase. Protein phosphatase-2B (a Ca^{2+}-calmodulin-dependent enzyme) is the most specific phosphatase and to-date is only known to dephosphorylate the α-subunit of phosphorylase kinase, inhibitor-1 and myosin light chains at a significant rate (Ingebritsen and Cohen, 1983).

Most of the work on insect adenylate cyclases has been carried out with nervous tissue, but by no means exclusively. It appears that

adenylate cyclases are as widespread in invertebrates as they are in vertebrates (Smith and Combest, 1985). Cyclic nucleotide dependent protein kinases have been obtained from various arthropod tissues (Kuo *et al.*, 1971; Liu *et al.*, 1981). The two principal protein kinases present in these tissues are A-kinases and G-kinases. In fact, most A-kinases can be activated by higher molar concentrations of cyclic GMP and most G-kinases by cyclic AMP. It has been suggested by Kuo and Greengard (1969) that all the biochemical and physiological effects of the two nucleotides are mediated through their specific kinases. The high level of cyclic GMP in insect tissues (Ishikawa *et al.*, 1969) and changes in protein kinase activity during the course of development of silkworm eggs (Takahashi *et al.*, 1975), support the hypothesis that the two cyclic nucleotide-dependent protein kinases may play distinct roles in insect metabolism.

Extensive characterizations have been made on both the A- and G-kinases in whole pupae of the silkworm, *Bombyx mori* (Nishiyama *et al.*, 1975; Takai *et al.*, 1976; Inoue *et al.*, 1976). The cyclic AMP dependent kinase purified from this source appears to be identical to the mammalian enzyme. Further, the insect and mammalian enzymes are completely exchangeable in their ability to phosphorylate muscle glycogen phosphorylase kinase and synthase, resulting in activation and inactivation of the respective enzymes (Nishiyama *et al*, 1975; Takai *et al.*, 1976; Inoue *et al.*, 1976). Both cyclic nucleotide kinases have also been purified from pharate adults of *Ceratitis capitata* (Garcia *et al.*, 1983; Haro *et al.*, 1983) and *Drosophila* embryos (Tsuzuki and Kiger 1975; Olsen *et al.*, 1982).

In addition to the typical A-kinase, a protein kinase has been isolated from the thoracic integument of Melanoplus sanguinipes and a similar kinase from the fat body of the adult cockroach, Periplaneta americana, which display equal high affinities for both cyclic AMP and cyclic GMP (Takahashi and Hanaoka, 1977; Vardanis, 1980).

The first indication of protein kinase activity in insect nervous tissue was reported by Kuo and co-workers (1971) in Hyalophora cecropia silkmoth heads. A-kinases have also been isolated in the central nervous system of Galleria mellonella, where two soluble types were isolated, and also in the nerve cord of Manduca sexta larvae, where only one type was isolated and most of its activity in this case was associated with the particulate fraction (Tsuzuki and Newburgh, 1974; Albin and Newburgh, 1975).

The amount of G-kinase increases progressively (up to 4-fold) during the eight days of the fifth larval instar in Bombyx fat body. Neither the G- or A-kinase is found in the first 48 hours after oviposition. At three days, only the G-kinase is detectable, whereas by six days both forms are present in equal amounts and by eight days only the A-kinase is present (Takahashi, 1976).

Limited studies have focused on the phosphorylated endogenous substrates of protein kinase in arthropods. In synaptosomal fractions of Drosophila heads, several phosphorylated proteins have been identified that are affected by cyclic AMP as well as by calcium-calmodulin (Kelly, 1981). Phosphorylation of three of these

proteins is enhanced by cyclic AMP, while a fourth is reduced. Treatment of synaptosomal fractions with EGTA results in the loss of several phosphoproteins which can be rephosphorylated by addition of calcium and calmodulin. The identity and function of these phosphoproteins is unknown. Cyclic AMP-dependent thiophosphorylation has also been observed in homogenates of Drosophila heads, the major substrate for thiophosphorylation being the regulatory subunit of the Drosophilia type II cyclic AMP-dependent protein kinase (Muller et al., 1988). In the adult female locust, vitellin, a phosphorylated storage protein of eggs, serves as a good in vitro substrate for fat body A-kinase (Pines and Applebaum, 1978). In addition, three prominent proteins from the larval brain of Manduca sexta have been isolated, phosphorylation of which are markedly stimulated by cyclic AMP (Combest and Gilbert, 1986). The major phosphoprotein detected was apparently independent of cyclic nucleotides and calcium-calmodulin. The phosphorylation of two of the protein kinase substrates was stimulated by exogenous calcium and calmodulin. Incubating, the salivary glands of the lone star tick, Amblyomma americanum, with dopamine or cyclic AMP and theophylline, an inhibitor of phosphodiesterase, significantly increased the amount of phosphate incorporated into at least twelve proteins of whole tick salivary glands (McSwain et al., 1985).

Correlations between A- and G-kinases and a defined physiological role are few. The effects of the compound diamide in blocking amino acid uptake and protein secretion in locust fat body are correlated with the potent inhibition of G-kinase (Harry et al., 1978). In the

cockroach, injected extracts of corpora cardiaca increase cyclic AMP in the fat body and dissociate the holoenzyme of cyclic AMP-dependent protein kinase into regulatory and active catalytic subunits. It is suggested that the A-kinase may be involved in the hyperglycemic action of copora cardiaca extract (Takahashi et al., 1983). It has also been observed in the snail, Aplysia that an A-kinase is involved in short-term learning mechanisms by modifying K^+-channel conductivity and subsequent Ca^{2+} influx in presynaptic endings (Kandel and Schwartz, 1982).

In addition, Ca^{2+}-calmodulin-dependent protein kinases and phospholipid-dependent protein kinase C have been identified in insects (Ashida and Wyatt, 1979; Kuo et al., 1980; Morishima et al., 1985; Orr et al., 1985; Combest and Gilbert, 1986).

There is very little information on phosphoprotein phosphatases in arthropods. The protein phosphatase activity in salivary glands of the tick Amblyomma americanum is inhibited by micromolar Ca^{2+} and by rabbit skeletal muscle heat-stable inhibitor protein, and activated by histone H-1. The crude enzyme could be separated by anion exchange chromatography at pH 7.2 into two peaks of activity. Both of these peaks were inhibited by micromolar Ca^{2+}. Separation of the crude enzyme by anion exchange chromatography at pH 7.5 produced four peaks of activity, all of which where inhibited by micromolar Ca^{2+} when phosphorylase a and histone H-1 were substrates. However, they were only 50% inhibited when exhaustively phosphorylated histone H-2b was the substrate. Further purification of these peaks by gel filtration

resulted, from each of the four peaks, in a single peak of activity which was inhibited by micromolar calcium and a heat-stable inhibitor protein in all cases (McSwain et al., 1985; Williams et al., 1988).

In a number of insect species, glycogen phosphorylase has been shown to exist in two interconvertible forms: phosphorylase a, whose activity is largely independent of AMP, and phosphorylase b, which is active only in the presence of high levels of AMP (Steele, 1982). Recently in the locust fat body, in addition to the a and b forms, a third form of glycogen phosphorylase was identified, which is referred to as phosphorylase ab (Van Marrewijk et al., 1988). It is generally assumed that a phosphorylase kinase catalyses the incorporation of phosphate from ATP-Mg^{2+} into phosphorylase b and multisubstrate protein phosphatases reverse the reactions by the hydrolysis of the phosphoester bond in phosphorylase a as in mammalian systems (Cohen, 1983). The following lines of evidence support this assumption: (i) phosphorylase activities independent (a) and dependent (b) on cyclic AMP were measured in a great number of insect tissues (Steele, 1982); (ii) the a and b forms of the enzyme were separated by ion exchange chromatography from Phormia regina (Childress and Sacktor, 1970) Bombyx mori (Yanagawa and Horie, 1977) and Drosophila melanogaster (Dombradi et al., 1986); (iii) the level of active phosphorylase a rises during development or in response to injury (Stevenson and Wyatt, 1964), flight (Childress and Sacktor, 1970; Van Marrewijk et al., 1980), cold (Hayakawa and Chino, 1982) and starvation (Siegert and Ziegler, 1983) in intact insects; (iv) the activation also occurs in crude homogenates, requires ATP-Mg^{2+} (Wiens

and Gilbert, 1967) and is catalysed by phosphorylase kinase (Hansford and Sacktor, 1970; Yanagawa and Horie, 1978; Ashida and Wyatt 1979; Pallen and Steele, 1988); (v) the inactivation of phosphorylase a was observed after prolonged incubation of insect fat body at 25-30°C (Weins and Gilbert, 1967; Steele, 1969; Ziegler et al., 1979; Hayakawa and Chino, 1982) and the presence of phosphorylase phosphatase in *Manduca sexta* was demonstrated (Ashida and Wyatt, 1979).

Several reports on the phosphorylase kinase in insect fat body have been published (Steele, 1982), but little is known about the phosphorylase phosphatase in that tissue, the activity of the latter enzyme in fat body homogenate being extremely low (Ashida and Wyatt, 1979). The fat body phosphorylase phosphatase from the pupae of the silkworm, *Philosamia cynthia*, essentially resembles that reported for mammalian muscle phosphorylase phosphatase (Brautigan et al., 1982). The enzyme is stimulated by Mn^{2+} or Mg^{2+} but is not affected by Ca^{2+} (Hayakawa, 1985). The fat body phosphorylase phosphatase appears to be much more sensitive to Zn^{2+} than mammalian phosphatases, a situation which is almost equivalent to that reported for phosphotyrosine phosphatases of some mammalian tumor cells (Hayakawa, 1985). In addition, the fat body phosphorylase phosphatase is inhibited by low concentrations of ATP, even in the presence of Mg^{2+} (Hayakawa, 1985). The sensitivity to inhibition by ATP resembles that reported for mammalian protein phosphatase 2A (Ingebritsen and Cohen, 1983; Ingebritsen et al., 1983).

CHAPTER TWO

MATERIALS AND METHODS

1. Materials

A. Solvents

Chloroform	-	Analar grade (BDH Chemicals Limited).
Ethanol	-	Redistilled.
Methanol	-	Redistilled.
Hexane	-	Analar Grade (BDH Chemicals Limited).
Butan-1-ol	-	Reagent Grade (BDH Chemicals Limited).
Acetone	-	Redistilled (BDH Chemicals Limited).
Diethyl ether	-	Dried over sodium-lead alloy for 24 hours and redistilled prior to use over reduced iron (BDH Chemicals Limited).
Toluene	-	Analar Grade (Koch-Light Laboratories).
Water	-	Glass Redistilled.
Acetonitrile	-	Analar Grade (BDH Chemicals Limited).

B. H.p.l.c. Solvents

Methanol - H.p.l.c. Grade (Fisons).

1,1-dichloroethane - H.p.l.c. Grade (Rathburn Chemicals).

Water - Glass Redistilled.

All h.p.l.c. solvents were filtered using a Millipore type HA (0.45μm) filter and degassed under vacuum prior to use.

C. Insect Diet Ingredients

Agar; Difco Laboratories.

Casein; BDH Limited.

Wheat Germ (Bemax); Local Chemist.

Dried active yeast; Distillers Co. Ltd.

Corn oil ('Fry Crisp and Dry'); Local Supermarket.

D. Chromatographic Adsorbents

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

Kieselgel G, (type 60) used for thin-layer chromatography (E. Merck).

Celite - washed prior to use with methanol and chloroform (Koch-Light).

Sep-Pak Cartridges - (Millipore Ltd./Waters Chromatography Division).

E. **Ecdysteroids**

Ecdysone; Simes, Milan, Italy.

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

2-Deoxyecdysone - isolated by Dr. R.E. Isaac.

5α-2-Deoxyecdysone - prepared as outlined in the Methods Section.

2-Deoxyecdysone 22-phosphate - isolated by Dr. R.E. Isaac.

F. **Radiochemicals**

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

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

G. Antiserum

DHS 1-13.5 Antiserum was a gift from Professor J.D. O'Connor, University of California, Los Angeles, California, U.S.A.

H. *Spodoptera littoralis* (Boisd.)

The strain of *S. littoralis* (Lepidoptera) used in this work was obtained from May and Baker Limited, Ongar Research Station, Ongar, Essex.

I. Buffers

MES buffer: 0.2M 2-(N-morpholino)ethane sulphonic acid adjusted to pH 5.8 with dilute sodium hydroxide (Hopkin and Williams, Chadwell Heath, Essex, U.K.).

Borate buffer pH 8.4: 6.18g boric acid, 9.54g sodium tetraborate, 4.31g sodium chloride per litre of water.

Hepes buffer: 0.037M 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid adjusted to pH 7.5 with dilute sodium hydroxide (Sigma).

Isotonic Hepes buffer: 0.037M Hepes buffer containing 0.3M sucrose.

Hypotonic Hepes buffer: 0.037M Hepes buffer containing 0.05M sucrose.

Phosphate buffer: $KHPO_4$ (0.1M) adjusted to pH 7.5 with KH_2PO_4 (0.1M).

Imidazole buffer: 0.02M adjusted to pH 7.5 with sodium hydroxide (Sigma).

Ringers solution: (130mM NaCl, 4.6mM KCl, 1.9mM $CaCl_2$; Bodenstein, 1946).

J. Reagents for Investigation of Ecdysone 20-Monooxygenase

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase from yeast, nicotinamide dinucleotide cofactors, cyclic AMP-dependent protein kinase (rabbit muscle), dibutyryl cyclic AMP, cyclic AMP, alkaline phosphatase [Type III, bacterial (*E. coli*)] and 1,10 phenanthroline from Sigma.

Chymostatin, pepstatin and antipain were from Scientific Marketing Associates, London.

K. Others

Aryl sulphatase enzyme preparation (Type-HI) from *Helix pomatia* was purchased from Sigma.

Tween 20 was purchased from Koch-Light Laboratories.

2. Methods

A. Growth of Insects

Spodoptera littoralis (cotton leafworm) were 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 blue kitchen roll. Coarse netting covered the top, held in place with elastic bands. Approximately 60 pupae of mixed sex were kept in one bin. These emerged and mated in the bin. Adults were fed on a 5% (w/v) sucrose solution which was absorbed onto cotton wool in a petri dish. The females laid eggs in batches on the blue kitchen roll lining or on the underside of the netting. The eggs laid on the underside of the netting were transferred gently with a paint brush to a small plastic container (112x82x42mm). Areas of blue kitchen roll containing eggs were cut out and stapled to the underside of the fine netting covering the small plastic container. The fine netting was held in place by the lid which had a large hole cut from the centre. A narrow strip of diet was placed in the small plastic container on the second day. This was replenished as required when the previous diet was consumed or deteriorated.

When the larvae which hatched from the eggs, reached the 3rd instar they were transferred, with a paint brush, to large plastic containers (10x15x30cm). These contained a bed of vermiculite and were covered with a lid which had three large holes, closed with netting for ventilation. The large plastic container also contained a strip of diet which was replaced every day to prevent fungal growth. The larvae were transferred to fresh large plastic containers twice a week, to prevent infection and to avoid the presence of excess moisture. The larvae were reared in this manner until they neared the end of the 6th and final larval instar. During the sixth instar, the larvae grew rapidly for 3 days and then stopped feeding. At this point the larvae were transferred to a clean large plastic container containing vermiculite and were given no further diet. The larvae decreased in size and weight and after the sixth day of the instar they shed their old cuticle and pupated. After the pupae had tanned and hardened they were transferred to a petri dish. Here they were incubated for 3 days prior to being transferred to the adult cages, thus continuing the life cycle.

The sex of the pupae was determined by examining the rear abdomen. The male pupae have two small swellings corresponding to the male genital aperture.

The small plastic containers were disposable and not used a second time. All other boxes and instruments were washed in a dilute bleach solution (5%) and then rinsed thoroughly with water.

Under these conditions the complete life cycle took approximately 6 weeks.

B. Preparation of Insect Diet

An artificial agar-based diet was prepared as described by McKinley (1970). The following ingredients were mixed thoroughly together, first by hand, then using a mechanical mixer.

Ingredient mix (637.5g)

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

While the ingredients were mixing, agar (75g) was placed in the inner compartment of a double boiler along with 2250ml of glass distilled water and boiled for 30 mins. It was then left to cool to 80°C prior to being mixed with the "ingredient mix". Choline chloride (3.75g) and the "antibiotic plus vitamin mix" (22.5g) were then added to the total mix. This was poured into 5 polythene storage containers and stirred till set.

When the diet had cooled and set, the lids were placed on the containers and the diet stored at 4°C until required. The diet was not stored for more than two weeks before use.

Wesson Salt Mix

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

Vitamin Mix

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

C. Administration of Radioactive Cholesterol

Radioactive cholesterol was administered to the pupae by injection. The [1α,2α-$^3H_2$]cholesterol was purified by t.l.c. prior to injection. The cholesterol was emulsified with ten times its weight of Tween 20 in insect 'Dipteran' Ringer solution. The pupae were injected with 2μl of emulsion ventrally between the 1st and 2nd abdominal segments using a Hamilton 25μl syringe. The syringe needle (gauge 26S) and area to be injected were cleaned with ethanol prior to injection. After injection the wound was covered with molten, low melting point paraffin wax (BDH), which set hard. The chance of infection and losses due to bleeding were thus minimal. Further development of the insects was not affected by this treatment.

D. Extraction of Insects

A typical extraction procedure for the ovaries and eggs of *Spodoptera* is outlined in Fig. 1. The material to be extracted was homogenised using a Potter-Elvehjem homogenizer in 70% (v/v) methanol/water for approximately 3 minutes. The homogenate was then

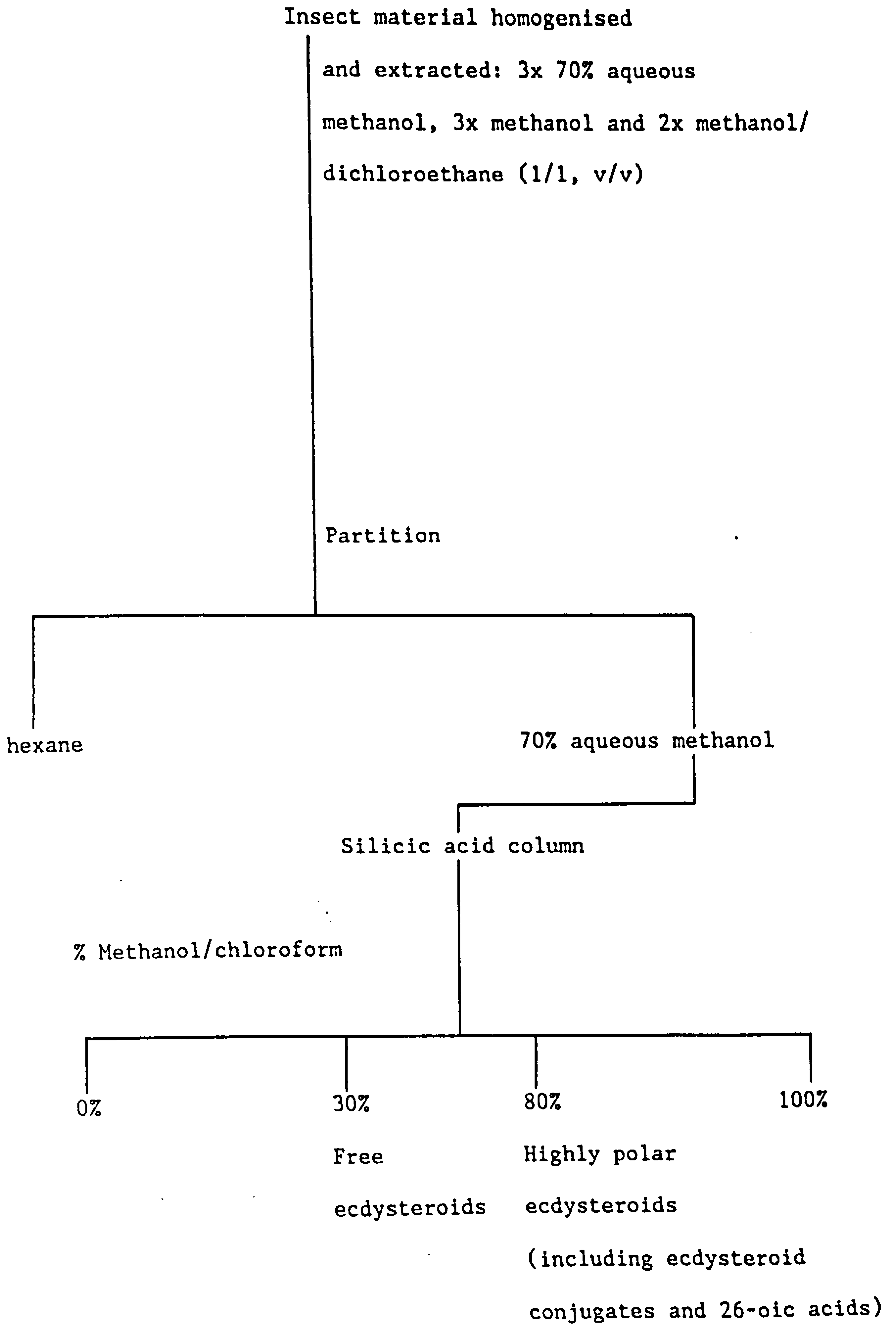


Fig. 1 Summary of a typical ecdysteroid extraction procedure for the ovaries and eggs of Spodoptera littoralis.

centrifuged at 2500xg for 10 min to pellet the residue and the solvent removed. The residue was re-extracted twice with 70% (v/v) methanol/water, three times with methanol and twice with 50% (v/v) methanol/dichloroethane as previously described.

The combined supernatants were evaporated to dryness azeotroping any remaining water with butan-1-ol and the extract partitioned between counter-saturated 70% (v/v) methanol/water and hexane. Both the 70% (v/v) methanol/water and the hexane phase were back extracted once. The 70% (v/v) methanol/water phase contained the ecdysteroids and the hexane phase the sterols. The 70% (v/v) methanol/water phase was evaporated to dryness, any remaining water was azeotroped with butan-1-ol.

E. Separation of Free and Highly Polar Ecdysteroids

The residue from the 70% (v/v) methanol/water phase was redissolved in methanol and evaporated onto Celite (a diatomaceous earth) under vacuum. The dried Celite plus sample was then loaded onto the top of a silicic acid (70-230 mesh, Kieselgel 60 reinst) column prepared as a slurry in chloroform (100mg sample/10g column). The column was developed in a stepwise manner with increasing percentage of methanol in chloroform (Dinan and Rees, 1981).

Eluting Solvent	Compound Eluted
Chloroform	Sterols and apolar compounds
30% (v/v) Methanol/Chloroform	Free ecdysteroids
80% (v/v) Methanol/Chloroform	Highly polar ecdysteroids (including ecdysteroid conjugates and 26-oic acids)
Methanol	Residual polar material

The volume of each fraction was ten times the weight of adsorbant employed.

F. Thin-layer Chromatography (t.l.c.)

For purification and separation of sterols, Kieselgel G t.l.c. plates were prepared by spreading a 1:2 (w/v) mixture of silica gel:water as a slurry over the plates (20cmx20cm; 0.5mm thick). The plates were dried at 110°C for 60 min, allowed to cool, washed by development in diethyl ether and after air drying were reheated as before.

After application of samples, plates were developed twice with chloroform in tanks lined with chromatography paper. The plates were dried and sprayed with a solution of 1% (w/v) berberine sulphate in methanol:acetone (1:1, v/v) followed by examination under U.V. light (254nm). In the purification of radioactive cholesterol, the band of

radioactivity which co-migrated with the authentic marker was carefully removed. The radioactive cholesterol was eluted from the silica with dry redistilled diethyl ether using a scintered funnel.

G. High-performance Liquid Chromatography (h.p.l.c.)

A Waters Associate high-performance liquid chromatography system was used, incorporating a single model 510 pump for isocratic elution, and two pumps in conjunction with a gradient controller (model 680) for gradient elution. The samples were introduced onto the column via a model U6K injector. A model 440 or 441 U.V. detector was used.

Two types of reversed-phase columns were employed: a µBondapak C_{18} Radial-Pak cartridge (particle size 10µm; 10cm long x 8mm internal diameter; Waters Associates) and a Nova-pak C_{18} Radial-Pak cartridge (particle size 4µm, 10cm long x 8mm internal diameter; Waters Associates). Adsorption chromatography employed a µBondapak NH_2 Radial-Pak cartridge (particle size 10µm; 10cm long x 8mm internal diameter; Waters Associates). The Radial-Pak cartridges were used with a Z-module Radial Compression module (Waters). For both reversed-phase and adsorption chromatography a Waters precolumn module was employed with small 'RCSS guard pak CN' cartridges.

For the monitoring of radioactive fractions, 1ml fractions were collected. When aqueous methanol was used as eluent, scintillant (4ml; Scintran Cocktail T, BDH) was added directly. When the eluent

contained any chlorinated solvents, the solvent was evaporated, using a Gyro-vap centrifugal vacuum evaporator (Uniscience, London) before the addition of scintillant to avoid excessive quenching.

H. Enzymic Hydrolysis

The polar ecdysteroid conjugate fraction from silicic acid column chromatography was occasionally hydrolysed by *Helix pomatia* hydrolases prior to further analysis. The biological sample was evaporated to dryness under nitrogen and dissolved in 0.2M MES buffer pH 5.4 (1ml). To this, a crude so called arylsulphatase preparation from *Helix pomatia* (1mg) dissolved in MES buffer (1ml) was added and incubated at $37^{o}C$ for 13hr. The reaction was terminated by the addition of chilled ethanol and kept at $4^{o}C$ for 30 min. to precipitate the proteins. The suspension was centrifuged at 2,500xg for 5 min., the residue re-extracted twice with ethanol, and the combined supernatants evaporated to dryness under vacuum. For each enzymic hydrolysis, a control incubation consisting of enzyme and buffer only was carried out. Generally, the samples were further purified by chromatography on C_{18} Sep-pak cartridges.

I. C_{18} Sep-Pak Cartridges

For the further purification or desalting of samples prior to h.p.l.c., radioimmunoassay, mass spectrometry, or nuclear magnetic resonance spectrometry, C_{18} Sep-pak cartridges (Millipore) were often

used. The procedure for using the cartridges in the purification of ecdysteroids (Greenwood, 1981) is summarised below.

The cartridge was first 'activated' by washing with methanol (4ml) which was then removed with water (10ml). The sample to be purified was dissolved in 10% (v/v) methanol in water (2x1ml) and loaded onto the cartridge. 10% (v/v) Methanol in water (4ml) was then passed through the cartridge to remove any salts or proteins. Conjugates and polar material were eluted with 30% (v/v) methanol in water (4ml) and the "free" ecdysteroids eluted with 60% (v/v) methanol in water (5ml). The cartridge was then eluted with methanol(4ml) and acetonitrile (3ml) to remove less polar material. The fractions were collected and evaporated to dryness under vacuum prior to further purification or analysis.

J. Radioimmunoassay of Ecdysteroids

(i) Antiserum

A single antiserum has been used during the course of this research, produced by the administration of a hapten-thyroglobulin conjugate to rabbits.

DHS 1-13.5 (Soumoff *et al.*, 1981) 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 than the nucleus. The optimal dilution for this antiserum was found to be 1 in 1500 giving approximately 50% binding of the [$^{3}H_{2}$]ecdysone employed.

(ii) **Assay Procedure**

A series of micro-centrifuge tubes (Sarstedt) were set up, each containing increasing amounts of ecdysone (in duplicate) ranging from 0-1000pg. This constituted the standard curve. The concentration of a stock solution of ecdysone in methanol was accurately determined spectrophotometrically at 254nm (E_{254} = 12,400 for ecdysone in methanol). A second set of tubes was used for aliquots of the biological sample, and the solvent was removed using a Gyro-vap centrifugal vacuum evaporator. Both the standard curve and biological samples were treated in the same way from this point onwards. [23,24-$^{3}H_{2}$]ecdysone (100µl) in borate buffer (0.1M, pH 8.4) was added to each tube and vortex mixed. Each tube contained an exact amount of radioactivity (approximately 4500cpm/tube). Antiserum (100µl) diluted with 5% (v/v) inactivated rabbit serum (Wellcome Research Laboratories, Beckenham, Kent, U.K.) in borate buffer was added to each tube. After gentle vortex mixing, the tubes were incubated for 18 hours at 4°C together with a control tube containing only [23,24-$^{3}H_{2}$]ecdysone (100µl).

After the incubation, saturated ammonium sulphate solution in borate buffer (200µl) was added to each tube and vortex mixed. These were allowed to stand for 20 min. at 4°C to precipitate the protein. The tubes were then centrifuged at 10,000g for 7 min. at 4°C in a 'Koolspin' refrigerated centrifuge (Burkard Scientific, Middlesex, U.K.) and the supernatant discarded. The pellet was then washed with a solution of 50% saturated ammonium sulphate in borate buffer

(500μl). These were allowed to stand for a further 20 min. at 4°C to re-precipitate the protein. The tubes were again centrifuged and the supernatant removed. The pellets were resuspended in water (125μl) and Aquasol scintillant (NEN; 1ml) was added. The tubes were vortex mixed and placed in stoppered 20ml glass scintillation vials for radioassay.

(iii) Analysis of Results

The results of the radioassays represent the amount of [23,24-$^3H_2$]ecdysone bound to the antiserum (expressed in c.p.m.). The results from the ecdysone standard tubes are then collated to produce a standard curve which can be expressed graphically by plotting the percentage of radioactive ecdysone bound to the antiserum relative to the "blank" tubes containing no standard ecdysone (ordinate) against the amount of ecdysone, plotted on a log scale. The results from the biological samples can then be calculated, in pg ecdysone equivalents, by reading from the standard curve. The concentrations of biological samples were chosen to correspond to the most accurate region of the standard curve.

K. Mass Spectrometry

Mass spectra were obtained using a VG Micromass 7070H mass spectrometer linked to a Finnigan INCOS 2300 data system operated by Mr. M. Prescott and Dr. R.P. Evershed of the Biochemistry Department, University of Liverpool. Negative-ion fast atom bombardment (f.a.b.)

mass spectra were determined with the spectrometer incorporating an adapted saddle-field source atom gun (Ion Tech Ltd., Teddington, Middlesex, U.K.) operated at 8-9kV and a tube current of 1-2mA, using a primary atom beam of xenon (99.98%, BDH). The mass spectrometer was operated at low resolution (about 1000), at an accelerating voltage of 3kV (scanning m/z 40-900). Samples were dissolved in methanol for transfer to the matrix (glycerol) on the f.a.b. probe tip, excess methanol being removed using a gentle stream of warm air before f.a.b.

L. N.M.R. Spectroscopy

Fourier-transform ^{1}H-n.m.r. spectra were recorded on a Bruker 400MHz instrument (Science and Engineering Research Council, High Field NMR Service, Department of Chemistry, University of Sheffield, U.K.). Samples were dissolved in deuterated methanol and spectra were calibrated with respect to tetramethylsilane.

M. Preparation of 5α-2-Deoxyecdysone

2-Deoxyecdysone naturally occurs as the 5β-compound. The 5α-2-deoxyecdysone was synthesized as an h.p.l.c. marker from 2-deoxyecdysone.

The 5β-2-deoxyecdysone (0.2mg) was dissolved in 1% (w/v) potassium carbonate in 90% aqueous methanol. The solution was incubated at 50°C in a heating block for 6 hours (Mori et al., 1968). The resulting mixture of 5α- and 5β-2-deoxyecdysone was purified by C_{18} Sep-pak and reversed-phase h.p.l.c. The h.p.l.c. system employed was a Nova-pak C_{18} Radial-Pak cartridge eluted at 1ml min^{-1} with a 45 minute linear gradient of methanol in water changing from 50:50 (v/v) to 70:30 (v/v), which gave retention times of 23 minutes and 28 minutes, respectively, for the authentic 5β- and 5α-2-deoxyecdysone.

N. Silylation of Glassware

All glassware was silylated once prior to use. Glassware was rinsed with dimethyldichlorosilane solution (about 2% (v/v) in 1,1,1-trichloroethane, BDH) and baked at 100°C for one hour. The glassware was then rinsed with hexane and baked as before, prior to a final rinse with distilled water. The glassware was dried, ready for use. Glassware was resilylated approximately every eight months.

O. Liquid Scintillation Counting

Radioactive samples were assayed using a Beckman LS9800 or an LKB-Wallac 1219 Spectra Beta scintillation counter. The scintillant used was either Scintran Cocktail T (BDH) for general use or Aquasol (NEN) for radioimmunoassay.

P. Ecdysone 20-Monooxygenase Assays

(i) Preparation of Subcellular Fractions

Fat body from larvae 88 hours into the 6th instar, which lasts 6 days under our conditions, was used, since that time corresponds to high ecdysone 20-monooxygenase activity in that tissue.

For investigation of the subcellular location of the monooxygenase fat body (approximately 115mg/insect) from 14 insects was dissected in ice-cold isotonic Hepes buffer containing 0.1M potassium fluoride and homogenized using a Potter-Elvehjem homogenizer in the same buffer (2.4ml). Fluoride was included to inhibit protein phosphatase activity and hence, keep the 20-monooxygenase in the phosphorylated state, the apparent active form. A portion (0.4ml) of the homogenate was kept for enzyme assays and the remainder (2.0ml) subjected to differential centrifugation as follows, each pellet being resuspended in 1.5ml of isotonic Hepes buffer containing 0.1M potassium fluoride. The homogenate was centrifuged at 600g for 5 minutes in a Koolspin refrigerated centrifuge (Burkard Scientific) (Cell debris/nuclear fraction; N) and the supernatant centrifuged at 8800g for 15 minutes in a Micro Centaur centrifuge (MSE). The pellet was resuspended (mitochondrial fraction; M) and the supernatant centrifuged at 100,000g for 90 minutes in a Sorvall OTD-65 ultracentrifuge (Dupont Instruments). The resuspended pellet constituted the microsomal fraction (P) and the supernatant the cytosol fraction (S). All the foregoing operations were carried out at 0-4°C.

For the routine preparation of intact fat body tissue, fat body mitochondrial fraction and fat body microsomal fraction, the fat body was dissected in ice-cold isotonic Hepes buffer unless otherwise stated.

For the routine preparation of intact fat body, the tissue was dissected washed with ice-cold isotonic Hepes buffer and blotted dry with filter paper. The fat body was weighed, divided into suitable portions and resuspended in relevant buffer.

For the routine preparation of fat body mitochondrial fraction, the post - 600g supernatant was centrifuged at 8800g for 15 minutes at 4°C and the pellet was washed with isotonic Hepes buffer and re-centrifuged. The mitochondrial fraction was then resuspended in either isotonic Hepes buffer to give intact mitochondria or hypotonic Hepes buffer to give broken mitochondria.

For routine preparation of fat body microsomal fraction, the post - 8800g supernatant (prepared with a 10 min. centrifugation at 4°C) was centrifuged at 100,000g for 90 minutes at 4°C. The microsomal fraction was then resuspended in relevant buffer.

For routine preparation of combined fat body mitochondria and microsomal fraction, the post - 600g supernatant was centrifuged at 100,000g for 90 minutes and the pellet resuspended in hypotonic Hepes buffer to burst the mitochondria. All the foregoing tissue/sub-cellular preparations were subjected to one or more preincubations prior to assay of ecdysone 20-monooxygenase activity.

(ii) Ecdysone 20-monooxygenase Assay

The standard assay for ecdysone 20-monooxygenase activity contained the following in Hepes buffer ("isotonic" in experiments where the mitochondria were intact, but "hypotonic" when broken mitochondria were used); total volume 300µl: [$^3H_2$]ecdysone (0.25µCi; 4.22pmol), NADPH (9.68µmol) $NADP^+$ (0.74µmol), glucose-6-phosphate (9.5µmol), glucose-6-phosphate dehydrogenase (0.6IU) [all in total volume, 100µl), the enzyme preparation to be assayed (50 or 100µl) and additional buffer (150 or 100µl). After incubation at 37°C for 30 min, the reaction was stopped by the addition of chilled ethanol (1ml) and small amounts (approximately 4ng each) of unlabelled ecdysone and 20-hydroxyecdysone added. The mixtures were placed in the freezer for 30 minutes to precipitate out protein and then centrifuged for 10 min at 8,800g in a Micro Centaur centrifuge at 4°C. the supernatant was removed and evaporated to dryness under vacuum in a Gyro-vap centrifugal vacuum evaporator. The residue was redissolved in methanol:water (1:1, v/v; 100µl) and 50µl subjected to h.p.l.c. on a radial compression module, Nova-pak C_{18} Radial-Pak cartridge eluted at a flow rate of 1ml/min with a linear gradient (30 min) of methanol in water changing from 7:13 (v/v) to 4:1 (v/v). Fractions were collected every minute and following the addition of Scintran Cocktail T (4ml) scintillation fluid were radioassayed. In all the incubations 20-hydroxyecdysone. was the only detectable product, and the radioactivity detected in it was expressed as a percentage of the total recovered in ecdysone and 20-hydroxyecdysone.

For determination of ecdysone 20-monooxygenase activity during investigation of the subcellular location of this enzyme system, the standard assay conditions were modified such that the total volume of the mixture was 200μl: enzyme preparation to be assayed (100μl) and [$^3H_2$]ecdysone substrate plus cofactors and NADPH-generating system (100μl).

In all experiments, the recovery of the initial radioactivity was generally greater than 90% and all incubations were carried out in duplicate on a single tissue preparation. The results given are presented as the mean unless stated and in each case the range of results for duplicate incubations was less than 10%.

Q. Protein Estimation - the Tannin Micromethod for Protein Determination. (Mejbaum - Katzenellenbogen and Dobryszycka, 1959)

(i) Reagents

Tannin: 2ml of phenol was mixed with 1M HCl (98ml) at 80°C, into which 10g of tannin (Sigma) was dissolved. The solution was stored in a brown bottle away from sunlight. Gum Arabic: 0.1g gum arabic (Acacia; Sigma) was dissolved in warm water (100ml). Standard protein solution: Bovine serum albumin was dissolved in 0.9% NaCl solution to give a stock solution (100μg/ml).

(ii) **Procedure**

The protein solution in 0.9% aqueous NaCl (0.25ml) was placed in a 5ml test tube and warmed in a water bath at 30°C for 5 minutes. The tannin reagent (0.25ml) (pre-warmed at 30°C) was then added, the solution vortex-mixed and the mixture incubated at 30°C for 10 minutes. Gum arabic solution (2ml) was then added and the solution vortex mixed. The mixture was then cooled to room temperature and the optical density (650nm) 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 the protein content of the biological samples could be determined.

R. **Abbreviations**

t.l.c.	-	thin-layer chromatography
h.p.l.c.	-	high-performance liquid chromatography
R.I.A.	-	radioimmunoassay
cyclic AMP	-	cyclic adenosine monophosphate
ATP	-	adenosine triphosphate
ppm	-	parts per million
chol	-	cholesterol
2dE	-	2-deoxyecdysone
2dE 22P	-	2-deoxyecdysone 22-phosphate

u.v.	-	ultra violet
f.a.b.	-	fast atom bombardment
p.m.r.	-	proton magnetic resonance
n.m.r.	-	nuclear magnetic resonance

CHAPTER THREE

SOME PROPERTIES OF ECDYSONE 20-MONOOXYGENASE

1. Introduction

Ecdysone 20-monooxygenase occurs primarily, based on studies with the last-instar larvae of *Calliphora vicina*, *Manduca sexta*, *Locusta migratoria* and *Schistocerca gregaria*, in the fat body, Malpighian tubules, midgut, integument and possibly the gonads (Smith, 1985). In *Spodoptera littoralis*, ecdysone 20-monooxygenase occurs in the fat body and Malpighian tubules, and to a lesser extent in the gut (Milner, 1984; Zimowska *et al*., 1985). It is also fairly clear that other tissues do not possess ecdysone 20-monooxygenase activity, including the prothorasic glands, muscle, ventral nerve cord and imaginal discs, but at present due to conflicting data, it remains to be established whether other tissues such as, salivary glands, foregut, hindgut and oenocytes possess ecdysone 20-monooxygenase activity, and if so, to what extent (Smith, 1985).

This enzyme system has been reported to be either largely mitochondrial or microsomal or to have dual location in both subcellular fractions (Weirich *et al*., 1984). The specific activity of ecdysone 20-monooxygenase in various tissues of locusts (Johnson and Rees, 1977b; Feyereisen and Durst, 1980) and tobacco hornworm (Nigg *et al*., 1976; Smith *et al*., 1983) undergoes developmental variation during the final larval instar and exhibits a distinct peak. In *Spodoptera littoralis* the peak of monooxygenase activity occurs approximately 88hr. into the sixth final larval instar (Fig. 1) under our culture conditions (Milner, 1984).

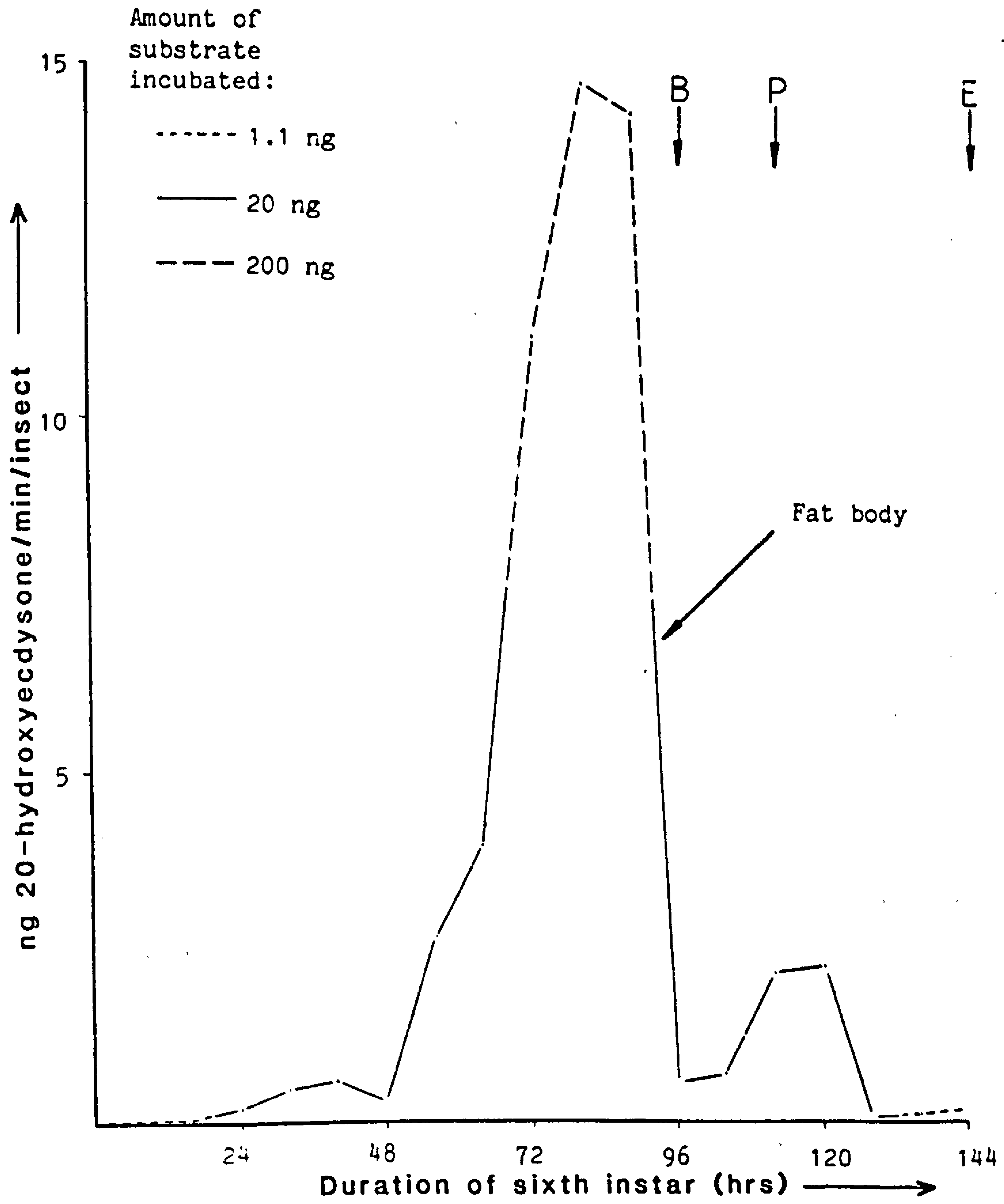


Fig 1. Titre of ecdysone 20-monooxygenase activity in fat body homogenate during the sixth larval instar of S. littoralis,

(Milner, 1984)

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

A potential mechanism for the rapid modulation of ecdysone 20-monooxygenase is the reversible phosphorylation of the enzyme system. This is discussed in more depth in the following chapters.

This chapter concerns the subcellular localization of ecdysone 20-monooxygenase in the fat body of 6th instar Spodoptera littoralis and the effect of possible modulators of phosphorylation-dephosphorylation on fat body preparations. The modulators of phosphorylation-dephosphorylation examined include choleratoxin, forskolin and A23187 (a Ca^{2+} ionophore). The activity of the G stimulatory-GTP complex is ended by the hydrolysis of GTP to GDP, which is catalysed by the enzyme GTPase. The activity of GTPase is inhibited, by the toxin produced by the cholera bacillus, choleratoxin. The toxin thereby prolongs the life of the G stimulatory-GTP complex, so that the cell produces cyclic AMP continually, even in the absence of an external signal (Berridge, 1985). Forskolin is an organic molecule isolated from roots of the Indian herb Coleus forskohlii. Forskolin activates adenylate cyclase which catalyses the conversion of ATP to cyclic AMP (Berridge, 1985). Calcium ionophores are molecules that shield the electric charge of a calcium ion and thus make it permeable to the cell membrane. Calcium ionophores in this way mimic the action of inositol trisphosphate by introducing free calcium into the cell (Berridge, 1985).

2. Experimental and Results

A. Subcellular localization of ecdysone 20-monooxygenase in the fat body of Spodoptera littoralis

The subcellular location of ecdysone 20-monooxygenase in the fat body of Spodoptera was first examined by differential centrifugaton of a homogenate. The ecdysone 20-monooxygenase activity was determined in individual fractions and was compared with the profiles of (i) citrate synthase (E.C. 4.1.3.7); (ii) NADPH-cytochrome P-450 (c) reductase (E.C. 1.6.2.4) and (iii) pyruvate kinase (E.C. 2.7.1.40). Enzymes (i), (ii) and (iii) can be used as marker enzymes for mitochondrial, microsomal and cytosolic fractions, respectively.

Citrate synthase was assayed by following the rate of change in extinction at 412nm (Alp et al., 1976) in a Cecil recording spectrophotometer (model CE599) at 25°C. The assay medium (pH 7.5; final volume 2ml) for citrate synthase consisted of 50mM Tris-HCl, 0.2mM 5,5'-dithiobis-(2-nitrobenzoic acid), 0.1mM acetyl-CoA, 0.5mM oxaloacetate and suitably diluted homogenate (100μl). The assay was initiated by the addition of the oxaloacetate.

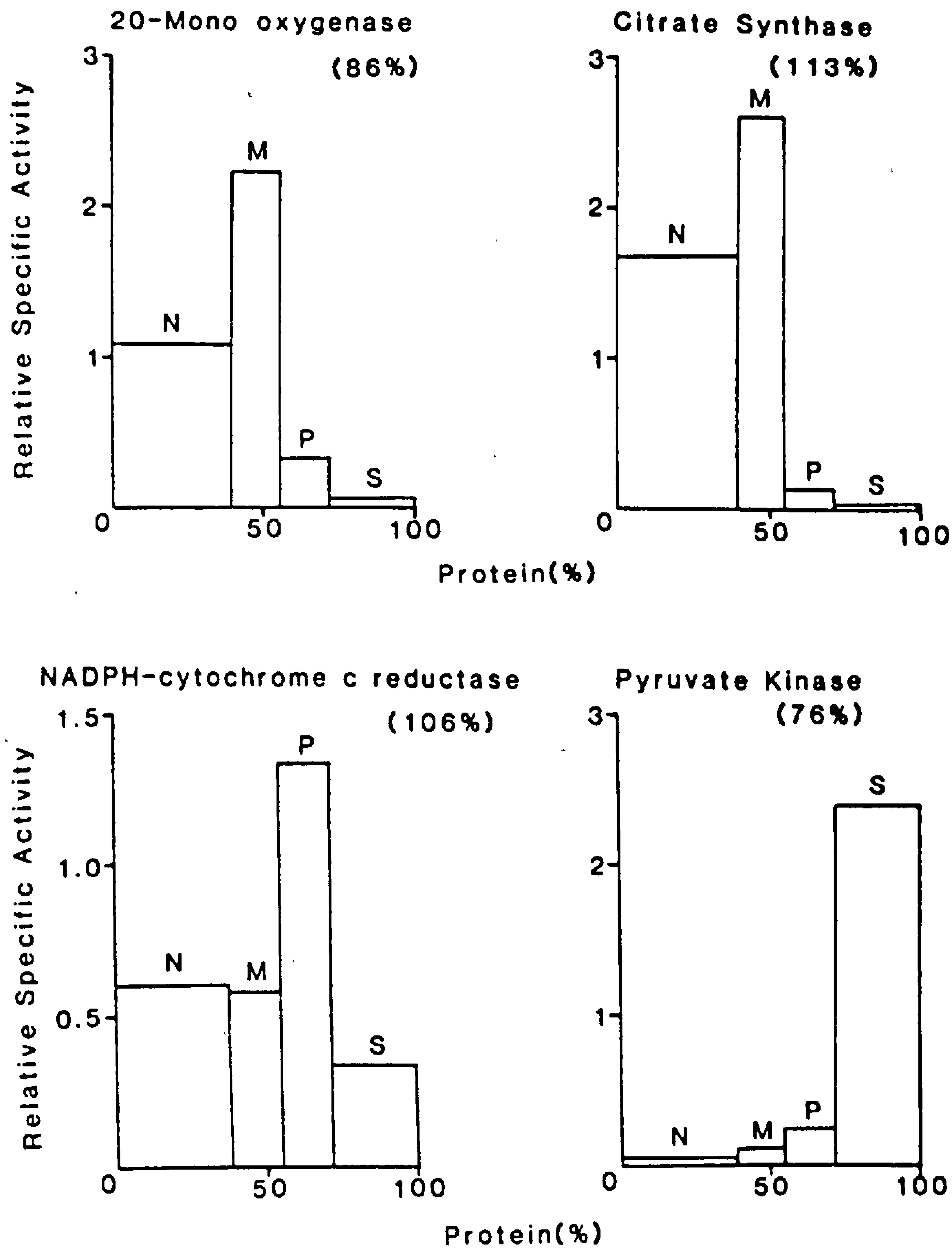
Pyruvate kinase was assayed by following the rate of change of extinction at 340nm (Zammit et al., 1978) in the same spectrophotometer at 25°C. The assay medium (pH 7.35; final volume 2ml) for pyruvate kinase consisted of 160mM triethanolamine hydrochloride, 10mM $MgCl_2$, 80mM KCl, 0.17mM NADH, 5mM ADP, 2mM phosphoenolpyruvate, 25μg of lactate dehydrogenase and suitably diluted homogenate (100μl). The assay was initiated by the addition of the phosphoenolpyruvate.

NADPH-cytochrome c reductase was assayed by following the rate of change of extinction coefficient at 550nm (Greenwood and Rees, 1984) in the same spectrophotometer at 25°C. The assay medium (pH 7.35; final volume 2ml) for NADPH-cytochrome c reductase consisted of 50µM cytochrome c, 0.33mM neutralized KCN, 1.0mM EDTA, 48mM potassium phosphate, 0.1mM NADPH and 0.33mM Tris buffer and suitably diluted homogenate (100µl). The assay was initiated by the addition of the NADPH.

In all the assays, the results for the control incubations without oxaloacetate, phosphoenolpyruvate and NADPH, respectively, were subtracted from the actual individual reaction values. The reaction in each case was linear during the period of assay. The results are expressed (Fig. 2) as the relative specific activity plotted against the protein in each fraction expressed as a percentage of the total protein present in all fractions. The relative specific activity is obtained by dividing the enzymic activity in the fraction (expressed as percentage of the total activity in all fractions) by the percentage of protein in that fraction.

A considerable proportion of the total activities of the ecdysone 20-monooxygenase, citrate synthase, and NADPH-cytochrome c reductase [NADPH-cytochrome P-450 (c) reductase] was present in the cell debris/nuclear fraction (N), presumably owing to incomplete homogenization of the tissue. The apparent low activity of pyruvate kinase in that fraction may be due to lack of permeability of components of the coupled enzyme assay. The majority of the total

Fig. 2 Subcellular location of ecdysone 20-monooxygenase activity



Fractions: N-cell debris/nuclear (600g pellet); M-mitochondrial (8800g pellet); P-microsomal (100,000g pellet); S-cell sap (100,000g supernatant).

The values in parentheses indicate the percentage recoveries of the summed enzymic activities in the fractions relative to that in the initial homogenate.

ecdysone 20-monooxygenase activity in the post-cell debris/nuclear fraction was present in the mitochondrial fraction, in agreement with the citrate synthase fractionation profile and quite distinct from the fractionation of "microsomal" membranes as assayed by NADPH-cytochrome c reductase. However, a small proportion of ecdysone 20-monooxygenase activity also occurs in the microsomal fraction and apparently cannot be accounted for merely by mitochondrial contamination, as reflected by the citrate synthase profile. The presence of NADPH-cytochrome c reductase activity in the cell sap fraction(S) probably indicated insufficient centrifugal force to sediment the entire "microsomal" membrane population (Morello et al., 1971).

B. Modulation of ecdysone 20-monooxygenase activity in intact tissue

Fat body tissue was prepared in isotonic Hepes buffer and divided into eight fractions (8 x 0.08g). Each fraction was resuspended in isotonic Hepes buffer (500μl) and preincubated for 1hr. at 37°C, unless otherwise stated, after the addition (Table 1) of the following: (i) 0.35M KF in isotonic Hepes buffer (100μl) at 4°C, (ii) 0.35M KF in isotonic Hepes buffer (100μl), (iii) 0.35M KCl in isotonic Hepes buffer (100μl) at 4°C and (iv) 0.35M KCl in isotonic Hepes buffer (100μl) [each preparation was preincubated in duplicate]. The whole preparations were then assayed for ecdysone 20-monooxygenase activity.

