Characterisation of Juvenile Hormone Esterase in *Drosophila melanogaster*

by

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Declaration

The research carried out in the course of this investigation and the results presented in this thesis are, except where acknowledged, the original work of the author.

Peter Malcolm Campbell

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Table of Contents

Summary 1

Chapter 1 Introduction and Literature Review

1.1 Introduction 3

1.2 The Structures and Distribution of Juvenile Hormones 3

1.3 Functions of JH 5

1.4 Physiology and Hormonal Regulation of Insect Metamorphosis 6

1.4.1 Physiology of Metamorphosis 6

1.4.2 Hormonal Regulation of Metamorphosis 7

1.4.3 Mechanisms Underlying Hormonal Control of Metamorphosis 9

1.4.3.1 Timing of Metamorphosis 9

1.4.3.2 Mechanisms for JH Action 9

1.4.3.3 Mechanisms for Ecdysone Action 10

1.5 Regulation of JH Titre 11

1.5.1 Synthesis of JH 11

1.5.2 JH Carrier Proteins 12

1.5.3 JH Degradation 13

1.5.3.1 Definition of "JH-Specific" Hydrolysis 13

1.6 Physiology of JH Hydrolysis 14

1.6.1 Temporal Profile of JH hydrolysis 14

1.6.2 Spatial Profile of JH Hydrolysis 15

1.6.3 Regulation of JH Hydrolysis 16

1.6.4 JH Hydrolysis in Diptera 17

1.7 Biochemistry of Juvenile Hormone Esterases 18

1.7.1 *In Vitro* Inhibitors of JHE 18

1.7.2 Purification of JHEs 19

1.7.3 Kinetic Characterisation of JHEs 20

1.7.4 Intraspecific and Interspecific Differences Among JHEs 21

1.8 Molecular Characterisation of JHE 23

1.9 Aims and Outline of This Study 23

Chapter 2 Identification and Expression Pattern of JH Degrading Enzymes

2.1	Introduction	25
2.2	Materials and Methods	26
2.2.1	Drosophila Strains, Culture and Sample Preparation	26
2.2.1.1	Strains and Culture	26
2.2.1.2	Sample Preparation	26
2.2.2	JHE Assay, Electrophoretic and Protein Detection Procedures	27
2.2.2.1	Protein Determination	27
2.2.2.2	Native Polyacrylamide Gel Electrophoresis (PAGE)	27
2.2.2.3	Kinetic Experiments Using Native PAGE	27
2.2.2.4	Isolation of JHE Activity After Native PAGE	27
2.2.2.5	Thin Layer Chromatography to Identify JH Hydrolytic Products	28
2.2.2.6	Partition Assay of JH Hydrolytic Activities	28
2.2.3	pH, Isoelectric and Inhibition Properties of JHE	29
2.2.3.1	pH Profile of JHE Activity	29
2.2.3.2	Isoelectric Focusing	29
2.2.3.3	Effect of Inhibitors on JHE	30
2.3	Results	30
2.3.1	Comparison of the Anodal Esterases of *Drosophila melanogaster* and *Drosophila virilis*	30
2.3.1.1	Pattern of Expression of the Anodal Esterases	30
2.3.1.2	Biochemical Properties of the Anodal Esterases	31
2.3.2	Characterisation of JH Hydrolysis	31
2.3.2.1	Localisation of JHE After Native PAGE	31
2.3.2.2	Developmental Profile of JHIII Hydrolysis	31
2.3.2.3	Subcellular Localisation of JH Hydrolytic Activities	32
2.3.2.4	Biochemical Properties of JHE	33
2.4	Discussion	33
2.4.1	Comparisons Among the Anodal Esterases and JHE	33
2.4.1.1	Comparison Between p-Esterase and EST20	33
2.4.1.2	Association Between p-Esterase/EST20 and JHE	34
2.4.2	Expression Pattern, Roles and Specificity of JH Hydrolysing Enzymes	35
2.4.2.1	Expression Pattern of JH Hydrolysing Enzymes	35
2.4.2.2	Role of JH Hydrolysis in Regulation of JH Titre	36
2.4.2.3	Specificity of JH Hydrolysing Enzymes	37
2.4.3	Biochemical Properties of JHE	37

Chapter 3 Biochemical Characterisation of Purified JHE

3.1 Introduction 39

3.2 Materials and Methods 40
3.2.1 Chemicals 40
3.2.2 JHE Assay, Electrophoretic and Protein Detection Procedures 40
3.2.2.1 JHE Activity Assay 40
3.2.2.2 Electrophoretic Procedures 40
3.2.2.3 Protein Detection and Sequencing Procedures 41
3.2.3 Purification of JHE 41
3.2.3.1 Initial Purification Steps 41
3.2.3.2 Affinity Purification of JHE 42
3.2.3.3 Conventional Purification 42
3.2.4 Kinetic Characterisation of JHE 43
3.2.4.1 Kinetic Analysis of JHIII Hydrolysis and Inhibition by JH Isoforms 43
3.2.4.2 Kinetic Properties with α-Naphthylacetate 44
3.2.4.3 Identification of JH Structural Features which Promote Binding to JHE 44
3.2.4.4 Interaction of JHE with Lipophorin 45

3.3 Results 45
3.3.1 Purification of JHE 45
3.3.2 Properties of Purified JHE 46
3.3.2.1 Identification of JHE by Two-Dimensional PAGE 46
3.3.2.2 Molecular Weight Estimation 47
3.3.2.3 Stability of Purified JHE 47
3.3.2.4 N-Terminal Sequencing 47
3.3.3 Kinetic Characterisation of JHE 48
3.3.3.1 Kinetic Analysis of JHIII Hydrolysis and Its Inhibition by JH Isoforms 48
3.3.3.2 Kinetic Properties with α-Naphthylacetate 48
3.3.3.3 Binding Affinities for Other JH Analogues and Isoforms 48
3.3.3.4 Interaction of JHE with Lipophorin 52

3.4 Discussion 52
3.4.1 Purification and Physical Properties of JHE 52
3.4.2 Comparison of *D. melanogaster* JHE with Other Esterases 54
3.4.3 Kinetic Characterisation of JHE 54
3.4.3.1 JH-Specificity of JHE 54
3.4.3.2 Characterisation of Substrate Binding by JHE 55
3.4.3.3 Interaction of JHE with Lipophorin 57

Chapter 4 General Discussion

4.1 Introduction 59

4.2 The Specificity of JH Hydrolysing Enzymes and Naphthylacetate Hydrolysis 59
- 4.2.1 JH-Specificity of *D. melanogaster* Enzymes 59
- 4.2.2 The Relationship Between JH Hydrolysis and Naphthylacetate Hydrolysis 60

4.3 General Model for JH Regulation in Diptera 60
- 4.3.1 General Pattern of Expression of JH hydrolysis in Diptera 61
- 4.3.2 Correlation of JH Hydrolysis with JH Production and Titre Changes 62
  - 4.3.2.1 The Prewandering Period of the Final Larval Instar 62
  - 4.3.2.2 Prepupae/Pupae 62
  - 4.3.2.3 Adults 63
  - 4.3.2.4 Further Analysis of JH Titre Regulation 64
- 4.3.3 Compartmentalisation and Relative Contributions to JH Hydrolysis of JHE and JHEH 64
- 4.3.4 The Role of Lipophorin in JH Titre Regulation 66

4.4 Roles and Regulation of JH Isoforms 67
- 4.4.1 Identification and Functions of JH Isoforms 67
- 4.4.2 Differential Regulation of JH Isoforms 68
  - 4.4.2.1 Synthesis of JH Isoforms 68
  - 4.4.2.2 Degradation of JH Isoforms 68
  - 4.4.2.3 Interactions Among Carrier Binding, Synthesis and Degradation of JH Isoforms 69
  - 4.4.2.4 Future Directions for the Analysis of JH Isoform Roles and Regulation 69

4.5 Structure and Regulation of JHE 70

4.6 Conclusions 71

References 73

Appendix 89

Summary

Juvenile hormone (JH) is one of the main morphogenic hormones of insects and degradation of the hormone is believed to play an important role in its regulation in many insects. The two main routes for JH degradation are hydroysis by JH esterase (JHE) and JH epoxide hydrolase (JHEH). A major focus of research has been on juvenile hormone esterase (JHE) found in the haemolymph of final instar lepidopteran larvae, although considerable hydrolysis of JH occurs in other tissues and at other life stages. In contrast, no comprehensive study of JH degradation has been undertaken in any dipteran species. However, the available evidence suggests that very little JH degrading activity is found in the haemolymph of Diptera and several dipteran JHEs exhibit atypical patterns of sensitivity to inhibitors. These data suggested that both the expression pattern and the biochemical properties of dipteran JH degrading enzymes may differ from other insect orders, and therefore, that the role of JH degradation may be different.

The aim of this project was to comprehensively characterise JH degradation in the dipteran, *Drosophila melanogaster*, with special emphasis on JHE. The first objective was to identify the JH degrading enzymes of *D. melanogaster* and the second was to determine their expression patterns and biochemical properties in order to determine their role in the regulation of JH titre. These data were compared with other data to determine whether a general model could be developed for dipteran JH titre regulation. Finally, the roles of JH hydrolysis in the regulation of JH titre were compared among Diptera, Lepidoptera and other insects.

Two approaches were taken to identify the JH degrading enzymes of *D. melanogaster*. The first was to investigate the properties of an esterase, denoted EST20, which can be visualised after native polyacrylamide gel electrophoresis using artificial esterase substrates, α- and ß-naphthylacetate. EST20 is shown to be a likely homologue of the p-esterase of *Drosophila virilis*, which has been claimed to be the JHE of this species. However, this study shows that JH hydrolytic activity is associated with neither EST20 in *D. melanogaster* nor the p-esterase in *D. virilis*.

The second approach was to use a radiometric assay which identified two JH hydrolysing enzymes, JHE and JHEH. The developmental profile of JH hydrolytic activity in *D. melanogaster* correlates inversely with profiles of JH titre and production. Key features are prewandering and prepupal maxima of JH hydrolytic activity in whole body homogenates of final instar larvae. This developmental profile of expression is similar to the lepidopteran pattern suggesting that the role of JH hydrolysis is essentially similar during this period. In *D. melanogaster*, the prewandering maximum consists mainly of JHEH whereas the prepupal peak consists mainly of JHE. However, the relative contributions of JHE and JHEH at these times differs both among the Diptera and in other orders.

JHE from the prepupal stage was purified to homogeneity by selective precipitations, isoelectric focussing, anion exchange and gel filtration chromatography, and its N-terminal sequence was determined. Its K_M and V_{max} for JHIII hydrolysis are 89 nM and 0.59 µmol/min/mg, respectively, indicating a specificity constant of 6.8×10^6 $M^{-1}sec^{-1}$. JHE also hydrolyses α-naphthylacetate with a K_M of 120 µM and a specificity constant of 5.9×10^5 $M^{-1}sec^{-1}$, 12 fold lower than for JHIII.

Two forms of juvenile hormone, JHIII and JHIIIbisepoxide (JHB_3) may have roles in the higher Diptera. Further kinetic experiments showed that binding in the active site of JHE is promoted by the features found in JHIII and JHB_3 and reduced by almost any departure from these, suggesting that both molecules are *in vivo* substrates. Studies of the interaction of JHE with the JH carrier, lipophorin, indicate that competition with lipophorin for binding of JH may prevent JH hydrolysis at some life stages. However, lipophorin may assist in the clearance of JH from the organism at other times.

The developmental profiles of both JHE and JHEH expression and the kinetic properties of JHE indicate that JH is the *in vivo* substrate for these enzymes and that JH degradation plays an important role in the regulation of JH titre in *D. melanogaster*. Comparison with data from the literature enabled the development of a generalised model for the expression pattern and role of JH degradation in the Diptera. The model indicates that the expression pattern and role of JH degradation in the Diptera is more similar to the well characterised lepidopteran system than was previously apparent. Finally, the roles of the dipteran JH isoforms, JHIII and JHB_3 are considered. It is proposed that the JH isoforms may be differentially regulated by a combination of differential synthesis and differential hydrolysis, and that hydrolysis may be modulated by differential binding of JH isoforms to lipophorin.

Chapter 1

Introduction and Literature Review

1.1 Introduction

Juvenile hormone (JH) is one of several important insect hormones whose interplay regulates development and reproduction (Gupta, 1990a; Riddiford, in press, for reviews). JH has been described as the "inhibitory" or "status quo" hormone of arthropods and is proposed to modify the action of moulting hormone (ecdysteroids, Willis, 1981). Thus, it is believed to inhibit progress from one developmental program to the next, such as from a larval program to a pupal one or from a pupal to an adult program (Willis, 1990; Tata, 1993). The JH titre is therefore critical for regulation of developmental events. However, the role of JH in larval Diptera remains conjectural, partly because JH has different effects and sensitive periods in dipteran larvae compared with other species (Richard and Gilbert, 1991; Riddiford, in press).

At certain times in development a reduced JH titre is required and in many insects both reduced synthesis of JH and increased JH degradation are important to affect this change (de Kort and Granger, 1981; Hammock, 1985; Roe and Venkatesh, 1990, for reviews). Temporally and spatially regulated JH degrading enzymes are most thoroughly characterised in the Lepidoptera, with juvenile hormone esterase (JHE) studied most intensively. In contrast, the dipteran JH degrading enzymes are poorly characterised, although the available evidence suggests that the patterns of expression and properties of dipteran JH degrading enzymes differ markedly from other insects. These observations have lead to the speculation that the roles of JHE may differ between Lepidoptera and Diptera.

The aim of my project was to comprehensively characterise JH degradation in a dipteran and compare and contrast the physiological and biochemical properties of the JH degrading enzymes in Diptera with those in Lepidoptera, with special emphasis on JHE. Having defined the dipteran JH degrading enzymes a further aim was to assess the relative contributions of degradation to regulation of JH titre between Diptera and Lepidoptera.

This chapter reviews the relevant literature. The roles of JH are discussed with particular emphasis on its regulation of metamorphosis. Mechanisms of JH titre regulation via changes in synthesis, interactions with JH carrier proteins and activity of degrading enzymes are reviewed. The degradation of JH is reviewed in greater detail, reflecting the emphasis of this study.

1.2 The Structures and Distribution of Juvenile Hormones

Juvenile hormones are closely similar sesquiterpenoid compounds produced by insects. The most common isoform is JHIII (methyl (2E,6E)-10,11-epoxy-3,7,11-trimethyl 2,6-dodecadienoate) which is found in all of the nine insect orders investigated (Schooley *et al.*, 1984). Other isoforms of JH are very similar to this molecule,

differing slightly by the substituents on the acid moiety (Fig. 1.1). All are esters of methanol, have a 10(R),11 epoxide group, and a 2,3 double bond. The 2,3 double bond is conjugated with the ester group leading to increased resistance of the ester to hydrolysis.

The Lepidoptera is the only order in which four isoforms of JH (JH0, JHI, JHII, and 4-methyl JHI) have been identified reliably (Schooley *et al.*, 1984), with the exception of one report of JHI in an Hemipteran (Numata *et al.*, 1992). JH0, JHI, and JHII differ from JHIII by the replacement of one or more of the methyl side-chains of JHIII with ethyl side-chains and 4-methyl JHI has an additional methyl side chain.

Key aspects of the JH system may be common to all arthropods (Cusson *et al.*, 1991). It has been proposed that methylfarnesoate (MF), which differs from JHIII only by the lack of the 10,11 epoxide group, or a similar molecule is the ancestral form of JH. MF is found in Crustacia (Gupta, 1990b; Cusson *et al.*, 1991) and its titre varies with size and reproductive status through development in a manner which suggests that MF has functions similar to JH in insects (Borst and Tsukimra, 1992; Laufer *et al.*, 1993). In the Myriapoda there is evidence, suggesting the presence of JH-like compounds but none have been isolated (Gupta, 1990b). Unidentified JH-like compounds are produced by Chelicerata which are probably similar to insect JHs because insect JHs and agonists have JH-like and anti-JH-like effects, respectively, in several species (Bonaric and Juberthie, 1990), and developmentally regulated JHE and juvenile hormone epoxide hydrolase (JHEH) activities are found in one chelicerid (Roe and Venkatesh, 1990). In addition, it is possible that JH-like compounds will be found outside the arthropod group because ecdysteroids are found in annelids and nematodes and these hormones may have evolved together with a JH-like endogenous control mechanism (Gupta, 1990b).

JHIII bisepoxide (JHB_3) has been found only in higher Diptera and differs from JHIII by epoxidation of the 6,7 double bond (Richard *et al.*, 1989b; Hearlt *et al.*, 1993). Both *D. melanogaster* and *Calliphora vomitoria* produce JHIII as a minor product and JHB_3 as the major product, whereas JHB_3 is reported to be the only JH isoform produced in *Lucilia cuprina* (LeFevere *et al.*, 1993). There is some dispute about the identification of the JHIII product and the available evidence on the functional roles of JHIII and JHB_3 does not resolve whether both isoforms are physiologically relevant in the higher Diptera. Evidence that supports a role for JHIII includes the similarity of developmental changes of its titre in *Drosophila hydei* and *D. melanogaster* with those in other insects (Bührlen *et al.*, 1984; Bownes and Rembold, 1987; Sliter *et al.*, 1987). In addition, the JH carrier in *D. melanogaster* haemolymph has a greater binding affinity for JHIII than any other JH carrier (Shemshedini and Wilson, 1988). The *L. cuprina* JH carrier also has a high affinity for JHIII, with preliminary results suggesting a low affinity for JHB_3 (Dr. S. C. Trowell, pers. comm.). However, in *D. melanogaster* adults, JHB_3 production is temporally regulated while JHIII and MF are produced at relatively constant rates, suggesting that the latter may merely be precursors for JHB_3 (Altaratz *et al.*, 1991).

In *D. melanogaster*, different bioassays provide examples of both greater sensitivity to JHIII over JHB_3 and vice versa (Saunders *et al.*, 1990; Richard *et al.*, 1989b; Section 4.4.1), perhaps indicating different, but specific roles for both JHIII and JHB_3. However, non-physiological JH isoforms are sometimes more potent than physiological isoforms in bioassays, possibly due to less rapid degradation of the non-physiological isoform (Richard *et al.*, 1989b). Investigations of the metabolism of

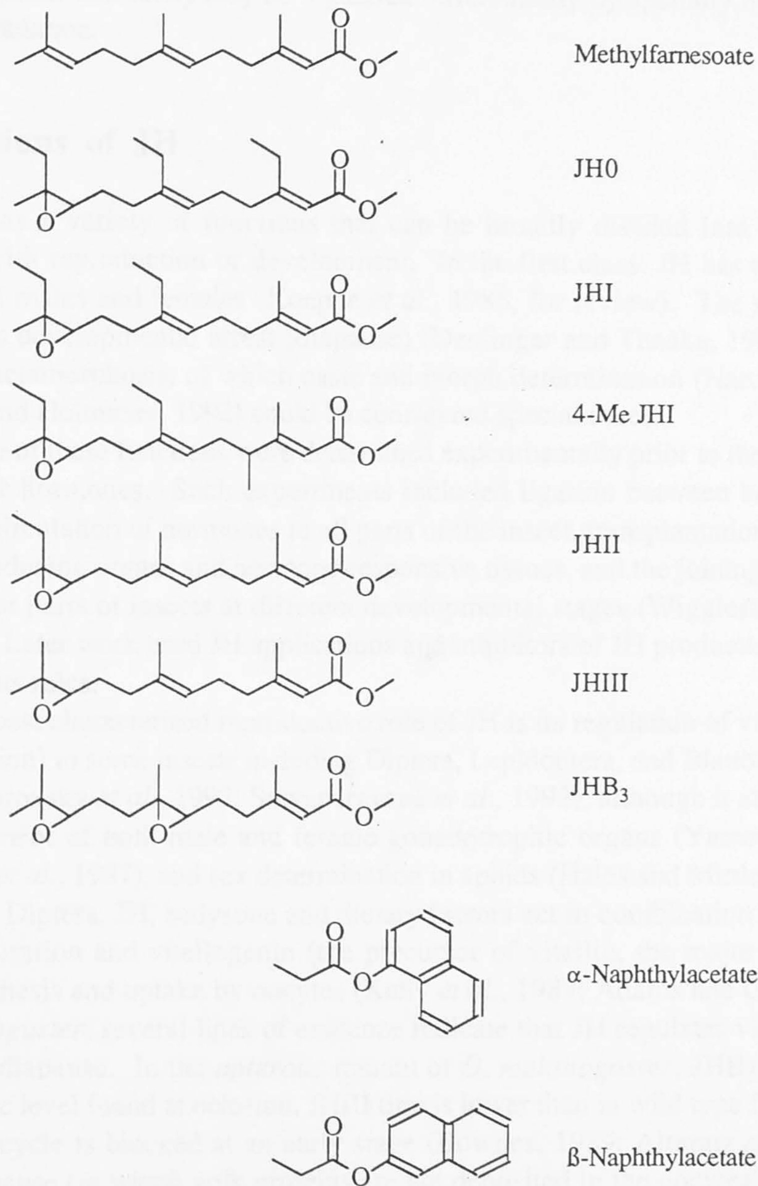


Figure 1.1 JH Isoforms and the Artificial Esterase Substrates
α- and ß-Naphthylacetate

JHIII and JHB_3 suggest the presence of more than one JHEH with different specificities for JHIII and JHB_3. In *D. melanogaster* larvae, cell fractions differ in their relative rates of epoxide hydrolysis of JHIII and JHB_3, although JHIII is more rapidly hydrolysed in all fractions (Casas *et al.*, 1991; Harshman *et al.*, 1991). These findings suggest that JHIII and JHB_3 may be regulated differentially by spatially or temporally specific degradation.

1.3 Functions of JH

JH has a variety of functions that can be broadly divided into two classes associated with reproduction or development. In the first class, JH has reproductive roles in both males and females (Koeppe *et al.*, 1985, for review). The second class encompasses developmental arrest (diapause) (Denlinger and Tanaka, 1989; de Kort, 1990) and metamorphosis, of which caste and morph determination (Hartfelder *et al.*, 1993; Zera and Holtmeier, 1992) could be considered special cases.

Some of these functions were determined experimentally prior to the isolation of JH and other hormones. Such experiments included ligation between body parts to prevent the circulation of hormones to all parts of the insect, transplantation of putative hormone-producing organs and hormone-responsive tissues, and the joining of different live insects or parts of insects at different developmental stages (Wigglesworth, 1954, for review). Later work used JH applications and inhibitors of JH production to further characterise its roles.

The best characterised reproductive role of JH is its regulation of vitellogenesis (egg production) in some insects including Diptera, Lepidoptera, and Blattodea (Tobe *et al.*, 1985; Borovsky *et al.*, 1992; Satyanarayana *et al.*, 1992), although it also regulates the development of both male and female gonadotrophic organs (Yamomoto *et al.*, 1988; Kelly *et al.*, 1987), and sex determination in aphids (Hales and Mittler, 1988). In adult female Diptera, JH, ecdysone and dietary factors act in combination to stimulate ovarian maturation and vitellogenin (the precursor of vitellin, the major insect yolk protein) synthesis and uptake by oocytes (Kelly *et al.*, 1987; Adams and Gerst, 1992). In *D. melanogaster*, several lines of evidence indicate that JH regulates vitellogenesis and ovarian diapause. In the *apterous* mutant of *D. melanogaster*, JHB_3 production remains at the level found at eclosion, JHIII titre is lower than in wild type flies, and the vitellogenic cycle is blocked at an early stage (Bownes, 1989; Altaratz *et al.*, 1991). Ovarian diapause (in which yolk proteins are not deposited in the oocytes) is reversed by topical application of JH and corpora allata removed from non-diapausing flies exhibit higher rates of *in vitro* JHB_3 production (Saunders *et al.*, 1990).

The metamorphic role of JH has been stated generally as maintenance of the current developmental state of the insect. Its removal and/or non-production prior to the pupal and adult moults permits redifferentiation in response to the signal to moult (Willis, 1981; Kumaran, 1990). Programs of gene expression specific for each life stage and cell type are determined during the embryonic stage. Thus, in holometabolous larvae, ecdysone with low JH causes a moult during which cells change from their larval programs of gene expression to their pupal programs of gene expression. Similarly, in pupae, ecdysone with low JH causes a moult during which cells change from their pupal programs of gene expression to their adult programs of gene expression. The presence of JH at a moult prevents progress to the next program of

gene expression, but the absence of JH permits progress. Regulation of metamorphosis is the best characterised role of JH and is discussed in greater detail below.

1.4 Physiology and Hormonal Regulation of Insect Metamorphosis

1.4.1 Physiology of Metamorphosis

In order for arthropods to grow, periodic shedding of old cuticle (moulting) must occur. In the holometabolous insects, which includes the Diptera and Lepidoptera, a series of larval stages or instars are separated by moults (three for the Diptera-Cyclorrapha (higher flies); generally around five for the Lepidoptera), followed by a moult to the pupal form, and then a moult to the adult form. The final larval instar is divided into a feeding phase and a post-feeding or "wandering" phase, during which larvae seek a suitable site to undergo the pupal moult. In the cyclorraphous Diptera the pupal and adult moults occur within the puparium, a unique hard case which is formed by contraction and tanning of the cuticle of the final larval instar (pupariation). A "prepupal" phase, which should be regarded as a continuation of the larval stage (Roberts and Gilbert, 1986), commences with pupariation and ends when the epidermis separates from the puparium (pupal apolysis), about twelve hours later in *D. melanogaster*. Pupal apolysis is regarded as the equivalent of the pupal moult in other insects even though the pupa does not break out of the larval cuticle until much later. The pupa to adult moult (adult apolysis) occurs when the pupal cuticle separates from the underlying epidermis but the "pharate adult" does not immediately break out of the puparium (eclosion). In *D. melanogaster*, adult apolysis occurs about 34-50 hours after pupariation and eclosion occurs about 100 hours after pupariation (Fraenkel and Bhaskaran, 1973; Bainbridge and Bownes, 1981).

In holometabolous insects, almost all larval tissues are replaced after metamorphosis by pupal then adult tissues (Wigglesworth, 1954; Madhavan and Schneiderman, 1977). Those adult tissues which have larval homologues can be derived either from larval cells or imaginal (adult) anlage (set aside) cells. Homologous tissues in adult insects whose larvae do not grow rapidly, such as Coleoptera and Lepidoptera, are derived from larval cells. That is, the cells which formed larval tissues redifferentiate under the influence of an altered hormonal environment to form the adult tissues. However, in insects whose larvae grow rapidly such as Diptera, many larval cells hypertrophy, so a separate stock of diploid anlage cells are set aside during embryogenesis for redifferentiation. These are found scattered, in small clusters or as rings within various tissues such as the gut, trachea and abdominal epidermis. At metamorphosis the anlage cells grow, proliferate, differentiate and replace the larval cells of these tissues (Pearson, 1972; Madhavan and Schneiderman, 1977).

The specialised, adult structures which have no larval homologues such as eyes, wings, legs and antennae are derived from a special class of imaginal cells, the imaginal disc cells. Growth and differentiation of these cells occurs during the larval stage by invagination into the lumen of the imaginal discs. During metamorphosis the imaginal discs are incorporated into the complete pupa with evagination and growth of the specialised structures (Madhavan and Schneiderman, 1977).

Thus, except for the specialised structures, the pupal and adult cuticles of lepidopterans are secreted by the same epidermal cells as produced the larval cuticle. In contrast, the entire adult cuticle of dipterans is secreted by different cells from the larval cuticle (Pearson, 1972; Roseland and Schneiderman, 1979). The adult abdominal epidermis is derived from abdominal anlage cells ("abdominal histoblasts"), and unlike other insects, the imaginal discs provide the cells of the general epidermis of the adult head and thorax in addition to the specialised adult structures.

In *D. melanogaster*, the imaginal discs and abdominal histoblasts differ by both the time at which they recommence cell division after embryogenesis and the time at which they replace larval tissues (Madhavan and Schneiderman, 1977). Imaginal discs recommence cell division during the first or second instar, are incorporated into the head and thorax during the larva to pupa metamorphosis, and secrete first a pupal then an adult cuticle. In contrast, the abdominal histoblasts do not divide between the emergence of the larva from the egg and five hours after pupariation. The abdominal histoblasts then spread out to cover the abdomen just after head eversion (larva-pupa apolysis), replace the larval epidermis, and then secrete the adult abdominal cuticle (Madhavan and Schneiderman, 1977; Roseland and Schneiderman, 1979). Thus, the pupal cuticle of the head and thorax is largely secreted by imaginal disc-derived cells but the pupal cuticle of the abdomen is secreted by the larval epidermal cells. Unlike *D. melanogaster*, the abdominal histoblasts of other higher Diptera such as *Dacus tryoni* and *Calliphora erythrocephala* recommence cell division during the larval stage along with the other imaginal cells (Anderson, 1964; Pearson, 1972).

1.4.2 Hormonal Regulation of Metamorphosis

A generalised scheme for the regulation of metamorphosis in holometabolous insects is presented below (Roe and Venkatesh, 1990; Riddiford, in press, for reviews). All moults are initiated by ecdysone, JH determines the outcome of the moult and several other hormones also have roles. Release of the moult-initiating pulse of ecdysone from the prothoracic glands is triggered by the release of prothoracicotrophic hormone (PTTH) from a set of neurosecretory cells in the brain. Ecdysone is then converted to more active metabolites, ecdysteroids, in the fat body and various target tissues. An early event in the moulting process is the separation of the old cuticle from the epidermis (apolysis). Moulting concludes with the shedding of the old cuticle (ecdysis) caused by eclosion hormone, a peptide released from other neurosecretory cells when the ecdysteroid titre has fallen to a low level.

However, during the final instar, at least one pulse of ecdysone occurs which does not immediately cause a moult but is involved in the commitment to metamorphosis. After the final larval moult JH titres decline as a result of decreased synthesis and increased degradation. PTTH is released in response to very low JH titre and other physiological or environmental cues (critical weight or critical ratio of body dimensions in some Lepidoptera, Jones *et al.*, 1981), causing a small pulse of ecdysone release (Watson *et al.*, 1987). In both Lepidoptera and Diptera this is a major endocrine event with major changes in gene expression (Cherbas, 1993) and in the types of RNA synthesised (Shaaya, 1993). The ecdysteroids, together with low JH titre, cause commitment to the production of pupal rather than larval proteins at the next ecdysone pulse, as well as a behavioural change from feeding to wandering (Dominick and Truman, 1985).

Pupation is initiated by the next pulse of ecdysone which coincides with a pulse of JH production in Lepidoptera (Baker *et al.*, 1987) and *D. melanogaster* (Richard *et al.*, 1989a). In *Manduca sexta*, this JH pulse prevents adult differentiation of the imaginal discs during the pupal moult (Kiguchi and Riddiford, 1978). Pupariation occurs at this time in the higher Diptera. The JH titre immediately falls again, before the pupal moult occurs, and in Lepidoptera this is due to decreased synthesis and increased degradation (Roe and Venkatesh, 1990). In *D. melanogaster* a pulse of ecdysone occurs about twelve hours after pupariation, coinciding with pupal apolysis. During the pupal stage there is an ecdysone pulse without JH, which causes the adult moult and stimulates the expression of adult-specific genes (Riddiford, in press).

Although the available information suggests that the patterns of JH and ecdysone titre changes in *D. melanogaster* are similar to those found in the Lepidoptera (Bownes and Rembold, 1987; Sliter *et al.*, 1987; Richard *et al.*, 1989a; Altaratz *et al.*, 1991; Dai and Gilbert, 1991; Riddiford, in press; Fig. 1.2), two lines of evidence indicate that the commitment to pupation in Diptera occurs at an earlier stage than for insects such as the Lepidoptera. Firstly, Lepidoptera and other insects can be induced to undergo supernumary larval moults by the application of JH in the final instar. In contrast, JH application to final instar dipterans delays pupariation, which can be rescued with ecdysone injection (Srivastava and Gilbert, 1969), but supernumary larval moults cannot be induced (Sehnal and Zdarek, 1976). Secondly, starved, final instar larvae of Diptera pupate in concert with fed larvae and become undersized but fertile adults (Bakker, 1959; Srivastava and Gilbert, 1969), unlike starved Lepidoptera which experience delayed pupation or supernumary larval moults (Cymborowski *et al.*, 1982).

The time at which different *D. melanogaster* tissues are sensitive to JH treatment varies. Exposure to high levels of JH analogues in the first and early second instar, but not later, prevents the adult development of the head and thorax (Riddiford and Ashburner, 1991). In contrast, JH and JH analogues applied to late larvae or prepupae do not affect pupariation but severely inhibit the adult development of the abdomen (Bhaskaran, 1972; Postlethwait, 1974). When JH is applied to prepupae of *D. melanogaster* most of the normal proliferation and redistribution of abdominal histoblast cells occurs (Dr. L. M. Riddiford, pers. comm.), yet these cells produce an abnormal cuticle which lacks bristles and resembles a second larval or pupal cuticle. However, it remains to be determined whether the profile of proteins secreted by the epidermal cells most closely resembles a larval, pupal or adult pattern. In summary, the period of sensitivity of tissues to JH coincides with the commencement of postembryonic cell division in the imaginal cells from which the tissues are derived (Section 1.4.1, Riddiford and Ashburner, 1991).

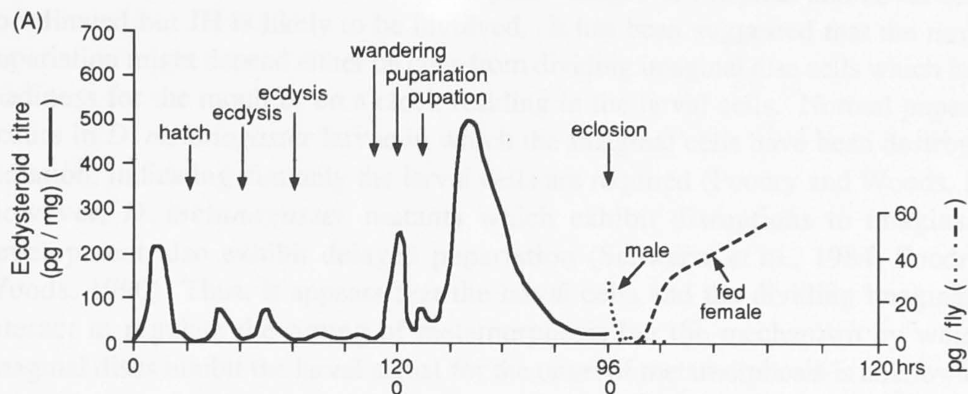
The ability to induce supernumary larvae in the Lepidoptera but not the Diptera appears to be a consequence of their different mode of metamorphosis. Lepidoptera largely rely on redifferentiation of larval cells at the pupal moult and this redifferentiation can be inhibited by JH. In contrast, Diptera replace at least the epidermal cells of the head and thorax at this moult (Section 1.4.1) and it appears that the commitment to this process is made much earlier in larval development. At the the pupa to adult moult, the anti-metamorphic effect of JH on dipteran pupae is typical of other insects, with the earliest pupal applications of JH causing retention of pupal characteristics in the greatest number of structures (Srivastava and Gilbert, 1969).

Figure 1.2 Comparison of the Ecdysteroid Titre, JH Titre and JH Production During Development of *D. melanogaster*

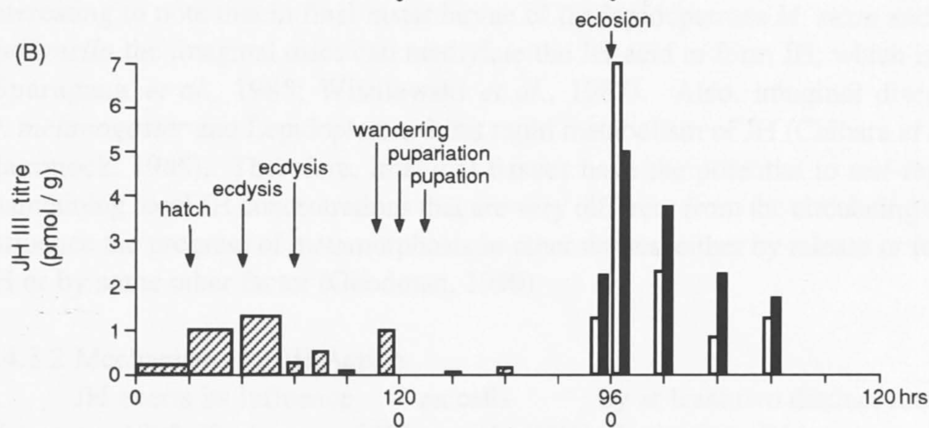
(A) shows the titre of ecdysteroids during development. The figure is reproduced from Riddiford (in press) and the data were compiled from ten sources cited therein.

(B) shows the titre of JHIII during development. Solid bars indicates male titres and open bars indicate female titres. The figure is reproduced from Riddiford (in press) and the data were compiled from Bownes and Rembold (1987) and Sliter *et al.* (1987).

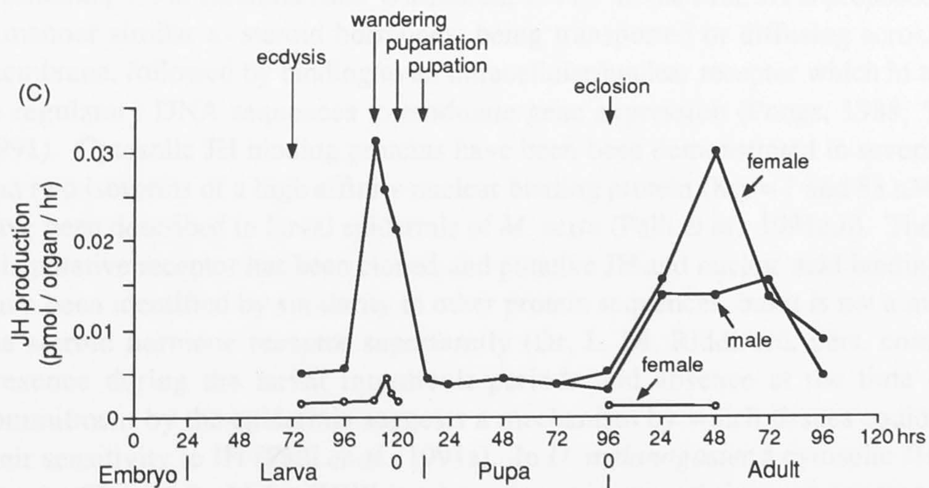
(C) shows *in vitro* JH production by brain/ring gland/ventral ganglion complexes or adult corpora allata aligned with the profiles of ecdysteroid and JHIII titres. Solid symbols indicate JHB3 production and open symbols indicate JHIII production. The data were compiled from Richard *et al.* (1989a), Altaratz *et al.* (1991) and Dai and Gilbert (1991). Pupal age is indicated from pupariation although the larva/pupa transition occurs at pupation (pupal apolysis), twelve hours later (Section 1.4.1).



(A)
Ecdysteroid titre (pg / mg) (——)
pg / fly (·····---)
hatch
ecdysis
ecdysis
wandering
pupariation
pupation
eclosion
male
fed female
0
120
0
96
0
120 hrs



(B)
JH III titre (pmol / g)
eclosion
hatch
ecdysis
ecdysis
wandering
pupariation
pupation
0
120
0
96
0
120 hrs



(C)
JH production (pmol / organ / hr)
ecdysis
wandering
pupariation
pupation
eclosion
female
male
female
0 24 48 72 96 120 24 48 72 96 24 48 72 96 120 hrs
0
0
Embryo
Larva
Pupa
Adult

1.4.3 Mechanisms Underlying Hormonal Control of Metamorphosis

1.4.3.1 Timing of Metamorphosis

It is not known how the metamorphic changes of imaginal and larval cells are coordinated but JH is likely to be involved. It has been suggested that the timing of pupariation might depend either on cues from dividing imaginal disc cells which indicate readiness for the moult or on a clock residing in the larval cells. Normal pupariation occurs in *D. melanogaster* larvae in which the imaginal cells have been destroyed by radiation, indicating that only the larval cells are required (Poodry and Woods, 1990). However, *D. melanogaster* mutants which exhibit disruptions to imaginal disc development also exhibit delayed pupariation (Schwartz *et al.*, 1984; Poodry and Woods, 1990) Thus, it appears that the larval cells and the dividing imaginal cells interact to regulate the timing of metamorphosis but the mechanism by which the imaginal discs inhibit the larval signal for the onset of metamorphosis is unknown. It is interesting to note that in final instar larvae of the lepidopterans *M. sexta* and *Galleria mellonella* the imaginal discs can methylate the JH-acid to form JH, which is released (Sparagana *et al.*, 1985; Wisniewski *et al.*, 1987). Also, imaginal discs in both *D. melanogaster* and Lepidoptera exhibit rapid metabolism of JH (Chihara *et al.*, 1972; Hammock, 1985). Therefore, imaginal tissues have the potential to self-regulate by maintaining local JH concentrations that are very different from the circulating titre, or to influence the progress of metamorphosis in other tissues, either by release or removal of JH or by some other factor (Goodman, 1990).

