CHAPTER 1

ISOZYMES, ESTERASES AND BIOCHEMICAL ADAPTATION

> "In the world as it really is, the environment is neither constant nor uniform, and no genotype is a paragon of adaptedness in all environments. At any one time level, diverse genotypes are needed to exploit the environments varying in space. They are also needed to maintain the adaptedness to environments varying in time."
>
> Dobzhansky (1970)

1.1 MECHANISMS OF BIOCHEMICAL ADAPTATION

The various studies to be reported in this thesis represent a preliminary analysis of the biochemical diversity produced by genetic variation at two highly polymorphic esterase loci of the cactophilic drosophilid, *Drosophila buzzatii*, a member of the *mulleri* subgroup of the *repleta* species group. It represents one phase of a larger multidisciplinary programme to assess the adaptive significance of allozymic variation in this species, (reviewed in Barker, 1977; 1982). Taken in isolation, the biochemical studies reported herein constitute a highly reductionist approach to the study of adaptation, and consequently are very conducive to the kinds of interpretation which were lucidly, if colourfully, criticised by Gould and Lewontin (1979) in their critique of the adaptationist programme. It may be possible partially to circumvent these difficulties by considering the results in the context of the physiology of the organism, the environment in which it feeds and breeds, and the links which may be established with other members of the genus *Drosophila*. If differences between allozymic variants measured using *in vitro* techniques can be used to make predictions of *in vivo* differences between different genotypes, and if these predictions can be further supported by ecological data, then the case for a selectively maintained polymorphism

will be greatly strengthened.

In studying the properties of individual protein molecules, it is deceptively easy to overlook the fact that they are components of a highly integrated system; the scope for alteration is likely to be limited at any point in time. As Darwin (1859) noted in the first edition to "The Origin of Species"

> "That natural selection will always act with extreme slowness, I fully admit. Its action depends on there being places in the polity of nature, which can be better occupied by some of the inhabitants of the country undergoing modification of some kind".

At the molecular level, in addition to the restraints imposed by physiological function, those imposed by protein architecture also must be considered. Essentially, we do not know how great are the constraints which circumscribe permissible changes in the primary, secondary, tertiary (and where appropriate quaternary and quintinary) structures of proteins. It seems that an apparently adaptive alteration in one enzymic property is frequently accompanied by covariation of some other property. An example of this is the higher heat stability of orthologous homologues of enzymes from endotherms as compared with ectotherms (Somero, 1978). The price paid for this added thermal stability is an increased enthalpy and free energy of activation of the enzyme.

1.1.1 Some Processes in Molecular Evolution

It is a commonly held view that one mechanism of protein evolution has been gene duplication and subsequent divergence of function (MacIntyre, 1976). A number of alternative paths may be followed subsequent to a duplication event. In the simplest case, the extra activity provided by the duplicated locus may be sufficient to counter the environmental challenge. The conventional view is that of a gene duplication followed by subsequent divergence of function, and Ohno (1970) has suggested that multiple-locus isozymes represent an early step in this process. There is some evidence of two relatively recent gene duplications in the *mulleri* subgroup of *Drosophila*, and *D. buzzatii* appears to

manifest both of these. We have presented evidence of a duplicated *Alcohol dehydrogenase* locus in *D. buzzatii* (Oakeshott *et al.*, 1982), and have suggested that the duplication is restricted to members of the *mulleri* subgroup. These initial results were confirmed and extended by Batterham *et al.* (1982, 1983) working with *D. mojavensis* and an extensive array of other species of the *repleta* group. These duplicate loci have diverged to the extent of temporal differentiation, in that the *Adh-1* locus is expressed primarily in the larval/pupal stages and very little in the adult fly, while *Adh-2* appears to show a fairly constant level of expression. However, there is little evidence of spatial differentiation, *viz.* with respect to tissue specific expression. Another striking example of a recently duplicated locus is that of two β-esterases in members of the *repleta* species group (Zouros *et al.*, 1982). This duplication exists in *D. buzzatii* (Chapter 2), and there has been both temporal and spatial differentiation of the two loci, although in the haemolymph where both enzymes occur to some extent it is possible to detect a presumptive interlocus heterodimer (Zouros *et al.*, 1982; and the present study). Since these two loci are very active at certain stages of development, these enzymes should present an excellent opportunity for the study of ontogenic and tissue specific gene regulation at the molecular level.

Another adaptive response, which has been observed in experimental bacterial populations, is the evolution of a new enzyme. One mechanism whereby this might be achieved was proposed by Koch (1972), who suggested that the gene might evolve from a pool of "silent" duplicate genes. The recent discoveries made possible by advances in molecular biology are consistent with such a model. We now know that many eukaryote genomes do indeed harbour a pool of "silent" duplicated genes, which have commonly been designated as pseudogenes. The best known examples of these are the globin pseudogenes of man (Proudfoot and Maniatis, 1980) and mouse (Nishioka *et al.*, 1980). The discovery of the "split" nature of many eukaryote protein coding genes (for review see Breathnach and Chambon, 1982), and the observation that some exon regions code for specific

protein domains, (e.g. the central exon of the globin gene codes for the region of protein which binds the haem moiety (Craik *et al.*, 1980), and the product of the third exon encodes the amino acid residues involved in α-β contact and cooperativity of oxygen binding (Eaton, 1980)) has led to the suggestion that new genes could evolve by a process of exon "shuffling" (Gilbert, 1978; Tonegawa *et al.*, 1978).

1.1.2 Isozymes and Protein Adaptation

My main concern in this review is with the roles which isozymes might play in adaptation. Since the term isozyme was first introduced by Markert and Møller (1959) to denote multiple molecular forms of enzymes, the widespread use of electrophoretic and chromatographic techniques has revealed a variety of mechanisms for their production. In a recent review of the status of the isozyme concept, Markert (1977) identified 7 different varieties of isozymes:

(i) Genetically independent proteins encoded by separate loci e.g. mitochondrial and cytosollic malate dehydrogenases.

(ii) Polymeric enzymes where sub-units are coded for by separate loci, and the isozymes are generated by the formation of interlocus heteropolymers e.g. lactate dehydrogenase.

(iii) Isozymes encoded by allelic variants at a single locus.

(iv) Formation of a polymeric series based on a single sub-unit e.g. glutamate dehydrogenase.

(v) Post-translational modification of the initial protein, e.g. phosphorylation, acetylation or addition of sialic acid residues.

(vi) Partial proteolysis of an original polypeptide. If precursor and product both have enzymic activity, then they may validly be considered as isozymes.

(vii) Conformational isozymes e.g. allosteric modification resulting from cofactor binding.

Regardless of their method of production, isozymes frequently show ontogenetic or cell specific differences in expression, and also different distributions at the subcellular level. Markert (1977) interpreted the ubiquity of isozymic variation as a mechanism for providing metabolic adaptability: "Isozymes endow the organism with greater metabolic flexibility, versatility and precision. One molecular form of an enzyme is simply just not good enough to produce the most effective biological organisation". This view of isozymes has great intuitive appeal, and has been widely accepted, but despite the enormous literature pertaining to the subject, Markert (1977) was forced to conclude: "...but with very few exceptions we do not yet know the specific functional significance of individual isozymes".

One of the most fruitful areas of research in the field of biochemical adaptation has been the study of the roles which enzymes, and especially isozymic variants, play in thermal acclimation and acclimatisation. The manifold effects which temperature exerts on biochemical processes make it an ideal parameter to work with (Hochachka and Somero, 1973), and I believe that the molecular solutions which natural selection has provided to meet the challenge of changes in environmental temperature provide the best currently available model for the study of biochemical adaptation. The solutions can be divided into a number of different classes, which will be considered separately below. Hochachka and Somero (1973) defined three ways in which enzyme activity may be adjusted to compensate for the effects of temperature changes:

(i) the "Quantitative strategy", or changes in concentration of pre-existing enzymes.

(ii) the "Qualitative strategy", or changes in the types of enzymes present.

(iii) The "Modulation strategy", or changes in the activities of pre-existing enzymes.

Clearly the three strategies are not mutually exclusive, and indeed the type of strategy employed may differ for different classes of enzymes.