Fluoride is a known inhibitor of endogenous phosphoprotein phosphatases (Hauschildt, 1986). The activity of ecdysone

Table 1 **Effect of the presence of fluoride during preincubation on the activity of ecdysone 20-monooxygenase**

Preincubation conditions (1hr.)	Monooxygenase activity (pmole/hr. per mg protein)
(i) KF ($4^{\circ}C$)	1.77
(ii) KF	1.18
(iii) KCl ($4^{\circ}C$)	0.76
(iv) KCl	0.60

20-monooxygenase was 2-fold greater when KF rather than KCl was present in the preincubation [incubations (ii) and (iv), Table 1] presumably by inhibition of the decrease in monooxygenase activity by endogenous phosphoprotein phosphatases by fluoride. The results for the control incubations [(i) and (iii), Table 1] indicated that the action of fluoride is not simply on the monooxygenase assay. A much reduced effect of fluoride is also observed when the preincubation was carried out at 37° compared with 4°C [incubations (i) and (ii), Table 1], suggesting possibly, that the fluoride is not completely inhibiting the relevant phosphoprotein phosphatase or that there is greater denaturation of the enzyme at 37°C.

In addition to fluoride, the effect on intact tissue of other modulators of protein phosphorylation-dephosphorylation were investigated during the preincubation. Neither forskolin, choleratoxin, A23187 (a Ca^{2+} ionophore), dibutyryl cyclic AMP, dibutyryl cyclic GMP or 1-oleoyl 2-acetyl glycerol (a diacyl glycerol) evoked a significant increase in monooxygenase activity, when present during the preincubation.

3. Discussion

The specific activity of ecdysone 20-monooxygenase, in various tissues of Spodoptera littoralis undergoes developmental variation in the final larval instar and exhibits a distinct peak (Fig. 1). The differences observed in the specific activity of the ecdysone 20-monooxgyenase between some experiments, in this and the following

two chapters, may be explained by variation in the exact stage of development, coupled with the major changes in activity of the monooxygenase during the final larval instar.

The subcellular location of ecdysone 20-monooxygenase in the fat body of Spodoptera littoralis shows that with the exception of the post-cell debris/nuclear fraction, the majority of the monooxygenase activity is in the mitochondrial fraction, with a small, but significant, proportion of the enzymic activity in the microsomal fraction. The possibility that the activity of the microsomal and mitochondrial ecdysone 20-monooxygeanse can be modulated by changes in its phosphorylation state is examined in chapters four and five, respectively.

The effect of fluoride and other phosphorylation-dephosphorylation modulators was investigated on fat body tissue. Since fluoride is a known inhibitor of phosphoprotein phosphatases (Beg and Brewer, 1981), the observation that ecdysone 20-monooxygenase activity is much higher following preincubation of fat body tissue with KF than with KCl [Table 1], suggests that in the absence of fluoride, endogenous phosphoprotein phosphatase partially dephosphorylates the monooxygenase leading to reduced activity. Other promotors, A23187 (a Ca^{2+} ionophore), 1-oleoyl 2-acetyl glycerol (a diacyl glycerol), dibutyryl cyclic AMP, dibutyryl cyclic GMP and choleratoxin, which are known to stimulate artificially the intracellular protein kinase system, had no significant effect when present in the preincubation. As demonstrated earlier the majority of

the ecdysone 20-monooxygenase activity in the fat body tissue is in the mitochondria, in which cyclic AMP and cyclic GMP would not be expected to have an effect on enzymic activity. The same applies to the promotors of cyclic AMP, such as choleratoxin and forskolin. Thus, the negative effect of these promotors on fat body tissue is not surprising in view of the small proportion of microsomal ecdysone 20-monooxygenase activity. However, the Ca^{2+} ionophore and diacyl glycerol would increase the intracellular calcium concentration which may have an indirect effect on the mitochondrial calcium level. Fluoride will readily penetrate the cell and mitochondrial membrane, exerting a direct effect on the endogenous phosphoprotein phosphatase. The exact significance of these data is uncertain and must await more definitive evidence.

CHAPTER FOUR

MODULATION OF ECDYSONE 20-MONOOXYGENASE IN THE MICROSOMAL FRACTION

1. Introduction

The activity of ecdysone 20-monooxygenase in the fat body of Spodoptera littoralis undergoes developmental variation during the final, 6th, larval instar and exhibits a distinct peak. Possible regulatory mechanisms for these long-term changes in enzymic activity have been previously considered (Chapter 1). In addition, a potential mechanism for the rapid modulation of ecdysone 20-monooxygenase activity is the reversible phosphorylation of the enzyme system. Cyclic AMP-, cyclic GMP-, and cyclic AMP/cyclic GMP-dependent protein kinases have been reported in insects (Smith, 1985). In addition, Ca^{2+}-calmodulin-dependent protein kinases and phospholipid-dependent protein kinase C have been identified (Kuo et al., 1980; Combest and Gilbert, 1986).

In the fat body of Spodoptera, the majority of the ecdysone 20-monooxygenase activity occurs in the mitochondria, with a small amount associated with the microsomal fraction (see Chapter 3). The microsomal ecdysone 20-monooxygenase was investigated initially rather than the mitochondrial monooxygenase. This is because many microsomal and soluble enzymes have been demonstrated to be regulated by covalent modulation, whereas such modulation has only been recognised, albeit incompletely elucidated, in two mitochondrial enzyme complexes.

Evidence is now presented for reversible activation-inactivation of the more experimentally amenable microsomal ecdysone 20-monooxygenase, consistent with changes in its extent of

phosphorylation. The possibility that the activity of the microsomal monooxygenase can be modulated by changes in its phosphorylation state was examined by subjecting the microsomal fraction to various preincubation conditions before assay of the enzymic activity.

2. Experimental and Results

A. Purity of the microsomal sub-cellular fraction

In the routine preparation of the microsomal sub-cellular fraction, the mitochondrial sub-cellular fraction was centrifuged at 8,800g for 10 min. compared with 15 min. in the preparation of the microsomes in the investigation of the sub-cellular localization of ecdysone 20-monooxygenase (Chapter 3). In this experiment the purity of the routinely prepared microsomal sub-cellular fraction was determined by estimating the citrate synthase and ecdysone 20-monooxygenase activities, because of the 5 min. difference in centrifugation time in the two procedures.

The fat body was homogenized in 0.15M KF in isotonic Hepes buffer (2.5ml) and divided into two fractions (2x1ml). Each fraction was then centrifuged at 8,800g for 10 and 15 min., respectively. The microsomal fractions were then prepared by centrifugation (2x0.7ml; 100,000g for 90 min.) and resuspended in 150mM KF in isotonic Hepes buffer (0.5ml). Aliquots of the microsomal fractions (100μl and 50μl, respectively) were then assayed for ecdysone 20-monooxygenase and citrate synthase activities [Table 1]. Citrate synthase is a marker enzyme for the mitochondrial fraction and was assayed as described by Alp *et al.*, (1976).

Table 1: Purity of the microsomal sub-cellular fraction

Time (min.) of centrifugation at 8,800g	Citrate synthase activity (μmoles/min.)	Ecdysone 20-mono-oxygenase activity (pmol/hr. per mg protein)
(i) 10	20.6	8.43
(ii) 15	20.2	8.06

The differing centrifugation times caused an apparent 2% variation in citrate synthase activity and a 4% variation in ecdysone 20-monooxygenase activity [Table 1]. These results indicate no great variation in either mitochondrial contamination or ecdysone 20-monooxygeanse activity of the microsomal fraction, due to the 5 min. difference in centrifugation time, during preparation of the mitochondrial sub-fraction.

B. Linearity of the ecdysone 20-monooxygenase assay with time

The fat body microsomal fraction was prepared and resuspended in 300mM KF in Hepes buffer. Aliquots (50μl) were assayed for ecdysone 20-monooxygenase activity after the addition of Hepes buffer (150μl), with assay incubation times of 0, 10, 20, 30, 40 and 50 min. The results (Fig. 1) show that the ecdysone 20-monooxygenase assay is linear up to an assay incubation time of at least 50 minutes, which is well beyond the 30 minute incubation period for the monooxygenase assay.

C. Linearity of the ecdysone 20-monooxygenase assay with increasing amounts of microsomal protein

The fat body microsomal fraction was prepared and resuspended in 150mM KF in Hepes buffer. Aliquots (100, 80, 60, 50, 40 and 20μl) of the resuspended microsomal fraction were diluted with 150mM KF in Hepes buffer to give a total volume of 100μl in each. Following the further addition of Hepes buffer (100μl) the preparations were assayed for ecdysone 20-monooxygenase activity. The results (Fig. 2) show that the ecdysone 20-monooxygenase activity is linear with increasing concentrations of microsomal protein.

Fig 1 Linearity of the mitochondrial ecdysone 20-monooxygenase assay with time

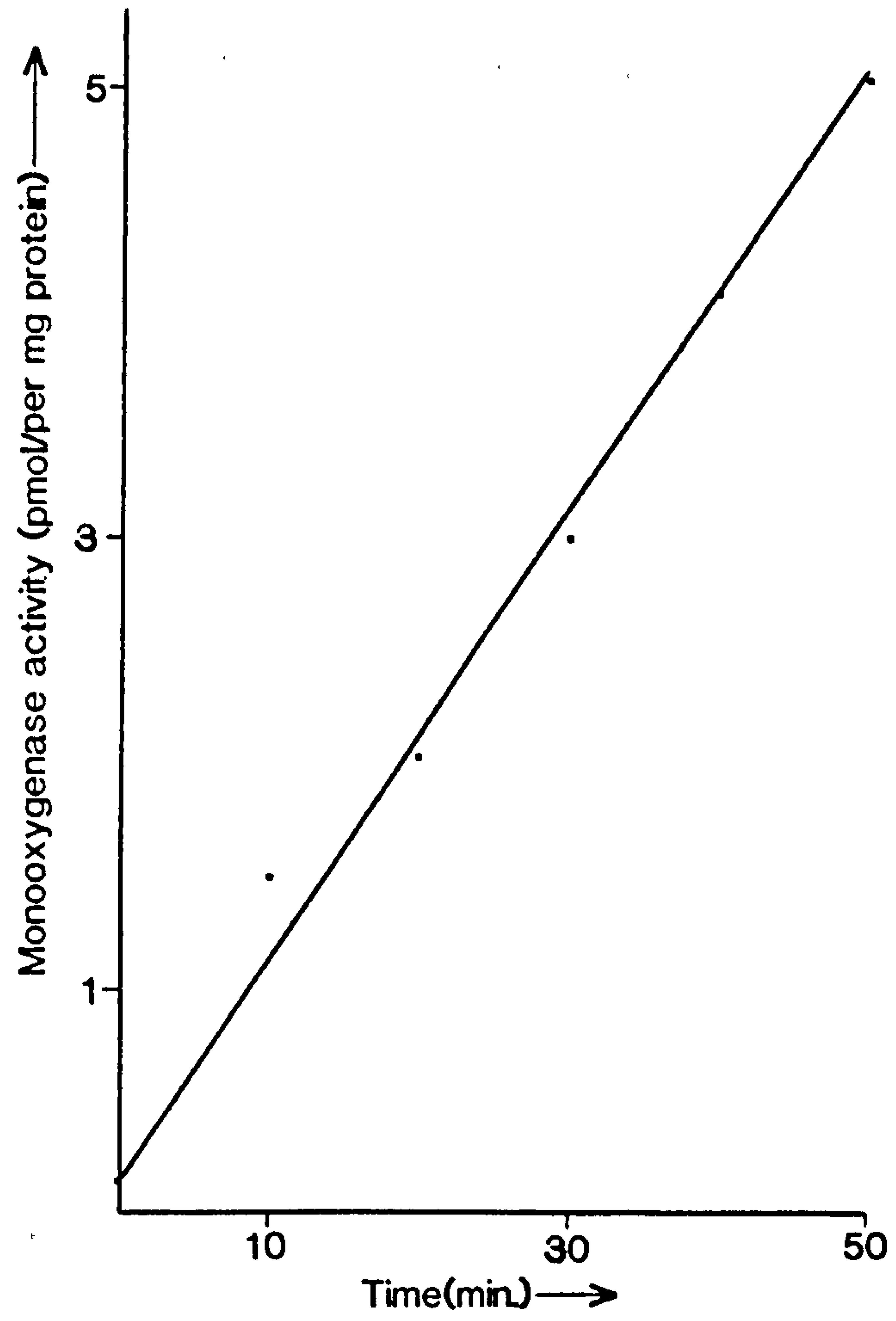
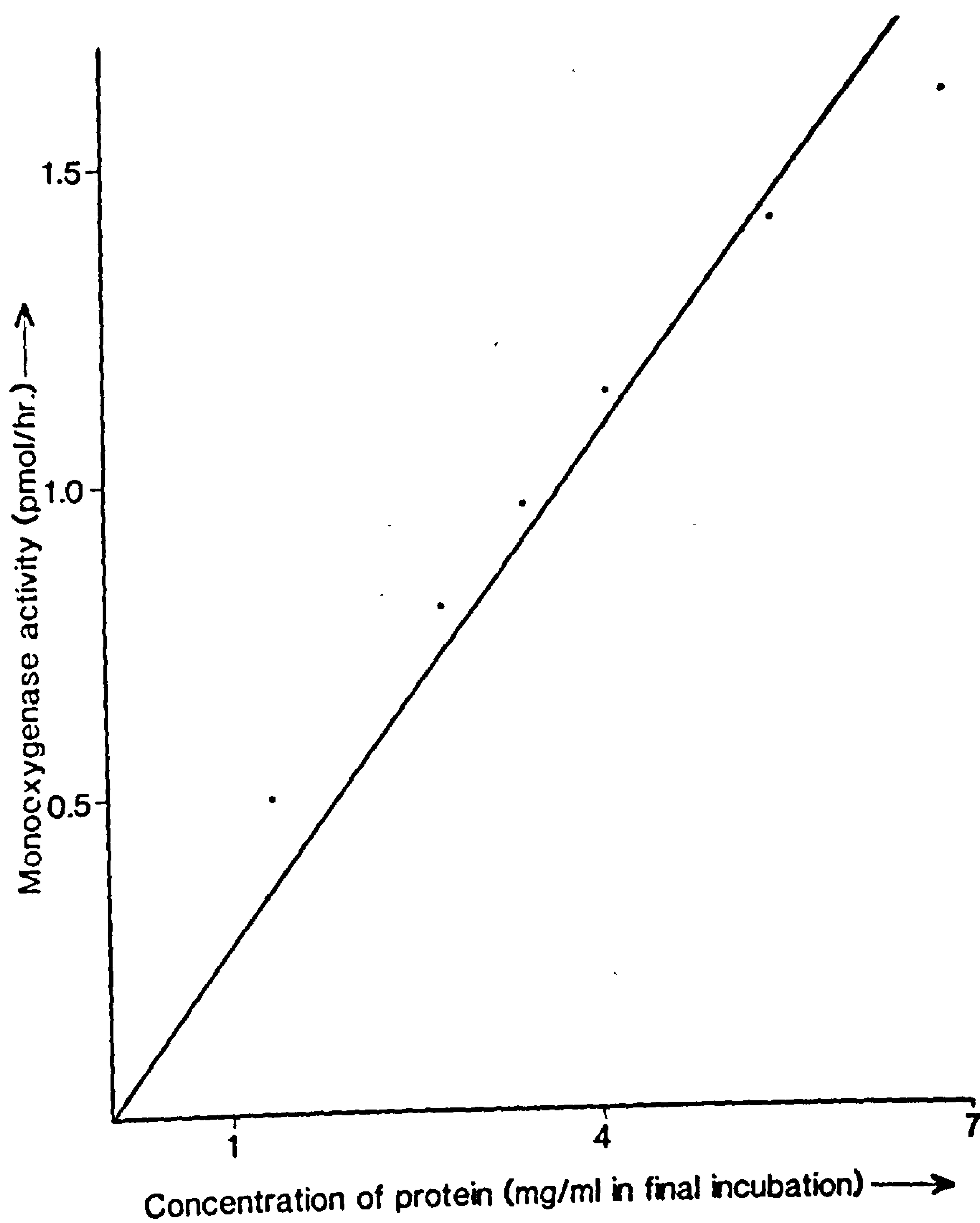


Fig 2 Linearity of ecdysone 20-monooxygenase activity with increasing concentrations of microsomal protein



D. Effect of the presence of fluoride during preincubation on the activity of ecdysone 20-monooxygenase

The fat body microsomal fraction prepared in Hepes buffer was resuspended in either 0.15M KCl or 0.15M KF in Hepes buffer. Aliquots (100μl) were preincubated for 1hr. at 37°C and, following the addition (100μl) of Hepes buffer to incubations (i) and (iii), of 0.15M KF in Hepes buffer to (ii), and of 0.15M KCl in Hepes buffer to (iv) [Table 2], the whole preparations were assayed for ecdysone 20-monooxygenase activity.

Fluoride is a known inhibitor of endogenous phosphoprotein phosphatases (Hauschildt, 1986). The activity of ecdysone 20-monooxygenase was 2-fold greater, when KF rather than KCl was present in the preincubation [incubations (i) and (iii), Table 2], as a result of prevention of the decrease in monooxygenase activity. The results for the control incubations [(ii) and (iv), Table 2] indicated that the action of fluoride is not simply on the monooxygenase assay components.

Phosphate buffer is also a known inhibitor of phosphoprotein phosphatases (Sanghvi et al., 1981). The presence of phosphate buffer in the preincubation, also results in a greater monooxygenase activity, than for Hepes buffer (results not shown) again as a result of prevention of the decrease in monooxygenase activity by phosphoprotein phosphatases.

Table 2: Effect of the presence of fluoride during preincubation on the activity of ecdysone 20-monooxygenase

Preincubation conditions (1hr.)	Addition before monooxygenase assay	Monooxygenase activity (pmole/hr. per mg protein
(i) KCl	-	0.99
(ii) KCl	KF	1.35
(iii) KF	-	1.92
(iv) KF	KCl	1.87

Table 3: Effect of the presence of exogenous alkaline phosphatase during preincubation on the activity of ecdysone 20-monooxygenase

Preincubation conditions (1hr.)	Addition before monooxgenase assay	Monooxygenase activity (pmole/hr. per mg protein)*
(i) -	-	0.077 ± 0.002
(ii) Alkaline phosphatase	-	0.017 ± 0.001
(iii) -	Alkaline phosphatase	0.035 ± 0.004
(iv) Inactivated alkaline phosphatase	-	0.067 ± 0.006

*Results are the means ± SEM of three separate experiments using different tissue preparations, with each type of incubation done in duplicate.

E. Effect of the presence of exogenous alkaline phosphatase during preincubation on the activity of ecdysone 20-monooxygenase

The fat body microsomal fraction was prepared and resuspended in 0.3M KF in Hepes buffer. Aliquots (50µl) were then preincubated for 1hr. at 37°C with the addition [Table 3] of Hepes buffer (100µl) to incubations (i) and (iii), E. coli alkaline phosphatase in Hepes buffer (20iu; 50µl) and Hepes buffer (50µl) to (ii), and E. coli alkaline phosphatase in Hepes buffer (20iu; 50µl) inactivated by the addition of 120mM EDTA and 600mM NaH_2PO_4 in Hepes buffer (50µl) to (iv). After the preincubation, the following additions [Table 3] were made: Hepes buffer (50µl) to incubations (i), (ii) and (iv), and E. coli alkaline phosphatase in Hepes buffer (20iu; 50µl) to incubation (iii). The whole preparations were then assayed for ecdysone 20-monooxygenase.

In this experiment, E. coli alkaline phosphatase, which is not inhibited by fluoride, was used to hydrolise phosphate covalently bound to monooxygenase (Mellgren et al., 1977), before assay of enzymic activity. The presence of E. coli alkaline phosphatase in the preincubation significantly decreased the monooxygenase activity by 4-fold, over the preincubation in Hepes alone [incubations (i) and (ii), Table 3]. Inhibition of alkaline phosphatase by EDTA and NaH_2PO_4 (Goodwin et al., 1982) eliminated this effect [incubation (iv), Table 3]. It has been reported that alkaline phosphatase can hydrolise $NADP^+$ and glucose-6-phosphate (Goodwin et al., 1982), both of which are components of the assay. This does not account for the results obtained in incubation (ii), since alkaline phosphatase present only during the monooxygenase assay, has as smaller effect then when present during the preincubation as well [incubation (ii) and (iii), Table 3].

F. Effect of the presence of exogenous protein kinase during preincubation on the activity of ecdysone 20-monooxygenase

The fat body microsomal fraction was prepared and resuspended (0.5ml) in Hepes buffer, then preincubated for 30 min. at 37°C to allow removal of phosphate covalently bound to the monooxygenase. Following addition of 0.6M KF in Hepes buffer (0.5ml), aliquots (50μl) of the microsomal preparation were preincubated for 1hr. at 37°C after the addition of one or more [Table 4] of the following: (a) 300μM dibutyryl cyclic AMP, 24mM $MgCl_2$ and 12mM ATP in Hepes buffer (50μl), (b) cyclic AMP-dependent protein kinase (50iu) in Hepes buffer (50μl), (c) cyclic AMP-dependent protein kinase inhibitor (0.1mg) in Hepes buffer (50μl), or (d) Hepes buffer to give a total preincubation volume of 200μl in each case. The whole preparations were then assayed for ecdysone 20-monooxygenase activity [Table 4].

In this series of incubations, the dephosphorylated microsomal fraction was preincubated in the presence of cyclic AMP-dependent protein kinase (from rabbit muscle) and appropriate cofactors, in the presence of KF, to effect possible rephosphorylation of the ecdysone 20-monooxygenase, before assay of its activity. The presence, during preincubation with protein kinase, of MgATP, dibutyryl cyclic AMP and fluoride [incubation (i), Table 4] resulted in a significant 4-fold increase in monooxygenase activity, compared with the control incubation [(v), Table 4]. Omission of the cyclic AMP-dependent protein kinase [incubation (ii), Table 4] or the dibutyryl cyclic AMP, MgATP [incubation (iii), Table 4] during the preincubation did not lead to enhanced activity. Similarly, preincubation with dibutyryl

Table 4: Effect of the presence of exogenous protein kinase during preincubation on the activity of ecdysone 20-monooxygenase

Second preincubation conditions (1hr.)	Monooxygenase activity (pmol/ hr. per mg protein)*
(i) Dibutyryl cyclic AMP, MgATP, protein kinase	0.063 ± 0.005
(ii) Dibutyryl cyclic AMP, MgATP	0.016 ± 0.002
(iii) Protein kinase	0.018 ± 0.002
(iv) Dibutyryl cyclic AMP, MgATP, protein kinase, protein kinase inhibitor	0.019 ± 0.002
(v) -	0.017 ± 0.001

*Results are the means ± SEM of three separate experiments using different tissue preparations, with each type of incubation done in duplicate.

cyclic AMP, MgATP, cyclic AMP-dependent protein kinase, and fluoride in the presence of cyclic AMP-dependent protein kinase inhibitor [Ashby and Walsh, 1972; incubation (iv), Table 4] prevents the elevation of the monooxygenase activity, confirming that the stimulation is due to the cyclic AMP dependent protein kinase.

G. Effect of the presence of putative endogenous phosphoprotein phosphatase during preincubation on the activity of ecdysone 20-monooxygenase

The fat body was homogenized in Hepes buffer (6ml) and centrifuged for 15 min. at 8,800g. The supernatant was divided into six fractions and preincubated for 30 min. at 37°C unless otherwise stated after the addition (Table 5) of the following: (i) 0.6M KF in Hepes buffer (100μl), (ii) Hepes buffer (100μl) at 4°C, (iii) Hepes buffer (100μl), (iv) 2.4mM $CaCl_2$ plus calmodulin (10 units) in Hepes buffer (50μl) and 264mM $MgCl_2$ in Hepes buffer (50μl), (v) 264mM $MgCl_2$ in Hepes buffer (50μl) and Hepes buffer (50μl), and (vi) 2.4mM $CaCl_2$ plus calmodulin (10 units) in Hepes buffer (50μl) and Hepes buffer (50μl). Following the preincubation, 0.6M KF in Hepes buffer (100μl) was added [Table 5] to (ii), (iii), (iv), (v) and (vi), and Hepes buffer (100μl) to (i). The microsomal fractions were then prepared by centrifugation (100,000g for 90min.), resuspended in 50mM KF in Hepes buffer (1ml) and recentrifuged under the same conditions. The microsomal fractions were resuspended in 75mM KF in Hepes buffer (0.5ml) and aliquots (200μl) were assayed for ecdysone 20-monooxygenase activity [Table 5].

Table 5: Effect of the presence of putative endogenous phosphoprotein phosphatase during the preincubation on the activity of ecdysone 20-monooxygenase

Preincubation conditions (30 min.)	Monooxygenase activity (pmol/hr. per mg protein)
(i) KF	0.42
(ii) $4^{o}C$	0.30
(iii) -	0.17
(iv) Mg^{2+}, Ca^{2+}-calmodulin	0.07
(v) Mg^{2+}	0.09
(vi) Ca^{2+}-calmodulin	0.07

Table 6: Effect of the presence of putative endogenous phosphoprotein phosphatase in conjunction with calcium ions and calmodulin during the preincubation on the activity of ecdysone 20-monooxygenase

Preincubation conditions (30 min.)	Addition before monooxygenase assay	Monooxygenase activity (pmol/hr. per mg protein)
(i) KF	-	0.30
(ii) -	KF	0.14
(iii) Ca^{2+}-calmodulin	KF	0.07
(iv) KF, Ca^{2+}-calmodulin	-	0.29

This series of incubations, was to investigate the presence of phosphoprotein phosphatase activity. Preincubation of a microsomal plus supernatant fraction at 37°C [incubation (iii), Table 5] reduced the monooxygenase activity approximately two and a half-fold, over a control incubation [incubation (i), Table 5] containing KF to inhibit any endogenous phosphoprotein phosphatases (Beg and Brewer, 1981). A much reduced effect was observed when the preincubation was carried out at 4°C [incubation (ii), Table 5] compared with 37°C. It is known that some Type II phosphoprotein phosphatases (Ingebristen and Cohen, 1983) are stimulated by calcium ions in conjunction with calmodulin and by Mg^{2+} ions. Addition of either Ca^{2+}-calmodulin or Mg^{2+} to the microsomal plus supernatant preparation during preincubation, resulted in a further reduction in ecdysone 20-monooxygenase activity [incubations (v) and (vi), Table 5].

In another experiment [Table 6] the fat body was homogenised in Hepes buffer (4ml) and centrifuged for 15 min. at 8,800g. The supernatant was divided into four fractions and preincubated at 37°C for 30 min. after the addition (Table 6) of the following: (i) 1.25M KF in Hepes buffer (50μl) and Hepes buffer (100μl), (ii) Hepes buffer (150μl), (iii) 2.5mM $CaCl_2$ in Hepes buffer (50μl), calmodulin (10 units) in Hepes buffer (50μl) and Hepes buffer (50μl), (iv) 2.5mM $CaCl_2$ in Hepes buffer (50μl), calmodulin (10 units) in Hepes buffer (50μl) and 1.25M KF in Hepes buffer (50μl). Following the preincubation 625mM KF in Hepes buffer (100μl) was added to (ii) and (iii), while Hepes buffer (100μl) was added to (i) and (iv). The microsomal fractions were pelleted at 100,000g, resuspended in 50mM KF

in Hepes buffer (1ml), repelleted at 100,000g, and resuspended in 75mM KF in Hepes buffer (0.5ml). Aliquots (200μl) were then assayed for ecdysone 20-monooxygenase.

The Ca^{2+}-calmodulin decreases the ecdysone 20-monooxygenase 2-fold, over the control incubation [incubations (iii) and (ii), Table 6] as expected, but only in the absence of fluoride [incubation (iv), Table 6]. This indicates that the Ca^{2+}-calmodulin is stimulating a phosphoprotein phosphatase and not a Ca^{2+}-calmodulin-dependent protein kinase.

H. Effect of the presence of putative endogenous protein kinase during preincubation on the activity of ecdysone 20-monooxygenase

The fat body microsomal fraction was prepared and resuspended in Hepes buffer (0.5ml); the original 100,000g supernatant was also kept for subsequent use. The microsomal preparation was then preincubated for 30 min. at 37°C to allow removal of phosphate covalently bound to the monooxygenase, after which 0.6M KF in Hepes buffer (0.5ml) was added. Aliquots (50μl) of this microsomal preparation were then further preincubated for 1hr. at 37°C after the addition [Table 7] of one or more of the following: (a) 100,000g supernatant (50μl), (b) 300μM cyclic AMP in Hepes buffer (50μl), (c) 24mM $MgCl_2$ plus 12mM ATP in Hepes buffer (50μl), or (d) Hepes buffer to give a total preincubation volume of 200μl in each case. Following the preincubations, the whole preparations were assayed for ecdysone 20-monooxygenase activity.

Table 7: Effect of the presence of putative endogenous protein kinase in the preincubation on the activity of ecdysone 20-monooxygenase

Second preincubation conditions (1hr.)	Monooxygenase activity (pmol/hr. per mg protein)
(i) Cyclic AMP, MgATP, supernatant	3.44
(ii) Cyclic AMP, MgATP	1.02
(iii) MgATP	1.80
(iv) Cyclic AMP	1.72
(v) -	1.56
(vi) Supernatant	1.17

This series of incubations was to investigate the presence of an appropriate protein kinase in the supernatant. The presence of the 100,000g supernatant, MgATP, cyclic AMP and fluoride in the second preincubation [incubation (i), Table 7] resulted in a 2-fold increase in monooxygenase activity compared with a control incubation containing fluoride only [incubation (v), Table 7]. Omission of the 100,000g supernatant [incubation (ii), Table 7] or the cyclic AMP and MgATP [incubation (vi), Table 7] during the preincubation did not result in enhanced monooxygenase activity.

I. <u>Effect of the presence of putative endogenous phosphoprotein phosphatase and protein kinase during preincubation on ecdysone 20-monooxygenase activity in the presence of protease inhibitors</u>

Fat body from 36 insects was homogenised in Hepes buffer (6ml) containing a mixture of protease inhibitors [chymostatin, pepstatin, antipain, all at a concentration of 82.5μg/ml, and 1,10-phenanthroline (1.8mM)]. The homogenate was then centrifuged for 15 min. at 8,800g at 4°C and the supernatant was divided as follows: 3x 1ml fractions (section I, Table 8) and 1x 3ml fraction (section II, Table 8).

In section I, the effect of endogenous, phosphoprotein phosphatase was investigated. The three fractions (1ml) were preincubated for 1hr. at 37°C after the addition [Table 8] of the following: (i) Hepes buffer (100μl), (ii) and (iii) 0.5M KF in Hepes buffer (100μl). After the first preincubation, the mixtures were centrifuged at 100,000xg for 90 min. at 4°C and the microsomal

Table 8: Effect of the presence of putative endogenous phosphoprotein phosphatase and protein kinase on the activity of ecdysone 20-monooxygenase in the presence of protease inhibitors

First preincubation conditions (microsomes and supernatant)	Second preincubation conditions [1hr.] (microsomes)	Monooxygenase activity (pmole/hr. per mg protein)
I. Effect of phosphoprotein phosphatase (60 min.)		
(i) Inhibitors*	Inhibitors + KF	0.10
(ii) Inhibitors + KF	Inhibitors + KF	0.39
(iii) Inhibitors + KF	KF	0.35
II. Effect of protein kinase (30 min.)		
(iv) Inhibitors	Supernatant (including inhibitors + KF), cyclic AMP, MgATP	0.34
(v) Inhibitors	Supernatant (including inhibitors + KF)	0.13
(vi) Inhibitors	Inhibitors + KF	0.13

*Inhibitors refers to a mixture of protease inhibitors: chymostatin, pepstatin, antipain and 1,10-phenanthroline

fractions resuspended [Table 8] as follows: (i) and (ii) inhibitors (at the same concentrations as previously stated) in Hepes buffer (0.5ml) and (iii) Hepes buffer (0.5ml). 0.75M KF in Hepes buffer (50µl) was then added to each preparation before being subjected to a second preincubation for 1hr. at 37°C. Aliquots (200µl) of the mixtures were then assayed for ecdysone 20-monooxgyenase activity.

In section II, the effect of endogenous protein kinase was investigated. The original 8,800xg supernatant (3ml) containing inhibitors was preincubated at 37°C for 30 min., after which 0.8M KF in Hepes buffer (300µl) was added. After sub-division of the preparation into three equal portions (1100µl each), centrifugation at 100,000xg yielded 'supernatant' and 'microsomal' fractions which were resuspended either in respective 'supernatant fractions' [0.5ml; incubations (iv) and (v), Table 8] or in inhibitors (at the same concentrations previously stated) in Hepes buffer [0.5ml; incubation (vi), Table 8]. The preparations were then subjected to a second preincubation for 1hr. at 37°C after addition [Table 8] of the following: (iv) 0.75mM cyclic AMP, 60mM $MgCl_2$ and 30mM ATP in Hepes buffer (50µl), (v) Hepes buffer (50µl) and (vi) 0.75mM KF in Hepes buffer (50µl) [total volume, 550µl]. Aliquots (200µl) of the mixtures were then assayed for ecdysone 20-monooxygenase activity.

The effects of endogenous phosphoprotein phosphatase and protein kinase were evaluated in the presence of protease inhibitors to ascertain whether or not proteases have a significant complicating effect on the foregoing experiments.

In the examination of the effect of endogenous phosphoprotein phosphatase (Table 8, Section I), it is apparent that preincubation for 30 min. in the absence of KF results in a dramatic drop in ecdysone 20-monooxygenase activity [(i) and (ii), Table 8]. Furthermore, preincubation of microsomal fraction with KF, but in the absence of protease inhibitors, did not result in a significant change in monooxygenase activity [(ii) and (iii), Table 8].

In the examination of the effect of endogenous protein kinase [Table 8, Section II), the results show that addition of supernatant, cyclic AMP, MgATP, and KF to the preincubation mixture results in approximately 2.5-fold increase in monooxygenase activity [(iv) and (vi), Table 8], which is not observed when cyclic AMP and MgATP is omitted [(iv) and (v), Table 8].

J. <u>Time course of activation of microsomal ecdysone 20-monooxygenase by incubation with supernatant</u>

The fat body microsomal fraction was prepared and resuspended in Hepes buffer (0.5ml). It was then preincubated for 30 min. at 37°C to allow removal of phosphate covalently bound to the monooxygenase, after which 0.6M KF in Hepes buffer (0.5ml) was added. Duplicate aliquots (50µl) of the preparation were then preincubated for 0, 10, 30 or 60 min. at 37°C after the addition of the following: (a) 300µM cyclic AMP, 24mM $MgCl_2$ and 12mM ATP in Hepes buffer (50µl), (b) 100,000g supernatant (50µl), and (c) Hepes buffer, to give a total preincubation volume of 200µl in each case (●). Two control

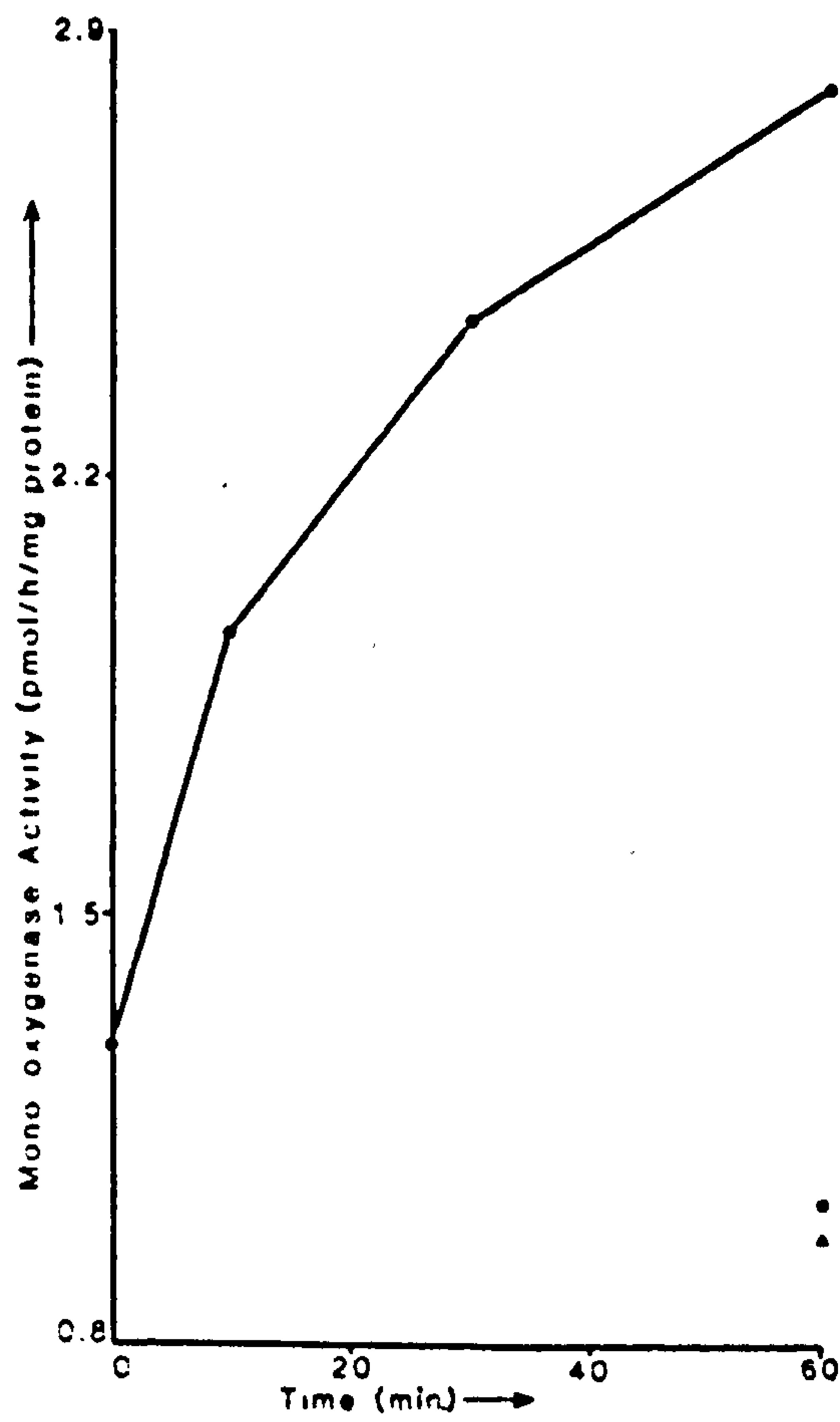
incubations were also carried out involving preincubation for 1hr. at $37^{o}C$ in the absence of cyclic AMP, and MgATP (■) or supernatant, cyclic AMP, and MgATP (▲) [Fig. 3]. Immediately following the preincubations, the whole preparations were assayed for ecdysone 20-monooxygenase activity [Fig. 3].

The results show that the activation of ecdysone 20-monooxygenase increased with the time of preincubation and that it is dependent on the presence of supernatant, cyclic AMP and/or MgATP.

K. <u>Effect of the presence of cyclic AMP-dependent protein kinase inhibitor during preincubation on the activation of ecdysone 20-monooxygenase</u>

The fat body microsomal fraction prepared and resuspended (0.5ml) in Hepes buffer was preincubated for 30 min. at $37^{o}C$ to allow removal of phosphate covalently bound to the monooxygenase, followed by the addition of 0.6M KF in Hepes buffer (0.5ml). Aliquots (50μl) of the preparation were then preincubated for 1hr. at $37^{o}C$ after the addition [Table 9] of one or more of the following: (a) 300μM dibutyryl cyclic AMP, 24mM $MgCl_2$, and 12mM ATP in Hepes buffer (50μl), (b) cyclic AMP-dependent protein kinase (50iu) in Hepes buffer (50μl), (c) 100,000g supernatant (50μl), (d) cyclic AMP-dependent protein kinase inhibitor (0.1mg) in Hepes buffer (50μl), or (e) Hepes buffer to give a total preincubation volume of 200μl in each case. Following the preincubation, the whole preparations were assayed for ecdysone 20-monooxygenase activity [Table 9].

Fig 3. Time course of activation of microsomal ecdysone 20-monooxygenase by incubation with supernatant.



Preincubation conditions

- ● KF, supernatant, cyclic AMP, MgATP
- ■ KF, supernatant
- ▲ KF

Table 9: Effect of cyclic AMP-dependent protein kinase inhibitor on the activation of ecdysone 20-monooxygenase

Second preincubation (1hr.)	Monooxygenase activity (pmol/hr. per mg protein)
(i) Supernatant	0.29
(ii) Supernatant, MgATP, dibutyryl cyclic AMP	0.58
(iii) Supernatant, MgATP, dibutyryl cyclic AMP, protein kinase inhibitor	0.23
(iv) Protein kinase, MgATP, dibutyryl cyclic AMP	1.11
(v) Protein kinase, MgATP, dibutyryl cyclic AMP, protein kinase inhibitor	0.29
(vi) Protein kinase inhibitor	0.29

This experiment was to investigate the effect of a cyclic AMP-dependent protein kinase inhibitor (Ashby and Walsh, 1972) on the activation of ecdysone 20-monooxygenase by either supernatant or exogenous protein kinase. The presence of 100,000g supernatant, MgATP, and dibutyryl cyclic AMP in the preincubation [(ii), Table 9] results in a two-fold increase in the ecdysone 20-monooxygenase activity, over the control incubation containing protein kinase inhibitor [(vi), Table 9]. Similarly, a four-fold increase in monooxygenase activity is observed following preincubation in the presence of cyclic AMP-dependent protein kinase, MgATP and dibutyryl cyclic AMP [Table 9, (iv) and (vi)]. However, addition of cyclic AMP-dependent protein kinase inhibitor during preincubation with either 100,000g supernatant [Table 9, (ii) and (iii)] or cyclic AMP-dependent protein kinase [Table 9, (iv) and (v)] eliminates this effect. As observed earlier [Table 7], ecdysone 20-monooxygenase activation was not observed by preincubation with 100,000g supernatant in the absence of MgATP and cyclic AMP [Table 9, (i) and (ii)].

L. Reactivation by putative endogenous protein kinase (supernatant fraction) of alkaline phosphatase-incubated ecdysone 20-monooxygenase (microsomal fraction)

The fat body was homogenized in 105mM KF in Hepes buffer (2ml) and centrifuged at 8,800g for 15 min. The supernatant was divided into four fractions (0.5ml) and the microsomal fractions prepared as usual. The microsomal fractions were then resuspended in 105mM KF in Hepes buffer (0.5ml) and preincubated for 30 min. at 37°C after the

addition [Table 10] of the following: (i) and (iii) Hepes buffer (100μl), (ii) and (iv) E. coli alkaline phosphatase (20iu) in Hepes buffer (50μl) and Hepes buffer (50μl) to give a total preincubation volume of 600μl in each case. Following the preincubation, 1.05M NaH_2PO_4 plus 210mM EDTA in Hepes buffer (100μl) was added to each preparation to inactivate the alkaline phosphatase. Aliquots (200μl) from preparations (i) and (ii) [Table 10] were then assayed for ecdysone 20-monooxygenase activity.

Preparations (iii) and (iv) [Table 10] were centrifuged at 100,000g and the microsomal fractions resuspended in 105mM KF in Hepes buffer [0.5ml; (iii)] and 100,000g supernatant already containing KF [0.5ml; (iv), Table 10] respectively. The preparations were then subjected to a further preincubation for 1hr. at 37°C after the addition [Table 10] of the following: (iii) Hepes buffer (100μl), (iv) 84mM $MgCl_2$, 42mM ATP, and 1.05mM cyclic AMP in Hepes buffer (100μl), to give a total preincubation volume of 600μl in each case. Following the preincubation, Hepes buffer (100μl) was added to both preparations to provide the same total volume as for (i) and (ii) [Table 10]. Aliquots (200μl) from preparations (iii) and (iv) were then assayed for ecdysone 20-monooxygenase activity.

The results show that preincubation with E. coli alkaline phosphatase resulted in a marked reduction in ecdysone 20-monooxygenase activity [(i) and (ii), Table 10]. However, after inactivation of the alkaline phosphatase and re-sedimentation of the microsomes, a second preincubation of the latter in the presence of

Table 10: Reactivation of alkaline phosphatase-inactivated ecdysone 20-monooxygenase by putative endogenous protein kinase*

First preincubation conditions (30 min.)	Second preincubation conditions (1hr.)	Addition before mono-oxygenase assay	Monooxygenase activity (pmol/hr. per protein)
(i) -	Assayed directly		0.31
(ii) Alkaline phosphatase	Assayed directly		0.06
(iii) -	Hepes[a]	Hepes	0.24
(iv) Alkaline phosphatase	Supernatant, MgATP, cyclic AMP[a]	Hepes	0.39

[a]Microsomal fractions were re-isolated before the second preincubation to avoid dilution of the supernatant fraction in (iv) and to remove most of the alkaline phosphatase in case inhibition by EDTA/NaH_2PO_4 was incomplete.

*KF was present throughout the experiment in all incubations.

100,000g supernatant, MgATP and cyclic AMP reversed this effect resulting in a one and a half-fold increase in monooxygenase activity, compared to a control incubation lacking 100,000g supernatant, MgATP and cyclic AMP [(iii) and (iv), Table 10]. Examination of the two control incubations [(i) and (iii), Table 10] indicates that there is loss of approximately one third of the monooxygenase activity during sedimentation of the microsomal fraction and subsequent 1hr. preincubation. This can only be partly accounted for in terms of loss of total microsomal protein.

3. Discussion

In the routine preparation of the microsomal sub-cellular fraction, the mitochondrial sub-cellular fraction was centrifuged at 8,800g for 10 min. [Table 1] compared with 15 min. in the preparation of the microsomes in the sub-cellular localization of ecdysone 20-monooxygenase (Chapter 3). This difference in centrifugation time did not affect appreciably the mitochondrial contamination or the monooxygenase activity, of the microsomal fraction [Table 1].

Fig. 1 and Fig. 2 demonstrate that the ecdysone 20-monooxygenase activity is linear with respect to time and microsomal protein. The foregoing data are consistent with a role for reversible phosphorylation in the modulation of microsomal ecdysone 20-monooxygenase activity.

Since fluoride is a known inhibitor of phosphoprotein phosphatases (Beg and Brewer, 1981), the observation that ecdysone 20-monooxgyenase activity is much higher following preincubation of microsomal fraction with KF than with KCl [Table 2], suggests that in the absence of fluoride, endogenous phosphoprotein phosphatase partially dephosphorylates the monooxygenase leading to reduced activity. This is corroborated by the finding that preincubation of the microsomal fraction with alkaline phosphatase, resulted in a significantly greater than 4-fold decrease in monooxygenase activity [Table 3]. This effect was eliminated by inhibition of the alkaline phosphatase with NaH_2PO_4 and EDTA.

Approximately 4-fold greater monooxygenase activity was observed following preincubation of microsomal fraction in the presence of cyclic AMP-dependent protein kinase, cyclic AMP and MgATP, compared to a control incubation lacking the cyclic AMP-dependent protein kinase [Table 4]. The enhanced activity was not observed in the presence of a cyclic AMP-dependent protein kinase inhibitor (Table 4). These observations are consistent with the premise that enhanced phosphorylation of the monooxygenase results in increased activity.

Evidence was also obtained for the presence in Spodoptera fat body of appropriate cytosolic phosphoprotein phosphatase and protein kinase activities, necessary for modulation of the phosphorylation state of ecdysone 20-monooxygenase. Results presented in Table 5 and 6 are consistent with the occurrence in the cytosol of such a phosphatase, which appears to be stimulated by Ca^{2+}-calmodulin and/or

by Mg^{2+} ions, as reported for some mammalian type II phosphoprotein phosphatases (Ingebristsen and Cohen, 1983). Similarly, monooxygenase activity was enhanced by incubation of microsomes with supernatant fraction and KF, but only in the presence of cyclic AMP and MgATP under the in vitro conditions employed [Table 7]. Enhancement of ecdysone 20-monooxygenase activity increased over a preincubation period up to one hour, in a manner suggesting an enzymic mechanism (Fig. 3). However, such increase in monooxygenase activity was not observed when protein kinase inhibitor was added together with either supernatant or cyclic AMP-dependent protein kinase in the presence of MgATP and dibutyryl cyclic AMP [Table 9]. Furthermore, when the microsomal fraction was first preincubated with alkaline phosphatase to remove covalently bound phosphate from the monooxygenase, followed by incubation with supernatant, MgATP and cyclic AMP, ecdysone 20-monooxygenase activity could be restored [Table 10]. These results indicate that increased phosphorylation of the monooxygenase results in enhanced activity and that the endogenous protein kinase is cyclic AMP-dependent.

The results given in Table 8 show that the effects which can be attributed to endogenous cytosolic phosphoprotein phosphatase and protein kinase are also demonstrable in the presence of a mixture of protease inhibitors during the whole experiment, indicating that the changes observed in monooxygenase activity in this work are not complicated unduly by protease action.

The combined data, provide indirect evidence that the microsomal ecdysone 20-monooxygenase exists in two forms, an active phosphorylated form and an inactive dephosphorylated form interconvertible by a cyclic AMP-dependent protein kinase and a phosphoprotein phosphatase, which may be stimulated by Ca^{2+}-calmodulin and/or by Mg^{2+} ions. However, confirmation of this hypothesis awaits more direct evidence, including the incorporation of ^{32}P-labelled phosphate from ATP into the ecdysone 20-monooxygenase system.

CHAPTER FIVE

MODULATION OF ECDYSONE 20-MONOOXYGENASE IN THE MITOCHONDRIAL FRACTION

1. Introduction

The activity of ecdysone 20-monooxygenase in the fat body of Spodoptera littoralis undergoes developmental variation during the final, 6th, larval instar and exhibits a distinct peak. Possible regulatory mechanisms for these long-term changes in enzymic activity have been previously considered (Chapter 1). In addition, a potential mechanism for the rapid modulation of ecdysone 20-monooxygenase activity is the reversible phosphorylation of the enzymic system.

In the fat body of Spodoptera, the majority of the ecdysone 20-monooxygenase activity occurs in the mitochondria with a small amount associated with the microsomal fraction (see Chapter 3). Indirect evidence has been furnished demonstrating the feasibility of modulating the activity of the microsomal ecdysone 20-monooxygenase in a manner commensurate with reversible changes in its phosphorylation state (Chapter 4). Such a control mechanism has been documented for only two vertebrate mitochondrial enzyme systems, the pyruvate dehydrogenase complex (Wieland, 1983) and branched-chain 2-oxoacid dehydrogenase complex (Jones and Yeaman, 1986).

Evidence is now presented for reversible activation-inactivation of the mitochondrial monooxygenase commensurate with modulation of its activity by phosphorylation-dephosphorylation. The possibility that the activity of the mitochondrial ecdysone 20-monooxygenase can be modulated by changes in its phosphorylation state was examined by subjecting the mitochondrial fraction to various treatments before assay of the enzymic activity.

2. Experimental and Results

A. Linearity of the ecdysone 20-monooxygenase assay with time

The fat body mitochondrial fraction was prepared and resuspended in 300mM KF in isotonic Hepes buffer. Aliquots (50μl) were assayed for ecdysone 20-monooxygenase activity after the addition of isotonic Hepes buffer (150μl) with assay incubation times of 0, 10, 20, 30, 40 and 50 min.

The results (Fig. 1) show that the ecdysone 20-monooxygenase assay is linear up to an assay incubation time of at least 50 minutes, which is well beyond the 30 minute incubation period set for the monooxygenase assay.

B. Linearity of the ecdysone 20-monooxygenase assay with increasing amounts of mitochondrial protein

The fat body mitochondrial fraction was prepared and resuspended in 150mM KF in isotonic Hepes buffer. Aliquots (100, 80, 60, 50, 40 and 20μl) of the resuspended mitochondrial fraction were diluted with 150mM KF in isotonic Hepes buffer to give a total volume of 100μl in each case. Following the further addition of isotonic Hepes buffer (100μl) the preparations were assayed for ecdysone 20-monooxygenase activity. The results (Fig. 2) show that the ecdysone 20-monooxygenase activity is linear with increasing concentrations of mitochondrial protein.