1.4.3.2 Mechanisms for JH Action

JH exerts its influence on cells by at least two distinct mechanisms (Kumaran, 1990; Kochman and Wieczorek, 1991). In the first, JH is proposed to act in a manner similar to steroid hormones, being transported or diffusing across the cell membrane, followed by binding to an intracellular/nuclear receptor which in turn binds to regulatory DNA sequences to modulate gene expression (Pongs, 1988; Segraves, 1991). Cytosolic JH binding proteins have been been demonstrated in several species and two isoforms of a high affinity nuclear binding protein (K_D = 7 and 88 nM for JHI) have been described in larval epidermis of *M. sexta* (Palli *et al.*, 1991a,b). The gene for this putative receptor has been cloned and putative JH and nucleic acid binding regions have been identified by similarity to other protein sequences, but it is not a member of the steroid hormone receptor superfamily (Dr. L. M. Riddiford, pers. comm.). Its presence during the larval intermoult periods and absence at the time of pupal commitment by the epidermis suggests a mechanism by which tissues could regulate their sensitivity to JH (Palli *et al.*, 1991a). In *D. melanogaster* a cytosolic JH binding protein (K_D = 6.7 nM for JHIII) has been found in several tissues presumed to be JH sensitive and not in tissues presumed to be insensitive (Shemshedini *et al.*, 1990). Furthermore, the binding affinity of the protein for JHIII is 10-fold higher in strains which are susceptible to the JH analogue, methoprene than in mutants exhibiting resistance to methoprene, strongly suggesting that the binding protein is the JH receptor (Shemshedini and Wilson, 1990).

The second mechanism by which JH appears to exert its influence on cells appears to be mediated by membrane protein/second messenger systems and involves activation of protein kinase C (Yamomoto *et al.*, 1988; Sevala and Davey, 1989). However, the presumptive intracellular receptor was also found in one of these tissues,

suggesting that both mechanisms of response to JH may operate in a single tissue (Shemshedini *et al.*, 1990).

A third mechanism by which JH may exert its effects is by modulation of polyamine levels, but this proposal remains highly conjectoral. The anti-metamorphic effect of JH may be a consequence of a block to redifferentiation caused by a block to chromatin structural changes (Willis, 1981, 1990). This effect has been proposed to be mediated by polyamines, which in vertebrates regulate DNA, RNA and protein synthesis, modulate the phosphorylation of specific proteins, and are essential for cell growth and differentiation (Strambi *et al.*, 1993). Although few studies have been performed in insects, the following examples indicate that polyamines are likely to play key, albeit poorly understood, roles in the response of insect cells to ecdysone and JH. In both a bovine system and *D. melanogaster*, JH can modulate the induction of ornithine decarboxylase (ODC), the first key enzyme in the biosynthesis of polyamines (Birnbaum and Gilbert, 1990), and in the house cricket *Acheta domesticus*, both ecdysone and JH modulate polyamine synthesis, ODC activity and phosphorylation of specific proteins (Strambi *et al.*, 1992, 1993). Polyamine levels peak in *Tenebrio molitor* epidermis just before pupal ecdysis between the last larval ecdysone peak and a period of JH sensitivity (Besson *et al.*, 1986), and in *M. sexta* fat body and neural tissue fluctuations in polyamine concentrations and ODC activity appear to be associated with periods of growth, differentiation and proliferation during metamorphosis (Birnbaum *et al.*, 1988).

1.4.3.3 Mechanisms for Ecdysone Action

In *D. melanogaster* regulation of gene expression by ecdysteroids has been extensively studied, together with the effect of JH on this expression. The temporally regulated "puffs" in the giant polytene chromosomes of the salivary glands of third instar larvae and early pupae have served as a model for ecdysone-induced changes (Andres and Thummel, 1992, for review). A well defined sequence of early and late puffs occur in response to three pulses (pre-wandering, pupariation and pupal apolysis) of ecdysone and these puffs correspond to the activation of genes. The early gene products include proteins whose sequence includes DNA binding domains, supporting the proposal that these are gene regulators which turn on the late genes, while possibly also inhibiting their own production. This model is further supported by the finding that antibodies to one of the early gene products binds to many of the early and late ecdysone-induced puffs (Andres and Thummel, 1992).

The puffing sequence can be reproduced *in vitro* by appropriately timed incubations with ecdysone-containing and ecdysone-free media. JH has been shown to modify the competence of salivary glands to respond to ecdysone (Richards, 1978), indicating that many of the genes regulated by ecdysone are also regulated by JH (and presumably by tissue specific factors). The prepupal ecdysone-induced puffing sequence can be inhibited by JH with the JH sensitive period occurring prior to prepupal ecdysone exposure. However, the late larval puffing sequence is not influenced by JH. The coincidence of the JH sensitive periods for salivary gland chromosomal puffs and adult abdominal defects (Postlethwait, 1974) suggests that the prepupal period is generally important for expression of JH regulated genes.

1.5 Regulation of JH Titre

In order to regulate the variety of processes described above, the abundance of JH itself must be tightly regulated. Regulation of JH titre is achieved by a balance between synthesis and degradation. However, JH carrier molecules can modulate these two processes, and other mechanisms such as sequestration and excretion may also contribute to modulation of the JH titre (de Kort and Granger, 1981; Hammock, 1985; Tobe and Stay, 1985; Roe and Venkatesh, 1990, for reviews).

1.5.1 Synthesis of JH

JH is produced in a specialised organ, the corpus allatum. In dipteran larvae, however, the corpus allatum is part of a composite organ, the ring gland, which includes the prothoracic gland and the corpus cardiacum. Although the corpus allatum and regulation of JH synthesis have been studied in many insects (Tobe and Stay, 1985; Goodman, 1990, for reviews), JH synthesis in Diptera has only recently received detailed attention (Richard *et al.*, 1989a,b, 1990; Richard and Gilbert, 1991; Altaratz *et al.*, 1991; Dai and Gilbert, 1991; Duve *et al.*, 1992).

Regulation of JH synthesis by the nervous system has been demonstrated in many insect orders, mediated by the products of neurosecretory cells and possibly conventional electrical impulses. Regulation occurs both at several early steps in its biosynthetic pathway and at the terminal steps (Goodman, 1990; Borovsky *et al.*, 1992). Terminal steps in the JH biosynthetic pathway involve the esterification of farnesoate to MF and 10,11 epoxidation (and 6,7 epoxidation for JHB_3), although the order of these steps appears to vary between species (Tobe and Stay, 1985). In *D. melanogaster*, JH production appears to be regulated both by the availability of JH precursors and the activity of the oxidase(s) which adds the epoxide group(s). Feedback inhibition of JH production by JH occurs at least at two levels. JHB_3 stimulates the brain to produce an allatostatic factor which is transfered to the corpus allatum via nervous connections (Altaratz *et al.*, 1991), but JHIII and JHB_3 also inhibit JH production by ring glands which are isolated from the brain (Richard and Gilbert, 1991).

Temporal regulation of JH production is one of the major factors causing variation of JH titre (Goodman, 1990). In *D. melanogaster*, regulation of JH production has been investigated in final instar larvae and adults (Fig. 1.2). In late wandering larvae a peak of JH synthesis occurs which declines abruptly one or several hours after pupariation (Richard *et al.*, 1989a; Dai and Gilbert, 1991) and in adults JH synthesis rises then falls during the first four days after eclosion (Altaratz *et al.*, 1991; Dai and Gilbert, 1991). The latter peak in females coincides with the onset of vitellogenesis, histolysis of the larval fat body and changes in sexual behaviour. These two periods of JH synthesis coincide with known peaks of JH titre in *D. melanogaster* (Bownes and Rembold, 1987; Sliter *et al.*, 1987), and other species have peaks of JH titre during equivalent periods (Roe and Venkatesh, 1990).

The temporal regulation of synthesis differs between JH isoforms. The products of the corpus allatum of adult female *D. melanogaster* include between 3 and 23% JHIII and 1 to 14% MF. The absolute amounts of MF and JHIII do not change greatly, whereas the dominant *in vitro* product, JHB_3, is temporally regulated, suggesting that MF and JHIII are merely precursors of JHB_3 (Altaratz *et al.*, 1991). In

final instar larvae, however, JHIII and MF production rise and fall approximately in concert with JHB_3, although JHB_3 is the major product (Richard *et al.*, 1989a), suggesting that all these JH forms could have a role at this life stage (Fig. 1.2). However, the form of JH released from the corpus allatum is not necessarily the biologically active form. For example, the corpus allatum of prepupal *M. sexta* is unable to carry out esterification of JH-acid, and the corpus allatum of prepupal *G. mellonella* synthesises JH but also has a high activity of JHE. Thus, both species release JH-acid which is esterified to active JH in other tissues (Sparagana *et al.*, 1985; Wisniewski *et al.*, 1987).

1.5.2 JH Carrier Proteins

At least four classes of high affinity, JH-specific carrier proteins are found in the haemolymph of insects (Goodman, 1990; Trowell, 1992, for reviews). Most insects, including Diptera, use lipophorin as their JH carrier. Lipophorin is a high molecular weight lipoprotein (about 300 kDa) which is abundant in haemolymph. It is the major carrier for lipids but also binds JH at a single specific site. In contrast, the lepidopteran JH carrier proteins have typical molecular weights of 25-35 kDa and are not associated with significant amounts of lipid.

JH carriers have at least two roles, the first being to retain JH in solution. Although JH is soluble at physiological concentrations it adsorbs to *in vitro* surfaces and sustained *in vitro* JH production by corpora allata of the cockroach, *Nauphoeta cinerea*, requires the presence of the JH carrier (Lanzrein *et al.*, 1993). Thus, it is assumed that JH would also adsorb to *in vivo* surfaces, which might prevent its effective circulation and result in a steep gradient of JH concentration away from its site of production (Trowell, 1992).

A second proposed role is modulation of the activity of JH degrading enzymes (Sanburg *et al.*, 1975) and two types of interaction with these enzymes have been described. The first type of interaction is a simple mass action model, in which the relative abundance and kinetic behaviour of both the carrier and JH degrading enzymes determine the rate of JH degradation. When the JHE titre is low the carrier is proposed to stabilise JH in solution but enhance the clearance of JH when JHE activity is high (Hammock *et al.*, 1987). However, in haemolymph of *T. ni* at times of maximal JHE activity it is the rate of dissociation of JH from the carrier rather that the abundance of JHE or JH which limits the rate of JH hydrolysis (Abdel-Aal and Hammock, 1988). The second type of interaction is known only in the blattodean, *Leucophaea maderae*, and requires direct contact for non-competitive inhibition of JHE by lipophorin (Engelmann, 1984; Engelmann *et al.*, 1988).

Differences between JH isoforms in their binding affinities for the carrier and degrading enzymes suggest a mechanism for the independent regulation of titres of JH isoforms, although separate roles for JH isoforms have not been demonstrated. Thus, one isoform could be preferentially degraded while the other is protected by preferential binding to the carrier. This has been shown for the lepidopteran, *G. mellonella* in which only a portion of the total JHII is degraded but hydrolysis eliminates JHIII from circulation at defined developmental stages (Szolajska, 1991).

1.5.3 JH Degradation

The activities of JH hydrolysing enzymes often vary through development in an inverse relationship to JH titre, suggesting that they play an important role in the regulation of JH titre (Hammock, 1985). The two main degradative pathways for JH are hydrolysis of the ester and the epoxide groups by JHE and JHEH, respectively (de Kort and Granger, 1981; Hammock, 1985; Tobe and Stay, 1985; Roe and Venkatesh, 1990, for reviews). The products of hydrolysis of JH by JHE and JHEH are JH-acid and JH-diol, respectively, and sequential hydrolysis of JH by both enzymes can yield JH-acid-diol (Fig. 1.3).

Oxidation and conjugation have also been proposed to inactivate JH. In several Diptera an NADPH dependent oxidase was described as metabolising JH (Slade and Zibitt, 1972; Ajami and Riddiford, 1973; Yu and Terriere, 1978a), but it now appears that this enzyme catalyses the terminal step in the biosynthetic pathway for JHB_3. It is found in the *D. melanogaster* ring gland, consistent with a biosynthetic function, but not in the fat body or brain (Richard *et al.*, 1989b) and the peak activities in *Musca domestica* occur at the times of expected peak JHB_3 synthesis in late larval and adult stages (Yu and Terriere, 1978a). *In vivo* conjugation of the JH hydrolytic products has been demonstrated in many species, with JH-diol and JH-acid being recovered after glucosidase or sulphatase treatments of polar JH metabolites (Roe and Venkatesh, 1990). Conversion to more polar products by hydrolysis and conjugation may be important for excretion of JH as no unmetabolised JH was found in the faeces of *M. sexta* (Slade and Zibitt, 1972). Conjugation may also be important to ensure that hydrolysed JH is not converted back to JH, as JH-acid can be remethylated to form JH, at least in *M. sexta* imaginal discs (Sparagana *et al.*, 1985).

The hydrolytic pathways for JH metabolism have been characterised in most detail, with JHE subjected to intensive investigation. These pathways are reviewed in greater detail below.

1.5.3.1 Definition of "JH-Specific" Hydrolysis

An enzyme which can hydrolyse JH *in vitro* does not necessarily have specificity for JH *in vivo* or a physiological role in regulation of JH titre so it is important to demonstrate that the enzyme is "JH-specific" (Hammock, 1985). The possibility that an enzyme is not specific can be illustrated by two observations. Firstly, many esterases and epoxide hydrolases have the capacity to hydrolyse substrates *in vitro* that have no relationship to their biological functions. For example, more than twenty esterases in *D. melanogaster* can hydrolyse the non-physiological ester, naphthylacetate (Healy *et al.*, 1991). Secondly, the commonly used assays for JH hydrolysis usually include JH in considerable excess of physiological concentrations and may not indicate an ability to hydrolyse JH at low, *in vivo* concentrations. It should be noted also that resistance to inhibition by the organophosphate diisopropylfluorophosphate (DFP) has sometimes been regarded as diagnostic for JHE. However, while most JHEs are resistant, this alone has no direct bearing on whether JH is the *in vivo* substrate (Hammock, 1985).

Hammock (1985) has therefore suggested six criteria which singly or in combination could support the application of the term "JH-specific esterase". These criteria, however, can be applied with equal justification to an epoxide hydrolase. Two physiological criteria require demonstration that the enzyme is essential for the clearance of JH from an insect's body, or that increases of enzyme titre correlate with declines of JH titre. Four biochemical criteria include demonstration that the enzyme has a low K_M

for JH, hydrolyses JH with a high K_{cat}/K_M ratio, rapidly hydrolyses JH in the presence or absence of JH carrier protein (indicative of a low K_M), or, most rigorously, shows a higher K_{cat}/K_M ratio for JH than for an alternative substrate such as α-naphthylacetate. An additional criterion suggested by Abdel-Aal and Hammock (1988) uses the relationship between structure and activity of enzyme inhibitors or alternative substrates to indicate specificity. Enzymes with some or all of these of these properties are discussed below.

1.6 Physiology of JH Hydrolysis

1.6.1 Temporal Profile of JH Hydrolysis

JH hydrolysis has been studied extensively in the Lepidoptera, with the main focus on hydrolysis in the haemolymph and the events leading up to the pupal moult. During this period JH hydrolysis in the haemolymph occurs exclusively by JHE (Jones, 1986; Roe and Venkatesh, 1990). Key features of this period are the general occurrence of two maxima in the profile of haemolymph JHE activity which coincide with minima in the profiles of JH titre and production (Jones *et al.*, 1982, 1990; Fescemyer *et al.*, 1986; Zimowska *et al.*, 1989). The prewandering JHE maximum appears to coincide with the commitment to pupation (Jones, 1985) and the second JHE maximum occurs immediately prior to pupation and is required for ecdysis (Jones and Hammock, 1985). JHE inhibitors applied at these times cause an increase in JH titre and a disruption to development, suggesting that the function of JHE is to remove JH at these times and that JH removal is required for correct development (Jones, 1986). In one lepidopteran, *Lymantria dispar*, there is an earlier JHE peak, just after ecdysis to the last instar, and a mid pupal peak in addition to the usual two last larval instar peaks, with a decline in JH titre associated with each peak of JHE. In this species JHE activity is higher in females while JH titre is higher in males for most of this larval/pupal period (Tanaka *et al.*, 1989).

The inverse correlation of JH hydrolysis with JH titre and production also occurs in adult females in two insect orders, Blattodea (Tobe *et al.*, 1985) and Diptera (Shapiro *et al.*, 1986; Borovsky *et al.*, 1992) and probably also occurs in Lepidoptera (Venkatesh *et al.*, 1988). In these examples high JH titres and production levels at the onset of vitellogenesis are followed by reduced JH synthesis and titre and increased JHE activity prior to oviposition. This suggests that JHE also contributes to the reduction of JH titre in its adult reproductive roles.

However, studies of carefully timed final instar larvae of *M. sexta* (Nijhout, 1975; Roe and Venkatesh, 1990) and *T. ni* (Jones *et al.*, 1990) and adults of *Diploptera punctata* (Tobe *et al.*, 1985) and *Aedes aegypti* (Shapiro *et al.*, 1986) have shown that the JH titre drops to low levels before haemolymph JHE activity rises. Nevertheless JHE inhibitors cause disruptions to metamorphosis and egg production (Jones, 1985; Shapiro *et al.*, 1986; Roe and Venkatesh, 1990), and in prewandering *T. ni*, the JH titre has been shown to rise slightly when JHE is inhibited (Jones *et al.*, 1990), indicating that JHE is required even when JH titres are low. Quantities of pure JHE, comparable with the final instar JHE maxima, injected into earlier larval stages of *M. sexta*, when JH is normally high, cause symptoms of only slightly reduced JH titre (Philpott and Hammock, 1990). These results suggest that the role of the highest haemolymph JHE activities is to scavenge the last traces of circulating JH after

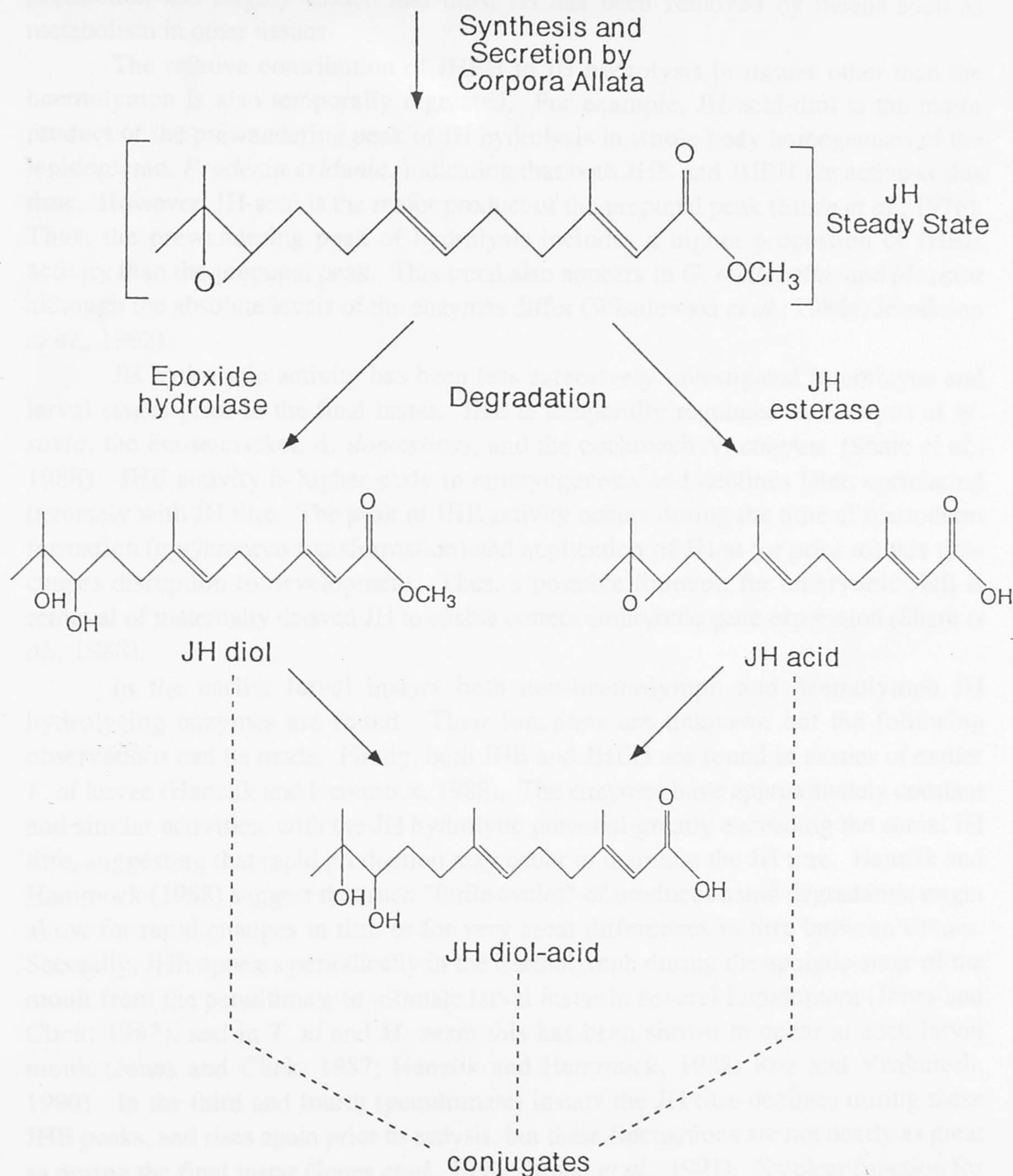


Figure 1.3 Control of Steady State Levels of Juvenile Hormone

Control of JH titre is generally achieved through both regulated JH synthesis in the corpus allatum and degradation. The two main routes for degradation are hydrolysis of the epoxide group and the ester bond by JH epoxide hydrolase and JH esterase, respectively, yielding the products JH-diol and JH-acid. Further hydrolysis may occur yielding JH-diol-acid. All these products can be conjugated to form more polar products which are excreted.

production has largely ceased and most JH has been removed by means such as metabolism in other tissues.

The relative contribution of JHEH to JH hydrolysis in tissues other than the haemolymph is also temporally regulated. For example, JH-acid-diol is the major product of the prewandering peak of JH hydrolysis in whole body homogenates of the lepidopteran, *Prodenia eridania*, indicating that both JHE and JHEH are active at this time. However, JH-acid is the major product of the prepupal peak (Slade *et al.*, 1976). Thus, the prewandering peak of hydrolysis includes a higher proportion of JHEH activity than the prepupal peak. This trend also appears in *G. mellonella* and *M. sexta* although the absolute levels of the enzymes differ (Wisniewski *et al.*, 1986a; Jesudason *et al.*, 1992).

JH hydrolytic activity has been less extensively investigated in embryos and larval stages prior to the final instar. JHE is temporally regulated in embryos of *M. sexta*, the house cricket, *A. domesticus*, and the cockroach *N. cinerea* (Share *et al.*, 1988). JHE activity is higher early in embryogenesis and declines later, correlating inversely with JH titre. The peak of JHE activity occurs during the time of blastoderm formation (egg/embryo transformation) and application of JH at (or prior to) this time causes disruption to development. Thus, a possible function for embryonic JHE is removal of maternally derived JH to enable correct embryonic gene expression (Share *et al.*, 1988).

In the earlier larval instars both non-haemolymph and haemolymph JH hydrolysing enzymes are found. Their functions are unknown but the following observations can be made. Firstly, both JHE and JHEH are found in tissues of earlier *T. ni* larvae (Hanzlik and Hammock, 1988). The enzymes have approximately constant and similar activities, with the JH hydrolytic potential greatly exceeding the actual JH titre, suggesting that rapid production may occur to maintain the JH titre. Hanzlik and Hammock (1988) suggest that such "futile cycles" of production and degradation might allow for rapid changes in titre or for very great differences in titre between tissues. Secondly, JHE appears periodically in the haemolymph during the apolytic stage of the moult from the penultimate to ultimate larval instar in several Lepidoptera (Jones and Click, 1987), and in *T. ni* and *M. sexta* this has been shown to occur at each larval moult (Jones and Click, 1987; Hanzlik and Hammock, 1988; Roe and Venkatesh, 1990). In the third and fourth (penultimate) instars the JH titre declines during these JHE peaks, and rises again prior to ecdysis, but these fluctuations are not nearly as great as during the final instar (Jones *et al.*, 1990; Strand *et al.*, 1991). No clear function for JHE during these periods could be demonstrated either by the application of JHE inhibitors or JH analogues (Jones and Click, 1987; Hanzlik and Hammock, 1988).

1.6.2 Spatial Profile of JH Hydrolysis

The JHE that is recovered from the haemolymph is believed to be synthesised in the fat body and exported. In *T. ni*, JHE activity increases in the fat body at the time of the prepupal peak but following the prepupal haemolymph peak JHE continues to be found in the fat body while its export to the haemolymph is blocked (Wozniak and Jones, 1990).

Metabolism of JH at sites other than the haemolymph has received less attention. Various studies have identified JHE and/or JHEH activities in dissected tissues such as fat body, epidermis, midgut, brain, suboesophageal ganglion, tracheae, corpora allata

and cardiaca, silk glands, gonads, and imaginal discs (Roe and Venkatesh, 1990) but only in final instar larvae of the lepidopterans, *M. sexta*, *G. mellonella* and *T. ni*, have developmental changes of these enzymes within tissues been investigated. In *M. sexta* the specific activities of JHE and JHEH are similiar in the fat body, integument, midgut, and brain, with JHE and JHEH in these tissues and JHE in the haemolymph each accounting for about one third of the total JH hydrolytic activity (Jesudason *et al.*, 1992). The study indicates that sufficient JH metabolism occurs in tissues to account for JH titres falling whenever JH production declines. In most tissues of *M. sexta*, prewandering and prepupal peaks of JHE and JHEH occur in concert. However, there is also evidence of tissue- and stage-specific regulation of these enzymes. For example, in the integument, the prepupal JHE peak is greater than the prewandering peak but the opposite occurs for JHEH, and neither JHE nor JHEH have a prepupal peak in the fat body (Jesudason *et al.*, 1992). A similar pattern was observed in tissues of *G. mellonella*, with JHEH activities generally higher earlier in the final instar and JHE generally higher later in the final instar (Wisniewski *et al.*, 1986a).

The high levels of JH hydrolysis in tissues suggest that specific tissues could maintain much lower local JH concentrations than occur in general circulation. For example, the increase in brain JHE activity at the time of a small JH peak in post wandering *G. mellonella* larvae may function to protect neuroblasts which are involved in intense, JH-sensitive formation of neurons (Wisniewski *et al.*, 1986b). Thus, spatial differences of JH hydrolysis allow the possibility of great variation of JH titre between tissues and independent regulation of the response of specific tissues to the endocrine environment.

1.6.3 Regulation of JH Hydrolysis

Regulation of JHE activity occurs at the levels of synthesis, export from the site of production, specific degradation and modulation of its activity by interactions with other molecules. Regulation of JHEH, however, has not been investigated to the same extent. In *M. domestica* adults, 3 days of exposure to phenobarbital causes JHEH activity to be induced by 78% (Yu and Terriere, 1978b), and in *D. melanogaster*, selection by exposure to cut orange over 20 generations caused JHEH, but not JHE, to be increased by 80% in adults (Ottea *et al.*, 1988). While these results suggest that JHEH can be regulated, it is not clear how they relate to regulation of JH titre under natural conditions.

JHE is inducible by its substrate JH at certain times in larvae, pupae or adults of Lepidoptera, Coleoptera and Blattodea (Roe and Venkatesh, 1990). Induction of JHE by JH occurs indirectly in prewandering *T. ni* and *M. sexta* but more directly in postwandering larvae. Topically applied JH induces JHE in the abdomens of prewandering larvae but only if they are not head-ligated. In contrast, induction of JHE by JH occurs in postwandering larvae with or without head ligation, indicating that a neuroendocrine (head) factor(s) other than JH is required at the earlier, but not the later, stage (Roe and Venkatesh, 1990).

A JHE-repressive peptide was identified initially from final instar larvae of the lepidopteran, *Pseudaletia separata*, in which metamorphosis is not initiated due to parasitism by the wasp, *Apantales kariyai* (Hayakawa, 1990, 1991). The peptide is also present in the haemolymph of the unparasitised host during the penultimate instar and may be a previously undescribed hormone (Hayakawa, 1992). In all three lepidopteran

species tested, the peptide reduces weight gain in the final instar and delays pupation but it is not clear whether JHE repression is the cause or a consequence of the block to growth and development (Hayakawa and Yasuhara, 1993).

The *T. ni* JHE must be glycosylated before it is exported from its site of synthesis in the fat body. However, after the prepupal peak of haemolymph JHE, high levels of JHE continue to be present in the fat body and a factor other than synthesis or glycosylation in pupae prevents its export to the haemolymph (Wozniak and Jones, 1990).

Lepidopteran larvae also have a specific mechanism for the removal of JHE from the haemolymph. Exogenous *Heliothis virescens* JHE has a half life of only 1.2-3.6 hours in the haemolymphs of *H. virescens* or *M. sexta*, being rapidly and specifically taken up into lysosomes of haemocytes and pericardial cells at the developmental times when endogenous JHE activity is known to fall (Booth *et al.*, 1992; Ichinose *et al.*, 1992a,b). JHE taken into the pericardial cells could not be recovered from homogenised pericardial complexes without the use of a protease inhibitor cocktail, indicating that JHE is rapidly degraded (Ichinose *et al.*, 1992b).

Lipophorin, described in section 1.5.2, may interact directly with JHE as a non-competitive inhibitor (Engelmann *et al.*, 1988) and other endogenous inhibitors or activators of JHE may exist. *M. sexta* JHE is greatly activated in the presence of molar concentrations of a wide range of water-miscible organic solvents (Croston *et al.*, 1987). The solvents might act at a relatively hydrophobic modifier site that has an *in vivo* activation role. However, other lepidopteran JHEs were only activated modestly or inhibited under these conditions.

1.6.4 JH Hydrolysis in Diptera

JH degradation in many insects plays a specific, albeit not fully understood, role in the regulation of JH. In the Diptera, however, the role of JH degradation is far from clear. The use of the non-physiological JHI until the early 1980s and the small number of studies limit generalisation about dipteran JH metabolism. With this caveat in mind however, some comments on the temporal and spatial patterns of expression of dipteran JH hydrolysing enzymes can be made.

The available evidence from larvae suggests that prewandering and prepupal peaks of hydrolysis may occur. Microsome preparations of *M. domestica* larvae exhibit a prewandering peak of JHEH activity (Yu and Terriere, 1978b) and JH hydrolysis occurs in final instar larvae and pupae of *Drosophila virilis* with a minimum of activity just prior to pupariation (Rauschenbach *et al.*, 1991). A prepupal peak of JHE activity occurs in *D. hydei* (Klages and Emmerich, 1979) and rates of JH metabolism in whole pupae of *M. domestica*, *Chaoborus americanus*, and *D. melanogaster* are comparable to other orders (Ajami and Riddiford, 1973). JH hydrolysis is temporally regulated in two adult female dipterans. In *A. aegypti*, JHE activity varies inversely with JH titre in response to a blood meal (Shapiro *et al.*, 1986; Borovsky *et al.*, 1992) and this is similar to temporal variations in JHE and JH titres observed in other insect orders during cycles of egg production (Renucci *et al.*, 1984). In *D. melanogaster*, microsomal JHEH activity was found in 2 day old whole adult virgin females but not in 6 day old virgin females, whereas microsomal JHE activity is similar at both ages (Ottea *et al.*, 1988). While temporal regulation of JH production in *D. melanogaster* has been

demonstrated (Altaratz *et al.*, 1991), it is not known whether hydrolysis of JH also plays a part in the regulation of JH titre during vitellogenesis in this species.

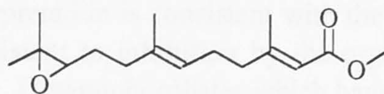
Non-haemolymph metabolism of JH may be of particular importance in the Diptera. The haemolymph of *Sarcophaga bullata* (Weirich and Wren, 1976), *D. hydei* (Klages and Emmerich, 1979) and *D. melanogaster* (Wilson and Gilbert, 1978; Shemshedini and Wilson, 1988) larvae contains little JH hydrolytic activity, unlike most other insects investigated. Nevertheless, JHE and JHEH activities are present in whole *D. melanogaster* (Wilson and Gilbert, 1978), microsome preparations of *M. domestica* larvae (Yu and Terriere, 1978a), and the body wall and fat body of *D. hydei* larvae (Klages and Emmerich, 1979). The finding of multiple forms of JHEH in *D. melanogaster* final instar larvae with differing activities towards JHIII and JHB_3 and differing subcellular localisations might reflect local regulation of JH within tissues, perhaps with differential regulation of JHIII and JHB_3 (Casas *et al.*, 1991; Harshman *et al.*, 1991). Most JHE and JHEH activities occur in the microsomal fraction of whole, female *M. domestica* (Yu and Terriere, 1978a), suggesting an intracellular location. Furthermore, in adult *M. domestica*, haemolymph JHE activity was the lowest found in a comparative study across six insect orders, yet the half life of injected JHI was lowest in this species (de Kort *et al.*, 1979). These observations suggest that considerable JH degrading activity occurs in non-haemolymph sites. Although JH degradation appears to be generally absent or low in the haemolymph of larvae and adults, JHE occurs in the haemolymph of *D. hydei* prepupae and persists though declining through the pupal stage (Klages and Emmerich, 1979). However, other pupal tissues were not investigated.

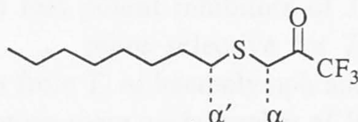
In *D. virilis* an electrophoretically detected esterase (denoted "p-esterase" for pupal esterase) has been extensively studied since the early 1970s and is proposed to be JHE. Several lines of evidence led to this proposal: it is induced by JH and JH analogues; it follows the expected developmental profile for JHE, with predominantly pupal expression; JHI appeared to competitively inhibit its hydrolysis of naphthylacetate in an "in gel" assay; JH hydrolytic activity was only observed in homogenates with p-esterase activity; and it was the only naphthylacetate esterase not to be inhibited by DFP (Rauschenbach *et al.*, 1987, 1991 and earlier references therein). Furthermore, after anion exchange chromatography of pupal homogenates, JH hydrolytic activity was recovered from two fractions, one of which also contained the p-esterase (Dr. I. Y. Rauschenbach, pers. comm.).

1.7 Biochemistry of Juvenile Hormone Esterases

1.7.1 *In Vitro* Inhibitors of JHE

The active sites of lepidopteran JHEs have been extensively probed using trifluoromethylketones (Fig. 1.4), a class of esterase inhibitors (Roe *et al.*, 1990 and references therein). The trifluoromethylketone group mimics the transition state of ester hydrolysis and therefore binds with high affinity in the active site of esterases (Linderman *et al.*, 1993). Several series of compounds which incorporate the trifluomethylketone group were tested as inhibitors of lepidopteran JHEs to uncover determinants of specific binding to JHE. Aliphatic trifluoromethylketones with chain lengths similar to the backbone of JH were found to be especially good inhibitors and





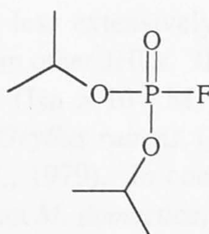


Figure 1.4 Structures of Esterase Inhibitors Compared with JHIII

The structures of the trifluoromethylketone inhibitor, OTFP and the organophosphorus inhibitor, DFP are compared with JHIII. The positions of α and α′ methyl substitutions which enhance the activity of OTFP are indicated (Linderman *et al.*, 1987).

quite selective for JHE over other esterases; the most effective inhibitor (3-(octylthio)-1,1,1-trifluoropropan-2-one, OTFP) had a chainlength of twelve. It also incorporates a sulphur atom into the chain which is proposed to mimic the 2,3 double bond (Linderman *et al.*, 1987). Neither the addition of side chains at C7 and C11, nor the introduction of further double bonds into the chain, enhances inhibition, although these changes were expected to better mimic the structure of JH (Linderman *et al.*, 1989). Addition of an α- or α'-methyl group, however, does enhance inhibition by OTFP but an α-ethyl addition reduces inhibition (Linderman *et al.*, 1987). This was interpreted as indicating that a small hydrophobic pocket exists adjacent to the carbonyl binding site of JHE which favours binding of the C3 methyl substitution found on JH but not larger groups. This interpretation is consistent with the observation that most non-dipteran JHEs are very resistant to inhibition by the organophosphate, DFP ($I_{50} > 10^{-3}$ M) (Hammock, 1985). Organophosphates which have less bulk adjacent to the phosphate than DFP are effective inhibitors of JHEs, suggesting that poor inhibition by DFP is due to steric hinderance (Roe *et al.*, 1990).

Recently, OTFP and α-methylOTFP have been modified by replacement of the carbonyl moiety with an alcohol moiety (Linderman *et al.*, 1993). The modified compounds are each less potent inhibitors of JHE than their parent compounds. However, they are more selective for *T. ni* JHE over α-naphthylacetate hydrolysing esterases from *T. ni* haemolymph and electric eel acetylcholine esterase (AChE) and so may prove more useful probes of JHE function *in vivo*. The selectivity of these compounds for JHE suggests that hydrogen bonding plays a different role in the binding of the JH transition state than in the transition state binding of other esterases.

Dipteran JHEs have been less extensively investigated but their profiles of sensitivity to inhibitors differ from other JHEs. JHEs from non-dipteran insect orders are generally resistant to DFP ($I_{50} > 10^{-3}$ M, Hammock, 1985), although two exceptions are the orthopterans, *Gryllus rubens*, ($I_{50} = 10^{-5}$ M, Zera *et al.*, 1992), and *Locusta migratoria* (Peter *et al.*, 1979). In contrast, the JHEs of the dipterans, *S. bullata* and *Phormia regina* but not *M. domestica*, are sensitive to DFP (I_{50} about 10^{-4} M, Yu and Terriere, 1978a) and *D. virilis* may have both DFP-sensitive and DFP-resistant forms of JHE (Dr. I. Y. Rauschenbach, pers. comm.). *M. domestica* JHE exhibits an atypical pattern of sensitivity to a range of organophosphates, carbamates and other esterase inhibitors (Mumby *et al.*, 1979; Sparks and Hammock, 1980) and *Culex pipiens pipiens* JHE is resistant to several effective inhibitors of other JHEs (Hooper, 1976). Taken together these atypical inhibitor sensitivities suggest structural differences between the active sites of dipteran and non-dipteran JHEs.

1.7.2 Purification of JHEs

JHE has been purified to homogeneity from larval haemolymph of seven species of Lepidoptera but not from any other order. Classical chromatographic techniques have been used to purify JHEs from *M. sexta* (Couldron *et al.*, 1981), *T. ni* (Yuhas *et al.*, 1983; Rudnicka and Jones, 1987; Wozniak *et al.*, 1987), *G. mellonella* (Rudnicka and Kochman, 1984), and *L. dispar* (Valaitis, 1991; 1992). Recoveries and enrichment factors were 1-40% and 383-4000 fold. Each of these purifications required four or five steps, with most including either polyethyleneglycol (PEG) or ammonium sulphate precipitation followed by gel filtration and ion exchange chromatography, and several

included hydroxylapatite chromatography. Particularly successful methods used in the more recent purifications involved concanavalin A binding, which relies on the presence of polysaccharides on the surface of JHE (Valaitis, 1992), and preparative isoelectric focussing (IEF, Rudnicka and Jones, 1987; Wozniak *et al.*, 1987).

Affinity chromatography has been used to purify JHEs from the haemolymph of *M. sexta*, *Bombyx mori*, *H. virescens* and *Heliothis zea* (Abdel-Aal and Hammock, 1985; 1986; Abdel-Aal *et al.*, 1988), and from haemolymph and whole organism homogenate of prepupal larvae of *T. ni* (Hanzlik and Hammock, 1987). This method uses trifluoromethylketone compounds (Section 1.7.1) as both the ligand and the eluting agent (OTFP). Recoveries from haemolymph were 50-98% with 585-1240 fold enrichment, whereas the recovery and enrichment factor from *T. ni* larval homogenate were 10% and 1340 fold, respectively.

All the purified JHEs consist of a single polypeptide in the range of 62-68 kDa which is typical of other esterases (Cygler *et al.*, 1993), and the JHEs of *M. sexta*, *T. ni* and *L. dispar* are monomeric.