1.1.2.1 Changes in Enzyme Concentration: The Quantitative Strategy

Somero (1978) noted that during thermal acclimation, and evolutionary adaptation to new thermal regimes, decreasing environmental temperatures were accompanied by increasing levels of the enzymes involved in the major pathways of energy metabolism. In contrast, for digestive and detoxifying enzymes, acclimation to warm environments was accompanied by increased levels of activity. These different patterns for different classes of enzyme were rationalised as follows: as temperature is decreased, the rate of enzyme catalysed reactions decreases. Energy production can be maintained in the face of this loss of catalytic activity if enzyme levels are increased. However, as temperature is increased, the need for nutrients and the rate of production of toxic metabolic by-products increase, and so elevated levels of digestive and detoxifying enzymes are required.

Although temperature adaptation almost invariably involves changes in enzyme concentration, the strategies can usually be further divided into two groups: Those where the adaptive response is an intrinsic property of a single protein molecule, and those where genetically different proteins are involved. The former group belong to the "Modulation Strategy" whereas the latter represent the "Qualitative Strategy".

1.1.2.2 "Modulation" as an Adaptive Strategy

Instantaneous rate compensation appears to be a common property of many enzymes. In the case of temperature, this mechanism has been termed positive thermal modulation (Hochachka and Somero, 1973), and involves a compensatory increase in K_m with increasing temperature. Interestingly, wholesale changes in K_m do not appear to have been employed as an adaptive strategy on evolutionary time scales (Somero, 1978), and the explanation suggested for this was that regulatory sensitivity is lost to an enzyme which is saturated with substrate under all conditions. Once again however, the strategy employed may depend on the class of enzyme, since a study of digestive enzymes in differently adapted organisms showed no evidence of compensatory changes in K_m

(Hofer *et al.*, 1975). Although natural selection appears to have promoted alterations in catalytic efficiency rather than enzyme-substrate affinity as an evolutionary adaptation to different thermal regimes, Graves and Somero (1982) did detect temperature compensatory differences in K_m for four closely related fish species.

Allied to this intrinsic capacity of individual proteins to instantaneously compensate for environmental changes is the theoretical modelling of the properties of enzymes embedded in pathways (Kacser and Burns, 1981). The studies of Kacser and Burns (1981) indicate that, as the number of enzymes in a pathway is increased, the effect of reducing the activity of any one enzyme on flux through the pathway is negligible. It appears that metabolic pathways as well as individual proteins are intrinsically buffered against environmental changes.

Another method of molecular adaptation which has been detected in studies of thermal acclimation is the formation of catalytically distinct variants of a single protein species. To my knowledge this unusual strategy has been detected only once, in a study of pyruvate kinase of Alaskan king-crab (Somero, 1969). Two kinetically distinct forms of pyruvate kinase were found, one with hyperbolic kinetics and minimal K_m at $5^{\circ}C$, and another with sigmoidal kinetics and minimal K_m at about $12^{\circ}C$. No evidence could be found for the existence of isozymic variants in the conventional sense, and Somero (1969) concluded that the two forms of enzyme resulted from a temperature mediated change in protein configuration.

The examples cited above give some evidence of the molecular basis of the metabolic homeostasis which is seen to be a common property of both endo- and ectothermic organisms. Enzymes and enzyme pathways have evolved in such a way that they possess a substantial catalytic safety factor, which buffers them against rapid changes in both the intra- and extracellular environment. Despite this reserve capacity, however, there is some evidence that isozymes are, in some instances, an important adaptive mechanism.

1.1.2.3 Isozymes: The Qualitative Strategy

1.1.2.3.1 Multiple Locus Isozymes as an Adaptive Strategy

The two types of isozymic variant most commonly investigated are the multiple locus isozymes and allozymes. Probably the strongest evidence of the capacity of multiple-locus isozymes to provide alternative modes of biochemical adaptation comes from studies of thermal acclimation in the rainbow trout, *Salmo gairdneri*. This species is tetraploid, and consequently may not be typical of the majority of animals. However, the extra copy of the genome is ideally suited as a genetic pool from which natural selection has the opportunity to promote different strategies of adaptation. A particularly striking example of the use of multiple locus isozymes in thermal adaptation is provided by the season-specific production of acetylcholinesterase variants in rainbow trout (Baldwin and Hochachka, 1970). Kinetic analyses suggested the existence of different molecular forms of acetylcholinesterase in trout acclimated to different temperatures. The acetylcholinesterase of fish acclimated to low temperature exhibited a minimal K_m in the range 0-8°C, and a rapid increase in K_m at temperatures above 10°C. Conversely, trout acclimated to 18°C showed a minimal K_m in the region 15-20°C, and a very rapid increase in K_m at temperatures below 10°C. Electrophoretic analysis revealed the presence of two acetylcholinesterase isozymes: in fish acclimated to 2°C one isozyme was present, at 18°C another, electrophoretically distinct, isozyme was found, and in trout acclimated to 12°C both isozyme species were present.

Other data for the rainbow trout indicate that this multiple isozyme variant strategy of adaptation is not unique to the acetylcholinesterase isozyme system. Moon and Hochachka (1971) provided evidence of a similar phenomenon for isocitrate dehydrogenase isozymes. However, not all enzymes show this pattern of season-specific isozyme switching. Two energetically important enzymes, lactate- and malate dehydrogenase were found to be remarkably eurythermal, with quite flat K_m *vs* temperature curves over the species'

biological thermal range. In spite of these elegant examples of the potential for multiple locus isozymes to solve some of the problems associated with living in a heterogeneous environment, it is by no means clear that isozymes are commonly employed in that capacity. In an attempt to address this question, Somero (1974; Somero and Soule, 1974) investigated the degree of isozymic and allozymic variation in several species of eurythermal and stenothermal marine teleosts, and concluded that temperature variation was not a sufficient explanation for the existence of complex isozymic systems in fishes. Available data indicate that this issue can probably not be reduced to a simple generalisation. Different strategies of biochemical adaptation have been employed by different organisms, and as noted earlier, different mechanisms of adaptation have been adopted by the various types of enzymes. Before considering these points in greater detail it is necessary to consider one further genetic mechanism for potential adaptation, the class of isozymes encoded by allelic variants of a single locus.

1.1.2.3.2 Allozyme Variation as a Mechanism of Adaptation

Over the past 15 years there has been much research and debate on the nature and significance of allozymic variation (Wills, 1981 for a recent review). Athough we still have no clear idea of what proportion of allozyme variation is adaptive rather than neutral, there are a number of examples of enzyme polymorphism which appear to be maintained by some form of balancing selection. One of the most intensively studied of these is the alcohol dehydrogenase polymorphism of *D. melanogaster* (for reviews see van Delden, 1982, and Gibson and Oakeshott, 1982). Other likely cases of balanced enzyme polymorphism include the amylases of *D. melanogaster* (de Jong and Scharloo, 1976) the α-glycerophosphate dehydrogenase of *D. melanogaster* (Miller *et al.*, 1975), a leucine-aminopeptidase of the mussel *Mytilus edulis* (Koehn, 1978) and the muscle lactate dehydrogenase (LDH-B) of the killifish *Fundulus heteroclitus* (Place and Powers, 1979).

The precise mechanism of balancing selection has not been established for any of these polymorphisms, though in every case environmental heterogeneity (e.g. alcohol type (Adh), carbohydrate resource (Amy), temperature (α-Gpdh and LDH-B) and osmolarity (LAP)) has been suggested as a likely factor. Various aspects of the biochemical nature of allozyme variants are considered in greater detail in Chapters 4 and 5 of this thesis. In the context of mechanisms of biochemical adaptation, one of the most interesting early observations was that enzymes which were presumed to act on exogenous substrates (Group II enzymes) tended to be more polymorphic than single substrate enzymes, such as those of intermediary metabolism (Group I enzymes) (Gillespie and Kojima, 1968; Kojima *et al.*, 1970). A number of studies have confirmed the generality of this observation, and the "substrate-specificity hypothesis", with its implicit assumption of selection operating in heterogeneous environments (with respect to substrate) appeared to provide an attractive explanation for at least a proportion of the allozymic variation observed. This hypothesis was criticised by a number of authors (Zouros, 1975; Selander, 1976; Singh, 1976) and after an extensive review of the literature on allozyme polymorphism Singh (1976) concluded that the majority of difference in substrate specificity was between isozyme loci rather than allelic variants of particular enzymes, and that selectively neutral alleles were proportionately more common in Group II loci. As a global explanation of polymorphism at loci coding for non-specific enzymes, the substrate-specificity hypothesis appears to have failed, but in the absence of detailed study of individual loci it seems premature to dismiss the concept completely.