Fig 1 Linearity of the mitochondrial ecdysone 20-monooxygenase assay with time

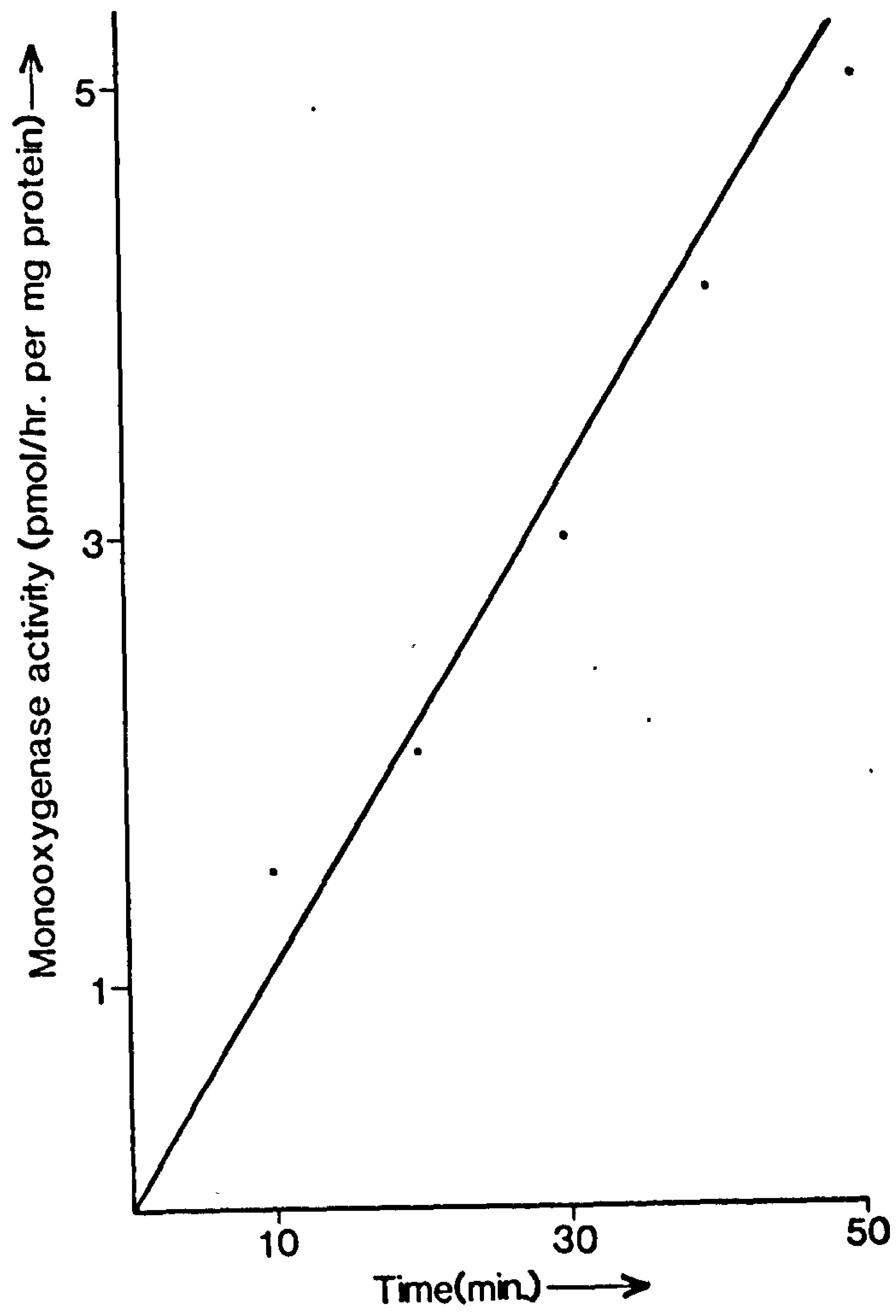
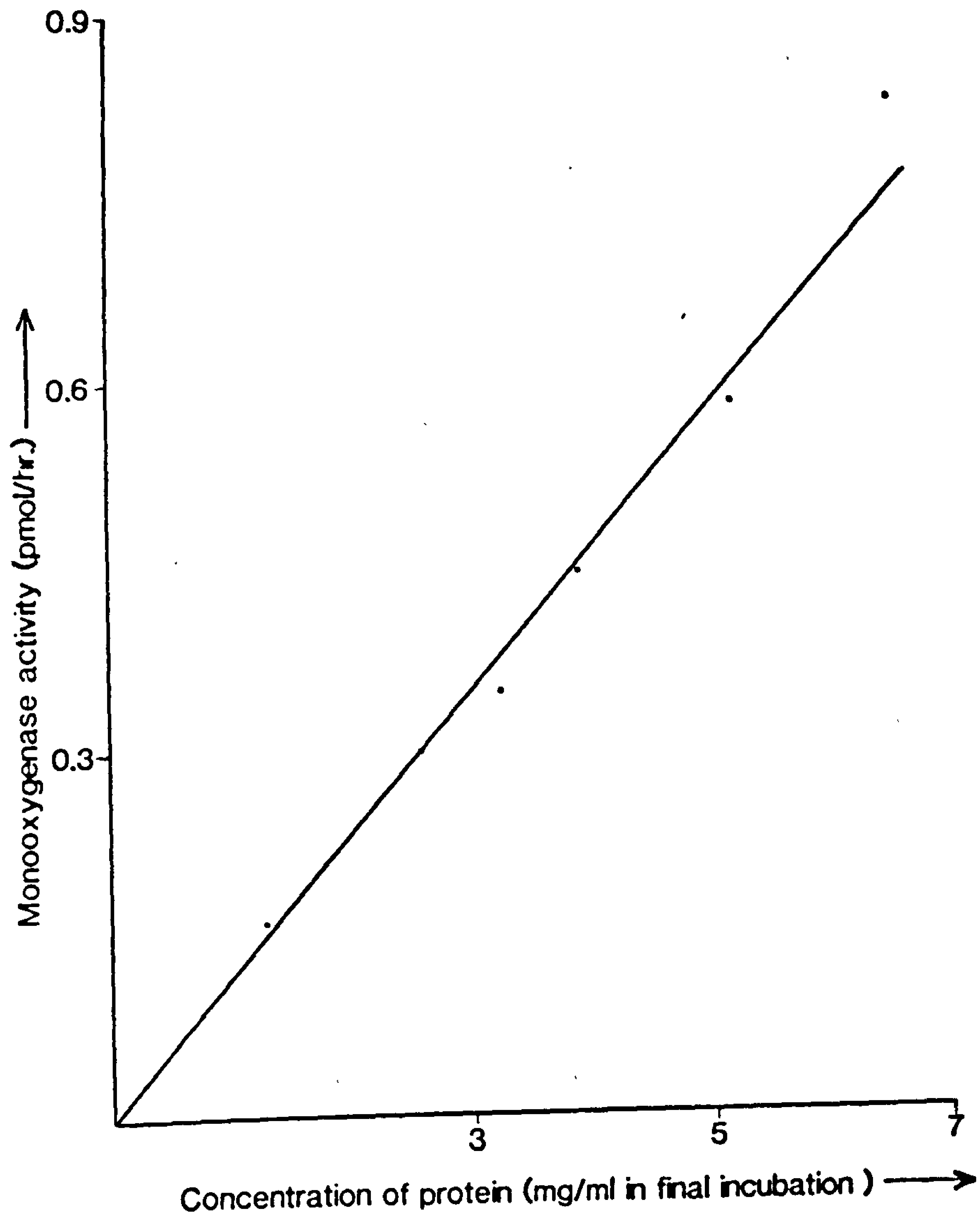


Fig 2 Linearity of ecdysone 20-monooxygenase activity with increasing concentrations of mitochondrial protein



C. Effect of the presence of fluoride during preincubation on the activity of ecdysone 20-monooxygenase

The fat body mitochondrial fraction prepared in isotonic Hepes buffer was resuspended in either 0.15M KCl or 0.15M KF in isotonic Hepes buffer. Aliquots (100µl) were preincubated for 1hr. at 37°C and following the addition (100µl) of isotonic Hepes buffer to incubations (i) and (iii), of 0.15M KF in isotonic Hepes buffer to (ii), and of 0.15M KCl in isotonic Hepes buffer to (iv) [Table 1], the whole preparations were assayed for ecdysone 20-monooxygenase activity.

Fluoride is a known inhibitor of endogenous phosphoprotein phosphatases (Hauschildt, 1986). The activity of ecdysone 20-monooxygenase was 4- to 5-fold greater when KF rather than KCl was present in the preincubation [incubations (i) and (iii), Table 1], as a result of prevention of the decrease in monooxygenase activity. The results for the control incubations [(ii) and (iv), Table 1] indicated that the action of fluoride is not simply on the monooxygenase assay components.

D. Effect of temperature and the presence of fluoride and Mg^{2+} during preincubation on the activity of ecdysone 20-monooxgenase

The fat body mitochondrial fraction was prepared and resuspended in isotonic Hepes buffer. Aliquots (100µl) were preincubated for 1hr. at 37°C unless otherwise stated after the addition [Table 2] of the following: (i) isotonic Hepes buffer (100µl), (ii) 0.15M KF in isotonic Hepes buffer (100µl), (iii) isotonic Hepes buffer (100µl) at 4°C and (iv) 33mM $MgCl_2$ in isotonic Hepes buffer (100µl). Following

Table 1: Effect of the presence of fluoride during preincubation on the activity of ecdysone 20-monooxygenase

Preincubation conditions (lhr.)	Addition before monooxgenase assay	Monooxygenase activity (pmol/hr. per mg protein)
(i) KCl	-	0.23
(ii) KCl	KF	0.57
(iii) KF	-	1.09
(iv) KF	KCl	1.09

Table 2: Effect of temperature and the presence of fluoride and Mg^{2+} during the preincubation on the activity of ecdysone 20-monooxygenase

Preincubation conditions (lhr.)	Monooxygenase activity (pmol/hr. per mg protein)
(i) -	0.56
(ii) KF	1.48
(iii) 4°C	3.64
(iv) Mg^{2+}	0.31

the preincubation, the whole preparations were assayed for ecdysone 20-monooxygenase activity.

Again, the presence of KF during the preincubation resulted, indirectly, in a greater ecdysone 20-monooxygenase activity [(i) and (ii), Table 2] although not as high as when preincubation occurred in the absence of KF at $4^{o}C$ [(ii) and (iii), Table 2]. Some type II protein phosphatases are stimulated by Mg^{2+} ions (Ingebritsen and Cohen, 1983), however the presence of Mg^{2+} ions in the preincubation had negligible apparent effect on the already low activity of the monooxygenase.

E. Effect of the presence of exogenous alkaline phosphatase during preincubation on the activity of ecdysone 20-monooxygenase

The fat body mitochondrial fraction prepared in 0.15M KF in isotonic Hepes buffer was resuspended in 0.3M KF in hypotonic Hepes buffer and preincubated for 15 min. at $4^{o}C$ to allow the mitochondria to burst. Aliquots (50μl) were then preincubated at $37^{o}C$ for 1hr. with addition [Table 3] of the following: (i) hypotonic Hepes buffer (150μl), (ii) hypotonic Hepes buffer (150μl), (iii) *E. coli* alkaline phosphatase in hypotonic Hepes buffer (20iu; 50μl) and hypotonic Hepes buffer (100μl), or (iv) *E. coli* alkaline phosphatase in hypotonic Hepes buffer (20iu; 50μl) inactivated by the addition of 120mM EDTA and 600mM NaH_2PO_4 in hypotonic Hepes buffer (50μl) and hypotonic Hepes buffer (50μl). After the preincubation the following additions [Table 3] were made: (i) and (iv) hypotonic Hepes buffer (50μl), (ii) and (iii) 120mM EDTA and 600mM NaH_2PO_4 in hypotonic Hepes buffer (50μl). The whole preparations were then assayed for ecdysone 20-monooxygenase activity.

Table 3: **Effect of the presence of exogenous alkaline phosphatase during the preincubation on the activity of ecdysone 20-monooxygenase**

Preincubation conditions (1hr.)	Addition before monooxygenase activity	Monooxygenase activity (pmol/hr. per mg protein)
(i) -	-	0.73
(ii) -	EDTA, NaH_2PO_4	0.81
(iii) Alkaline phosphatase	EDTA, NaH_2PO_4	0.44
(iv) Inactivated alkaline phosphatase	-	0.81

In this experiment the mitochondria were burst in the presence of hypotonic buffer to allow access to the E. coli alkaline phosphatase, which is not inhibited by fluoride, to hydrolise phosphate covalently bound to the monooxygenase (Mellgren et al., 1977) before assay of enzymic activity. The presence of alkaline phosphatase in the preincubation reduced the monooxygenase activity 2-fold compared with a control incubation [(ii) and (iii), Table 3]. However, when the alkaline phosphatase was inactivated by the presence of EDTA and NaH_2PO_4, the monooxygenase activity was similar to that in the control incubation without alkaline phosphatase [(ii) and (iv), Table 3]. Prior to the ecdysone 20-monooxygenase assay, EDTA and NaH_2PO_4 were added [(ii) and (iii), Table 3] to ensure constant compositions of the mixtures, for the assay. This is important, since it has been reported that alkaline phosphatase can hydrolyse $NADP^+$ and glucose-6-phosphate (Goodwin et al., 1982) both of which are components of the assay. It was also shown that EDTA and NaH_2PO_4 had a negligible effect [(i) and (ii), Table 3] when present in the monooxygenase assay.

F. Effect of the presence of protein kinase during preincubation on the activity of ecdysone 20-monooxygenase

The 600g supernatant from a fat body homogenate in isotonic Hepes buffer was divided into two equal portions, the mitochondrial fractions prepared simultaneously, and then resuspended in either isotonic Hepes buffer or hypotonic Hepes buffer, and preincubated at 4^oC for 15 min. to allow the latter mitochondria to burst. Both mitochondrial preparations were then preincubated for 30 min. at 37^oC

to allow removal of phosphate covalently bound to the monooxygenase after which 0.6M KF in Hepes buffer (0.5ml) was added. All additions were made in isotonic or hypotonic buffer to the "intact" or "broken" mitochondrial preparations, respectively. Aliquots (50μl) of the mitochondrial preparations were then preincubated at 37°C for 60 min. after the addition [Table 4] of one or more of the following: (a) 300μM dibutyryl cyclic AMP in Hepes buffer (50μl), (b) 24mM $MgCl_2$ plus 12mM ATP in Hepes buffer, (c) cyclic AMP-dependent protein kinase (50iu) in Hepes buffer (50μl), or (d) Hepes buffer to give a total preincubation volume of 200μl in each case. Following the preincubations, the preparations were assayed for ecdysone 20-monooxygenase activity.

In this series of incubations the dephosphorylated "broken" and "intact" mitochondrial fractions were preincubated in the presence of a cyclic AMP-dependent protein kinase (from rabbit muscle) and appropriate cofactors in the presence of KF to effect possible rephosphorylation of the ecdysone 20-monooxygenase, before assay of its activity. Preincubation of the "broken" mitochondria with protein kinase, dibutyryl cyclic AMP, and MgATP in the presence of KF results in a 2-fold increase in ecdysone 20-monooxygenase activity, compared with a control incubation [(i) and (iii), Table 4]. Omission of protein kinase [(ii), Table 4] or of dibutyryl cyclic AMP, and MgATP [(iv), Table 4] did not result in enhanced activity. As expected, an analogous enhancement of monooxygenase activity following preincubation of intact mitochondria with protein kinase and necessary cofactors was not observed [(i) and (iii), Table 4].

Table 4: Effect of the presence of protein kinase on the activity of ecdysone 20-monooxygenase

Preincubation Conditions (1hr.)	Monooxygenase activity (pmol/hr. per mg protein)	
	Intact mitochondria	Broken mitochondria
(i) Dibutyryl cyclic AMP, MgATP, protein kinase	0.76	1.5
(ii) Dibutyryl cyclic AMP, MgATP	0.68	0.67
(iii) -	0.68	0.75
(iv) Protein kinase	0.59	0.58

G. Reactivation by exogenous protein kinase of alkaline phosphatase-incubated ecdysone 20-monooxygenase

The fat body mitochondrial fraction prepared in 0.3M KF in isotonic Hepes buffer was resuspended in 0.3M KF in hypotonic Hepes buffer, and preincubated for 15 min. at $4^{o}C$ to allow the mitochondria to burst. Aliquots (50μl) were then preincubated at $37^{o}C$ for 30 min. with [Table 5] either (i) hypotonic Hepes buffer, (50μl) or (ii) to (vi) E. coli alkaline phosphatase (20iu; 50μl) in hypotonic Hepes buffer. The preparations were then subjected to a second preincubation at $37^{o}C$ for 60 min. after the addition [Table 5] of one or more of the following: (a) hypotonic Hepes buffer (100 or 50μl), (b) 0.6M NaH_2PO_4, 120mM EDTA in hypotonic Hepes buffer (50μl), (c) cyclic AMP-dependent protein kinase (50iu), 0.3mM dibutyryl cyclic AMP, 24mM $MgCl_2$, 12mM ATP in hypotonic Hepes buffer (50μl), (d) 0.3mM dibutyryl cyclic AMP, 24mM $MgCl_2$, 12mM ATP in hypotonic Hepes buffer (50μl), (e) cyclic AMP-dependent protein kinase in hypotonic Hepes buffer (50iu; 50μl). The total preincubation volume in each case was 200μl. Following the preincubation, the preparations were assayed for ecdysone 20-monooxygenase activity.

In this series of incubations, carried out with a "broken" mitochondrial preparation the effect was investigated of the removal by E. coli alkaline phosphatase of covalently bound phosphate from the ecdysone 20-monooxygenase, followed by reactivation of the enzyme with cyclic AMP-dependent protein kinase (from rabbit muscle) and appropriate cofactors to effect possible rephosphorylation of the monooxygenase. The initial preincubation with alkaline phosphatase

Table 5: Reactivation by exogenous protein kinase of alkaline phosphatase-incubated ecdysone 20-monooxygenase in a broken mitochondrial preparation

First preincubation (30 min.)	Second preincubation (60 min.)	Monooxygenase activity (pmol/hr. per mg protein)
(i) -	-	1.19
(ii) Alkaline phosphatase	NaH_2PO_4, EDTA	0.64
(iii) Alkaline phosphatase	-	0.73
(iv) Alkaline phosphatase	NaH_2PO_4, EDTA, dibutyryl cyclic AMP, MgATP, protein kinase	2.48
(v) Alkaline phosphatase	NaH_2PO_4, EDTA, dibutyryl cyclic AMP, MgATP	0.83
(vi) Alkaline phosphatase	NaH_2PO_4, EDTA, protein kinase	0.73

resulted in a 2-fold reduction in monooxygenase activity [(ii) and (iii), Table 5] compared to the control incubation [(i), Table 5]. The effect of the phosphatase was the same irrespective of whether or not it was inhibited by NaH_2PO_4 and EDTA (Goodwin _et al._, 1982) during the second preincubation period. Inhibition of the alkaline phosphatase with NaH_2PO_4 and EDTA after the first preincubation period followed by a second preincubation with protein kinase, dibutyryl cyclic AMP, and MgATP resulted in an almost 4-fold increase in monooxygenase activity [(iv) and (ii), Table 5]. This enhanced activity is approximately twice that of the control incubation not treated with alkaline phosphatase, presumably reflecting a greater degree of phosphorylation of the enzyme system [(i) and (iv), Table 5]. Omission of protein kinase [(v), Table 5] or of dibutyryl cyclic AMP, and MgATP [(vi), Table 5] during the second preincubation period did not result in restoration of monooxygenase activity.

3. Discussion

Fig. 1 and Fig. 2 demonstrate that ecdysone 20-monooxygenase activity is linear with respect to time and mitochondrial protein.

The foregoing data are consistent with a role for reversible phosphorylation in the modulation of ecdysone 20-monooxygenase activity. Since fluoride is a known inhibitor of phosphoprotein phosphatases (Beg and Brewer, 1981), the observation that ecdysone 20-monooxygenase activity is much higher following preincubation of "intact" mitochondria fraction with KF than with KCl [Table 1] suggests that in the absence of fluoride endogenous phosphoprotein

phosphatase partially dephosphorylates the monooxygenase leading to reduced activity. It is notable that the activity of the monooxygenase is 2-fold greater following preincubation at 4°C in the absence of KF than at 37°C in the presence of KF, the exact significance of this is uncertain [Table 2]. Since preincubation in the absence of KF resulted in low monooxygenase activity, a further effect of Mg^{2+} on phosphoprotein phosphatase was difficult to demonstrate [Table 2].

It is apparent that alkaline phosphatase appreciably reduced ecdysone 20-monooxygenase activity in fluoride-prepared broken mitochondria [Table 3]. This effect was eliminated by inhibition of the alkaline phosphatase by NaH_2PO_4 and EDTA. This supports the premise that phosphatase action on ecdysone 20-monooxygenase can decrease the activity of the latter by removal of covalently bound phosphate.

The observed increase in ecdysone 20-monooxygenase activity in "broken" mitochondria, but not "intact" ones, following preincubation with cyclic AMP-dependent protein kinase in the presence of dibutyryl cyclic AMP, MgATP and KF [Table 4], is consistent with the notion that enhanced phosphorylation of the monooxygenase results in an increase in activity. In the "intact" mitochondrial preparation, the ecdysone 20-monooxygenase system would be inaccessible to the protein kinase, thus, indicating also that the former enzymic activity is intramitochondrial. Of course, *in vivo* a cyclic AMP-dependent protein kinase would not be expected to be involved in phosphorylation of a mitochondrial protein. When covalently bound phosphate was

removed from "broken" mitochondria by incubation with alkaline phosphatase, resulting in reduced ecdysone 20-monooxygenase activity, the latter could be restored, and even enhanced, by incubation with cyclic AMP-dependent protein kinase and appropriate co-factors [Table 5]. Again, this is consistent with the feasibility of deactivation-activation of mitochondrial monooxygenase by dephosphorylation-phosphorylation.

The combined data, provide indirect evidence that the mitochondrial ecdysone 20-monooxygenase can exist in two forms, an active, phosphorylated form and an inactive, dephosphorylated form, which are interconvertible by a phosphoprotein phosphatase and a protein kinase. Although it has been possible in this work to demonstrate effects on the monooxygenase that can be ascribed to endogneous phosphoprotein phosphatase, so far, using a variety of potential effectors of protein kinase including cyclic AMP, cyclic GMP and Ca^{2+}-ionophores, we have been unable to demonstrate possible effects of such endogenous kinases on the monooxygenase. In the case of the mammalian mitochondrial enzyme systems where covalent modification has been established, the pyruvate dehydrogenase complex and the branched chain 2-oxoacid dehydrogenase complex, relevant endogenous phosphoprotein phosphatase and protein kinase activities have been demonstrated (Wieland, 1983; Jones and Yeaman, 1986). Confirmation of this hypothesis must await more definitive evidence including direct demonstration of the occurrence of an appropriate mitochondrial protein phosphatase(s) and protein kinase(s), as well as demonstration of the incorporation of labelled phosphate from ATP into a component(s) of the ecdysone 20-monooxygenase enzyme system.

It has been shown that ecdysone 20-monooxygenase has dual sub-cellular localization, but it remains to be definitively demonstrated that the microsomal ecdysone 20-monooxygenase is a distinct enzyme and not an artifact of preparation.

CHAPTER SIX

THE EFFECTS OF CYTOCHROME P-450 INHIBITORS ON THE ACTIVITY OF ECDYSONE 20-MONOOXYGENASE

1. Introduction

The multiple form, membrane-bound cytochrome P-450 corresponds to a single polypeptide that contains an iron protoporphyrin moiety located in a hydrophobic pocket of the apoprotein. Four pyrrole nitrogen atoms are bound to the iron; the fifth ligand is considered to be a thiolate anion (from a cysteine residue). The sixth coordination position is responsible for the binding of ligands such as oxygen and cyanide (Ray, 1967). The cytochrome P-450-dependent monooxygenase system catalyses a wide variety of oxidative reactions such as aliphatic oxidation, epoxidation, O-dealkylation, N-dealkylation, deamination, and sulphoxidation etc. In insect cytochrome-P-450 dependent monooxygenase systems the result of oxidation of endobiotics includes activation (e.g. ecdysone to 20-hydroxyecdysone, methylfarnesoate to juvenile hormone, etc.), or metabolism of different hormones and pheromones. The result of oxdiation of xenobiotics is metabolic detoxification or suicide activation (Hodgson, 1983).

Cytochrome P-450 isoenzymes co-exist as hydrophobic proteins in the same cell type and require membrane phospholipids to provide their functions. Most microsomal forms of cytochrome P-450 have wide substrate specificity, the rate at which a particular substrate is oxidized may differ from one substrate to another (Hodgson, 1983). Some mitochondrial cytochrome P-450 isoenzymes metabolize steroids only, and do not participate in xenobiotic metabolism (Pelkonen *et al.*, 1985). Mitochondrial forms of cytochrome P-450 generally exhibit

a high degree of specificity for substrates and in product formation (Jefcoate, 1986). In the fat body of Spodoptera littoralis, ecdysone 20-monooxygenase activity is located primarily in the mitochondria with a small, but significant, proportion apparently in the microsomal subcellular fraction (Chapter 3).

The possibilities for inhibition of cytochrome P-450 hydroxylation reactions are: 1) Electron diversion (paraquat, diquat); 2) Apoprotein interaction; 2.1) Reversible complex formation (proadifen, amphetamine, methylenedioxyphenyl); 2.2) Covalent binding to the apoprotein (zineb, parathion); 3) Catalytic site interaction; 3.1) Reversible ligand formation (metyrapone, fenarimol, triadimefon, clotrimazole); 3.2) Covalent binding to the prosthetic haeme (secobarbital, norethisterone, norgestrel, bencyclane; 4) Substrate binding site interaction (perfluorinated hydrocarbons) [Wilkinson and Murray, 1984].

Of particular interest is the inhibition of cytochrome P-450 by reversible ligand formation with haeme. A variety of nitrogen heterocycles can interact directly with the haeme moiety of cytochrome P-450 and can promote profound inhibitory effects on hydroxylation both in vitro and in vivo, several of which have been commercially developed for clinical use as antimycotic agents (eg. clotimazole, miconazole (R. Khal et al., 1980; Niemegeers et al., 1981) and histamine H_2-receptor antagonists eg. cimetidine (Knodell et al., 1982). A large number of nitrogen hetrocycle analogues have also been introduced as commercial fungicides and appear to act by inhibiting

the sterol 14-demethylase involved in fungal ergosterol biosynthesis (Baldwin, 1983). These include pyrimidines (fenarimol), imidazoles (imazalil) and triazoles (triadimefon). The mechanism of action of the nitrogen heterocycles appears to result from direct ligand interaction between sp^2 or sp^3 electrons of the nitrogen atom with the fifth or sixth ligand of the haeme iron of cytochrome P-450, the same ligand responsible for oxygen binding (Wilkinson _et al._, 1972). The ligand binding to cytochrome P-450 depends primarily on the presence of a relatively unhindered nitrogen atom (Rogerson _et al._, 1977). Lipophlicity also strongly influences inhibitory potency, which suggests hydrophobic bonding is an additional determinant of binding efficiency (Murray _et al._, 1982).

In this Chapter, the effect of various cytochrome P-450 inhibitors on the ecdysone 20-monooxygenase system was investigated in a fat body broken mitochondrial plus microsomal preparation.

2. Experimental and Results

The cytochrome P-450 inhibitors were divided into three groups for convenience according to the industrial or academic establishment(s) which kindly provided them.

A. Ecdysone 20-monooxygenase incubations

In all the ecdysone 20-monooxygenase incubations in this Chapter, the fat body mitochondrial plus microsomal fraction prepared in 0.15M

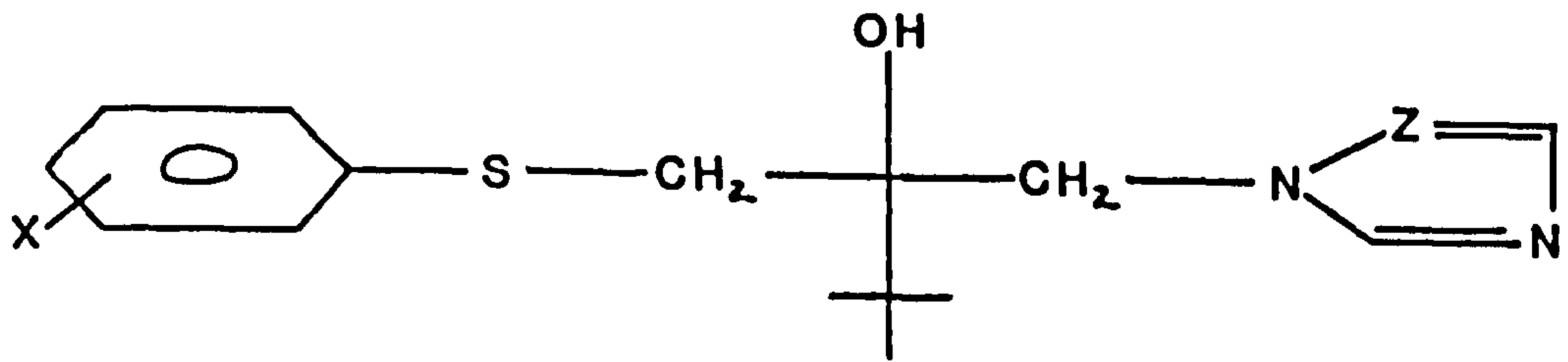
KF in isotonic Hepes buffer was resuspended in 0.15M KF in hypotonic Hepes buffer and preincubated for 15 min. at 4°C to allow tho mitochondria to burst. Aliquots were then preincubated for 15 min. at 37°C with the individual cytochrome P-450 inhibitors, together with control incubations containing Hepes buffer and 0.75% aqueous methanol. All the inhibitors were solubilized in 0.75% aqueous methanol. After the preincubation, the whole preparations were then assayed for ecdysone 20-monooxgyenase activity.

B. Group I Inhibitors

The group I inhibitors were investigated at a final concentration in the monooxygenase assay of 10ppm (parts per million). The broken mitochondrial plus microsomal fraction was prepared as described previously. Aliquots (100μl) were preincubated as stated with the addition [Table 1] of the following: (i) hypotonic Hepes buffer (100μl), (ii) 0.75% aqueous methanol (100μl), (iii) to (x) inhibitors 1-8, respectively, (100μl) at a concentration of 30ppm. All incubations were then assayed for ecdysone 20-monooxygenase activity. Inhibitors 1-8 were kindly provided by Rhone-Poulenc Ltd. and their structures are given in Fig. 1.

The 0.75% aqueous methanol [incubation (ii), Table 1] appears to have a nominal effect on the ecdysone 20-monooxygenase activity compared with the control incubation [(i), Table 1], but this is within experimental error. Inhibitor 8 (propiconazole), Inhibitor 4 and Inhibitor 5 were the most potent inhibitors giving 88%, 85% and

Fig 1 Group I inhibitor structures (provided by Rhone-Poulenc Ltd)

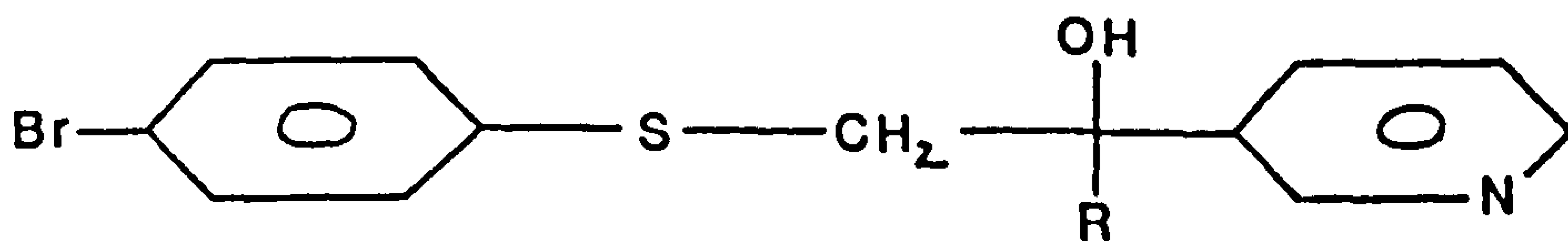


Inhibitor 1: X= 4-Cl Z= N

Inhibitor 2: X= 4-CH Z=N

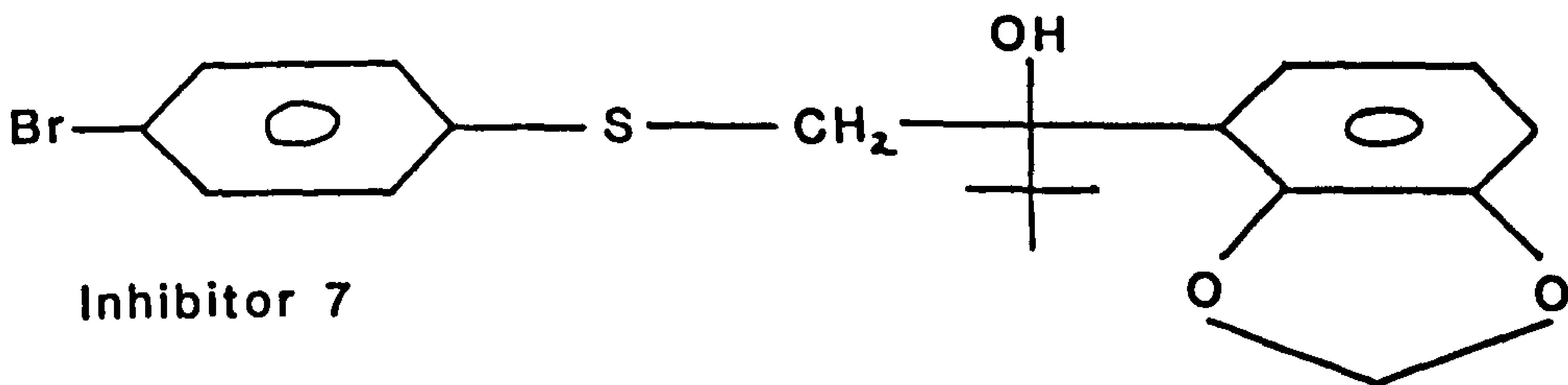
Inhibitor 3: X= 3-CH Z=N

Inhibitor 4: X= 4-Br Z=CH

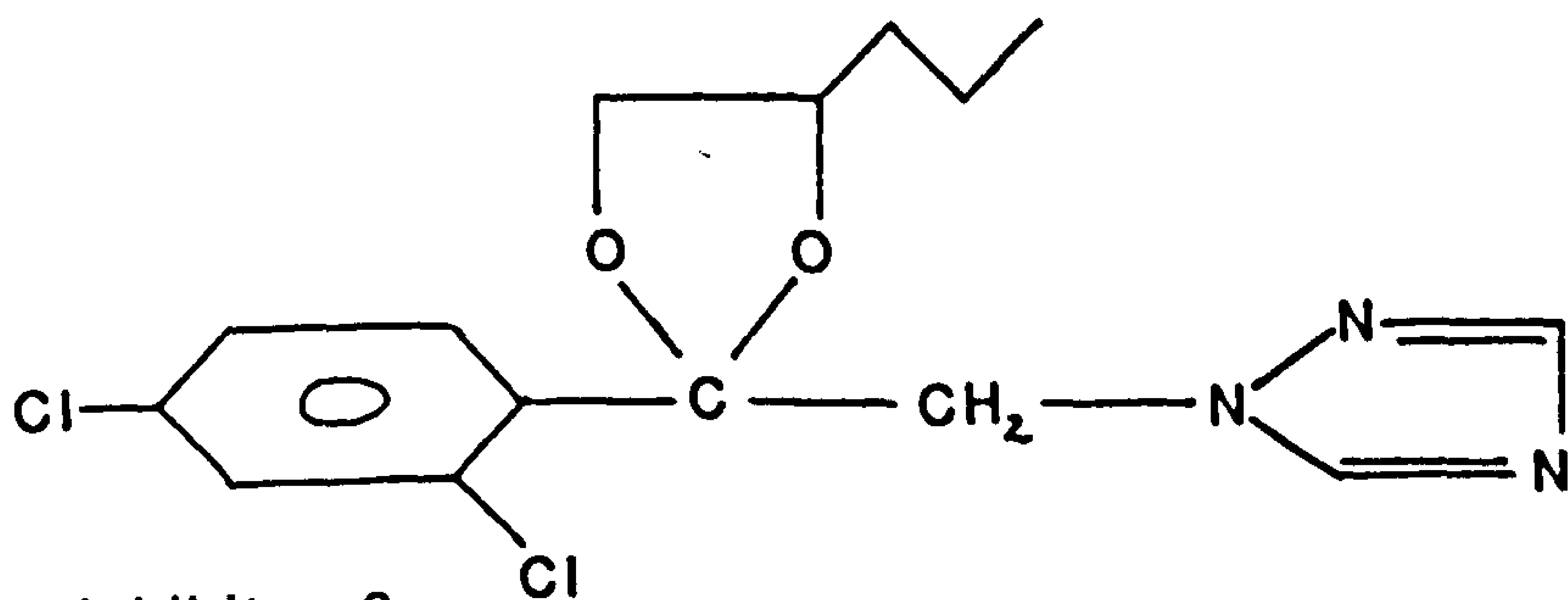


Inhibitor 5: R= sec-butyl

Inhibitor 6: R= ter-butyl



Inhibitor 7



Inhibitor 8 (propiconazole)

Table 1 <u>Effect of the presence of Group I inhibitors on the activity of ecdysone 20-monooxygenase at a final concentration of 10ppm</u>

Preincubation conditions (15 min.)		Monooxygenase activity (pmole/hr. per mg protein)	Percentage inhibition of monooxygenase activity
(i)	-	3.01	0
(ii)	0.75 aqueous methanol	2.84	0
(iii)	Inhibitor 1	1.26	56
(iv)	Inhibitor 2	1.04	64
(v)	Inhibitor 3	1.20	58
(vi)	Inhibitor 4	0.44	85
(vii)	Inhibitor 5	0.38	86
(viii)	Inhibitor 6	1.86	35
(ix)	Inhibitor 7	2.73	3
(x)	Inhibitor 8	0.36	88

86% inhibition, respectively, of the monooxygenase activity [(x), (vi) and (vii), Table 1]. Inhibitors 1 to 3 all gave in the region of 50% inhibition of activity [(iii), (iv) and (v), Table 1] with inhibitor 6 giving 35% inhibition of monooxgyenase activity [(viii), Table 1]. The least potent inhibitor, having a negligible effect, was inhibitor 7 which only gave 3% inhibition of enzymic activity [(ix), Table 1]. This is also the only inhibitor without a nitrogen atom (Fig. 1).

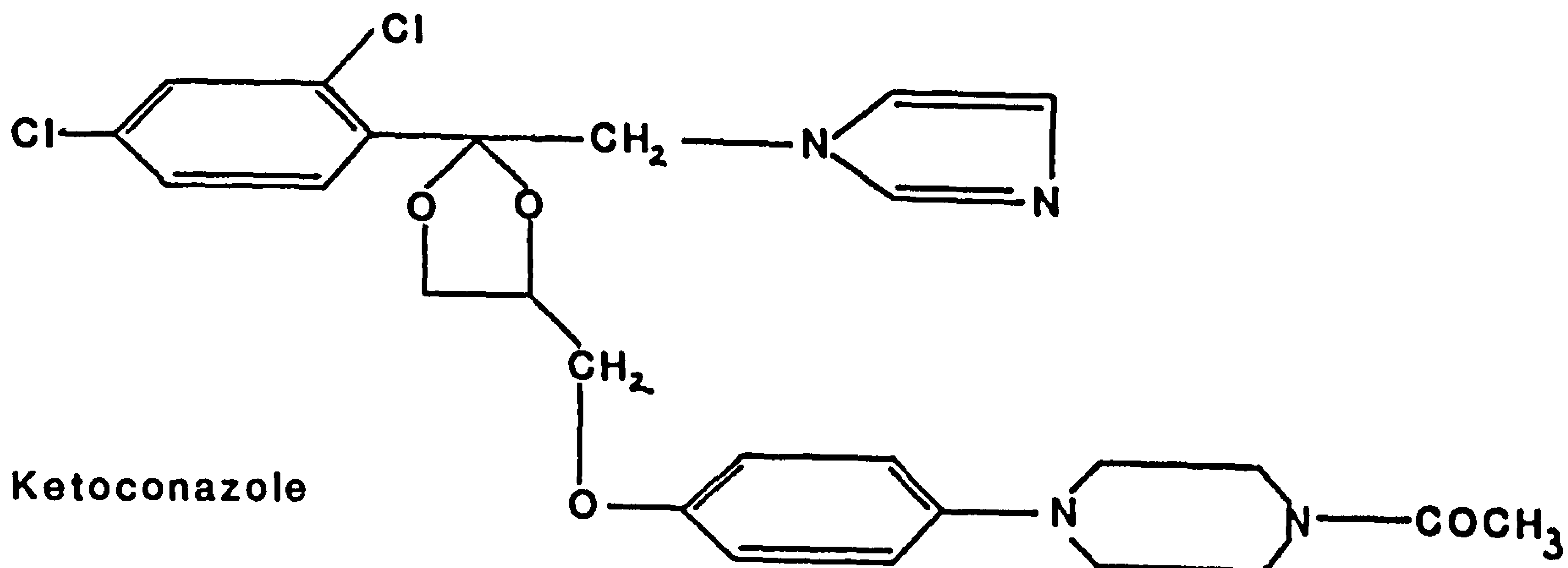
C. Group II inhibitors

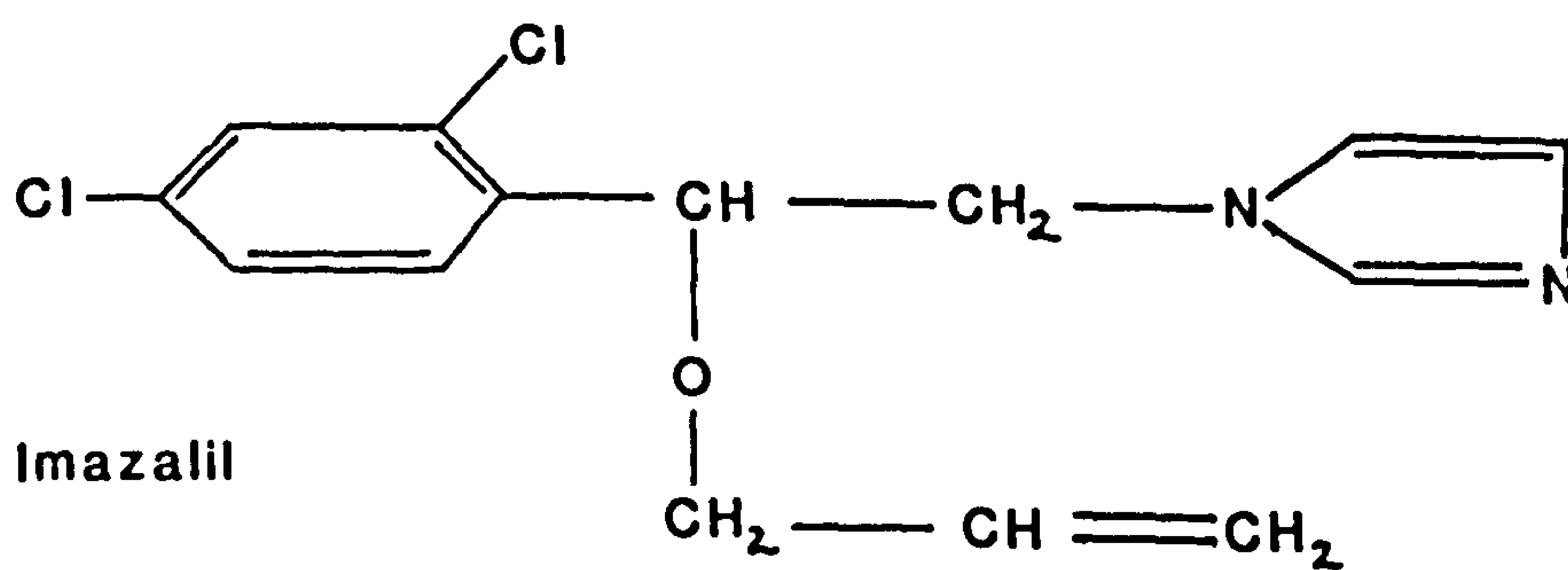
I. Effect of Group II inhibitors at a final concentration in the monooxygenase assay of 10ppm

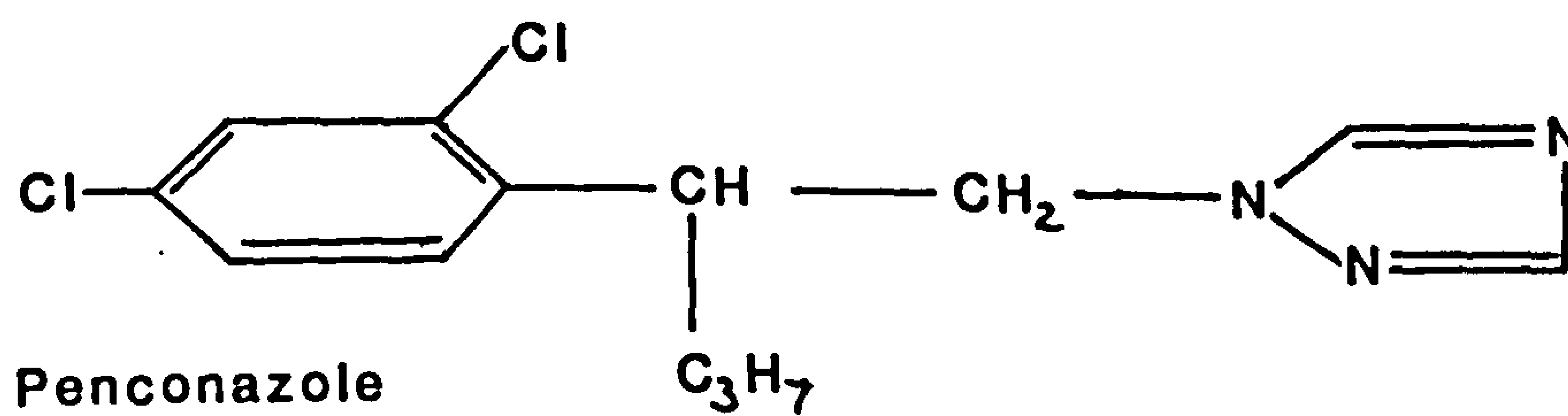
The broken mitochondrial plus microsomal fraction was prepared as described previously. Aliquots (100μl) were preincubated as stated with the addition [Table 2] of the following: (i) hypotonic Hepes buffer (100μl), (ii) 0.75% aqueous methanol (100μl), (iii) to (viii) various inhibitors (100μl) at a concentration of 30ppm. All incubations were then assayed for ecdysone 20-monooxygenase activity.

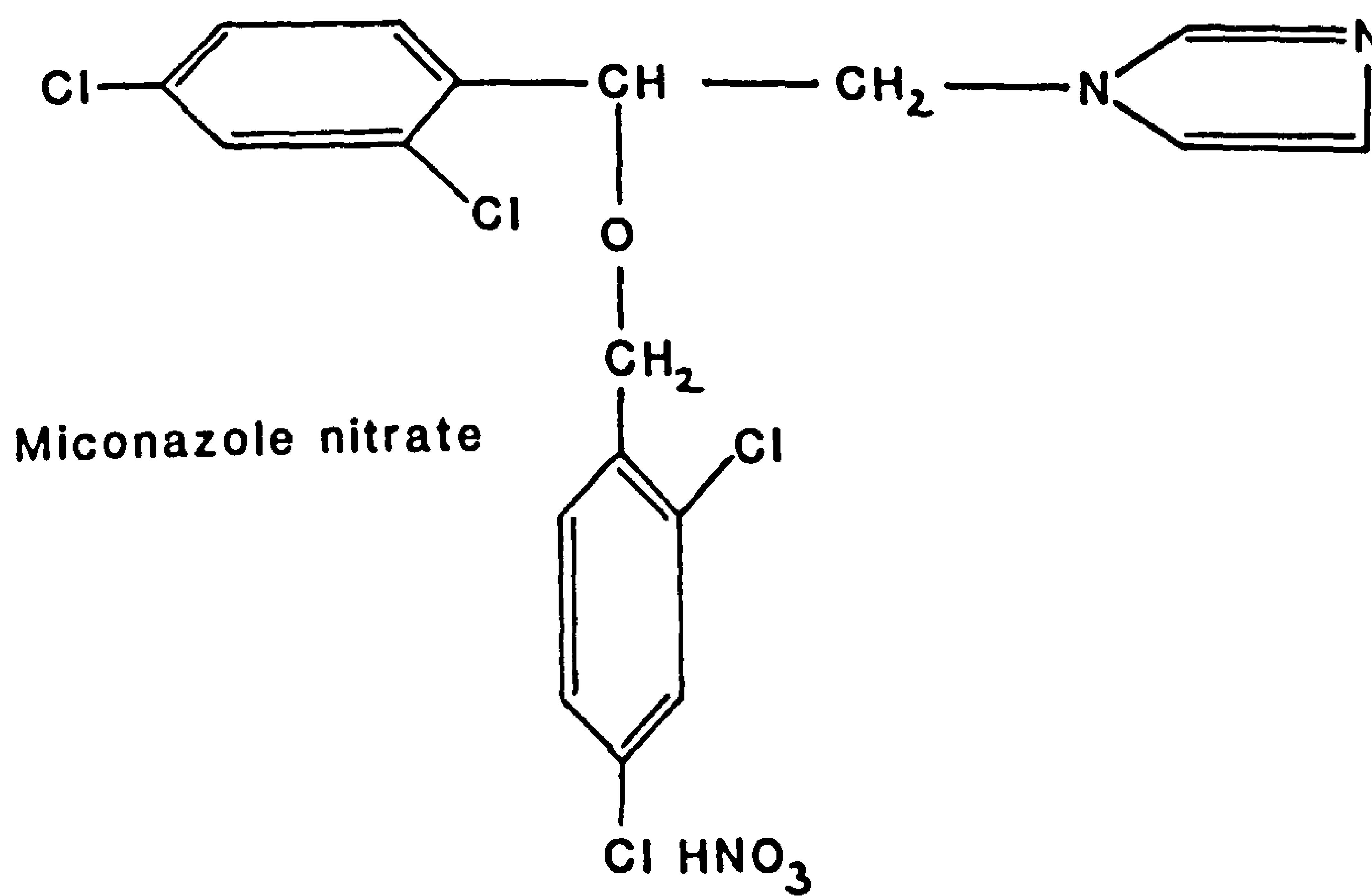
Ketoconazole, imazalil, azaconazole, penconazole and miconazole nitrate were kindly provided by Janssen Pharmaceuticals (Vanden-Bossche *et al*., 1987; Hart *et al*., 1988) and S.S.P.-11 was kindly provided by Dr. H. Akai. The structures of the inhibitors, with the exception of S.S.P.-11, are given in Fig. 2.

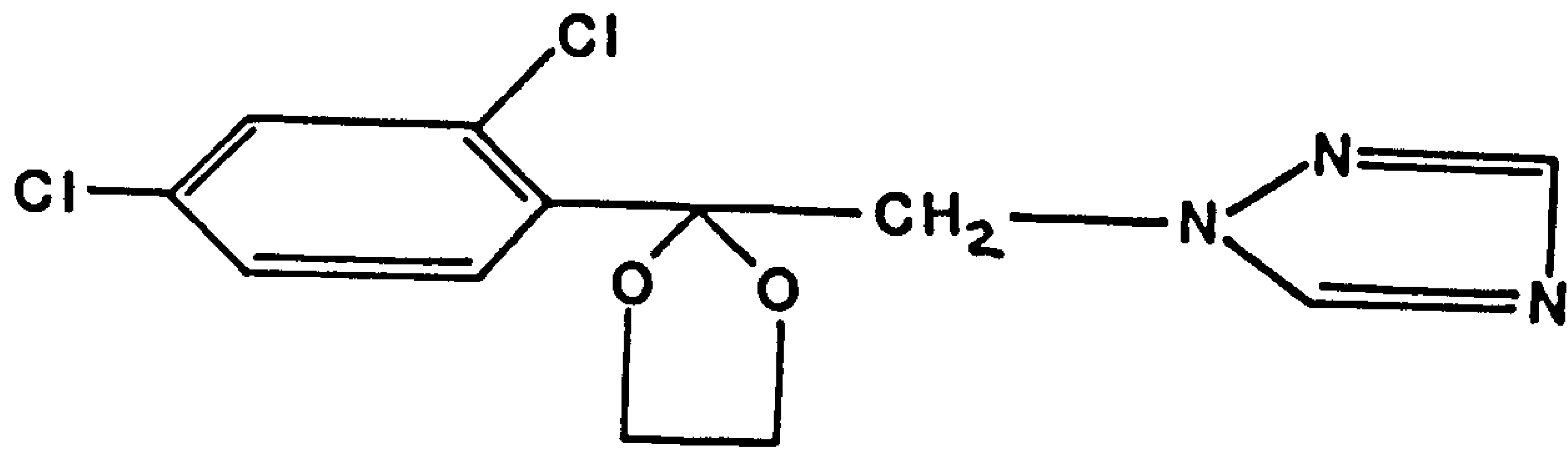
Fig 2. Group II inhibitor structures











Azaconazole

Table 2 **Effect of the presence of Group II inhibitors on the activity of ecdysone 20-monooxygenase at a final concentration of 10ppm**

Preincubation conditions (15 min.)		Monooxygenase activity (pmole/hr. per mg protein)	Percentage inhibition of monooxygenase activity
(i)	-	2.21	0
(ii)	0.75% aqueous methanol	1.86	0
(iii)	Ketoconazole	0.35	81
(iv)	Imazalil	0.35	81
(v)	S.S.P.-11	0.88	53
(vi)	Azaconazole	0.78	58
(vii)	Penconazole	0.65	65
(viii)	Miconazole nitrate	2.20	0

Again, the 0.75% aqueous methanol [incubation (ii), Table 2] appears to have a nominal effect on the ecdysone 20-monooxygenase activity compared with the control incubation [(i), Table 2] but this is within experimental error.

Ketoconazole and imazalil were the most potent of all the inhibitors giving 81% inhibition of the monooxygenase activity [(iii) and (iv), Table 2]. Penconazole gives 65% inhibition of the monooxygenase activity at 10ppm [incubation (vii), Table 2]. S.S.P.-11 and azaconazole both give in the region of 50% inhibition of the monooxygenase activity [(v) and (vi), Table 2]. Miconazole nitrate appears to have no effect on the monooxygenase activity at 10ppm, making it the least potent inhibitor [(viii), Table 2].

II. Effect of the most potent Group II inhibitors at a final concentration in the monooxygenase assay of 1ppm

The broken mitochondrial plus microsomal fraction was prepared as described previously. Aliquots (100µl) were preincubated as stated with the addition [Table 3] of the following: (i) hypotonic Hepes buffer (100µl), (ii) 0.75% aqueous methanol (100µl); (iii) hypotonic Hepes buffer (100µl), (iv) to (vi) various inhibitors (100µl) at a concentration of 3ppm. All incubations were then assayed for ecdysone 20-monooxygenase activity.

Table 3 Effect of the presence of ketoconazole, imazalil and S.S.P.-11 on the activity of ecdysone 20-monooxygenase at a final concentration of 1ppm

Preincubation conditions (15 min.)		Monooxygenase activity (pmole/hr. per mg protein)	Percentage inhibition of monooxygenase activity
(i)	-	2.32	0
(ii)	0.75% aqueous methanol	2.24	0
(iii)	inactivated monooxygenase	0.06	100
(iv)	Ketoconazole	0.37	86
(v)	Imazalil	0.37	86
(vi)	S.S.P.-11	1.29	44

In incubation (iii) [Table 3], before preincubation the subcellular preparation was heated for 10 min. at 100°C to destroy the enzymic activity.