1.7.3 Kinetic Characterisation of JHEs

Kinetic analyses of non-purified JHEs have been performed using diluted haemolymph. The JHEs exhibited K_Ms of around 100 nM in the Lepidoptera (Wing *et al.*, 1984), 1570 nM and 670 nM for two JHEs in the blattodean *L. maderae* (Gunawan and Engelmann, 1984), 500 nM in the coleopteran *Leptinotarsa decemlineata* (Kramer and de Kort, 1976), and 47-81 nM in the orthopteran *G. rubens* (Zera *et al.*, 1992). The use of unpurified enzyme generally prevents complete kinetic analysis because interference from carriers or other modulators of JHE activity may occur and quantification of the amount of enzyme in such mixtures is often difficult. An exception is the study of Abdel-Aal and Hammock (1988) in which OTFP was used as a stoichiometric inhibitor of JHE in whole *T. ni* haemolymph to determine the enzyme molar equivalency. Thus, effects of JH binding by both the carrier and JHE were included in the kinetic analysis, and a K_{cat} of 0.53 sec^{-1} and a K_M of 70.6 nM for the hydrolysis of JHII were determined.

The only dipteran JHE subjected to kinetic analysis is from cytosol of *D. hydei* late larval integument (Bisser and Emmerich, 1981). The apparent K_M for JHI is 640 nM and competition data suggest a similar affinity for JHIII and, surprisingly, a greater affinity for the ethyl ester of JHIII. However, it is not known whether any competing JH-binding proteins were present in the cytosol preparation. Affinity for MF appears to be about ten-fold lower than for JHIII and about 100 fold lower for the JH-analogue, methoprene.

Kinetic analysis reveals that most purified JHEs from Lepidoptera have very low K_Ms for JH (21-360 nM) and quite low turnover numbers (K_{cat}, 0.49-4.33 sec^{-1}) (Rudnicka and Kochman, 1984; Abdel-Aal and Hammock, 1985; Hanzlik and Hammock, 1987; Rudnicka and Jones, 1987; Wozniak *et al.*, 1987; Abdel-Aal *et al.*, 1988; Valaitis, 1991, 1992; Ward *et al.*, 1992). One preparation of *T. ni* JHE had a higher K_M (1130 nM, JHI; 402 nM, JHIII) and lower K_{cat} (0.046 sec^{-1}, Yuhas *et al.*, 1983) but was nine-fold less enriched than other *T. ni* JHE preparations. Maximum *in vivo* JH concentrations in insects are generally very low (1-100 ng/ml or 3.6-360 nM, Schooley *et al.*, 1984) and so the K_Ms of the lepidopteran JHEs are similar to the maximum JH titres. The specificity constants (K_{cat}/K_M) of these enzymes

(1.4-58×10^6 $M^{-1}sec^{-1}$) determine the rate of hydrolysis when the substrate concentration is much lower than the K_M (Abdel-Aal and Hammock, 1988), and approach the diffusion limited maximum value (10^8-10^9 $M^{-1}sec^{-1}$, Stryer, 1981). These data indicate that lepidopteran JHEs are close to optimally adapted for efficient hydrolysis of JH from low concentrations, which is consistent with their proposed role as scavengers of trace amounts of JH at times in development when very low JH titres are required (Roe and Venkatesh, 1990).

1.7.4 Intraspecific and Interspecific Differences Among JHEs

The haemolymphs of several lepidopterans and two orthopterans contain multiple, closely similar forms of JHE which can be separated by high resolution IEF. However, the nature of the isozyme variation is not always clear and the function of intraspecific isozyme diversity, if any, is unknown (Wing *et al.*, 1984; Zera *et al.*, 1992).

It has been hypothesised that variants of *T. ni* JHE reflect differences in glycosylation or may be a consequence of aggregation as kinetic differences are small (Jones *et al.*, 1986; Hanzlik and Hammock, 1987). However, glycosylation differences probably do not account for the electrophoretic variation as the glycosylation of each variant is heterogeneous. These variants do not represent precursors and mature forms of JHE because they are coordinately induced by JH in the fat body, suggesting that they either derive from a common, hypothetical and undetectable precursor or from different transcripts (Wozniak and Jones, 1990). In *H. virescens* two variants differ by an N-terminal extension of two amino acids on one variant (Hanzlik *et al.*, 1989) and slight differences in amino acid composition also occur between JHE variants in both *T. ni* (Wozniak *et al.*, 1987) and *L. dispar* (Valaitis, 1991). The *T. ni* JHEs are immunologically indistinguishable and the two variants in *L. dispar* have conservative amino acid substitutions in homologous peptides, suggesting that they are allelic variants. Purified *M. sexta* JHE which is homogeneous by electrophoretic (Abdel-Aal and Hammock, 1985) and N-terminal sequence (Dr. T. N. Hanzlik, pers. comm.) criteria has two apparent K_Ms and a point of inflection on inhibitor curves which indicates two equally abundant active sites. The authors suggest that *M. sexta* JHE has two active sites but it is also possible that the preparation contained equal amounts of two similar JHEs. No known esterase has more than one active site per subunit (Cygler *et al.*, 1993), although this feature has been postulated for AChE (Small, 1990).

Kinetic analysis reveals two forms of the JHE active site in diluted *H. virescens* haemolymph (K_Ms = 16 nM and 250 nM, Wozniak and Jones, 1987). There are also two electrophoretic variants but only the lower K_M can be detected after elution of either electrophoretic variant from IEF gels. Thus, it is unclear whether these results indicate separation of JHE from a modifier molecule or loss of activity by a higher K_M variant during electrophoresis. In *G. mellonella* two JHE variants copurify but can be separated by IEF. At least one difference between these variants has an influence on the active site because they differ in their ability to hydrolyse naphthylacetate (Rudnicka and Kochman, 1984).

In spite of attempts to identify functional differences between intraspecific variants, little information is available. The same haemolymph variants appear throughout development in *M. sexta* and *T. ni* (Hanzlik and Hammock, 1988; Jesudason *et al.*, 1990). In the cricket *G. rubens* the presence of a second haemolymph

JHE variant correlated with wing morph, suggesting a possible functional difference between the two variants (Zera *et al.*, 1992). Non-haemolymph JHE variants in some cases exhibit the same distribution of isoelectric points as the haemolymph variants (Wozniak and Jones, 1990) but in other cases they are different (Hanzlik and Hammock, 1987; Sparks *et al.*, 1989).

In the Diptera little data are available on intraspecific variation. However, soluble and membrane associated JHEs in *M. domestica* exhibit different stability and inhibitor sensitivities, which may reflect a functional difference between two variants (Sparks and Hammock, 1980).

The lepidopteran JHEs also exhibit substantial interspecific differences. JHEs from two *Heliothis* species are antigenically distinct, reflecting considerable differences in substrate selectivity, inhibitor sensitivity and response to organic co-solvents (Section 1.6.3, Croston *et al.*, 1987; Abdel-Aal *et al.*, 1988). However, within the superfamily Noctuoidea, some, but not all, JHEs exhibit immunological cross-reactivity with *T. ni* JHE (Wozniak and Jones, 1987; Valaitis, 1991). Immunological relationships outside the Noctuoidea were investigated using antisera raised against JHEs of *Heliothis virescens* (Noctuioidae), *M. sexta* (Sphingoidae) and *T. ni* (Noctuioidae) to probe western blots of JHEs from these three species, *Heliothis zea* (Noctuioidae) and *Bombyx mori* (Bombycoidea) (Hanzlik, 1988). *H. virescens* antibodies detected all five JHEs, *M. sexta* antibodies recognised both *B. mori* and some of the Noctuoidea JHEs, but *T. ni* antibodies only recognised the JHEs from the same superfamily. These complex immunological relationships suggest that while some epitopes are conserved between all the lepidopteran JHEs, considerable interspecific differences occur. The apparently conflicting data might be resolved in part by considering the epitopes recognised by particular antisera. For example, antibodies generated against *L. dispar* JHE from haemolymph recognise JHEs from other species, yet fail to recognise *L. dispar* JHE from the fat body which lacks carbohydrate, suggesting that this antiserum mainly recognises the carbohydrate moiety (Valaitis, 1992).

Amino acid sequence data also suggest considerable difference among the lepidopteran JHEs. Sequences of peptide fragments of *L. dispar* JHE totalling more than 300 residues (over 50% of the whole protein) were difficult to align with the *H. virescens* complete JHE sequence (Valaitis, 1991) and there is little similarity between the three published N-terminal sequences of lepidopteran JHEs (*H. virescens*, complete cDNA, Hanzlik *et al.*, 1989; *L. dispar*, 69 residues, Valiatis, 1992; *M. sexta*, 15 residues, Venkatesh *et al.*, 1990).

Several lines of evidence suggest that dipteran JHEs will differ substantially from the lepidopteran JHEs. Firstly, the lepidopteran JHEs show considerable interspecific differences and are expected to show even greater divergence from JHEs of other insect orders. Secondly, the inhibitor sensitivities of lepidopteran and dipteran JHEs differ (Section 1.7.1). Thirdly, the cDNA of *H. virescens* JHE (Hanzlik *et al.*, 1989) does not hybridise with *D. melanogaster* genomic DNA (Ms. S. J. Dent, pers. comm.), and fourthly, *H. virescens* JHE antibodies do not cross-react with *D. melanogaster* proteins (Ms. M. M. Dumancic, pers. comm.).

1.8 Characterisation of the JHE Gene

The JHE gene has been isolated and characterised from one lepidopteran, *H. virescens*. Its inferred amino acid sequence shows about 24% identity with several esterases including AChE (Hanzlik *et al.*, 1989). High levels of conservation occur around particular features that identify JHE as a member of a multigene family which includes other carboxylesterases, cholinesterases and lipases. X-ray structures have been determined for two members of the family and this has enabled functional roles of conserved sequences to be inferred (Cygler *et al.*, 1993). Generally conserved features include the residues and position of the catalytic triad in the primary sequence, hydrophobic core-forming residues, residues involved in salt bridges, cysteine residues forming disulphide bridges, and residues at the edges of secondary structural elements. In *H. virescens* JHE, the catalytic triad suggested by sequence comparison (Ser_{201}, Glu_{332}, and His_{446}) is supported by *in vitro* mutagenesis experiments (Ward *et al.*, 1992). *H. virescens* JHE lacks one of the generally conserved cysteine pairs but its location in a surface loop near the active site suggests that its role might be substrate binding rather than maintainance of structure.

The catalytic triad mechanism used for hydrolysis by the esterases/lipases is found in a number of enzyme families. It is proposed that the esterase/lipase family is part of a larger group of hydrolases which is characterised by a common structural feature, the "α/ß hydrolase fold" (Ollis *et al.*, 1992). This structure determines a consistent spacial arrangement of the catalytic triad, and is proposed to have been conserved among enzymes no longer exhibiting detectable sequence similarity and having diverse substrates. The serine proteases use the same catalytic mechanism as the esterase/lipase family although the order of the triad residues in the primary sequence and other structural features are different. Therefore, the use of a catalytic triad by the serine proteases as well as two other classes of hydrolases is believed to be an example of convergent evolution (Ollis *et al.*, 1992).

Among the cloned esterases only AChE and JHE have well defined *in vivo* substrates and so these enzymes are of particular importance for the understanding of structure/function relationships in this multigene family.

1.9 Aims and Outline of This Study

The aim of this project was to characterise JH degradation in *D. melanogaster*. The physiological and biochemical properties of the JH degrading enzymes in Diptera are compared with those in other insect orders, with particular emphasis on JHE. Having defined the dipteran JH degrading enzymes a further aim was to assess the relative contributions of degradation to regulation of JH titre between Diptera and Lepidoptera.

No systematic study of JH hydrolysis had been performed previously in *D. melanogaster* although JHE and JHEH were known to occur in larvae, pupae and adults (Ajami and Riddiford, 1973; Wilson and Gilbert, 1978; Ottea *et al.*, 1987). In other Diptera, JHE is temporally regulated in *D. hydei* prepupae (Klages and Emmerich, 1979) and during vitellogenesis in adult female *A. aegypti* (Shapiro *et al.*, 1986) and has a low K_M for JHI in larval epidermis of *D. hydei* (Bisser and Emmerich, 1981). Together these data suggest that *D. melanogaster* JHE may have a role in the regulation of JH titre as has been established in other orders (Section 1.5). However,

little or no JHE is found in the haemolymph of Diptera, in contrast to most other insects (Section 1.6), and dipteran JHEs have an atypical pattern of inhibitor sensitivity (Section 1.7.1), suggesting that both the expression pattern and biochemical properties of the dipteran JHEs may be atypical.

Chapter two describes the identification and physiological characterisation of JH hydrolytic enzymes in *D. melanogaster*. Two approaches were taken to the identification of the JH hydrolysing enzymes, the first being to determine whether any of the electrophoretically detected esterases of *D. melanogaster* is JHE. In *D. virilis* the electrophoretically detected p-esterase was proposed to be JHE (Rauschenbach *et al.*, 1987) and preliminary work suggested that an homologous esterase may be present in *D. melanogaster* (Healy *et al.*, 1991). An esterase, EST20, visualised after native polyacrylamide gel electrophoresis (native PAGE) using histochemical techniques was found to be very similar to the p-esterase of *D. virilis*. However, it was also found to not exhibit JH hydrolysing activity. The second approach was to use a radiometric assay with JHIII as the substrate (Hammock and Roe, 1985). Two JH hydrolysing enzymes, JHE and JHEH were identified using this approach. Investigation of the temporal regulation of these enzymes indicated that the activities of both enzymes increase at times of known declines of JH titre, thereby satisfying one of the proposed physiological criteria for JH-specificity (Section 1.5.3.1). Further work then concentrated on characterisation of JHE from the time of its maximum expression.

Chapter three describes the purification and kinetic characterisation of JHE. An affinity purification procedure previously used for lepidopteran JHEs was applied to the purification of *D. melanogaster* JHE but did not yield active enzyme. A conventional chromatographic protocol for the purification of JHE was then developed and the protein purified by this procedure was used for N-terminal amino acid sequencing and kinetic studies. Kinetic experiments were used to assess the specificity of JHE for JH, JHE selectivity between JH isoforms, the features of the JH molecule that determine JHE specificity, and the nature of any interaction between JHE and the JH carrier, lipophorin. JHE was found to have a low K_M for JHIII and a high K_{cat}/K_M, confirming that it is capable of efficient hydrolysis of JH at low, physiological concentrations. It has high binding affinity for both JHIII and the natural stereoisomer of JHB_3, indicating that either or both of these JH isoforms could be the *in vivo* substrate for JHE. Experiments with a wide variety of JH analogues indicated that most features of the JH molecule contribute to recognition by the active site of JHE but the 10,11 epoxide in the natural R conformation is of particular importance. Lipophorin was found to reduce the rate of JH hydrolysis by JHE but the effect was due only to competition for binding of JH. Together these results indicate that JHE is specific for JH by each of the proposed biochemical criteria (Section 1.5.3.1).

In Chapter four the results from chapters two and three are combined with data from the literature to develop a model of JH hydrolysis in Diptera. This model is compared and contrasted with JH hydrolysis in Lepidoptera and other insect orders. JH specificity and an inverse relationship between the developmental profiles of the JH hydrolytic enzymes and the profiles of JH production and JH titre indicate an important role for JH hydrolysing enzymes in the regulation of JH titre. This proposal is further supported by similarities between the patterns of JH hydrolysis in the Diptera and Lepidoptera although there are some important differences. The possibility of differential regulation of the JH isoforms, JHIII and JHB_3, through an interplay of regulated hydrolysis, production and carrier binding, is discussed.

Chapter 2

Identification and Expression Pattern of JH Degrading Enzymes

2.1 Introduction

The work described in this chapter had two aims. The first was to identify the JH degrading enzymes of *D. melanogaster* and the second was to determine their importance for regulation of JH titre. The available data indicate that while JHE and JHEH both occur in Diptera, including *D. melanogaster*, their patterns of expression and physical properties differ from those seen in other insects (Sections 1.6.4 and 1.7.1). This chapter describes two approaches used to identify and characterise JH degrading enzymes in *D. melanogaster*.

The first approach was to investigate esterases of *D. melanogaster* fractionated by electrophoresis and visualised using the artificial esterase substrate, naphthylacetate. Electrophoretically detected esterases from the lepidopteran, *Hyalophora gloveri*, and the dipteran, *Drosophila buzzatii*, have been postulated to be JHE on the grounds of induction by JH, elution of JH hydrolytic activity from gel slices after electrophoresis, or expression at times of presumed reduction of JH titre (Whitmore *et al.*, 1972; East, 1982). Furthermore, the most extensive study of a dipteran enzyme believed to be involved with JH degradation is the electrophoretically detected "p-esterase" of *D. virilis* (Rauschenbach *et al.*, 1987, 1991, and references cited therein). Fractionation of esterases by native PAGE reveals similarities in the number, relative mobility and substrate specificity of esterases among *Drosophila* species and these properties enabled initial identification of homologues of *D. melanogaster* EST6 in other *Drosophila* species, including *D. virilis* (Oakeshott *et al.*, 1990). The substrate specificity and expression pattern of the p-esterase, together with its high mobility on native PAGE, are consistent with the properties of the "anodal" esterases described in five *Drosophila* species, including *D. melanogaster* (Berger and Canter, 1973; Rauschenbach *et al.*, 1977; Healy *et al.*, 1991). Thus, my initial hypothesis was that the JHE of *D. melanogaster* would be found among the anodal esterases of this species.

In the second approach, JH hydrolysis was measured directly using a radiometric assay which detects both JHE and JHEH. This approach has been used extensively to identify JH hydrolysing enzymes in many insect orders including the Diptera (Hammock, 1985; Hammock and Roe, 1985). Therefore, this technique was used to identify the JH hydrolysing enzymes in whole body homogenates of *D. melanogaster* both prior to, and after, electrophoretic and subcellular separations.

The second aim of this study was to determine the importance of JH degradation for regulation of JH titre. Hammock (1985) suggested two physiological criteria for determining the JH specificity of enzymes that hydrolyse JH *in vitro* (Section 1.5.3.1). One of these requires that increases of enzyme activity correlate with declines of JH titre and this criterion is fulfilled by JHEs from haemolymph of final instar lepidopterans (Section 1.6.1). Among the Diptera, these criteria have only been satisfied for JHE in adult female *A. aegypti*, although comparison of data between dipteran species suggests that increased JH hydrolysis may also occur during the final instar of dipterans at times

of reduced JH titre (Section 1.6.4). In this study the developmental profiles of JHE and JHEH from *D. melanogaster* were determined using a radiometric assay and compared with data from the literature for the developmental profiles of JH titre and production.

2.2 Materials and Methods

2.2.1 Drosophila Strains, Culture and Sample Preparation

2.2.1.1 Strains and Culture

The *D. melanogaster* strain 12I11.2 (Cooke *et al.*, 1987) which expresses the anodal esterases more reliably than other strains (Healy *et al.*, 1991 and unpublished data) was used, except where *Est6*^{null} *ry* (Oakeshott *et al.*, 1987) is indicated. *Est6*^{null} *ry* carries a null allele for *Est6* which produces no EST6 protein. The strain of *D. virilis* used was Texmelucan.

Flies were routinely raised in a 12 : 12 hour light : dark cycle at 25°C, 65% humidity, on agar-cornmeal-molasses medium. Cultures were kept at low density by allowing only a small number of eggs to be laid in bottles. Staged larvae were cultured from eggs laid within two hours. Pupae were staged by the flotation method of Mitchell and Mitchell (1964). Adults were staged by collecting those which emerged within a twelve hour period. Males and females were then aged separately.

The effect of culture density on esterase expression was determined after counting 10-200 eggs into vials containing 13 ml of medium. Prepupae/pupae were collected from the vials every twelve hours until all larvae had pupariated.

To determine the fate of esterases at eclosion, pupae that had been staged to within two hours were incubated until about half the adults had emerged. The remaining pharate adults, the discarded pupal cases and the newly emerged adults were collected and homogenised as described below.

2.2.1.2 Sample Preparation

Except where indicated, all samples were frozen in liquid nitrogen and stored at -70°C prior to homogenisation in 100 mM sodium phosphate buffer, pH 6.8 containing 0.5 mM phenylthiourea (PTU), using 10 µl per organism. Homogenates were centrifuged at 15,000*g* for 10 minutes and aliquots of the supernatant stored at -20°C.

Subcellular fractions of *D. melanogaster* were prepared by the method of Dr. V. K. Walker (pers. comm.) from duplicate, staged cultures. Fresh flies were homogenised at 4°C using a loose fitting teflon-glass homogeniser in 50 mM Tris-HCl, pH 7.5 containing 25 mM KCl, 5 mM Mg acetate, 350 mM sucrose, 0.5 mM PTU, and 1 mM dithiothrietol. The homogenate was centrifuged at 80*g* for 15 minutes to remove debris; 1,500*g* for 10 minutes to pellet nuclei; 17,000*g* for 20 minutes to pellet mitochondria; and 120,000*g* for 60 minutes to pellet microsomes. The nuclear and mitochondrial pellets were washed by resuspension in the homogenising buffer and recentrifuged at 1,500*g* and 17,000*g*, respectively. The microsomal pellet was rinsed with homogenising buffer. All pellets were resuspended in homogenising buffer and stored at -20°C prior to use. No attempt was made to solubilise the subcellular fractions other than the disruption that would occur by one freeze-thaw cycle.

2.2.2 JHE Assay, Electrophoretic and Protein Detection Procedures

2.2.2.1 Protein Determination

Protein concentrations were determined by the method of Bradford (1976) using the Biorad Protein Assay kit, except for native PAGE experiments for which the method of Markwell *et al.* (1978) was used. The latter reaction was performed in a total volume of 215 µl and the absorbance was measured at 670 nm. Bovine serum albumin (BSA) was used as a standard in both assays.

2.2.2.2 Native Polyacrylamide Gel Electrophoresis (PAGE)

The non-denaturing discontinuous gel system of Vernick *et al.* (1988) was used as described by Healy *et al.* (1991). Triton X-100 was omitted from the gels, except where indicated, because it has been reported to inhibit JHE activity in some insects (Hammock, 1985). Gels of 1 mm thickness were used to ensure rapid diffusion of substrates and inhibitors into the gel. Ester hydrolysis of naphthylacetate was detected using either 5.4 x 10^{-4} M α- and ß-naphthylacetate or 5.4 x 10^{-4} M ß-naphthylacetate alone. After visualisation of the esterases, gels were fixed in 40% methanol, 10% acetic acid, 3% glycerol solution and dried on paper under vacuum.

The sensitivity of esterases to the inhibitors diisopropylfluorophosphate (DFP; Sigma) and octylthiotrifluoropropanone (OTFP; a gift from the laboratory of Dr. B. D. Hammock) was assessed by including 10^{-4} M solutions of these inhibitors in the washing and staining buffers.

2.2.2.3 Kinetic Experiments Using Native PAGE

Kinetic experiments to test whether JH inhibits naphthylacetate hydrolysis by esterases of *D. melanogaster* were performed as described by Rauschenbach *et al.* (1987), with some modifications. Aliquots of pupal homogenates (100 µg protein) containing the anodal esterases were fractionated by electrophoresis, as above. Triplicates of excised gel lanes were added to 10 ml of a vigorously mixed staining solution containing JHIII (Sigma, at a final concentration of 0, 2.5, 5.0 or 7.5 x 10^{-4} M added in 0.5 ml ethanol) and ß-naphthylacetate (at 0.8, 1.3, 2.7, 5.4 or 8.1 x 10^{-4} M). Initial rates of reaction were determined by stopping one reaction in each triplicate set after 10, 20, or 30 minutes. The relative intensity of staining was determined by measuring the reflectance of individual esterase bands in dried gels at 520 nm using a Shimadzu CS-930 scanning densitometer. The data for zero JH allowed the calculation of the K_M of EST20 for ß-naphthylacetate. In order to determine the specificity of any inhibition of naphthylacetate hydrolysis by JH, olive oil (0-200 µl) was substituted for the JHIII.

A similar experiment was performed using a *D. virilis* pupal homogenate, except that JHI (Sigma) was used in place of JHIII. Two JHI concentrations (10^{-4} M and 5 x 10^{-4} M) and two amounts of olive oil (2 µl and 25 µl) were tested for inhibitition of ß-naphthylacetate (2.7 x 10^{-4} M) hydrolysis by the p-esterase.

2.2.2.4 Isolation of JHE Activity After Native PAGE

In order to determine whether JHE activity was associated with any of the naphthylacetate hydrolysing esterases, JHE activity was assayed directly from gel slices after native PAGE. Homogenates of 0-12 hour pupae (1 mg protein) were fractionated by native PAGE as described above. Each lane was sliced into at least 12 pieces and the

JHE extracted from each gel slice by homogenisation in 1 ml of 100 mM sodium phosphate buffer, pH 8.0. The resulting slurry was centrifuged at 10,000*g* for 10 minutes at 4°C and the supernatant stored at -20°C before being subject to the JHE partition assay described below.

2.2.2.5 Thin Layer Chromatography to Identify JH Hydrolytic Products

Thin layer chromatography (TLC) was used qualitatively to detect the products of JH metabolism generated by various life stages and subcellular fractions of *D. melanogaster* and *D. virilis*. The TLC system used was one of several described by Ottea *et al.* (1988). Enzyme samples (15-150 µg protein) were incubated at 30°C with [10-^{3}H]-JHIII (5 µM, 4200 Bq; NEN) in 100 mM sodium phosphate buffer, pH 8.0 at a final volume of 100 µl. JH and its products were extracted three times (>96% ^{3}H recovered) with ethylacetate and separated on plastic-backed silica-60 A254 TLC plates (Merck, 70 x 80 mm), using hexane/ethylacetate (7 : 4) as the solvent system. The developed plates were sprayed with scintillant (0.4% w/v 2,5-diphenyloxazole (NEN) in 1-metnaphthalene) and air dried for 30 minutes. The locations of ^{3}H-JH and its products were determined by exposure to X-ray film (Fuji XR100) for 20-40 hours (Shapiro *et al.*, 1986). The relative mobilities (Rfs) for JH-diol, JH-acid, JHIII and n-butaneboronic acid (BBA) derivatised JH-diol (see below) were 0.11, 0.22, 0.50, and 0.61 respectively, which are in close agreement with the values obtained by Ottea *et al.* (1988) for this system.

Derivatising agents (BBA and diazomethane) and modification of the solvent system were used to confirm the identity of the products of JH metabolism (Hammock and Roe, 1985; Ottea *et al.*, 1988). BBA reacts with vicinal diols and thereby increases the mobility of JH-diol and JH-acid-diol during TLC. Diazomethane reacts with carboxylic acids to form the corresponding methyl ester and thereby converts JH-acid and JH-acid-diol to JH and JH-diol, respectively. The predominant products of JH metabolism were found to be JH-acid and JH-diol, which permits the simultaneous determination of JHE and JHEH by the partition assay described below.

2.2.2.6 Partition Assay of JH Hydrolytic Activities

The JH hydrolytic activities due to JHE and JHEH were measured simultaneously and quantitatively by a simple modification of the commonly used radiometric partition assay of Hammock and Roe (1985). This assay was performed as described except that the buffer pH was increased to 8.0 and the volumes of the substrate solution added and samples taken were 2 and 100 µl, respectively. pH 8.0 is near optimal both for dipteran JHEHs which have very broad pH optima (Yu and Terriere, 1978b; Harshman *et al.*, 1991), and for *D. melanogaster* JHE (determined below). One half of the reaction was stopped in the usual manner. The other half was treated likewise except that 2 mM BBA (final conc.) was included. In this assay unreacted JH is extracted into an organic phase whereas all the JH-acid remains in the aqueous phase.

The percentage of JH-diol retained in the aqueous phase of the standard assay was determined in the following experiment. ^{3}H-JH-diol was generated using homogenates of 90 hour larvae. The reaction was partitioned or extracted in ethylacetate three times, as above, to recover the total JH-diol generated. JH-diol from both the organic phase of the partition and the entire reaction were separated from JH and other metabolites by TLC. JH-diol was quantified by liquid scintillation counting of the

appropriate regions of the TLC plate. 56±5% (SE, of 7 determinations) of the JH-diol was found in the aqueous phase, which is in good agreement with the previously determined value of 54.1±2% S.D. (Share and Roe, 1988). Following derivatisation by BBA, all the JH-diol was found in the organic phase. The half reaction with BBA thus contains only JH-acid in the aqueous phase, whereas the half reaction without BBA contains the JH-acid plus 56% of the JH-diol in the aqueous phase. JHE activity is calculated from the radioactivity in the aqueous phase with BBA and the JHEH activity is calculated from the difference between samples with and without BBA. JHE activity was linear until 64% of the JH was consumed, whereas JHEH activity was linear until 33% of the JH was consumed.

2.2.3 pH, Isoelectric and Inhibition Properties of JHE

2.2.3.1 pH Profile of JHE Activity

Homogenates of *D. melanogaster* pupae (mixed ages, 1 pupa per 10 μl) were prepared as described previously except that the homogenisation buffer was 10 mM sodium phosphate, pH 6.8. Aliquots of homogenate (20 μl), or water controls, were added to 180 μl of a 100 mM buffer (see below) at the experimental pH. Hydrolytic activity was determined using the partition assay (Section 2.2.2.6) except that no BBA was used. The ammonium hydroxide in the stop solution (Hammock and Roe, 1985) is sufficient to exceed the capacity of a 100 mM buffer. The experimental buffers were citric acid-phosphate (7 buffers, pH 4.0-7.0), Imidazole-HCl (4 buffers, pH 6.6-8.0), and Tris-HCl (4 buffers, pH 7.6-9.0).

2.2.3.2 Isoelectric Focusing

Isoelectric focusing (IEF) was performed in agarose in order to facilitate the recovery of JH hydrolysing enzymes from the gel. IEF gels containing 1% w/v agarose-IEF (Pharmacia), 12% w/v sorbitol and 6.3% v/v ampholyte solution (Pharmalyte pH 3-10 or pH 4-6.5, Pharmacia) were cast 1 mm thick on 65 x 145 mm plastic sheets (GelBond, Pharmacia). 12 hour pupae were homogenised in water (10 per 100 μl) with a few crystals of PTU. This homogenate (30 μl) or isoelectric point marker proteins (Broad pI or Low pI Calibration Kit, Pharmacia) were applied to the gel on filter paper wicks which were removed after about 45 minutes of focussing. Homogenates were focussed at 24°C using the Pharmacia Flat Bed Electrophoresis Apparatus FBE3000 for a total of 90 minutes at 8 watts constant power (approximately 1,500 volt-hours). After focussing, the lane containing marker proteins was removed and stained using Coomassie Brilliant Blue R-250. Lanes containing fractionated homogenate were tested for JH hydrolysing activity. When using broad range ampholytes the lanes were cut into 5 mm slices. When using narrow range ampholytes the lane was sliced lengthways: one part was incubated with α- and ß-naphthylacetate to detect esterase activity as described in section 2.2.2.2; the other was sliced into 5 mm slices or 2 mm slices in the region of the gel expected to contain JHE. JH hydrolysing activities were extracted from gel slices using a motor-driven pestle in 200 μl of 100 mM sodium phosphate buffer, pH 8.0. The resultant slurry was centrifuged and the supernatant assayed for JH hydrolysis using the partition assay (Section 2.2.2.6).

2.2.3.3 Effect of Inhibitors on JHE

Homogenates of 12 hour pupae (15 μg total protein) were incubated with solutions of the esterase inhibitors DFP and OTFP (10^{-4} and 10^{-5} M) in assay buffer for 10 minutes. The incubated samples and untreated controls were then assayed by the partition method (Section 2.2.2.6). DFP was stored for five days as a 100 μM solution in ethanol at 4°C prior to use. Fresh DFP from some sources has been reported to contain a potent but unstable inhibitor of lepidopteran JHEs. Storage in ethanol solution destroys the inhibitor without affecting the potency of the DFP against other esterases (Hanzlik and Hammock, 1987). The ethanol-stored DFP (1 mM) used in this study had no effect on the JHE activity in the haemolymph of the lepidopteran, *G. mellonella*, previously shown to be insensitive to DFP (Rudnicka and Kochman, 1984).

2.3 Results

2.3.1 Comparison of the Anodal Esterases of *Drosophila melanogaster* and *Drosophila virilis*

2.3.1.1 Pattern of Expression of the Anodal Esterases

Pupae of *D. melanogaster* and *D. virilis* exhibit very similar electrophoretic profiles of naphthylacetate hydrolysing esterases (Fig. 2.1), suggesting that homologous esterases of these two species could be readily identified. The p-esterase of *D. virilis* has been characterised as preferentially hydrolysing ß-naphthylacatate over α-naphthylacetate, having high mobility on native PAGE, responding to increased larval crowding with reduced expression in pupae and having predominantly pupal expression (Rauschenbach *et al.*, 1977). Of the three anodal esterases of *D. melanogaster* which preferentially hydrolyse ß-naphthylacetate (EST19, EST20 and EST21; Healy *et al.*, 1991), the Rf of EST20 (0.91) corresponds most closely to that of the p-esterase of *D. virilis* (0.91). EST19 and EST21 were not present in the samples used for this experiment but are shown in figures 2.2, 2.3 and 2.4.

Increasing the culture density consistently decreased the expression of EST20 in *D. melanogaster* (Fig. 2.1). In contrast, other esterases either responded inconsistently or were unaffected by culture density (compare Fig. 2.1, 2.2 and 2.3). Whereas low culture density was usually sufficient to obtain EST20 activity in pupae, it was often not sufficient to ensure expression of EST19 or EST21. Although three replicate experiments revealed some variation in the extent of crowding required to reduce EST20 expression, vials seeded with 30 to 100 eggs showed decreased levels of expression in all three experiments. EST20 thus resembles the p-esterase of *D. virilis* in respect of crowding effects.

The developmental profiles of EST19, EST20 and EST21 are shown in figure 2.2. Although these esterases show some expression in larvae and adults, they are predominantly expressed in pupae (this study and Healy et al., 1991), as is the p-esterase of *D. virilis* (Rauschenbach *et al.*, 1977).

At the end of pupal life some *D. melanogaster* esterases including EST20, EST21 and the α-naphthylacetate hydrolysing EST22 remain in the discarded pupal case, whereas others are found in the newly emerged adult. In contrast, in *D. virilis* the p-esterase is retained in newly emerged adults (Rauschenbach *et al.*, 1977), although two other esterases are found in the pupal case (Fig. 2.3).

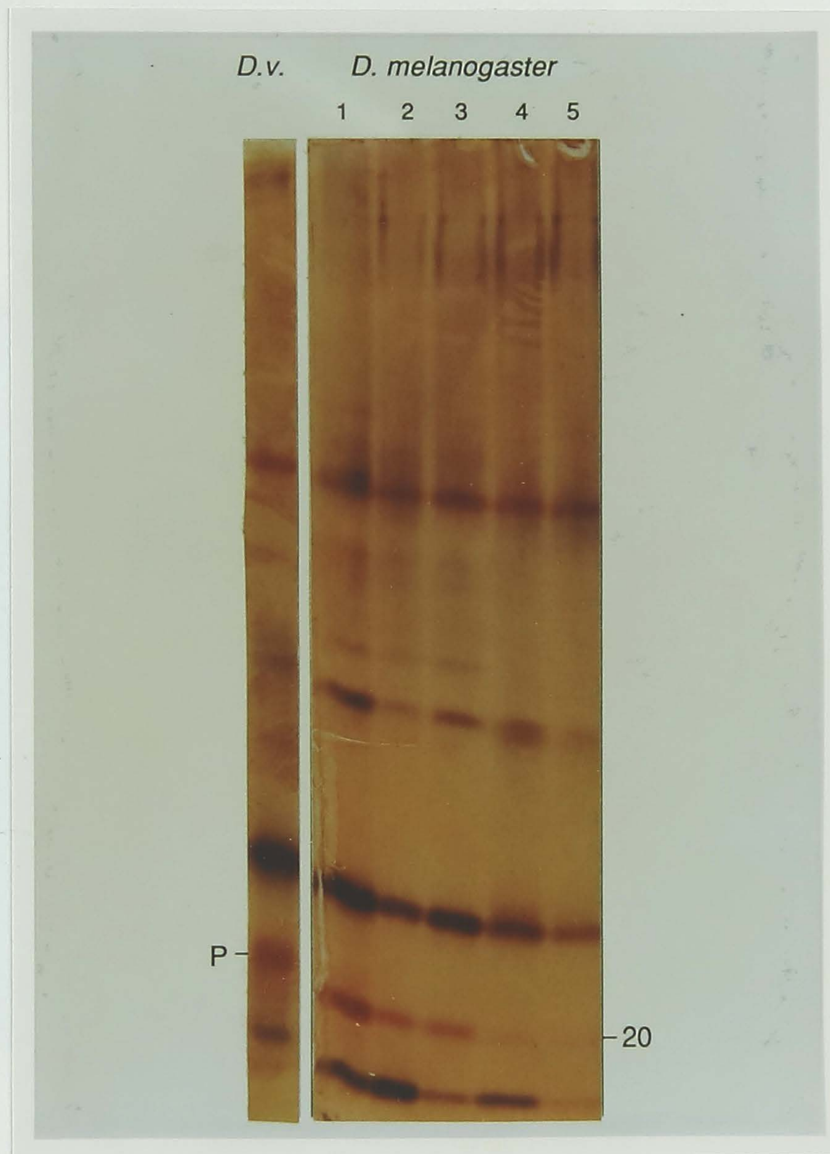


Figure 2.1 Comparison Between the Esterases of *D. virilis* and *D. melanogaster* Pupae Showing the Effect of Larval Culture Density on EST20 Expression.

Esterases were separated by native PAGE and detected using the *in vitro* substrates α- and ß-naphthylacetate. Brown and red bands indicate esterases which preferentially hydrolyse α- and ß-naphthylacetate, respectively. The left lane (D.v) shows the esterases of *D. virilis* pupae (200 μg protein) aligned with the esterases of *D. melanogaster*. The locations of the *D. virilis* p-esterase (P) and the *D. melanogaster* esterase 20 (20) are indicated. The remaining lanes show the effect of larval culture density on esterase activities in pupal homogenates (200 μg protein) of *D. melanogaster*. Organisms were cultured as described in section 2.2.1.1. For lanes 1 to 5, vials were seeded with 10, 30, 60, 100 or 200 eggs, respectively.

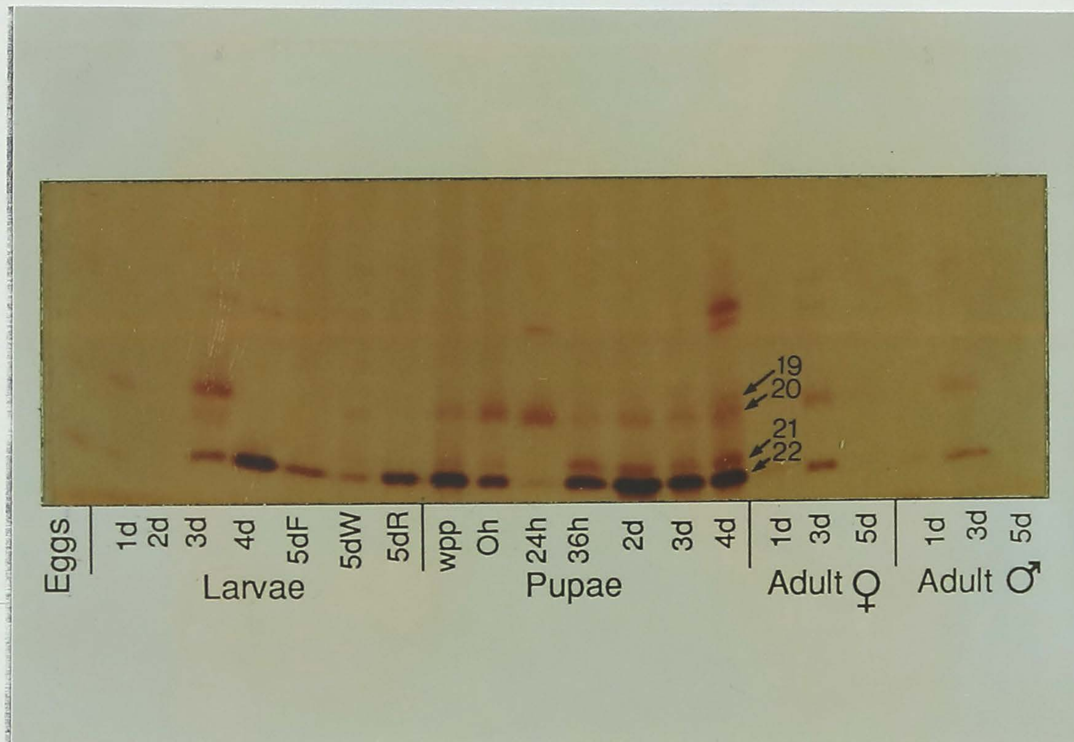


Figure 2.2 Developmental Profile of Anodal Esterases of *D. melanogaster*.