Laboratory experiments designed to test for a relationship between level of genetic polymorphism and degree of environmental heterogeneity (Powell, 1971; McDonald and Ayala, 1974) showed that average heterozygosity was lower for populations maintained in a constant environment as compared with those in more variable environments.

Myers (1978) has argued that isozymes and allozymes might represent alternate forms of protein adaptation. She advanced the hypothesis that constant or predictable environments would favour the selection of multiple-locus isozyme systems whereas variable or unpredictable environments would favour allozymic variation. Myers (1978) surveyed the literature and found that the single-substrate (Group I) enzymes were consistent with her hypothesis: higher levels of isozymic variation were accompanied by lower levels of allozymic variation. However, she found that this relationship was not true of Group II enzymes, and further that there were differences in the patterns of variability between groups of organisms. She concluded that "...different groups of organisms may be experiencing or responding to environmental variability in different ways which should also be kept in mind when general patterns of enzyme adaptation are sought...."

1.1.3 Concluding Comments

This brief review has been concerned with biochemical adaptation as it is manifest both at the level of the enzyme molecule and also at the level of the genome. One thread links all these observations, and that is, in the course of evolution there appear always to be multiple solutions to the problems of adaptation to environmental change or uncertainty. This is no doubt to be expected since different classes of proteins will be subject to different constraints, both in terms of their internal architecture and also in their interactions with other macromolecules and cellular structures. Similarly different "classes" of organisms may be subject to different types of selective pressure and so different genetic strategies may be utilised. Under these circumstances, seeking global explanations for specific phenomena is futile: certainly we know that not all variation is adaptive, but only a locus by locus analysis will establish what proportion is. In this context esterase enzymes are particularly intriguing, though somewhat refractory to analysis. In any organsim there is almost invariably a multiplicity of esterase isozymes,

and some proportion of these commonly exhibit genetic polymorphism (Nevo, 1978). Singh (1976) clearly believed that most variation at esterase loci is likely to be selectively neutral, but such a conclusion seems premature until more detailed analyses of specific polymorphisms become available.

1.2 NON-SPECIFIC ESTERASES

The non-specific ester hydrolases (E.C. 3.1.1) have been the subject of extensive investigation since the existence of multiple esterase isozymes was first described by Markert and Hunter (1959). Most species exhibit a great diversity of esterase enzymes which frequently differ in ontogenetic or tissue-specific distribution. The ubiquity of esterase isozymes, coupled with their ease of detection, and the fact that they frequently exhibit genetic polymorphism (Nevo, 1978) has made them particularly useful tools in population genetic studies. There are now a number of examples of esterases for which quite strong circumstantial evidence exists indicating selectively balanced polymorphism. Koehn (1969, 1970) described an esterase polymorphism in the fish *Catostomus clarkii*, for which there was marked clinal variation in gene frequency. He found that enzyme activity varied as a function of temperature in a manner consistent with the observed cline in frequency of the alleles encoding the allozymes. Koehn *et al.* (1971) described temperature dependent differences in activity between allozymic variants at an esterase locus in another fish, *Notropis stramineus*. Thermal selection was also strongly implicated in the maintenance of esterase polymorphism in the barnacle, *Balanus amphitrite* (Nevo *et al.*, 1977). Circumstantial evidence for the selective maintenance of the *Esterase-6* polymorphism in *Drosophila melanogaster* was provided by Oakeshott *et al.* (1981) who found parallel clines in the frequency of the $Est\text{-}6^{1.00}$ allele on three continents. Despite their genetic interest, however, studies of esterase polymorphisms have been frustrated by lack of information concerning the physiological function of these enzymes.

1.2.1 Studies of *Drosophila* Esterases

The esterases of *Drosophila* species were first investigated by Wright (1963) and Beckman and Johnson (1964) in *D. melanogaster*. Since then there have been numerous reports of genetic variation at esterase loci in *Drosophila* species (e.g. Johnson *et al.*, 1966; Kojima *et al.*, 1970; Powell, 1975), and they have been widely employed in population genetic studies. The adaptive significance of polymorphism at esterase loci has long been the subject of controversy, and in some instances conflicting results have been reported. For example, the early studies of the *Esterase-5* polymorphism of *D. pseudoobscura* failed to provide any evidence of selective differences between genotypes at the *Est-5* locus (Yamazaki, 1971), whereas other workers have produced contrary results (Marinkovic and Ayala, 1975; Arnason, 1981).

There have been a number of biochemical studies of *Drosophila* esterases; the *Esterase-5* enzyme of *D. pseudoobscura* (Narise and Hubby, 1966), the α- and β-esterases of *D. virilis* (Narise, 1973a,b), and *Esterase-6* of *D. melanogaster* (Danford and Beardmore, 1979 ; Mane *et al.*, 1983).

There are two large-scale studies of *Drosophila* esterases currently in progress. The first of these, by Korochkin and co-workers, has been concerned with the developmental genetics of esterases in *D. virilis* and related species, and especially with the ejaculatory bulb esterase (*Est-s*) of adult males (reviewed in Korochkin, 1980). The enzyme is transferred in a waxy plug from the male to the female at the time of ejaculation, but no specific function has yet been ascribed to it. The *Est-s* gene has been extensively characterised by genetic studies. It is one of a group of esterase loci located on the second chromosome of *D. virilis*. Genetic mapping localised the *Est-s* gene to a region extremely close to the α-esterase loci (Korochkin, 1980). The *Est-s* gene has recently been cloned (Yenikolopov *et al.*, 1983) and nucleotide sequence studies should soon help to clarify the evolutionary relationships between the *D. virilis* esterase isozymes. Genes which modify the expression

of *Est-5* have been located on the X, IV and V chromosomes in *D. virilis*.

The other major study of a *Drosophila* esterase is that of Richmond and co-workers on the *Est-6* of *D. melanogaster* (e.g. Richmond *et al.*, 1980; Gilbert, 1981; Gilbert and Richmond, 1982a,b). The enzyme is widespread through the tissues of adult *D. melanogaster*, but is found primarily in the anterior ejaculatory duct of adult males (Sheehan *et al.*, 1979). It is transferred from the male to the female in the seminal fluid at the time of mating. Studies with a null activity mutant of *Est-6* suggest that the enzyme affects female productivity and the timing of remating (Gilbert *et al.*, 1981; Gilbert and Richmond, 1982a). For the first time in a study of *Drosophila* esterases, a likely *in vivo* substrate, the lipid *cis*-vaccenyl acetate, has been suggested (Mane *et al.*, 1983). It appears that the widespread polymorphism at the *Est-6* locus is maintained by a complex form of sexual selection (Gilbert and Richmond, 1982b). Genes located on the X-chromosome regulate the expression of *Est-6*, and its expression is to some extent modulated by juvenile hormone and 20-hydroxyecdysone (Richmond and Tepper, unpublished MS.). Further studies of the *Est-6* polymorphism in *D. melanogaster*, and of the orthologous homologues of the enzyme in other species closely related to *D. melanogaster*, hold great promise for the experimental evaluation of the significance of gene regulation phenomena in the evolutionary process.

1.2.2 Esterases in Other Insect Species

Whereas the study of esterases in *Drosophila* species has been primarily concerned with fundamental questions relating to developmental and evolutionary genetics, there have been many other studies of esterases in insects, particularly agricultural pests and disease vectors. Interest in esterases in these species was, in large part, stimulated by the early discovery by van Asperen and Oppenoorth (1959), and Oppenoorth and van Asperen (1960) of an association between organophosphate resistance and the activity of a carboxylesterase. Subsequently, it has been recognised that one of the mechanisms of detoxification

of xenobiotics is through hydrolysis of ester linkages (Dauterman, 1976; Dauterman and Hodgson, 1978). There are now several instances in which insecticide resistance has been strongly linked to the action of carboxylesterases. For example, resistance to the organophosphate ester, malathion in the mosquito, *Culex tarsalis* (Matsumura and Brown, 1961) and the two-spotted spider mite (Matsumura and Voss, 1965) has been shown to be mediated through a carboxylesterase. Devonshire (1977) has investigated the properties of an esterase in the aphid, *Myzus persicae*, which appears to be responsible for conferring insecticide resistance.