Again, the 0.75% aqueous methanol [incubation (ii), Table 3] appears to have a nominal effect on the ecdysone 20-monooxygenase activity compared with the control incubation [(i), Table 3] but this is within experimental error. Incubation (iii) [Table 3] in which the ecdysone 20-monooxygenase has been inactivated gives the basal monooxygenase activity and can be regarded as 100% inhibition. This is used as a basis when calculating the percentage inhibition of the monooxygenase activity by the inhibitors in Table 3. The monooxygenase activity present is presumably due to some inefficiency in the experimental separation of ecdysone and 20-hydroxyecdysone in the monooxygenase assay.

Ketoconazole and imazalil were the most potent of the inhibitors both giving 86% inhibition at a concentration as low as 1ppm [(iv) and (v), Table 3]. The lower percentage inhibition at the higher concentration of 10ppm [(iii) and (iv), Table 2] may be due to differences in the specific activity of the ecdysone 20-monooxygenase activity in the two experiments (see Chapter 3). S.S.P.-11 gives 44% inhibition of the monooxygenase activity at the lower inhibitor concentration of 1ppm [(vi), Table 3].

III. Effect of different concentrations of ketoconazole on the monooxygenase assay

The broken mitochondrial plus microsomal fraction was prepared as described previously. Aliquots (100μl) were preincubated as stated with the addition [Table 4] of the following: (i) hypotonic Hepes buffer (100μl), (ii) 0.75% aqueous methanol (100μl), (iii) hypotonic Hepes buffer (100μl), and (iv) to (viii) ketoconazole at a concentration of 30,3, 0.3, 0.03 and 0.003ppm, respectively (100μl) (concentrations given are those solutions added). All incubations were then assayed for ecdysone 20-monooxygenase activity.

In incubation (iii) [Table 4], before preincubation, the subcellular preparation was heated for 10 min. at 100°C to destroy the enzymic activity.

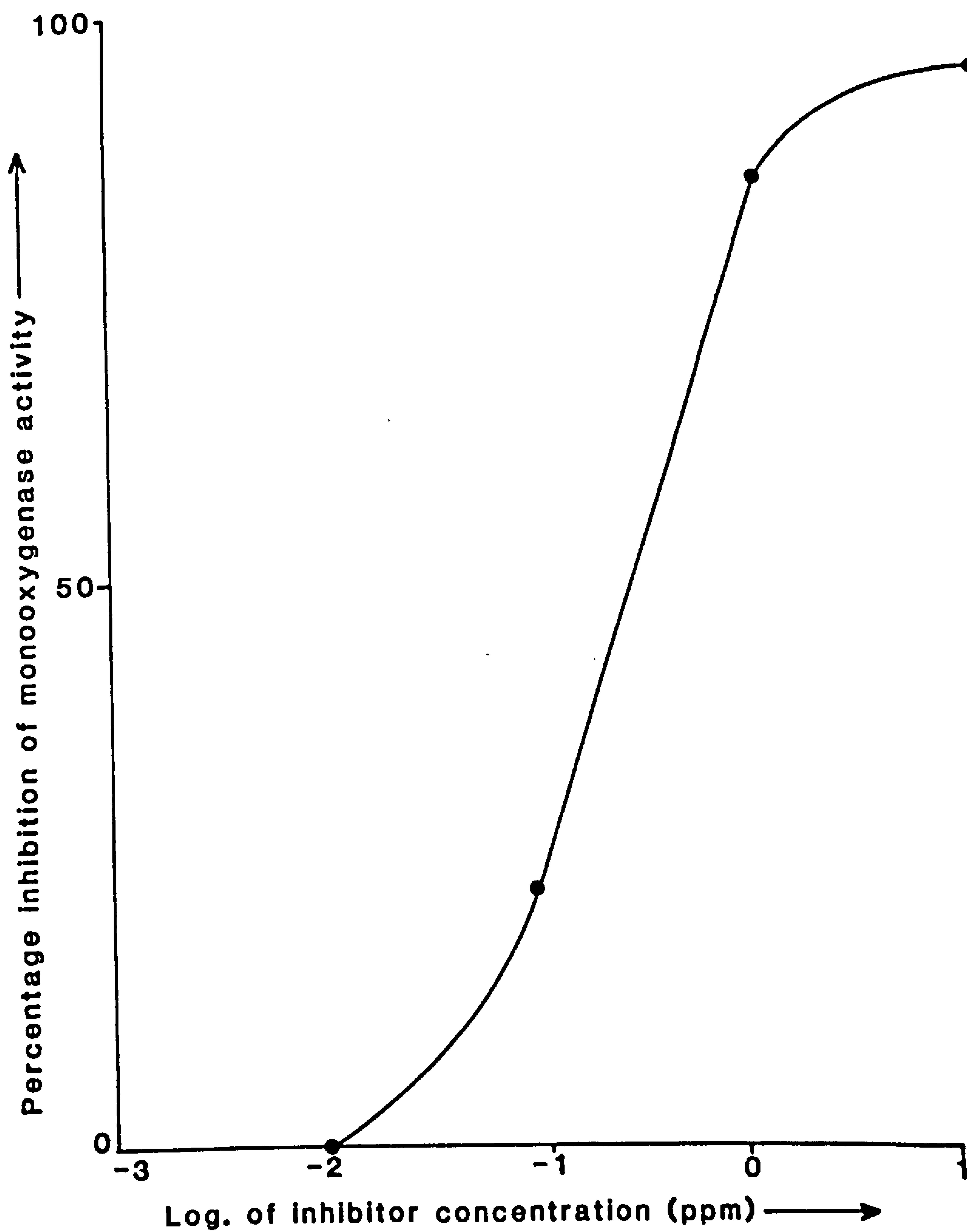
The 0.75% aqueous methanol [incubation (ii), Table 4] has a negligible effect on the ecdysone 20-monooxygenase activity compared with the control incubation [(i), Table 4]. Incubation (iii) [Table 4], in which the ecdysone 20-monooxygenase has been inactivated gives the basal monooxygenase activity and can be regarded as 100% inhibition. This is used as a basis when calculating the percentage inhibition of the monooxygenase activity by the inhibitors in Table 4. As expected ketoconazole shows a decrease in the percentage inhibition of ecdysone 20-monooxygenase activity with decreasing inhibitor concentration (Fig. 3, Table 4]. Ketoconazole has an I_{50} value of 0.26ppm (Fig. 3).

Table 4 **Effect of the presence of different concentrations of ketoconazole on the activity of ecdysone 20-monooxygenase**

Preincubation conditions[a] (15 min.)	Monooxygenase activity (pmole/hr. per mg protein)	Percentage inhibition of monooxygenase activity
(i) -	4.16	0
(ii) 0.75% aqueous methanol	4.14	0
(iii) inactivated monooxygenase	0.06	100
(iv) 10ppm	0.13	98
(v) 1ppm	0.52	89
(vi) 0.1ppm	3.20	23
(vii) 0.01ppm	4.14	0
(viii) 0.001pm	4.09	1

[a]Inhibitor concentrations given are the final concentrations in the monooxygenase assay.

Fig 3. Effect of the presence of different concentrations of ketoconazole on the activity of ecdysone 20-monooxygenase.



IV <u>Effect of different concentrations of imazalil on the monooxygenase assay</u>

The broken mitochondrial plus microsomal fraction was prepared as described previously. Aliquots (100μl) were preincubated as stated with the addition [Table 5] of the following: (i) hypotonic Hepes buffer (100μl), (ii) 0.75% aqueous methanol (100μl), (iii) hypotonic Hepes buffer (100μl) and (iv) to (viii) imazalil at a concentration of 30, 3, 0.3, 0.03 and 0.003ppm, respectively (100μl) (concentrations given are those solutions added). All incubations were then assayed for ecdysone 20-monooxygenase activity.

In incubation (iii) [Table 5], before preincubation, the subcellular preparation was heated for 10 min. at 100°C to destroy the enzymic activity.

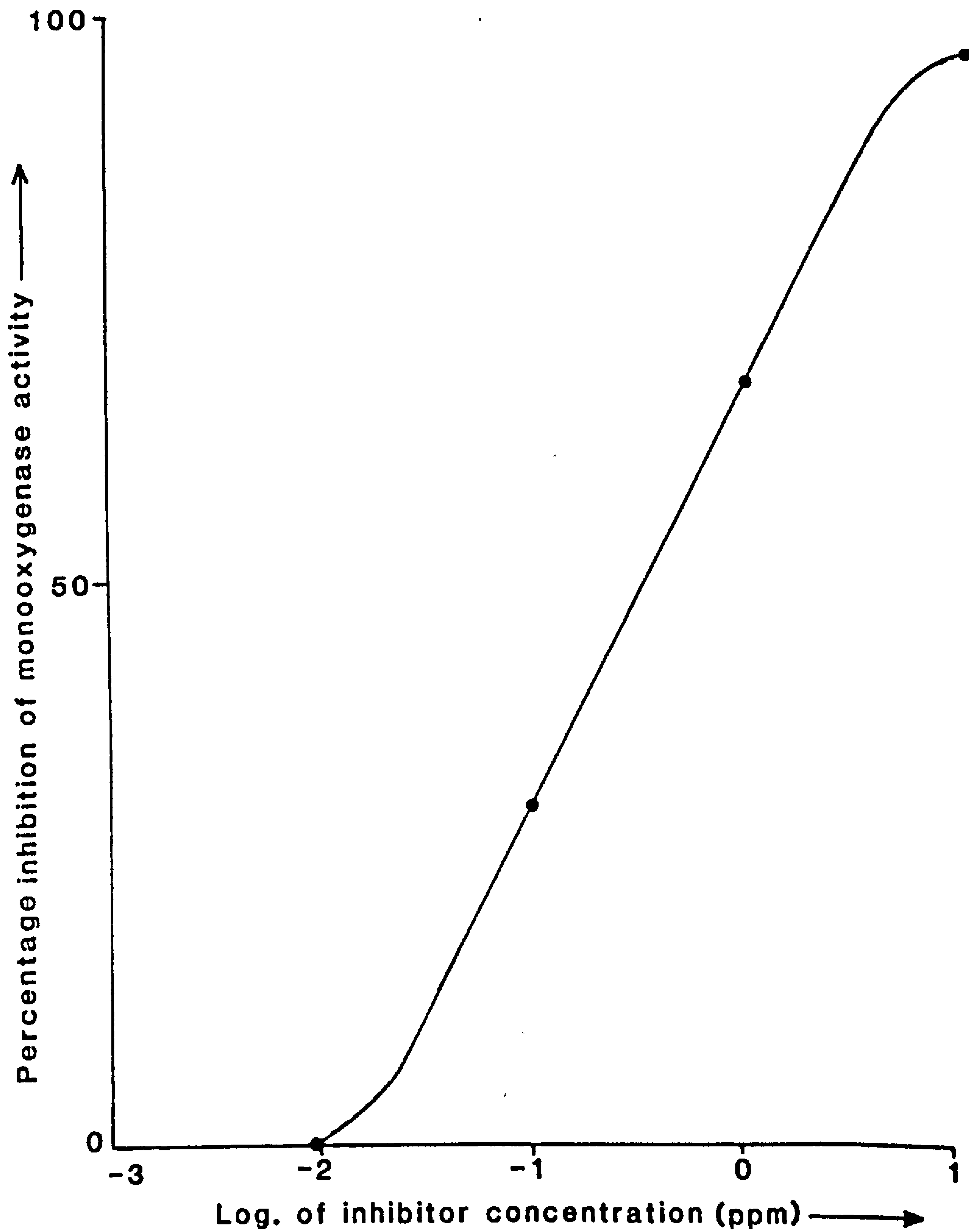
The 0.75% aqueous methanol [incubation (ii), Table 5] has a negligible effect on the ecdysone 20-monooxygenase activity compared with the control incubation [(i), Table 5]. Incubation (iii) [Table 5], in which the ecdysone 20-monooxygenase has been inactivated gives the basal monooxygenase activity and can be regarded as 100% inhibition. This is used as a basis when calculating the percentage inhibition of the monooxygenase activity by the inhibitors in Table 5. As expected imazalil shows a decrease in the percentage inhibition of the ecdysone 20-monooxygenase activity with decreasing inhibitor concentration [Fig. 4, Table 5]. Imazalil has an I_{50} value of 0.32ppm (Fig. 4) which is similar to that for ketoconazole (Fig. 3).

Table 5 Effect of the presence of different concentrations of imazalil on the activity of ecdysone 20-monooxygenase

Preincubation conditions[a] (15 min.)		Monooxygenase activity (pmole/hr. per mg protein)	Percentage inhibition of monooxygenase activity
(i)	-	4.84	0
(ii)	0.75% aqueous methanol	4.60	0
(iii)	inactivated monooxygenase	0.17	100
(iv)	10ppm	0.23	99
(v)	1ppm	1.55	69
(vi)	0.1ppm	3.29	30
(vii)	0.01ppm	4.58	0
(viii)	0.001ppm	4.81	0

[a]Inhibitor concentrations given are the final concentrations in the monooxygenase assay.

Fig 4.Effect of the presence of different concentrations of imazalil on the activity of ecdysone 20-monooxygenase.

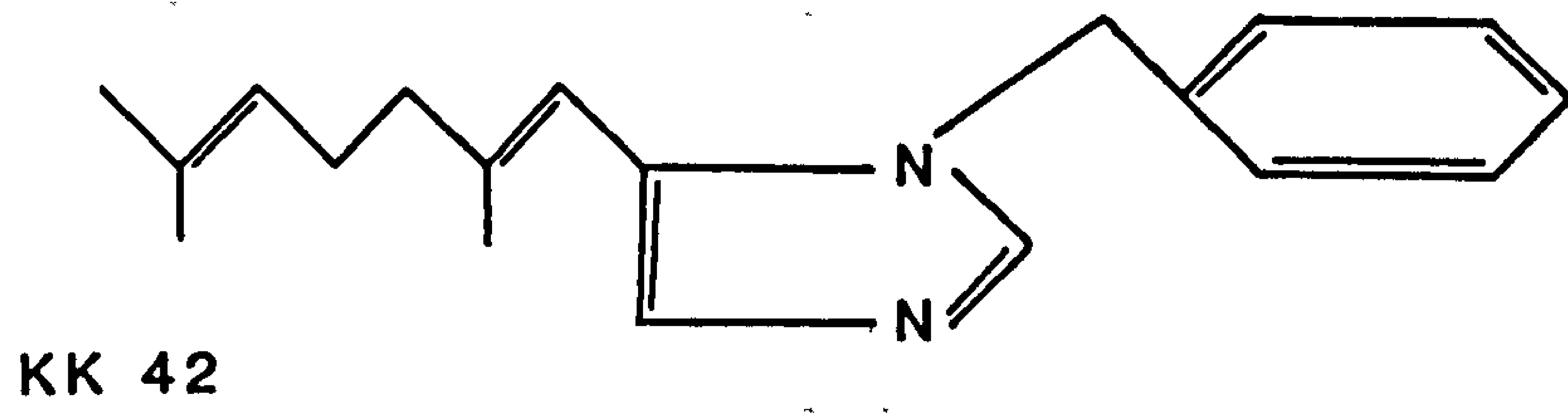


D. <u>Group III inhibitors</u>

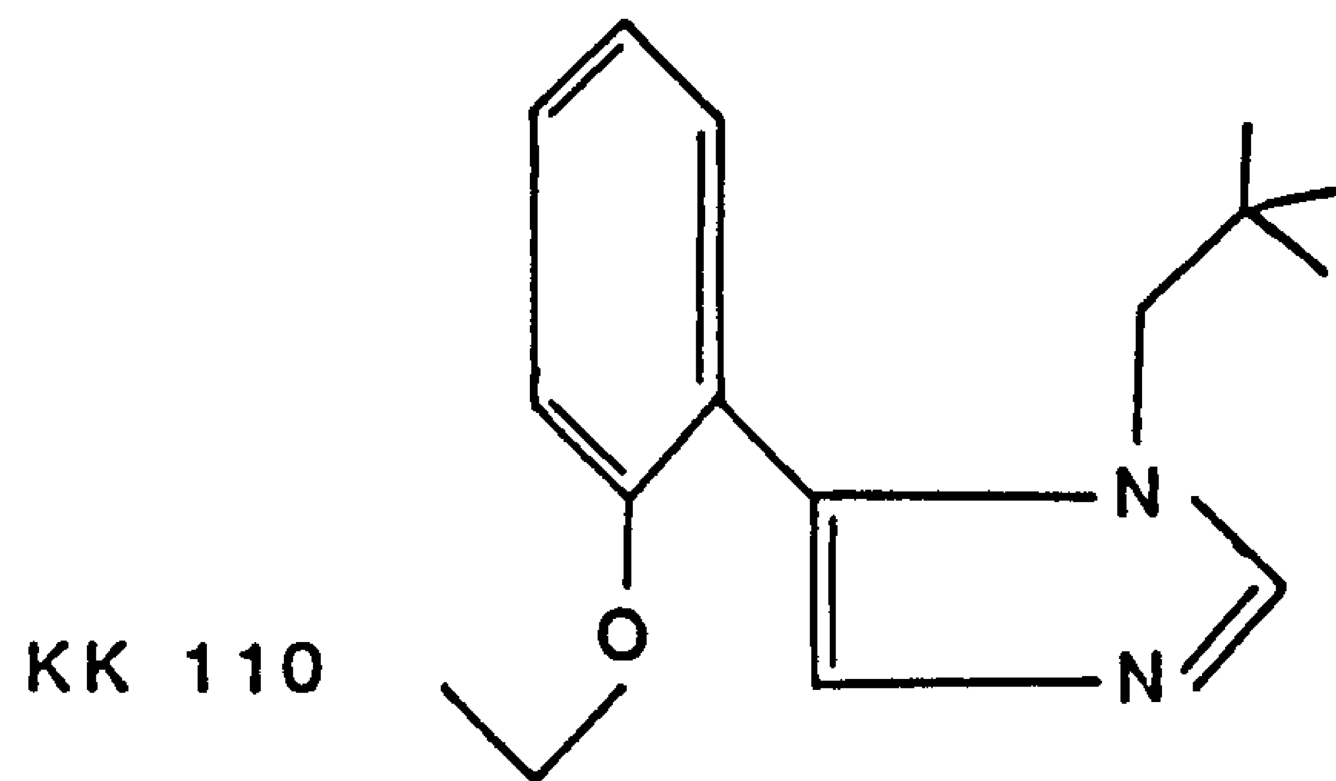
The group III inhibitors were investigated at a final concentration in the monooxygenase assay of 1ppm. The broken mitochondrial plus microsomal fraction was prepared as described previously. Aliquots (100µl) were preincubated as stated with the addition [Table 6] of the following: (i) hypotonic Hepes buffer (100µl), (ii) 0.75% aqueous methanol (100µl), (iii) hypotonic Hepes buffer (100µl), (iv) to (vii) various inhibitors (100µl) at a concentration of 3ppm. All incubations were then assayed for ecdysone 20-monooxygenase activity. KK-42, KK-110 and KK-135 were kindly provided by the Department of Agricultural Chemistry, Kyushu University, Japan (Yamashita *et al.*, 1987; Kadono-Okuda *et al.*, 1987; Roussel *et al.*, 1987; Kuwano *et al.*, 1988). PIM was kindly provided by the Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary (Napoli and Counsell, 1977; Fisher and Mayer, 1982; Belai *et al.*, 1988). The structures of all group III inhibitors are given in Fig. 5.

In incubation (iii) [Table 6], before preincubation, the subcellular preparation was heated for 10 min. at 100°C to destroy the enzymic activity. Again, the 0.75% aqueous methanol [incubation (ii), Table 6] appears to have a nominal effect on the ecdysone 20-monooxygenase activity compared with the control incubation [(ii), Table 6]. Incubation (iii) [Table 6] in which the ecdysone 20-monooxygenase has been inactivated gives the basal monooxygenase activity and can be regarded as 100% inhibition. This is used as a basis when calculating the percentage inhibition of the monooxygenase activity by the inhibitors in Table 6.

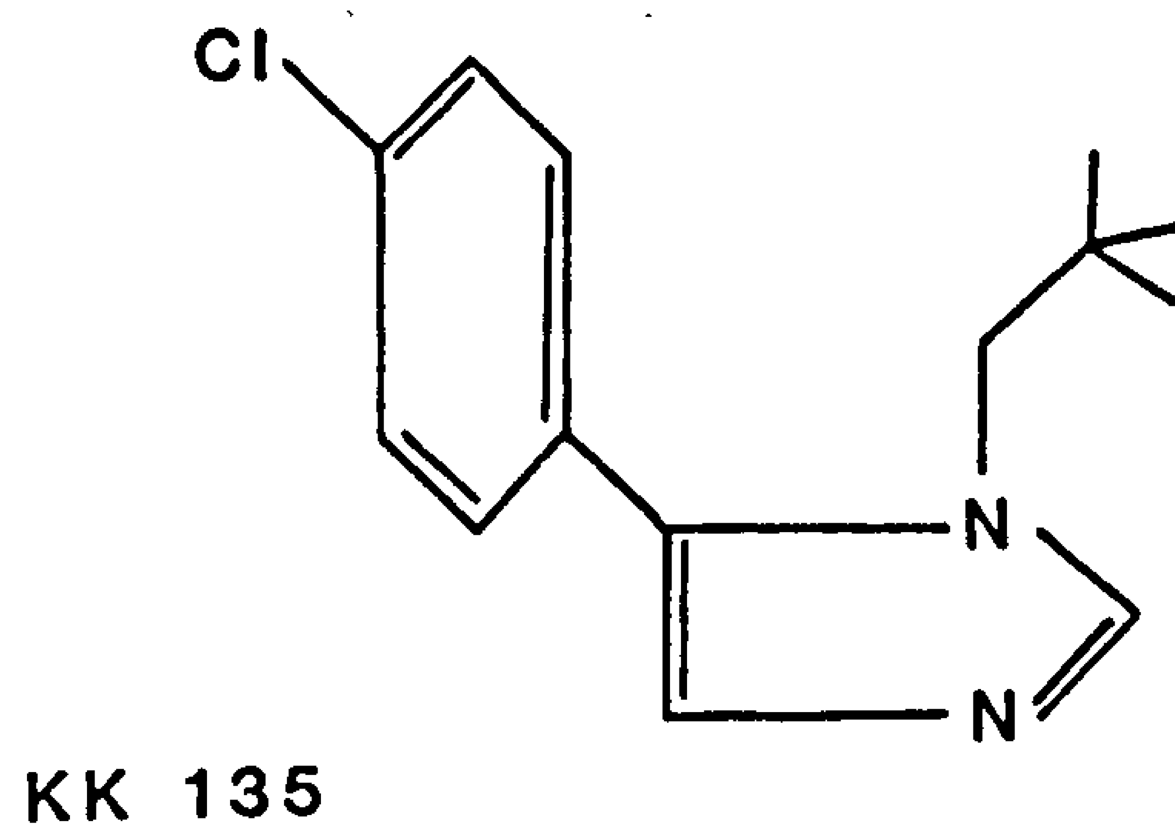
Fig 5. Group III inhibitor structures



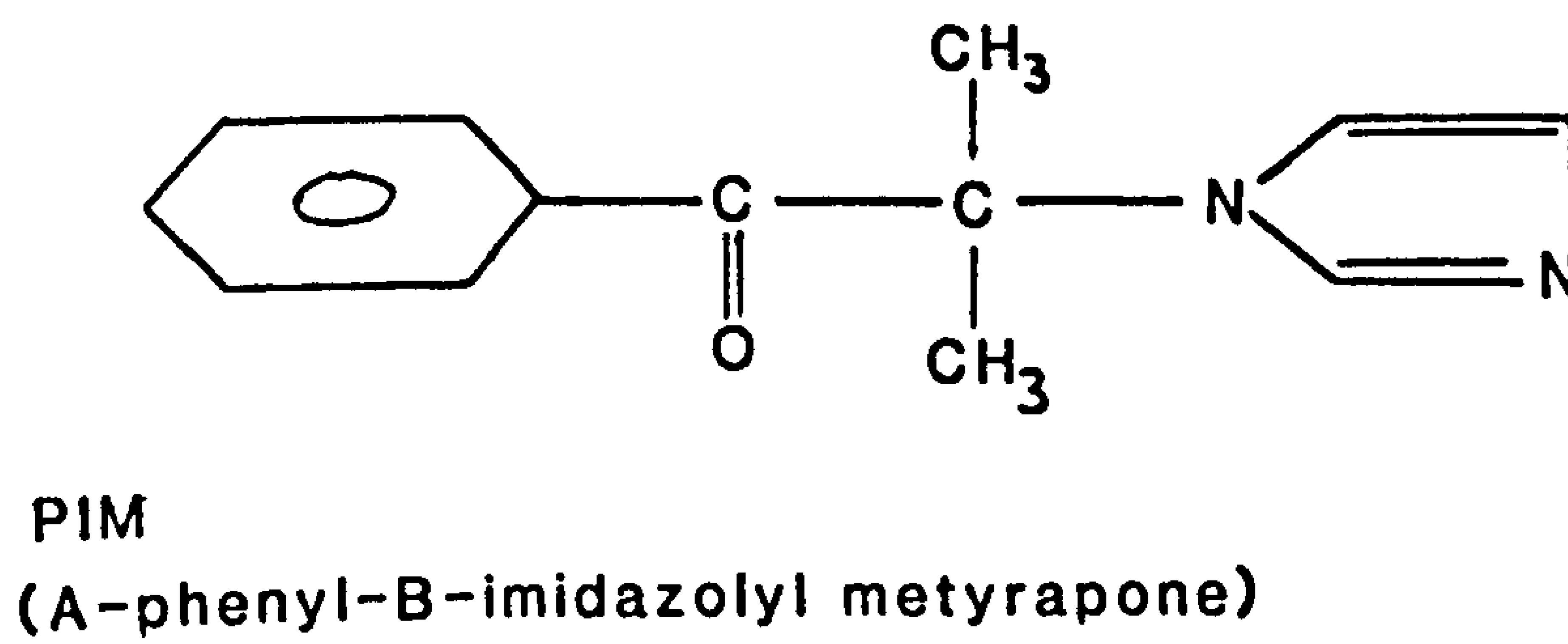
KK 42



KK 110



KK 135



PIM
(A-phenyl-B-imidazolyl metyrapone)

Table 6 **Effect of the presence of Group III inhibitors on the activity of ecdysone 20-monooxygenase at a final concentration of 1ppm**

Preincubation conditions (15 min.)		Monooxygenase activity (pmole/hr. per mg protein)	Percentage inhibition of monooxygenase activity
(i)	-	2.32	0
(ii)	0.75% aqueous methanol	2.24	0
(iii)	inactivated monooxygenase	0.06	100
(iv)	KK-42	2.28	0
(v)	KK-110	0.52	79
(vi)	KK-135	1.06	54
(vii)	PIM	0.86	63

KK-110 is the most potent inhibitor [(v), Table 6] giving 79% inhibition of monooxygenase activity at a concentration of 1ppm. KK-135 and PIM give 54% and 63% inhibition of monooxygenase activity [(vi) and (vii), Table 6], while KK-42 has no inhibitory effect at a concentration of 1ppm [(iv), Table 6].

3. Discussion

Little information is available on the group I inhibitors, but with the exception of inhibitor 7, which has a negligible effect on the ecdysone 20-monooxygenase activity [Table 1] at a concentration of 10ppm, all are nitrogen heterocycles. A variety of nitrogen heterocycles are known to interact directly, through reversible binding with the haeme moiety of cytochrome P-450 and can thus promote inhibitory effects (Wilkinson and Murray, 1984). Two of the most potent inhibitors in group I, inhibitor 4 and inhibitor 8 [Table 1] are azoles. Inhibitor 8 is propiconazole which is marketed commercially as a cereal fungicide.

All the group II inhibitors with the exception of S.S.P.-11, are azoles and can thus interact directly with the haeme moiety of cytochrome P-450. These azoles are all antifungal agents and have been shown in fungi to inhibit the cytochrome P-450-dependent 14α-demethylation of lanosterol or 24-methylenedihydrolanosterol (Vanden-Bossche _et al._, 1987).

The two most potent inhibitors in this group are ketoconazole and imazalil which have I_{50} values of 0.26 and 0.32ppm, respectively,

for the inhibition of ecdysone 20-monooxygenase activity [Table 6 and 7]. Ketoconazole is an oral azole antimycotic used in the treatment of mycoses. It is a potent inhibitor of cytochrome P-450-dependent lanosterol 14α-demethylase in Saccharomyces cerevisiae (Vanden-Bossche et al., 1985) and Candida albicans (Vanden-Bossche et al., 1980). At higher concentrations, ketoconzole also affects the microsomal sterol 17,20-lyase system in rat testis (Vanden-Bossche et al., 1985) and the lanosterol 14α-demethylase system in rat liver microsomes (Willemsens et al., 1980). Imazalil is used in seed treatment as a fungicide. The other azoles present in group II also effectively inhibit ecdysone 20-monooxgyenase activity with the exception of miconazole nitrate which has no inhibitory effect [Table 2].

In the group III inhibitors, KK-42, KK-110 and KK-135 are all 1,5-disubstituted imidazoles. All three inhibitors have been demonstrated to possess anti-juvenile hormone and anti-ecdysteroid action (Kuwano et al., 1988). In particular, KK-42 has been reported to inhibit ecdysteroid synthesis in the prothoracic glands of Bombyx mori and depressed the total amount of ecdysteroids released by the prothoracic glands of Locusta migratoria in vitro (Yamashita et al., 1987; Kadono-Okuda et al., 1987; Roussel et al., 1987). In contrast to these findings, KK-42 had no affect on the ecdysone 20-monooxygenase activity at a concentration of 1ppm [Table 6], but KK-110 and KK-135 gave 79% and 54% inhibition, respectively, of the monooxygenase activity at the same concentration [Table 6]. It has been suggested that KK-42, KK-110 and KK-135 may act via the imadazole part of the molecule on the cytochrome P-450 isoenzymes involved in methylfarnesoate epoxidation and ecdysone 20-hydroxylation (Kando-Okuda et al., 1987).

Metyrapone is a pyridine type cytochrome P-450 inhibitor (Liebman, 1969). The B-pyridyl ring of metyrapone interacts directly with the haeme moiety of cytochrome P-450. The A ring of metyrapone can be replaced with a phenyl ring, without loss of activity, to give the fourth inhibitor of Group III, A-phenyl B-triazolyl metyrapone [PIM] (Napoli and Counsell, 1977). Metyrapone, in particular, and PIM have been demonstrated to be cytochrome P-450 inhibitors in a number of insect species affecting both ecdysteroids and juvenile hormones (Bollenbacher et al., 1977b; Feyereisen and Durst, 1978; Fisher and Mayer, 1982; Belai et al., 1988). PIM gives 63% inhibition of the ecdysone 20-monooxygenase activity at a concentration of 1ppm [Table 6].

In conclusion, all the most active inhibitors tested are nitrogen heterocycles. Thus, they probably interact directly, through reversible ligand formation, with the haeme moiety of cytochrome P-450. Many of the inhibitors are closely related and their different potency might be connected with the different secondary structure of the apoprotein of the cytochrome P-450 isoenzymes, which are integral membrane globular proteins (Hudecek and Anzenbacher, 1985). Thus, some substrates/inhibitors can reach via the substrate channel(s), the substrate-binding site in the protein, while others cannot.

CHAPTER SEVEN

PROFILE OF ECDYSTEROIDS DURING OOGENESIS AND EMBRYOGENESIS

1. Introduction

It is now well established that ecdysteroids not only occur in immature stages of insects, but also in the ovaries of maturing adult females and in the eggs (Hoffmann and Lagueux, 1985).

Insect ovaries and eggs contain a wide variety of ecdysteroids including ecdysone, 2-deoxyecdysone, 20-hydroxyecdysone, 26-hydroxyecdysone and 20,26-dihydroxyecdysone either as conjugates, primarily phosphate, or in the free form (Hagedorn, 1985). Ecdysteroid 22-fatty acyl esters have also been isolated from Arachida (ticks, Connat *et al*., 1984; Crosby *et al*., 1986) and more recently from insects (Hoffmann *et al*., 1985; Whiting and Dinan, 1988; Slinger and Isaac, 1988).

The ovaries of reproductively competent female insects synthesize ecdysteroids. This can occur during pupal development, as in *Bombyx mori* (Ohnishi *et al*., 1977) or in adult insects such as in *Aedes aegypti* (Hagedorn *et al*., 1975) or *Locusta migratoria* (Lagueux *et al*., 1977).

In many species, the bulk of the ovarian ecdysteroids are transferred to the ooplasm of eggs (Goltzene *et al*., 1978) where they may have a function during embryonic development (Hoffmann, 1986). However, in others, an appreciable amount of the ovarian ecdysteroids are released into the maternal haemolymph where they might play a role in the female (Hoffmann, 1986). Ovarian ecdysteroids are thought in

higher insect orders to play a role in vitellogenin synthesis and in lower insect orders to play a role in the early events of embryogenesis (Hagedorn, 1985). There exists at present a large amount of evidence that one of the functions of egg ecdysteroids is in the control of embryonic moults (see Chapter 1). In several insect species, a correlation between moulting events in the embryo and the concentration of moulting hormone activity was clearly evident (Imboden and Lanzrein, 1982; Fournier and Radallah, 1988). Another function of embryonic ecdysteroids in insects may be the control of diapause. In the eggs of Bombyx mori the level of moulting hormone activity is diminished during diapause and re-elevated upon resumption of embryonic development (Ohnishi et al., 1971; Coulon, 1988). There is also a correlation between the concentration of moulting hormone activity and mitotic activity in the late stages of embryogenesis in Schistocerca gregaria (Scalia et al., 1987). However, the status of ovarian-embryonic ecdysteroids generally seem to differ form one species to another, so that it is difficult to find a general principle that holds in this field (see Chapter 1).

In some insect species (eg. Locusta, Schistocerca and Bombyx) the peaks of free ecdysteroids at least during early embryogenesis may result from hydrolysis of ecdysteroid conjugates of maternal origin rather than de novo synthesis (Hagedorn, 1985). Schistocerca embryos contain a phosphatase enzyme which can hydrolyse ecdysteroid 22-phosphates to release mainly ecdysone (Isaac et al., 1983b). This does not exclude the possibility that during later stages of development, the embryos synthesize ecdysteroid de novo once the prothoracic glands (or other tissues capable of ecdysone biosynthesis) have differentiated (see Chapter 1).

To elucidate the function of ecdysteroids in insect ovaries and eggs, qualitative and quantitative studies on the ecdysteroids during development are necessary. In this chapter, the major ecdysteroids from developing Spodoptera littoralis ovaries and eggs were first identified and the changes in their titre and composition during oogenesis and embryogenesis analysed. Ovarian/egg ecdysteroids were first labelled from [$^3H_2$]cholesterol administered to pupae to assist in their isolation and identification.

2. Experimental and Results

A. The tritiated metabolite profile in developing ovaries from Spodoptera adults derived from [1α,2α-$^3H_2$]cholesterol-injected pupae

In a typical experiment, 16 female S. littoralis pupae were synchronized at pupation (± 4 hrs) and prepared for injection after 7 days of pupal development, which lasts nine days under our conditions. Each pupae was injected with 4μCi of [1α,2α-$^3H_2$]cholesterol (50Ci/mmol) ventrally between the first and second abdominal segments (see Chapter 2). After 5 days of further development, 3 days after the adults had emerged and just prior to egg deposition, the ovaries were dissected. The female pupae were allowed to emerge with an equal number of males for mating.

The ovaries were extracted by the method described in Chapter 2 and the distribution of the radioactivity after separation on a silicic acid column is shown in Fig. 1.

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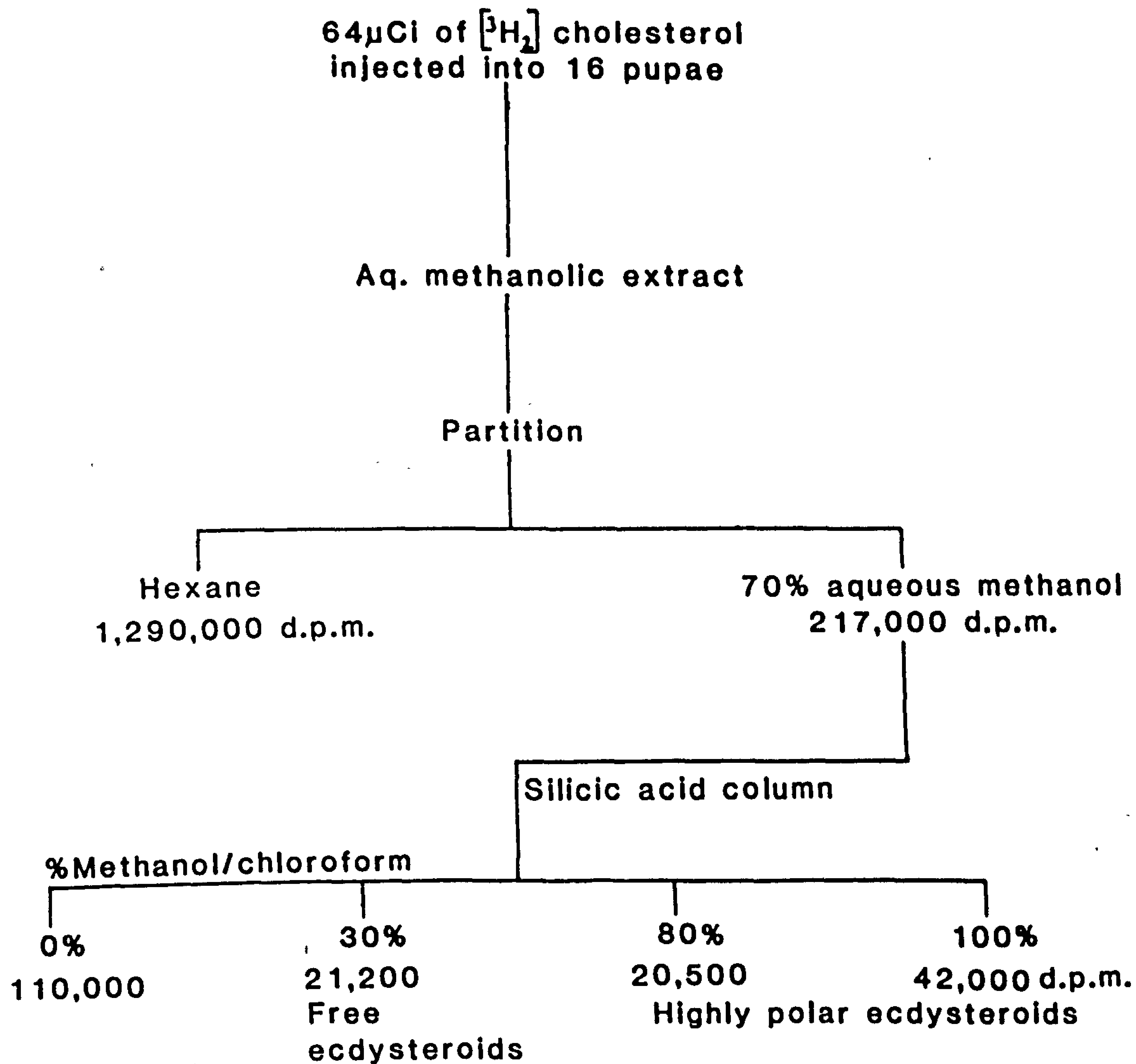
Fig 1.Summary of distribution of radioactivity during extraction of day 3 ovaries from adults derived from [$^3H_2$] cholesterol-injected pupae.



The free ecdysteroid fraction (30% methanol/chloroform silicic acid column fraction) was analysed by reversed-phase h.p.l.c. (Table 1; system 1) with collection of fractions every minute for radioassay. The radiochromatogram together with the positions of elution of authentic marker compounds is shown in Fig. 2. All radiochromatograms in this chapter were obtained by collecting one-minute fractions and plotted out on a computer program. There are a number of radioactive peaks present, with the major one co-chromatographing with authentic 2-deoxyecdysone.

Both the 80% and 100% methanol/chloroform silicic acid column fractions (highly polar ecdysteroid fraction) were analysed by ion-suppression reversed-phase h.p.l.c. (Table 1; system 4) with collection of fractions for radioassay. The radiochromatogram, together with the positions of elution of authentic marker compounds, is shown in Fig. 3. A single peak occurs in both the 80% and 100% silicic acid column fractions that co-chromatographs with authentic 2-deoxyecdysone 22-phosphate. Thus, both fractions were combined from this point onwards.

The highly polar ecdysteroid fraction was treated with the *Helix pomatia* hydrolases (crude aryl sulphatase enzyme preparation) and the products of hydrolysis purified on a C_{18} Sep-pak cartridge (see Chapter 2). The ecdysteroids released by hydrolysis were then analysed by reversed-phase h.p.l.c. (Table 1; system 1) with collection of fractions for radioassay (Fig. 4). The hydrolysed fraction contains a single radioactive peak which co-chromatographs with 2-deoxyecdysone.

TABLE 1 **High-performance liquid chromatography systems used in this chapter**

System 1: Nova-pak C_{18} Radial-pak cartridge eluted at 1ml $min.^{-1}$ with a 30 minute linear gradient of methanol in water changing from 35:65 (v/v) to 100:0 (v/v).

System 2: Nova-pak C_{18} Radial-pak cartridge eluted isocratically at 1ml $min.^{-1}$ with methanol in water 45:55 (v/v).

System 3: Nova-pak C_{18} Radial-pak cartridge eluted at 1ml $min.^{-1}$ with a 45 minute linear gradient of methanol in water changing from 50:50 (v/v) to 70:30 (v/v).

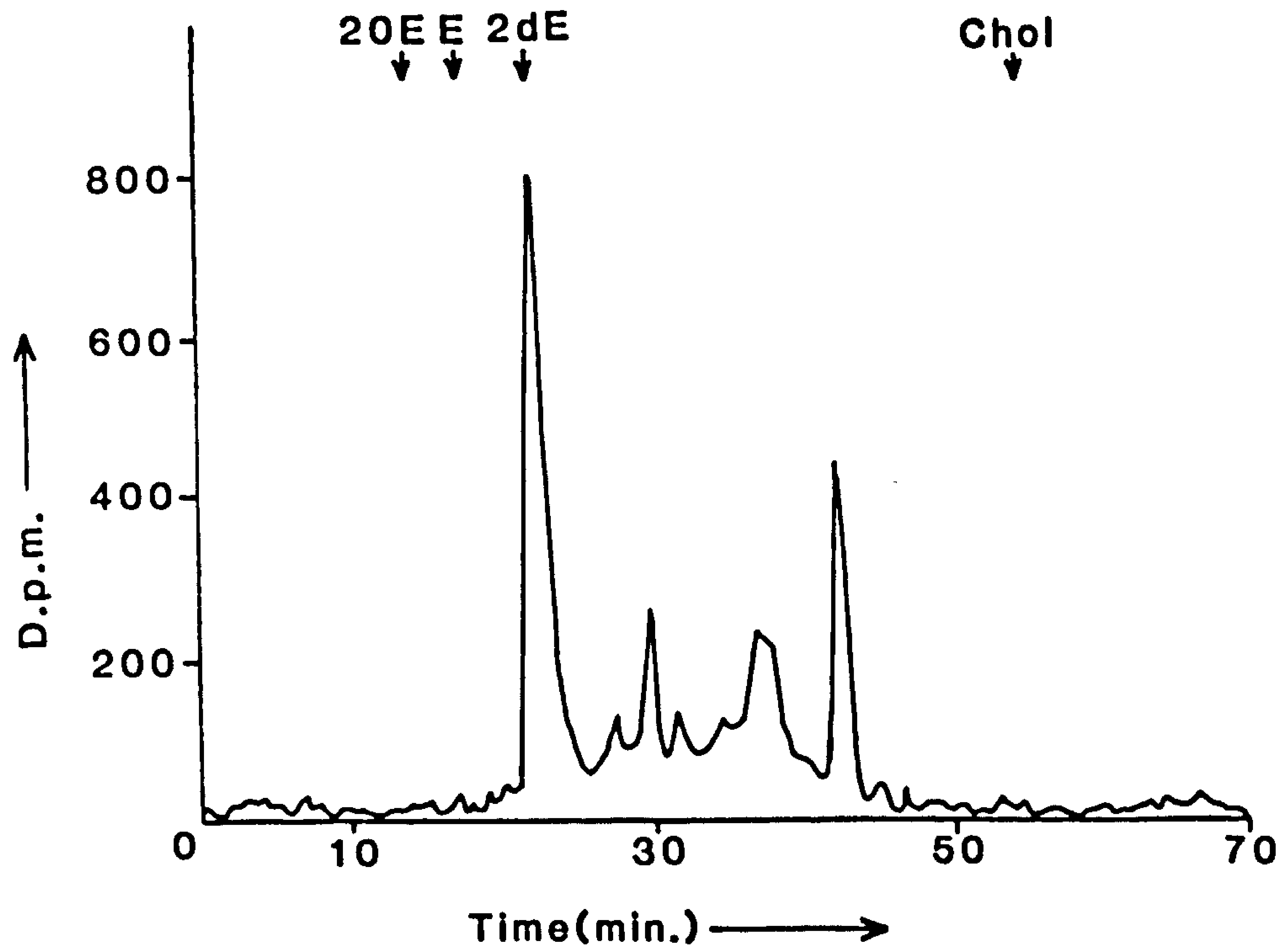
System 4: Nova-pak C_{18} Radial-pak cartridge eluted at 1ml $min.^{-1}$ with a 30 minute linear gradient of methanol in 0.03M sodium dihydrogen phosphate buffer pH 5 changing from 20:80 (v/v) to 70:30 (v/v).

System 5: Nova-pak C_{18} Radial-pak cartridge eluted at 1ml $min.^{-1}$ with a 30 minute linear gradient of methanol in 0.03M sodium dihydrogen phosphate buffer pH 5 changing from 40:60 (v/v) to 50:50 (v/v).

System 6: Nova-pak C_{18} Radial-pak cartridge eluted isocratically at 1ml $min.^{-1}$ with methanol in 0.03M sodium dihydrogen buffer pH 5, 45:55 (v/v).

System 7: μBondapak NH_2 Radial-pak cartridge eluted isocratically at 1ml $min.^{-1}$ with methanol in dichloroethane 8:92 (v/v).

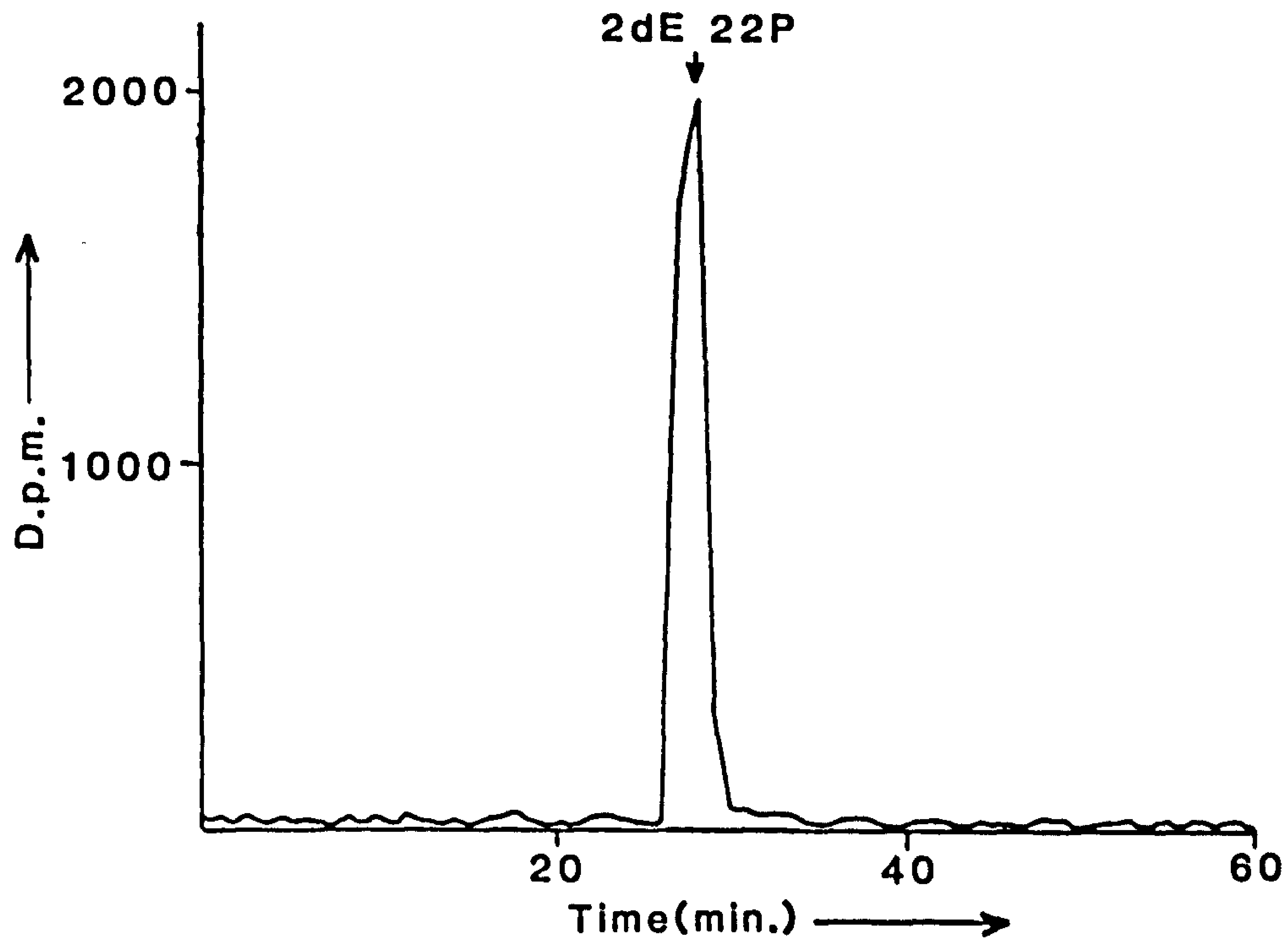
Fig 2. Reversed-phase h.p.l.c. radiochromatogram (system 1) of the tritiated free ecdysteroids isolated from ovaries of adults derived from [$^3H_2$]cholesterol-injected pupae.



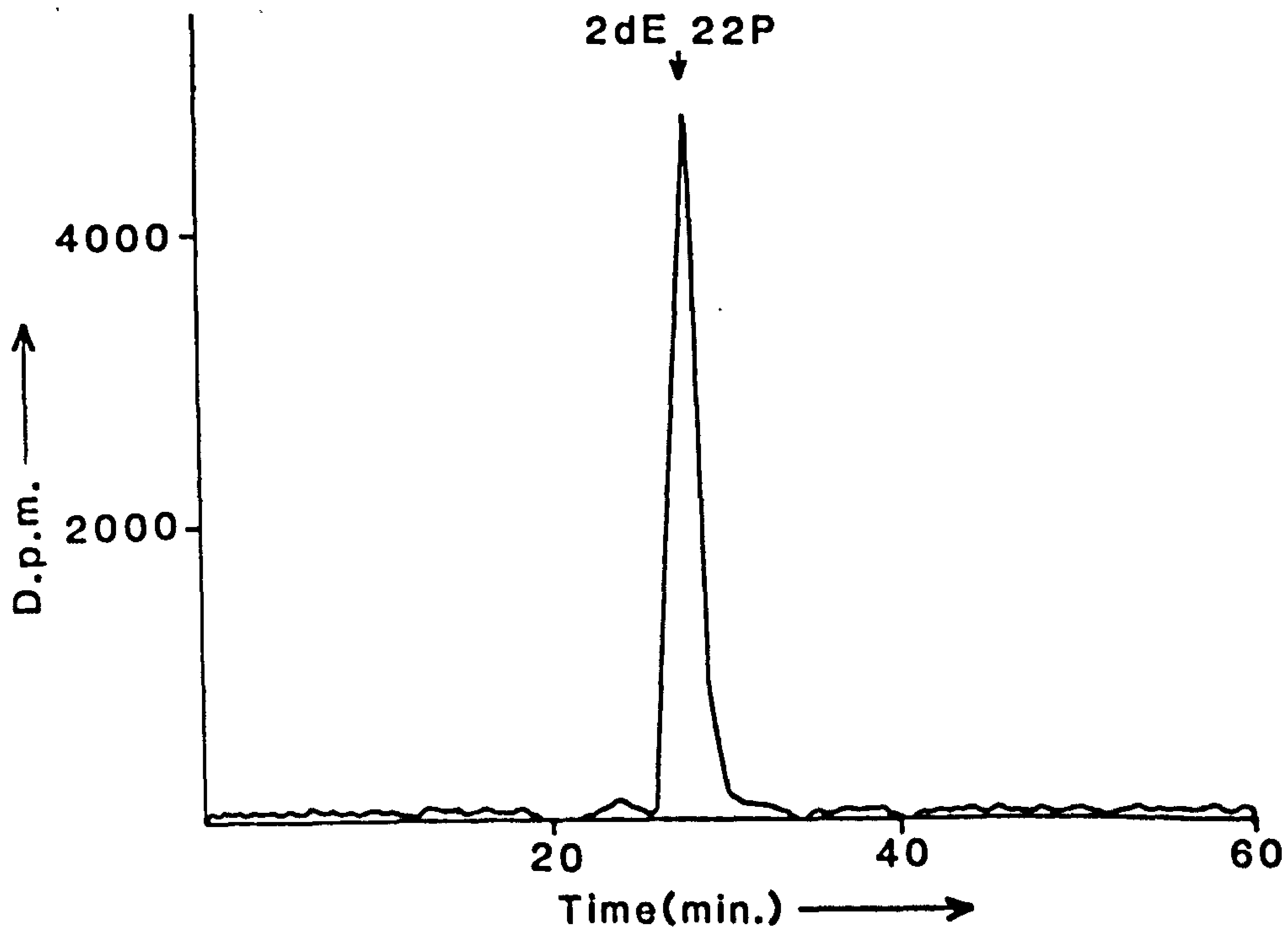
Arrows indicate the retention times of authentic samples.