Homogenates (100μg total protein) of *D. melanogaster* raised at low culture density were separated by native PAGE. Ages are shown in hours (h) or days (d). Five day larvae were feeding (F), wandering (W), or resting (R). Esterase activity was detected using α- and ß-naphthylacetate. The anodal region only is shown (Rf 0.6-1.0). The white prepupal stage is indicated (wpp).

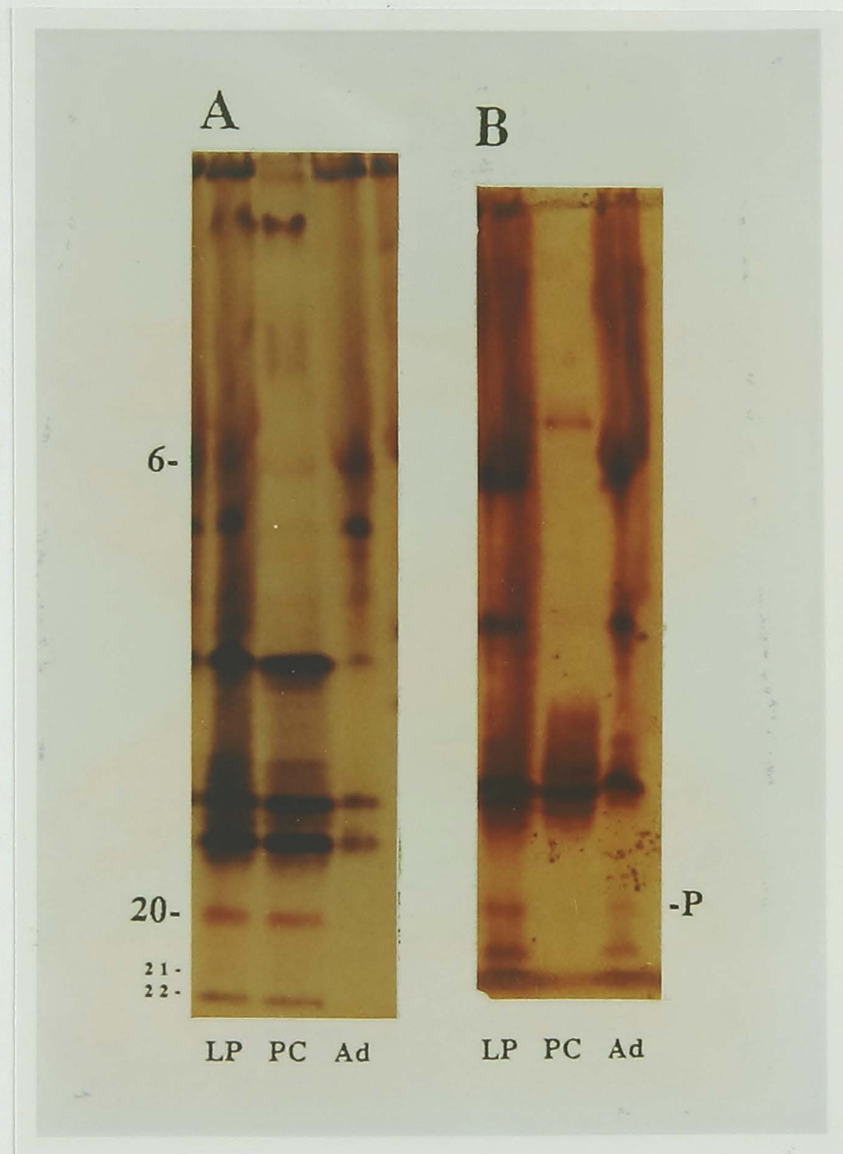


Figure 2.3 Comparison of Esterases Retained After Adult Ecdysis or discarded with the Pupal Case

Prepupae were staged to within two hours then incubated until about half the adults had emerged. The remaining pharate adults, the discarded pupal cases and the newly emerged adults were collected, homogenised, separated by native PAGE and stained for esterase activity as described in sections 2.2.1 and 2.2.2.2.

Panel A shows esterases separated from *D. melanogaster* and panel B shows esterases separated from *D. virilis*. LP indicates late pupae (pharate adults), PC indicates discarded pupal cases, and Ad indicates newly eclosed adults. Two organism/pupal case equivalents were loaded in each lane. Thus, the esterases present in LP represent the sum of esterases present in PC and Ad. The locations of EST6, EST20, EST21, EST22 and the p-esterase are indicated

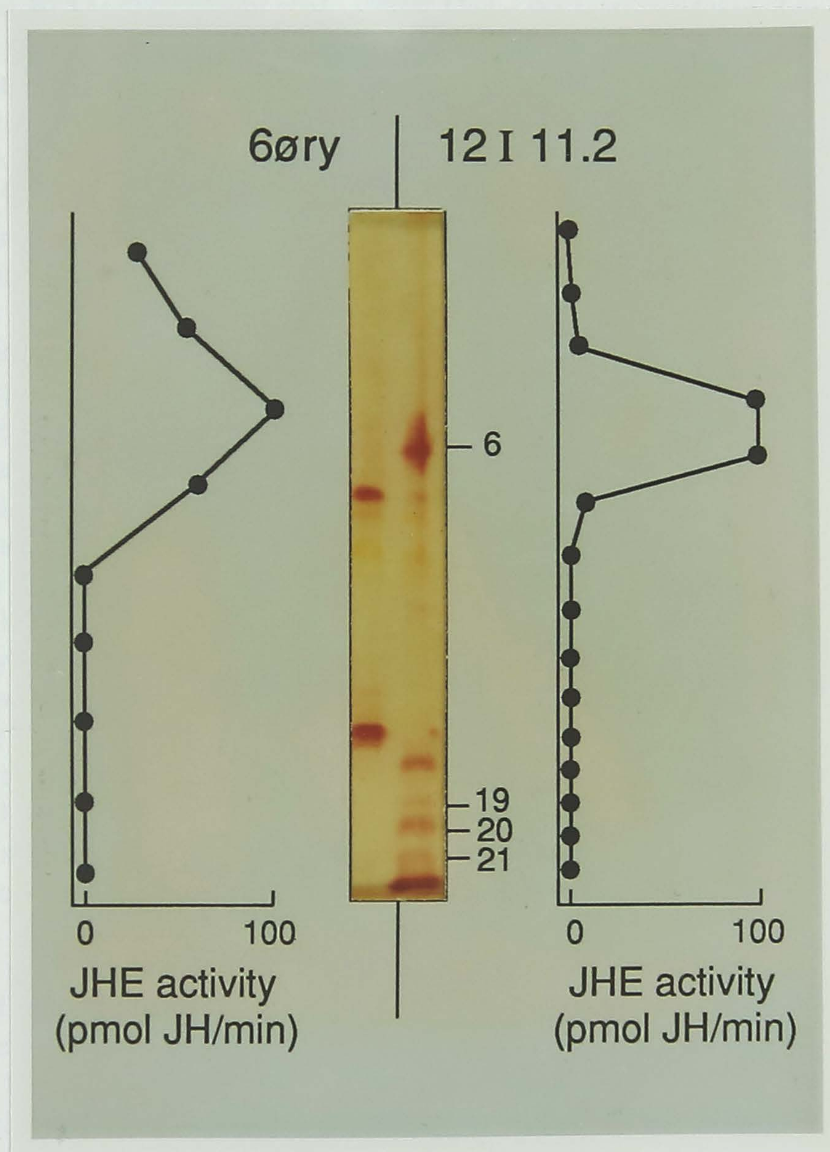


Figure 2.4 JHE Activity Isolated from Polyacrylamide Gel Slices.

Homogenates of *D. melanogaster* pupae (1 mg protein), strains *Est6*^{null} *ry* (6øry) and 12I11.2, were separated by native PAGE and the JHE activity extracted from gel slices was measured using the partition assay method. 100 µg samples of the same homogenates were separated by native PAGE and esterases were detected using ß-naphthylacetate.

2.3.1.2 Biochemical Properties of the Anodal Esterases

The *D. melanogaster* naphthylacetate hydrolysing esterases most sensitive to inhibition by 10^{-4} M OTFP are EST19, EST20 and EST21, although these enzymes are resistant to 10^{-4} M DFP (Healy *et al.*, 1991). Under the same conditions I find that the *D. virilis* p-esterase is resistant to DFP (as reported by Rauschenbach *et al.*, 1987) but is completely inhibited by OTFP (not tested by Rauschenbach *et al.*, 1987).

The K_M of *D. melanogaster* EST20 for ß-naphthylacetate hydrolysis is $2.9 \pm 0.6 \times 10^{-4}$ M (SE from 4 determinations), which is similar to the value of 1.0×10^{-4} M determined for the *D. virilis* p-esterase (Rauschenbach *et al.*, 1987). The intensity of staining of other esterases was unaffected by the change in naphthylacetate concentration in the conditions of this experiment, indicating that these esterases are operating at their maximum velocity at the concentrations of naphthylacetate used.

Rauschenbach *et al.* (1987) reported that naphthylacetate hydrolysis by the p-esterase of *D. virilis* is competitively inhibited by JH. However, I was unable to demonstrate competitive inhibition of ß-naphthylacetate hydrolysis by soluble amounts of JH using either *D. melanogaster* or *D. virilis* homogenates. Hydrolysis of naphthylacetate by EST20 and EST21 of *D. melanogaster* (as measured by staining intensity) was reduced by 15-55% in the presence of insoluble quantities of JHIII, although hydrolysis by other esterases was not affected. This effect occurred when the ß-naphthylacetate concentration was $1.3\text{-}5.4 \times 10^{-4}$ M and the concentration of JHIII, if soluble, would have been $2.5\text{-}7.5 \times 10^{-4}$ M.

In order to test the specificity of this reduction in hydrolysis, a substance expected to have similar physical properties to JH, namely olive oil, was substituted for JH. 25 µl olive oil reduced EST20 hydrolysis by 18%, and 100 µl olive oil reduced it by 50%, suggesting that the effect is not JH-specific. Hydrolysis by other esterases was reduced to a lesser extent and 5 µl or less olive oil had no effect on any of the esterases.

A similar experiment was performed with the p-esterase of *D. virilis* using JHI instead of JHIII because JHI had been claimed to cause competitive inhibition of the p-esterase (Rauschenbach *et al.*, 1987). Neither 10^{-4} M JHI nor 2 µl olive oil had any effect on hydrolysis by the p-esterase. However, both 5×10^{-4} M JHI and 25 µl olive oil markedly reduced hydrolysis by the p-esterase in comparison to the other esterases, suggesting that the effect of JHI on hydrolysis by the p-esterase is not specific.

2.3.2 Characterisation of JH Hydrolysis

2.3.2.1 Localisation of JHE After Native PAGE

In view of the non-specific inhibition of naphthylacetate hydrolysis by JH, proteins were extracted from gels after native PAGE of pupal homogenates and assayed for JHE directly using the partition assay. For *D. melanogaster* no activity was recovered from the region of the gels corresponding to the anodal esterases, but about 70% of the applied JHE activity was recovered (with or without 0.1%Triton X-100) at an Rf of 0.36 (Fig. 2.4). As the Rf of *D. melanogaster* JHE is close to that of the ß-naphthylacetate staining enzyme EST6 (0.34), an *Est6*^{null} strain was used to determine whether JHE and EST6 could be separated. JHE activity from the *Est6*^{null} strain was recovered at the same Rf as in the 12I11.2 strain and the level of JHE activity

was similar. Only a trace of naphthylacetate hydrolysis was detected in the region of the gel from which JHE was recovered (Fig. 2.4).

As described above, it is possible to alter the culture conditions of larvae in order to produce pupae either expressing or lacking the anodal esterases. I find that the level of JHE activity in homogenates of pupae of the same age is similar whether anodal esterases are present or absent, for both *D. melanogaster* and *D. virilis* .

2.3.2.2 Developmental Profile of JHIII-Hydrolysis

Two experiments were performed to identify the JHIII hydrolysing enzymes in *D. melanogaster* at different developmental stages. The first used TLC qualitatively to identify the enzymes responsible for JH hydrolysis at three developmental stages (Fig. 2.5). In ninety hour larvae, JH-diol was found together with a trace of JH-acid, indicating that JHEH is the predominant JH hydrolytic enzyme at this stage. In twelve hour pupae, JH-acid was found together with traces of JH-diol (and sometimes a trace of an unidentified metabolite which remained near the origin), indicating that JHE is the predominant JH hydrolytic enzyme at this stage. In five day adult females, similar amounts of JH-acid and JH-diol were found, indicating the presence of both JHE and JHEH. JH-acid-diol was not found at this stage, indicating that JH-diol is not a good substrate for JHE, and that JH-acid is not a good substrate for the JHEH, a conclusion also reached by Ottea *et al.* (1988). Also, in twelve hour pupae of *D. virilis*, JH-acid was found together with a trace of JH-diol, indicating that, as with *D. melanogaster*, JHE is the predominant JH hydrolytic enzyme at this stage.

A more complete quantitative developmental profile of JH hydrolytic activities in *D. melanogaster* was obtained using the partition assay. Two periods of high activity were revealed (Fig. 2.6). The first occurs in 70 to 92 hours old larvae and is predominantly due to JHEH (86% of total hydrolysis). Late in the final larval instar both JHE and JHEH activities fall, reaching a minimum in white prepupae (stage P1 of Bainbridge and Bownes, 1981) and zero hour pupae (stage P3-4(i)). The second and major peak of JH hydrolytic activity is due mainly to JHE (also 86% of total hydrolysis) and occurs in 6 to 12 hour pupae. This is when pupal apolysis (the pupal moult, stage P4(ii)) occurs. After this time hydrolytic activity declines, reaching a low level by the end of the pupal stage. Adults of both sexes exhibit low (albeit underestimated in these results, see below) levels of JH hydrolytic activity, with JHE activity being similar in both males and females and remaining constant. JHEH activity rises slightly in older adults, about twice as much in females as males, which may be correlated with different roles of JH in sex-specific reproductive events (Koeppe *et al.*, 1985).

A control experiment demonstrated that storage at -20°C for 5 days reduces JHE activity in homogenates of fresh male and female adult flies by 52% and 55% respectively ($n = 4$, $p < 0.01$). No further loss of adult JHE activity was observed after refreezing and a further 8 days of storage at -20°C. No significant losses ($n = 4$, $p > 0.05$) in the activities of JHE or JHEH were found for homogenates of 90 hour old larvae, 12 hour old pupae, or for JHEH in adult flies assayed before and after freezing.

2.3.2.3 Subcellular Localisation of JH Hydrolytic Activities

Nuclear, mitochondrial, microsomal and soluble fractions were prepared from 90 hour larvae, 12 hour pupae and 5 day female adults of *D. melanogaster* and the levels of JH hydrolytic activities in each were determined using the partition assay (Table 2.1).

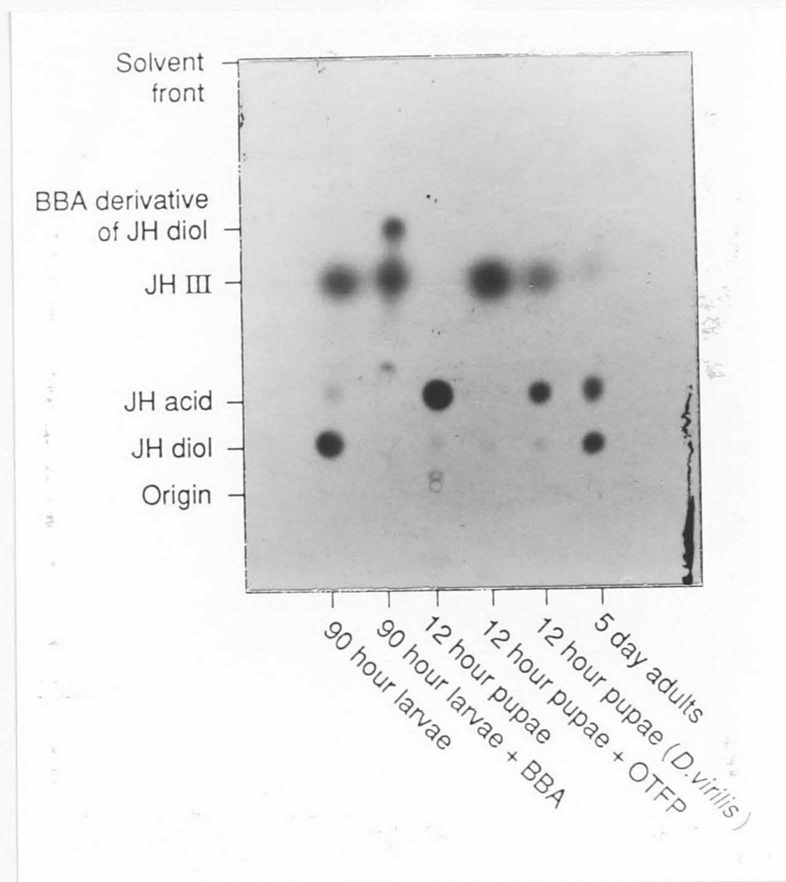


Figure 2.5 Thin Layer Chromatography of the Products of JH-Hydrolysis.

The products of JH-hydrolysis at defined developmental stages were visualised by TLC followed by fluorography, as described in section 2.2.2.5. OTFP (10^{-4}M), where indicated, was added to the reaction mixture prior to the start of the incubation. OTFP removes all JHE activity, eliminating JH-acid production. 1-butaneboronic acid (BBA), where indicated, was added to the reaction extract before it was applied to the TLC plate. The BBA derivative of JH-diol has an increased mobility. Results shown were obtained with *D. melanogaster* except where *D. virilis* is indicated.

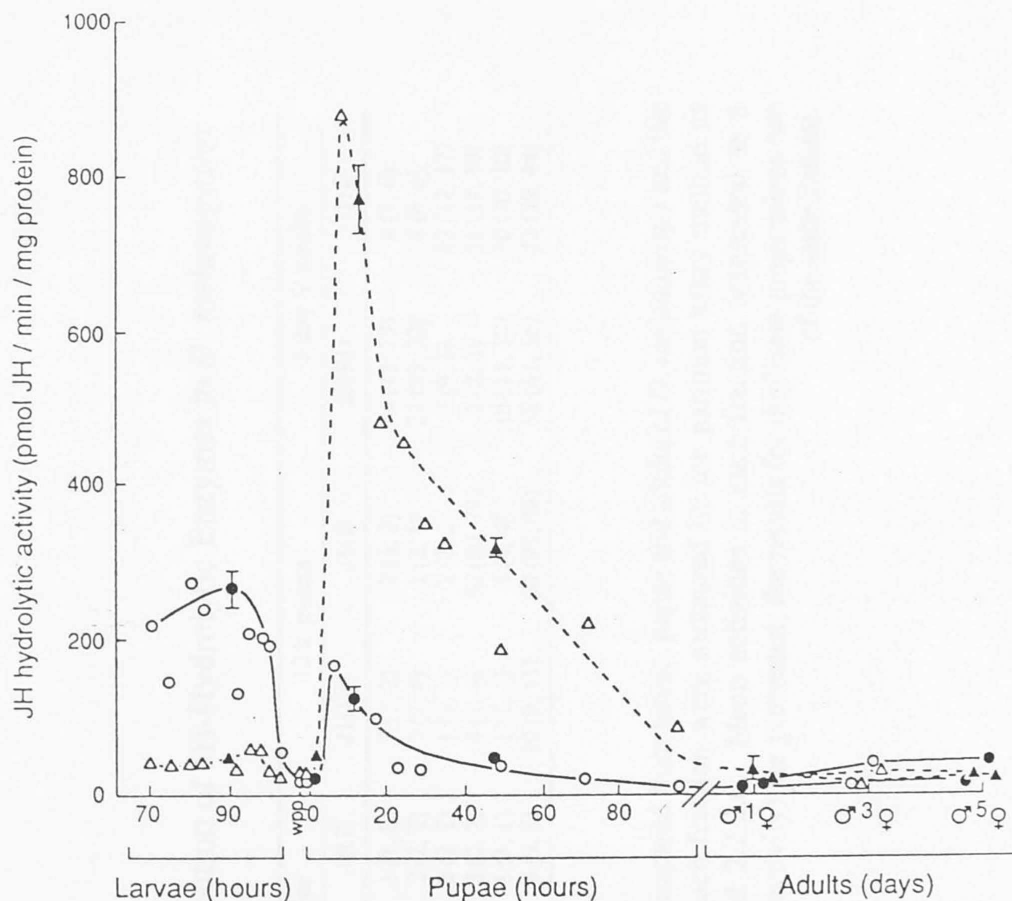


Figure 2.6 Developmental Profile of JHE and JHEH Activities in *D. melanogaster*.

Specific activities of JHE and JHEH were determined simultaneously for homogenates of *D. melanogaster* using the partition assay as described in section 2.2.2.6. Triangles indicate JHE activity and circles indicate JHEH activity. Solid symbols represent the mean result for four independent homogenates; standard errors are shown only where they exceed the size of the symbol. Open symbols represent single data points. The white prepuparium stage is indicated by "wpp".

Table 2.1 Subcellular Distribution of JH-Hydrolytic Enzymes in *D. melanogaster*

	90 h larvae		12 h pupae		5 day ♀ adults	
Fraction	JHEH	JHE	JHEH	JHE	JHEH	JHE
Nuclear	6 (6, 5)	1 (1, 0)	2 (2, 2)	2 (3, 2)	14 (18, 19)	4 (1, 6)
Mitochondrial	50 (47, 52)	2 (2, 2)	3 (2, 4)	1 (2, 1)	27 (32, 22)	6 (6, 6)
Microsomal	17 (18, 16)	2 (2, 2)	1 (1, 1)	2 (2, 2)	7 (9, 5)	12 (12, 12)
Soluble	5 (6, 4)	3 (3, 2)	4 (5, 4)	80 (80, 79)	1 (2, 0)	11 (12, 10)
Washes	14 (14, 14)	1 (1, 1)	1 (1, 1)	4 (4, 4)	10 (11, 10)	10 (10, 10)
Total	92 (91, 92)	8 (9, 8)	10 (9, 11)	90 (91, 89)	58 (61, 56)	42 (39, 44)

Subcellular fractions were prepared from larvae, pupae and adults of *D. melanogaster* and the activities of JHE and JHEH in each fraction were measured by the partition assay method as described in sections 2.2.1.2 and 2.2.2.6. Mean activities in each fraction (expressed as a percentage of total JH-hydrolytic activity) are presented; the results for duplicate preparations of the same culture are given in parentheses.

In larvae, 92% of the total JH hydrolytic activity was due to JHEH. JHEH in the mitochondrial and the microsomal fractions contributed 50% and 17%, respectively, to the total JH hydrolytic activity recovered at this life stage. In pupae, 80% of the total JH hydrolytic activity recovered was JHE from the soluble fraction. In adults, JHE and JHEH contributed more equally to total JH hydrolytic activity; the highest levels of JHEH were found in the mitochondrial fraction (27% of total hydrolysis) but JHE was distributed approximately equally between the microsomal and soluble fractions (12% and 11% of total hydrolysis, respectively).

2.3.2.4 Biochemical Properties of JHE

Varying the hydrogen ion concentration of the assay solution over a range from pH 4.0 to 9.0 revealed no buffer-specific effects on the activity of JHE from pupae of *D. melanogaster*. Below pH 6.0 considerable non-enzymic, acid hydrolysis of JH occurred. Over the pH range from 6.0 to 8.6, JHE activity almost doubled, increasing linearly with increasing pH. No further change in activity was observed at pH 9.0. This result is very similar to the profile of activity variation with pH found for *D. hydei* JHE (Bisser and Emmerich, 1981).

After IEF (pH 3-10), JH hydrolysis was observed between pH 5.2 and pH 5.85 for homogenates of 12 hour pupae of both *D. melanogaster* and *D. virilis*. Using narrow range ampholytes (pH 4-6.5), the pIs of the soluble JHE and JHEH from 12 hour pupae of *D. melanogaster* were found to be 5.41±0.04 and 6.25±0.13 (SE, 3 determinations), respectively (Fig. 2.7). The pI of JHE from *D. melanogaster* coincides with an α-naphthylacetate staining esterase not previously observed by native PAGE or IEF (Healy *et al.*, 1991) and similarly the pI of JHE of *D. virilis* coincides with an esterase which preferentially hydrolyses α-naphthylacetate over ß-naphthylacetate.

The sensitivity of JHE to the esterase inhibitors OTFP and DFP was determined for homogenates of 12 hour pupae of both *D. melanogaster* and *D. virilis*. Greater than 95% of the JHE activity in homogenates from both species was removed by 10^{-5} M OTFP. 10^{-4} M DFP removed all JHE activity from *D. melanogaster* homogenates and 10^{-5} M DFP removed 87±3% (SE, 3 determinations). 10^{-4} M DFP removed 94±2% JHE activity (SE, 4 determinations) from *D. virilis* homogenates and 10^{-5} M DFP removed 50±5% (SE, 4 determinations).

2.4 Discussion

2.4.1 Comparisons Among the Anodal Esterases and JHE

2.4.1.1 Comparison Between p-Esterase and EST20

This study provides several lines of evidence suggesting that the p-esterase of *D. virilis* is homologous to EST20 of *D. melanogaster*. The two esterases have similar relative mobility on native PAGE, substrate preference, kinetics with ß-naphthylacetate and sensitivity to the inhibitors DFP and OTFP. Both enzymes are predominantly expressed in pupae and respond to increased larval crowding with reduced pupal expression. The only property studied which differs between the two enzymes is their fate at eclosion, the p-esterase being retained in the adult whereas EST20 is discarded with the pupal case. The interpretation of homology between the p-esterase and EST20

is supported by studies in other esterase gene/enzyme systems. In particular, the homologues of *D. melanogaster* EST6 identified initially on the basis of shared electrophoretic properties and substrate preferences have now been confirmed by genetic and molecular biological studies (Oakeshott *et al.*, 1990).

Fewer data were collected for EST19 and EST21 of *D. melanogaster*, but they also share properties with the *D. virilis* p-esterase and anodal esterases in other *Drosophila* species. Shared properties include substrate preference, high mobility on native PAGE, predominantly pupal expression, retention in the pupal case at adult eclosion (EST21 only), resistance to DFP inhibition and a requirement for low crowding of larvae for expression (Berger and Canter, 1972; 1973; Osterbur and Steiner, 1979). The evolutionary relationship between the anodal esterases has not been studied but mapping of the encoding genes may resolve this question.

2.4.1.2 Association Between p-Esterase/EST20 and JHE

The claim that the *D. virilis* p-esterase is JHE was based largely on three observations: JHI appeared to competitively inhibit its hydrolysis of ß-naphthylacetate; JH hydrolytic activity was only observed in homogenates with p-esterase activity; and its activity followed the developmental profile for JHE (Rauschenbach *et al.*, 1987; 1991). However, as discussed below, my results on each of these points are not consistent with either EST20 of *D. melanogaster* or the p-esterase of *D. virilis* being JHE.

Considering first the issue of inhibition by JH, this study did not reveal competitive inhibition by JH of any of the naphthylacetate hydrolysing enzymes of either *Drosophila* species. Large quantities of JH do reduce naphthylacetate hydrolysis by EST20 of *D. melanogaster* and the p-esterase of *D. virilis*. However, this effect is neither specific, since it can be duplicated using olive oil, nor competitive, since it only occurs with greater than soluble amounts of JH. The concentrations of JHI reported by Rauschenbach *et al.* (1987) to cause competitive inhibition of the *D. virilis* p-esterase (0.8-3.8 x 10^{-4} M) are higher than the aqueous solubility of JH (3-5 x 10^{-5} M; Pratt, 1975; Kramer and de Kort, 1976). I suggest that the reduction in staining of some esterases in the presence of insoluble JH may occur because naphthylacetate is partitioned between the undissolved JH and the aqueous phase, thereby reducing its aqueous concentration. Staining would be reduced only for those esterases which are hydrolysing naphthylacetate at less than their maximum rates at the lower concentration. This hypothesis is supported by the observation that the K_Ms of EST20 and p-esterase (2.4 x 10^{-4} M and 1 x 10^{-4} M (Rauschenbach *et al.*, 1987), respectively) are higher than for other *Drosophila* esterases which preferentially hydrolyse ß-naphthylacetate; EST6 has a K_M of about 3 μM (Mane *et al.*, 1983) and the K_Ms of most other ß-esterases of *Drosophila* species are also in the micromolar range (White *et al.*, 1988). The K_Ms of EST20/p-esterase are therefore similar to the naphthylacetate concentration used for gel staining (5 x 10^{-4} M), so any reduction in naphthylacetate concentration would be expected to reduce the rates of hydrolysis by these enzymes, while only large reductions in naphthylacetate concentration would be expected to reduce the rates of hydrolysis by the other ß-naphthylacetate hydrolysing esterases. Thus, if JH and olive oil do dissolve some naphthylacetate, EST20 and p-esterase would be expected to exhibit the greatest reduction in staining intensity, as is observed.

My results also contrast with those of Rauschenbach *et al.* (1987) in respect of the reported congruence of JHE and the p-esterase/EST20 activities. Thus, I find that

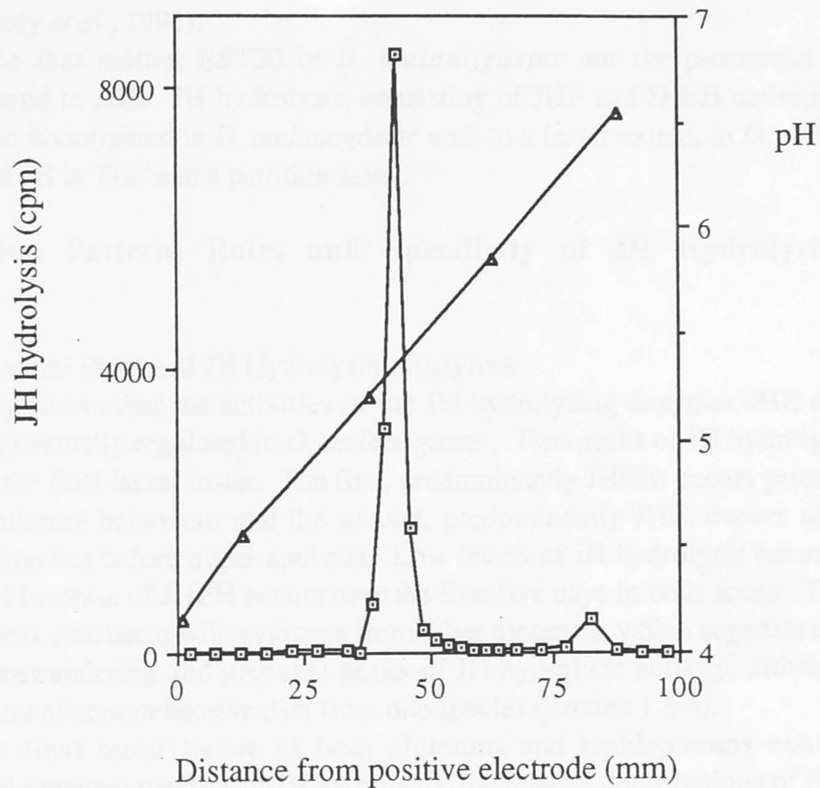


Figure 2.7 Isoelectric Focussing of JH Hydrolytic Enzymes

Homogenates of whole *D. melanogaster* pupae (three organism equivalents in 30μl) and isoelectric point marker proteins (Low pI Calibration Kit, Pharmacia) were subjected to isoelectric focussing using narrow range ampholytes as described in section 2.2.3.2. This figure shows a typical experiment in which JHE focussed at pH 5.4 and JHEH focussed at pH 6.3.

JHE activity is similar in homogenates of *D. virilis* pupae with and without p-esterase activity and in homogenates of *D. melanogaster* pupae in which EST20 is present or absent. In addition, JHE in *D. melanogaster* does not have the same developmental profile as any of the esterases previously observed by naphthylacetate staining after native PAGE (Healy *et al.*, 1991).

I conclude that neither EST20 of *D. melanogaster* nor the p-esterase of *D. virilis* correspond to JHE. JH hydrolysis, consisting of JHE and JHEH activities, has therefore been investigated in *D. melanogaster* and, to a lesser extent, in *D. virilis* using radiolabeled JH in TLC and a partition assay.

2.4.2 Expression Pattern, Roles and Specificity of JH Hydrolysing Enzymes

2.4.2.1 Developmental Pattern of JH Hydrolysing Enzymes

This study shows that the activities of the JH hydrolysing enzymes JHE and JHEH are developmentally regulated in *D. melanogaster*. Two peaks of JH hydrolytic activity occur in the final larval instar. The first, predominantly JHEH, occurs prior to the onset of wandering behaviour and the second, predominantly JHE, occurs after puparium formation but before pupal apolysis. Low levels of JH hydrolysis occur in adults but a small increase of JHEH occurs over the first five days in both sexes. This expression profile is consistent with evidence from other dipterans, which suggests that they also have prewandering and prepupal peaks of JH hydrolytic activity, although there is no other set of comprehensive data from one species (Section 1.6.4).

Although final instar larvae of both dipterans and lepidopterans exhibit prewandering and prepupal maxima of JH hydrolysis, the relative contributions of JHE and JHEH both within and between orders may be different. In *D. melanogaster* JH hydrolysis in prewandering larvae is predominantly due to JHEH associated with the membranous fractions of cells, and JH hydrolysis in prepupae is due predominantly to soluble JHE. However, in final instar larvae of *D. hydei*, JH-acid, the product of JHE, is a major metabolite of JHI in the fat body and body wall (29% and 39%, respectively). JH-acid-diol is also a significant product but JH-diol was not found (Klages and Emmerich, 1979). It remains to be determined whether the apparently higher proportion of JHE activity in *D. hydei* larvae compared with *D. melanogaster* indicates poor hydrolysis of the non-physiological JHI by JHEH, localisation of JHE to the two tissues tested, or a real difference between these two species. In Lepidoptera, both JHE in haemolymph and JHE and JHEH in other tissues are important at both prewandering and prepupal stages, although evidence from three lepidopterans suggests a trend for a higher proportion of JHEH at the prewandering stage compared with the prepupal stage (Section 1.6.1). Therefore, the relative activities of the two hydrolytic enzymes at each stage seems variable, but a notable contrast is the prominence of haemolymph JHE in prewandering lepidopterans and its absence in prewandering dipterans.

Although the available evidence suggests that the temporal patterns of JH hydrolysis are similar among dipterans, the reported activities are very different between three *Drosophila* species. The highest JHE activities reported for *D. hydei* and *D. virilis* prepupae/pupae are less than in *D. melanogaster* by approximately 2-3 or 5 orders of magnitude, respectively (Klages and Emmerich, 1979; Rauschenbach *et al.*, 1991). The maximum activity of *D. hydei* JHE was certainly underestimated because a non-physiological isoform, JHI, was used 180-fold lower than its K_M concentration

(Bisser and Emmerich, 1981) whereas a saturating concentration of JHIII was used with *D. melanogaster* JHE. For *D. virilis*, comparison between the reported enzyme activities and the assay method suggests that a calculation error may have caused activities to be understated by a factor of at least 1000. In contrast, the results of this study indicate that JHE activities in *D. virilis* pupal homogenates are about a tenth of the activity found in *D. melanogaster*. Thus, it is possible that the levels of JH hydrolytic activities are similar between these three *Drosophila* species.

2.4.2.2 Role of JH Hydrolysis in Regulation of JH Titre

Comparison between the JHIII titres of *D. melanogaster* (Bownes and Rembold, 1987; Sliter *et al.*, 1987) and peak levels of JH hydrolysis suggests that the hydrolytic capacity is sufficient to entirely remove JH within several milliseconds. Even the minimum levels of hydrolysis represent a high capacity, although the *in vivo* titres of JHB_3 are not known and may exceed the JHIII titre (Richard *et al.*, 1989b). Two explanations for a high JH hydrolytic capacity are proposed. Firstly, virtually all JH may be bound to lipophorin in final instar *D. melanogaster* (Shemshedini and Wilson, 1988), suggesting that a very high capacity for hydrolysis may be required for mass action to favour JH hydrolysis over JH binding to lipophorin. Alternatively, a high rate of hydrolysis may be matched by a high rate of synthesis, as proposed for larvae of *T. ni*, to enable large and rapid changes of JH titre to occur in response to relatively small changes in the rates of synthesis or hydrolysis (Hanzlik and Hammock, 1988). However, the second proposal seems less likely because maximum *in vitro* rates of JH production by *D. melanogaster* ring glands are less than 0.05 pmol/hour/gland (Richard *et al.*, 1990), a much lower rate of synthesis than the hydrolytic capacity.

The parallels between the expression pattern of JH hydrolysing enzymes in *D. melanogaster*, other dipterans and the lepidopterans suggest that JH hydrolysis plays an important role in the regulation of JH titre, as proposed for other insects (Section 1.5). Furthermore, JHIII hydrolytic capacity through development is negatively correlated with the profile of JHIII titre (Bownes and Rembold, 1987; Sliter *et al.*, 1987; Fig. 2.8). Although there are no data on the developmental profile of JHB_3 titre, hydrolysis is also negatively correlated with the profiles of *in vitro* JHIII and JHB_3 production (Richard *et al.*, 1989a; Altaratz *et al.*, 1991; Dai and Gilbert, 1991; Fig. 2.8). The prewandering peak of JHIII hydrolytic activity in *D. melanogaster* coincides with a low JH titre. Conversely, the low levels of JH hydrolysis in post-wandering larvae and at the white prepuparium stage coincide with maximum JH production and an elevated JH titre. One (or several) hour(s) after the formation of the puparium the production of JH by the ring gland declines rapidly. Total JH hydrolysis changes about 26-fold from its minimum to its second and larger maximum value within 6 hours of the prepupae becoming bouyant (between 6 and 12 hours after the formation of the puparium). Thus, the greatest and most rapid change in hydrolysis of JH immediately preceeds the pupal moult (Bainbridge and Bownes, 1981) towards the end of a brief period (from immediately prior to the formation of the puparium up to about 12 to 15 hours later) identified as critical for the development of adult abdominal morphology, and during which *D. melanogaster* is most sensitive to topical application of JH or JH analogues (Bhaskaran, 1972; Postlethwait, 1974). JH hydrolytic activity remains high though declining and JH titre is low during most of the pupal stage. In adults an elevated JH titre and JH synthesis coincide with low hydrolytic activity.

Figure 2.8 Comparison of the Ecdysteroid Titre, JH Titre, JH Hydrolysis and JH Production During the Development of *D. melanogaster*

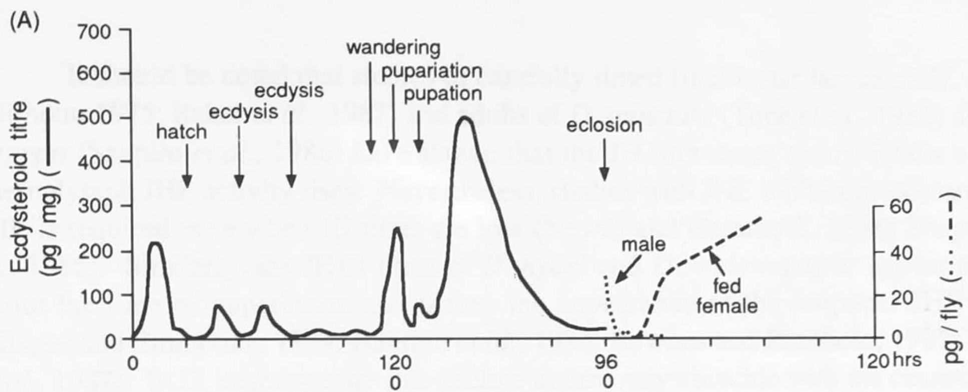
This figure combines the data presented in figure 1.2 (A, B and D) and figure 2.6 (C). Pupal age is indicated from pupariation although the larva/pupa transition occurs at pupation (pupal apolysis), twelve hours later (Section 1.4.1).

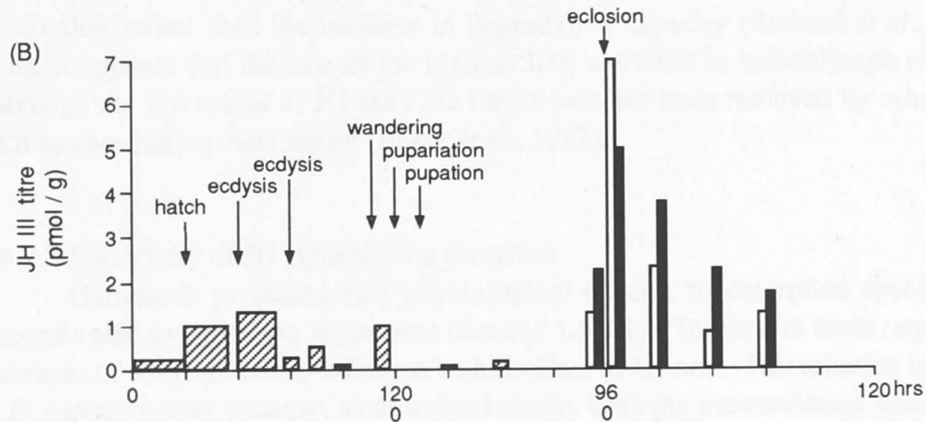
(A) shows the titre of ecdysteroids during development and is reproduced from Riddiford (in press). The data were compiled from ten sources cited therein.