In general, most organisms exhibit a great diversity of developmental and tissue specific patterns of esterase activities. In the cockroach, *Periplaneta americana*, Cook *et al.* (1969) described six carboxylesterases in the gastric secretion. Subsequent analyses (Hipps and Nelson, 1974) identified seven esterases in the mid-gut and gastric caecum, of which four were partially purified for further investigation. Electrophoretic analyses of the mosquito *Culex tarsalis* revealed up to 18 esterases (Houk *et al.*, 1979), though these showed differences in expression attributable to tissue type, age and sex of the individual. There are numerous other examples of the wealth of diversity in insect esterases, and the gut and haemolymph commonly contain up to 10 electrophoretically distinct isozyme forms. These enzymes frequently exhibit differences in substrate specificity (e.g. Hipps and Nelson, 1974; Ahmad, 1976), and crude extracts are usually capable of hydrolysing an enormous array of ester compounds, including fatty acid esters, acyl esters of naphthol, p-nitrophenol and 4-methylumbelliferone, lactones and tri-, di- and mono-acylglycerides. If mammalian carboxylesterases are included, this list can be extended to include many other types of ester compound (Section 1.2.3 below). The ubiquity of esterase isozymes in the gut has promoted the idea that some of these are involved in the digestion of dietary lipids. Treatment of insects with insecticides which are potent *in vivo* inhibitors of esterase activity results in impaired digestion and/or absorption of ingested food (Colhoun, 1960; Turunen, 1977). Nonetheless, no particular esterase has yet been convincingly

shown to have any digestive function.

A third area which has sparked considerable interest in insect carboxylesterases in recent years has been the discovery that one of the mechanisms for modulating the titre of juvenile hormone (JH) is esterolytic hydrolysis by haemolymph esterases. Initial studies by Whitmore and others (Whitmore *et al.*, 1972, 1974, 1975) established that a class of fast migrating carboxylesterases could be induced by treatment with juvenile hormone, and that these enzymes were capable of hydrolysing the hormone to the biologically inactive JH-acid. In the past ten years JH metabolism has been examined in many insects (for reviews, see Riddiford and Truman, 1978; de Kort and Granger, 1981) and JH-esterases have been demonstrated in most, but not all, of the species examined. It appears that many non-specific esterases are capable of hydrolysing free JH, but since the hormone is usually transported in the haemolymph as a lipoprotein-hormone complex, it is probable that only the JH-specific esterases have any *in vivo* significance for the regulation of JH titre. The role of esterases in JH metabolism in *Drosophila* remains to be clarified. The investigations to date (Ajami and Riddiford, 1973; Wilson and Gilbert, 1978) suggest that ester hydrolysis may not be an important mechanism of JH regulation in *Drosophila*, or the higher diptera in general. However, the significance of juvenile hormone for the regulation of larval development and vitellogenesis, and its possible role in controlling the expression of protein coding genes, ensures that much more research on JH-esterases will be done in the near future, particularly with the increased availability of rapid and sensitive assays for JH level and JH binding sites during development.

1.2.3 The Problem of Esterase Function

The ubiquity and great diversity of esterase isozymes strongly suggests that they have many different physiological roles. Nevertheless, in very few instances is the function of any particular esterase known. In insects, with the exception of acetylcholine esterase (Hall and Kankel, 1976) and the

juvenile-hormone specific esterases discussed previously, the natural physiological substrates remain essentially uncharacterised. Drawing on published results of studies of insect and mammalian enzymes, a list of suggested functions of esterases has been compiled (Table 1.1), and I have attempted to use this as a guide for the studies reported in this thesis.

That it has been possible, or indeed necessary, to ascribe such an extensive array of functions to the non-specific esterases is due largely to their lack of substrate specificity. It has been possible to differentiate between esterases to some extent on the basis of their activity on panels of synthetic esters, but one can never be sure that the observed patterns have any biological significance. Mammalian carboxylesterases have frequently been found to be associated with the microsomal fraction, and this has led to the suggestion that they may be hydrolytic counterparts to the microsomal mixed function oxidase system (see reviews by Krisch, 1971; Dauterman, 1976; Dauterman and Hodgson, 1978). The role of non-oxidative enzymes in the metabolism of insecticides (Ahmad and Forgash, 1976) and especially the frequent occurrence of insecticide resistance conferred by esterases (Section 1.2.2) is strong circumstantial evidence that some of these enzymes evolved as part of the natural detoxification system.

Similarly, it is difficult to avoid the conclusion that some esterases are part of the digestive system. Their frequent occurrence in the alimentary tract, and particularly in the gastric secretion (e.g. Cook *et al.*, 1969), coupled with the observation that esterases and lipases often have overlapping substrate specificities (Oosterbaan and Jansz, 1965; Hipps and Nelson, 1974) is suggestive of some role in the digestion and/or absorption of dietary lipids.

The extraordinary diversity of functions in which esterases have been implicated may in part reflect their apparent evolutionary relationship with the serine hydrolase family of enzymes (Dixon, 1966; Hartley, 1979). The variety of catalytic functions fulfilled by the serine hydrolases suggests that they have been a rich source for the acquisition of novel activities in

Table 1.1 Some possible functions of non-specific esterases

Function	Source
1. Digestion/Absorption	
1.1 Fatty acid esters	Lombardo *et al.* (1980), Geering and Freyvogel (1975)
1.2 Other esters, *e.g.* sterol esters, vitamin esters	Lombardo and Guy (1980)
2. Detoxification	
2.1 Volatile esters	
2.2 Natural xenobiotics, *e.g.* secondary plant compounds	Robinson (1979), pp.435-436
2.3 Synthetic xenobiotics, *e.g.*	Dauterman (1976), Ahmad and Forgash (1976)
3. Regulation of hormone titre	Riddiford and Truman (1978), de Kort and Granger (1981)
4. Acylase	
4.1 Peptide hydrolysis/synthesis	Goldberg and Fruton (1969)
4.2 Amino-acyl transferase	Krenitsky and Fruton (1966)
4.3 Acyl transferase	Pilz *et al.* (1966)
5. Thiol ester hydrolase	Drummond and Stern (1961)
6. Reproductive function	Richmond *et al.* (1980)
7. Lipid mobilization	Gilbert *et al.* (1965)
8. Wax deposition/transport	Locke (1974)
9. Amidase	Heyman and Mentlein (1981)

the course of evolution.

1.2.4 The Problem of Classification

Since esterases of known biochemical function such as the acetylcholine esterases and juvenile hormone esterases are also capable of hydrolysing the naphthol esters which are commonly used for the histochemical detection of non-specific esterases, the classification of these enzymes is rather ambiguous.

1.2.4.1 The Mammalian Classification System

In an attempt to formalise the classification of esterases, Aldridge (1953) proposed a division of non-specific esterases into two classes according to their sensitivity to low levels of the organophosphate diethyl-p-nitrophenyl phosphate (paraoxon, E600). This system has been revised and extended (Holmes and Masters, 1967; Pearse, 1972). In essence, the classification involves an initial division into specific and non-specific esterases on the basis of substrate specificity and sensitivity or resistance to a variety of inhibitors. Non-specific esterases can then be further subdivided according to their pattern of inhibition by these compounds. There are basically three types of inhibitor used, those specific for; a) cholinesterases, (e.g. eserine), b) serine hydrolases, (e.g. organophosphates such as diethyl-p-nitrophenyl phosphate) and c) sulphydryl enzymes (e.g. p-chloromecuribenzoate and Hg^{++} ions).

Thus, lipases (E.C. 3.1.1.3) are active on emulsions of long chain fatty acids and are inhibitor resistant. Cholinesterases (E.C. 3.1.1.7 and 3.1.1.8) show high activity on esters of choline, and are sensitive to low concentrations of eserine. Non-specific esterases are eserine resistant but may be divided into three sub-groups; carboxylesterases (E.C. 3.1.1.1, Group B esterases) are sensitive to diethyl-p-nitrophenyl phosphate and resistant to p-chloromecuribenzoate, aryl esterases (E.C. 3.1.1.3, Group A esterases) are resistant to diethyl-p-nitrophenyl phosphate, but sensitive to

p-chloromecuribenzoate, and acetyl esterases (E.C. 3.1.1.6, Group C esterases) are resistant to both of these inhibitors.