Fig 3. Reversed-phase h.p.l.c. radiochromatogram (system 4) of the tritiated highly polar ecdysteroids isolated from ovaries of adults derived from [$^3H_2$] cholesterol -injected pupae.

80% silicic acid column fraction

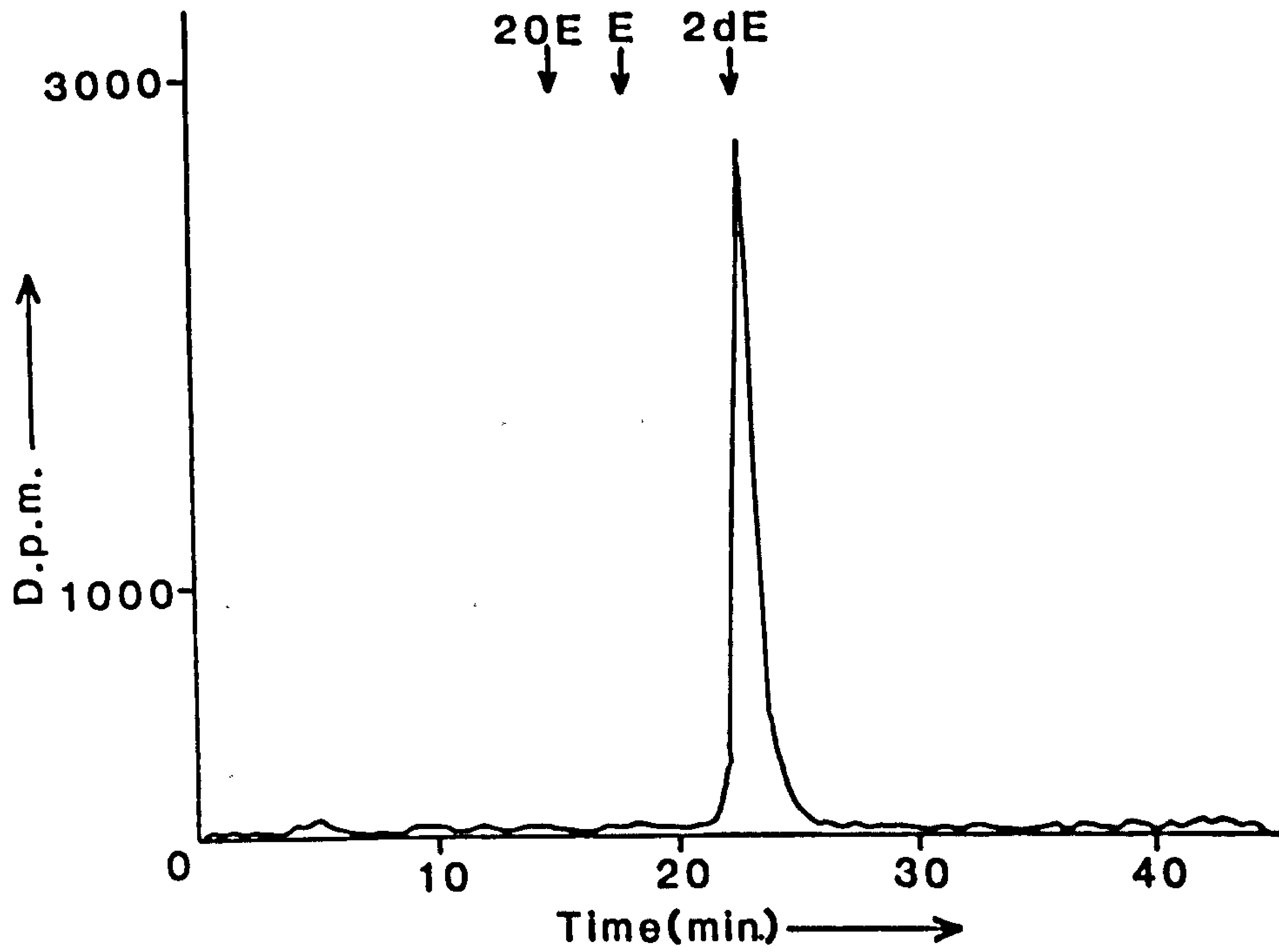


100% silicic acid column fraction



Arrows indicate the retention times of authentic samples.

Fig 4. Reversed-phase radiochromatogram (system 1) of the tritiated ecdysteroids released upon treatment of ovarian highly polar ecdysteroid metabolites with <u>Helix</u> hydrolases.



Arrows indicate the retention times of authentic samples.

B. <u>The tritiated metabolite profile in newly laid eggs from Spodoptera adults derived from [1α,2α-$^3H_2$]cholesterol-injected pupae</u>

In a typical experiment, 19 female <u>S. littoralis</u> pupae were injected with [1α,2α-$^3H_2$]cholesterol (76μCi total) as described in section A and then maintained with an equal number of males for mating. 3 Days after the adults had emerged, eggs were collected up to 4 hr. after deposition and stored at -20°C until required.

The newly laid eggs were extracted by the method described in Chapter 2 and the distribution of the radioactivity after separation on a silicic acid column is shown in Fig. 5.

The free ecdysteroid fraction (30% methanol/chloroform silicic acid column fraction) was analysed by reversed-phase h.p.l.c. (Table 1; system 1) with collection of fractions for radioassay (Fig. 6). The single radioactive peak present in this fraction co-chromatographed with authentic 2-deoxyecdysone. Further evidence was obtained for the identity of the radioactive metabolite, by co-chromatography (Fig. 7) with authentic 2-deoxyecdysone, on adsorption h.p.l.c. (Table 1; system 7).

The highly polar ecdysteroid fraction (combined 80% and 100% methanol/chloroform silicic acid column fractions) was analysed (Fig. 8) by ion suppression reversed-phase h.p.l.c. (Table 1; system 4) with collection of fractions for radioassay. The fraction contains a major radioactive peak which co-chromatographs with authentic 2-deoxyecdysone 22-phosphate; smaller radioactive peaks were also evident.

Fig 5. Summary of distribution of radioactivity during extraction of newly laid eggs from adults derived from [$^3H_2$] cholesterol-injected pupae.

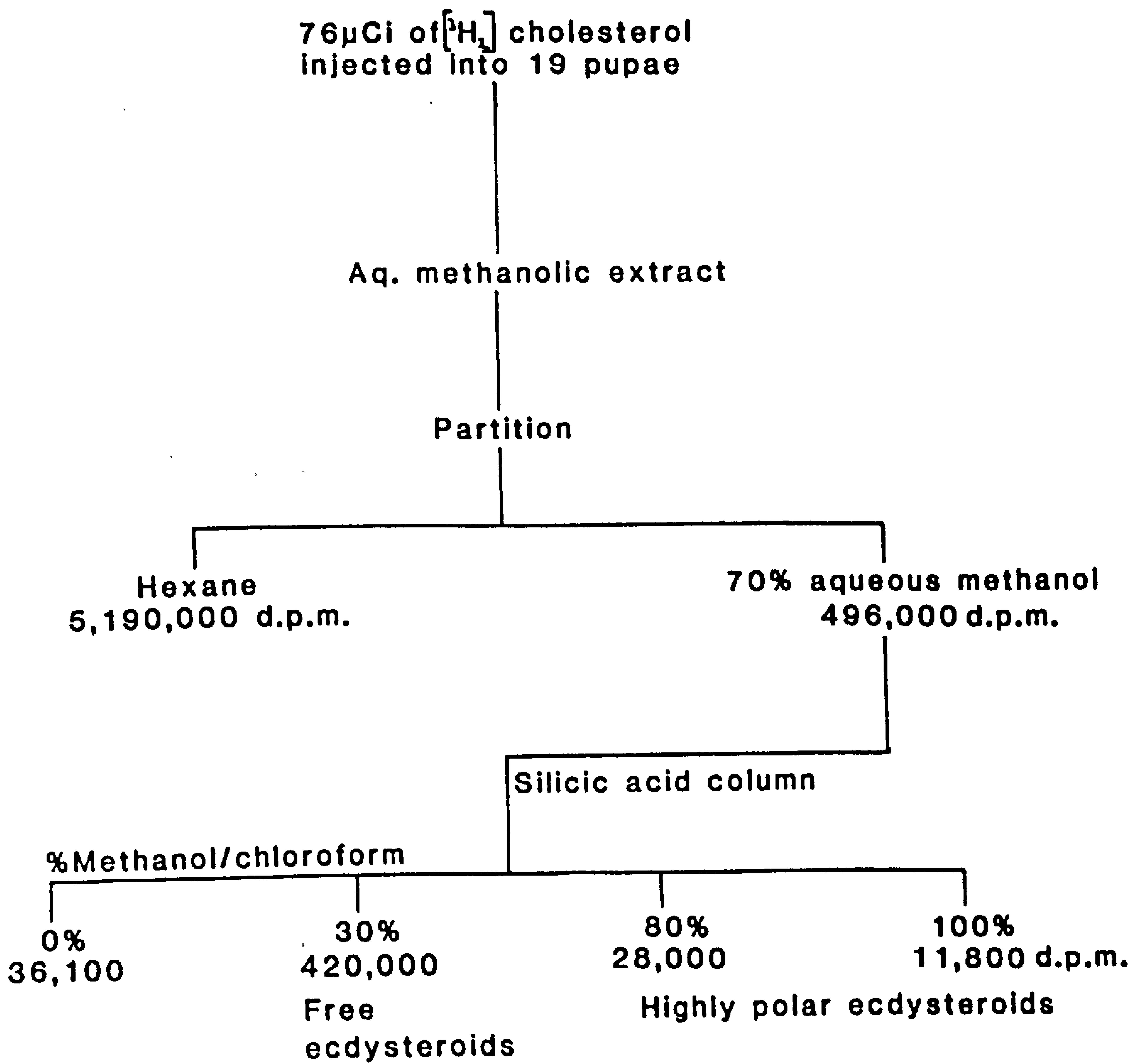
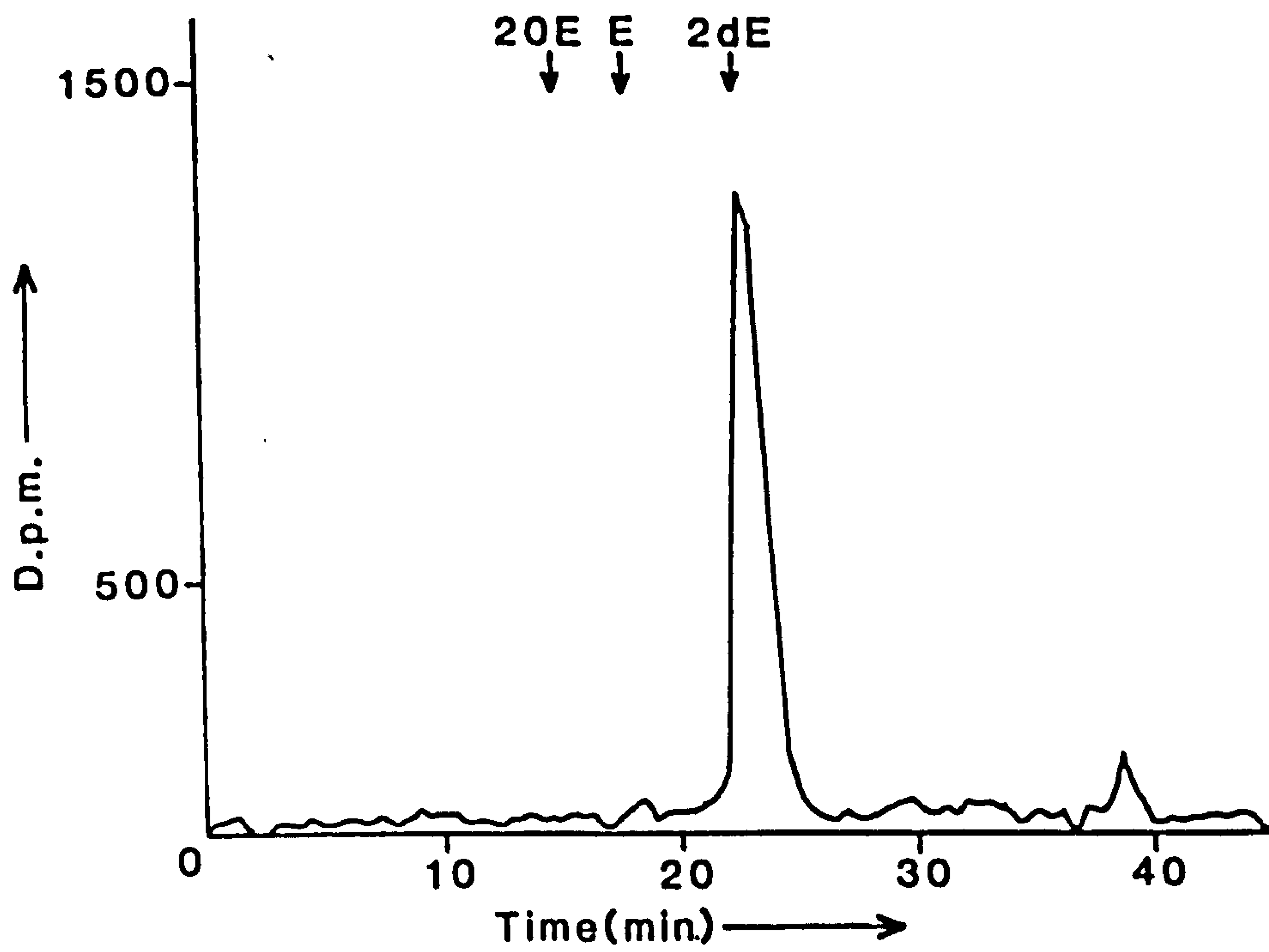
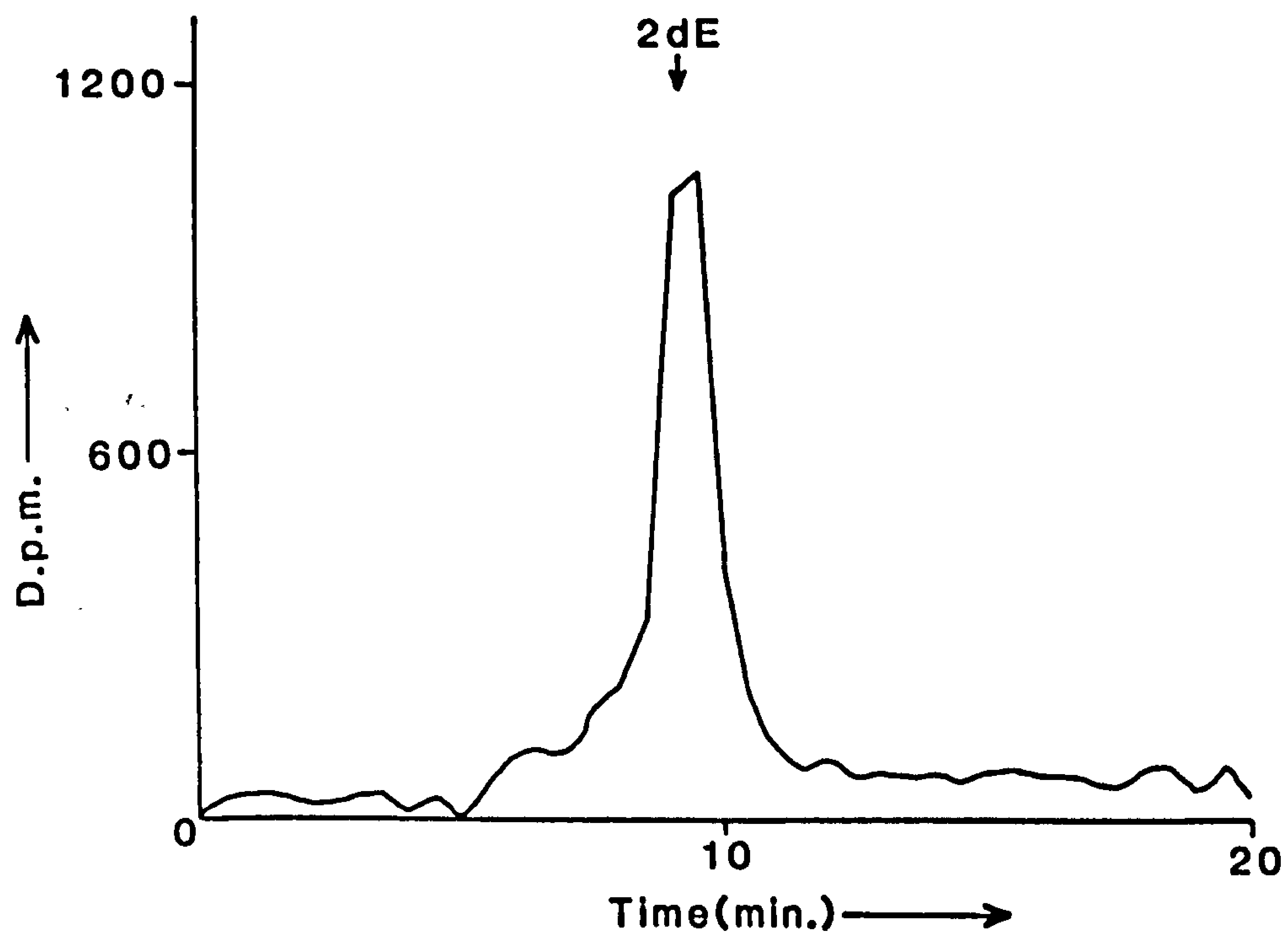


Fig 6. Reversed-phase h.p.l.c. radiochromatogram (system 1) of the tritiated free ecdysteroids isolated from newly laid eggs of the adult derived from [$^3H_2$]cholesterol-injected pupae.



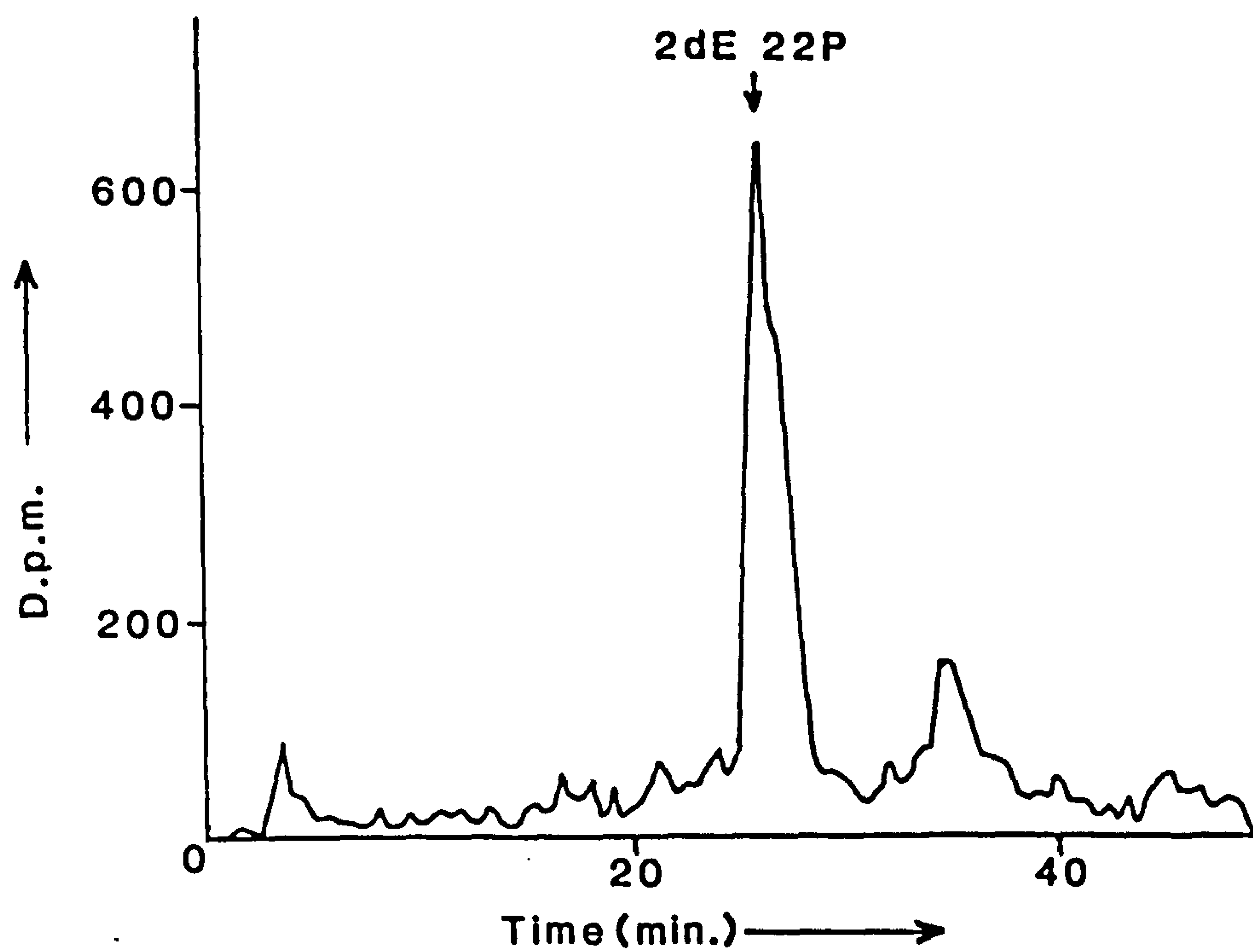
Arrows indicate the retention times of authentic samples.

Fig 7. Adsorption h.p.l.c. radiochromatogram (system 7) of the tritiated free ecdysteroids isolated from newly laid eggs of the adult derived from [$^3H_2$] cholesterol-injected pupae.



Arrows indicate the retention times of authentic samples.

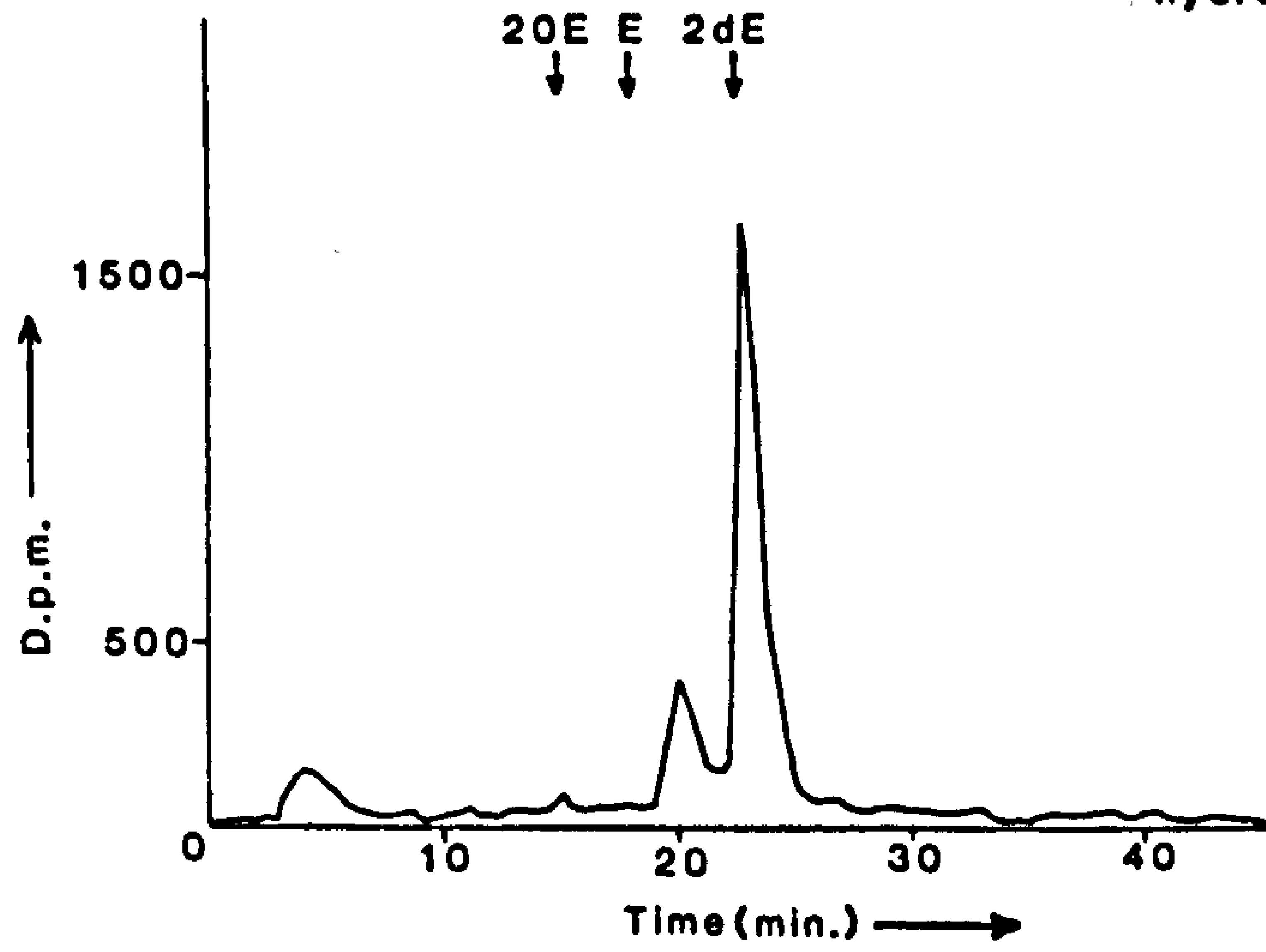
Fig 8. Reversed-phase h.p.l.c. radiochromatogram (system 4) of the tritiated highly polar ecdysteroids isolated from newly laid eggs of the adult derived from $[^3H_2]$ cholesterol-injected pupae.



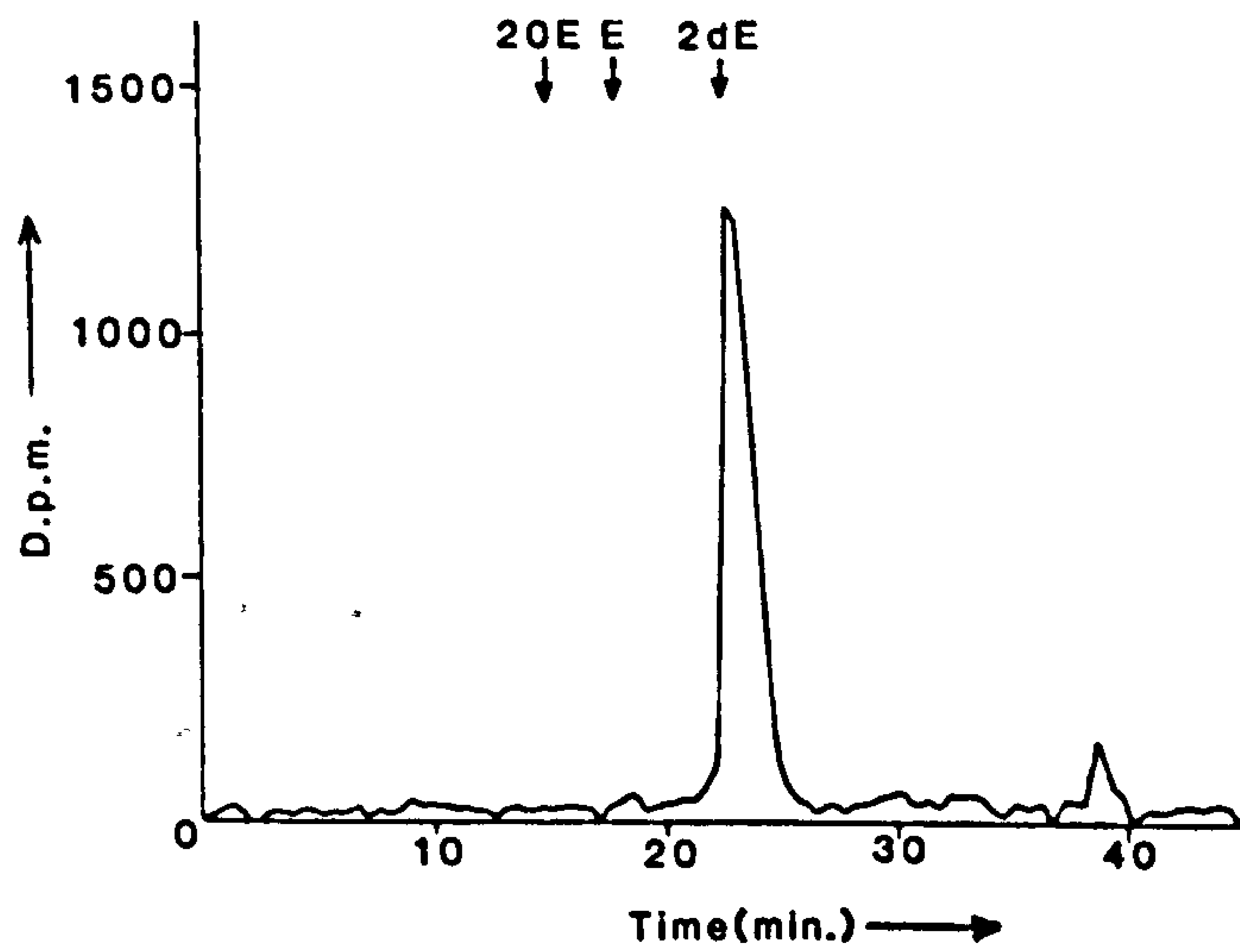
Arrows indicate the retention times of authentic samples.

Fig 9. Comparison of the tritiated free ecdysteroids released by enzymic hydrolysis of the highly polar fraction from newly laid eggs and ovaries, with free ecdysteroid metabolites from newly laid eggs (reversed-phase h.p.l.c. system 1).

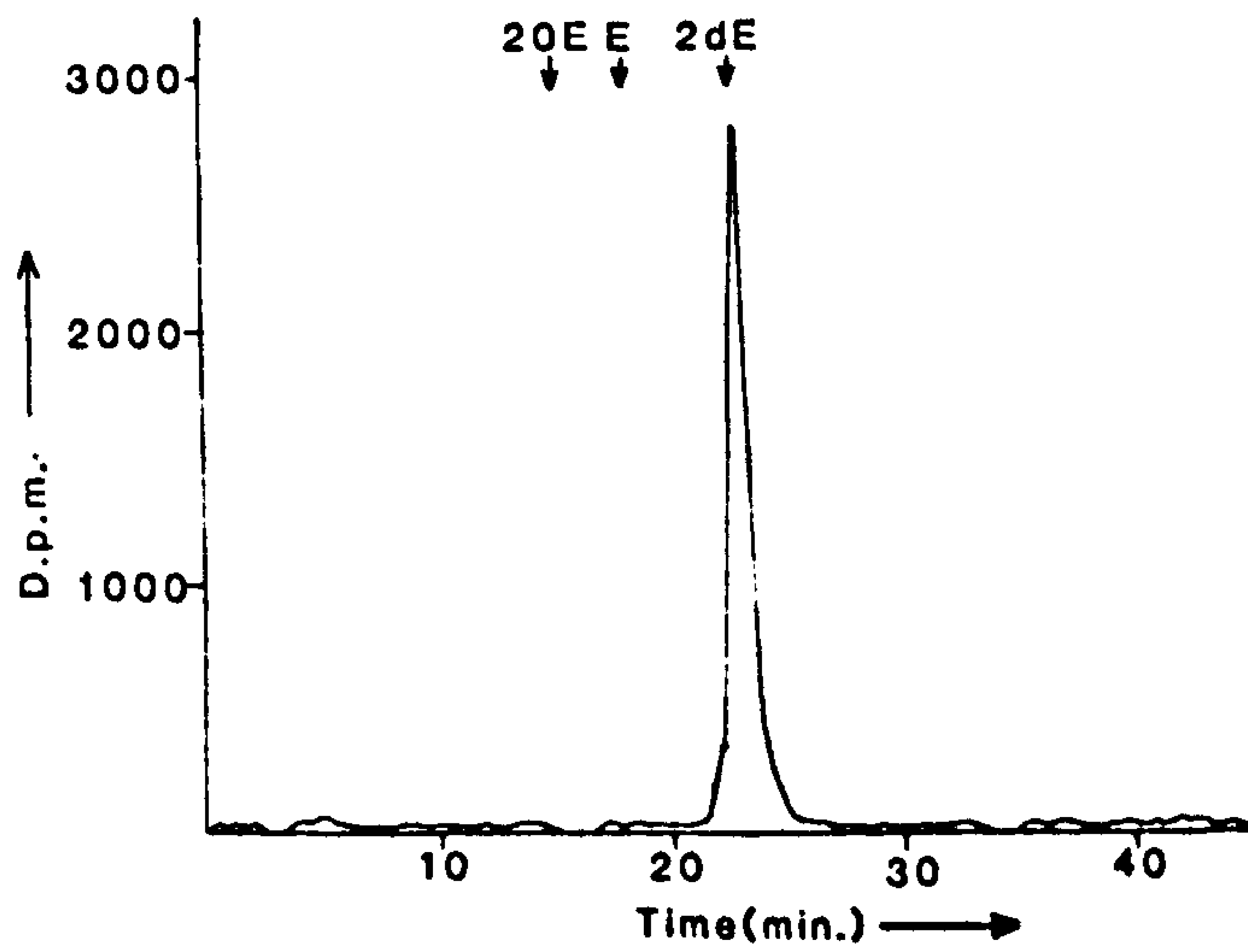
Newly laid egg highly polar ecdysteroid metabolites after Helix hydrolysis



Newly laid egg free ecdysteroid fraction



Ovarian highly polar ecdysteroid fraction after Helix hydrolysis



Arrows indicate the retention times of authentic samples.

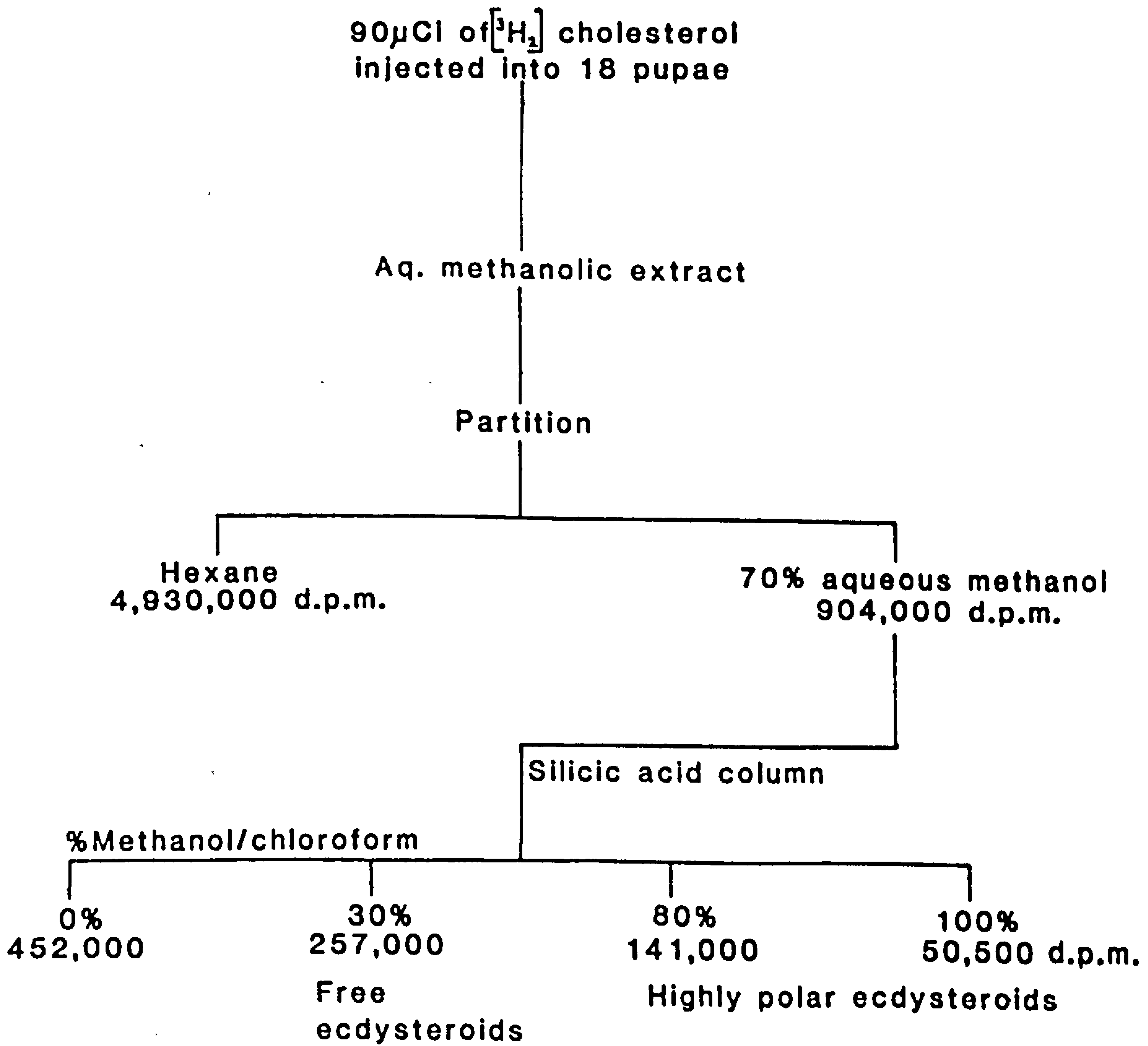
The highly polar ecdysteroids were treated with the *Helix pomatia* hydrolases (crude aryl sulphatase enzyme preparation) and the products of hydrolysis purified by C_{18} Sep-pak cartridge. The hydrolysed highly polar ecdysteroids were then analysed by reversed-phase h.p.l.c. (Table 1; system 1) together with authentic markers, the tritiated newly laid egg free ecdysteroid fraction and the hydrolysed tritiated ovarian highly polar ecdysteroid fraction (Fig. 9). The fraction contains 2 major radioactive peaks. The major radioactive peak, as expected, co-chromatographs with 2-deoxyecdysone and the single radioactive peak from both the newly laid egg free ecdysteroid fraction and the hydrolysed tritiated ovarian highly polar ecdysteroid fraction. The other radioactive metabolite present is unknown, but the corresponding conjugate does not appear as a major peak in Fig. 8.

C. **The tritiated metabolite profile in day 3 developing eggs from Spodoptera adults derived from [1α,2α-$^{3}H_{2}$]cholesterol-injected pupae**

In a typical experiment, 18 female *S. littoralis* pupae were injected with [1α,2α-$^{3}H_{2}$]cholesterol (90μCi total) as described in section A and then maintained with an equal number of males for mating. Eggs were collected up to 4 hr. after deposition and incubated for a further 3 days, which is just prior to hatching, when they were frozen at -20°C until required.

The day 3 eggs were extracted by the method described in Chapter 2 and the distribution of the radioactivity after separation on a silicic acid column is shown in Fig. 10.

Fig 10. Summary of distribution of radioactivity during extraction of day 3 developing eggs (just prior to hatching) from adults derived from $[^3H_2]$ cholesterol-injected pupae.



The free ecdysteroid fraction (30% methanol/chloroform silicic acid column fraction) was analysed by reversed-phase h.p.l.c. (Table 1; system 1) together with authentic markers and the tritiated newly laid egg free ecdysteroid fraction (Fig. 11). Two radioactive peaks were present, the major one co-chromatographs with authentic 2-deoxyecdysone and the single peak in the tritiated newly laid egg free ecdysteroid fraction. The second peak co-chromatographed with 5α-2-deoxyecdysone (see Chapter 2).

The highly polar ecdysteroid fraction (combined 80% and 100% methanol/chloroform silicic acid column fractions) was analysed by ion-suppression reversed-phase h.p.l.c. (Table 1; system 4) together with authentic markers and the tritiated ovarian highly polar ecdysteroid fraction (Fig. 12). The single radioactive peak present in this fraction co-chromatographed with 2-deoxyecdysone 22-phosphate and the radioactive metabolite present in the tritiated ovarian highly polar ecdysteroid fraction.

The highly polar ecdysteroids were treated with Helix pomatia hydrolyases, the products of hydrolysis purified by C_{18} Sep-pak cartridge and analysed by reversed-phase h.p.l.c. (Table 1; system 1) together with authentic markers and the tritiated newly laid egg free ecdysteroid fraction (Fig. 13). The single radioactive peak present, as expected, co-chromatographed with authentic 2-deoxyecdysone and the radioactive peak present in the tritiated newly laid egg free ecdysteroid fraction.

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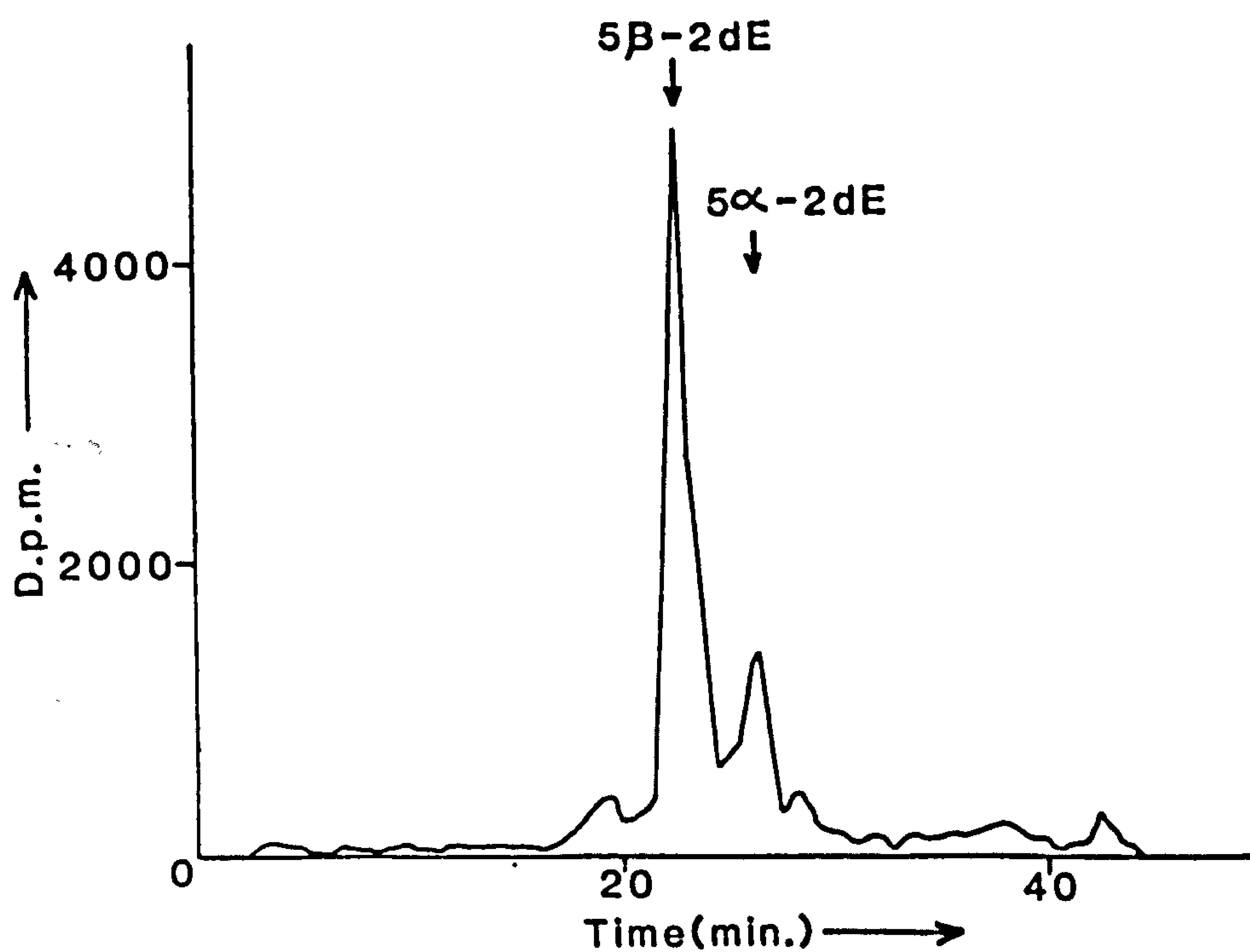
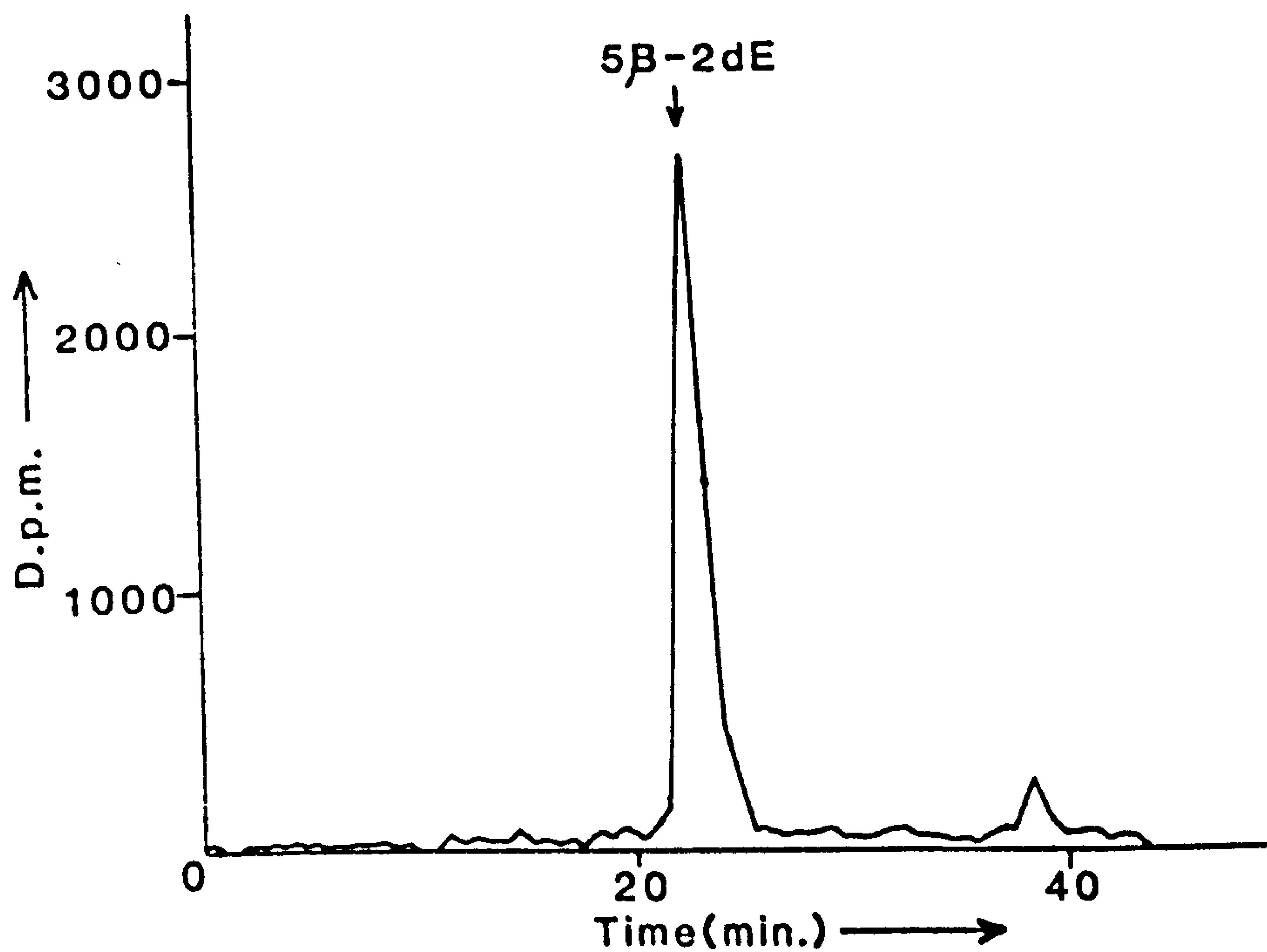
Fig 11.Comparison of free tritiated ecdysteroid metabolites isolated from either newly laid or day 3 developed eggs of adults derived from $[^3H_2]$ cholesterol-injected pupae (reversed-phase h.p.l.c. system 1).

Day 3 egg free ecdsteroid metabolites



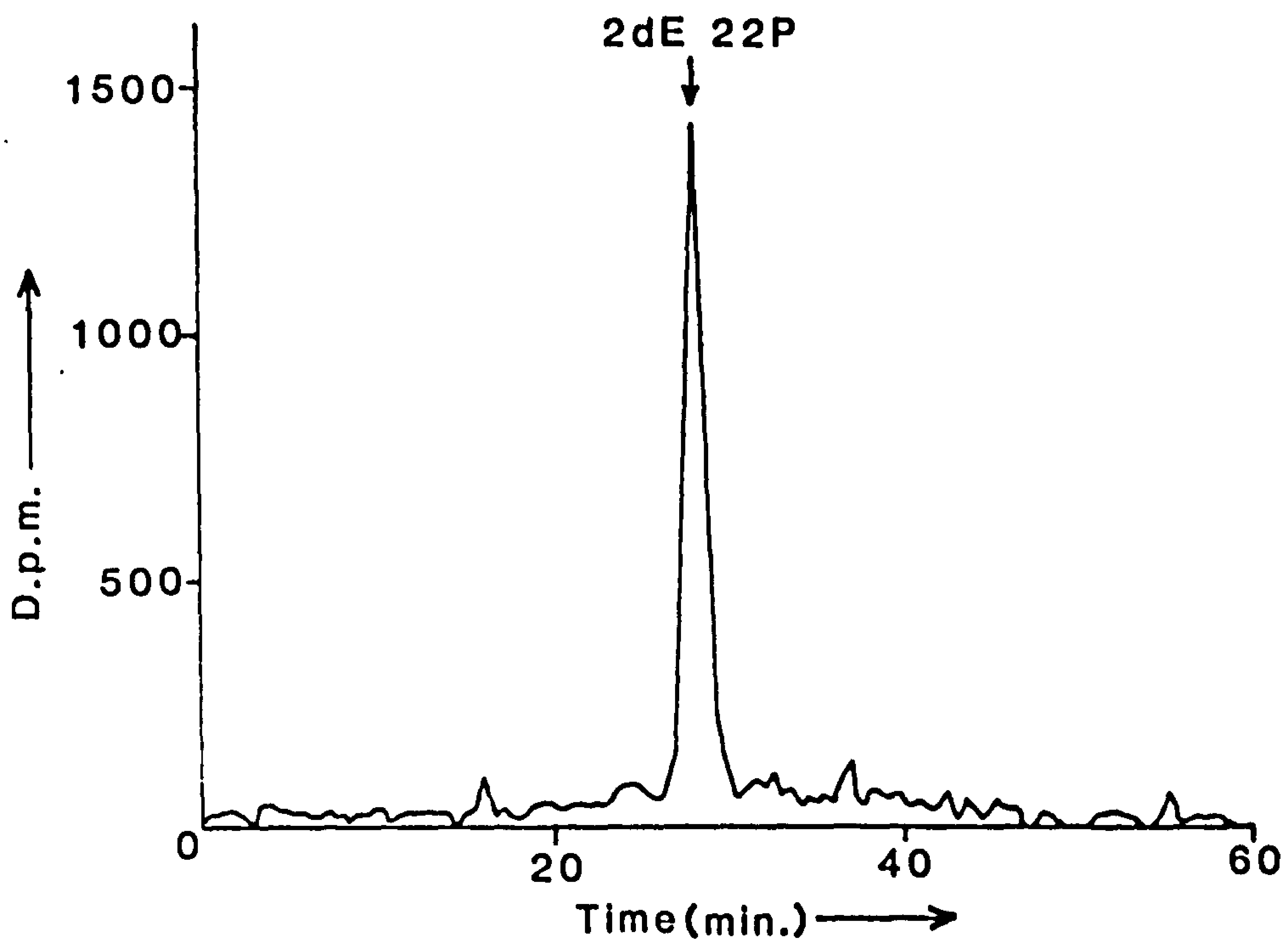
Newly laid free ecdysteroid metabolites



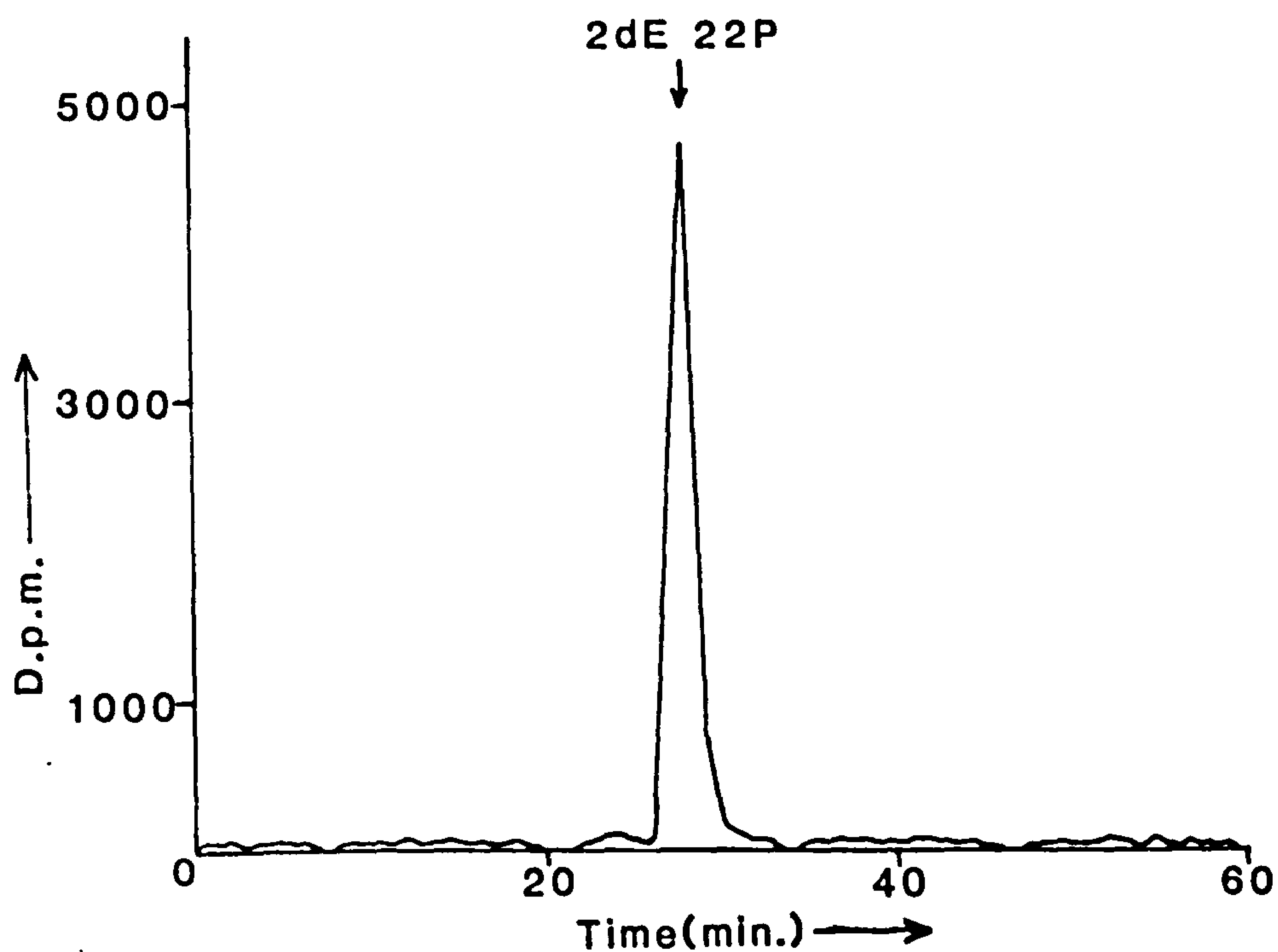
Arrows indicate the retention times of the authentic samples.