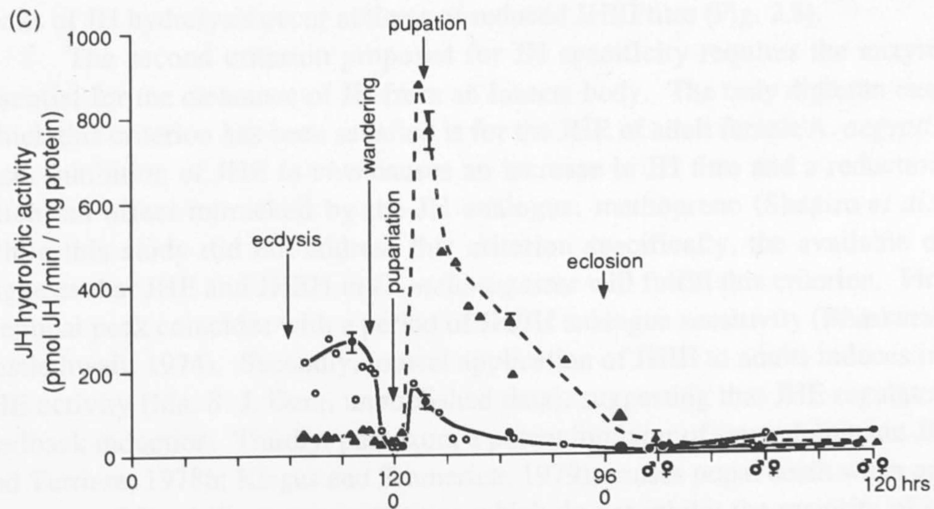
(B) shows the titre of JHIII during development and is reproduced from Riddiford (in press). Solid bars indicates male titres and open bars indicate female titres. The data were compiled from Bownes and Rembold (1987) and Sliter *et al.* (1987).

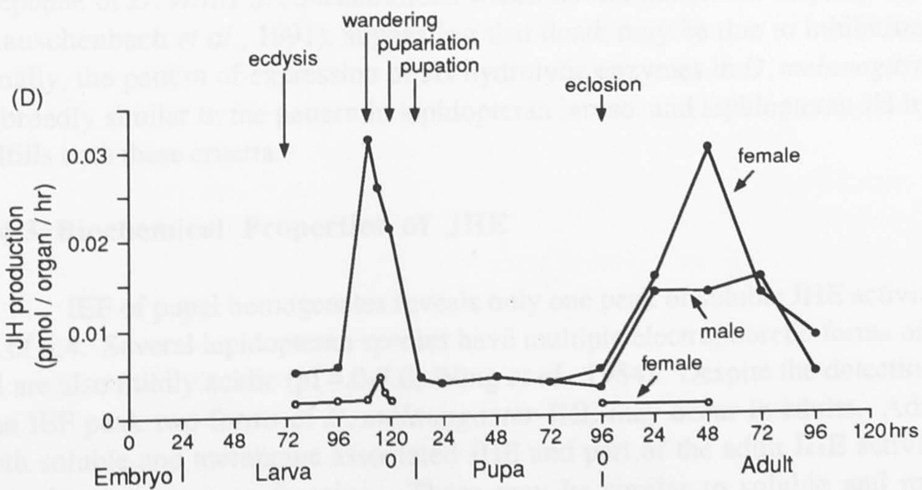
(C) shows the activities of JHE (triangles) and JHEH (circles) during development. The developmental time axis has been altered slightly from figure 2.6 to align with the other data.

(D) shows *in vitro* JH production by brain/ring gland/ventral ganglion complexes or adult corpora allata. Solid symbols indicate JHB_3 production and open symbols indicate JHIII production. The data were compiled from Richard *et al.* (1989a), Altaratz *et al.* (1991) and Dai and Gilbert (1991).


(A)
Ecdysteroid titre (pg / mg) (——)
pg / fly (········)
hatch
ecdysis
ecdysis
wandering
pupariation
pupation
eclosion
male
fed female
0 120 0 96 0 120 hrs


(B)
JH III titre (pmol / g)
hatch
ecdysis
ecdysis
wandering
pupariation
pupation
eclosion
0 120 0 96 0 120 hrs


(C)
JH hydrolytic activity (pmol JH / min / mg protein)
ecdysis
wandering
pupariation
pupation
eclosion
♂♀
0 120 0 96 0 120 hrs


(D)
JH production (pmol / organ / hr)
ecdysis
wandering
pupariation
pupation
eclosion
female
male
female
0 24 48 72 96 120 0 24 48 72 96 0 24 48 72 96 120 hrs
Embryo
Larva
Pupa
Adult

It should be noted that studies of carefully timed final instar larvae of *M. sexta* (Nijhout, 1975; Baker *et al.*, 1987) and adults of *D. punctata* (Tobe *et al.*, 1985) and *A. aegypti* (Shapiro *et al.*, 1986) have shown that the JH titre drops to low levels before haemolymph JHE activity rises. Nevertheless, studies with JHE inhibitors indicate that JHE is required even when JH titres are low (Newitt and Hammock, 1986; Shapiro *et al.*, 1986). Similarly, the JHIII titres of *D. hydei* and *D. melanogaster* appear to fall about the time of pupariation and before the appearance of the prepupal JHE peak (Klages and Emmerich, 1979; Bührlen *et al.*, 1984; Bownes and Rembold, 1987; Sliter *et al.*, 1987). In *D. melanogaster* this decline in titre may coincide with the cessation of production rather than the increase in degradative capacity (Richard *et al.*, 1989a). Thus, it appears that the role of the highest JHE activities in haemolymph may be to scavenge the last traces of JH after the larger part has been removed by other means such as constitutive metabolism (Baker *et al.*, 1987).

2.4.2.3 Specificity of JH Hydrolysing Enzymes

Hammock proposed two physiological criteria to determine specificity of enzymes able to hydrolyse JH *in vitro* (Section 1.5.3.1). The first of these requires that increases of enzyme activity correlate with declines of JH titre. This criterion is satisfied in *D. melanogaster* because, as described above, both the prewandering and prepupal peaks of JH hydrolysis occur at times of reduced JHIII titre (Fig. 2.8).

The second criterion proposed for JH specificity requires the enzyme to be essential for the clearance of JH from an insects body. The only dipteran example in which this criterion has been satisfied is for the JHE of adult female *A. aegypti*. In this case, inhibition of JHE *in vivo* causes an increase in JH titre and a reduction in egg hatch, an effect mimicked by the JH analogue, methoprene (Shapiro *et al.*, 1986). While this study did not address this criterion specifically, the available evidence suggests that JHE and JHEH in *D. melanogaster* will fulfill this criterion. Firstly, the prepupal peak coincides with a period of JH/JH analogue sensitivity (Bhaskaran, 1972; Postlethwait, 1974). Secondly, topical application of JHIII to adults induces increased JHE activity (Ms. S. J. Dent, unpublished data), suggesting that JHE regulates JH via feedback induction. Thirdly, paraoxon, a potent inhibitor of several dipteran JHEs (Yu and Terriere, 1978b; Klages and Emmerich, 1979), causes pupal death when applied to prepupae of *D. virilis* at concentrations which do not inhibit the majority of esterases (Rauschenbach *et al.*, 1991), suggesting that death may be due to inhibition of JHE. Finally, the pattern of expression of JH hydrolytic enzymes in *D. melanogaster* larvae is broadly similar to the pattern in lepidopteran larvae, and lepidopteran JH hydrolysis fulfills both these criteria.

2.4.3 Biochemical Properties of JHE

IEF of pupal homogenates reveals only one peak of soluble JHE activity, with a pI of 5.4. Several lepidopteran species have multiple electrophoretic forms of JHE but all are also mildly acidic (pI 4.0-7.0; Wing *et al.*, 1984). Despite the detection of only one IEF peak two forms of *D. melanogaster* JHE may occur in adults. Adults have both soluble and membrane associated JHE and part of the adult JHE activity is lost from homogenates on freezing. These may be similar to soluble and membrane

associated forms of JHE in the dipteran, *M. domestica*, which exhibit different stability and inhibitor sensitivities (Sparks and Hammock, 1980).

Previous inhibitor studies have suggested that JHEs in Diptera may differ structurally from those of other insect orders (Section 1.7.1). In this study, JHEs from *D. melanogaster* and *D. virilis* were sensitive to both of the inhibitors tested. One compound, OTFP, is an esterase transition state analogue that shows high specificity for the JHEs of Lepidoptera (Hammock *et al.*, 1984; Abdel-Aal and Hammock, 1986). OTFP inhibited the JHEs of both *Drosophila* species at 10^{-5} M, although several other esterases of *D. melanogaster* are also inhibited by this concentration (Healy *et al.*, 1991). The other compound, DFP is an organophosphate and both compounds act by binding to the active site serine of carboxylesterases. It has been suggested that resistance to inhibition by DFP is a universal characteristic of JHEs (Hammock, 1985). However, the JHEs of the dipterans in this study, as well as *S. bullata* and *P. regina* (Yu and Terriere, 1978a), but not *M. domestica* (Sparks and Hammock, 1980), are sensitive to inhibition by DFP. Since several of the soluble, naphthylacetate hydrolysing esterases of *Drosophila* are also resistant to DFP (Healy *et al.*, 1991), it would appear that DFP resistance is neither a unique nor universal characteristic of JHEs.

Chapter 3

Biochemical Characterisation of Purified JHE

3.1 Introduction.

My aim in this section of the work was to characterise *D. melanogaster* JHE biochemically to further assess its role in JH metabolism. To achieve this overall aim, four specific issues were addressed.

Firstly, a purification protocol for JHE from *D. melanogaster* was developed as the use of purified JHE would eliminate possible interference from other molecules during kinetic characterisation. JHE has been purified from seven lepidopteran species (Section 1.7.2) but only in *T. ni* has it been purified from starting material other than haemolymph. Moreover, it has not been purified from any species outside the Lepidoptera. Two approaches to the purification of *D. melanogaster* JHE were used in this study. The first was an affinity chromatography technique based on the binding of an esterase transition state analogue, an approach which has been used to purify JHE from five lepidopterans. Conventional chromatographic techniques were used in the second approach and JHEs from four lepidopterans have been purified using such techniques. Both approaches have yielded sufficient active lepidopteran JHE for further characterisation (Section 1.7.2).

Secondly, the purified esterase was characterised kinetically to test its JH-specificity against Hammock's (1985) biochemical criteria (Section 1.5.3.1). These criteria relate directly to the action of JHE *in vivo*, addressing the issues of whether JH is likely to be the predominant *in vivo* substrate and whether the binding affinity and catalytic efficiency of JHE are sufficiently high to remove JH at the low *in vivo* concentrations. Kinetic analysis of lepidopteran JHEs revealed that they exhibit low K_Ms and high specificity constants (K_{cat}/K_M) for JH (Section 1.7.3). These values indicate that the enzyme is well adapted to efficient hydrolysis of JH at low concentrations, possibly in competition with other high affinity JH binding proteins.

Thirdly, the features of the JH molecule which promote binding by JHE were determined. In particular, the unresolved issue of the relative importance of JHIII versus JHB_3 was addressed by binding studies intended to determine whether JHE exhibited specificity for one of these isoforms over the other. Recent studies have investigated JH synthesis, JH metabolism by JHEH, and some *in vivo* effects of these two JHs (Section 1.2). Taken together these studies indicate an important role for JHB_3. However they are equivocal with regard to the role of JHIII.

The final aim was to determine the mode of interaction between JHE and lipophorin, the haemolymph JH carrier. Two distinct models for this interaction (Section 1.5.2) were tested using kinetic experiments. The first is a mass action model which depends only on the relative abundance and kinetic properties of the carrier and degrading enzymes to determine the rate of JH degradation. The second model requires direct contact between JHE and the carrier and has only been demonstrated in one species in which the carrier is also a non-competitive inhibitor of JHE.

3.2 Materials and Methods

3.2.1 Chemicals

The JH isoforms and analogues not previously described in Chapter 2 or referenced otherwise were synthesised in the laboratory of Professor R. W. Rickards of The Australian National University (see Rickards and Thomas, 1992 for synthesis of four stereoisomers of JHB_3). OTFP was synthesised by Dr. C. P. Whittle (methods of Abdel-Aal and Hammock, 1986), *L. cuprina* lipophorin was purified by density gradient centrifugation by Dr. S. C. Trowell (Trowell, 1992) and *D. melanogaster* EST6 was purified by Ms. M. M. Dumancic (Division of Entomology, C.S.I.R.O.). Commercially produced compounds were S-methoprene, hydroprene (provided by Zoecon), JHI, JHII and JHIII (Sigma).

3.2.2 JHE Assay, Electrophoretic, and Protein Detection Procedures

3.2.2.1 JHE Activity Assay

The JHE partition assay described in section 2.2.2.6 was modified for the monitoring of JHE activity through its purification and for the kinetic studies of JHE. For the purification the assay volume was reduced to 100 µl, BBA was not used and 0.5 mg/ml BSA (Sigma) was included. The addition of BSA was necessary to retain enzyme activity when the enzyme preparation contained little other protein. For the kinetic experiments some further changes were made. The ^{3}H-JHIII concentration was varied, potential inhibitors were included in the assay, as described in section 3.2.4, and the assay tubes were pretreated with 2% polyethyleneglycol ("Carbowax" Compound 4000, Union Carbide) to reduce adsorption of JH (and its analogues) to the glass (Giese *et al.*, 1976). This technique has been shown to reduce the proportion of very dilute JHIII bound to glassware from 78% to 44%, although surface binding of JH probably remains the main source of error in assays performed with less-than-saturating JH concentrations (Lanzrein *et al.*, 1993).

3.2.2.2 Electrophoretic Procedures

Native PAGE

Staining for naphthylacetate hydrolysis and extraction of JHE activity from native PAGE were performed as described in sections 2.2.2.2 and 2.2.2.4. Electrophoresis was performed at a constant 5 W per gel (70 x 80 x 0.75 mm) with circulating iced water for cooling.

Denaturing PAGE

Denaturing (SDS) PAGE was performed by the method of Laemmli (1970) using 12% 40:1 acrylamide/bis-acrylamide. Electrophoresis was performed at room temperature with circulating water for cooling at a constant 150-200 V. Gels were 70 x 80 x 0.75 or 1 mm.

Two Dimensional PAGE

Samples were firstly fractionated by native PAGE. Replicate lanes of the native PAGE gel were stained for naphthylacetate hydrolysis, or total protein, or sliced and assayed for JHE activity. A gel piece corresponding to the region with JHE activity was

excised from an unstained lane, incubated with denaturing gel sample buffer (Laemmli, 1970) for approximately 15 minutes, then subjected to SDS-PAGE as the second dimension.

All polyacrylamide gels described in this chapter were air-dried between cellophane using a Promega Gel Drying Kit.

3.2.2.3 Protein Detection and Sequencing Procedures

After fractionation by PAGE, proteins were detected by the silver staining method of Merril *et al.* (1980) using the Biorad silver staining kit. The protein content of solutions was determined by the method of Bradford (1976) using the Biorad Protein Assay kit, with BSA as the standard.

N-terminal amino acid sequencing of purified JHE (20 μg of Fr6, Section 3.3.1) was performed by Edman degradation by the Biomolecular Resource Unit at the John Curtin School of Medical Reseach, Australian National University. The EMBL database (Release 27.0) was searched for sequences with similarity to the N-terminal amino acid sequence of JHE (Devereux *et al.*, 1984).

3.2.3 Purification of JHE

Two approaches were taken to the purification of JHE, an affinity chromatography procedure and a conventional, chromatographic approach. The initial steps (homogenisation, ammonium sulphate and acetone precipitations) were common to both approaches.

3.2.3.1 Initial Purification Steps

Preparation of Homogenates

The *D. melanogaster* strain 12I11.2 (Cooke *et al.*, 1987) was cultured in population cages under the conditions described in section 2.2.1.1 except that the food was regularly moistened to ensure that most larvae pupated on the walls of the cage. Wandering larvae and pupae were removed from the walls of the cage by washing with a strong jet of water. Pupae were aged by the flotation method of Mitchell and Mitchell (1964) to 12±6 hours (the time of maximum JHE activity, Section 2.3.2.2) and then stored at -70°C.

100 g of frozen pupae were homogenised on ice in a Sorvall blender with a few milligrams of PTU and 500 ml of a pH 4.5 buffer (10 mM KOH adjusted with acetic acid). The homogenate was clarified by centrifugation at 13,000*g*, 4°C for 20 minutes followed by filtration through glass wool. The pH of this solution was then raised by the addition of an equal volume of 100 mM sodium phosphate buffer, pH 7.0 containing 10% glycerol.

Ammonium Sulphate Precipitation

Solid ammonium sulphate was added slowly to the homogenate with stirring at 4°C until 30% saturation was reached. The solution was stirred gently for a further 60 minutes and then centrifuged for 20 minutes at 13000*g* at 4°C. The supernatant was brought to 60% saturation by slow addition of solid ammonium sulphate, stirred for a further 60 minutes and then the precipitate was pelleted as above. The supernatant was

discarded and the pellet was resuspended in 100 mM sodium phosphate buffer, pH 7.0 containing 10% glycerol to a final volume of 52 ml.

Acetone Precipitation

Acetone (0^oC) was slowly added to the resuspended ammonium sulphate pellet with vigorous swirling in an ice/water slurry until a final concentration of 40% (v/v) was reached. The resulting solution was centrifuged at 9000*g* for 15 minutes at 0^oC in 30 ml Corex tubes. The supernatant was dialysed overnight at 4^oC against 3 l of 10 mM imidazole/Cl buffer, pH 7.0 containing 10% glycerol. This material was concentrated to less than 50 ml using aquacide III (Calbiochem 17852).

3.2.3.2 Affinity Purification of JHE

Two versions of trifluoromethylketone affinity matrix were made (Fig. 3.1). Briefly, 1,4 butanedithiol was reacted with 3 bromo 1,1,1 trifluoropropanone, to produce 3-[(4'-mercapto)butylthio]-1,1,1-trifluoropropan-2-one, which was then coupled to either Thiopropyl Sepharose 6B (Pharmacia, Hanzlik, 1988) or Epoxy-activated Sepharose 6B (Pharmacia, Abdel-Aal and Hammock, 1986) following the manufacturers instructions. The former incorporates a disulphide bond into the linker between the sepharose bead and the trifluoromethylketone moiety. Reduction of the disulphide bond enables greater recovery of bound protein but recovery of enzyme activity is not expected by this method (Hanzlik, 1988).

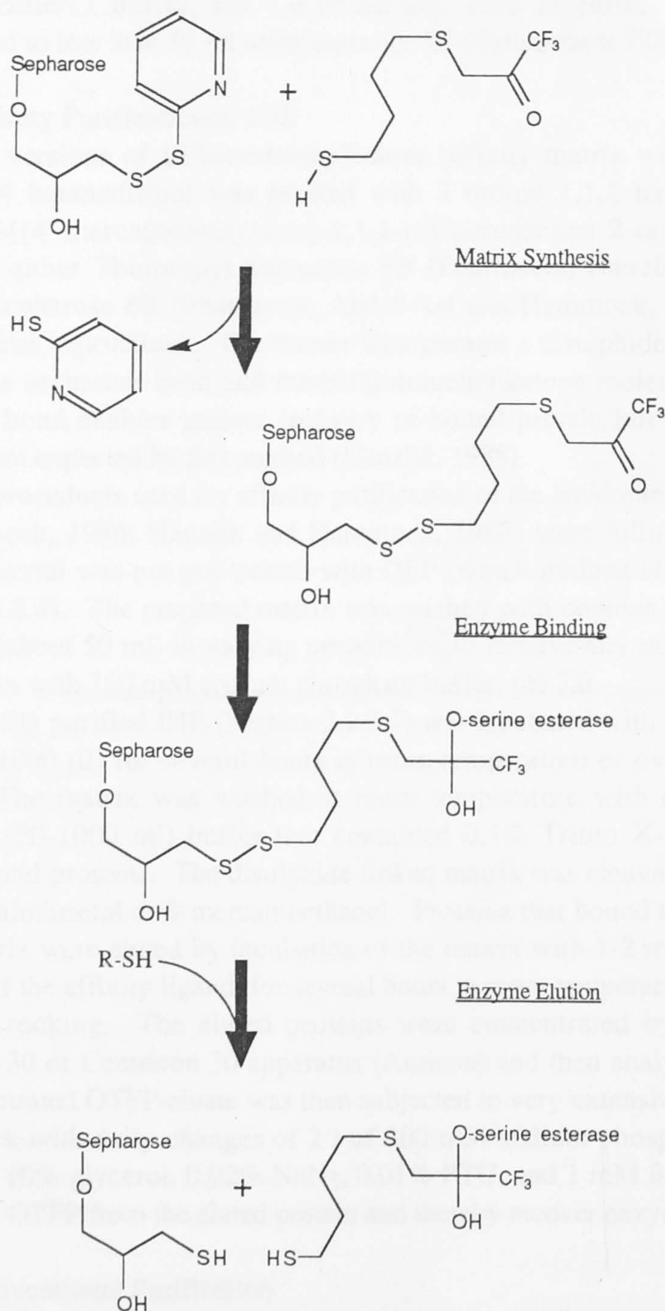
The procedures used for affinity purification of the lepidopteran JHEs (Abdel-Aal and Hammock, 1986; Hanzlik and Hammock, 1987) were followed, except that the starting material was not pre-treated with DFP (which inhibits *D. melanogaster* JHE, Section 2.3.2.4). The prepared matrix was washed with copious quantities of ethanol and water (about 50 ml) in varying proportions (3:1, 1:1, 1:3) to remove any unbound ligand before equilibration with 100 mM sodium phosphate buffer, pH 7.0.

Partially purified JHE (Section 3.2.3.1) was incubated with either affinity matrix type (100-1000 μl) for several hours at room temperature or overnight at 4^oC, with rocking. The matrix was washed at room temperature with copious volumes of phosphate (50-1000 ml) buffer that contained 0.1% Triton X-100, then treated to remove bound proteins. The disulphide linked matrix was cleaved by incubation with 20 mM dithiothrietol or ß-mercaptoethanol. Proteins that bound to the non-disulphide linked matrix were eluted by incubation of the matrix with 1-2 ml of 10^{-3} M OTFP, a free form of the affinity ligand, for several hours at room temperature, then overnight at 4^oC, with rocking. The eluted proteins were concentrated by ultrafiltration in a Centriprep 30 or Centricon 30 apparatus (Amicon) and then analysed by SDS-PAGE. The concentrated OTFP eluate was then subjected to very extensive dialysis at 4^oC (up to one week with daily changes of 2 l of 200 mM sodium phosphate buffer, pH 7.4, containing 10% glycerol, 0.02% NaN_3, 0.01% PTU, and 1 mM ß-mercaptoethanol) to remove the OTFP from the eluted protein and thereby recover enzyme activity.

3.2.3.3 Conventional Purification

Isoelectric Focussing

Isoelectric Focussing (IEF) was performed using a Biorad "Rotofor" apparatus. Three ml of ampholytes (Pharmalyte pH 5-6, Pharmacia) and 10% glycerol were added to partially purified JHE (Section 3.2.3.1), bringing the total volume to 50 ml, the volume of the Rotofor focussing chamber. In accordance with the manufacturer's



A
Sepharose
+
CF_3
Matrix Synthesis
Enzyme Binding
O-serine esterase
R-SH
Enzyme Elution

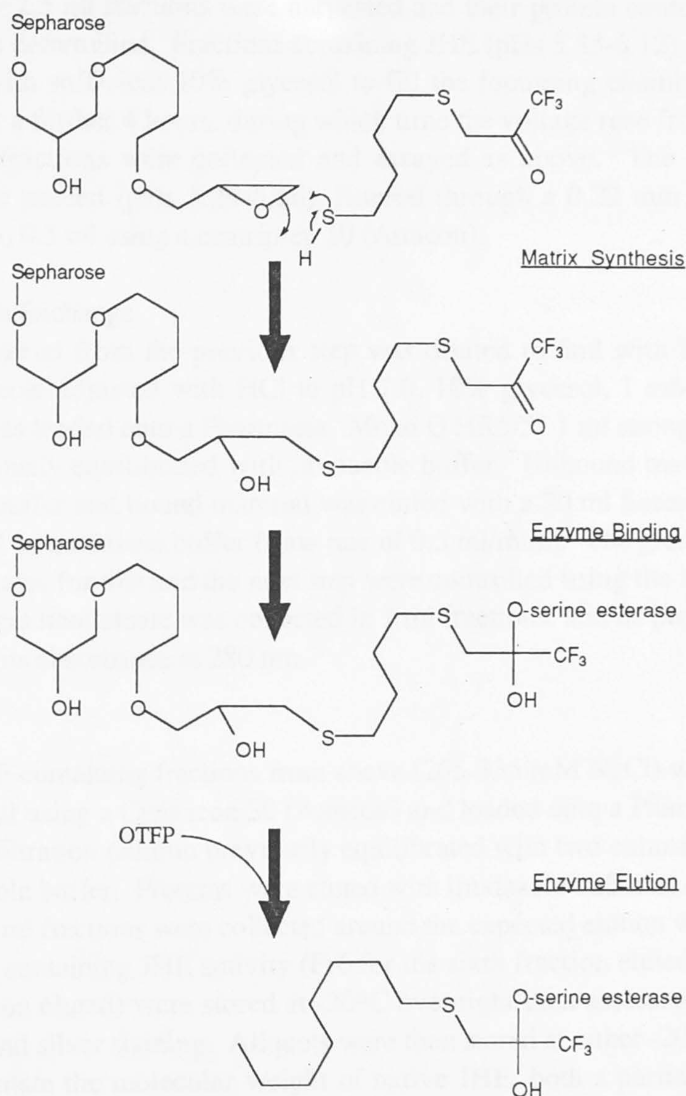


Figure 3.1 Synthesis and Use of Matrices for Trifluoromethylketone Affinity Chromatography

3-[(4'-mercapto)butylthio]-1,1,1-trifluoropropan-2-one was coupled to either Thiopropyl Sepharose 6B (A, Hanzlik, 1988) or Epoxy-activated Sepharose 6B (B, Abdel-Aal and Hammock, 1986). Esterases bind to the trifluoromethylketone group covalently through their active site serine. In (A), a disulphide bond is incorporated into the linker between the sepharose bead and the trifluoromethylketone group. Esterases are recovered by reduction of the disulphide bond. In (B), esterases are recovered from the non-disulphide linked version by slow exchange for OTFP which also contains the trifluoromethylketone moiety. Extensive dialysis is then required to remove OTFP from the active site and recover enzyme activity.

instructions an antifreeze solution at -8^oC was circulated through the apparatus to achieve a temperature in the focussing chamber of around 2^oC. The sample was focussed for 4 hours at 12 watts, during which time the voltage rose from 300 to 880 volts. Twenty 2.5 ml fractions were harvested and their protein content, pH and JHE activities were determined. Fractions containing JHE (pHs 5.35-6.12) were returned to the Rotofor with sufficient 10% glycerol to fill the focussing chamber. These were refocussed for a further 4 hours, during which time the voltage rose from 1600 to 1900 volts. Then fractions were collected and assayed as above. The JHE containing fractions were pooled (pHs 5.29-5.56), filtered through a 0.22 mm membrane, and concentrated to 0.5 ml using a centriprep 30 (Amicon).

Mono Q Anion Exchange

The material from the previous step was diluted to 5ml with imidazole buffer (0.1 M imidazole adjusted with HCl to pH 7.0, 10% glycerol, 1 mM dithiothrietol). The sample was loaded onto a Pharmacia "Mono Q HR5/5" 1 ml strong anion exchange column previously equilibrated with imidazole buffer. Unbound material was eluted with 6 ml of buffer and bound material was eluted with a 20 ml linear gradient of 0 to 500 mM NaCl in imidazole buffer (flow rate of 0.5 ml/min.). The gradient for this step and the flow rates for this and the next step were controlled using the Pharmacia FPLC system. The gradient eluate was collected in 1 ml fractions, and its protein content was monitored by its absorbance at 280 nm.

Gel Filtration

The JHE containing fractions from above (265-335 mM NaCl) were concentrated to about 100 μl using a Centricon 30 (Amicon) and loaded onto a Pharmacia "Superose 6" 30 ml gel filtration column previously equilibrated with two column volumes of the above imidazole buffer. Proteins were eluted with imidazole buffer at a flow rate of 0.5 ml/min and 1 ml fractions were collected around the expected elution volume (16.5 ml). The fractions containing JHE activity (Fr6 for the sixth fraction eluted, and Fr7 for the seventh fraction eluted) were stored at -20^oC overnight then assessed for purity using SDS-PAGE and silver staining. Aliquots were then stored at either -20^oC or -70^oC.

To estimate the molecular weight of native JHE, both a partially purified JHE preparation and a mixture of known proteins (Biorad Gel Filtration Standards) were separated on a 320 ml Pharmacia "Superdex 200" gel filtration column using imidazole buffer and a flow rate of 1.0 ml/min.

3.2.4 Kinetic Characterisation of JHE

Kinetic experiments were all performed using material conventionally purified to near homogeneity (Fr7, characterised in sections 3.3.1.2 and 3.3.2.1).

3.2.4.1 Kinetic Analysis of JHIII Hydrolysis and Inhibition by JH Isoforms

The type of inhibition and the inhibition constants were determined for three isoforms of JH (JHI, JHB_3, and MF). In each experiment, assays (Section 3.2.2.1) were performed with various concentrations of both the JHIII and inhibitor. A series of JHIII concentrations (0.0555, 0.0715, 0.1, 0.1665, 0.5 μM, 3871 cpm/pmol) were chosen to yield equally spaced data points on a Lineweaver-Burke (double reciprocal) plot and span the K_M concentration of JHIII. ^{3}H-JHIII was added to the assay solution

in 1 μl ethanol whereas the JH isoforms were added in 1 μl dimethylsulphoxide (DMSO). Reactions were started by the addition of 3 μl of JHE (fraction 7 diluted 1/20 in 5 mg/ml BSA). Incubation times for these experiments were 20 minutes, during which time a maximum of 20% of the substrate was consumed and the reaction rates were linear. Each inhibition experiment also provided an estimate of the Michaelis constant (K_M) for hydrolysis of JHIII by JHE. For each isoform, the experiment was repeated three times, providing three data sets.

After graphical determination of the type of inhibition, an overall fit of each set of data was made to the appropriate rate equation (in this case competitive inhibition) using the appropriate computer program of Cleland (1979). An example of the computer analysis is shown in the appendix. Weighted means of the inhibition constants from each data set were then calculated. The reaction rate per mole of JHE (K_{cat}) for JHIII was calculated using the specific activity of Fr6 measured soon after purification because the aliquots of Fr7 used for these experiments had been stored for a few days at -20°C, resulting in the loss of 97% of the activity measured immediately after purification. However, no loss of activity occurred during subsequent storage at -70°C. The constants K_M and K_I are independent of the absolute amount of enzyme used in each experiment and so needed no correction.

3.2.4.2 Kinetic Properties with α-Naphthylacetate

α-Naphthylacetate was tested as an inhibitor of JHE as above (Section 3.2.4.1) and the properties of α-naphthylacetate as a substrate for JHE were determined spectrophotometrically using the method of Mastrapaolo and Yourno (1981). Substrate at a final concentration of 25, 50, 100, 200 or 400 μM was added in 1 μl of 2-methoxyethanol to 75 μl of 100 mM sodium phosphate buffer, pH 8.0 in a quartz microcuvette. α-Naphthylacetate slowly hydrolyses in water so a background rate was determined before 1 μl JHE (Fr7 diluted 1/5 in 10 mg/ml BSA) was added to start the enzymic reaction. This background rate was subtracted from the reaction rate with enzyme and the published extinction coefficient for α-naphthol (24.0 $mM^{-1}cm^{-1}$ at 235 nM) was used to calculate absolute rates. The temperature of the spectrophotometer chamber was a constant 33°C after equilibration. The small reaction volume was necessary in order to conserve JHE but evaporation from the cuvette during assays caused poor reproducibility. To minimise this effect reaction times were reduced (typically 1-2 minutes) and the spectrophotometer chamber was humidified.

Two corrections were made to the measured reaction rates of α-naphthylacetate hydrolysis with Fr7 to account for the presence of a single, major contaminant in Fr7 which is absent from Fr6 and for loss of activity after purification. Firstly, the amount of JHE protein in Fr7 was estimated using the specific activity of the homogeneous Fr6 and the total measured activity of the two preparations. Secondly, the measured reaction rates were scaled up proportionately to reflect the original JHE activity of Fr7.

3.2.4.3 Identification of JH Structural Features which Promote Binding to JHE

In order to further define the specificity of binding by JHE, a wide range of JH isoforms and compounds sharing structural features with JH ("JHAs" for JH analogue; listed in Fig. 3.2) were tested as inhibitors of JHIII hydrolysis. JHE assays were performed as described in section 3.2.4.1 except that the JHA concentrations (10 μM final concentration added in 1 μl DMSO) and ^{3}H-JHIII concentration (0.056 μM, 3871 cpm/pmol) were fixed and reactions were started by the addition of 3 μl of JHE (Fr7

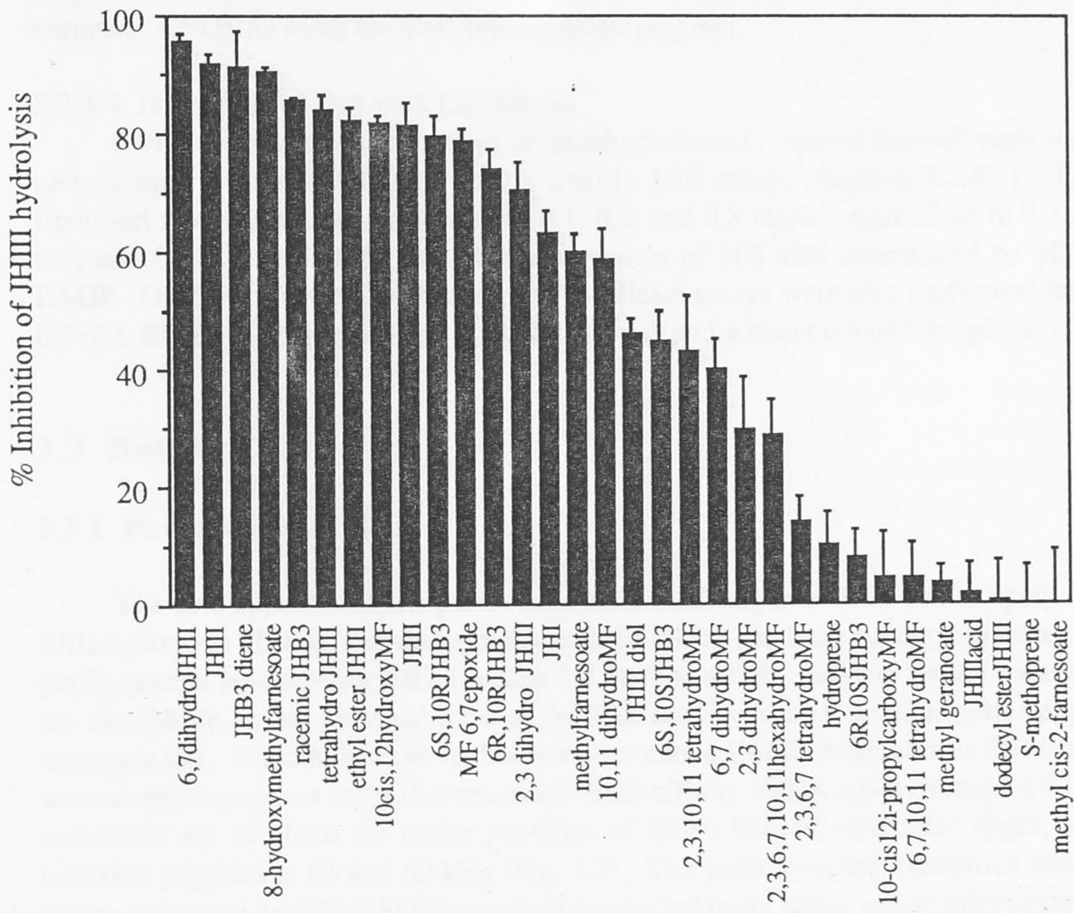


Figure 3.2 Inhibition of JHIII hydrolysis by JHAs

JHAs (10 μM) were incubated with JHIII (0.056 μM) and JHE as described in section 3.2.4.3 and the percent inhibition of JHIII hydrolysis was calculated. Standard errors of triplicate reactions are indicated.

diluted 1/20 in 5 mg/ml BSA). Each compound was tested in triplicate. Control assays lacked the JHA and inhibition was expressed as a percent reduction of the control activity. Where appropriate, data for JHAs sharing systematic differences were subjected to the arcsine transformation (Sokal and Rohlf, 1969) followed by analysis of variance (ANOVA) using the StatView computer program.

3.2.4.4. Interaction of JHE with Lipophorin

Combinations of concentrations of lipophorin from *L. cuprina* haemolymph were coincubated with JHIII (0.0555 to 0.5 μM) in JHE assays (Section 3.2.4.1). The lipophorin concentrations used were 0, 0.1, 0.2, and 0.3 mg/ml, equivalent to 0, 0.3, 0.6, and 0.9 μM, assuming the molecular weight of 300 kDa determined by SDS-PAGE (Dr. S. C. Trowell, pers. comm.). Triplicate assays were also performed with 6.7 nM JHIII (no lipophorin) and 5 μM JHIII (with and without 0.6 μM lipophorin).

3.3 Results

3.3.1 Purification of JHE

The first approach to the purification of *D. melanogaster* JHE was to apply the trifluoroketone affinity chromatography method which has been successfully used to purify several lepidopteran JHEs (Section 1.7.2). The affinity matrices (with or without the disulphide linker) removed most of the JHE activity from *D. melanogaster* pupal homogenates. Naphthylacetate hydrolysis after native PAGE revealed that EST6 and several other esterases were also removed. Both affinity matrix types released a fairly consistent set of about six major peptides, of 45-80 kDa in molecular weight, and included peptides at 66 and 60 kDa (Fig. 3.3). The yield from the disulphide linked matrix estimated by SDS-PAGE was much greater but many minor and smaller peptides were also detected. As enzymes released from the disulphide linked matrix are exposed to a strong reductant and the preparation contained many contaminants, no attempt was made to recover JHE activity. The esterase inhibitor OTFP was used to elute peptides from non-disulphide linked matrix. Extensive dialysis was used to remove OTFP from the eluate but no JHE activity was recovered after one day or one week. It was therefore not possible to readily determine which of the protein bands corresponded to JHE and an alternative purification protocol was subsequently developed to purify JHE.

A latter protocol used conventional chromatographic techniques and consisted of three selective precipitation steps followed by three high resolution chromatographic steps. The six steps were acid homogenisation, ammonium sulphate precipitation and acetone precipitation, followed by preparative isoelectric focussing (Biorad, Rotofor apparatus), anion exchange (Pharmacia, Mono Q) and gel permeation (Pharmacia, Superose 6) chromatography. Table 3.1 summarises the results of the purification of JHE from whole *D. melanogaster* pupae. Overall a 429-fold enrichment for JHE was achieved with a recovery of 0.64% (Fr6 only). However, the recovery is 1.4% if a fraction containing a single major contaminant is included (Fr6 plus Fr7), and the enrichment factor might be about double with respect to a pH 7.0 homogenate (see below). At no point in the purification was there evidence suggesting the presence of more than one JHE isoform.

In preliminary experiments homogenisation of pupae at pH 4.5 and pH 7.0 yielded similar JHE activities, but the pH 4.5 homogenate contained about half the soluble protein. Therefore initial homogenisation was performed at this pH. Ammonium sulphate precipitation generally achieved about two fold enrichment with about 75% yield and a large reduction in volume from about 900ml to 10-15 ml, which was necessary for the subsequent steps. In trials of acetone precipitation, it was found that concentrations of greater than 40% acetone were required to precipitate JHE and these concentrations also caused large losses of activity. Nevertheless precipitation with 40% acetone was included in the purification procedure because many other proteins were precipitated, leaving most of the JHE soluble and active (typically 70% yield and 4 fold enrichment). When used in the complete purification the ammonium sulphate and acetone precipitation steps gave a poorer yield and enrichment (17%/2.2 fold) than were usually obtained in trial experiments (typically about 50%/8 fold).