1.2.4.2 Classification of Insect Esterases

Unfortunately, there has been no thorough classification system elaborated for insect esterases, and attempts to apply the mammalian system frequently lead to ambiguity. Hipps and Nelson (1974), despite quite extensive characterisation of several esterases of the cockroach, *Periplaneta americana*, were forced to conclude that the conventional distinction between esterase and lipase was not applicable to these enzymes. Furthermore, although they could classify them as belonging to the Group B esterases, they still showed considerable differentiation of inhibition pattern with a variety of organophosphates. Similarly, Hooper (1976) found difficulty in classifying the esterases of the mosquito *Culex pipiens pipiens*, since the majority of the activity was both diethyl-p-nitrophenyl phosphate and p-chloromecuribenzoate sensitive, thus preventing any distinction between Group A and B esterases. This overlap in specificity is also known to some extent in mammalian systems, and was recognized by Oosterbaan and Jansz (1965) in their review of esterases and lipases, where they concluded; "The differences in mechanism of action between A-, B- and C-types of esterases may well be of a qualitative rather than of a quantitative character".

This overlap in classification is also found in the two major esterases of *Drosophila buzzatii* (Section 3.3.3). Narise (1973a) described significant differences between allozymic variants of the α- and β-esterase loci of *D. virilis* with respect to inhibition pattern. If one were forced to rely on the degree of inhibition at different concentrations of inhibitor to distinghish between carboxyl- and arylesterases as has been suggested by Aldridge (1953), then this type of genetic variation confounds the issue still further.

1.2.5 The Esterases of *D. buzzatii* in Relation to Selection

Adult *D. buzzatii* possess two esterases which are polymorphic in almost all populations examined to date (Barker and Mulley, 1976; Barker and East, unpublished). Available data strongly indicate that the variation at these loci is not neutral, but is maintained by some form of balancing selection. Multivariate analyses of macroenvironmental variables and spatial variation of gene frequencies showed significant associations at both of these esterase loci (Mulley *et al.*, 1979). The evidence generally was strongest for the *Esterase-2* locus, where significant association of allele frequencies was found with both geographical location, and with environmental variables after adjustment for location. In the case of the *Esterase-1* locus significant associations were found with location, but not with environmental variables after adjustment for location. Mulley *et al.* (1979) concluded that, in the case of *Est-2* at least, migration and genetic drift were not a sufficient explanation for the observed pattern of gene frequencies.

Further evidence for the action of selection derives from a temporal study of gene frequency variation in a single population of *D. buzzatii* (Barker, 1981, 1982; Barker, East and Weir, unpublished). Monthly samples were made over a four year period, and analyses indicated non-random variation in allele frequencies at both esterase loci. Simple, cyclical patterns of variation were not observed, but one of the four common alleles at the *Est-2* locus was found to show a significant decrease in frequency over the study period (Barker, 1981). In this and other studies (Barker, 1982), multivariate analyses of gene frequencies were made with a number of microenvironmental variables. Again both esterase loci showed significant associations with host plant chemistry and rot microflora, and there was some suggestion that density dependent selection might be important. The possible significance of microflora, particularly yeasts, was strengthened by laboratory and field experiments (Barker *et al.*, 1981a,b; Vacek, East and Barker, unpublished), which

indicated that both larvae and adults of *D. buzzatii* can discriminate between different cactophilic yeast species, and that *Est-2* genotypes may be differentially attracted (Barker *et al.*, 1981a,b). Perturbation of gene frequencies in a natural population of *D. buzzatii* (Barker and East, 1980) also strongly indicated the action of selection in maintaining equilibrium gene frequencies at the *Est-2* locus. Finally, there was some evidence for differential selection on genotypes at both esterase loci in a study of the effects of temperature shocks on *D. buzzatii* (Watt, 1981). Watt found that there were significant effects of low temperature shocks on allele frequencies at the *Est-1* locus, and effects of high temperature shocks at the *Est-2* locus.

The volume of evidence implying the action of selection on the two esterase loci of *D. buzzatii*, especially at the *Est-2* locus and the apparent effects of temperature, provided much of the impetus for the studies reported in this thesis.

CHAPTER 2

ONTOGENETIC AND TISSUE SPECIFICITY STUDIES OF THE MAJOR ESTERASE ACTIVITIES

2.1 INTRODUCTION

Although the non-specific carboxyl ester hydrolases are widespread in insect species, they remain poorly characterised. There have been extensive population genetic studies of esterase loci, especially in *Drosophila* species, although they have also proven to be useful genetic markers in other insects (Sell *et al.*, 1974; Townson, 1972). Genetic variants at esterase loci in *D. melanogaster* were among the first allozyme polymorphisms described in that species (Wright, 1963; Beckman and Johnson, 1964), and yet we still know remarkably little regarding the physiological function of any *Drosophila* esterase. If we are to have any hope of establishing whether or not allozymic variation is of adaptive significance then it is imperative that we understand the role which the enzymes fulfil in the physiology of the organsim (Clarke, 1975; Koehn, 1978). With the growing acceptance of the need for detailed study of individual loci according to the methodologies outlined by Clarke (1975) and Koehn (1978), population and evolutionary geneticists have increasingly turned to biochemical and physiological studies of allozyme polymorphism.

Since the function of non-specific esterases is poorly understood, most workers have restricted themselves to a description of the number of esterases which can be characterised by gel electrophoresis. One exception has been the extensive study of the ESTERASE-6 enzyme of *D. melanogaster* by Richmond and co-workers (Richmond *et al.*, 1980; Sheehan *et al.*, 1979). By using a null activity mutant of the *Esterase-6* locus as a physiological probe, they have been able to demonstrate that this enzyme has a function in reproduction, and

that the probable substrate of the enzyme is a lipid which is transferred from the male to the female at the time of mating (Gilbert and Richmond, 1982). Further, some physiological studies have been reported for cactophilic species of *Drosophila*. Kambysellis *et al.* (1968) investigated the tissue distribution of a number of esterases in *D. aldrichi* and *D. mulleri*. Recently, Zouros *et al.* (1982) reported on the tissue distribution and ontogenic expression of a number of esterases of the sibling species *D. mojavensis* and *D. arizonesis*.

Gel electrophoresis of *Drosophila* imagos frequently reveals two major non-specific esterases, one which preferentially hydrolyses α-naphthyl acetate and the other, β-naphthyl acetate (e.g. Johnson *et al.*, 1966; Sasaki and Narise, 1978). In *D. buzzatii*, a species which, in Australia, feeds and breeds on necrotic tissue of prickly pear plants of the genus *Opuntia*, these two esterases are polymorphic and are designated ESTERASE-2 and ESTERASE-1 respectively (Barker and Mulley, 1976; East, 1982).

A number of population genetic analyses of the two polymorphic esterases in *D. buzzatii* have indicated that allozymic variants may be of adaptive significance in this species (Section 1.2.5). As a first step in elucidating the nature of the differences between these variants and their relationship to genetic adaptation in *D. buzzatii*, the tissue distribution and pattern of developmental expression of the major non-specific esterases in this species were investigated.

2.2 MATERIALS AND METHODS

2.2.1 Insect Culture

D. buzzatii were cultured on a medium containing 5.6% sucrose, 10% yeast (*Saccharomyces cerevisiae*) and 1% agar w/v. Sucrose and yeast solutions were autoclaved separately, mixed and 7 ml dispensed into 75 x 27 mm vials. All individuals were derived by egg sampling from three population cages, each comprising approximately 300 pairs of mature *D. buzzatii*, homozygous for the $Est\text{-}1^{a}$ and $Est\text{-}2^{c}$ alleles. Larvae were cultured at a density of 40 indivduals per vial.