Fig 12. Comparison of highly polar tritiated ecdysteroid metabolites isolated from day 3 eggs and ovaries of the adult derived from $[^3H_2]$ cholesterol-injected pupae (reversed-phase h.p.l.c. system 4).

Day 3eggs highly polar ecdysteroid metabolites



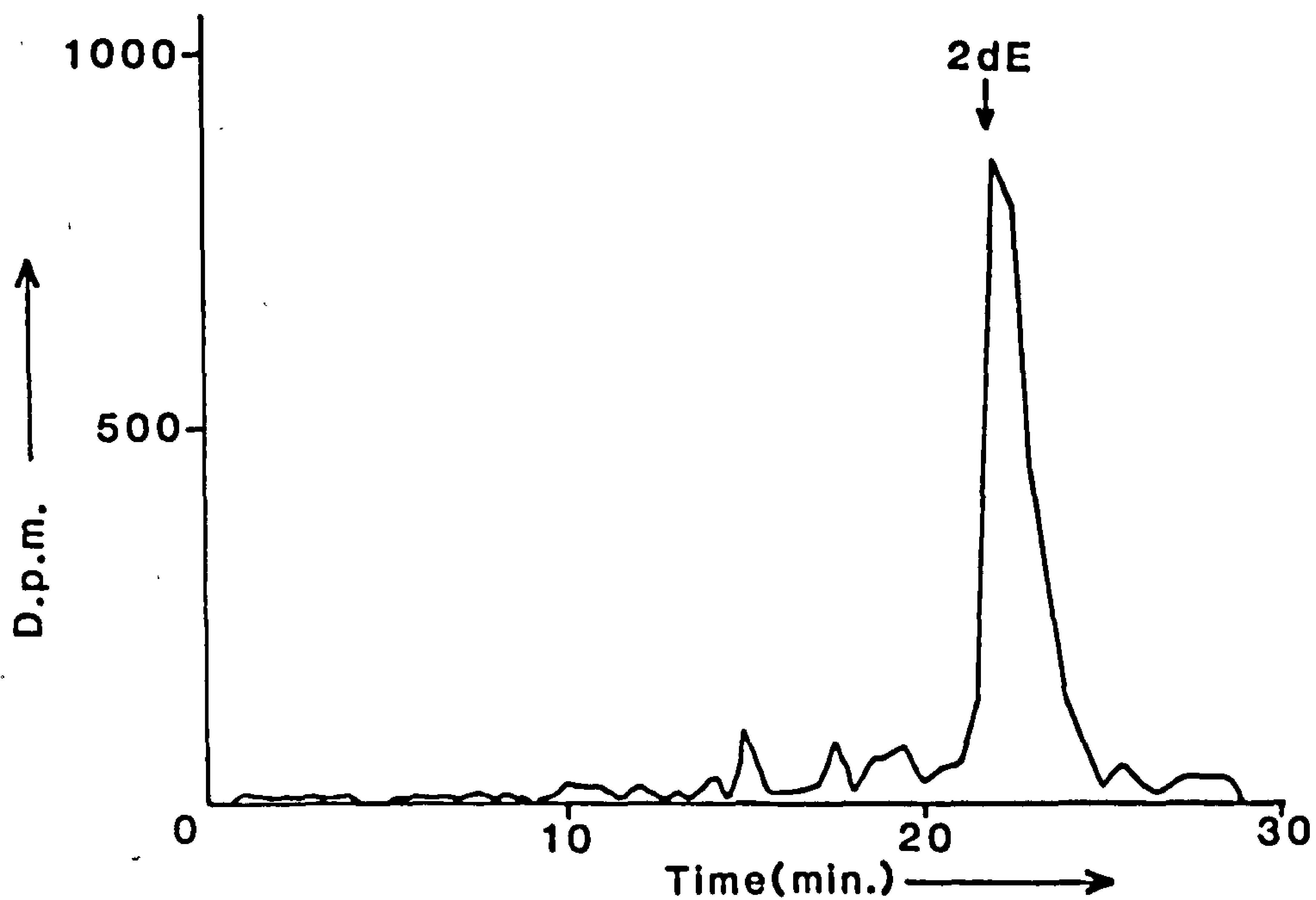
Ovarian highly polar ecdysteroid metabolites



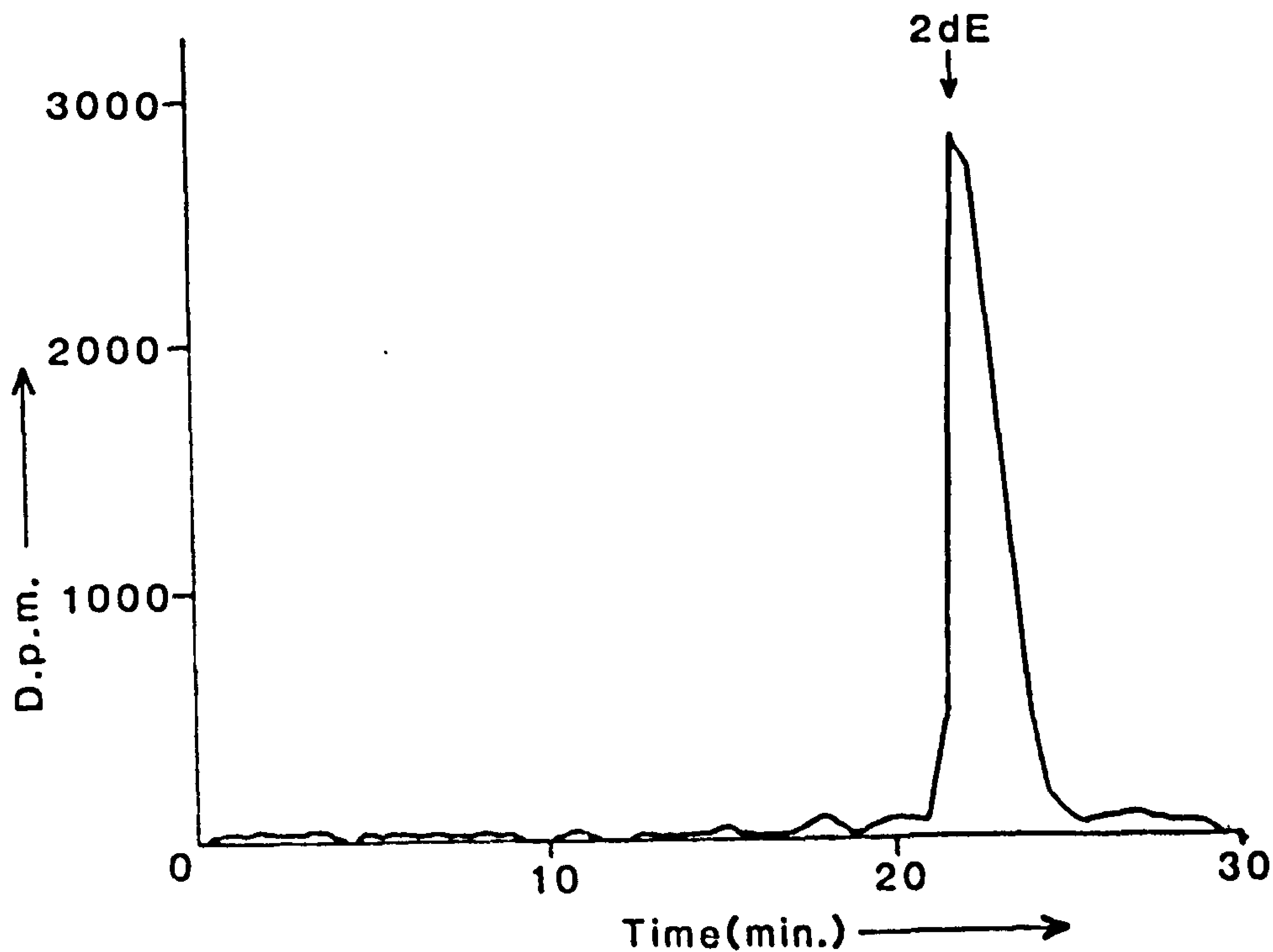
Arrows indicate the retention times of the authentic samples.

Fig 13. Comparison of the tritiated free ecdysteroids released by enzymic hydrolysis of the highly polar fraction from day 3 eggs with the free ecdysteroids from newly laid eggs (reversed-phase h.p.l.c. system 1).

Day 3 highly polar ecdysteroid metabolites after <u>Helix</u> hydrolysis



Newly laid egg free ecdysteroid metabolites



Arrows indicate the retention times of the authentic samples.

D. Identification of ovarian metabolites

To allow complete identification of the ovarian metabolites a few hundred micrograms of each metabolite are required for nuclear magnetic resonance and mass spectrometric analysis. To collect such an amount of these ecdysteroids, 540 developing ovaries were dissected from day 3 adult females (just prior to egg deposition), which had been mated with an equal number of males. The ovaries were stored at -20°C in methanol until required and were then extracted by the method described in Chapter 2. The weights of various fractions during the extraction are summarised in Fig. 14.

I. Purification of free ecdysteroids

The free ecdysteroid (30% methanol/chloroform silicic acid column fraction) was purified initially by reversed-phase h.p.l.c. (Table 1; system 1) using authentic markers and the tritiated ovarian free ecdysteroid fraction to identify the relevant u.v. absorbing peaks. A typical chromatogram is shown in Fig. 15; peak 1 co-chromatographs with authentic 2-deoxyecdysone and the major metabolite in the tritiated ovarian free ecdysteroid fraction. Peaks 1-3 were collected by h.p.l.c. using this system (Table 1; system 1). Each of the metabolites were then applied separately to an adsorption h.p.l.c. system (Table 1; system 7) for further purification.

Fig 14. Weights of fractions during extraction of day 3 female ovaries.

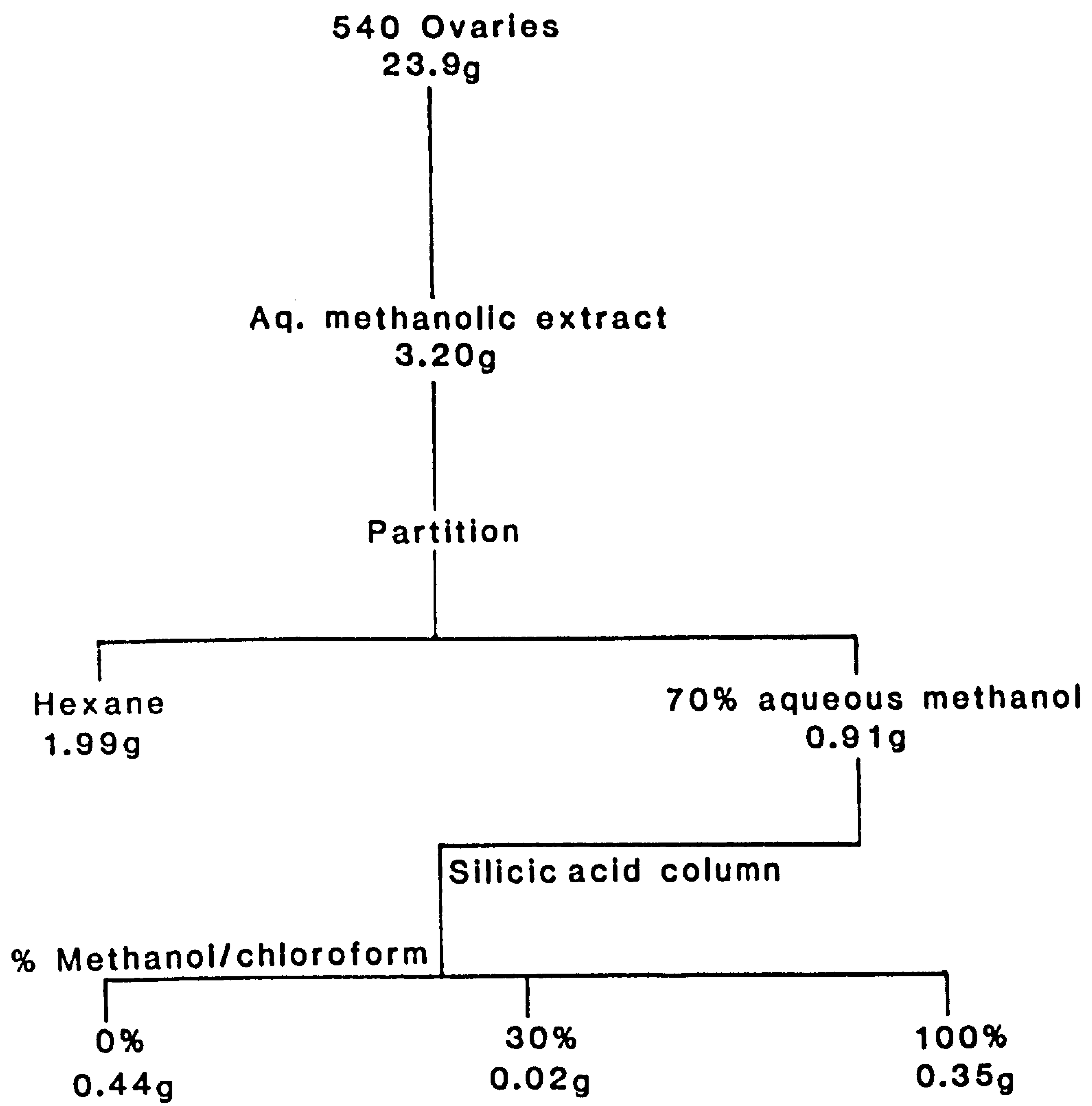
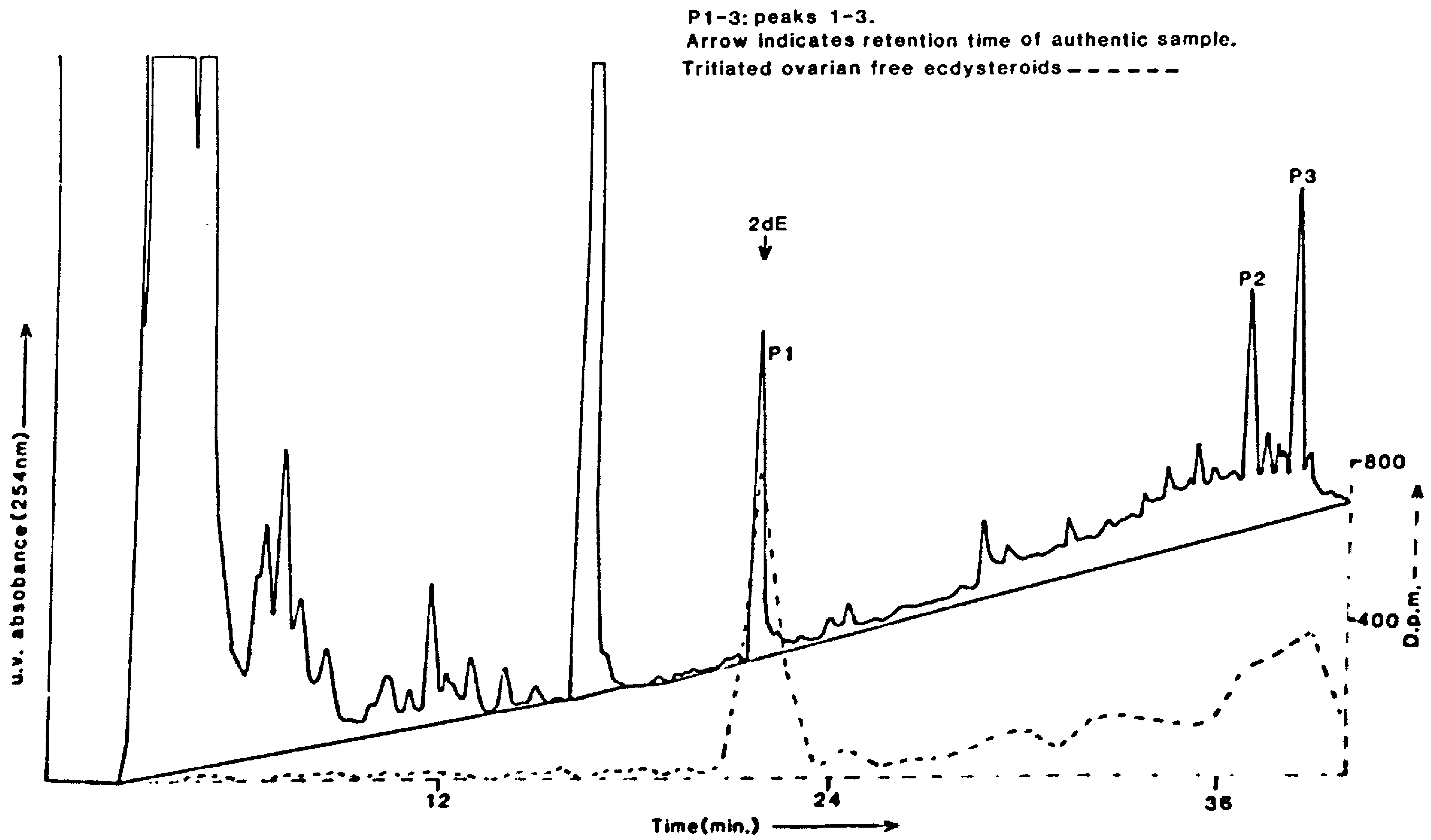


Fig 15. Purification of ovarian free ecdysteroids by reversed-phase h.p.l.c. (system 1)



II. Mass spectrometric analysis of free ecdysteroids

An aliquot of approximately 10μg of each of the purified metabolites was then analysed by negative-ion fast atom bombardment (f.a.b.) mass spectrometry using glycerol as the probe matrix (see Chapter 2).

In the negative-ion f.a.b. mass spectrum of metabolite 1 (Fig. 16) the major high mass ion is at m/z 447 which corresponds to the $[M-H]^-$, pseudomolecular ion, indicating a molecular weight of 448. This is substantiated by the presence of an ion at m/z 539 corresponding to M-H with the addition of glycerol. There is also a peak at m/z 469 corresponding to the M-H with the addition of sodium. This further indicates the structure of metabolite 1 as 2-deoxyecdysone. The mass spectra of metabolites 2 and 3 were difficult to interpret and were thus probably impure samples (see Appendix).

III. Analysis of the free ecdysteroid fraction from day 3 ovaries for the presence of ecdysone and 20-hydroxyecdysone

Since tissues of most, but not all, investigated insect species generally contain ecdysone and 20-hydroxyecdysone as significant ecdysteroid components (see Chapter 1) it was surprising that such ecdysteroids were not apparently detected in tritiated ovaries. Therefore, the ovarian free ecdysteroid fraction was analysed by reversed-phase h.p.l.c. using u.v. detection for the presence of

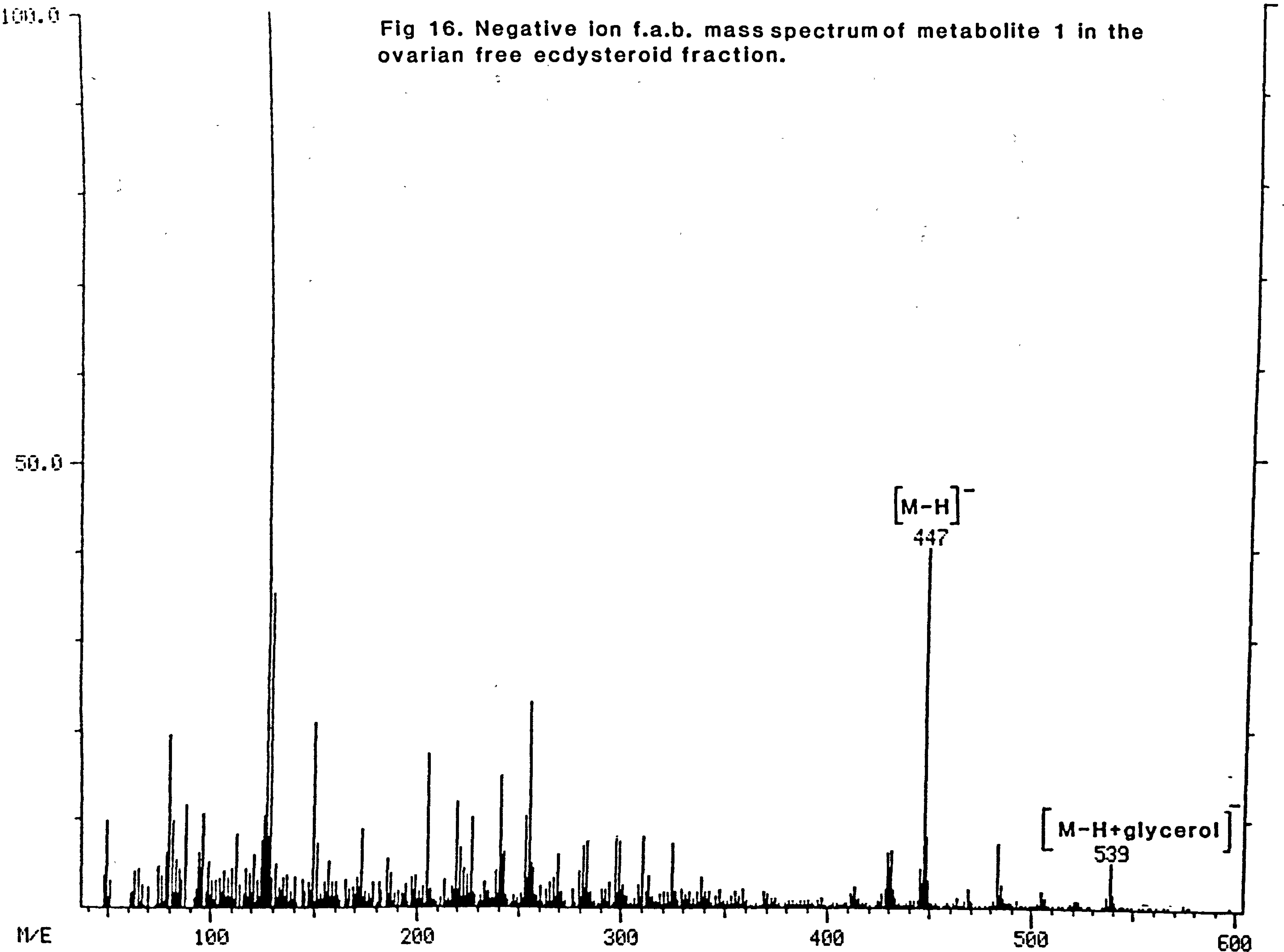


Fig 16. Negative ion f.a.b. mass spectrum of metabolite 1 in the ovarian free ecdysteroid fraction.

ecdysone and 20-hydroxyecdysone. The h.p.l.c. system employed was a Nova-pak C_{18} Radial pak cartridge (Table 1; system 2) which gave retention times of 9.36 min. and 18.9 min., respectively, for the authentic ecdysone and 20-hydroxyecdysone. Neither ecdysone or 20-hydroxyecdysone was detected by u.v. absorption in the ovarian free ecdysteroid fraction.

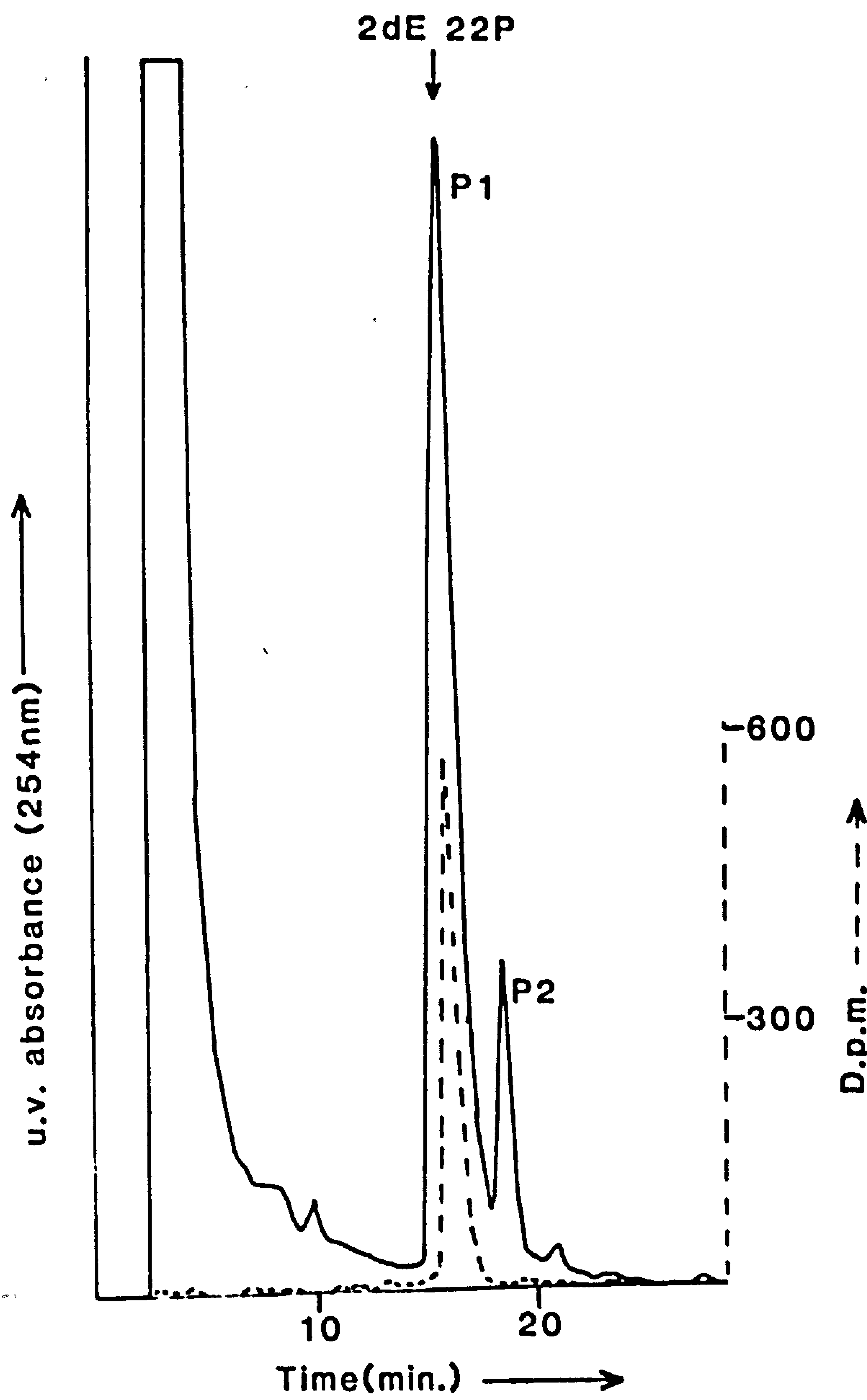
IV. Purification of highly polar ecdysteroids

The highly polar ecdysteroid fraction (combined 80% and 100% methanol/chloroform silicic acid column fractions) were initially purified by ion-suppression reversed-phase h.p.l.c. (Table 2; system 5) using authentic markers and the tritiated day 3 ovarian highly polar ecdysteroid fraction to identify relevant u.v. absorbing peaks. A typical chromatogram is shown in Fig. 17 and peaks 1 and 2 were purified using this system. Peak 1 co-chromatographs with authentic 2-deoxyecdysone 22-phosphate and the single metabolite in the tritiated day 3 highly polar ecdysteroid fraction. Each of the metabolites was then applied separately to a second reversed-phase ion-suppression h.p.l.c. system (Table 1; system 6) for further purification.

V. Mass spectrometric analysis of highly polar ecdysteroids

An aliquot of approximately 10μg of each of the purified metabolites was then analysed by negative-ion fast atom bombardment (f.a.b.) mass spectrometry using glycerol as the probe matrix (see

Fig 17. Purification of ovarian highly polar ecdysteroids by reversed-phase h.p.l.c. (system 5).



P1 and 2: Peaks 1 and 2
Arrow indicates retention time of authentic sample.
Tritiated ovarian highly polar ecdysteroids. - - - - -

Chapter 2). In the negative-ion f.a.b. mass spectra of metabolites 1 and 2 the major ion at high mass is at m/z 527, which corresponds to the $[M-H]^-$ ion, indicating a molecular weight of 448 (Fig. 18 and 19, respectively). The ions at 97 and 79 are a characteristic of phosphate (Isaac et al., 1982). Both metabolites have a molecular weight which corresponds to that of 2-deoxyecdysone 22-phosphate. Metabolite 2 does not co-chromatograph with authentic 3-epi-2-deoxy-ecdysone 22-phosphate on reversed-phase h.p.l.c. Thus, it was possible that it was the 5α-epimer of 2-deoxyecdysone 22-phosphate, with metabolite 1 being the 5β-epimer.

Hydrolysis of metabolite 2 by Helix pomatia hydrolases, followed by purification on a C_{18} Sep-pak cartridge, gave mainly the 5α-2-deoxyecdysone with some 5β-2-deoxyecdysone when analysed by reversed-phase h.p.l.c. (Table 1; system 1).

In addition, an aliquot of approximately 20μg of purified metabolite 1 was treated with the Helix pomatia hydrolases (crude aryl sulphatase enzyme preparation) and the products of hydrolysis purified on a C_{18} Sep-pak cartridge. An aliquot of approximately 10μg of the hydrolysed metabolite 1 was then analysed by negative-ion f.a.b. mass spectrometry using glycerol as the probe matrix (see Chapter 2). The major ion at high mass was at m/z 447, $[M-H]^-$, indicating a molecular weight of 448, which corresponds to that of 2-deoxyecdysone (Fig. 20). This is substantiated again by the presence of ions at m/z 539 and 469 corresponding to $[M-H]^-$ with the addition of glycerol and sodium, respectively.

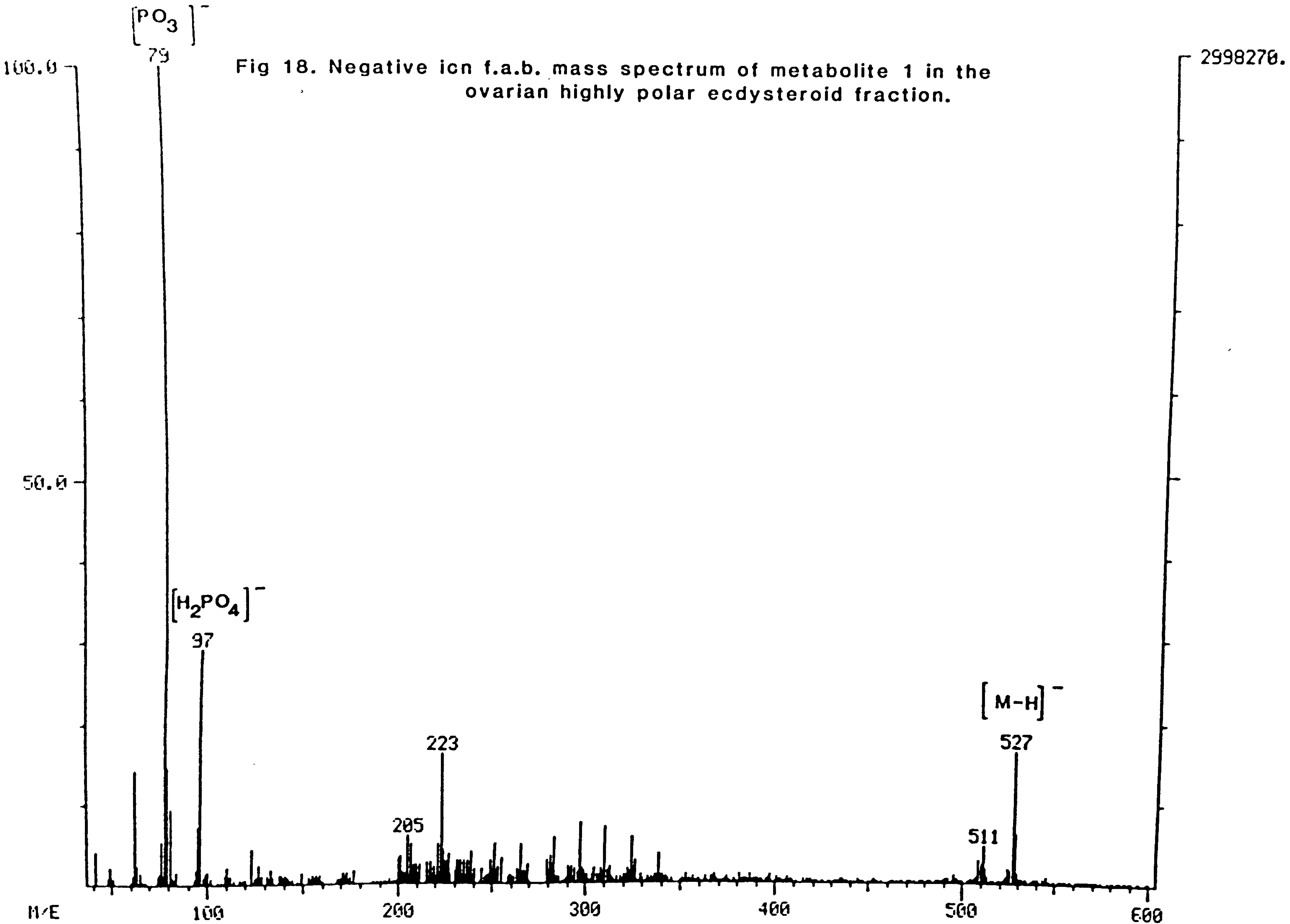


Fig 18. Negative icn f.a.b. mass spectrum of metabolite 1 in the ovarian highly polar ecdysteroid fraction.

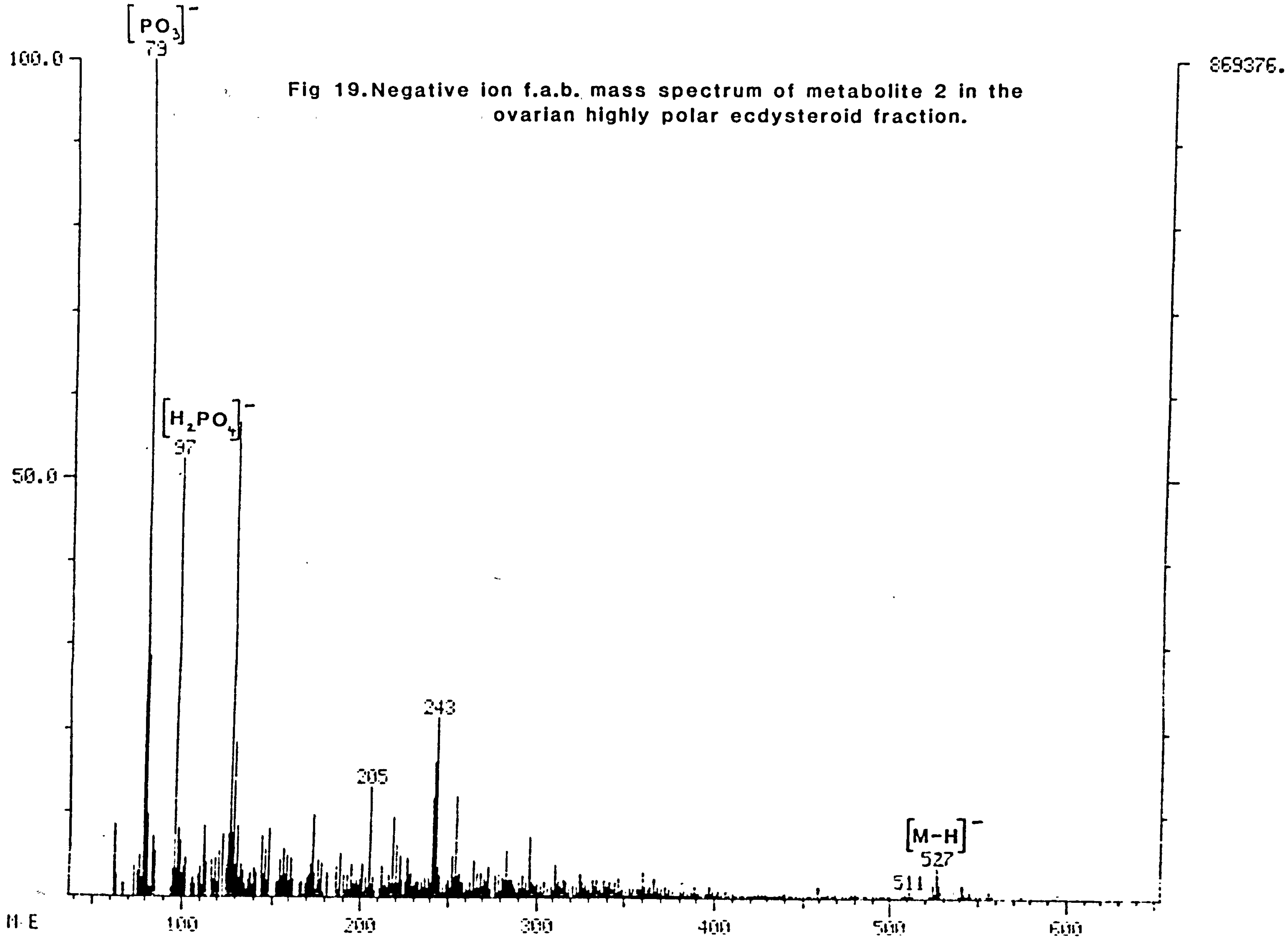


Fig 19. Negative ion f.a.b. mass spectrum of metabolite 2 in the ovarian highly polar ecdysteroid fraction.

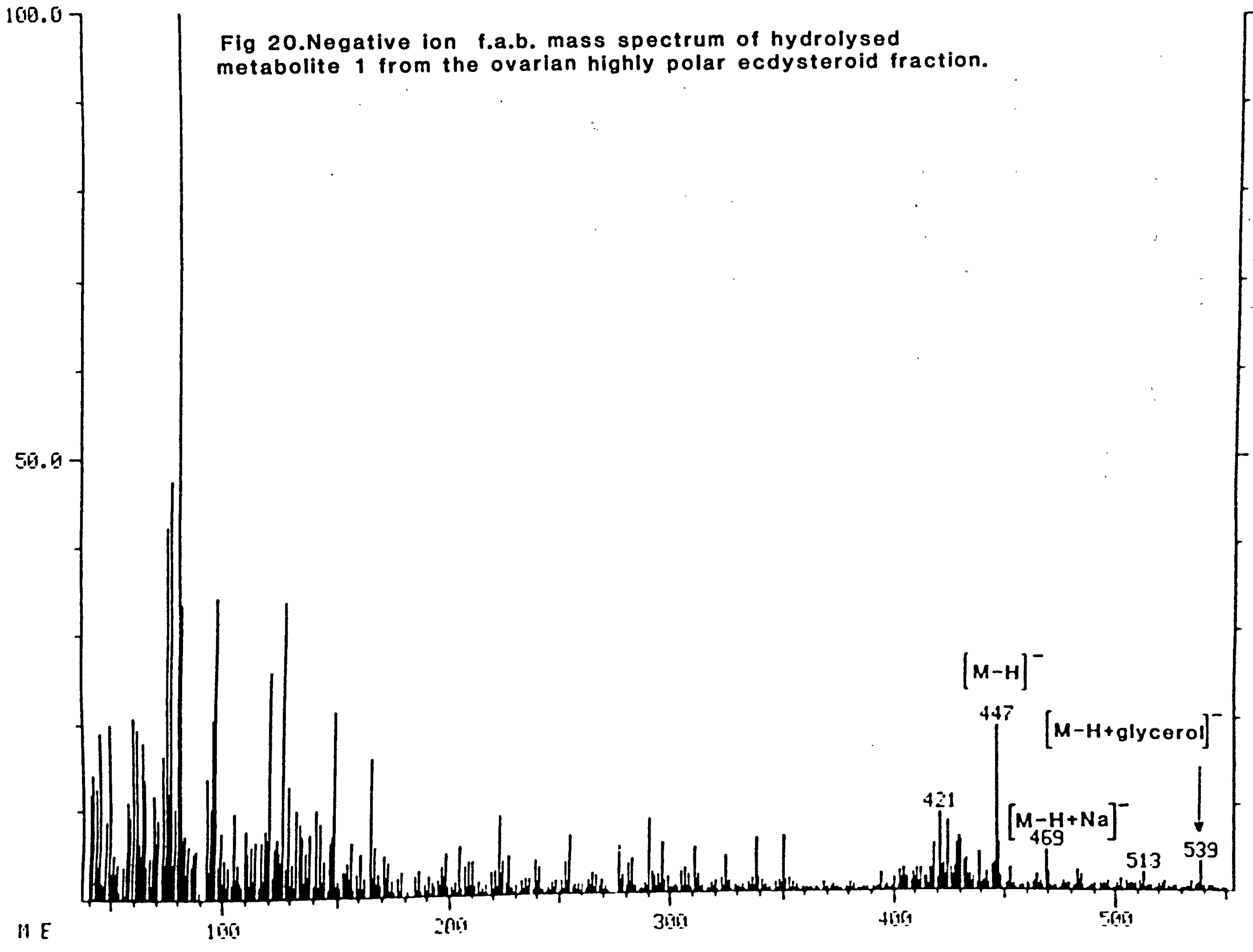


Fig 20. Negative ion f.a.b. mass spectrum of hydrolysed metabolite 1 from the ovarian highly polar ecdysteroid fraction.

VI. N.m.r. spectroscopic analysis of highly polar ecdysteroids

An aliquot of approximately 500μg of the putative 2-deoxyecdysone 22-phosphate (metabolite 1) from the highly polar ecdysteroid fraction isolated from the ovaries of Spodoptera was then further analysed by Fourier-transform nuclear magnetic resonance (n.m.r.) spectroscopy (see Chapter 2) to confirm its structure and the position of the phosphate moiety. The proton magnetic resonance spectrum (p.m.r.) of the ovarian metabolite (Fig. 21) was compared with the spectrum of authentic 2-deoxyecdysone 22-phosphate, obtained from published data, (Isaac et al., 1983a) using the same solvent [(^{2}H)methanol] (Table 2).

There are negligible differences between the spectra of metabolite 1 and the authentic 2-deoxyecdysone 22-phosphate, confirming the structure of the former and the position of the phosphate moiety.

E. Identification of egg metabolites

To allow complete identification of the egg metabolites a few hundred micrograms of each metabolite are required for nuclear magnetic resonance and mass spectrometric analysis. To collect such an amount of these ecdysteroids, approximately 41.2g of developing eggs, ranging from newly laid eggs to day 3 eggs just prior to hatching, were collected and stored at -20°C in methanol until required. The eggs were extracted by the method described in Chapter 2 and the weights of various fractions during the extraction are summarised in Fig. 22.

Fig 21. Fourier-transform n.m.r. spectrum of metabolite 1 from the ovarian highly polar ecdysteroid fraction.

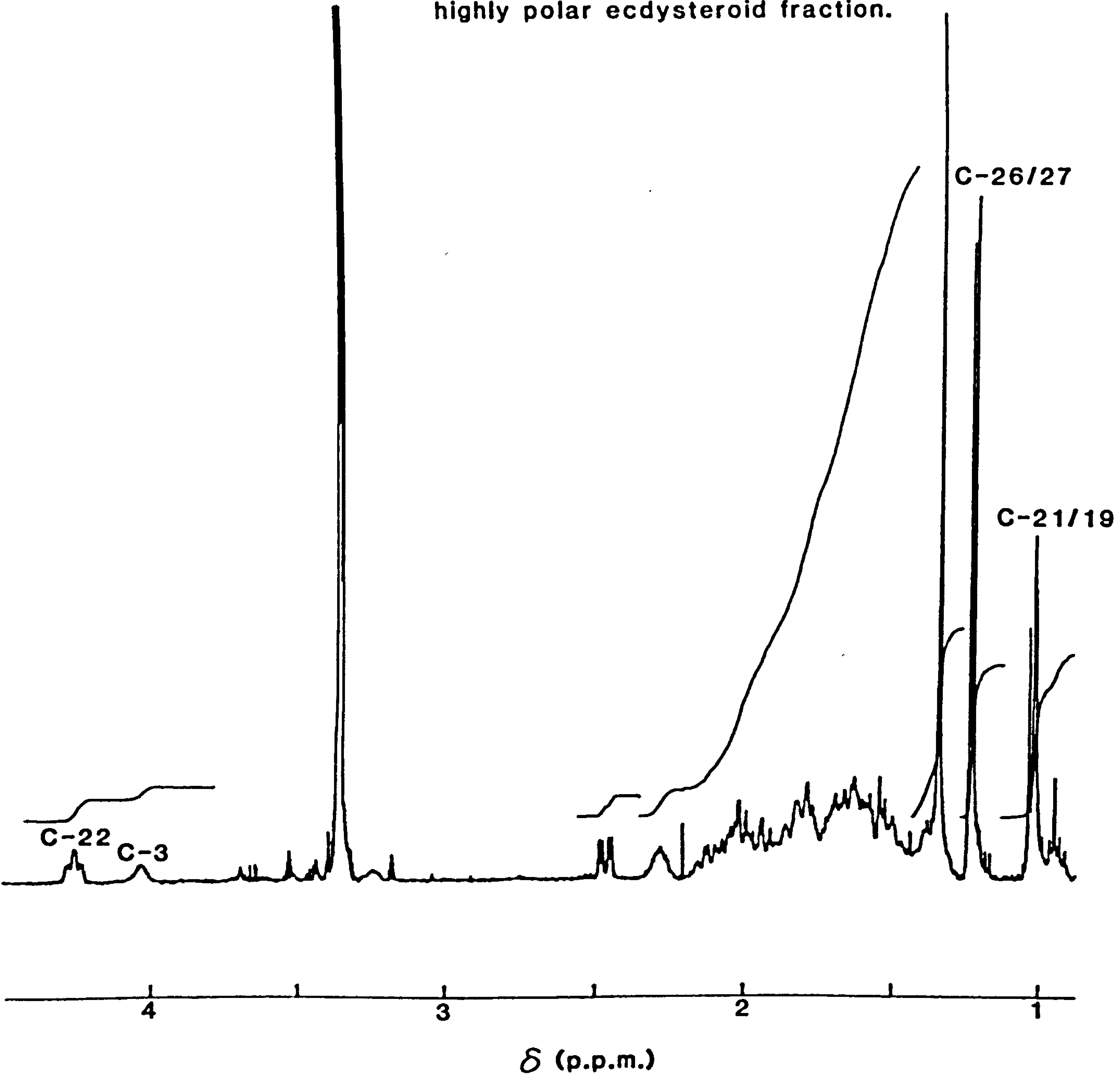


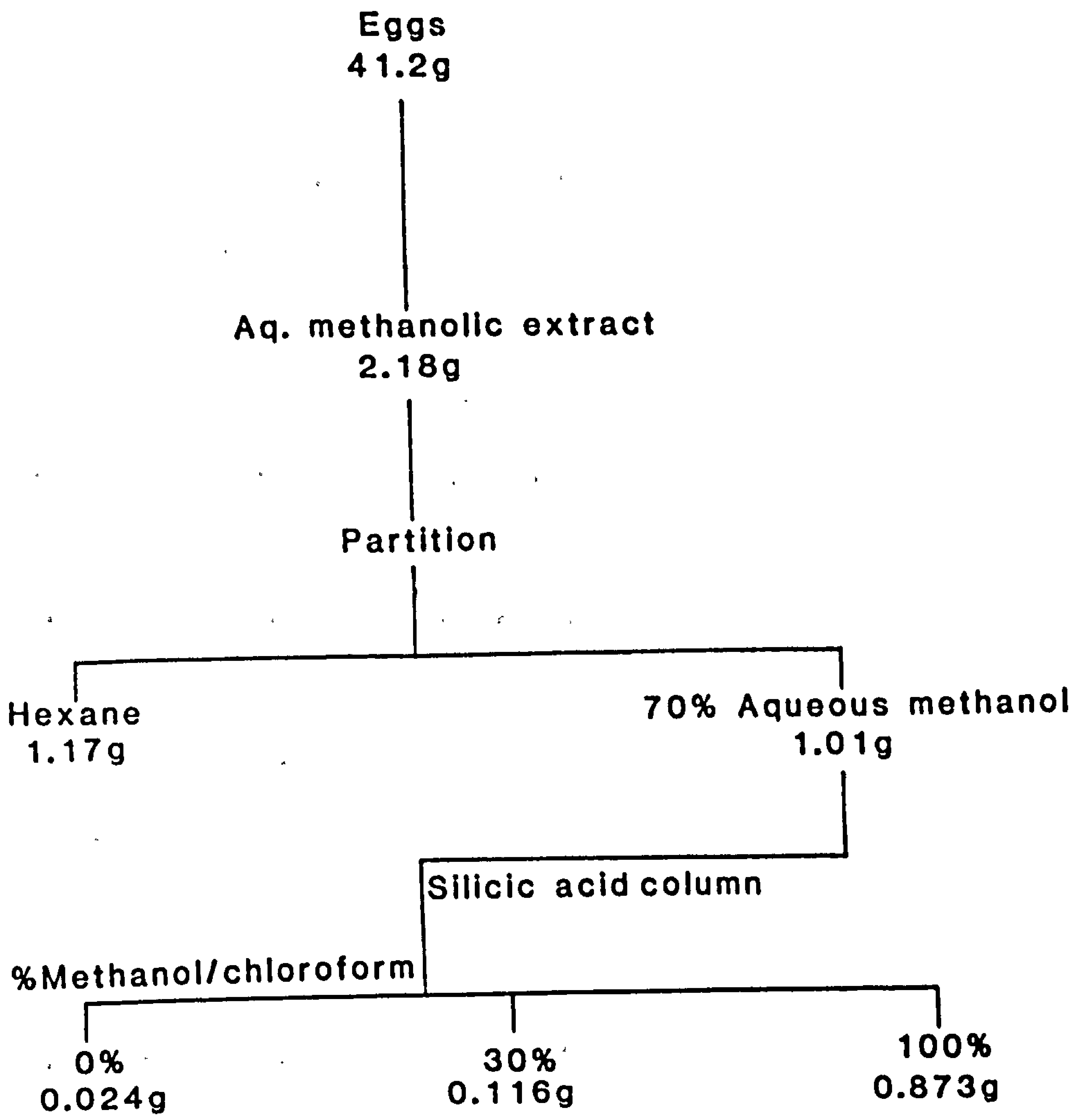
TABLE 2 **Chemical shifts of characteristic protons of putative 2-deoxyecdysone 22-phosphate (metabolite 1) of the highly polar ecdysteroids isolated from the ovaries of *S. littoralis* and of authentic 2-deoxyecdysone 22-phosphate**

P.m.r. spectra were recorded with (^{2}H)methanol as the solvent and tetramethysilane as reference. Chemical shifts are given downfield of tetramethylsilane.

	δ (p.p.m.)							
	Signal...	3H				1H		
Compound	Carbon atom(s)	C_{18}	C_{19}	C_{21}	C_{26}/C_{27}	C_3	C_{22}	C_7
Ovarian metabolite 1		0.78 (s)	1.01 (s)	1.01,1.03 (d,J 7Hz)	1.22,1.23 (2s)	4.02 (m,$W_{½}$ 18Hz*)	4.24 (m,$W_{½}$ 22Hz)	5.85 (d,J 1Hz)
Authentic 2-deoxy-ecdysone 22-phosphate		0.74 (s)	0.96 (s)	0.96,0.98 (d, J 8Hz)	1.17,1.19 (2s)	3.98 (m,$W_{½}$ 18Hz)	4.20 (m,$W_{½}$ 22Hz)	5.80 (d,J 2Hz)

*Width at half height

Fig 22. Weights of fractions during extraction of mixed day 0 to day 3 eggs.