JHE containing fractions from preparative IEF were refocussed to give a good yield and enrichment for this step (41%/22 fold, Fig. 3.4). JHE focussed at a pH of 5.4 under these conditions, the same pI as was found for JHE after analytical IEF of crude pupal homogenates (Section 2.3.4).

JHE binds to the Mono Q anion exchange column under conditions where most of the protein remaining after the IEF step is eluted without binding (Fig. 3.5). JHE was subsequently eluted with 265-335 mM NaCl, giving a yield of 79% and a 7 fold enrichment.

JHE eluted from the final gel filtration step (Superose 6, Fig. 3.6) in the 6th and 7th fractions (Fr6, Fr7). In the complete purification this step gave only a 1.2 fold enrichment (Fr6) and a yield of 26% (Fr6 and Fr7). However, Fr6 appeared to be homogeneous when analysed by silver stained SDS-PAGE (Fig. 3.7A) and it contained a single peptide at 66 kDa. Fr7 contained two peptides; one at a molecular weight of 66 kDa and another at 40 kDa. In both fractions the 66 kDa peptide stained with similar intensity and the JHE activity was similar, suggesting that the 66 kDa peptide is JHE. Fr6 was retained for N-terminal aminoacid sequencing and Fr7 was retained for kinetic experiments.

3.3.2 Properties of Purified JHE

3.3.2.1 Identification of JHE by Two-Dimensional PAGE

Fr7 was analysed by two-dimensional PAGE to confirm the identity of the 66 kDa peptide as JHE. Quadruplicate samples of Fr7 were first separated by native PAGE. One each of three of the replicate lanes was either cut into ten 5mm pieces and assayed for JHE activity, stained with α- and ß-naphthylacetate, or stained for total protein. Regions of equivalent Rf in three lanes contained JHE activity, exhibited naphthylacetate hydrolysis (α preference) and contained one of the main peptide bands (Fig. 3.7B). The Rf of this peptide on native PAGE corresponded with that previously determined for JHE in crude homogenates (Fig. 2.4). A gel piece corresponding to this Rf from the fourth lane was then subjected to SDS-PAGE with marker proteins (Biorad) and samples of Fr6 and Fr7, and stained for total protein. The mobility of the main peptide corresponded to a molecular weight of 66 kDa, confirming that the 66 kDa peptide in Fr6 and Fr7 is JHE (Fig. 3.7C). The identity of the 40 kDa peptide is unknown.

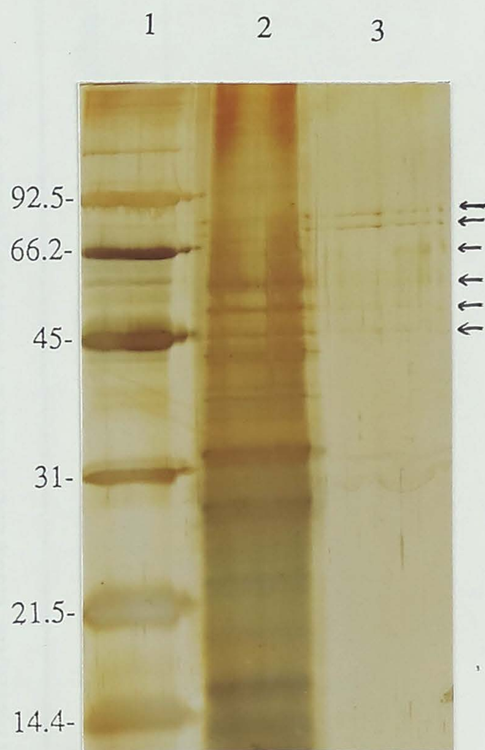


Figure 3.3 Proteins Isolated by Affinity Chromatography

Peptides from *D. melanogaster* pupae isolated by trifluoromethylketone affinity chromatography (Fig. 3.1), separated by denaturing PAGE and detected by silver staining as described in sections 3.2.2 and 3.2.3. Lane 1, molecular weight marker proteins with their weights (kDa) indicated; lane 2, peptides isolated using the disulphide linked matrix; Lane 3, peptides isolated using the non-disulphide linked matrix. Peptides common to lanes 2 and 3 are indicated.

Table 3.1. Purification of Juvenile Hormone Esterase from *D. melanogaster* Pupae

	Volume (ml)	Protein (mg)	Total activity (nmol/min)	Specific Activity (nmol/min/mg)	Recovery (%)	Purification factor
Homogenate pH4.5	900	2610	3600	1.4	–	–
$(NH_4)_2SO_4$ precipitate	52	1820	2880	1.5	80	1.1
40% acetone supernatant	65	200	601	3.0	17	2.2
IEF 1 Rotofor	17.5	36	344	9.5	10	6.9
IEF 2 Rotofor	27	3.85	252	66	7.0	48
Anion exchange Mono Q	3	0.42	200	480	5.5	345
Gel filtration Superose 6 -Fr6 (7)	1 (1)	0.039	23 (28)	590	0.64 (0.78)	429

NB. Bracketed figures refer to a fraction containing a major contaminant.

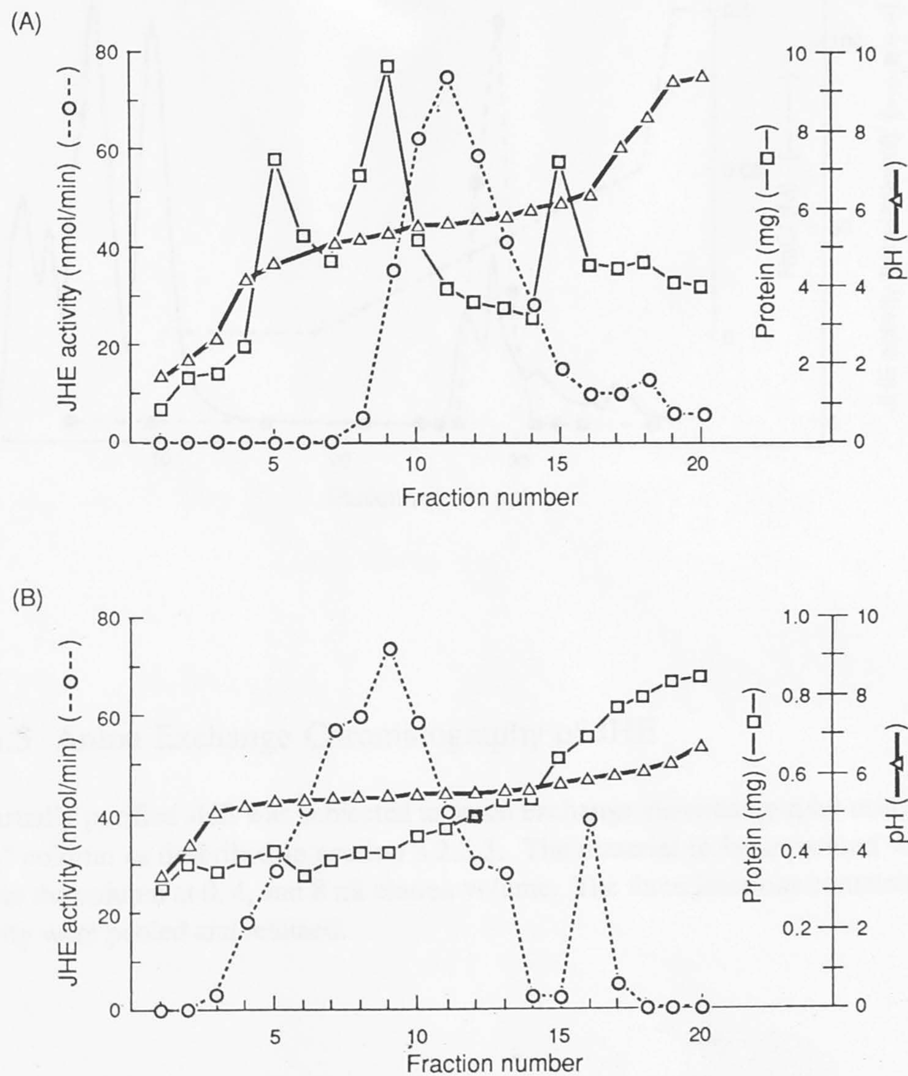


Figure 3.4 Preparative Isoelectric Focussing of JHE

(A) Partially purified JHE was subjected to isoelectric focussing using a "Rotofor" apparatus as described in section 3.2.3.3. (B) The fractions from (A) containing JHE activity (9-15) were pooled and refractionated. Fractions 5-13 were pooled and retained. The JH hydrolytic activity detected in fraction 16 was due to JHEH.

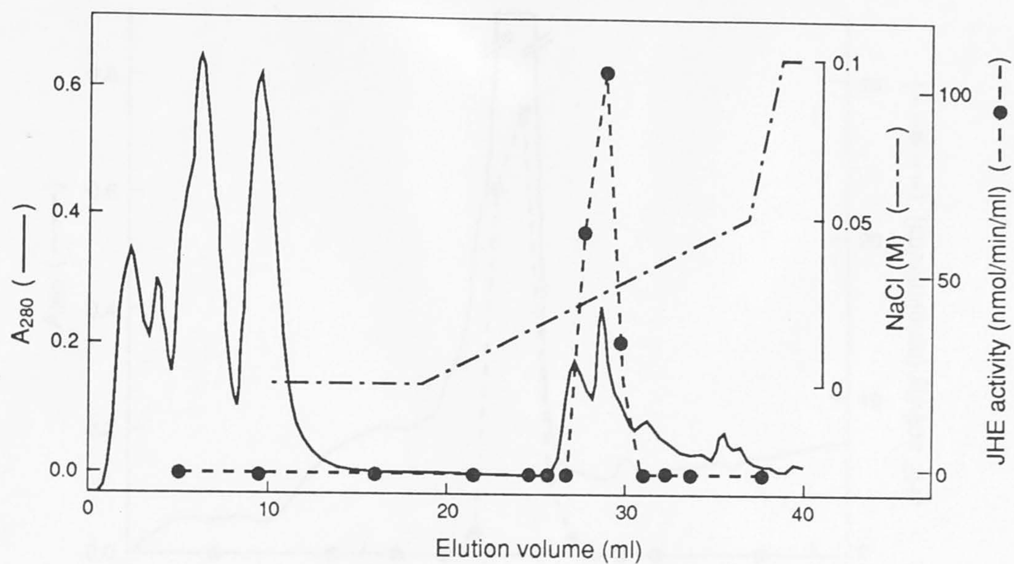


Figure 3.5 Anion Exchange Chromatography of JHE

Partially purified JHE was subjected to anion exchange chromatography using a "Mono Q" column as described in section 3.2.3.3. The material to be separated was loaded onto the column at 0, 4, and 8 ml elution volume. The three fractions containing JHE activity were pooled and retained.

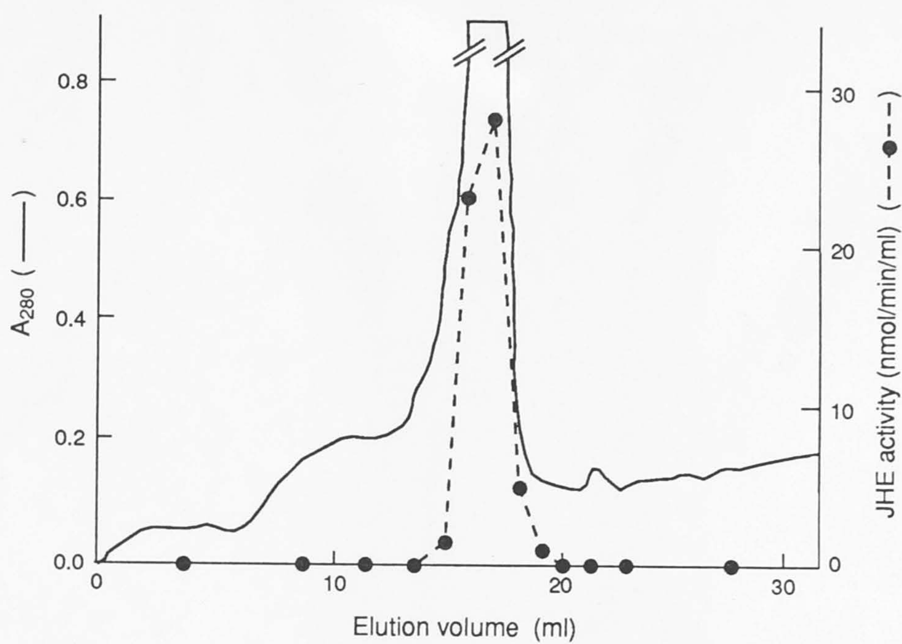


Figure 3.6 Gel Filtration of JHE

Partially purified JHE was subjected to gel filtration using a "Superose 6" column as described in section 3.2.3.3. The sixth and seventh fractions (Fr6 and Fr7) were retained.

A

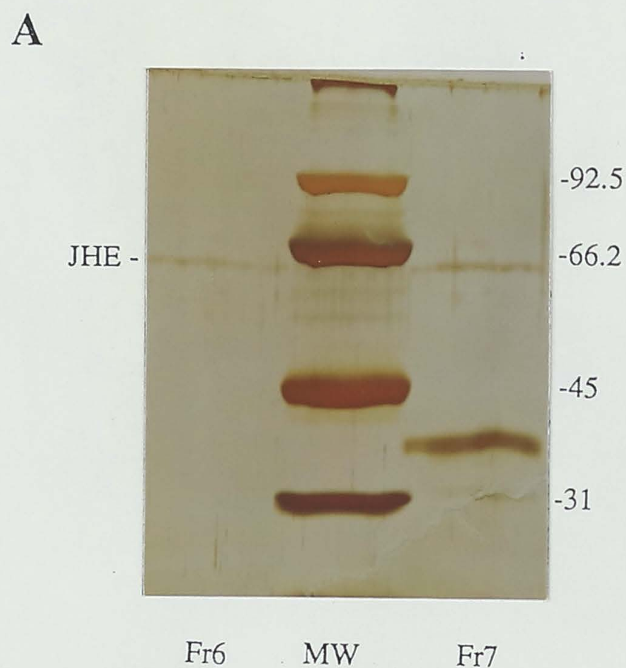


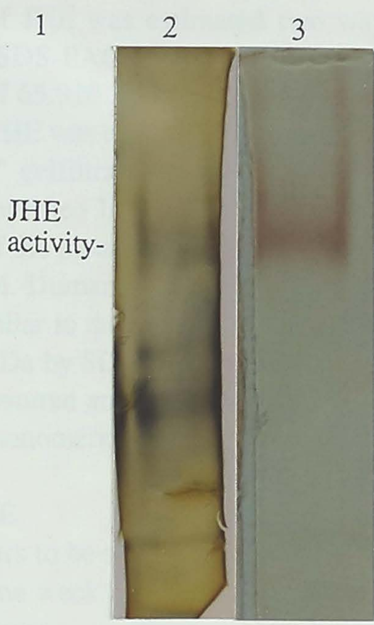
Figure 3.7 Analysis of Purified JHE by PAGE

(A) Purified JHE (Fr6 and Fr7) were separated by denaturing PAGE soon after purification and silver stained for total protein. The sizes of molecular weight marker proteins (MW) are indicated in kDa.

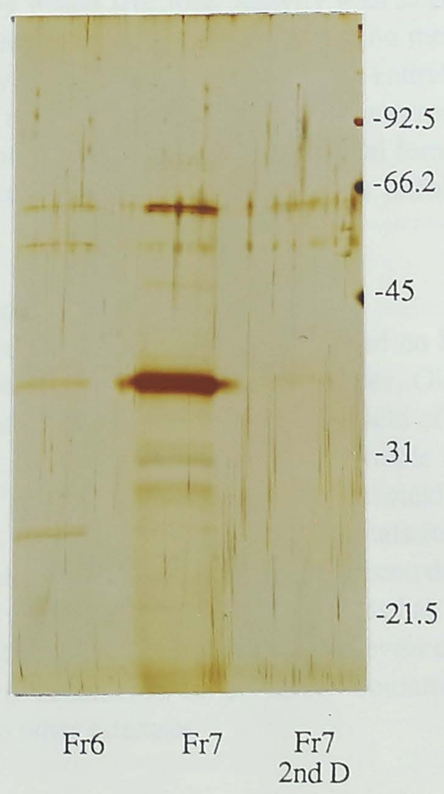
(B) Aliquots of Fr7 were separated by non-denaturing PAGE in four lanes. Lane 1 was cut into pieces. The pieces were assayed for JHE activity and the position of the recovered enzyme is indicated. Lane 2 was silver stained for total protein. Lane 3 was incubated with α- and ß-naphthylacetate to detect esterase activity. The brown stain indicates that JHE preferentially hydrolyses α-naphthylacetate. Lane 4 was cut into pieces and a gel piece corresponding to the location of the α-naphthylacetate and JH esterase activities was subjected to denaturing PAGE (shown in (C), Fr7 2nd D).

(C) Fr6 and Fr7 were separated by denaturing PAGE after storage for about one week at -20°C and silver stained for total protein.

B



C



3.3.2.2 Molecular Weight Estimation

The molecular weight of JHE was estimated two ways. Firstly, the relative mobility of purified JHE on SDS-PAGE compared with marker proteins (Biorad) indicated a molecular weight of 65.9±0.2 kDa (SE from 4 determinations). Secondly, the native molecular weight of JHE was estimated to be 54 kDa (1 determination) by use of a calibrated "Superdex 200" gelfiltration column (Pharmacia). Furthermore, the retention time of native, near-purified JHE on the Superose 6 column is only slightly longer than the retention time of the monomeric esterase, EST6 under similar conditions on the same column (Ms. M. M. Dumancic, unpublished data). This suggests that the molecular weight of JHE is similar to that of EST6 which is estimated to be 62 kDa by calibrated gel filtration, or 65 kDa by SDS-PAGE (Mane *et al.*, 1983). Thus, estimates of the molecular weight of denatured and native JHE are in fair agreement, indicating that the native form of JHE is monomeric.

3.3.2.3 Stability of Purified JHE

Purified JHE (Fr7) appears to be stable for over a year when stored at -70°C but loses all activity in less than one week at -20°C (Fr6 and Fr7). JHE lose activity on storage by at least two mechanisms.

The first results in the loss of a fragment of about 6 kDa. Although Fr6 appeared homogeneous soon after its preparation (Fig. 3.7A), a sample of Fr6 stored for about one week at -20°C revealed an additional 60 kDa peptide (Fig. 3.7C) which stained with the same intensity as the 66 kDa JHE band (a trace of the 40 kDa peptide was also revealed by longer staining). Similarly, after storage at -20°C, Fr7 also gained the 60 kDa peptide, with relatively less staining of the 66 kDa form of JHE. The reduction in the intensity of staining of JHE, together with the appearance of the 60 kDa peptide, suggests that JHE was degraded by the loss of a 6 kDa fragment.

The second mechanism by which JHE lose activity with storage has no effect on its molecular weight as estimated by SDS-PAGE. The second mechanism is inferred because loss of catalytic activity at -20°C cannot be attributed entirely to the mechanism described above. Thus, while most of the original activity was lost from a sample of Fr7 stored at -20°C, most of the JHE remained in the 66 kDa form (Fig. 3.7C). Loss of activity by this second mechanism presumably reflects unfolding of the native structure of JHE.

3.3.2.4 N-Terminal Sequencing

Ten rounds of the sequencing reaction were performed on Fr6 and yielded the following amino acid sequence: X, X, Gly, Glu, Val, Ile, Leu, Glu, Ala, X, where X indicates that the amino acid was not identified. The initial yield of the reaction was 70 pmoles, 23% of the amount of JHE estimated to be in the sample based on its protein content and molecular weight. The lower than expected initial yield may have been due to partial N-terminal blockage. However, high levels of signals for other amino acids were reported, suggesting that the low initial yield may have been due to degradation of JHE prior to sequencing. The sequence of seven amino acids has 100% identity with seven amino acids of plasmid R27 replication region, lower levels of similarity to other proteins, none of which were considered to be likely contaminants of the JHE preparation, and no similarity to other esterases.

3.3.3 Kinetic Characterisation of JHE

Four experiments were performed using JHE conventionally purified to near homogeneity (Fr7, Section 3.3.1). In the first, kinetic constants for JHIII hydrolysis and constants for inhibition by three JH isoforms were determined. In the second, the kinetic properties of α-naphthylacetate as both competitive inhibitor of JHIII hydrolysis and spectrophotometric substrate were determined. These first two experiments yielded kinetic parameters for a small range of alternative substrates. The third experiment yielded a ranking of the binding affinity of JHE for a much larger range of JH analogues and isoforms. In the fourth experiment, the mode of interaction between the JH carrier, lipophorin, and JHE was investigated.

3.3.3.1 Kinetic Analysis of JHIII Hydrolysis and Its Inhibition by JH Isoforms

The kinetic parameters for hydrolysis of racemic JHIII by *D. melanogaster* JHE are as follows (See figure 3.8 and the appendix for an example).

K_M	89±12 nM (SE for 13 determinations)
V_{max}	590 nmol/min/mg
K_{cat}	0.60 sec^{-1}
Specificity Constant (K_{cat}/K_M)	6.8 x 10^6 $M^{-1}sec^{-1}$

These data indicate that the V_{max}/K_{cat} is quite low but both the binding affinity and specificity constant of *D. melanogaster* JHE for JHIII are very high.

The kinetics of inhibition of JHIII hydrolysis by a selection of natural JH isoforms was investigated in order to determine whether JHE selectively binds a defined JH isoform. Each of these compounds exhibited competitive inhibition kinetics and no data set produced a significant fit to non-competitive inhibition kinetics. This indicates that JH isoforms bound to the same site as the substrate JHIII and that the K_I can be interpreted as a measure of binding affinity of JHE for these compounds. Inhibition constants (K_Is) (weighted means of three determinations with standard errors) were as follows (see figure 3.8 and the appendix for an example).

JHB_3 (optically racemic)	1.20±0.09 μM
MF	3.33±0.36 μM
JHI	3.34±0.47 μM

These K_Is are 13 to 38 fold higher than the K_M for JHIII above although, consistent with its probable role in Diptera, the K_I for the JHB_3 is the lowest.

3.3.3.2 Kinetic Properties with α-Naphthylacetate

The artificial substrate α-naphthylacetate was shown to inhibit JHIII hydrolysis competitively with a K_I of 120±30 μM (weighted mean of 3 determinations with standard error) and no data set produced a significant fit to non-competitive inhibition kinetics. Hydrolysis of naphthylacetate by JHE was demonstrated after native PAGE and the alpha form is hydrolysed preferentially over the beta form (Section 3.3.2.1, Fig. 3.7B). The kinetics of hydrolysis of α-naphthylacetate were also investigated spectrophotometrically (Fig. 3.9, Mastrapaolo and Yourno, 1981). These data were

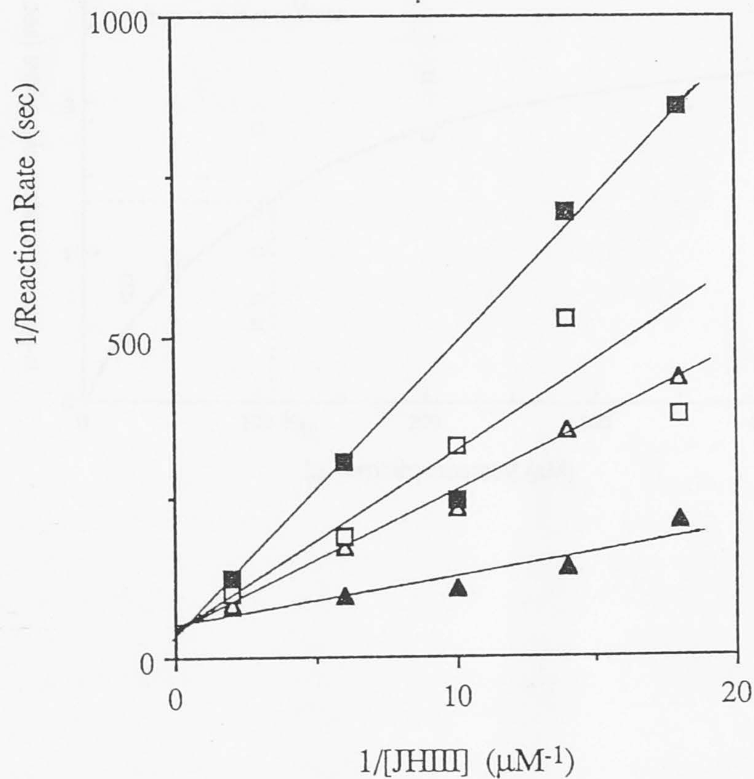


Figure 3.8 Inhibition of JHIII Hydrolysis by JHB_3: 1/V vs 1/[S] Plot

A typical experiment as described in section 3.2.4.1 to determine the nature of inhibition of JHIII hydrolysis, in this case by JHB_3, is illustrated. JHB_3 concentrations were zero (solid triangles), 3 μM (open triangles), 5 μM (open squares), and 8 μM (solid squares). Similar results were obtained when MF, JHI and α-naphthylacetate were tested as inhibitors of JHIII hydrolysis.

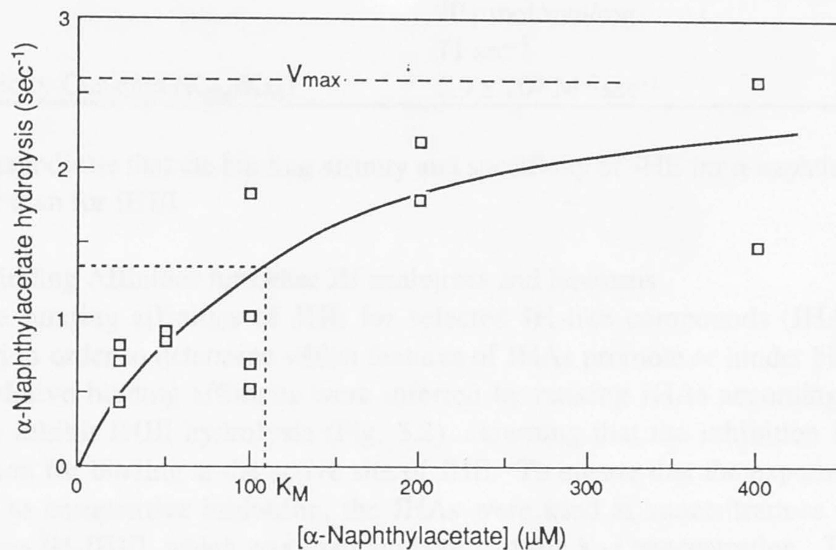


Figure 3.9 Hydrolysis of α-Naphthylacetate by JHE: V vs [S] Plot

Hydrolysis of α-naphthyacetate by JHE was monitored spectrophotometrically as described in section 3.2.4.2. The V_{max} and K_M for the reaction were estimated visually as indicated.

not analysed statistically because of the obvious scatter but graphical analysis of the results indicate the following approximate kinetic parameters.

K_M	120 μM
V_{max}	70 μmol/min/mg
K_{cat}	71 sec^{-1}
Specificity Constant (K_{cat}/K_M)	5.9 x 10^5 $M^{-1}sec^{-1}$

These data indicate that the binding affinity and specificity of JHE for α-naphthylacetate are lower than for JHIII.

3.3.3.3 Binding Affinities for Other JH analogues and Isoforms

The binding affinities of JHE for selected JH-like compounds (JHAs) were compared in order to determine which features of JHAs promote or hinder binding by JHE. Relative binding affinities were inferred by ranking JHAs according to their ability to inhibit JHIII hydrolysis (Fig. 3.2), assuming that the inhibition is due to competition for binding at the active site of JHE. To ensure that the experiment was sensitive to competitive inhibition, the JHAs were used at concentrations 180-fold higher than ^{3}H-JHIII, which was used at about half the K_M concentration. Two lines of evidence support the assumption of competition. Firstly, α-naphthylacetate binds at the same active site as JHIII (Section 3.3.3.2) and the JHAs are all more similar to JHIII than α-naphthylacetate. Secondly, the relative abilities of JHI, JHIII, MF and JHB_3 to inhibit JHIII hydrolysis are consistent with the inhibition/Michaelis constants calculated for these compounds (see below), and competitive inhibition by JHI, MF and JHB_3 has been rigorously demonstrated (Section 3.3.3.1):

Compound	K_I / K_M ± SE	% Inhibition of JHIII hydrolysis ± SE
JHI	3.34±0.47 μM	63.0±2.3%
MF	3.33±0.36 μM	60.0±1.8%
JHB_3	1.20±0.09 μM	85.5±0.4%
JHIII	89±12 nM	92.7±0.4%

Thus, JHI and MF have equal K_Is and were ranked as essentially the same in this experiment. JHB_3 has a lower K_I and caused greater inhibition, while JHIII, which has a K_M lower still, caused even greater inhibition when tested as a JHA.

The effects of JHAs on JHIII hydrolysis (Fig. 3.2) are here compared within sets of JHAs which share systematic differences from JHIII. The inhibition data are presented as means of triplicate experiments with standard errors.

1) Size of the carboxyl chain

The replacement of one or two of the methyl side chains of JHIII with ethyl groups produces the lepidopteran JH isoforms JHII or JHI, respectively. JHII (C11 ethyl group) has reduced ability to inhibit JHIII hydrolysis (81.3±2.3%), compared with an equal amount of non-radioactive JHIII (92.7±0.4%) and JHI (ethyl groups at C11 and C7) shows a further reduction (63.0±2.3%, Fig. 3.10A). However, little inhibition occurs if the JHIII backbone is truncated to 2/3 of its original length, as exemplified by methyl geranoate (3.5±1.7%). These data suggest that JHE binds most

effectively to molecules with carboxyl chains that are neither larger nor smaller than JHIII. In particular, the distal portion of the carboxyl chain which carries the epoxide group is required for high affinity binding.

2) Alternative alcohol moities

Replacement of the methyl alcohol moiety of JHIII with an ethyl group reduces inhibition only slightly compared with JHIII (82.1±1.2% and 92.7±0.4%, repectively). However, replacement with a longer alcohol group (dodecyl ester of JHIII) or the absence of any alcohol moiety (JH acid) gives essentially no inhibition (0.6±3.9% and 1.6±3.1%, respectively), indicating that a small alcohol moiety is an important requirement for high affinity binding (Fig. 3.10B).

3) Hydrogenation of the double bonds in JHIII and methyl farnesoate

The effect of hydrogenation of one or more double bonds was examined in JHIII and MF. Each of the four possible JHAs combining hydrogenation of the 2,3 and 6,7 double bonds of JHIII (Fig. 3.11A) and each of the nine possible JHAs combining hydrogenation of the three double bonds of MF (Fig. 3.11B) were tested for their ability to inhibit JHIII hydrolysis.

When an epoxide is present at carbons 10 and 11 (as in JHIII) hydrogenation of the 2,3 double bond reduces inhibition ($F_{1,9} = 131$, $p < 0.001$) whereas hydrogenation of the 6,7 double bond increases inhibition ($F_{1,9} = 26$, $p < 0.001$) and these effects are independent of one another ($F_{1,9} = 4.7$, $p > 0.05$). On average 2,3 hydrogenation decreases inhibition by 17% but 6,7 hydrogenation increases inhibition by 8%.

When there is no epoxide at carbons 10 and 11 (as in MF) the effects on inhibition of hydrogenation of the 2,3, 6,7, and 10,11 double bonds are more difficult to assess because all three pairwise interactions, as well as the three way interaction, are significant (Fig. 3.11B). Nevertheless, inspection of the sums of squares reveals that the 2,3 and 6,7 bonds exert the largest of the three main effects, with hydrogenation causing overall decreases in percentage inhibition of 10 and 28, respectively. By comparison, 10,11 hydrogenation has no independent (main) effect; instead its effects are manifest in the interaction terms, so its effects depend strongly on whether the 2,3 or 6,7 bonds are hydrogenated. When the 6,7 double bond is present, 10,11 hydrogenation increases percentage inhibition by 7, but when the 6,7 double bond is hydrogenated, 10,11 hydrogenation decreases percentage inhibition by 13. In contrast, when the 2,3 double bond is present, 10,11 hydrogenation decreases percentage inhibition by 24, but when the 2,3 double bond is hydrogenated, 10,11 hydrogenation increases percentage inhibition by 14.

The importance of the 2,3 double bond may be indicated by the absence of inhibition by the cis-2 isomer of MF (-2.4±5.3%). However, this modification probably causes the entire carboxyl chain to be displaced from its usual spatial relationship with the ester group.

Collating the results of these analyses, the presence of the 2,3 double bond promotes binding to JHE. The 6,7 double bond promotes binding if the 10,11 epoxide is absent but it reduces binding if the 10,11 epoxide is present. The greater inhibition by the entire JHIII-like (epoxidated) series compared with the MF-like series indicates that binding is more strongly promoted by the 10,11 epoxide group than by the presence or absence of any of the double bonds.

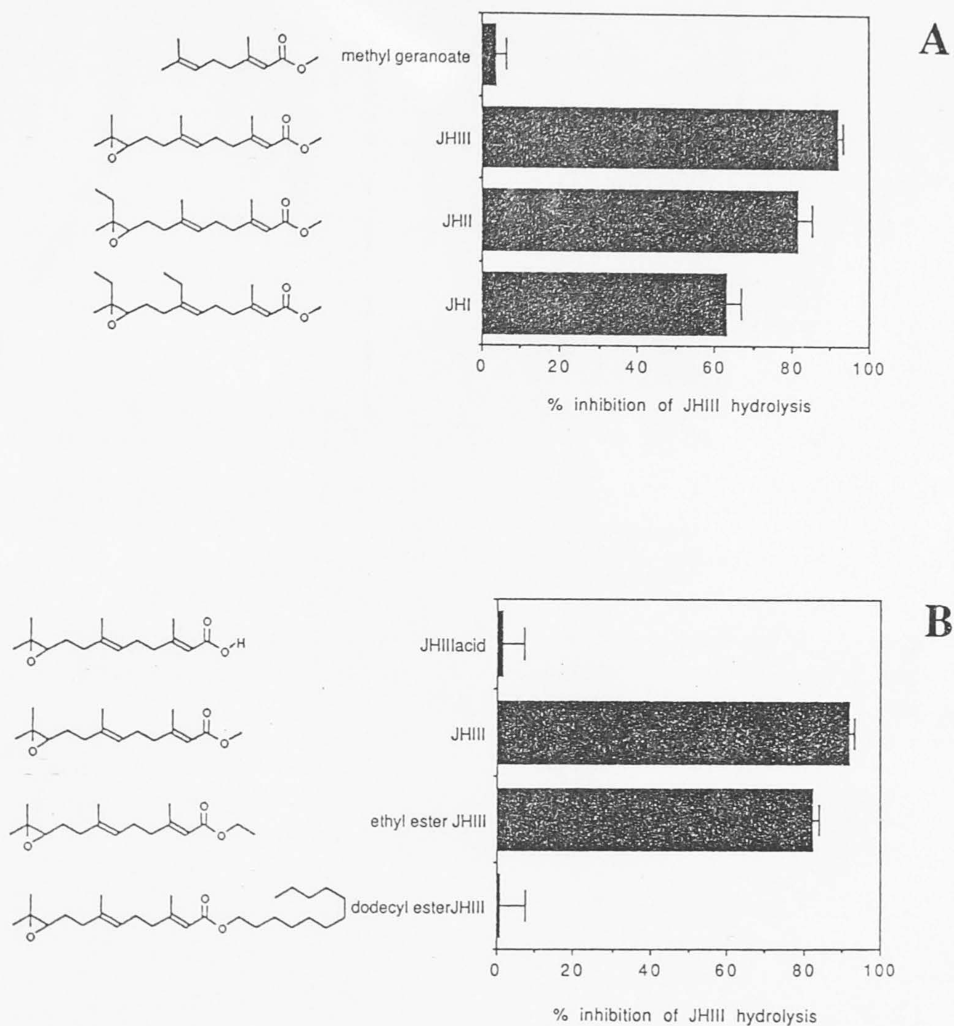


Figure 3.10 Effects of JHAs on JHE hydrolysis of JHIII -Size of Carboxyl Chain and Alcohol Moiety

JHAs (10 μM) were incubated with ^{3}H-JHIII (56 nM) and JHE as described in section 3.2.4.3 and the % inhibition of JHIII hydrolysis was calculated. (A) shows the effects by JHAs which differ in the size of the carboxyl chain and (B) shows the effects of JHAs which differ in the alcohol moiety.

A

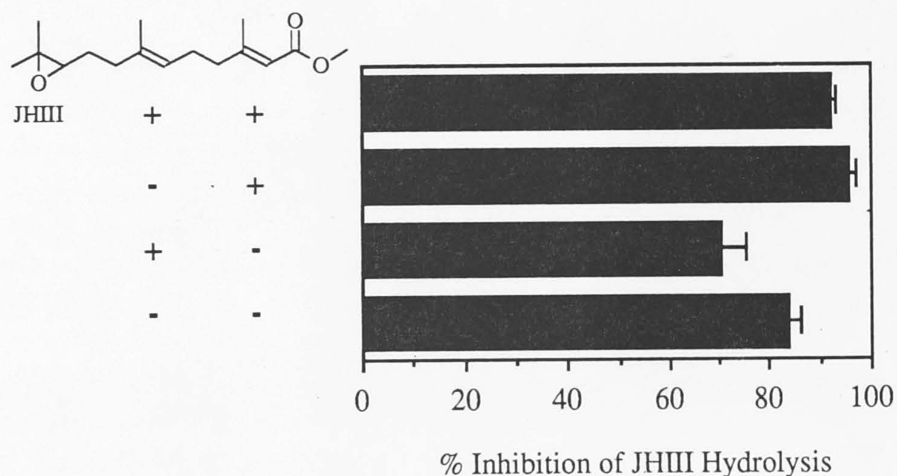
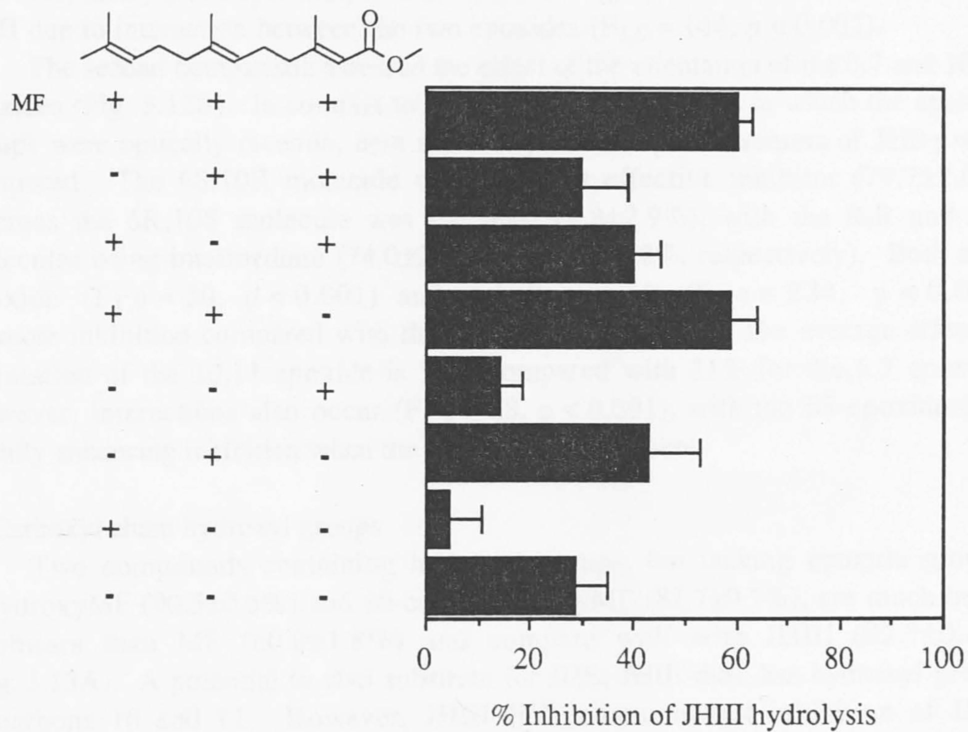


Figure 3.11 Effects of JHAs on JHE hydrolysis of JHIII -Effects of Double bonds

JHAs (10 μM) were incubated with ^{3}H-JHIII (56 nM) and JHE as described in section 3.2.4.3 and the percent inhibition of JHIII hydrolysis was calculated. (A) shows the effects of JHAs which differ by the presence or absence of double bonds when the 10,11 epoxide is present, as in JHIII and (B) shows the effects of JHAs which differ by the presence or absence of double bonds when the 10,11 epoxide is absent, as in MF. The table shows the results of analysis of variance of the data shown in panel B.