2.2.2 Sample collection

For the collection of large numbers of eggs, the food cups were replaced with jars containing 7 ml of an egg collection medium comprising 20% v/v cactus homogenate, 0.75% w/v agar, 1% v/v ethanol and 0.5% v/v glacial acetic acid. The surface of this medium was smeared with dead *Saccharomyces cerevisiae*. Flies were allowed to oviposit on this medium for three hours, yielding several thousand eggs. Samples of 1000 individuals were taken at 1, 12, 24 and 36 hours post-oviposition. Smaller numbers were taken at 24 hour intervals thereafter to provide 20-25 mg wet weight per sample. Sampling continued to 20 days post-ecolosion, and sexes of adults were analysed separately. No attempt was made to sex pre-adult stages. Eggs were dechorionated in 4% sodium hypochlorite and examined by transmitted light. Approximate stage of embryonic development was determined by comparison with Bownes' series for *D. melanogaster* (Bownes, 1975). Larval instar status was determined from mouthpart morphology.

2.2.3 Enzyme Distribution

Tissue distribution was determined in late third instar larvae. Tissues were sampled from individuals by dissection in ice-cold insect Ringers solution, and stored at -20^{o}C until assay.

The subcellular distribution of EST-1 and EST-2 was determined by differential centrifugation. Two grams of one day old adults were homogenised in 20 ml of ice-cold 0.1 M phosphate buffer, pH 7.0. The homogenate was filtered through glass wool to remove large tissue fragments, and to reduce the lipid content. This crude homogenate was then centrifuged at 800 g for 15 minutes, 12,000 g for 30 min and 100,000 g for one hour. The two low speed pellets were washed once in homogenising buffer and recentrifuged. All steps were carried out at 0-4^{o}C.

2.2.4 Assays

All samples for the developmental and tissue distribution studies were

prepared according to the same protocol. Each sample was homogenised in 0.5 ml of ice-cold 0.1 M phosphate buffer pH 7.0. The homogenate was centrifuged at 10,000 g for 15 min at 4°C. The supernatant was divided into three aliquots for subsequent analysis.

Protein was determined according to the method of Bradford (1976). Total esterase activity was measured by a modified version of the method of van Asperen (1962). Three mls of 10^{-4} M α-naphthyl acetate in 0.1 M phosphate buffer pH 7.0 were equilibrated at 25°C, then 0.1 ml of homogenate was added, the mixture was vigorously vortexed and incubated at 25°C for 20 min. The reaction was terminated and colour developed by the addition of 1 ml of a solution containing 5% w/v sodium dodecysulphate and 1% w/v diazo blue B salt. Absorption was measured at 600 nm on a Turner Model 380 spectrophotometer. Standard curves were prepared using α-naphthol in 0.1 M phosphate pH 7.0 and all assays were run in triplicate.

2.2.5 Electrophoretic Techniques

Since there is always a multiplicity of non-specific esterase activities in any particular sample, the contribution of each esterase to the total activity was determined by polyacrylamide gel (PAG) disc electrophoresis, histochemical staining and densitometric scanning of the stained gels. Electrophoresis was carried out according to the methods of Ornstein (1964) and Davis (1964) with a continuous Tris-glycine pH 8.3 buffer system in a Gradipore electrophoresis assembly, using 6% running gel and 3% spacer gel. The gels were stained according to the method of Kambysellis *et al.* (1968) except that α-naphthyl acetate only was used as substrate. Stained gels were fixed in 7% acetic acid and scanned on a Gelman integrating densitometer. All samples were treated in duplicate, and esterase zones were identified by R_f measurements, *i.e.* their mobility in the gel relative to the buffer front, which was marked by the dye bromophenol blue. Preliminary experiments were conducted to establish the conditions under which intensity of staining was directly proportional to esterase concentration and time of incubation. For the experiments, enzyme activity was adjusted for each gel, to fall within this linear range.

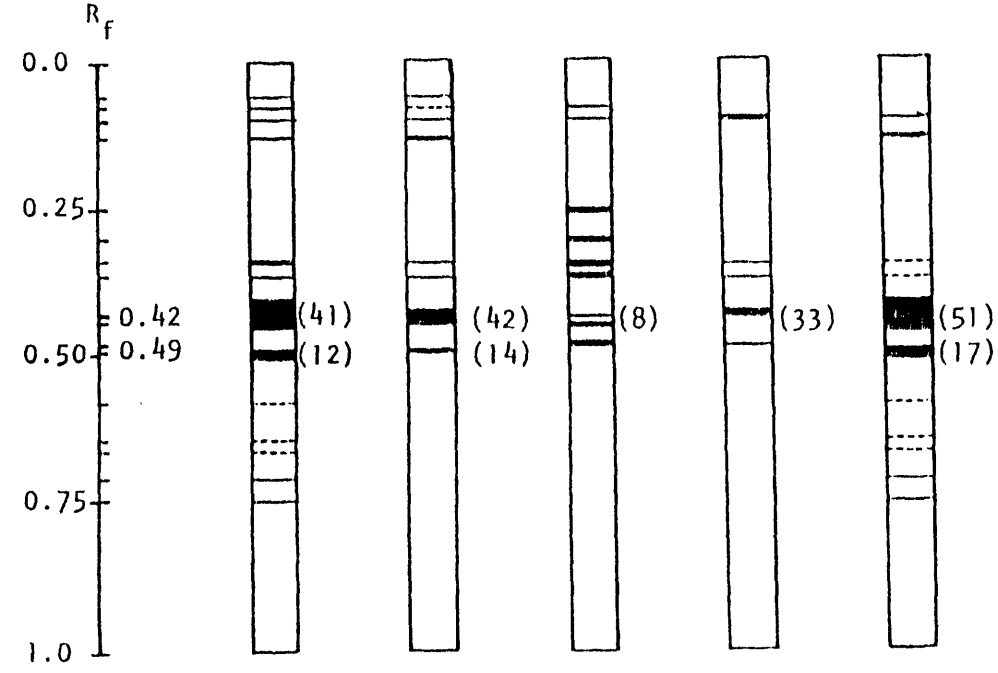
2.3 RESULTS

2.3.1 Subcellular Distribution

All subcellular fractions examined possessed esterase activity against α-naphthyl acetate. However, a comparison of the electrophoretic profiles of these fractions (Fig. 2.1, Table 2.1) indicated that each possessed a unique spectrum of esterase activities. Analysis by gel electrophoresis and densitometric scanning showed that in a crude homogenate of young imagos, in excess of 50% of the non-specific esterase activity could be attributed to the two enzymes, EST-1 (R_f = 0.49) and EST-2 (R_f = 0.42). Both of these enzymes appeared to be present in the 800 g pellet, though this represented only about 11% of the total activity recovered. It seems probable that most of this was due to contaminating supernatant, as the very soft pellet was difficult to wash thoroughly. The mitochondrial fraction, represented by the 10,000 g, pellet contained no detectable EST-1 activity and only trace amounts of EST-2, which again may be due to contamination given the very high level of activity of this enzyme in the original homogenate. The lysosomal fraction (100,000 g pellet) contained only a trivial amount of esterase activity, and within the limits of experimental error did not contribute significantly to the total activities of EST-1 and EST-2. By contrast the soluble fraction (100,000 g supernatant) showed a zymogram pattern almost identical to that of the crude homogenate, and contributed approximately 75% of the non-specific esterase activity recovered. The substantial recovery of EST-1 and EST-2 activities in this fraction strongly suggests a predominantly cytosollic or extracellular location for these two esterases.

2.3.2 Tissue Distribution

By contrast with crude homogenates of adult *D. buzzatii*, larvae from late final instar just prior to pupation exhibited three major esterase zones, and although a multiplicity of activities was detectable these three enzymes together accounted for almost 100% of the activity against α-naphthyl



Fraction I : Crude Homogenate
" II : 800 x g Pellet
" III : 10,000 x g Pellet
" IV : 100,000 x g Pellet
" V : 100,000 x g Supernatant

Fraction	I	II	III	IV	V
Specific* Activity	77.4	54.6	451.1	90.7	151.0
% Total Activity Recovered		10.8	10.2	5.1	73.8

Figure 2.1 Schematic representation of PAG disc gels showing the subcellular distribution of the non-specific esterases. Figures in parentheses are the area peak per cent of total integrator counts attributable to the relevant band. * Specific activity of the sample, expressed as nmoles of α-naphthol released per minute per mg. protein.