I. Purification of free ecdysteroids

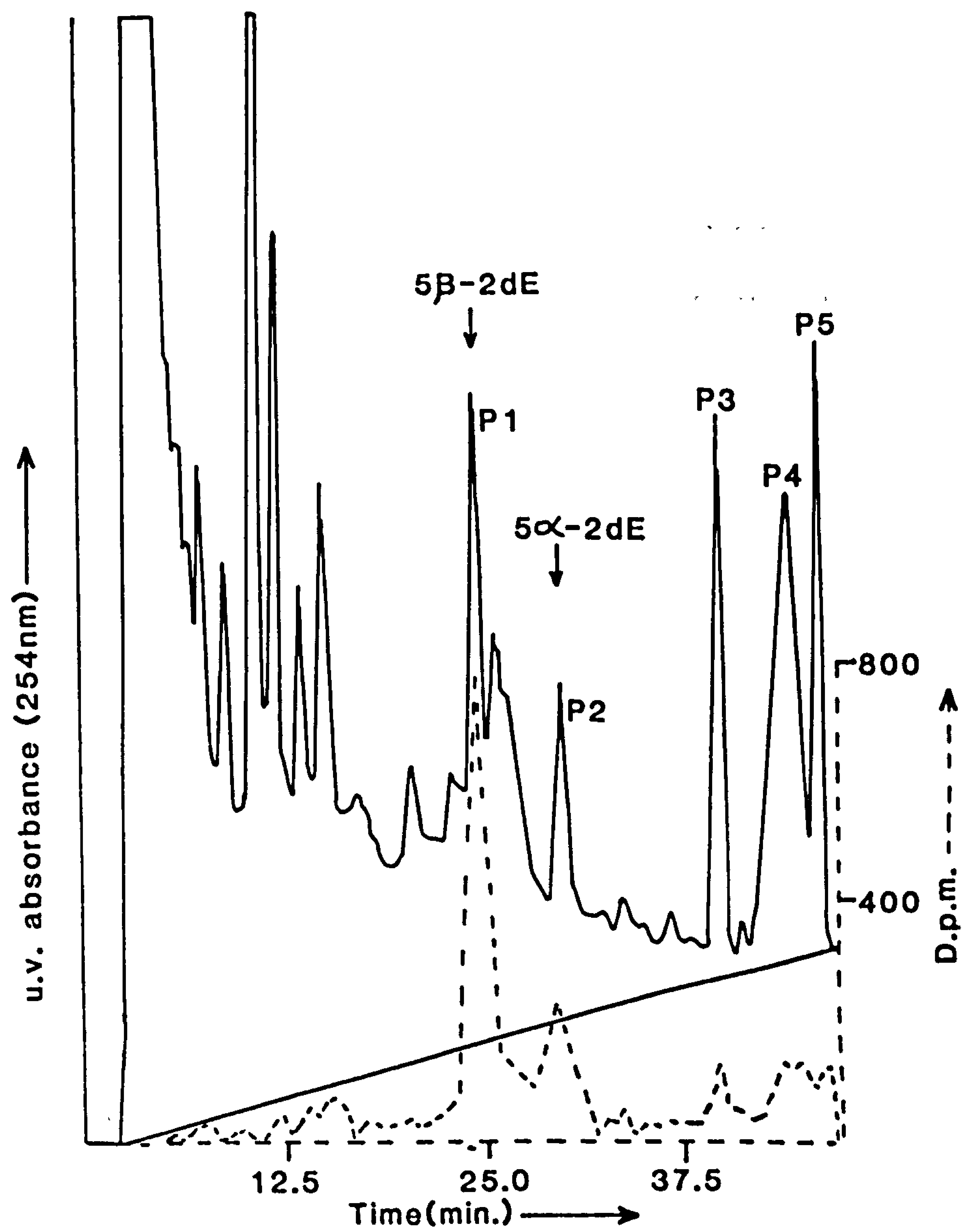
The free ecdysteroid fraction (30% methanol/chloroform silicic acid column fraction) was purified initially by reversed-phase h.p.l.c. (Table 1; system 3) using authentic markers and the tritiated free ecdysteroids from day 3 eggs to identify the relevant u.v. absorbing peaks. A typical chromatogram is shown in Fig. 23. Peaks 1-5 of the free ecdysteroids were collected by h.p.l.c. using this system (Table 1; system 3). Peaks 1 and 2 co-chromatograph with the authentic 5β-2-deoxyecdysone and 5α-2-deoxyecdysone (see Chapter 2) respectively, and also with the major and minor metabolites in the tritiated free ecdysteroid fraction from day 3 eggs (Fig. 23). Each of the metabolites was then applied separately to an adsorption h.p.l.c. system (Table 1; system 7) for further purification.

II. Mass spectrometric analysis of free ecdysteroids

An aliquot of approximately 10μg of each of the purified metabolites was then analysed by negative-ion f.a.b. mass spectrometry using glycerol as the probe matrix (see Chapter 2).

In the negative-ion f.a.b. mass spectra of metabolites 1 (Fig. 24) and 2 (Fig. 25) the major ion at high mass is at m/z 447, which corresponds to $[M-H]^-$; indicating a molecular weight of 448 as expected for 2-deoxyecdysone. The pseudomolecular ion for metabolite 1 is substantiated by the presence of an ion at m/z 539 corresponding to $[M-H + glycerol]^-$. This is consistent with previous data in which

Fig 23.Purification of free ecdysteroids from eggs by reversed-phase h.p.l.c. (system 3).



P1-5: Peaks 1-5

Arrows indicate retention times of authentic sample.

Tritiated free ecdysteroids from day 3 eggs. – – – –

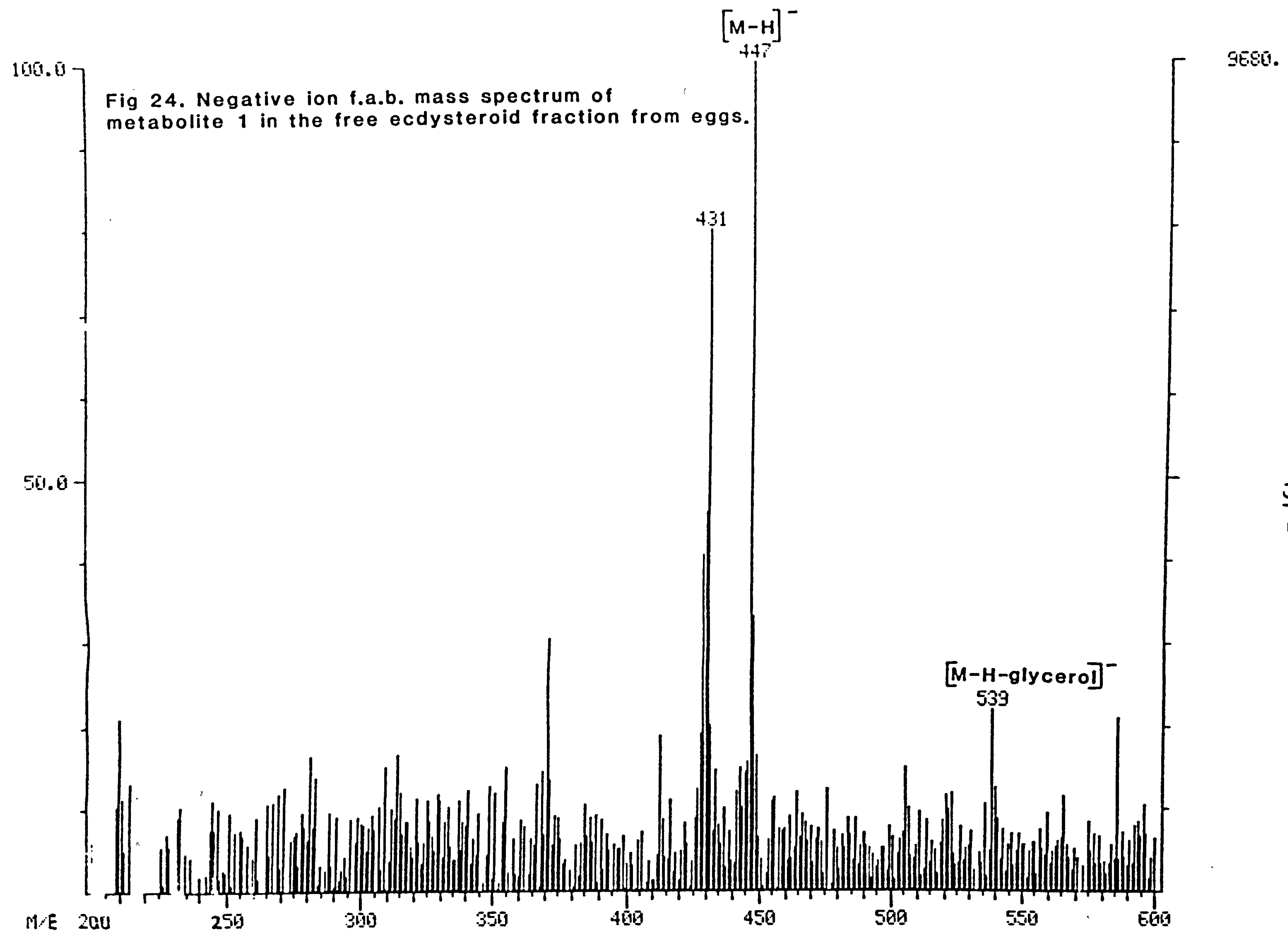


Fig 24. Negative ion f.a.b. mass spectrum of metabolite 1 in the free ecdysteroid fraction from eggs.

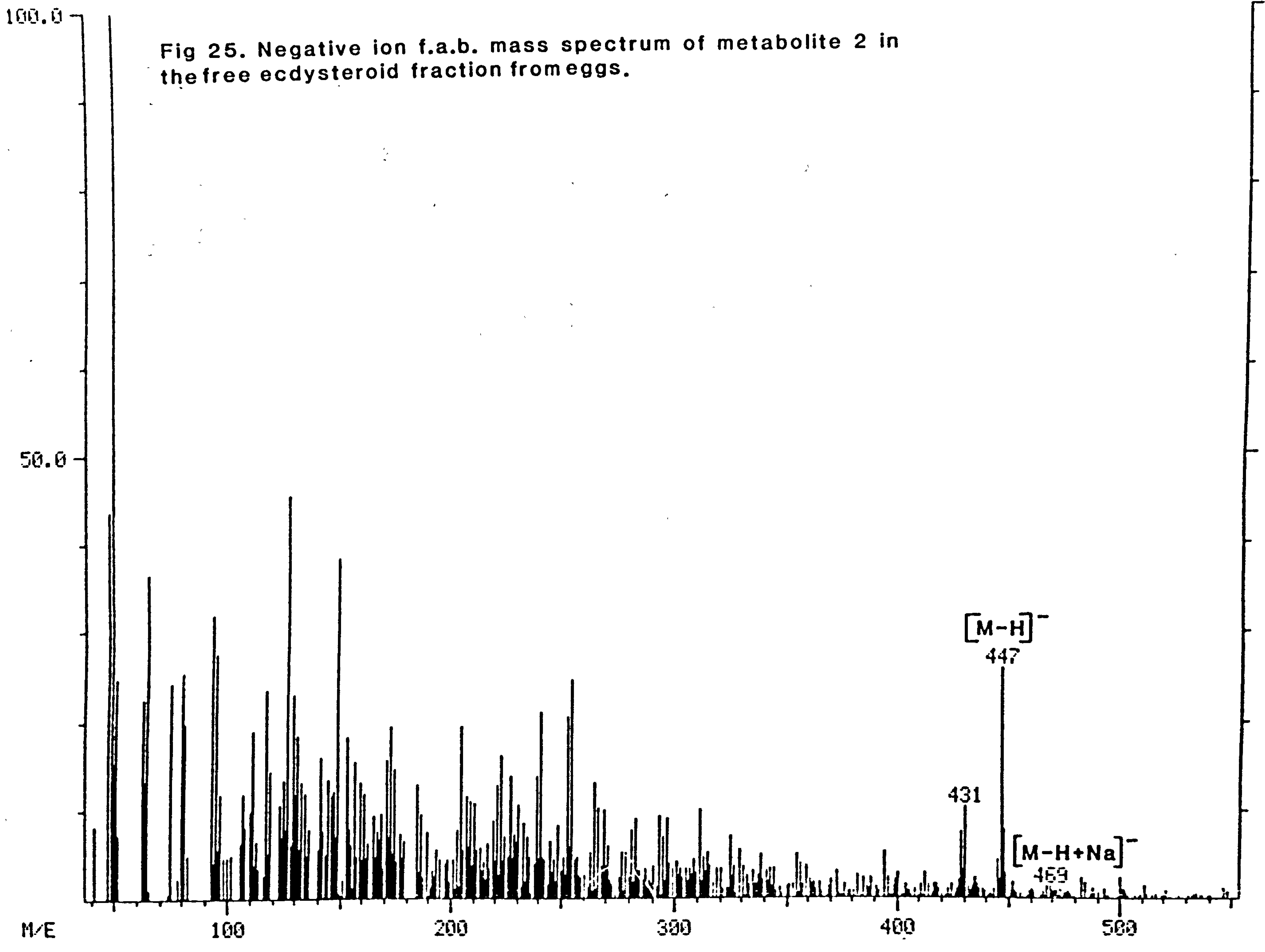


Fig 25. Negative ion f.a.b. mass spectrum of metabolite 2 in the free ecdysteroid fraction from eggs.

the 5α-2-deoxyecdysone (see Chapter 2) co-chromatographs with metabolite 2 and the tritiated peak 2 in the free ecdysteroid fraction from non radioactive and radioactive day 3 eggs (Fig. 23). This suggests that metabolites 1 and 2 are 5β- and 5α-2-deoxyecdysone, respectively. The mass spectra of metabolites 3-5 were difficult to interpret and were thus probably impure samples (see Appendix).

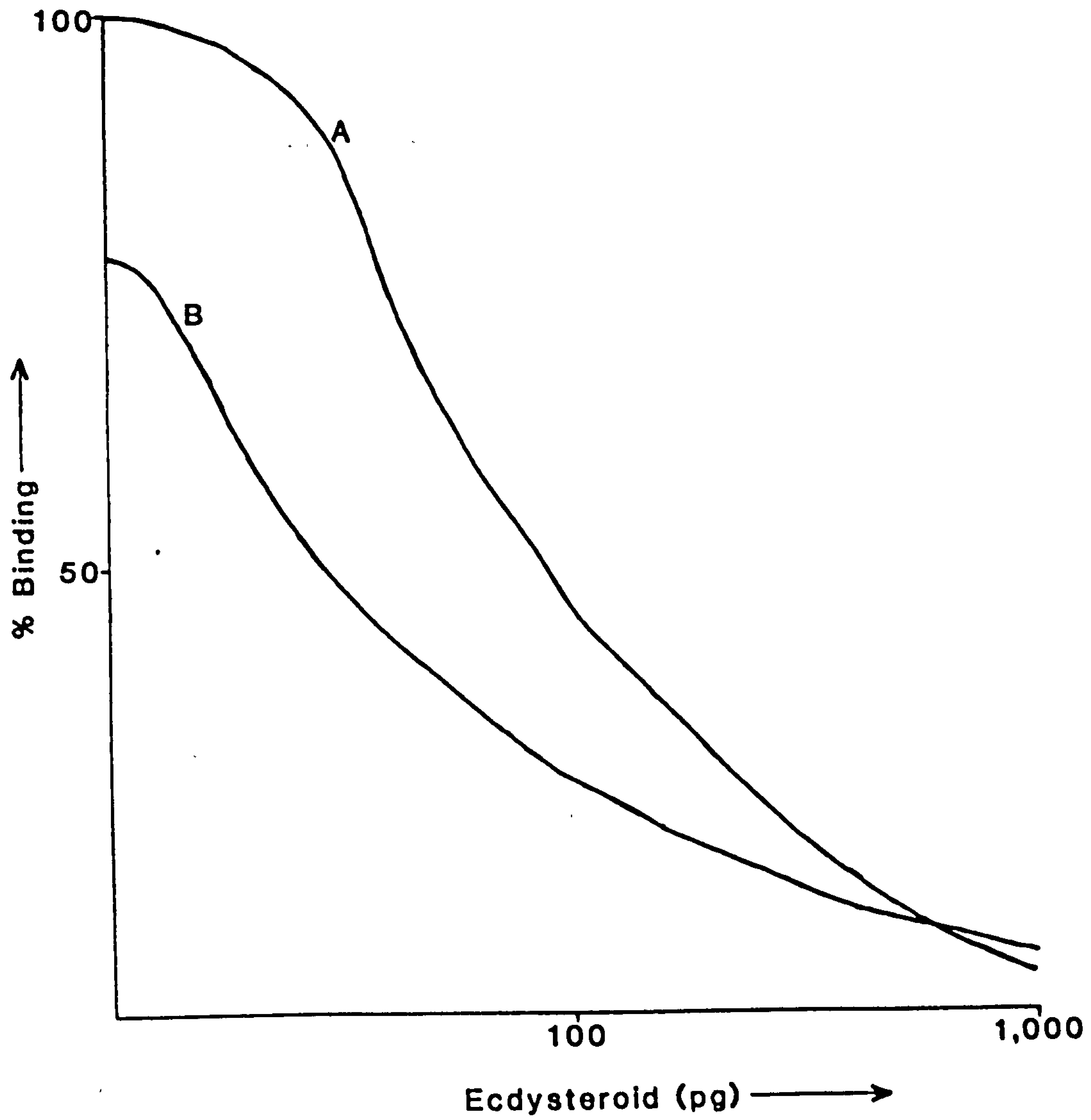
III. Analysis of the free ecdysteroid fraction for the presence of ecdysone and 20-hydroxyecdysone

The free ecdysteroids were analysed by h.p.l.c.-radioimmunoassay (R.I.A.) for the presence of ecdysone and 20-hydroxyecdysone.

Initially the cross-reactivities of 2-deoxyecdysone and ecdysone with DHS 1-13.5 antiserum were compared as the majority of the free ecdysteroid is 2-deoxyecdysone. This was determined by comparing a standard curve, prepared as previously (see Chapter 2), but using 2-deoxyecdysone instead of ecdysone, with a standard curve prepared at the same time using ecdysone. The cross-reactivities of the two ecdysteroids with the antiserum are very similar (Fig. 26) with 2-deoxyecdysone having slightly greater affinity for DHS 1-13.5. The cross reactivity factor of DHS 1-13.5 to 2-deoxyecdysone (ratio of the mass of 2-deoxyecdysone required to displace 50% of the labelled ecdysone to the mass of ecdysone required) is 0.36.

The free ecdysteroids were first analysed by reversed-phase h.p.l.c./R.I.A. (Table 1; system 1) for the presence of ecdysone and

Fig 26. Binding competition curve of ecdysone (A) and 2-deoxyecdysone (B) using DHS 1-13.5.



20-hydroxyecdysone, fractions being collected every minute for radioimmunoassay using DHS 1-13.5 antiserum. Authentic markers were used to identify the immunoreactive metabolites present. The h.p.l.c./R.I.A. chromatogram obtained is shown in Fig. 27. The major immunoreactive ecdysteroid co-chromatographs with 2-deoxyecdysone. There are also smaller peaks of immunoreactivity which co-chromatograph with ecdysone and 20-hydroxyecdysone.

The free ecdysteroids were then analysed by adsorption h.p.l.c./R.I.A. (Table 1; system 7) to further confirm the presence of ecdysone and 20-hydroxyecdysone, fractions being collected every minute for radioimmunoassay using DHS 1-13.5 antiserum (Fig. 28). Again the major immunoreactive ecdysteroid co-chromatographs with 2-deoxyecdysone. There are also smaller peaks of immunoreactivity which co-chromatograph with ecdysone and 20-hydroxyecdysone.

IV. Purification of highly polar ecdysteroids

The highly polar ecdysteroid fraction (combined 80% and 100% methanol/chloroform silicic acid column fractions) was initially purified by ion-suppression reversed-phase h.p.l.c. (Table 1; system 5) using authentic markers and the tritiated highly polar ecdysteroids from day 3 eggs to identify the relevant u.v. absorbing peaks. A typical chromatogram is shown in Fig. 29. Peak 1 co-chromatographs with authentic 2-deoxyecdysone 22-phosphate and the single metabolite

Fig 27. Analysis of free ecdysteroids from eggs on reversed-phase h.p.l.c./R.I.A. (system 1) for the presence of ecdysone and 20-hydroxyecdysone.

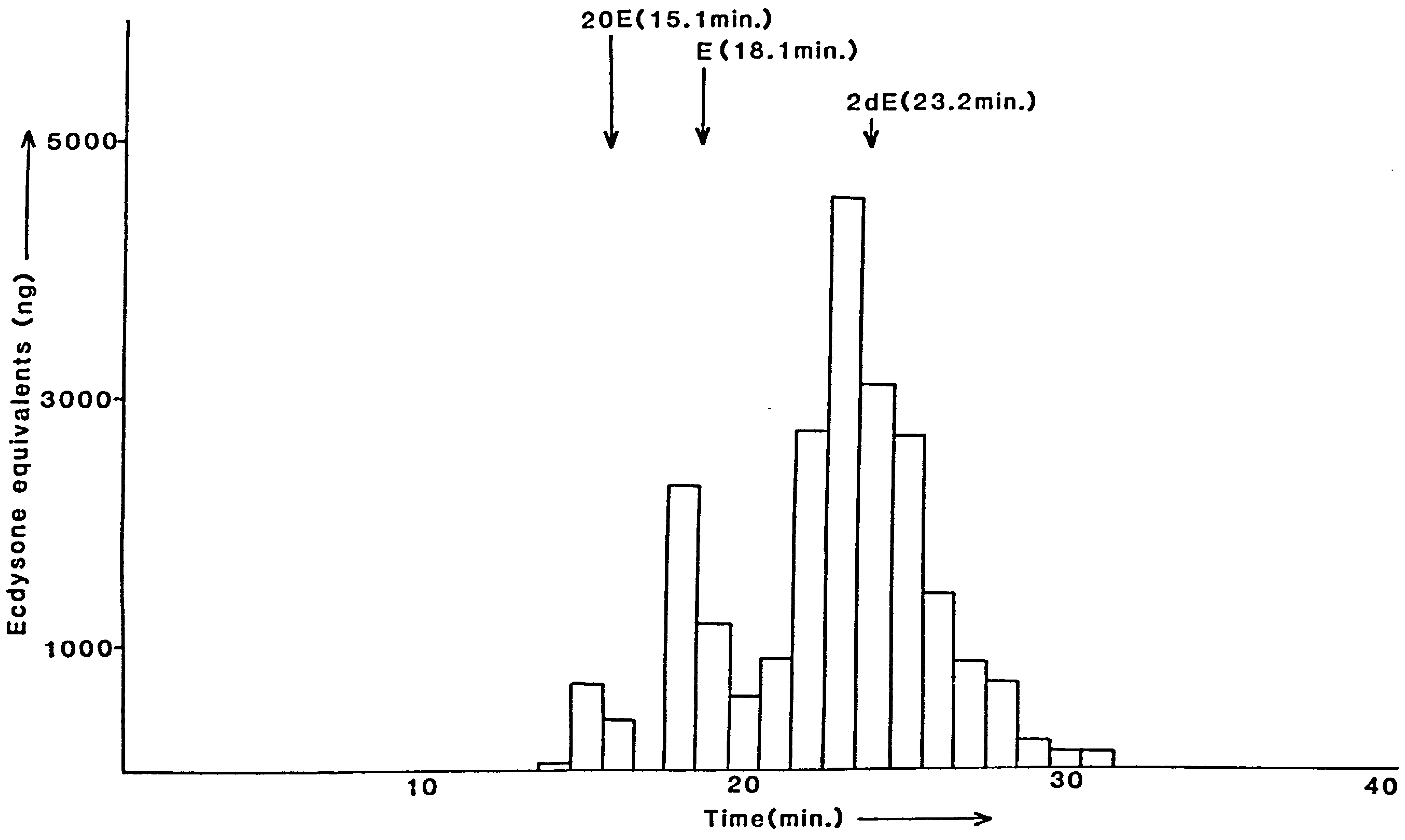


Fig 28. Analysis of free ecdysteroids from eggs on adsorption-h.p.l.c./R.I.A. (system 7) for the presence of ecdysone and 20-hydroxyecdysone.

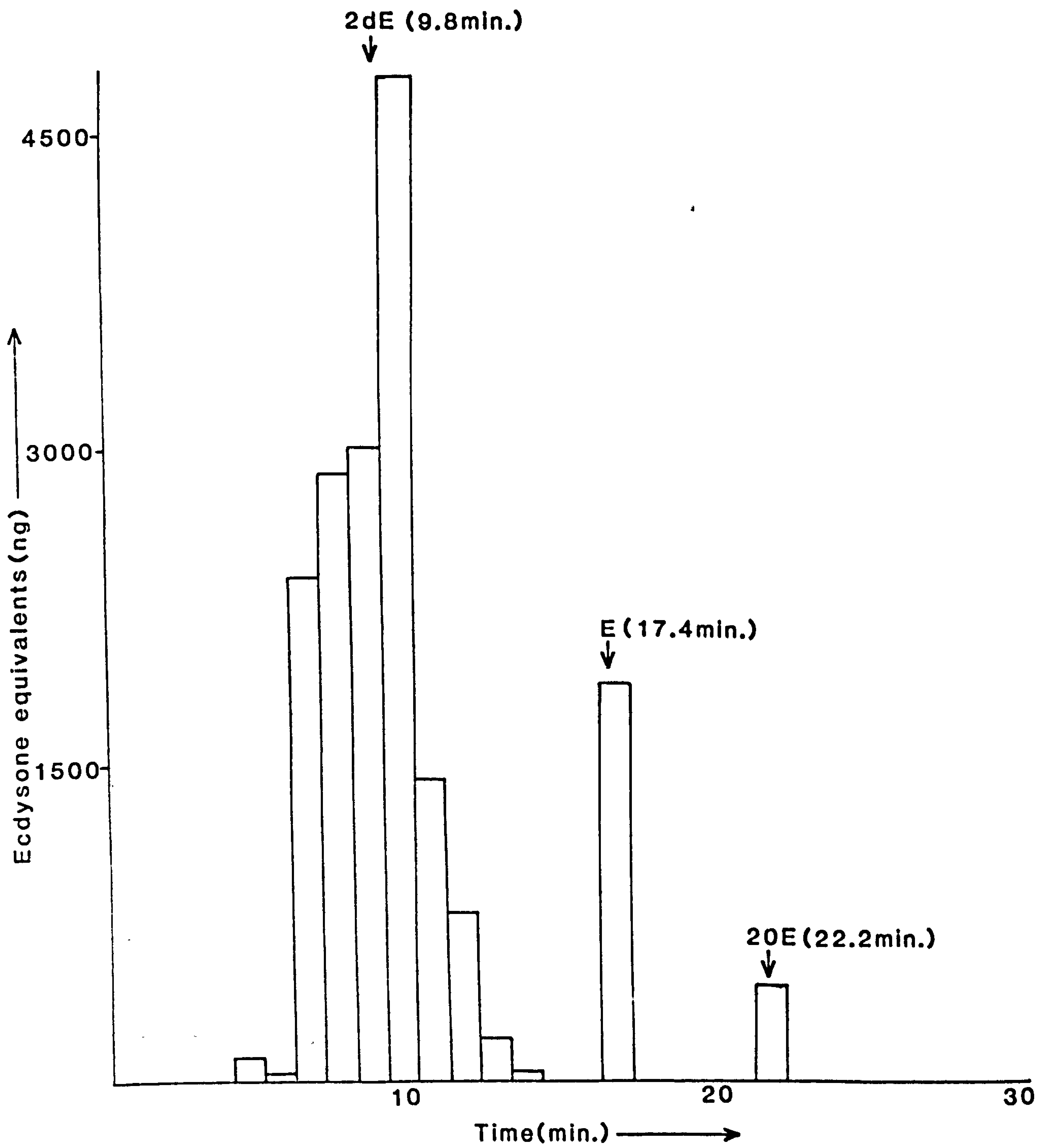
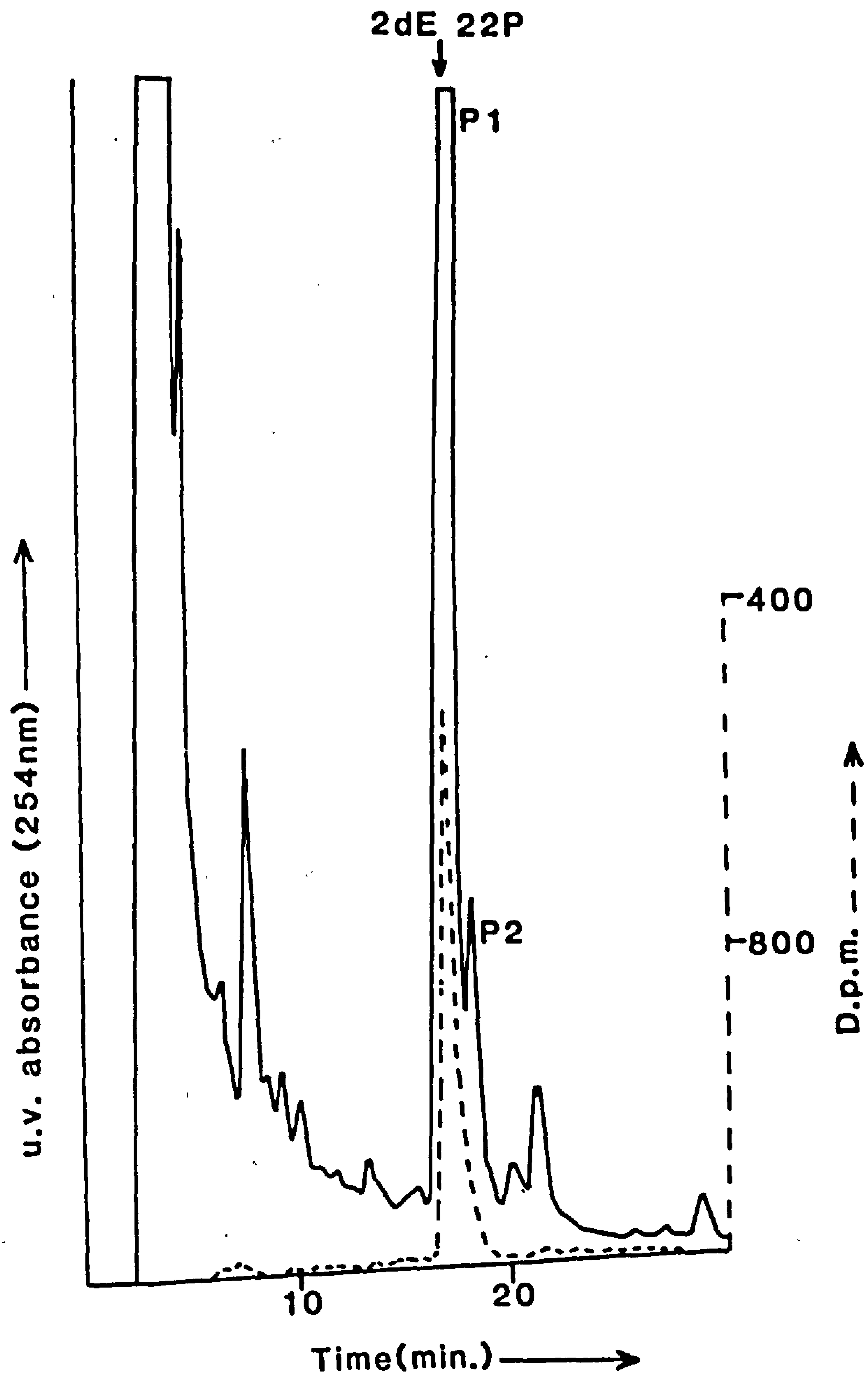


Fig 29. Purification of highly polar ecdysteroids from eggs by reversed-phase h.p.l.c. (system 5).



P1and 2: Peaks 1 and 2.
Arrow indicates retention time of authentic sample.
Tritiated highly polar ecdysteroids from day 3 eggs.- - - -

in the tritiated highly polar ecdysteroid fraction from day 3 eggs. Each of the metabolites was then applied separately to a second reversed-phase ion-suppression h.p.l.c. system (Table 1; system 6) for further purification.

V. Mass spectrometric analysis of highly polar ecdysteroids

An aliquot of approximately 10μg of each of the purified metabolites was then analysed by negative-ion f.a.b. mass spectrometry using glycerol as the probe matrix (see Chapter 2).

In the negative-ion f.a.b. mass spectra of metabolites 1 and 2 the major ion at high mass is at m/z 527, which corresponds to the pseudomolecular $[M-H]^-$ ion, indicating a molecular weight of 528 (Fig. 30 and 31, respectively) the same as that of 2-deoxyecdysone 22-phosphate. A significant ion is also observed at m/z 549 corresponding to $[M-H + Na]^-$. As before, the ions at m/z 97 and 79 are characteristic of phosphate. Metabolite 2 does not co-chromatograph with 3-epi-2-deoxyecdysone 22-phosphate. As before it was possible that it is the 5α-epimer of 2-deoxyecdysone 22-phosphate with metabolite 1 being the 5β-epimer. Metabolite 2 co-chromatographs with the ovarian metabolite 2 which was identified as the 5α-2-deoxy- ecdysone 22-phosphate (section D). In addition, the corresponding free 5α-2-deoxyecdysone was identified in the free ecdysteroid fraction from eggs (section E).

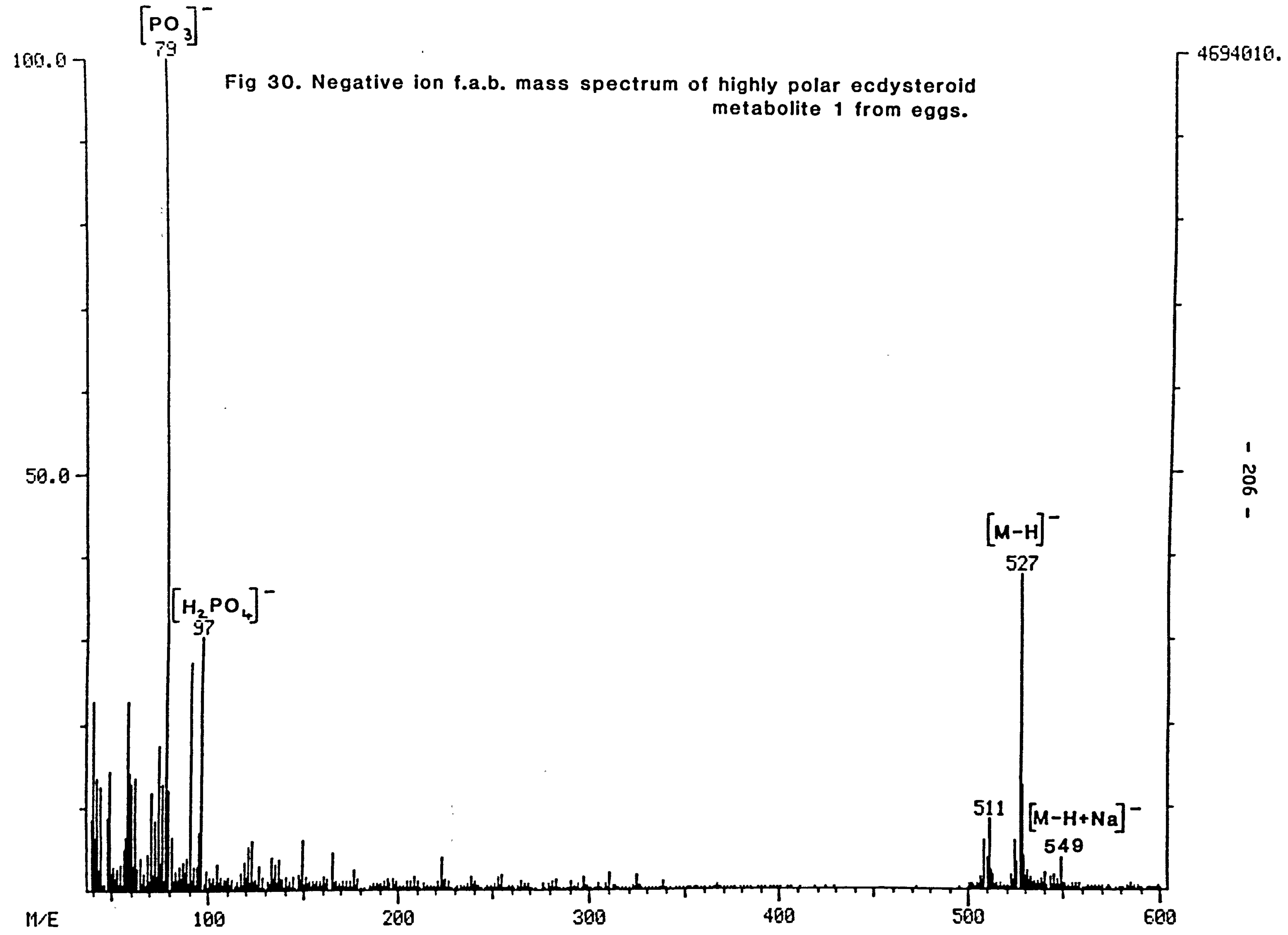


Fig 30. Negative ion f.a.b. mass spectrum of highly polar ecdysteroid metabolite 1 from eggs.

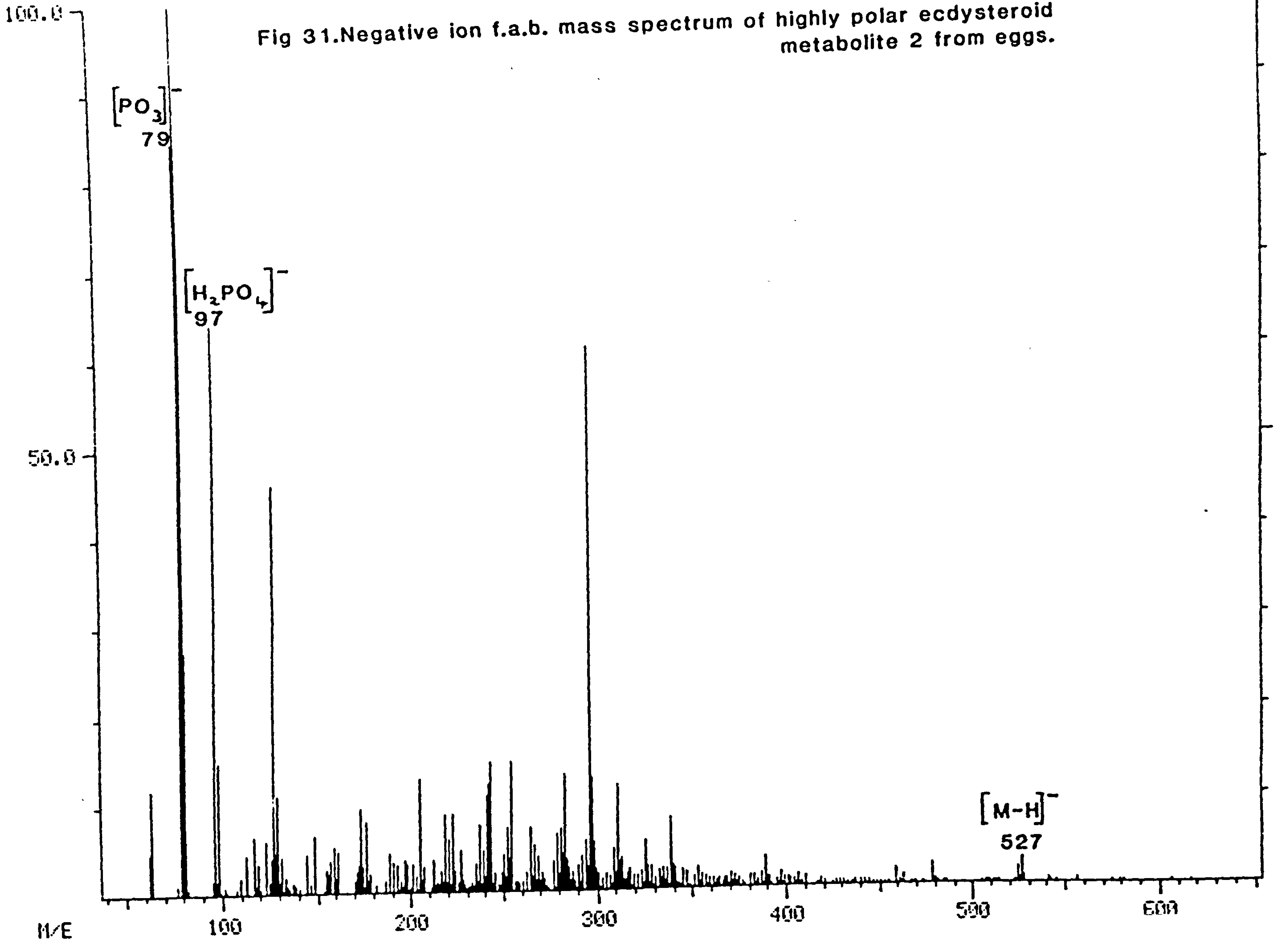


Fig 31.Negative ion f.a.b. mass spectrum of highly polar ecdysteroid metabolite 2 from eggs.

VI. **N.m.r. spectroscopic analysis of highly polar ecdysteroids**

An aliquot of approximately 900μg of the putative 2-deoxyecdysone 22-phosphate (metabolite 1) from the highly polar ecdysteroid fraction isolated from eggs of Spodoptera was then further analysed by Fourier-transform nuclear magnetic resonance (n.m.r.) spectroscopy (see Chapter 2) to confirm its structure and the position of the phosphate moiety. The proton magnetic resonance spectrum (p.m.r.) of the egg metabolite (Fig. 32) was compared with the spectra of the ovarian metabolite (section D) and that of the authentic 2-deoxyecdysone 22-phosphate, obtained from published data, (Isaac et al., 1983a) using the same solvent [(^{2}H)methanol] (Table 3). There are negligible differences between the spectra, confirming the structure of the egg metabolite as 2-deoxyecdysone 22-phosphate and identifying the position of the phosphate moiety.

F. **Distribution of radioactivity amongst the silicic acid column fractions in the ovaries and eggs**

36 female S. littoralis pupae were injected with [1α,2α-$^{3}H_{2}$]-cholesterol (108μCi total) as described in section A and then maintained with an equal number of males for mating. The ovaries were dissected from 10 adults and stored at -20°C until required. The remaining 21 female adults were allowed to lay eggs in two separate groups. The eggs laid by the first group (14 adults) were collected up to 4hr. after deposition and stored at -20°C until required. The eggs laid by the second group (12 adults) were collected up to 4hr. after

Fig 32. Fourier-transform n.m.r. spectrum of metabolite 1 from the highly polar ecdysteroid fraction from eggs.

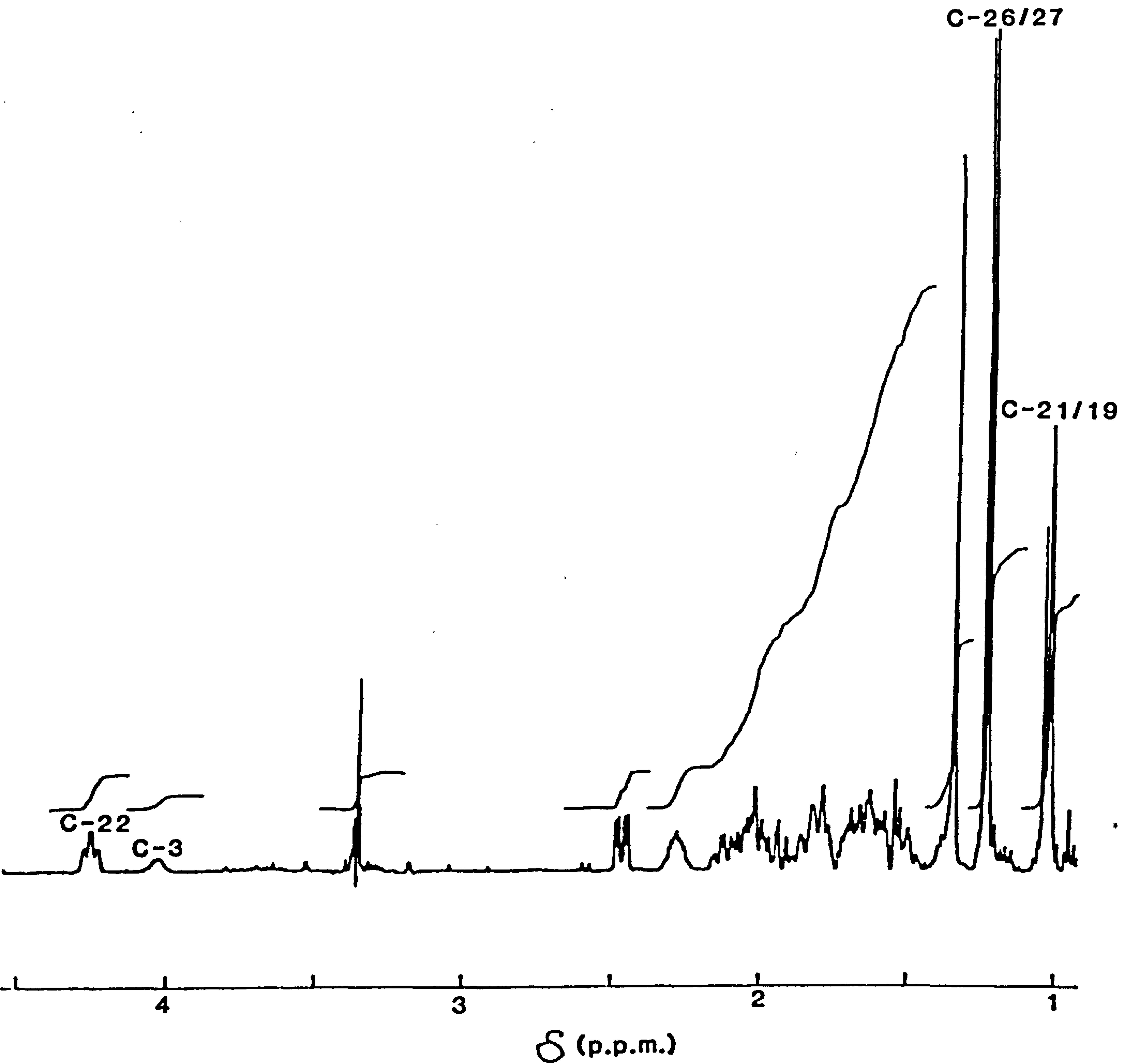


TABLE 3 Chemical shifts of characteristic protons of putative 2-deoxyecdysone 22-phosphate (metabolite 1) of the highly polar ecdysteroids isolated from the ovaries and eggs of *S. littoralis* and the authentic 2-deoxyecdysone 22-phosphate

P.m.r. spectra were recorded with (^{2}H]methanol as the solvent and tetramethylsilane as reference. Chemical shifts are given downfield of tetramethylsilane

	δ (p.p.m.)							
	Signal	3H				1H		
Compound	Carbon atom(s)	C_{18}	C_{19}	C_{21}	C_{26}/C_{27}	C_3	C_{22}	C_7
Egg metabolite 1		0.78 (s)	1.01 (s)	1.01,1.03 (d,J 7Hz)	1.22,1.23 (2s)	4.04 (m,$W_{1/2}$ 18Hz*)	4.25 (m,$W_{1/2}$ 22Hz)	5.85 (d,J 1Hz)
Ovarian metabolite 1		0.78 (s)	1.01 (s)	1.01,1.03 (d,J 7Hz)	1.22,1.23 (2s)	4.02 (m,$W_{1/2}$ 18Hz)	4.24 (m,$W_{1/2}$ 22Hz)	5.85 (d,J 1Hz)
Authentic 2-deoxy-ecdysone 22-phosphate		0.74 (s)	0.96 (s)	0.96,0.98 (d,J 8Hz)	1.17,1.19 (2s)	3.98 (m,$W_{1/2}$ 18Hz)	4.20 (m,$W_{1/2}$ 22Hz)	5.80 (d,J 2Hz)

*Width at half height

deposition and incubated for a further 3 days. The eggs were then frozen at -20°C and stored until required.

The developing ovaries, newly laid eggs and day 3 eggs were extracted by the method described in Chapter 2. The distribution of the radioactivity, in the free ecdysteroid fractions (30% silicic acid column fractions) and the highly polar ecdysteroid fractions (80% and 100% silicic acid column fractions) after separation on a silicic acid column is shown in Table 4.

The total radioactivity in 2-deoxyecdysone and the corresponding conjugate (Table 4) were obtained from reversed-phase h.p.l.c. analysis of the free and highly polar ecdysteroid fractions by calculating the radioactivity corresponding to each ecdysteroid metabolite relative to the total radioactivity recovered.

The ovaries (from day 3 adults) appear to contain a higher concentration of highly polar ecdysteroids, but this is apparently reversed in newly laid eggs in which there is an apparent higher concentration of free ecdysteroids. The difference is increased when the labelling in 2-deoxyecdysone is compared with that in the corresponding 22-phosphate. In eggs which had undergone 3 days of embryogenesis ie. just prior to hatching there appears to be an apparent slightly greater concentration of free ecdysteroids compared with the highly polar ecdysteroids. This difference is increased slightly when the labelling in 2-deoxyecdysone is compared with that in the corresponding 22-phosphate. Approximately, twice as much radioactivity is recovered corresponding to the 5β-2-deoxyecdysone compared with the 5α-epimer, in the free ecdysteroid fraction of day 3 eggs.

TABLE 4 **Distribution of radioactivity amongst silicic acid column fractions in the ovaries and eggs**

Stage of development	Free ecdysteroid fraction (d.p.m.)	Total d.p.m. in 2-deoxy ecdysone	Highly polar ecdysteroid fraction (d.p.m.)	Total d.p.m. in 2-deoxy ecdysone 22-phosphate
Ovaries	4,360	1,130	18,800	14,400
Newly laid eggs	60,400	41,600	5,830	2,560
Day 3 eggs	14,300	7,870 (3,000*)	11,200	7,060

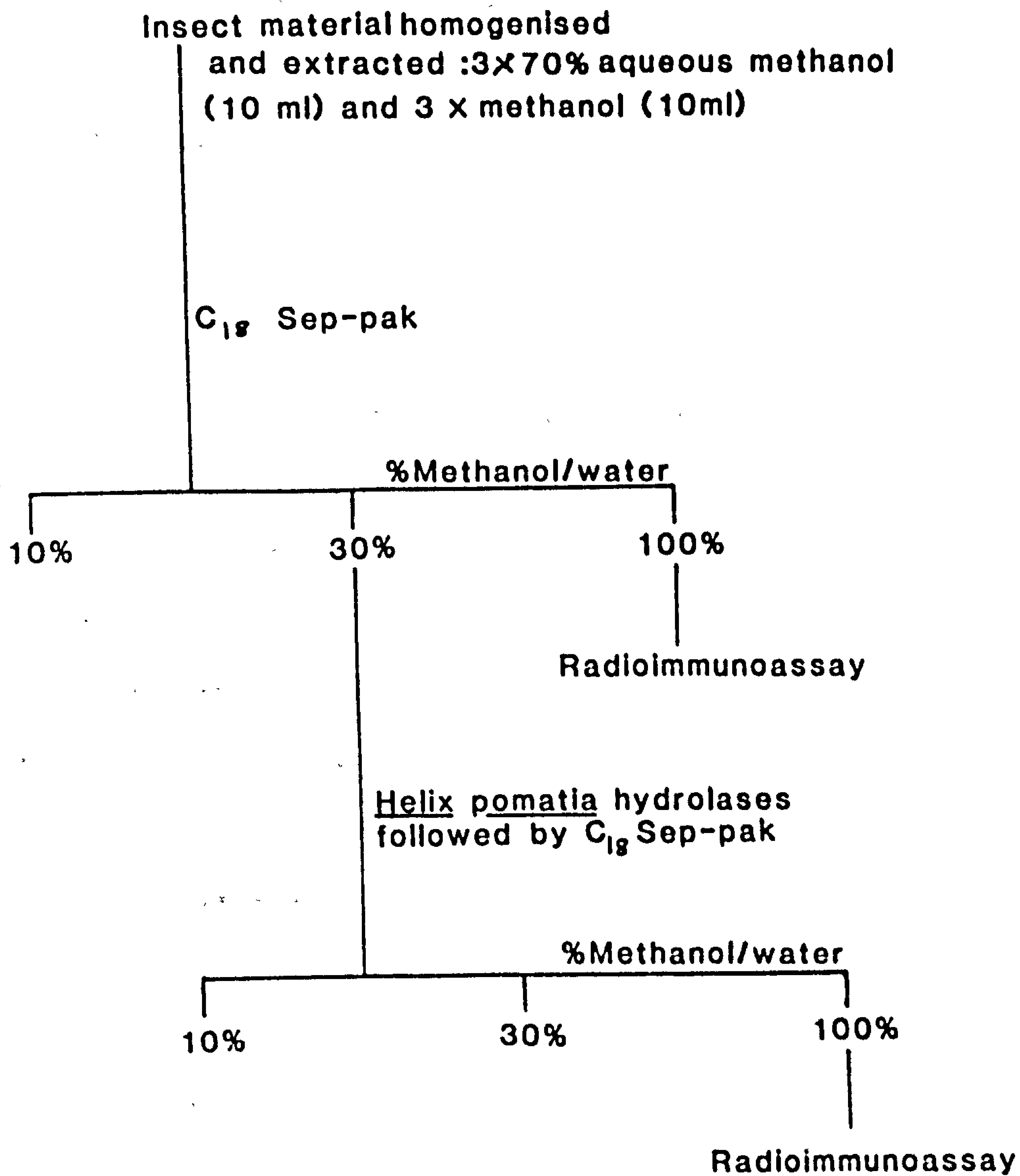
*Total d.p.m. in 5α-2-deoxyecdysone in parenthesis.

G. Profile of unlabelled ecdysteroids during oogenesis and embryogenesis

20 female S. littoralis pupae were synchronized (± 4hr.) at adult emergence. Duplicate samples of ovaries were each dissected after 24, 48 and 72hr. of ovarian development from 2 female adults which had been mated with an equal number of males. The ovaries were dissected in Ringers solution, washed once in Ringers solution and then water prior to being blotted dry with filter paper. Each duplicate batch of ovaries was weighed immediately and then freeze dried for 24hr. prior to being re-weighed. The samples were then stored at -20°C until required. The remaining 8 adults, which were mated with an equal number of males, were allowed to lay eggs. The eggs were collected up to 4hr. after deposition and incubated in five separate batches for 0, 24, 48 and 72hr. Four of the individual batches of eggs were then weighed after the respective times of development and stored at -20°C until required. The remaining batch of eggs was continually weighed throughout the 72hr. incubation period and was allowed to hatch, to ensure the eggs had developed normally.