Double Bonds	Degrees of Freedom	Mean Square	F-test	
2,3	1	205	8	*
6,7	1	1857	71	***
10,11	1	45	1.7	
2,3 x 6,7	1	362	14	**
2,3 x 10,11	1	853	33	***
6,7 x 10,11	1	249	10	**
2,3 x 6,7 x 10,11	1	344	13	**
Error	16	26		

*p < 0.05, **p < 0.01, ***p < 0.001

4) Alterations to the epoxide group(s)

Two types of comparison were used to assess the importance of epoxide groups in the binding of JH to JHE. Firstly, inhibition of JHIII hydrolysis was compared among the four possible JHAs which have either a double bond or an epoxide group between carbons 6 and 7 and carbons 10 and 11 (Fig. 3.12A). MF (no epoxides) was a poorer inhibitor (60.0±1.8%) than either the 6,7 epoxide of MF (78.5±1.3% inhibition) or the 10,11 epoxide (JHIII, 92.7±0.4%). Inhibition is promoted by both the 10,11 epoxide ($F_{1,9} = 357$, $p < 0.001$) or the 6,7 epoxide ($F_{1,9} = 11$, $p < 0.05$). However, JHB_3 (both epoxides, 85.5±0.4%) is a slightly less effective inhibitor than JHIII due to interaction between the two epoxides ($F_{1,9} = 144$, $p < 0.001$).

The second comparison assessed the effect of the orientation of the 6,7 and 10,11 epoxides (Fig. 3.12B). In contrast to the previous comparisons in which the epoxide groups were optically racemic, here the four possible optical isomers of JHB_3 were compared. The 6S,10R molecule was the most effective inhibitor (79.7±2.0%) whereas the 6R,10S molecule was the least (7.8±2.9%), with the R,R and S,S molecules being intermediate (74.0±2.0% and 44.6±3.2%, respectively). Both a 6S epoxide ($F_{1,9} = 50$, $p < 0.001$) and a 10R epoxide ($F_{1,9} = 234$, $p < 0.001$) promote inhibition compared with the opposite orientations. The average effect of orientation of the 10,11 epoxide is 51% compared with 21% for the 6,7 epoxide. However, interactions also occur ($F_{1,9} = 28$, $p < 0.001$), with the 6S epoxide only slightly enhancing inhibition when the 10R epoxide is present.

5) Carboxyl chain hydroxyl groups

Two compounds containing hydroxyl groups, but lacking epoxide groups, 8-hydroxyMF (90.5±0.5%) and 10-cis,12-hydroxyMF (81.7±0.7%), are much better inhibitors than MF (60.0±1.8%) and compare well with JHIII (92.7±0.4%) (Fig. 3.13A). A potential *in vivo* substrate for JHE, JHIII-diol, has hydroxyl groups at carbons 10 and 11. However, JHIII-diol causes modest inhibition of JHIII hydrolysis (46.0±1.5%), indicating that it does not bind strongly to JHE. This is consistent with the finding that little or no JH-acid-diol is produced when both JHE and JHEH are present (Section 2.3.2.2).

6) Insecticidal JHAs

The two JHAs tested that are registered insecticides were the juvenoids R/S-hydroprene and S-methoprene. These compounds mimic many of the effects of JH (Fig. 3.13B) but were poor inhibitors of JH hydrolysis (10.0±3.1% and 0.0±3.7%, respectively). Hydroprene and methoprene differ from JHIII by the presence of ethyl and isopropyl alcohol moities, respectively, the lack of an epoxide group, and the presence of an additional 4,5 double bond conjugated to the 2,3 double bond and the ester group. The first two features cause reduced inhibition of JHIII hydrolysis (Sections 3.3.3.3-2 and 3.3.3.3-4). 6S-JHB_3-2,3,4,5-diene is a good inhibitor (91.1±3.8%) suggesting that the third feature, additional conjugation, does not interfere with binding to JHE, although it would be expected to increase the rigidity and stability of the molecule. Poor binding to JHE is consistent with slow metabolism of these juvenoids in a range of insects (Bowers, 1982).

3.3.3.4 Interaction of JHE with Lipophorin

The double reciprocal plot of the velocity of JH hydrolysis versus substrate concentration yields a series of curves for reactions containing lipophorin rather than the straight line obtained in its absence (Fig. 3.14A). Rates of hydrolysis are reduced in the presence of lipophorin but it is clear that the V_{max} of the reaction is unaltered. Furthermore, there is no difference between the rate of hydrolysis with and without lipophorin when the JHIII concentration (5 μM) should be sufficiently high to saturate binding by both JHE and lipophorin (0.6 μM) ($p = 0.36$, $n = 3$).

The free concentration of JH in the presence of lipophorin was calculated to test the hypothesis that the reduced rates of JH hydrolysis could be explained simply by reduced substrate concentration. In order to bind in the active site of JHE, a JH molecule presumably must not be bound in the binding site of lipophorin. The dissociation constant of lipophorin (58 nM) and the concentrations of lipophorin and added JH were used to calculate the free concentrations of JH in the reactions, using the following formula.

$$[L_T] = K_D(f/1\text{-}f) + f[R_T] \quad \text{(Bennett and Yamamura, 1985)}$$

L_T is the total ligand concentration (JHIII), R_T is the total receptor concentration (lipophorin), K_D is its dissociation constant, $f = [LR]/R_T$, and LR is receptor bound to ligand. Solving the equations for [LR] yields the concentration of bound JHIII and free JHIII concentration is estimated by subtracting [LR] from L_T. Most of the calculated JH concentrations fell in the low nanomolar range and thus below the JH concentrations used in the reactions without lipophorin.

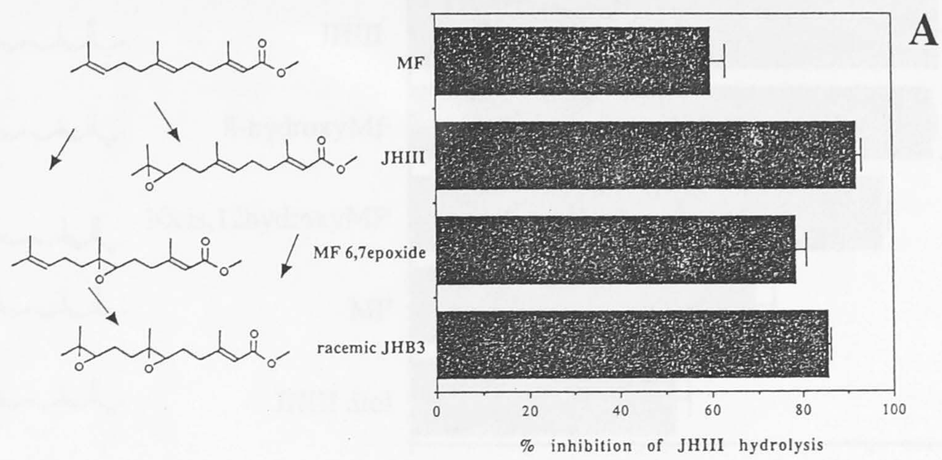
A double reciprocal plot using the calculated free JH concentrations (Fig. 3.14B) produced straight lines which lay close to the values predicted by extrapolation from the data collected in the absence of lipophorin. (Interestingly, these straight lines were fitted with correlation coefficients of 0.97-1.0 rather than the typical figure of 0.8-0.9, possibly due to reduced and less variable binding of JH to glassware). These results demonstrate that lipophorin does not inhibit JH hydrolysis by any mechanism other than by lowering the free concentration available to JHE.

Reaction rates for a single lower JH concentration (7.2 nM) without lipophorin were somewhat lower than would be predicted by extrapolation from the data collected in the absence of lipophorin whereas the reaction rates determined with lipophorin were somewhat higher than would be predicted. These departures from linearity may be due to a higher proportion of non-specific binding of JH to the glassware when JH is very dilute and the ability of lipophorin to prevent this effect. Lanzrein *et al.* (1993) found that carbowax treatment of glassware, as used in these experiments, reduces surface binding of JHIII (from 78% bound to 44%, for approx. 3.7 pM 10R-JHIII), but lipophorin and carbowax together eliminate such binding.

3.4 Discussion

3.4.1 Purification and Physical Properties of JHE

The first approach taken for the purification of *D. melanogaster* JHE was the affinity chromatography method of Abdel-Aal and Hammock (1986). Although JHE from *D. melanogaster* bound to the affinity matrices, successful application of this approach was prevented by the inability to pretreat starting material with DFP or recover



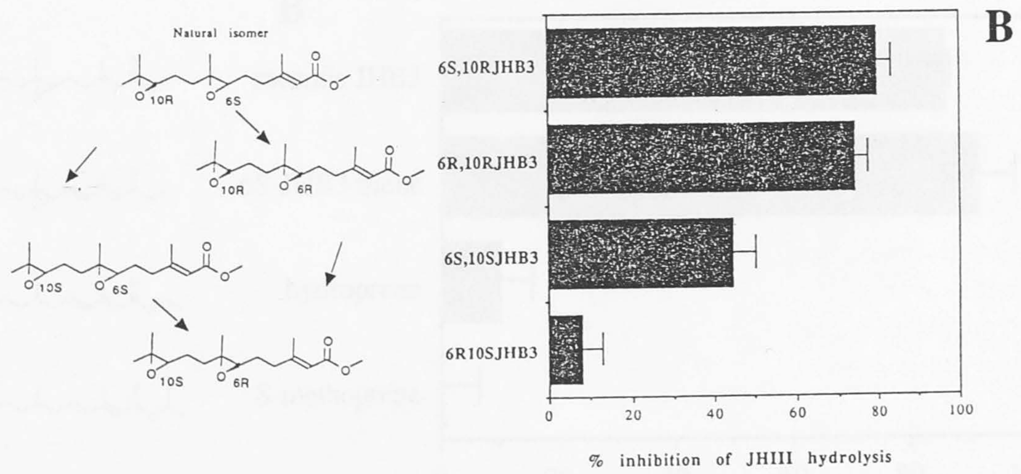


Figure 3.12 Effects of JHAs on JHE hydrolysis of JHIII -Effects of Epoxides

JHAs (10 µM) were incubated with ^{3}H-JHIII (56 nM) and JHE as described in section 3.2.4.3 and the percent inhibition of JHIII hydrolysis was calculated. (A) shows the effects of JHAs which differ by the presence or absence of 6,7 and 10,11 epoxide moieties and (B) shows the effects of JHAs which are optical isomers of JHB_3.

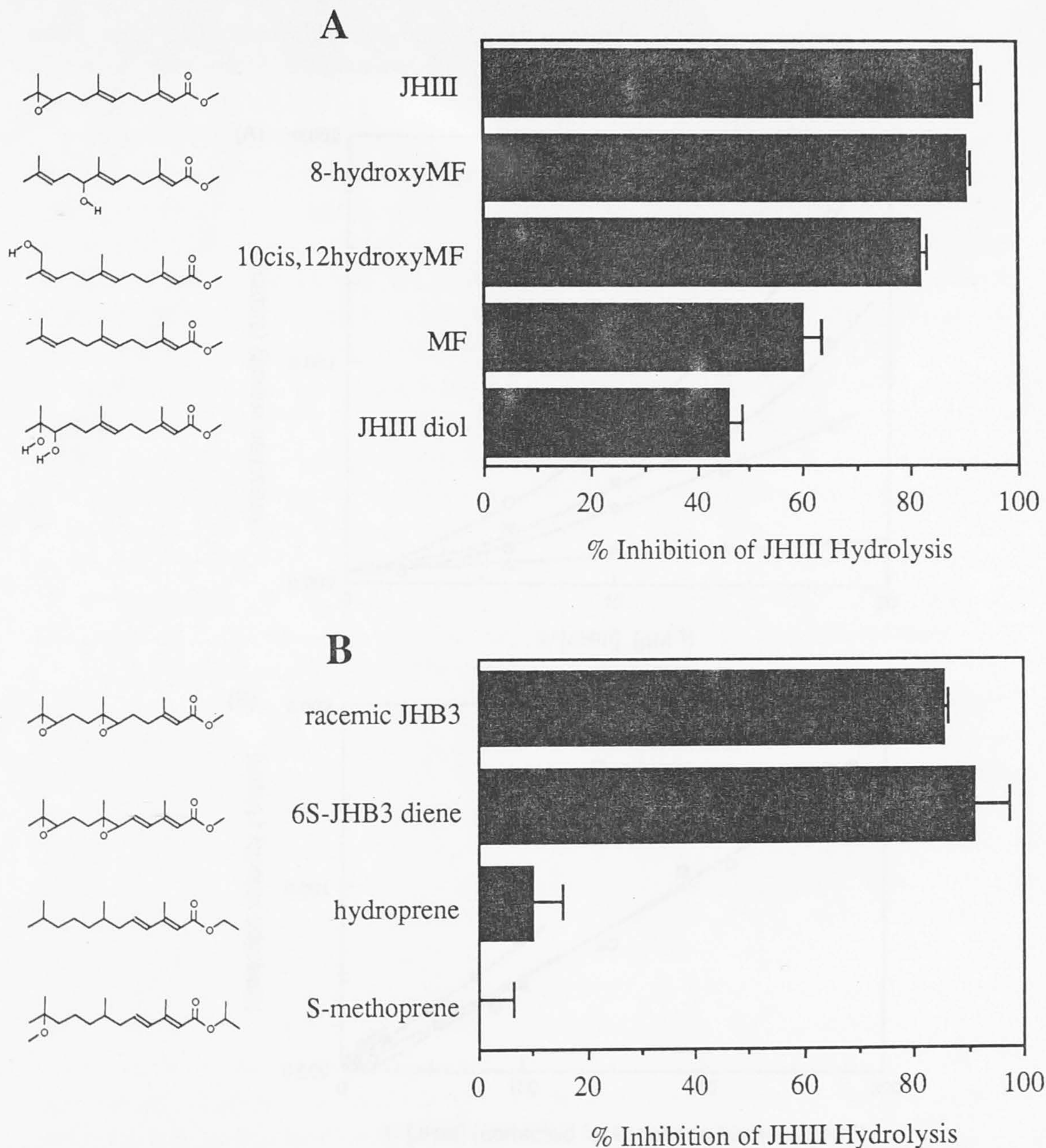


Figure 3.13 Effects of JHAs on JHE hydrolysis of JHIII -Hydroxyl Groups and Insecticidal JHAs

JHAs (10 μM) were incubated with ^{3}H-JHIII (56 nM) and JHE as described in section 3.2.4.3 and the percent inhibition of JHIII hydrolysis was calculated. (A) shows the effects by JHAs which have hydroxyl groups on the carboxyl chain and (B) shows the effects of two insecticidal JHAs compared with JHB_3 and JHB_3-diene.

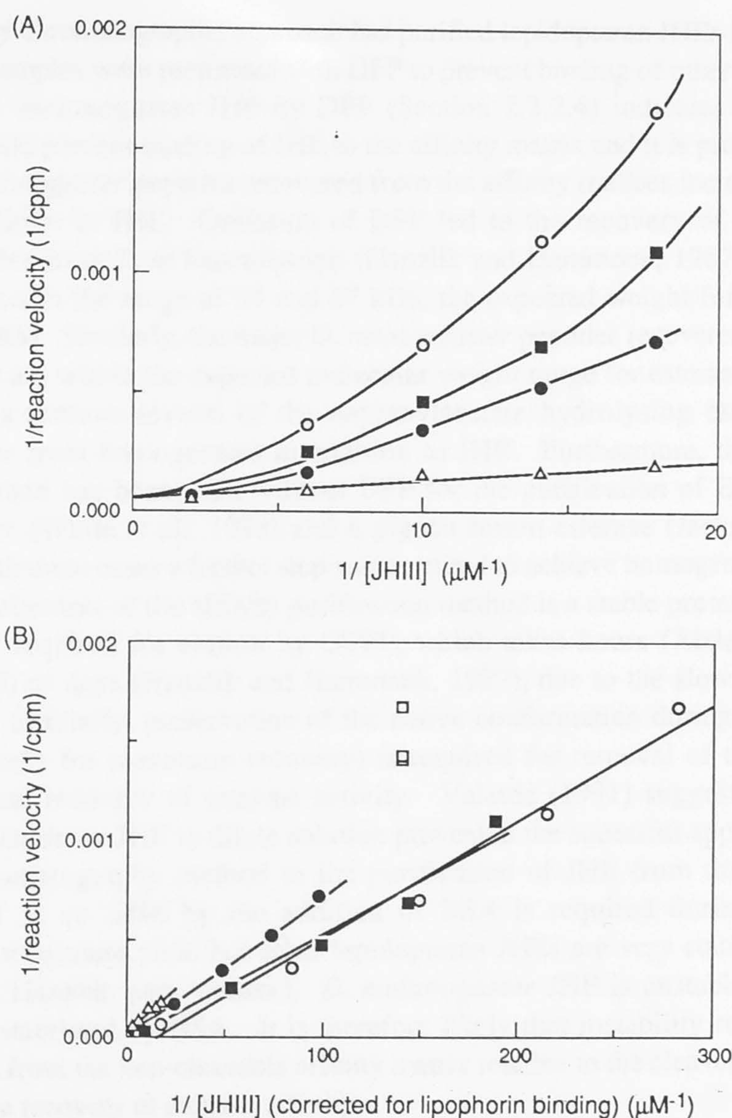


Figure 3.14 Inhibition of JHIII Hydrolysis by Lipophorin: 1/V vs 1/[S] Plot

Lipophorin was tested as an inhibitor of JHIII hydrolysis by JHE as described in section 3.2.4.4. Lipophorin concentrations were zero (open triangles), 0.1 mg/ml (solid circles), 0.2 mg/ml (solid squares), and 0.3 mg/ml (open circles). (A) shows the effect of lipophorin plotted against the concentration of added JHIII. (B) shows the same data plotted against the concentration of unbound JHIII which was calculated as described in section 3.3.3.4. In (B) the open squares indicate additional reactions which were performed without lipophorin.

enzyme activity, probably due to instability of JHE in dilute solution. The second approach used classical chromatographic techniques to purifiy *D. melanogaster* JHE to homogeneity.

The affinity chromatographic approach has purified lepidopteran JHEs in a single step only when samples were pretreated with DFP to prevent binding of other esterases. Inhibition of *D. melanogaster* JHE by DFP (Section 2.3.2.4) indicates that DFP pretreatment would prevent binding of JHE to the affinity matrix and it is probable that the major *D. melanogaster* peptides recovered from the affinity matices included other esterases in addition to JHE. Omission of DFP led to the recovery of about six additional peptides from *T. ni* haemolymph (Hanzlik and Hammock, 1987) that had molecular weights in the range of 54 and 67 kDa, the expected weight for esterases (Myers *et al.*, 1988). Similarly, the major *D. melanogaster* peptides recovered from the affinity matrices are within the expected molecular weight range for esterases and the affinity matrices remove several of the naphthylacetate hydrolysing esterases of *D. melanogaster* from homogenates in addition to JHE. Furthermore, the affinity purification method has been used without DFP for the purification of EST6 from *D. melanogaster* (White *et al.*, 1988) and a pigeon serum esterase (Jackson *et al.*, 1992), and in both these cases a further step was required to achieve homogeneity.

A key requirement of the affinity purification method is a stable protein. Native conformation is required for elution by OTFP, which takes hours (Abdel-Aal and Hammock, 1985) or days (Hanzlik and Hammock, 1987), due to the slow exchange rate of ligands. Similarly, preservation of the native conformation during extensive dialysis (1-2 weeks for maximum recovery) is required for removal of the eluting ligand, OTFP and recovery of enzyme activity. Valaitis (1991) suggests that the instability of *L. maderae* JHE in dilute solution prevented the sucessful application of the affinity chromatography method to the purification of JHE from this species. Stabilisation of *T. ni* JHE by the addition of BSA is required during affinity purification for maximum yield but other lepidopteran JHEs are very stable without BSA (Dr. T. N. Hanzlik, pers. comm.). *D. melanogaster* JHE is unstable in dilute solution and is stabilised by BSA. It is therefore likely that instability reduced the recovery of JHE from the non-cleavable affinity matrix relative to the cleavable version and prevented the recovery of enzyme activity.

In the second purification approach, classical chromatographic procedures were successfully applied to JHE from whole *D. melanogaster* pupae. After six steps, *D. melanogaster* JHE was homogeneous by the criterion of a single peptide (66 kDa) being visible after SDS-PAGE and silver staining for total protein. Furthermore, two dimensional PAGE revealed that JHE activity comigrated with the 66 kDa peptide.

The recovery of JHE from this procedure (0.64% for Fr6, or 1.4% for Fr6 plus Fr7) is within the range of recoveries of JHE by conventional purifications from lepidopteran haemolymph (1-40%, Section 1.7.2) but about 10 fold less than was achieved by the affinity chromatography method with whole *T. ni* larvae (10%, Hanzlik and Hammock, 1987). The enrichment factor for *D. melanogaster* JHE (Fr6, 429 fold) also falls within the range of conventional purifications of lepidopteran JHEs (383-4000 fold), although the starting material for the latter purifications was haemolymph. The specific activity of the *D. melanogaster* JHE (0.59 μmol/min/mg) falls at the lower end of the range for lepidopteran JHEs (0.48-4 μmol/min/mg). However, the specific activity for the *D. melanogaster* enzyme is probably an underestimate because of the large losses that occurred in the final purification step. Such losses also occurred during

a similar stage in the purification of *L. maderae* JHE (Valaitis, 1991) but an improved procedure which did not require gel filtration produced *L. maderae* JHE with 3.5 fold greater specific activity (Valaitis, 1992).

Although analytical IEF had revealed only one form of JHE in *D. melanogaster* pupae (Section 2.3.2.4), it remained possible that different forms might separate during chromatography. However, a single peak of JHE activity was recovered at each chromatographic step, providing no evidence of multiple forms. Multiple forms of JHE that occur in some other insects generally copurify and are only separated by isoelectric focussing (Section 1.7.4). The only exception is the use of concanvalin A-sepharose chromatography to separate unglycosylated and glycosylated forms of JHE from lepidopteran fat body, and these forms probably represent the unprocessed and mature JHE (Wozniak and Jones, 1990; Valaitis, 1992).

3.4.2 Comparison of *D. melanogaster* JHE with Other Esterases

Several lines of evidence suggest that *D. melanogaster* JHE is likely to be a member of the carboxyl/choline esterase multigene family (Section 1.8), although none of these provide sufficient evidence on their own. Firstly, its molecular weight (66 kDa) is similar to the weights of the lepidopteran JHEs and other esterases. Secondly, it is inhibited by OTFP and DFP which inhibit other members of this family and suggest that it has an active site serine. Thirdly, it hydrolyses carboxylester substrates, and lastly, JHE from *H. virescens* has been shown by sequence comparison to be a member of this family.

The N-terminal sequence of *D. melanogaster* JHE (7 residues) is not similar to any of the three published N-terminal sequences of lepidopteran JHEs (*H. virescens*, complete cDNA sequence, Hanzlik *et al.*, 1989; *M. sexta*, 15 residues, Venkatesh *et al.*, 1990; *L. dispar*, 69 residues including a possible insertion of 22-24 amino acids, Valaitis, 1992). However, there is no more similarity in this region among the lepidopteran JHE sequences than among the JHEs and other esterases or among other esterases (15-30%, Valaitis, 1992).

The conversion of purified *D. melanogaster* JHE from a 66 kDa peptide to a 60 kDa peptide may be similar to the single cleavage event observed for Acetylcholinesterase (AChE). Disulphide reduction of purified AChEs from *D. melanogaster* (70 kDa) yields a 55 kDa peptide as well as a 16 kDa peptide which contains the N-terminus of the protein. House fly AChE has similar sized fragments and the cleavage is believed to occur *in vivo*, or during purification (Fournier *et al.*, 1988). Similarly, AChE purified from vertebrates undergoes cleavage during storage. Therefore it has been proposed that the 75 kDa AChE protein of vertebrates is an inactive precursor of a protease which undergoes autolysis to a 25 kDa fragment with trypsin-like activity and a 50 kDa fragment which has carboxypeptidase activity and contains the AChE active site serine (Small, 1990). While the association of protease activity with AChE remains controversial (Wright *et al.*, 1993), it is an interesting possibility that JHE also has protease activity which causes the loss of a 6 kDa fragment during storage. However, such cleavage has not been observed in any other JHEs.

3.4.3 Kinetic Characterisation of JHE

3.4.3.1 JH-Specificity of JHE

Kinetic characterisation of JHE was carried out to determine whether JHE satisfied biochemical criteria for JH-specificity. A "JH-specific esterase" has been defined as an enzyme which has a low K_M for JH, hydrolyses JH with a high K_{cat}/K_M ratio, or, most rigorously, exhibits a higher K_{cat}/K_M ratio for JH than for an alternative substrate such as α-naphthylacetate (Section 1.5.3.1). The kinetic properties of the JH hydrolysing esterase purified from *D. melanogaster* in this study conforms to each of these criteria, indicating that this enzyme is indeed JH-specific.

The specific activity of *D. melanogaster* JHE (Fr6, at least 0.59 μmol/min/mg) indicates a low K_{cat} for JHIII (at least 0.61 sec^{-1}). However, the specificity constant (K_{cat}/K_M) of *D. melanogaster* JHE for JHIII is high (at least 6 x 10^6 $M^{-1}sec^{-1}$ which approaches the diffusion limited maximum value of 10^8-10^9 $M^{-1}sec^{-1}$, Stryer, 1981) due to the very low K_M (89 nM). The high specificity constant and low K_M for JHIII indicate that this enzyme is well adapted to the task of scavenging trace concentrations of JH as was proposed in section 2.4.2.2. These kinetic parameters for *D. melanogaster* JHE are within the range for lepidopteran JHEs (Wing *et al.*, 1984; Table 3.2). The high specificity constants indicate that both the *D. melanogaster* and lepidopteran esterases are specific for JH and the similar, low K_Ms suggest that they encounter similar, low JH concentrations *in vivo*.

Comparison of the kinetic parameters for hydrolysis of JHIII and the structurally different, artificial substrate, α-naphthylacetate, confirm the specificity of *D. melanogaster* JHE for JH. Naphthylacetate inhibition of JHIII hydrolysis is competitive, and both the K_M and K_I values are 120 μM. These results demonstrate that both JHE and α-naphthylacetate are hydrolysed by the same active site. Although JHE can hydrolyse α-naphthylacetate at a higher rate (70 μmol/min/mg) than JHIII (0.59 μmol/min/mg), the K_M of JHE for α-naphthylacetate is much higher than for JHIII and consequently the specificity constant (5.9 x 10^5 $M^{-1}sec^{-1}$) is 12 fold lower than for JHIII. *H. virescens* JHE is the only other example for which kinetic parameters have been determined for the hydrolysis of an artificial substrate, in this case a thioester, $C_6H_{13}OCH_2C(O)SCH_3$ (Ward *et al.*, 1992). In common with the *D. melanogaster* JHE, the *H. virescens* JHE has a higher K_{cat} for the artificial substrate compared with JHIII, a much higher K_M, and consequently a lower specificity constant (Table 3.2). Although the higher rates of hydrolysis with artificial substrates may seem counter-intuitive they can be explained because high binding affinity for the natural substrate is not compatible with rapid hydrolysis.

An additional criterion for the JH-specificity of JHE is its ability to hydrolysis JH in the presence of the JH carrier (Section 1.5.3.1). This criterion was proposed primarily because it is indicative of a low K_M but it also relates to *in vivo* hydrolysis, a major component of which, at least in Lepidoptera, occurs in the presence of high affinity carriers in the haemolymph. A non-specific esterase with a fortuitous ability to hydrolyse JH would be expected to hydrolyse JH only in the absence of competition for JH binding by a specific carrier (Sanburg *et al.*, 1975). The *D. melanogaster* JHE hydrolyses JH in the presence of the dipteran JH carrier, lipophorin, although the carrier comes from another species, *L. cuprina*. However, it is unclear whether coincubation of JHE and lipophorin can be used to model their interaction *in vivo* because it is unknown whether they occur in the same compartment. Lipophorin and JHE may be

located together in the haemolymph of dipteran pupae but in larvae and adults the JH degrading enzymes appear to be located in non-haemolymph tissues (Section 1.6.4).

3.4.3.2 Characterisation of Substrate Binding by JHE

Analysis of inhibition of JHIII hydrolysis by JH isoforms and analogues reveals that almost all deviations from the structure of JHIII reduce the affinity of binding to JHE. The overall size of the molecule is important and even small increases in size, such as the changes from methyl to ethyl side chains found in JHI and JHII, or increases in the size of the methyl ester moiety, decrease binding. While these results might suggest that molecules larger than JHIII are sterically hindered from binding in the active site, esters with larger alcohol moities are not absolutely precluded from hydrolysis, as demonstrated by the rapid hydrolysis of naphthylacetate. Conjugation of the 2,3 double bond with the ester group and the 10,11 epoxide in the R conformation both promote binding to JHE, consistent with all naturally occurring insect JHs having these features (Kindle *et al*., 1989; Hearlt *et al*., 1993). The strongest effect is promotion of binding by the 10R,11 epoxide but the results are equivocal with regard to the features at carbons 6 and 7 (discussed below).

Among the natural JH isoforms, *D. melanogaster* JHE has a higher affinity for JHIII and JHB_3 (which may have hormonal roles in higher Diptera) than for JHI and MF (which are probably not dipteran hormones). The affinity of *D. hydei* JHE for JHAs also has been investigated, with compounds tested as inhibitors of JHI hydrolysis by homogenates of larval integument (Bisser and Emmerich, 1981). The K_M of *D. hydei* JHE for JHI is 640 nM and the data suggest a similar affinity for JHIII. This contrasts with *D. melanogaster* JHE which has a seven-fold lower K_M for JHIII and a five-fold higher K_I for JHI. *D. hydei* JHE has a greater affinity for the JHIII ethyl ester than for JHIII, also in contrast with *D. melanogaster* JHE. These differences might indicate that the binding site of *D. hydei* JHE is more tolerant of bulk both on the carboxyl chain and the alcohol moiety. Alternatively, the affinity of *D. hydei* JHE for JHIII relative to the other compounds may have been underestimated if the homogenates contained JH binding proteins with high affinity and specificity for JHIII. Both the *D. melanogaster* and *D. hydei* JHEs exhibit more than ten-fold lower affinity for MF than for JHIII, suggesting that binding by both enzymes is strongly promoted by the 10,11 epoxide. This conclusion is supported by the low affinity of both enzymes for methoprene.

An *in vivo* role for JHIII is suggested by the 13 fold lower K_M of *D. melanogaster* JHE for JHIII than its K_I for JHB_3. This is not consistent with *in vitro* biosynthetic studies which suggest that JHB_3 may be the only functional isoform in the higher Diptera (Section 1.2). However, the JHIII used in these experiments was a racemate of two optical isomers while the JHB_3 was a racemate of four optical isomers. Thus, the greater dilution of the natural isomer of JHB_3 with unnatural isomers may have exaggerated the difference in binding affinity between the natural isomers of JHIII and JHB_3.

The data for modifications to the 6,7 bond are more difficult to interpret because the order in which modifications to the 6,7 bond promote binding is the reverse from expectations based on biosynthesis data. Thus, when the 10,11 epoxide is present (as in all insect JHs), a 6,7 double bond (as in JHIII) reduces binding affinity for JHE compared with a 6,7 single bond and the 6,7 epoxide (as in JHB_3) further reduces binding affinity. Nevertheless, the correct, 6S optical isomer of the 6,7 epoxide is

Table 3.2. Comparison of Kinetic Parameters for Purified JHEs with JH Isoforms and Artificial Substrates.

Species	Substrate	K_M (nM)	V_{max} (μmol/min/mg)	K_{cat} (sec^{-1})	K_{cat}/K_M ($10^6 M^{-1} sec^{-1}$)	Reference
H. virescens	JHIII	93-103	2.01#	2.18*	22*	Abdel-Aal *et al.*, 1988
H. virescens (baculovirus expressed)	JHIII $C_6H_{13}OCH_2(0)SCH_3$	30 46000	1.57 55.0	1.73 60.8	58 1.3	Ward *et al.*, 1992
H. zea	JHIII	125-197	4.00#	4.33*	39*	Abdel-Aal *et al.*, 1988
M. sexta	JHIII	24	1.08	1.17*	49*	Abdel-Aal and Hammock, 1985
T. ni	JHI JHIII	1130 402	0.058#	0.046*	0.41*	Yuhas *et al.*, 1983
T. ni (pI=5.4/5.5)	(10R,11S) JHII (10S,11R) JHII	352/324 156/139	2.03*/2.12* 0.92*/1.11*	2.2/2.3 1.0/1.2	6.3/7.1 6.4/8.6	Hanzlik and Hammock, 1987
T. ni	JHII	380 70.6	2.13# ND	2.34* 0.53	6.2* 7.5	Rudnicka and Jones, 1987 Abdel-Aal and Hammock, 1988
L. dispar	JHIII (10R,11S) JHII	360[a] 360[a]	0.76[a]# (2.65[b]#) 0.48[a]#	0.79[a]* 0.49[a]*	2.2[a]* 1.4[a]*	[a]Valaitis, 1991; [b]Valaitis, 1992
G. mellonella	JHI	21	0.92#	0.59*	28	Rudnicka and Kochman, 1984
D. melanogaster	JHIII α-naphthyl-acetate	89 120000	0.59 70	0.60 71	6.8 0.59	This study

#assuming near maximal activity with 5μM JH

*calculated from the reported data assuming that the preparation is fully active

ND not determined

preferred strongly to the 6R,7 epoxide, suggesting that JHB_3 is an *in vivo* substrate. However, it may be possible to rationalise these data as follows. The presence of a 6,7 single bond would increase the flexibility of the molecule which might enable the incorrect 10S,11 epoxide present in the racemic, experimental mixture to more strongly promote binding. In the absence of the (racemic)10,11 epoxide the natural 6,7 double bond is preferred as expected. Therefore I predict that the 6,7 double bond would have been preferred if 10R-JHIII and its hydrogenated analogues had been available for this experiment.

Binding of JHAs by JHE is promoted by the features found in natural JHIII and JHB_3 and reduced by almost any departure from these, suggesting that both molecules are *in vivo* substrates. However, two JHAs which contain an hydroxyl but lack an epoxide group exhibited unusually high binding affinity for JHE. The location of the hydroxyl groups near the positions of the epoxides of JHB_3 suggests either that the hydroxyls can fortuitously substitute for the epoxides in the binding site or that hydroxyls might be found in natural substrates. Incubation of JHB_3 with *D. melanogaster* cell fractions gives rise by epoxide hydrolysis to epoxy-diol and tetraol products of JHB_3 epoxide hydrolysis together with *cis*- and *trans*-tetrahydrofuran diols (Casas *et al.*, 1991). These compounds have the potential to be *in vivo* substrates for JHE but were not tested in this study. However, as JHIII-diol is not readily bound nor hydrolysed by JHE, the role of hydroxyl-containing substrates remains unclear.

3.4.3.3 Interaction of JHE with Lipophorin

While JHE activity is clearly regulated at the level of synthesis and degradation of JHE (Ichinose *et al.*, 1992a,b), it is possible that its activity is also modulated by interaction with other molecules such as the JH carrier. Two types of interaction with the JH carrier protein have been proposed (Section 1.5.2). The first type of interaction is a simple mass-action model: Sequestration of JH by the carrier could be expected to reduce the rate of hydrolysis. However, without the carrier JH could bind non-specifically at inaccessible sites *in vivo* and so might never be available either at its target sites or for degradation. Thus, the carrier might at one time protect JH from hydrolysis and at another time assist in making it available for hydrolysis.

A second type of interaction occurs in *L. maderae* in addition to mass-action effects. This interaction requires direct contact between between the carrier, lipophorin, and JHE. In this species lipophorin is also a non-competitive inhibitor of JHE; the rate of JH hydrolysis being reduced in the presence of lipophorin regardless of the JH concentration (Engelmann, 1984; Engelmann *et al.*, 1988).

L. cuprina lipophorin does not alter the V_{max} of JH hydrolysis by *D. melanogaster* JHE but it does reduce the rate of JH hydrolysis at lower substrate concentrations. This can be entirely accounted for by a reduction in the free concentration of JH. Thus, only the mass-action model is supported and there is no evidence of direct, inhibitory interaction between lipophorin and JHE in *D. melanogaster*. However, a direct interaction between the *D. melanogaster* lipophorin and JHE cannot be entirely discounted because *L. cuprina*, not *D. melanogaster*, lipophorin was used in this study. This could be tested either by purifying the *D. melanogaster* lipophorin or by immunoprecipitation of the *D. melanogaster* lipophorin using antibodies against *L. cuprina* lipophorin which cross-react (Dr. S. Trowell, pers. comm.).

It is not clear whether all the circulating JH in *D. melanogaster* is bound to lipophorin but this carrier has the lowest reported K_D for JHIII (1.5 nM) of any JH carrier, 59 fold lower than the K_M of JHE (Shemshedini and Wilson, 1988). The rapid dissociation rate between lipophorin and JHIII suggests that lower quantities of lipophorin might assist JHE and JHEH in the rapid clearance of JH *in vivo* but higher quantities could prevent hydrolysis. Knowledge of the developmental regulation of lipophorin in *D. melanogaster* is therefore necessary for better understanding of its role in JH titre regulation but this has not been reported.

The mass-action interactions between JHE and the JH carrier may differ between dipterans and lepidopterans because lepidopterans do not use lipophorin as their JH carrier (Trowell, 1992). The limited available data suggest that the K_Ds of lipophorin for JH are much lower than the K_Ms of JHEs in orders which use lipophorin as the JH carrier (Trowell, 1992). Furthermore, the concentrations of lipophorin appear to exceed JH titres by a considerable margin. Thus, lipophorin may be able to prevent hydrolysis by competition for binding (Trowell, 1992; King and Tobe, 1993). In contrast, Trowell (1992) suggests that the lepidopteran carrier may have limited effectiveness in protecting JH from hydrolysis because the K_Ds of these JH carriers and the K_Ms of lepidopteran JHEs are similar. However, in *T. ni* haemolymph, the slow dissociation rate of JH from the JH carrier limits the rate of JH hydrolysis (Abdel-Aal and Hammock, 1988). In the blattodean, *D. punctata* a temporally regulated decrease in lipophorin concentration is required for JH hydrolysis and a decline of JH titre (King and Tobe, 1993). In contrast, in several other insects, the titre of the carrier rises when JH titre falls and/or JHE activity increases, supporting the proposal that the carrier assists in the clearance of JH (Section 4.3.4). Thus, although the nature of the interactions may differ between species, JH carriers appear to have major roles in the regulation of JH titre.

Chapter 4

General Discussion

4.1 Introduction

This study has characterised JH hydrolysis in *D. melanogaster* and is the first systematic analysis of JH hydrolysis in any dipteran. Two enzymes have been identified as contributing to JH hydrolysis, JHE and JHEH, and this chapter discusses the evidence that these enzymes hydrolyse JH *in vivo*. The results presented in previous chapters are combined with the available data from the literature to develop a generalised model of the regulation of dipteran JH titre. The model considers the possible interaction of JH degradation with the JH carrier and JH synthesis, including the possibility of differential regulation of the JH isoforms, JHB_3 and JHIII. Dipteran JH regulation is compared with regulation in other insect orders, especially the Lepidoptera, to assess the universality of the model. Finally, unresolved issues and future directions for their resolution are discussed.

4.2 The Specificity of JH Hydrolysing Enzymes and Naphthylacetate Hydrolysis

4.2.1 JH-Specificity of *D. melanogaster* JH Hydrolysing Enzymes

Several lines of evidence suggest that both JHE and JHEH exhibit a high degree of specificity for JH as an *in vivo* substrate rather than functioning as general hydrolytic enzymes. Biological evidence for the JH-specificity of JHE includes maximal expression at the prepupal stage. This is a time when JH titre is known to fall, JH synthesis has ceased, interspecific comparisons suggest a requirement for low JH titre, and *D. melanogaster* are known to be sensitive to exogenous JH. Similarly, biological evidence that JHEH is JH-specific includes maximal expression during the prewandering period of the final larval instar when "it is becoming increasingly clear that there is an important endocrine event" (Cherbas, 1993), JH synthesis and titre are low, and low JH titre and increased JH hydrolysis are required in Lepidoptera (Roe and Venkatesh, 1990). The inducibility of JHE by JH in adults suggests that JH-specific JHE is involved in feedback regulation of JH titre, even though the activity of the enzyme is relatively low at this stage (Ms. S. J. Dent, pers. comm.).

Further evidence of the JH specificity of JHE is provided by its biochemical properties but the biochemical properties of JHEH were not characterised in this study. JHE has a very low K_M for JHIII and a high specificity constant, K_{cat}/K_M, which is greater for JHIII than for the artificial substrate, naphthylacetate. Furthermore, binding experiments demonstrated that JHE exhibits binding selectivity for JHIII and JHB_3 over other JH isoforms and analogues, and these are the JH isoforms most likely to be important in the higher Diptera.