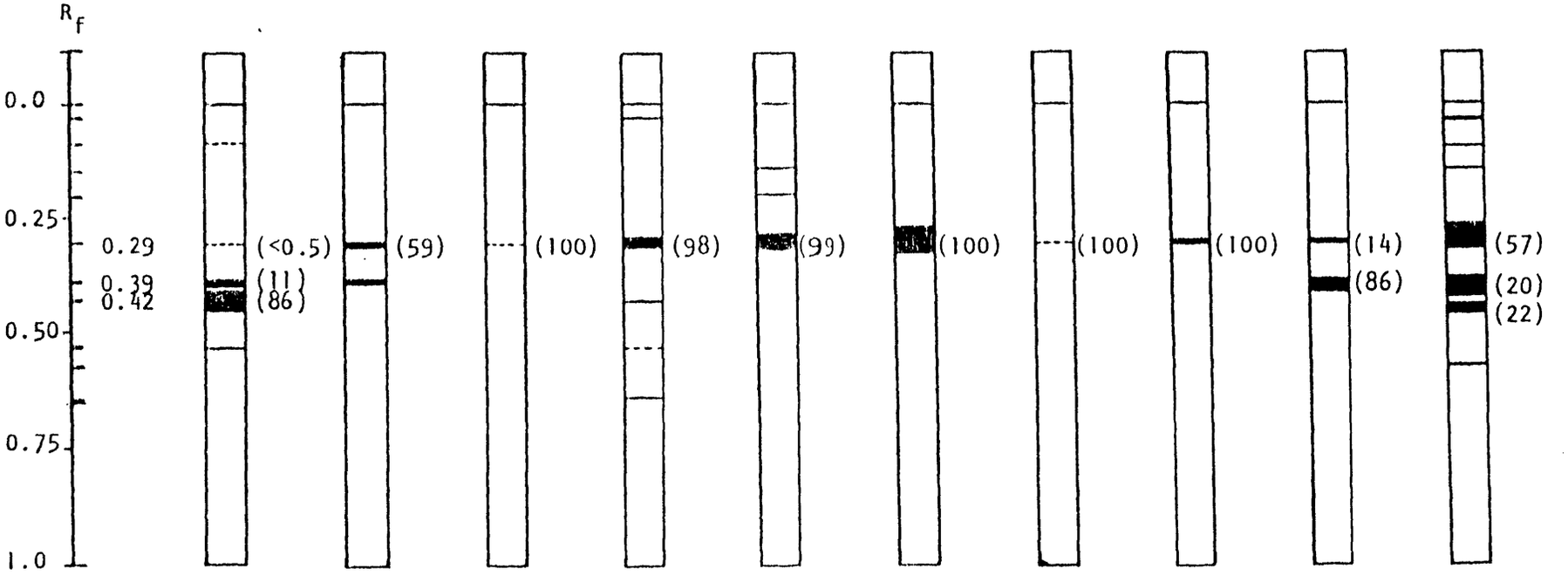
Table 2.1 Semi-quantitative estimation of the subcellular distribution of the non-specific esterases, based on enzyme assays and PAG disc gel electrophoresis

	Fraction	Specific activity	Total activity	% Total activity	*Est-2* activity	*Est-2* as % total *Est-2*	*Est-1* activity	*Est-1* as % total *Est-1*
I	Crude Homogenate	77.4	6026		2494.8		723	
II	800 x g Pellet	54.6	456	10.8	192.0	10.2	64.3	10.7
III	10,000 x g Pellet	451.1	429	10.2	36.0	1.9	0	
IV	100,000 x g Pellet	90.7	216	5.1	70.6	3.7	0	
V	100,000 x g Supernat.	151.0	3109	73.8	1588.7	84.2	534.7	89.3

acetate (Fig. 2.2, Table 2.2). However, when the tissue distribution of these enzymes was examined in late third instar larvae, they exhibited totally different patterns of expression. ESTERASE-1 (R_f = 0.42) was located almost exclusively in the haemolymph, and the only other tissue to show activity of this enzyme was the fat body, but it represented less than 1% of the esterase activity and was most likely due to contaminating haemolymph. ESTERASE-2 appeared to be very widely distributed, occurring in all tissues examined except haemolymph. However, the majority of EST-2 activity was located in the alimentary tract, with particularly high activity in the anterior segments, the gastric caeca, foregut and midgut. The other tissue to show substantial activity was the fat body, where essentially all of the detectable activity could be attributed to EST-2. The third esterase activity (R_f = 0.39) which we have called ESTERASE-J (EST-J), since it occurs only in juvenile or pre-adult life stages, was found to be restricted in its distribution almost exclusively to the carcass.

2.3.3 Ontogenic Expression

Multiple esterase zones were detected after PAG disc electrophoresis throughout the period of development examined, and a semiquantitative estimate of the pattern of expression of the three major esterases was derived as follows. The total enzyme activity in any given sample was sub-divided to each particular esterase according to the area peak per cent of integrator counts attributable to that enzyme zone after densitometric scanning of disc gels. The three major esterases showed quite different patterns of expression (Fig.2.3). ESTERASE-2 activity was detectable in all developmental stages from egg to adult. During embryonic development, EST-2 specific activity declined over the first 12 hours, but began to increase between 12 and 24 hours. By 36 hours after oviposition (approximately six hours after hatching), the specific activity had risen dramatically, and continued to rise until about half-way through development of the third larval instar.



Tissue	Haemo-lymph	Brain	Salivary Gland	Fat Body	Foregut	Midgut	Hindgut	Malp. Tubules	Carcass	Whole Larva
Specific Activity	0.03	0.83	0.86	0.54	1.96	2.29	1.65	3.08	0.41	
% Total Activity Recovered	†	4.4	3.1	19.9	22.5	28.8	0.7	5.0	15.7	

Figure 2.2 Schematic representation of PAG disc gels showing the tissue distribution of the non-specific esterases. Figures in parentheses are the area peak per cent of total integrator counts for the relevant bands. † Not quantitatively collected.

Table 2.2 Semi-quantitative estimation of the larval tissue distribution of the non-specific esterases, based on enzyme assays and PAG disc gel electrophoresis

Tissue Type	Specific activity	Total activity	% Total activity	*Est-2* activity	*Est-2* as % Total *Est-2*	*Est-1* activity	*Est-1* as % Total *Est-1*	*Est-J* activity	*Est-J* as % Total *Est-J*
Haemolymph	0.03	1.00	*	-	0	0.86	100	-	-
Brain	0.83	3.42	4.4	2.02	3.1	-	-	1.40	11.9
Salivary Gland	0.86	2.36	3.1	2.36	3.6	-	-	-	-
Fat Body	0.54	15.36	19.9	15.00	23.1	-	-	-	-
Foregut	1.96	17.33	22.5	17.09	26.4	-	-	-	-
Midgut	2.29	22.22	28.8	22.22	34.3	-	-	-	-
Hindgut	1.65	0.57	0.7	0.57	0.9	-	-	-	-
Malpighian Tubules	3.08	3.82	5.0	3.82	5.9	-	-	-	-
Carcass	0.41	12.09	15.7	1.72	2.7	-	-	10.37	88.1

* Not quantitatively collected.