The batches of ovaries and eggs were extracted by the method given in Fig. 33, which is a modified version of the method described in Chapter 2. The ovaries and eggs were extracted as described in Chapter 2, three times with 70% aqueous methanol (3 x 10ml) and three times with methanol (3 x 10ml). The combined fractions were fractionated by C_{18} Sep-pak cartridges (Chapter 2). An aliquot of the

Fig 33. Summary of the modified ecdysteroid extraction procedure for the ovaries and eggs of Spodoptera littoralis.



methanol fraction (free ecdysteroid fraction) was subjected to radioimmunoassay with DHS 1-13.5 antiserum (Chapter 2). The 30% aqueous methanol fraction (highly polar ecdysteroid fraction) was treated with the Helix pomatia enzyme preparation, and the free ecdysteroids released separated from any unhydrolysed metabolites on a C_{18} Sep-pak cartridge. An aliquot of the methanol fraction (free ecdysteroids released by Helix hydrolases) was subjected to radioimmunoassay with DHS 1-13.5 antiserum (Chapter 2).

The distribution of immunoreactivity, in the free ecdysteroids and the ecdysteroids released from polar conjugation by Helix hydrolases occurring at various stages of oogenesis and embryogenesis is given in Table 5. The ecdysteroid concentration during oogenesis (excluding unhydrolysable metabolites) remains relatively constant. This suggests that ovarian development occurs primarily during the first day following emergence from the pupae, since egg-laying only begins 3 days into oogenesis.

During embryogenesis, the weight of the eggs decreases by approximately one third (Table 6) probably due to water loss. This is taken into consideration in Table 6 where the ecdysteroid concentration is given per batch of eggs. The ecdysteroids appear to be passed into the eggs where the titre decreases early in embryogenesis (Table 6). Towards the end of embryogenesis the total ecdysteroid titre per batch of eggs rises dramatically again (Table 6). The profile of ecdysteroid metabolites during embryogenesis was confirmed in a repeat experiment (Table 7). The only difference is that the major peak in ecdysteroid concentration during embryogenesis

TABLE 5 Ecdysteroid profile during oogenesis and embryogenesis assayed by R.I.A. using the DHS 1-13.5 antiserum. (The cross reactivity of DHS 1-13.5 to 2-deoxyecdysone is 0.36)

Stage of development (Days)	Free ecdysteroids	Ecdysteroids released from polar conjugates	Total free ecdysteroids and ecdysteroids released from polar conjugates
Ovaries (oogenesis)			
1	$1.75 \pm 0.26^{\cdot}$ (6.60^{+})	$2.45 \pm 1.05^{\cdot}$ (9.03^{+})	$4.20 \pm 1.31^{\cdot}$ (15.63^{+})
2	$1.50 \pm 0.03^{\cdot}$ (5.50^{+})	$2.37 \pm 0.19^{\cdot}$ (8.67^{+})	$3.87 \pm 0.22^{\cdot}$ (14.17^{+})
3	$2.04 \pm 0.13^{\cdot}$ (7.91^{+})	$3.02 \pm 0.46^{\cdot}$ (11.60^{+})	$5.06 \pm 0.59^{\cdot}$ (19.51^{+})
Eggs (embryogenesis)			
0	142^{*}	108^{*}	250^{*}
1	7^{*}	34^{*}	41^{*}
2	35^{*}	90^{*}	125^{*}
3	147^{*}	117^{*}	264^{*}

$^{\cdot}$Values for ovaries are means and range of two complete determinations (μg ecdysone equivalents per gram wet weight of ovaries).

$^{+}$Values in parenthesis are μg ecdysone equivalents per gram dry weight of tissue.

*Values given are μg ecdysone equivalents per gram wet weight of eggs.

TABLE 6 **Ecdysteroid profile during embryogenesis per batch of eggs determined by R.I.A. using DHS 1-13.5 antiserum. (The cross reactivity of DHS 1-13.5 to 2-deoxyecdysone is 0.36)**

Stage of development (Days)	Weight of a single batch of eggs (grams)	Free ecdysteroids*	Ecdysteroids released from polar conjugates*	Total free ecdysteroids and ecdysteroids released from polar conjugates*
Eggs				
0	0.01284+	142	108	250
1	0.00951	5.18	25.2	30.4
2	0.00630	17.2	61.3	78.5
3	0.00422	48.3	86.7	135

*Values given are μg ecdysone equivalents per gram wet weight+ of day 0 egg tissue.

TABLE 7 Repeat ecdysteroid profile during embryogenesis per batch of eggs determined by R.I.A. using DHS 1-13.5 antiserum. (The cross reactivity of DHS 1-13.5 to 2-deoxyecdysone is 0.36)

Stage of development (Days)	Weight of a single batch of eggs (grams)	Free ecdysteroids*	Ecdysteroids released from polar conjugates*	Total free ecdysteroids and ecdysteroids released from polar conjugates*
Eggs				
0	0.01284+	144	115	259
1	0.00951	50	73.5	124
2	0.00630	104	97.1	201
3	0.00422	81.5	45.1	127

*Values given are µg ecdysone equivalents per gram wet weight+ of day 0 egg tissue.

occurs at day 2 compared with day 3 in the original experiment (Table 6). The immunoreactive response is more complicated, in that the cross-reactivity factor of DHS 1-13.5 to 2-deoxyecdysone was determined to be 0.36 (Fig. 26) and the major metabolite present throughout oogenesis and embryogenesis is 2-deoxyecdysone.

3. Discussion

In ovaries just prior to egg deposition, there is a complex mixture of metabolites in the free ecdysteroid fraction labelled from radioactive cholesterol. The major metabolite co-chromatographed with 2-deoxyecdysone on reversed-phase h.p.l.c. (Fig. 2). In the free ecdysteroid fraction of newly laid eggs a single tritiated metabolite is observed which co-chromatographs with 2-deoxyecdysone on both reversed-phase and adsorption h.p.l.c. columns (Figs. 6 and 7). The same tritiated metabolite appears in the free ecdysteroid fraction of day 3 eggs, just prior to hatching, again co-chromatographing with 2-deoxyecdysone on reversed-phase h.p.l.c. (Fig. 9). There is also a minor metabolite present which co-chromatographs with 5α-2-deoxyecdysone on the same system (Fig. 9). The structures of the metabolites co-chromatographing with 2-deoxyecdysone and 5α-2-deoxyecdysone on h.p.l.c. were confirmed by negative ion f.a.b. mass spectrometry (Figs. 16, 24 and 25). Ecdysone and 20-hydroxyecdysone were detected at low levels in a batch of eggs at different stages of embryogenesis by h.p.l.c./R.I.A. on reversed-phase and adsorption columns (Figs. 27 and 28). The cross reactivity factor of DHS 1-13.5 to 2-deoxyecdysone (ratio of the mass of 2-deoxyecdysone required to

displace 50% of the labelled ecdysone to mass of ecdysone required) was determined to be 0.36 (Fig. 26).

In ovaries (just prior to egg deposition), newly laid eggs and day 3 eggs, a single tritiated metabolite was present in the highly polar ecdysteroid fraction which co-chromatographs with 2-deoxyecdysone 22-phosphate on ion-suppression reversed-phase h.p.l.c. (Figs. 3, 8 and 12). Hydrolysis of this metabolite with Helix hydrolases gave a compound which co-chromatographed with 2-deoxyecdysone (Figs. 4, 9 and 13). The structure of this metabolite was confirmed by negative ion f.a.b. mass spectrometry and n.m.r. spectroscopy as the 22-phosphate of 2-deoxyecdysone (Figs. 18 and 30; Tables 2 and 3). A second metabolite occurs in both the ovaries and eggs in the highly polar ecdysteroid fraction which gives the same mass spectrum as 2-deoxyecdysone phosphate (Figs. 19 and 31). Hydrolysis of this metabolite with Helix hydrolases gives a compound which co-chromatographs with 5α-2-deoxyecdysone on reversed-phase h.p.l.c. This suggests that the second metabolite is 5α-2-deoxyecdysone 22-phosphate.

Generally, insect ovaries and eggs contain a complex mixture of different ecdysteroids whereas in S. littoralis there appear to be only four major ecdysteroids, 2-deoxyecdysone, 2-deoxyecdysone 22-phosphate and the corresponding 5α-epimers. The presence of 5α-epimers is unusual and it may be that they do not occur in vivo, but arise by keto-enol tautomerism during isolation. 2-Deoxyecdysone is more amenable to this than ecdysone in which there is strong steric

interaction between the C-2 hydroxyl group and the C-19 methyl in the 5α-epimer of ecdysone.

In Bombyx mori ovaries, 2-deoxyecdysone 22-phosphate, 2-deoxy 20-hydroxyecdysone 22-phosphate, 2,22-dideoxy-20-hydroxyecdysone-3-phosphate, bombycosterol-3-phosphate, ecdysone 22-phosphate and 20-hydroxyecdysone 22-phosphate in order of decreasing concentration have been isolated along with the corresponding free ecdysteroids (Ohnishi et al., 1989). As observed in Bombyx (Ohnishi, 1986), the amount of the conjugated ecdysteroids in Spodoptera ovaries was similar to that of the free ecdysteroids. At least a dozen different major ecdysteroids have been isolated from the ovaries and eggs of Manduca (Thompson et al, 1988), Locusta (Lagueux et al., 1984) or Shistocerca (Rees and Isaac, 1984).

In these insect species, ecdysone and 20-hydroxyecdysone are present as major metabolites, while in Spodoptera they only appear as minor components in the eggs (Figs. 27 and 28) and are not detectable by u.v. absorbance during h.p.l.c. analysis nor by the cholesterol labelling studies in the ovaries. However, in Bombyx (Ohnishi et al., 1989) and Locusta (Lagueux et al., 1984) ovaries, 2-deoxyecdysone 22-phosphate predominates over the corresponding ecdysone 22-phosphate.

In Spodoptera, the same ecdysteroids are present throughout oogenesis and embryogenesis, while in other species studied, the major ecdysteroids in early embryonating eggs and eggs near hatching are not

the same. In *Manduca* for example, the level of 26-hydroxyecdysone 26-phosphate, the major ecdysteroid in newly laid eggs, decreases during embryogenesis while a new ecdysteroid conjugate, 26-hydroxyecdysone 26-glucoside concurrently increases (Thompson *et al*, 1988). It is generally presumed that the ecdysteroid metabolites which accumulate towards the end of embryogenesis include inactivation products. The distribution of radioactivity amongst silicic acid column fractions in *Spodoptera* (Table 4) differ from the endogenous profile of ecdysteroids during oogenesis and embryogenesis (Table 5). In the distribution of radioactivity amongst the silicic acid column fractions of the ovaries, the labelling in the highly polar ecdysteroids is much greater than that in the free ecdysteroid fraction. The difference is increased when the labelling in 2-deoxyecdysone is compared with that in the corresponding 22-phosphate (Table 4). This is in contrast to the endogenous profile of ecdysteroids during oogenesis in which the difference in concentration between the free ecdysteroids and highly polar ecdysteroids is not as marked (Table 5). The reason for this is uncertain. Similar apparent discrepancies between the levels of labelling and endogenous ecdysteroid content occur for 2-deoxyecdysone and the corresponding conjugate in newly laid eggs. However, the results for the endogenous content of free and conjugated 2-deoxyecdysone in day 3 eggs are in good agreement with those for the labelling studies.

In the profile of endogenous ecdysteroids during oogenesis the total ecdysteroid concentration remains fairly constant in the ovaries, which suggests ecdysteroid synthesis occurs during the first day of ovarian development, since egg-laying only begins 3 days into oogenesis. The ecdysteroids appear to be passed into the eggs (Table 5). Since, the eggs decrease in weight due to water loss during embryogenesis, the ecdysteroid concentration was calculated per batch of eggs to compensate for this (Table 6). Early on in embryogenesis there is a sharp decrease in the total ecdysteroid content, but towards the end of embryogenesis the ecdysteroid titre per batch of eggs rises again (Table 6). The one day sampling interval used here would not allow the detection of rapid changes in ecdysteroid titre and, therefore, it is possible that other peaks in titre may occur which were not observed. The immunoreactive response could be further complicated if different ecdysteroids were transiently present at different stages of embryogenesis. However, the results of the labelling and h.p.l.c. analysis experiments indicate that 2-deoxyecdysone and its 22-phosphate conjugate were the only major ecdysteroid components.

In the mosquito, *Aedes aegypti*, it has been proposed that ecdysone synthesized in the ovaries is passed into the haemolymph and after 20-hydroxylation stimulates, together with juvenile hormone, the fat body to synthesize vitellogenin (Hagedorn *et al.*, 1975). In *Spodoptera*, as in *Bombyx*, there is as yet, no information regarding secretion of ecdysteroids from the ovaries into the haemolymph. The involvement of ecdysteroids in some phase of ovarian development in

the silkworm is suggested by the fact that exogenous ecdysteroids exert gonadotropic action on ovaries when administered to isolated pupal abdomens (Chatani and Ohnishi, 1976). In addition ecdysone and 20-hydroxyecdysone have been shown to induce meiotic reinitiation in the oocytes of Locusta (Lanot et al., 1987). The embryonic free ecdysteroids, especially ecdysone and 20-hydroxyecdysone are suggested to be involved in cycles of embryonic membrane formation/ cuticulogenesis in a number of insect systems (Hoffmann and Lagueux, 1985). The embryonic profile of free ecdysteroids in Spodoptera (Table 6) is similar to that observed in Calliphora where there is an initial peak of ecdysteroids early in embryogenesis, which occurs shortly before deposition of the typical cuticle, and after a transient decrease, the ecdysteroid titre rises again before hatching (Bordes-Alleaume and Sami, 1987). Establishing such a function for embryonic ecdysteroids in Spodoptera requires further research particularly as the major components are 2-deoxyecdysone and 2-deoxyecdysone 22-phosphate. Whether 2-deoxyecdysone has a physiological function per se is not known. In Calliphora, the ecdysteroids are essentially ecdysone and 20-hydroxyecdysone with no conjugated ecdysteroids present.

The embryonic profile for Spodoptera differs from those found in Locusta (Lagueux et al., 1984), Schistocerca (Scalia et al., 1987) or Manduca (Thompson et al., 1988) where the total ecdysteroid levels seem to remain relatively constant throughout embryogenesis, even if it is not the same ecdysteroid that predominates at the beginning and end of development. In the embryonic profile of Spodoptera, the

ecdysteroid content apparently increases towards the end of embryogenesis (Table 6). More information is required before it can be suggested that this is a result of *de novo* synthesis.

CHAPTER EIGHT

GENERAL DISCUSSION

The activity of ecdysone 20-monooxygenase in the fat body of Spodoptera littoralis undergoes developmental variation during the, final, 6th larval instar and exhibits a distinct peak (Milner, 1984). Evidence was provided for the feasibility of activation-inactivation of ecdysone 20-monooxygenase activity, from Spodoptera littoralis fat body, in a manner commensurate with possible, rapid, modulation of the enzyme's activity by reversible phosphorylation-dephosphorylation. Determination of the sub-cellular location of ecdysone 20-monooxygenase in the fat body of Spodoptera, showed that the majority of the monooxygenase activity, with the exception of the cell debris/nuclear fraction, occurs in the mitochondrial fraction, with a small but significant proportion of the enzymic activity in the microsomal fraction.

Indirect evidence suggests that both the microsomal and mitochondrial ecdysone 20-monooxygenase can exist in two forms, an active phosphorylated form and an inactive dephosphorylated form which are interconvertible by phosphoprotein phosphatase(s) and protein kinase(s). The microsomal, protein kinase is cyclic AMP dependent and the phosphoprotein phosphatase is stimulated by Ca^{2+}-calmodulin and/or by Mg^{2+} ions. It has been possible in this work to demonstrate effects on the mitochondrial monooxygenase that can be ascribed to endogenous phosphoprotein phosphatase(s), but so far using a variety of potential effectors of protein kinase, we have been unable to demonstrate possible effects of such endogenous kinases on the mitochondrial monooxygenase. Although this work provides evidence for the feasibility of rapid modulation of the microsomal and

mitochondrial ecdysone 20-monooxygenase system by a mechanism involving covalent modification, the physiological significance of this and of the dual location of the enzyme remains to be determined. The work extends the range of insect proteins for which covalent modification has been ascribed a role in modulation of activity (Smith and Combest, 1985; Combest and Gilbert, 1986).

Modulation by reversible phosphorylation- dephosphorylation is well established for a number of enzymes in the cytosol, but such a control mechanism has been documented for only two mitochondrial enzyme systems, the pyruvate dehydrogenase complex (Wieland, 1983; McCormack, 1985) and the branched-chain 2-oxoacid dehydrogenase complex (Randle et al., 1984; Jones and Yeaman, 1986). The mammalian pyruvate dehydrogenase complex (PDH complex) is located in the mitochondrial inner membrane-matrix space (Nestorescu et al., 1973). It catalyses the oxidative decarboxylation of pyruvate to acetyl-CoA. The reaction occupies a key position in cellular metabolism by controlling the supply of mitochondria with C_2 fragments arising from carbohydrates and amino acids which are either oxidized or converted to fat. The PDH complex, as mentioned, is subject to regulation by covalent modification (Wieland, 1983). In mitochondria, the PDH complex, kinase and phosphatase utilize only intra-mitochondrial substrates and effectors. The PDH phosphatase reaction requires Mg^{2+} and in the presence of Mg^{2+} is activated by Ca^{2+} over the physiological range (0.5-5μM; Km 0.8 μM), and may be inhibited by NADH (reversed by NAD) [Reed, 1981; Fuller and Randle, 1984; McCormack and Denton, 1984; McCormack, 1985]. The PDH kinase

reaction is inhibited by ADP (competitive with ATP) and by pyruvate (either non-competitive or uncompetitive with ATP); the inhibition by pyruvate is synergistic with ADP and enhanced by decreasing ratios of [ATP/ADP] (Linn et al., 1969; Pratt and Roche, 1979). The PDH kinase rate of reaction is increased by acetyl-CoA and by NADH and their effects are reversed by CoA and NAD, respectively. Activation by acetyl-CoA requires NADH but is not seen when maximal activation has been achieved with NADH . In mitochondria, the kinase reaction is, therefore, regulated by the intramitochondrial ratios of [ATP]/[ADP], [acetyl-CoA]/[CoA] and [NADH]/[NAD] and by intramitochondrial [pyruvate] (Kerbey et al., 1977; Cate and Roche, 1978; Ashour and Hansford, 1983; Fuller and Randle, 1984).

There are two hypothesis for the mechanism of the effects of acetyl-CoA, CoA, NADH and NAD on the rate of the PDH kinase reaction. The allosteric model involves direct interactions with PDH kinase but the evidence is based on work with artificial substrates (Reed, 1981) and has yet to be confirmed (Rahmatullah and Roche, 1985). The alternative model involves covalent modification of the PDH complex initiated by substrate interactions leading to reduction or reductive acetylation of lipoate. This model was first suggested by the observation that the PDH kinase reaction is also activated by low concentrations of pyruvate or by acetoin in the presence, but not in the absence of thiamin pyrophosphate (Cooper et al., 1974). The model was supported further by the dependence of the acetyl-CoA effect upon NADH , and by evidence that other reducing agents (dithiothreitol or dihydrolipoamide) may replace NADH (Cooper et al, 1975; Roche and

Cate, 1976; Cate and Roche, 1979). It is further supported by evidence that activation of the PDH kinase by acetyl-CoA is correlated with acetylation of the complex as opposed to the concentration of acetyl-CoA (Rahmatullah and Roche, 1985).

The covalent modification of glycogen phosphorylase has been demonstrated in the fat body of a number of insect species (Steele, 1982). The glycogen phosphorylase has been shown to exist in two interconvertible forms: phosphorylase a, whose activity is largely independent of AMP and phosphorylase b, which is active only in the presence of high levels of AMP (Steele, 1982). It is generally assumed that a cyclic AMP-dependent phosphorylase kinase catalyses the incorporation of phosphate from ATP-Mg^{2+} into phosphorylase b and that multisubstrate protein phosphatases reverse the reaction (Dombradi *et al.*, 1986). The fat body phosphorylase phosphatase from the pupae of the silkworm, *Philosamia cynthia*, essentially resembles that reported for mammalian muscle phosphorylase phosphatase (Brautigan *et al.*, 1982). The enzyme is stimulated by Mn^{2+} or Mg^{2+} but is not affected by Ca^{2+} (Hayakawa, 1985).

It has also been reported for *Calliphora* fat body *in vitro* that addition of forskolin which stimulates the plasma membrane adenylate cyclase system, led to decreased rates of conversion of ecdysone into 20-hydroxyecdysone and of the latter to other metabolites. Extracts prepared from larval brains of *Calliphora* also had the same effect as forskolin (Lehmann and Koolman, 1986). Initial comparison of the indirect evidence available suggests that increased cyclic AMP

concentration leads to opposite effects on the monooxygenase in Calliphora fat body system and in Spodoptera fat body microsomes.

The action of prothoracicotropic hormone can also be mimicked by forskolin. When forskolin was added to the prothoracic glands of Manduca sexta, an elevation of cyclic AMP level was observed which eventually resulted in an increased rate of ecdysone synthesis (Smith et al., 1984).

Also significant is indirect evidence that the activity of a related endoplasmic reticulum enzyme, cholesterol 7α-hydroxylase, may be enhanced by phosphorylation (Sanghvi et al., 1981; Goodwin et al., 1982). In contrast, some other mammalian microsomal cytochrome P-450 species can be inactivated by phosphorylation (Taniguchi et al., 1985; Pyerin et al., 1987). Of relevance is direct evidence that purified steroidogenic mitochondrial cytochrome P-450 (11β-hydroxylation) can be phosphorylated, leading to markedly increased P-450 11β-adrenodoxin affinity (Defaye et al., 1982).

In insect larvae ecdysone synthesis in prothoracic glands is controlled by a neuropeptide, prothoracicotropic hormone (see Chapter 1). It is generally assumed that the mode of action of tropins in insects and vertebrates is similar (Smith, 1985). In vertebrates, the tropin binds to specific receptors located on the surface of the steroid hormone-producing gland that leads to an elevation in cyclic AMP. The cyclic AMP, in turn, modulates probably via specific protein

All the most active inhibitors were nitrogen hetrocycles, the two most potent, ketoconazole and imazalil, gave I_{50} values of 0.26ppm and 0.32ppm, respectively. Ketoconazole is marketed commercially as an oral antimycotic in the treatment of mycosis and imazalil as a fungicide used in seed treatment.

It is assumed that these cytochrome P-450 inhibitors interact directly through reversible ligand formation, with the haeme moiety of cytochrome P-450. Many of the inhibitors are closely related and their different potency might be connected with the different secondary structure of the apoprotein of the cytochrome P-450 isoenzymes, which are integral membrane globular proteins (Hudecek and Azenbacher, 1985). Thus, some substrates/inhibitors can reach the substrate-binding site in the protein via the substrate channel(s), while others cannot.

Of particular interest are the group III inhibitors, KK-42, KK110, KK135 and PIM, which have all been demonstrated to possess anti-juvenile hormone and anti-ecdysteroid action at a concentration of approximately 10μg/larvae (Kuwano et al., 1988).

In particular, KK-42 has been reported to inhibit ecdysteroid synthesis in the prothoracic glands of *Bombyx mori* and depressed the total amount of ecdysteroids released by the prothoracic glands of *Locusta migratoria in vitro* (Yamashita et al., 1987; Kadono-Okuda et al., 1987; Roussel et al., 1987). In contrast, to these findings, KK-42 had no effect on the *Spodoptera* fat body ecdysone

20-monooxygenase activity at a concentration of 1ppm. Also of interest is PIM, which is a derivative of metyrapone in which the A ring is replaced by a phenyl ring without loss of activity in a number of systems. Both metyrapone and PIM have been widely demonstrated in a number of insect species to have anti-juvenile and anti-ecdysteroid action (Bollenbacher *et al.*, 1977b; Feyereisen and Durst, 1978; Fisher and Mayer, 1982; Belai *et al.*, 1988). PIM gives 63% inhibition of the *Spodoptera* fat body ecdysone 20-monooxygenase activity at a concentration as low as 1ppm.

The major ecdysteroids during oogenesis and embryogenesis in *Spodoptera littoralis* were analysed. It is well documented that the ovaries of reproductively competent female insects synthesize ecdysteroids (Hagedorn *et al.*, 1975; Ohnishi *et al.*, 1977; Lagueux *et al.*, 1977). In many species, the bulk of the ovarian ecdysteroids are transferred to the ooplasm of the eggs where they may have a function during embryonic development, while in others an appreciable amount of the ovarian ecdysteroids are released into the maternal haemolymph to play a role in regulation of vitellogenin synthesis (Hagedorn, 1975; Hoffmann, 1986). There is a large amount of indirect evidence that one of the functions of egg ecdysteroids is in the control of moulting. In several insect species a correlation between moulting events in the embryo and the concentration of moulting hormone activity was clearly evident (Lagueux *et al.*, 1979; Imboden and Lanzrein, 1982; Fournier and Radallah, 1988). Embryonic ecdysteroids may also be involved in the control of diapause in some insect species (Ohnishi *et al.*, 1971; Coulon, 1988). There is also a correlation

between the concentration of moulting hormone activity and mitotic activity in the late stages of embryogenesis in Schistocerca gregaria (Scalia et al., 1987).

The fate of [^{3}H]cholesterol injected into pupae of Spodoptera littoralis was investigated during oogenesis and embryogenesis to provide an indication of the types of ecdysteroids occurring. The presence of relatively small amounts of ecdysteroids in insects and the large pool of cholesterol, the distal precursor of ecdysone, makes the incorporation of label from administered cholesterol low. Therefore, caution is required in interpretation of these results as high levels of radioactivity must be administered to the insect. In ovaries which had undergone 3 days of ovarian development there is a complex mixture of tritiated metabolites. The major metabolite was identified as 2-deoxyecdysone. This metabolite was the only major free ecdysteroid in tritiated newly laid eggs. In tritiated eggs which had undergone 3 days of embryogenesis, ie. just prior to hatching, 2-deoxyecdysone and the corresponding 5α-epimer were the only major metabolites present in the free ecdysteroid fraction. The structures of these metabolites was confirmed by mass spectrometric analysis.

The only metabolite detected in the tritiated highly polar ecdysteroid fraction of the ovaries, day 0 eggs and day 3 eggs was 2-deoxyecdysone 22-phosphate. The structure was confirmed by mass spectrometric and nuclear magnetic resonance spectrometric analysis. In addition, the 22-phosphate of 5α-2-deoxyecdysone was identified by

h.p.l.c. and f.a.b. mass spectrometric analysis. The presence of 5α-epimers is unusual and it may be that they do not occur in vivo, but arise by keto-enol tautomerism during isolation.

Ecdysone and 20-hydroxyecdysone were present as minor metabolites in the free ecdysteroid fraction of eggs. This may suggest that 2-deoxyecdysone, and the corresponding conjugate are metabolized to some extent to the more active ecdysteroids ecdysone and 20-hydroxyecdysone when required. However, it appears that the C-2 hydroxylase has low activity in ovaries and developing eggs.

In Locusta migratoria approximately two thirds of the ovarian ecdysteroids correspond to 2-deoxyecdysone and one third to ecdysone, 20-hydroxyecdysone being only a minor metabolite (Dimarcq et al, 1987). The presence of 2-deoxyecdysone as the major ecdysteroid is thus not exclusive to Spodoptera, but the small concentration of ecdysone and 20-hydroxyecdysone is unusual. The major metabolite in Schistocera (Isaac and Rees, 1984) and Bombyx (Ohnishi et al., 1989) ovaries is 2-deoxyecydsone 22-phosphate.

Generally, insect ovaries and eggs contain a complex mixture of different ecdysteroids (Rees and Isaac, 1984; Lagueux et al, 1984; Thompson et al, 1988), whereas in S. littoralis there are only four major ecdysteroids, 2-deoxyecdysone, 2-deoxyecdysone 22-phosphate and the corresponding 5α-epimers. Furthermore, in Spodoptera the same ecdysteroids are present throughout oogenesis and embryogenesis while in other species studied, the major ecdysteroids in the ovaries and

newly laid eggs are not the same as in eggs near hatching (Rees and Isaac, 1984; Lagueux et al., 1984; Thompson et al., 1988). The ecdysteroid concentration and the ecdysteroid per ovary remain fairly constant during oogenesis which suggests that ecdysteroid synthesis may occur during the first day of ovarian development. Both free and conjugated ecdysteroids appear to be passed into the eggs. Early on in embryogenesis there is a sharp decrease in the total ecdysteroid concentration per batch of eggs. Towards the end of embryogenesis the ecdysteroid titre rises dramatically again, which suggests de novo synthesis of ecdysteroids. The one day sampling interval used here would not allow the detection of rapid variations in ecdysteroid titre, so it is possible other peaks in titre may occur which were not detected. The embryonic profile is similar to that observed in Calliphora (Bordes-Alleaume and Sami, 1987) where the initial peak in ecdysteroid titre is correlated with the deposition of the typical cuticle.

In Locusta migratoria, approximately 90% of the ecdysteroids are present in newly laid eggs as highly polar phosphate esters primarily 2-deoxyecdysone 22-phosphate (Lagueux et al., 1981). The large amount of conjugates present in L. migratoria newly-laid eggs are transformed during embryogenesis in the eggs, since at the time of hatching the eggs do not contain any detectable conjugated 2-deoxyecdysone. The vast majority of the 2-deoxyecdysone conjugates are hydrolysed and subsequently epimerized at C-3 and then phosphorylated at the 3α hydroxyl. After hydrolysis of the 2-deoxyecdysone conjugates, some 2-deoxyecdysone can be converted into ecdysone via the C-2 hydroxylase

system which is present in the L. migratoria embryo. It is unclear, however, if 2-deoxyecdysone is converted into ecdysone to any great extent at present (Dimarcq et al., 1987). The fate of the 2-deoxyecydsone and corresponding conjugate in Spodoptera remains to be determined as does their function.

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APPENDICES

APPENDIX 1

TRIVIAL AND I.U.P.A.C. EQUIVALENT NAMES

Appendix 1

Trivial and I.U.P.A.C. Equivalent Names

Ecdysteroids:

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-heptahydroxy-5β-cholest-7-en-6-one

3-epi-ecdysone = 2β, 3α, 14α, 22R, 25-pentahydroxy-5β-cholest-7-en-6-one

3-epi-20-hydroxyecdysone = 2β, 3α, 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

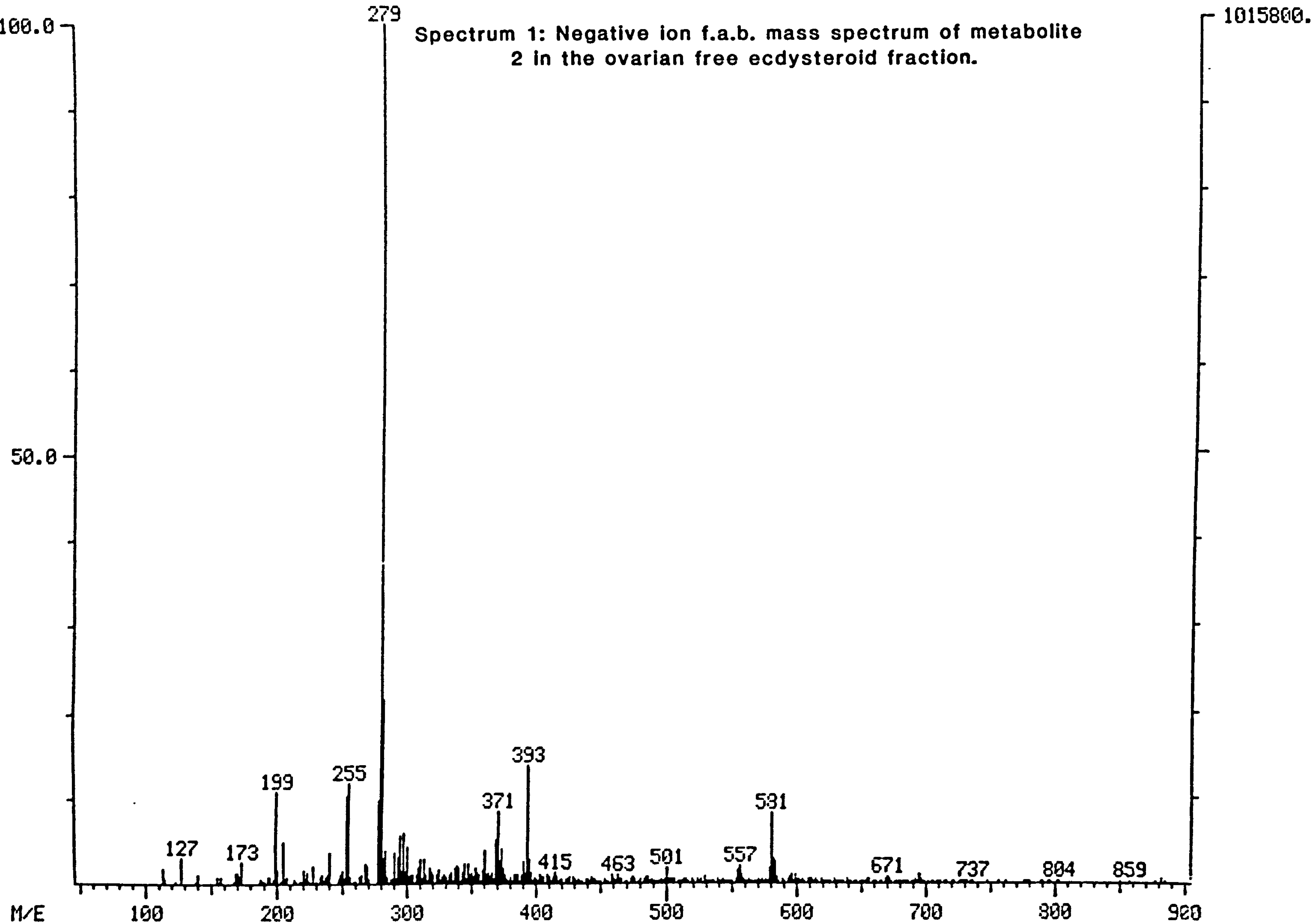
3-dehydroecdysone = 2β, 14α, 22R, 25-tetrahydroxy-5β-cholest-7-en-3,6-dione

3-dehydro-20-hydroxyecdysone = 2β, 14α, 20R, 22R, 25-pentahydroxy-5β-cholest-7-en-3,6-dione

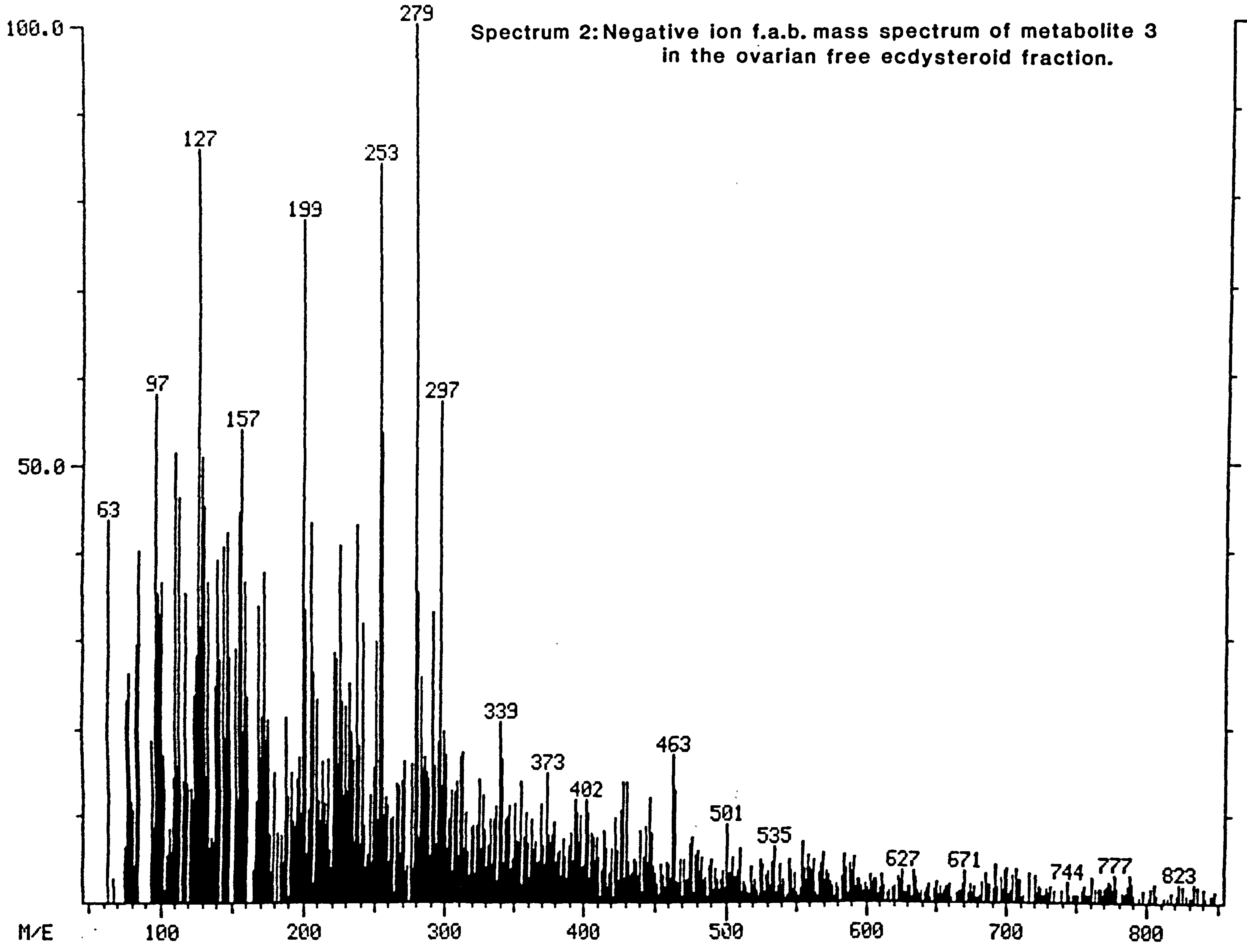
2-deoxyecdysone = 3β, 14α, 22R, 25-tetrahydroxy-5β-cholest-7-en-6-one

APPENDIX 2

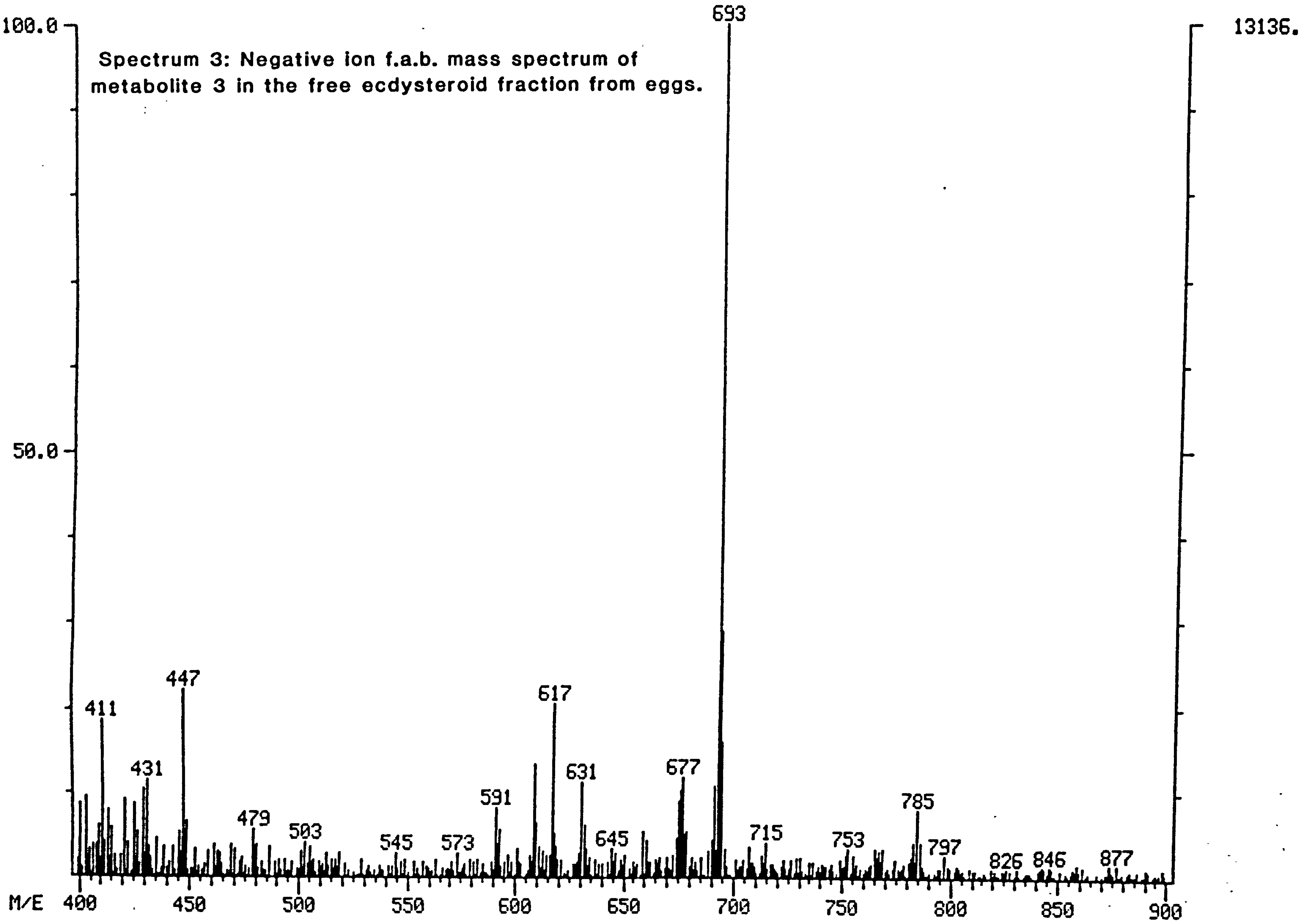
MASS SPECTRA



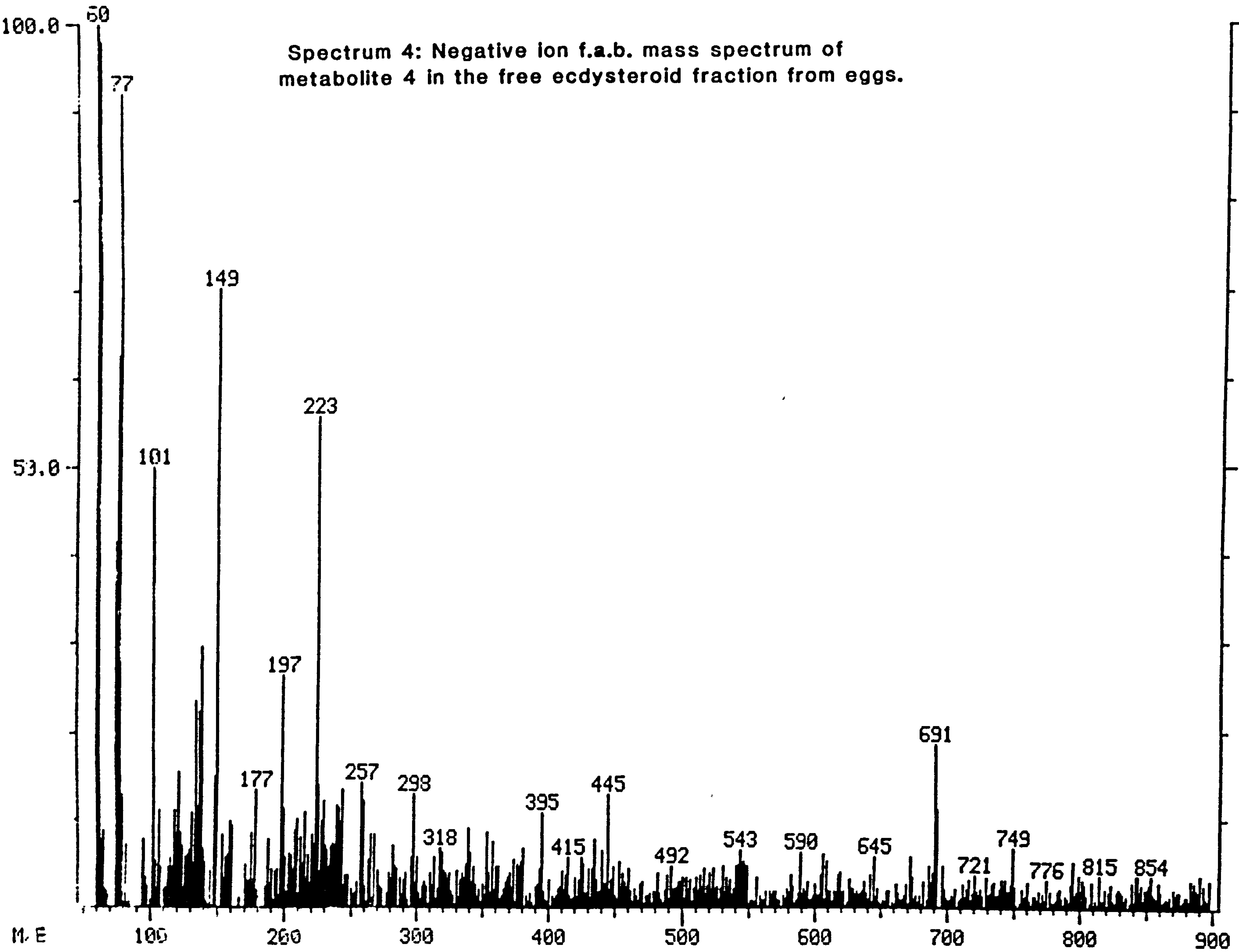
Spectrum 1: Negative ion f.a.b. mass spectrum of metabolite 2 in the ovarian free ecdysteroid fraction.



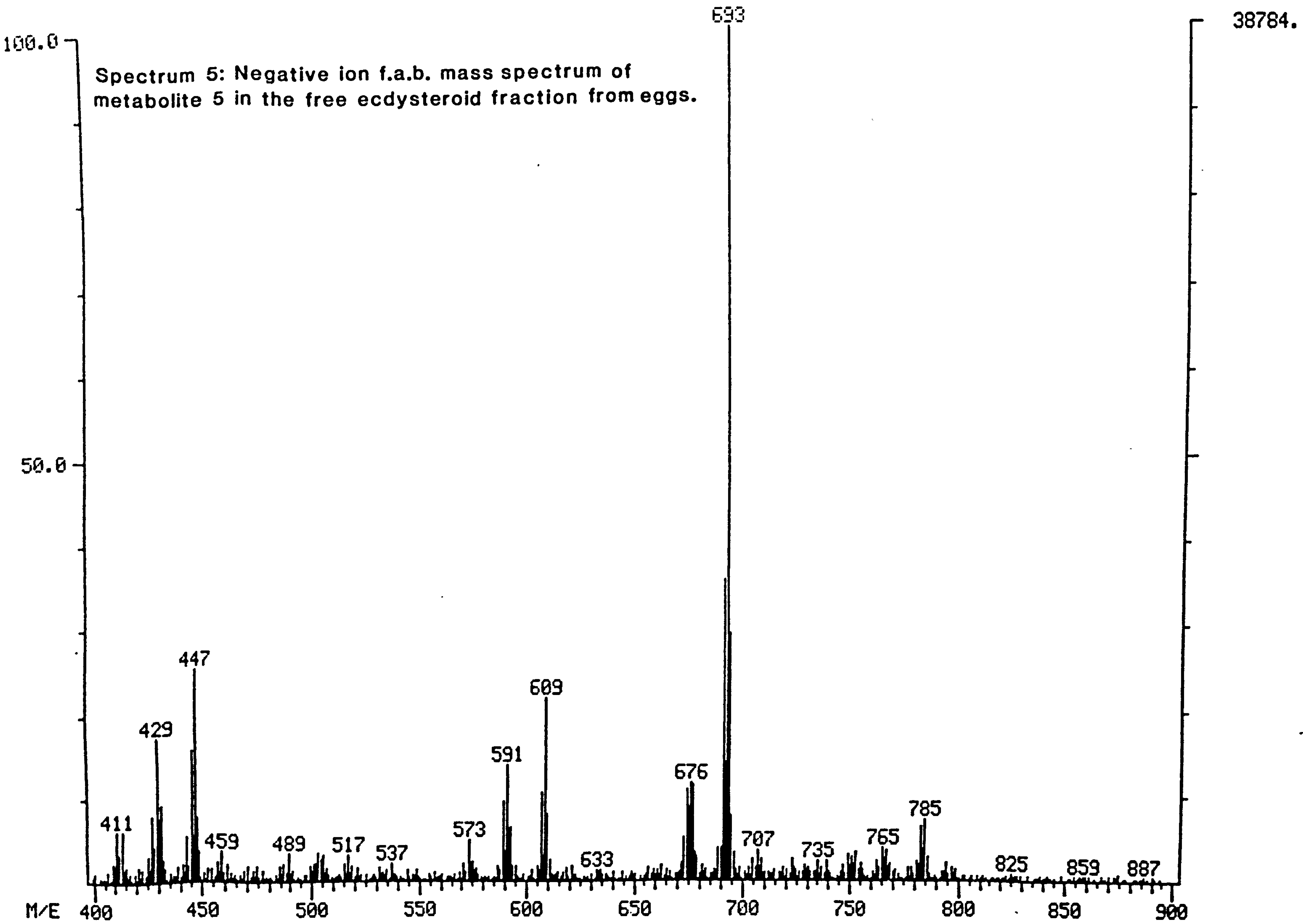
Spectrum 2: Negative ion f.a.b. mass spectrum of metabolite 3 in the ovarian free ecdysteroid fraction.



Spectrum 3: Negative ion f.a.b. mass spectrum of metabolite 3 in the free ecdysteroid fraction from eggs.



Spectrum 4: Negative ion f.a.b. mass spectrum of metabolite 4 in the free ecdysteroid fraction from eggs.



Spectrum 5: Negative ion f.a.b. mass spectrum of metabolite 5 in the free ecdysteroid fraction from eggs.

Ecdysteroid Metabolism in Spodoptera littoralis by Nigel Hoggard.

SUMMARY

The feasibility of the activation-inactivation of ecdysone 20-monooxygenase activity, from Spodoptera littoralis fat body, in a manner commensurate with possible modulation of the enzyme's activity, by reversible phosphorylation-dephosphorylation was investigated. Initially, the sub-cellular localization of the ecdysone 20-monooxygenase was determined in the fat body. It was found to be located primarily in the mitochondria with a small, but significant, proportion being apparently associated with the microsomal fraction.

Microsomal fractions showed higher ecdysone 20-monooxygenase activity after preincubation at 37°C in phosphate buffer rather than Hepes or Imidazole buffer systems. Similarly, activity of the monooxygenase was elevated following preincubation with fluoride, an inhibitor of phosphoprotein phosphatases. Preincubation with alkaline phosphatase, or with cyclic AMP-dependent protein kinase and appropriate cofactors, resulted in appreciable diminution or enhancement, respectively, in monooxygenase activity. Activation of ecdysone 20-monooxygenase activity could also be effected by incubation with a cytosolic fraction in the presence of cylcic AMP, ATP and fluoride; this activation was prevented by a cyclic AMP-dependent protein kinase inhibitor. Similarly, inactivation of the monooxygenase was achieved by preincubation with cytosol, the effect being enhanced by Ca^{2+}-calmodulin and/or by Mg^{2+} ions. Use of a mixture of proteinase inhibitors established that the results are not complicated by the effects of such enzymes. The combined results provide indirect evidence that the microsomal ecdysone 20-monooxygenase exists in an active phosphorylated form and an inactive dephosphorylated form, interconvertible by a cyclic AMP-dependent protein kinase and a phosphoprotein phosphatase.

Mitochondrial fractions also showed higher ecdysone 20-monooxygenase activity after preincubation with fluoride. Preincubation of a broken mitochondrial preparation with alkaline phosphatase resulted in appreciable diminution of monooxygenase activity, whereas activity was retained when the phosphatase was inhibited by EDTA and inorganic phosphate. Monooxygenase activity was increased when a broken mitochondrial preparation was incubated with cyclic AMP-dependent protein kinase and appropriate cofactors. Similarly, when the activity of the mitochondrial ecdysone 20-monooxygenase was reduced by incubation with alkaline phosphatase followed by inactivation of the phosphatase by EDTA and phosphate, there was an appreciable recovery of activity upon rephosphorylation of the enzyme system by incubation with protein kinase and appropriate cofactors. The combined results provide indirect evidence that the mitochondrial ecdysone 20-monooxygenase may also exist in an active phosphorylated form and an inactive dephosphorylated form, which are interconvertible by appropriate phosphoprotein phosphatase(s) and protein kinase(s). Of course, *in vivo* a cyclic AMP-dependent protein kinase would not be expected to be involved in modulation of the mitochondrial monooxygenase.

The effect of various known cytochrome P-450 inhibitors was investigated on a microsomal plus mitochondrial preparation from fat body. The most potent inhibitors were ketoconazole and imazalil which are both azoles with I_{50} values of 0.26ppm and 0.32ppm, respectively.

The major ecdysteroids occurring during oogenesis and embryogenesis in *Spodoptera littoralis* were analysed. Initially, the metabolism of injected [$^3H_2$]cholesterol was investigated. Four major metabolites were identified; 2-deoxyecdysone, 2-deoxyecdysone 22-phosphate and unusually the corresponding 5α-epimers. It is possible that the latter isomers do not occur *in vivo*, but arise by keto-enol tautomerism during isolation. 2-Deoxyecdysone and 2-deoxyecdysone 22-phosphate were present throughout oogenesis and embryogenesis. The structures were ascertained by mass spectrometric and nuclear magnetic resonance spectrometric analysis. In addition, small amounts of ecdysone and 20-hydroxyecdysone were identified during embryogenesis by h.p.l.c./R.I.A.

The changes in ecdysteroid titre and composition during oogenesis and embryogenesis were determined. In the ovaries of *Spodoptera*, the total ecdysteroid concentration remains almost constant. Appreciable amounts of ecdysteroids appear to be passed into the eggs where the titre per batch of eggs decreases early in embryogenesis. Towards the end of embryogenesis the total ecdysteroid titre per batch of eggs rises again.