4.2.2 The Relationship Between JH Hydrolysis and Naphthylacetate Hydrolysis

The relationship between JHE and naphthylacetate hydrolysing esterases has been contentious ever since the discovery that JH induces several naphthylacetate hydrolysing esterases in *Hyalophora gloveri* pupae which either hydrolyse JH or comigrate with JHE during native PAGE (Whitmore *et al.*, 1972). It was proposed that "general", naphthylacetate hydrolysing esterases might hydrolyse JH *in vivo* in the absence of a protective JH carrier, whereas the specific JHE could also hydrolyse JH bound to the carrier, and that these two classes could be distinguished by DFP inhibition of the general esterases (Sanburg, 1975). There have been several subsequent attempts to identify JHE among esterases separated by native PAGE and visualised with naphthylacetate (East, 1982; Rauschenbach *et al.*, 1987; this study). It is now clear that some purified JHEs cannot hydrolyse naphthylacetate (*L. maderae*, Gunawan and Engelmann, 1984; *G. mellonella* (pI = 5.0 variant), Rudnicka and Kochman, 1984, and *M. sexta*, Couldron *et al.*, 1981) whereas others can (*T. ni*, Hanzlik and Hammock, 1987; *G. mellonella* (pI = 4.75 variant), Rudnicka and Kochman, 1984), including *D. melanogaster* (this study).

Naphythylacetate hydrolysis by JHE is never more than a minor component of the total naphythylacetate hydrolysing capacity of insects. However, any compound which contains the ester moiety may have some binding affinity for JHEs, so long as it is not sterically hindered from access to the JHE active site. As naphthylacetate shares no other features with JH to promote specific binding, high rates of hydrolysis require high substrate concentrations. JHEs from *T. ni* and *H. virescens* have been shown to rapidly hydrolyse other artificial substrates but also require high substrate concentrations (Hanzlik and Hammock, 1987; Ward *et al.*, 1992).

In contrast, it is unlikely that non-specific esterases hydrolyse JH at low, *in vivo* concentrations, as the ester group of JH is both stabilised and unable to rotate, due to conjugation with the 2,3 double bond. Therefore, hydrolysis of JH requires the enzyme to provide greater activation energy but substrate binding is more sterically constrained. Thus, significant ester hydrolysis of JH is unlikely except by an enzyme with features that promote specific binding, such as found for *D. melanogster* JHE. This is supported by the finding that the only zone with JHE activity after native PAGE of *D. melanogaster* pupae corresponds to the specific JHE, even though greater than physiological concentrations of JH were used in the assay and several other esterases were present (Section 2.3.2.1). Furthermore EST6, one of the most active *D. melanogaster* esterases, has no activity with JHIII (Myers, 1990) although it is inducible with JH (Stein *et al.*, 1984; Dr. M. J. Healy and Ms. A. Cao, unpublished data)

4.3 General Model for JH Regulation in Diptera

The significance of lepidopteran JH hydrolysis to the regulation of JH titre is indicated, in part, by the strong conservation of its temporal and spatial expression patterns among lepidopterans. Therefore, in order to interpret the significance of JH hydrolysis in *D. melanogaster*, its physiological properties are compared here with data from other dipterans. The data from this study provide a framework to which the more

scattered data from other dipterans can be fitted to generate an overview of dipteran JH metabolism. Subsequent comparison of the pattern of expression of dipteran JH metabolism with the available data for JH synthesis and titre reveals a model for dipteran JH regulation that is broadly similar to that proposed for other orders.

4.3.1 General Pattern of Expression of JH hydrolysis in Diptera

The major observations from several studies of the temporal and subcellular expression patterns of JH hydrolysing enzymes in Diptera are as follows. Adults of *M. domestica* and *D. melanogaster* contain both JHE and JHEH activities, predominantly in the microsomal fraction (Yu and Terriere, 1978a; Ottea *et al.*, 1987) and not in the haemolymph (Wilson and Gilbert, 1978). JHEH in *S. bullata* and *P. regina* is also predominantly found in the microsomal fraction in adults (Yu and Terriere, 1978b). Most of the JHEH activity of larvae of *D. melanogaster* (Casas *et al.*, 1991; Harshman *et al.*, 1991) and *D. hydei* (Klages and Emmerich, 1979) appears to be mitochondrial and microsomal, supporting data from other insects that JHEH is generally membrane-bound (Roe and Venkatesh, 1990). No JH degrading enzymes are found in the haemolymph of *D. melanogaster* or *D. hydei* final instar larvae before pupariation (Wilson and Gilbert, 1978; Klages and Emmerich, 1979; Shemshedini and Wilson, 1988). However, JH degrading enzymes are found in the fat body and body wall of *D. hydei* larvae. A maximum of JHE activity occurs in the prepupal haemolymph of *D. hydei* four hours after the formation of the puparium but activity then declines gradually through the entire pupal stage (Klages and Emmerich, 1979). The above data are consistent with the finding that microsomes of *M. domestica*, *S. bullata* and *P. regina* larvae and adults contain higher activities of JH degrading enzymes than are present in pupal microsomes, however non-microsomal activities were only determined for adults (Yu and Terriere, 1978a,b). In *M. domestica* larvae, a prewandering peak of microsomal JHEH activity occurs (Yu and Terriere, 1978b). In *D. virilis* low JHE activity is found in final instar larvae but a maximum for JHE is found in pupae. In contrast, JHEH activity is highest earlier in final instar larvae. Thus, a minimum for JH hydrolysis occurs just prior to pupariation (Rauschenbach *et al.*, 1991). To these observations can be added the results of my study that prewandering and prepupal maxima of JH hydrolysis occur in *D. melanogaster*, and that JH degrading activities in larvae and adults are predominantly mitochondrial and microsomal, whereas the pupal activity is predominantly soluble JHE.

Taken together, these data suggest the following generalisations about the pattern of expression of JHE and JHEH in Diptera. In final instar larvae, JH hydrolysing enzymes are found in tissues other than the haemolymph and activity is greatest during the prewandering period. The relative contributions of JHE and JHEH to total activity vary among species but JHEH is always important. There is a minimum of activity late in the final instar, but a high level of soluble, circulating JHE activity occurs soon after the formation of the puparium and before the pupal moult, probably clearing JH from the whole organism. This JHE is retained, though declining in activity, through the pupal stage. In the adults low levels of both JHE and JHEH are found, though with different subcellular distribution.

4.3.2 Correlation of JH Hydrolysis with JH Production and Titre Changes

The developmental profile of JH hydrolysis correlates inversely with JH titre and production in *D. melanogaster* (section 2.4.2). Less complete data suggest that *D. hydei* exhibits a very similar inverse correlation of JH hydrolysis (Klages and Emmerich, 1979) with JH titre (Bührlen *et al.*, 1984) but JH production has not been investigated in *D. hydei*. JHIII was the only JH isoform detected when the developmental profiles of JH titres in *D. melanogaster* and *D. hydei* were determined. However, JHB_3 was neither proposed to be a hormone nor tested for at the time of these studies. Developmental changes in JH hydrolysis in *D. melanogaster* correlate well with critical endocrinological events. The relationships between changes of JH hydrolysis, production and titre and other processes during important periods in dipteran development are discussed below (and illustrated in Figure 2.8).

4.3.2.1 The Prewandering Period of the Final Larval Instar

In *D. melanogaster* at the prewandering stage a small peak of ecdysone occurs (Cherbas, 1993), JH titre is reduced (Bownes and Rembold, 1987; Sliter *et al.*, 1987), and this study now shows that JH hydrolysis is elevated. *D. hydei* also exhibits a reduction of JH titre just prior to the onset of wandering (Bührlen *et al.*, 1984). Although this has not been related to changes in rates of JH hydrolysis, JHE and JHEH occur in *D. hydei* tissues at this time (Klages and Emmerich, 1979) and a prewandering peak of microsomal JHEH occurs in *M. domestica* (Yu and Terriere, 1978b). In lepidopterans prewandering JHE reduces JH titre and leads to a prewandering ecdysone peak (Jones, 1985; Watson *et al.*, 1987).

These changes in endocrine status are associated with numerous changes in behaviour and gene expression in both dipterans and lepidopterans (Cherbas, 1993). Thus, the function of the prewandering ecdysone peak together with low JH titre appears to be induction of the first events in the onset of metamorphosis (Andres and Thummel, 1992; Shaaya, 1993). At the molecular level one of the first responses in insect epidermal cells is a brief cessation of RNA synthesis followed by the production of a novel RNA type (hetero-disperse nuclear RNA, hnRNA) (Shaaya, 1993). The hnRNA may have a role in the permanent repression of larval mRNA synthesis and/or the induction of pupal genes. The changes in RNA synthesis are observed in the lepidopterans, *M. sexta* and *Ephestia cautella*, and the dipteran *Calliphora vicina*. The importance of the low JH titre is indicated by application of the JH analogue, methoprene, which, if applied before the appearance of hnRNA, prevents these changes in RNA synthesis and delays the larval-pupal transformation .

4.3.2.2 Prepupae/Pupae

During the late larval/prepupal period two further ecdysone peaks occur in *D. melanogaster*. The first of these occurs just prior to puparium formation (Andres and Thummel, 1992) and coincides with high JH titre (Sliter *et al.*, 1987) and JH production (Richard *et al.*, 1989a). This study now shows that a minimum of JH degradation also occurs at this time. The second ecdysone peak occurs 10 hours later (Andres and Thummel, 1992) and coincides with a low JH titre (Bownes and Rembold, 1987) and the pupal apolysis (Bainbridge and Bownes, 1981). It also follows a cessation of JH synthesis (Richard *et al.*, 1989a) and this study now shows that a

maximum of JH degradation occurs at this time. An equivalent pattern of ecdysteroid peaks is also observed in the dipteran, *Sarcophaga crassipalpis* (Zdárek and Denlinger, 1987). In *D. hydei*, a peak of JH occurs after the onset of wandering. Late in the final instar JH titre falls ahead of the prepupal peak of haemolymph JHE activity which does not appear until shortly after pupariation and JH is barely detectable during the prepupal stage (Klages and Emmerich, 1979; Bührlen *et al.*, 1984).

These events in Diptera all correspond well with similar events in the Lepidoptera (Baker *et al.*, 1987): lepidopteran pupation is initiated by the coincidence of a pulse of ecdysone and a pulse of JH production and JH titre then falls to low levels ahead of the prepupal peak of JHE. The JH titre falls to essentially zero when the prepupal JHE appears, and this is followed by pupal apolysis.

The prepupal pulse of JH is believed to prevent adult differentiation of the imaginal discs during the pupal moult (Kiguchi and Riddiford, 1978). In both lepidopterans and dipterans, JH titres fall to low levels ahead of high levels of JHE. Thus, it appears that the function of elevated prepupal JHE activity is to scavenge for trace levels of JH after most has been removed by constitutive levels of the enzyme or other means (Baker *et al.*, 1987). Inhibition of the lepidopteran prepupal JHE peak causes larval-pupal intermediates and blocks ecdysis (Newitt and Hammock, 1986). In *D. virilis* the organophosphate esterase inhibitor paraoxon causes pupal death when applied to prepupae at concentrations which do not inhibit the majority of naphthylacetate hydrolysing esterases (Rauschenbach *et al.*, 1991). As paraoxon is a potent inhibitor of JHE in several dipterans (Yu and Terriere, 1978b; Klages and Emmerich, 1979) this effect may be due to inhibition of JHE.

The effects of inhibitors of JH hydrolysis have not been investigated *in vivo* in *D. melanogaster*. The identification of selective inhibitors of JHE and their use at this time in *D. melanogaster* may identify specific metamorphic changes which require JHE activity. These may include the secretion of the pupal abdominal cuticle and subsequent replacement of larval epidermal cells by abdominal histoblasts as this process is disrupted by JH application (Section 1.4.1).

During the pupal stage of *D. melanogaster* a further ecdysone peak occurs (Riddiford, in press) coinciding with pupal to adult apolysis (Bainbridge and Bownes, 1981). Pupal JH titre is low (Bownes and Rembold, 1987; Sliter *et al.*, 1987) and this study shows that JH hydrolysis is elevated though declining throughout this period. Similarly, JHE activity remains elevated though declining throughout the pupal stage of *D. hydei* and JH remains barely detectable (Klages and Emmerich, 1979; Bührlen *et al.*, 1984). Application of JH to pupae of the dipteran, *S. bullata* causes the secretion of a second cuticle with pupal characteristics (Srivastava and Gilbert, 1968; 1969). Thus, in common with Lepidoptera and other insects, the change from pupal to adult differentiation requires the absence of JH (Willis, 1981; Willis and Cox, 1984).

4.3.2.3 Adults

In *D. melanogaster*, JHIII titre rises rapidly after adult eclosion and remains high (Bownes and Rembold, 1987). Maximum JHB_3 production occurs in adult female *D. melanogaster* at the same time as other indicators of sexual maturity on day 2 (Altaratz *et al.*, 1991) and JH hydrolytic activity remains low during the first five days of the adult stage. Thus, an elevated JH titre correlates with elevated JH synthesis and low hydrolytic activity. However, mated and virgin *Drosophila* have not been compared for JH titre or JH hydrolysis. JH hydrolysis and production have also not

been investigated in adult *D. hydei* although JH titre rises less rapidly in female *D. hydei* than *D. melanogaster*, and less rapidly still in male *D. hydei*, reflecting their slower rates of sexual maturation (Bührlen *et al.*, 1984).

Although JHE and JHEH activities are lower in adult *D. melanogaster* than at other stages, JHE can be induced about 30% in females and 60% in males by topical application of JHIII (Ms. S. J. Dent, pers. comm.). Furthermore, JH synthesis appears to be required for stimulation of yolk protein uptake by oocytes (Saunders *et al.*, 1990), suggesting that regulation of JH titre by both JH synthesis and hydrolysis remains important in adults. Thus, JH synthesis and hydrolysis may vary in response to mating or other conditions which have not yet been tested.

In contrast to prepupae, more than half of the JHE as well as the JHEH activity is found in membranous cell fractions of whole adults (Section 2.3.2.3), suggesting that hydrolysis might mainly occur in specific tissues rather than throughout the organism. Thus, tissues may independently modulate the JH signal by hydrolysis or turn off the JH signal after it has reached its site of action.

JHE has a clear role in the regulation of JH during egg production in adult females of the dipteran *A. aegypti* (Shapiro *et al.*, 1986; Borovsky *et al.*, 1992) At the start and finish of a cycle of egg production JH titre is high and JHE activity is low, whereas the opposite occurs in the middle of the cycle. Inhibition of JHE causes an increase in JH titre and a reduction in egg hatch. Similar relationships between egg production, JH titre and JH hydrolysis have been observed in an orthopteran (Renucci *et al.*, 1984), a blattodean (Tobe *et al.*, 1985), and appear likely in a lepidopteran (Venkatesh *et al.*, 1988).

4.3.2.4 Further Analysis of JH Titre Regulation

In order to fully understand the role of hydrolysis in the regulation of JH titre, further investigation in several areas in required. The investigation of the pattern of expression of JH hydrolysing enzymes should be extended to include earlier larval stages, tissue localisation and the response of enzyme activities to mating. Determination of the effects of inhibition of JHE and JHEH *in vivo* would also contribute to understanding of the roles of these enzymes. However, care would be required for the interpretation of such experiments because other esterases or epoxide hydrolases may be inhibited. Paraoxon and OTFP (among others) have been used to inhibit JHEs *in vivo* but both inhibit numerous other *D. melanogaster* esterases (Healy *et al.*, 1991). To complement such data more detailed titres of both JHIII and JHB_3 throughout development are required.

4.3.3 Compartmentalisation and Relative Contributions to JH Hydrolysis of JHE and JHEH

The relative contributions of JHE and JHEH to prewandering activity vary among dipteran species although JHEH is always important. Comparison with *D. hydei* and Lepidoptera suggests that prewandering dipteran JH hydrolysing enzymes will be found in a wide range of tissues. In contrast it is probable that a large portion of the dipteran prepupal JHE will be found in the haemolymph because *D. melanogaster* JHE is soluble and in *D. hydei* and Lepidoptera it is found in the haemolymph. Comparison of the dipteran and lepidopteran developmental profiles of hydrolysis, synthesis, and titre suggest that the function of hydrolysis by both JHEH and JHE at both these times

is to clear JH from the whole organism (Section 4.3.2). In adult dipterans, low levels of both JHE and JHEH are found, though with different subcellular distribution.

While most studies of JH hydrolysis in insects have concentrated on haemolymph, a few studies of lepidopteran larvae have investigated hydrolysis in other tissues. These studies indicate that JHE and JHEH activities in tissues exhibit prewandering and prepupal peaks, suggesting that non-haemolymph JH hydrolysis may be as important as haemolymph hydrolysis (Section 1.6.2). Thus, the most obvious difference between JH hydrolysis in dipterans and lepidopterans is the lack of JHE in the haemolymph of prewandering dipterans.

The lack of JHE in the haemolymph of prewandering dipterans may not be unusual. The coleopteran, *Tenebrio molitor*, has a haemolymph JHE peak which coincides with pupal ecdysis and a JH sensitive period. Thus, its prepupal pattern resembles both Diptera and Lepidoptera (Connat, 1983). However, in common with the Diptera and unlike the Lepidoptera, no other haemolymph JHE peak occurs during the final larval instar. Whole larvae of another coleopteran, *Tribolium castaneum*, exhibit high levels of non-haemolymph JHEH activity (Connat, 1983), suggesting that the pattern of expression of JH hydrolytic enzymes in Coleoptera may more closely resemble the dipteran model than the lepidopteran model. While JHEH activities have not been studied in the same detail as JHE, JHEH occurs in whole organisms in conjunction with JHE at a variety of developmental stages in many insect orders (Slade and Zibitt, 1972; Ajami and Riddiford, 1973). Thus, it is not clear why prewandering dipterans and coleopterans lack haemolymph hydrolysis, or alternatively, why the Lepidoptera need it. It is also unknown whether JHE and JHEH differ in their functions.

JHE may be absent from general circulation at the prewandering stage of some insects including Diptera because the elimination of JH is only required by some tissues. For example, JH hydrolysis in imaginal discs from final instar *D. melanogaster* larvae appears to be sufficient to render these tissues insensitive to circulating JH, with much greater than physiological concentrations of JH required *in vitro* for inhibition of ecdysone-induced evagination (Chihara *et al.*, 1972). Alternatively, JH hydrolysis within restricted tissues may be sufficient to eliminate JH throughout the organism. The latter has not been shown in dipteran larvae but non-haemolymph metabolism appears to be sufficient for JH clearance from whole adult *M. domestica*; in a comparative study across six insect orders the haemolymph JHE activity of adult *M. domestica* was the lowest found, yet the half life of injected JHI was lowest in this species (de Kort *et al.*, 1979).

There is considerable variation in the relative titres of JHE and JHEH both within and between insect orders. However, there does appear to be a trend for greater hydrolysis by JHEH at the prewandering stage of the final instar and greater hydrolysis by JHE at the prepupal stage (Section 1.6.1). The significance of this observation is far from clear although there are several hypotheses. Firstly, different routes of hydrolysis may influence the ultimate fate of JH. Hydrolysis by JHEH causes absolute loss of JH from circulation because there is no known mechanism for reformation of the epoxide group from JH-diol. In contrast, JH-acid can be methylated by JH methyltransferase to reform JH in some tissues (Sparagana *et al.*, 1985). Thus, in the presence of JH-acid, some tissues may be able to maintain a locally elevated JH titre in spite of generally reduced titres, or influence other tissues by release of remethylated JH. Tissues with

JH methyl transferase activity include imaginal discs, suggesting a mechanism by which developing discs might influence the timing of metamorphosis.

In addition, differences between substrate specificities of JHE and JHEH may permit the selective hydrolysis of different JH isoforms. To date, there is evidence for more than one JH isoform in the Lepidoptera and the Diptera but no functional differences between JH isoforms have been demonstrated. In *D. melanogaster* larvae the relative rates of JHEH hydrolysis of JHB_3 and JHIII differ between subcellular fractions, suggesting that there is more than one JHEH variant and that the two JH isoforms might be hydrolysed at different rates at different sites *in vivo*.

Investigation of the tissue localisation of JHE and JHEH by dissection and assay of individual organs is an important future direction for research. However, high resolution analysis would require antibodies raised against purified enzymes. *D. melanogaster* JHEH has not been purified but the JHE purification protocol developed for the current study would enable the generation of JHE antibodies. Such approaches could be complemented with *in situ* RNA hybridisation studies once the genes for JHE and JHEH are isolated.

Determination of the tissue localisation of JHE and JHEH at times of minimum JH hydrolytic capacity is important to assess the relationship between local titres of JH within tissues and the circulating titres. It may be that the enzymes will be found at high concentration in restricted tissues which need to maintain low local JH concentrations in spite of high circulating titres. Kinetic characterisation of purified JHEHs would also be an important complement to the current study.

4.3.4 The Role of Lipophorin in JH Titre Regulation

This study has demonstrated that changing the relative concentrations of dipteran JHE and lipophorin modulates the rate of *in vitro* JH hydrolysis by JHE but it is not known whether lipophorin modulates JH hydrolysis *in vivo*. The *in vivo* concentration and JHIII binding kinetics of lipophorin from haemolymph of *D. melanogaster* wandering larvae have been determined but it is not clear whether this lipophorin titre is sufficient to bind all the JH because of uncertainty about the JHIII titre (Shemshedini and Wilson, 1988). Furthermore, the titre and binding kinetics of JHB_3 with lipophorin are unknown and it is also unknown whether lipophorin is developmentally regulated in *D. melanogaster*.

However, studies of the high affinity JH carrier protein of *A. aegypti* (presumably lipophorin) suggest that it has a role in the regulation of JH titre. A six-fold increase in JH carrier protein occurs within one hour of a blood meal, coinciding with a JH titre decline and a JHE peak which are required for egg production (Shapiro *et al.*, 1986; Thomas *et al.*, 1986 cited in Borovsky *et al.*, 1992). However, in *M. domestica*, lipophorin exhibits only slight titre variation through development (de Bianchi *et al.*, 1987).

The titres of JHE and the JH carrier also rise and fall in concert in haemolymph of the lepidopteran, *G. mellonella*, and the blattodean, *L. maderae*, (Ozyhar *et al.*, 1983; Engelmann *et al.*, 1988). In these examples the increase in carrier titre is proposed to assist in the clearance of JH. Therefore, interaction between JH carriers and hydrolytic enzymes appears to be important in at least three insect orders. However, the nature of the interaction differs between blattodeans. In contrast to *L. maderae*, a specific decline of lipophorin titre in *D. punctata* is proposed to be critical for clearance of JH from the

haemolymph (King and Tobe, 1993). The decline, which occurs just prior to oviposition, coincides with elevated haemolymph JHE and reduced JH titre. The titres of JH and lipophorin, the K_M of JHE, and the K_D of lipophorin for JH, lead to the conclusion that virtually all the JH would be bound and JH hydrolysis prevented *in vivo* if the decline in lipophorin titre did not occur. These contrasting examples demonstrate that careful consideration of all the kinetic parameters describing the behaviour of both the hydrolytic enzymes and the carrier, together with accurate titres of all components of the system, will be required for prediction of interactions. It should be noted also that developmental changes of lipophorin titre may occur due to changing requirements for another of its functions such as lipid transport (Trowell, 1992).

Whether circulating lipophorin affords protection to JH from intracellular enzymes depends in part on how readily JH moves between compartments. JH synthesised by the corpus allatum *in vitro* is protected from degradation by enzymes within the organ by lipophorin added to the incubation medium, suggesting that mass action effects for binding can act across tissue boundaries (Lanzrein *et al.*, 1993). However, it remains to be determined whether haemolymph JH carriers can afford protection from enzymes in other tissues.

4.4 Roles and Regulation of JH Isoforms

4.4.1 Identification and Functions of JH Isoforms

The identification of the functional JH isoform(s) of the higher Diptera remains inconclusive (Section 1.2), although as the major biosynthetic product, JHB_3 is probably functional . However, the role of JHIII is not yet defined. JHIII may be merely a precursor of JHB_3 that has no other function, or it may have a role which is more or less distinct from that of JHB_3.

Bioassays in *D. melanogaster* provide examples of both greater sensitivity to JHIII over JHB_3 and vice versa, suggesting that the two JH isoforms have different functions. JHB_3 has more potent effects on ovarian maturation than JHIII and it may be significant that the synthesis of JHB_3, but not JHIII, is developmentally regulated in adult females (Saunders *et al.*, 1990; Altaratz *et al.*, 1991). In contrast, JHIII is about ten-fold more potent than JHB_3 for production of various adult defects when topically applied to white prepupae and synthesis of both JHIII and JHB_3 is developmentally regulated at this stage (Richard *et al.*, 1989a,b). This might suggest that the role of JHIII is restricted to metamorphosis whereas JHB_3 has both a metamorphic and reproductive role. Such results should be treated with caution, however, because non-physiological JH isoforms and analogues are sometimes more potent, possibly due to less rapid degradation (Schwieter-Peyer, 1973; Wyatt *et al.*, 1987; Richard *et al.*, 1989b). The binding specificity of *D. melanogaster* JHE revealed in my study supports a function for both isoforms because JHIII and the natural optical isomer of JHB_3 are both bound with high affinity by JHE.

More than one JH isoform occur in at least two holometabolous insect orders (Diptera, if JHIII is functionally important *in vivo*, and Lepidoptera) and one hemimetabolous insect order (Hemiptera, Numata *et al.*, 1992). The appearance of more than one JH isoform in an insect may reflect a need for more complex regulation of physiological events in some orders (Bollenbacher, 1988; Cusson *et al.*, 1991). It

may be possible to infer any differences in the roles of the JH isoforms by observing temporal or spatial correlations between isoform-specific degradation, isoform titres, physiological events and isoform sensitivity in different bioassays. It is noteworthy that differential regulation of two ecdysteroids occurs in the lepidopteran, *Orgyia postica*, suggesting that ecdysteroid isoforms may also have different functions through development and between the sexes. However, no such functions have been defined (Gu *et al.*, 1992).

Although no distinct roles for JH isoforms have been demonstrated, differences in carrier binding, rates of production and rates of degradation suggest that JH isoforms are differentially regulated in both Diptera and Lepidoptera. These issues are discussed below.

4.4.2 Differential Regulation of JH Isoforms

4.4.2.1 Synthesis of JH Isoforms

Although developmental profiles for dipteran JHB_3 titre have not been determined, the available evidence suggests that differential regulation of JHIII and JHB_3 could occur at the level of synthesis and/or degradation. In final instar larvae of *D. melanogaster* production of JHB_3 and JHIII rise and fall in concert but in adult females the production of JHIII remains largely unchanged as the production of JHB_3 changes (Richard *et al.*, 1989a; Altaratz *et al.*, 1991). The presumed precursor of JHB_3, farnesoic acid, has no effect on *in vitro* JHB_3 production by corpora allata of adult *C. vomitoria* but does lead to an increase in JHIII production, suggesting that a 6,7 epoxidase could be regulated to determine whether the product of the pathway is JHIII or JHB_3 (Duve *et al.*, 1992).

Among the Lepidoptera the profile of JH isoform synthesis changes during development. For example, in *M. sexta* JH production shifts from exclusively 4-MeJHI and JH0 in embryos, to predominantly JHI and JHII in larvae, to JHII and JHIII in adults (Schooley and Baker, 1985). Also, JHII titre exceeds JHI at the start of the final instar but the reverse occurs for rest of the instar (Baker *et al.*, 1987).

4.4.2.2 Degradation of JH Isoforms

Selective degradation of JH isoforms can cause the actual ratio of JH isoforms to differ from the synthesised ratio (Szolajska, 1991). The relative abundance of JH isoforms could be developmentally regulated if multiple JH degrading enzymes with different isoform specificity are expressed at different times. Similarly, the relative abundance of JH isoforms could be spatially regulated by spatially regulated JH degrading enzymes. Isoform-specific degradation can be further modulated by selective protection of JH isoform(s) by the JH carrier (Szolajska, 1991).

The present study shows that the predominant route and site of JH hydrolysis changes through development, indicating the potential for developmental and spatial regulation of isoform selective degradation. Furthermore, the available evidence suggests that more than one JHEH variant occurs in larval tissues, that these differ in their specificities for JHIII and JHB_3, but that JHIII is generally the preferred substrate (Casas *et al.*, 1991; Harshman *et al.*, 1991). Prepupal JHE has a higher binding affinity for JHIII over JHB_3 which may also indicate selectivity for JHIII (Section 3.4.3.2), but there may be a different JHE variant in adult *D. melanogaster* (Section 2.4.3), and the maximum rate of hydrolysis of JHB_3 by JHE remains to be tested. JHEs from several

lepidopteran species also exhibit variation of K_M and/or specificity constants for different JH lepidopteran isoforms (Table 3.2), which may contribute to isoform specific regulation.

4.4.2.3 Interactions Among Carrier Binding, Synthesis and Degradation of JH Isoforms

Developmental and spatial regulation of particular JH isoforms may be achieved by a balance of synthesis and degradation, modulated by carrier binding, as is proposed for general JH regulation. *L. cuprina* and *D. melanogaster* lipophorins both have high affinities for JHIII, with preliminary results suggesting that *L. cuprina* lipophorin has a lower affinity for JHB_3 (Shemshedini and Wilson, 1988; Dr. S. C. Trowell, pers. comm.). Thus, abundant lipophorin might protect JHIII from hydrolysis but leave JHB_3 largely unbound and available for hydrolysis. At another time a lower lipophorin titre might not afford protection to either JHIII or JHB_3 (and may promote transport to the site of degradation). Hydrolytic enzymes might then hydrolyse their preferred substrate, JHIII. Thus, interaction between lipophorin and JH hydrolysing enzymes could cause the actual ratio of JHIII to JHB_3 to differ from the synthesized ratio.

In an analysis of the kinetics of binding of JH to JHE and the JH carrier in *T. ni* it was concluded that "even low levels of JHE could shift the relative titres of JH homologues observed if their relative equilibrium and kinetic dissociation constants with the JH binding protein differed" (Abdel-Aal and Hammock, 1988). Supporting the hypothesis that interaction between the carrier and hydrolytic enzymes can affect the relative abundance of natural JH isoforms is the observation that interaction between carriers and JHEs can result in preferential hydrolysis of one optical isomer of JH from experimental racemic mixes. Whether the physiological or non-physiological isomer is hydrolysed depends on whether the carrier or the esterase has the greater selectivity between isomers (Peter *et al.*, 1979; de Kort *et al.*, 1983; Schooley *et al.*, 1984; Meyer and Lanzrein, 1989).

Final instar larvae of the lepidopteran, *G. mellonella*, provide the most complete example of interaction between JH production, carrier binding and hydrolysis for the regulation of JH isoforms. Haemolymph titres of the JH carrier and JHE increase and decrease in concert (Ozyhar *et al.*, 1983), so the carrier has been proposed to assist in the clearance of JH from insect's tissues (Wing *et al.*, 1984; Trowell, 1992). However, three isoforms of JH (I, II, and III) are synthesised. Hydrolytic rates in the haemolymph and corpus cardiacum-corpus allatum complex are maximal with JHI and JHIII respectively, and JHII is most resistant to hydrolysis in both locations. JHII binding by the haemolymph carrier is slightly more stable than for JHI and about seven times more than for JHIII. Thus, preferential binding of JHII and hydrolysis of the other isoforms leads to JHII being the dominant form (Szolajska, 1991). The whole body content of JHII-acid follows the JHII titre indicating that only a portion of JHII is hydrolysed even with peak JHE activities. In contrast, high JHIII-acid titres are associated with the near total absence of JHIII, indicating that hydrolysis can effectively eliminate JHIII.

4.4.2.4 Future Directions for the Analysis of JH Isoform Roles and Regulation

A complete analysis of the regulation of JHIII and JHB_3 in *D. melanogaster* may contribute to the assessment of the function of each isoform. Important experiments yet to be performed include determination of the developmental profile of

the JHB_3 titre, the kinetics of JHB_3 binding by lipophorin, and the spatial expression pattern of JHE and JHEH.

An important experiment to complement the characterisation of JHIII hydrolysis by JHE (Section 3.4.3) is characterisation of hydrolysis of 6S,10R-JHB_3. While the K_M of JHE for JHIII is greater than the K_I for JHB_3, it is unknown how the K_{cat} compares between these two substrates. For JH concentrations which are much less than the K_M (such as occur *in vivo*) it is the specificity constant (K_{cat}/K_M) which determines the reaction rate (Abdel-Aal and Hammock, 1988). If the K_M and K_{cat} for JHB_3 are both higher than for JHIII, the specificity constant may be similar for the two isoforms. This would enable JHE to hydrolyse JHB_3 as efficiently as JHIII. If both JHIII and JHB_3 are *in vivo* substrates, a higher K_M for JHB_3 but similar specificity constant might reflect higher physiological titres of JHB_3.

Sufficient quantities of 6S,10R-JHB_3 to perform these experiments can now be separated from chemically synthesised, racemic JHB_3 (Hearlt *et al.*, 1993) and could be radiolabelled in the carboxyl chain. Alternatively, 6S,10R-JHB_3 radio-labelled in the methyl-alcohol moiety can be biosynthesised *in vitro* using *L. cuprina* corpora allata (Dr. P. East, pers. comm.). The JHE assay procedure for methyl-radio-labelled JH is essentially the same as used in present study (Hammock and Roe, 1985). Therefore, direct measurement of 6S,10R-JHB_3 hydrolysis by JHE is now possible.

4.5 Structure and Regulation of JHE

This study has analysed aspects of the regulation of JH by JHE at the protein level. There are now issues arising from this study which may best be addressed at the nucleic acid level, requiring isolation of the gene encoding JHE. This study has identified JH hydrolysis as an important factor in the regulation of JH titre. For deeper understanding of the regulation of JH titre it is now necessary to identify the factors involved in the temporal and spatial regulation of JH hydrolysis. One such factor is JH itself which may self-regulate by feedback induction of JHE. Other potential regulatory factors include ecdysone, "head factors" and tissue-specific transcription factors (Section 1.6.3).

Several approaches to the isolation of the *D. melanogaster* JHE gene have been pursued (Ms. S. J. Dent, Ms. M. M. Dumancic and Dr. M. J. Healy, unpublished data). However, the cDNA of *H. virescens* JHE (Hanzlik *et al.*, 1989) does not hybridise with *D. melanogaster* genomic DNA and antibodies raised against lepidopteran JHEs do not cross-react with *D. melanogaster* JHE, indicating that *D. melanogaster* and lepidopteran JHE are dissimilar. The gene may be obtained by probing DNA libraries with the oligonucleotides corresponding to the N-terminal amino acid sequence of the JHE purified in this study but the seven amino acid N-terminal sequence (Section 3.3.2.4) does not appear to be sufficient for unique identification of the JHE gene. However, more JHE could to be purified to obtain either a longer N-terminal sequence or internal amino acid sequence data after cleavage and separation of peptides. The peptide containing the presumed active site serine could be identified by treatment of JHE with radioactive DFP prior to cleavage, as has been achieved with other esterases (Ozols, 1987). Internal sequence data would enable the use of techniques including the polymerase chain reaction to obtain the DNA sequence between the N-terminus and the peptide containing the active site serine.

Isolation of the JHE gene would enable the identification of amino acid residues required for catalytic activity and substrate specificity, and RNA hybridisation would enable the site(s) of synthesis of JHE to be determined with high precision. Sequence comparison between the *H. virescens* and *D. melanogaster* JHE genes should enable the identification of conserved structural features of the protein. These are likely to include the residues of the catalytic triad and residues involved in maintainance of the secondary and tertiary structure of members of the esterase/lipase family (Section 1.8) but might also include shared features around the active site which confer JH specificity. The sequences of many esterases are known but unequivocal physiological roles are only known for JHE and AChE. Site directed mutagenesis experiments among the apparently dissimilar lepidopteran and dipteran JHEs and AChE could further help to identify features required for JH binding and others required for esterase function in general (Ward *et al.*, 1992).

It is probable that the regulation of JHE in *D. melanogaster* will prove as complex as it is in lepidopterans (Section 1.6). The results from this study, together with data from the literature, suggest that JHE is regulated in response to both ecdysone and JH because its activity rises from its minimum value to its maximum value between two pulses of ecdysone and shortly after a pulse of JH. Full characterisation of the regulation JHE may therefore provide insights into the interactions between these two hormones for the correct regulation of gene expression through the larval to pupal transition. Comparison between the *H. virescens* and *D. melanogaster* JHE genes may enable the identification of tissue-specific or hormone responsive elements, although these may diverged considerably between these two species. Another comparison which may prove fruitful is between the genes for JHE and EST6. Both genes are induced by JH in adult *D. melanogaster* and the gene for EST6 is well characterised (Ludwig *et al.*, 1993; Healy *et al.*, in preparation), but the JH responsive element(s) remain to be identified. Identification of such elements would assist in the identification and isolation of other JH regulated genes and in the construction of a model for the mechanism of JH action on gene expression. The availability of transformation technology for *D. melanogaster* embryos suggests the investigation of effects of specific disruptions to the expression of JHE by mutagenesis of the regulatory region of the JHE gene.

4.6 Conclusions

This study has identified two specific JH hydrolysing enzymes in *D. melanogaster*, JHE and JHEH. One of the enzymes, JHE, is highly specific for JHIII and JHB_3, the two isoforms of JH produced by the higher Diptera. The specificity of these enzymes and their temporal and spatial regulation indicate that dipteran JH hydrolysis, possibly modulated by interaction with the JH carrier, acts in a coordinated fashion with JH synthesis to regulate JH titre. Furthermore, the pattern of JH hydrolysis during metamorphosis closely matches the lepidopteran model (Roe and Venkatesh, 1990), except for the lack of prewandering, haemolymph JHE. These results suggest that the role of JH hydrolysing enzymes in Diptera is essentially similar to their role in other insects.

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Appendix

```
huxley% comp < jhbr3.dat

 Fit to:  v = V*A/(K(1+I/Kis) + A)

 weighting factor is 1.0
  1                18jhbr3.dat : v*1000 ; comp ; 10/92
  20.92941   0.15355   1.36277
  21.35919   0.16282   1.32720
  21.34247   0.16290   1.32506
  21.34143   0.16288   1.32504
  21.34140   0.16288   1.32504
  21.34145   0.16288   1.32504

        i          a        vexp        1/a       1/vexp      1/vopt       res

   0.0000     0.0555     4.8800    18.0180     0.2049     0.1844     0.5438
   0.0000     0.0715     7.2500    13.9860     0.1379     0.1536    -0.7396
   0.0000     0.1000     8.1700    10.0000     0.1224     0.1232    -0.0517
   0.0000     0.1665    10.8000     6.0060     0.0926     0.0927    -0.0121
   0.0000     0.5000    15.8000     2.0000     0.0633     0.0621     0.2975
   3.0000     0.0555     1.7000    18.0180     0.5882     0.4957     0.3173
   3.0000     0.0715     2.2300    13.9860     0.4484     0.3953     0.2999
   3.0000     0.1000     3.2900    10.0000     0.3040     0.2960     0.0886
   3.0000     0.1665     3.8000     6.0060     0.2632     0.1965     1.2896
   3.0000     0.5000    11.2000     2.0000     0.0893     0.0967    -0.8567
   5.0000     0.0555     1.3200    18.0180     0.7576     0.7033     0.1019
   5.0000     0.0715     1.9300    13.9860     0.5181     0.5564    -0.1327
   5.0000     0.1000     2.7100    10.0000     0.3690     0.4112    -0.2780
   5.0000     0.1665     3.8900     6.0060     0.2571     0.2657    -0.1259
   5.0000     0.5000     8.3300     2.0000     0.1200     0.1197     0.0227
   8.0000     0.0555     1.4200    18.0180     0.7042     1.0146    -0.4344
   8.0000     0.1000     1.9400    10.0000     0.5155     0.5840    -0.2276
   8.0000     0.1665     2.6700     6.0060     0.3745     0.3695     0.0367

   K =        0.162882   s.e.(K)     =    0.018895   w =    0.28010E+04
   V =       21.341446   s.e.(V)     =    1.112378   w =    0.80816E+00
 Kis =        1.325043   s.e.(Kis)   =    0.131502   w =    0.57828E+02
 K/Kis=       0.122926   s.e.(K/Kis)=     0.011608
 V/K =      131.023865   s.e.(V/K)   =    9.471806
 V/K*Kis =     173.612228   s.e.   =     11.788260

 variance =     0.25953E+00
 sigma =      0.509446


  program completed for     1 lines
```

Weighted mean K_{is} = 1·20 ± 0·09 μM.

Shown above is an example of the computer analysis of the data shown in figure 3.8. The absolute values of the reaction rates are different because the data shown in figure 3.8 has been corrected for loss of enzyme activity during storage (Section 3.2.4.1).

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82