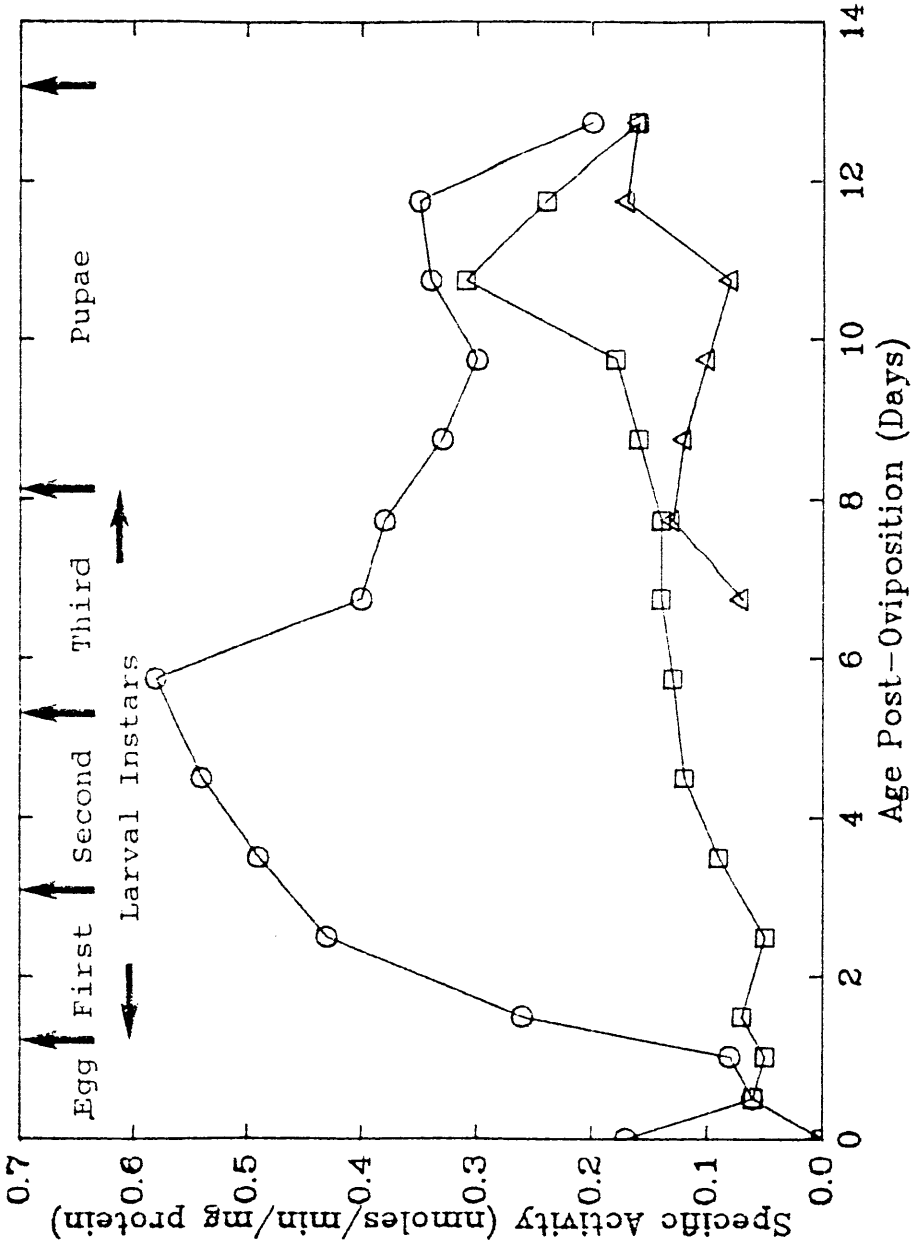


Figure 2.3 Developmental pattern of expression of the major non-specific esterases in pre-adult life-stages. (○) EST-2; (□) EST-1; (△) EST-3.

ESTERASE-2 activity decreased throughout the remainder of larval development and into the pupal phase, but increased slightly during development of the pharate adult. Since the soluble protein content of *D. buzzatii* varies with development (Fig. 2.4), as it does in other species of *Drosophila* (Dewhurst *et al.*, 1970; Church and Robertson, 1966), it is informative to examine the enzyme activity on a per individual basis (Fig. 2.6). Expressed in this way, EST-2 activity showed peaks early in third instar, early in pupal development and again during pharate adult development. After eclosion, the pattern of expression of EST-2 was similar in both sexes. There was a rapid increase in activity over the first 24 hours and activity remained high for 8-10 days, after which it declined and appeared to fluctuate.

ESTERASE-1 activity was not detectable in 0-4 hour eggs, but was present 12 hours after oviposition (Fig. 2.3). After hatching, EST-1 activity rose gradually throughout the whole of larval development. Activity continued to rise during formation of the pre-pupa and pupa, and showed an abrupt peak during early development of the pharate adult (Figs. 2.3 and 2.5). After eclosion, EST-1 activity was initially very high, but declined over a 10-14 day period, to stabilise at a level similar to that immediately prior to eclosion (Fig. 2.5). In general, males and females showed similar patterns of expression, though the activity in females was much higher in the period 5-10 days post-eclosion.

The third major esterase of *D.buzzatii* (EST-J), showed a very restricted pattern of expression, occurring only in pre-imaginal life stages (Figs. 2.3 and 2.5). ESTERASE-J was first detectable in mid third instar larvae. Activity rose rapidly to a peak in late third instar, declined during pupal development, then rose rapidly to a second peak late in development of the pharate adult. There was no detectable EST-J activity in the newly eclosed adult flies.

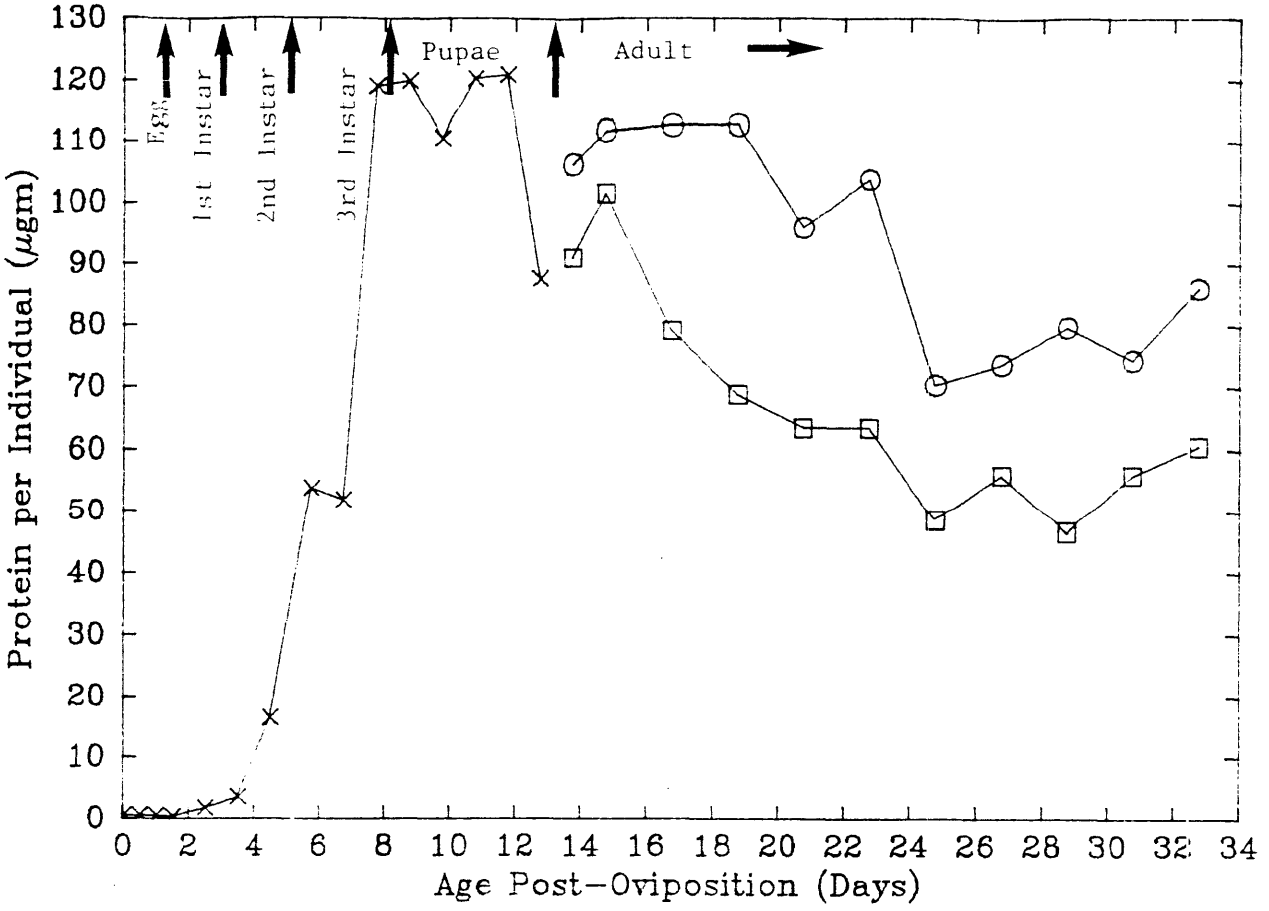


Figure 2.4 Soluble protein, expressed as ugm per individual, as a function of age in *D. buzzatii*. (X) Pre-adult; (○) adult female; (□) adult male.

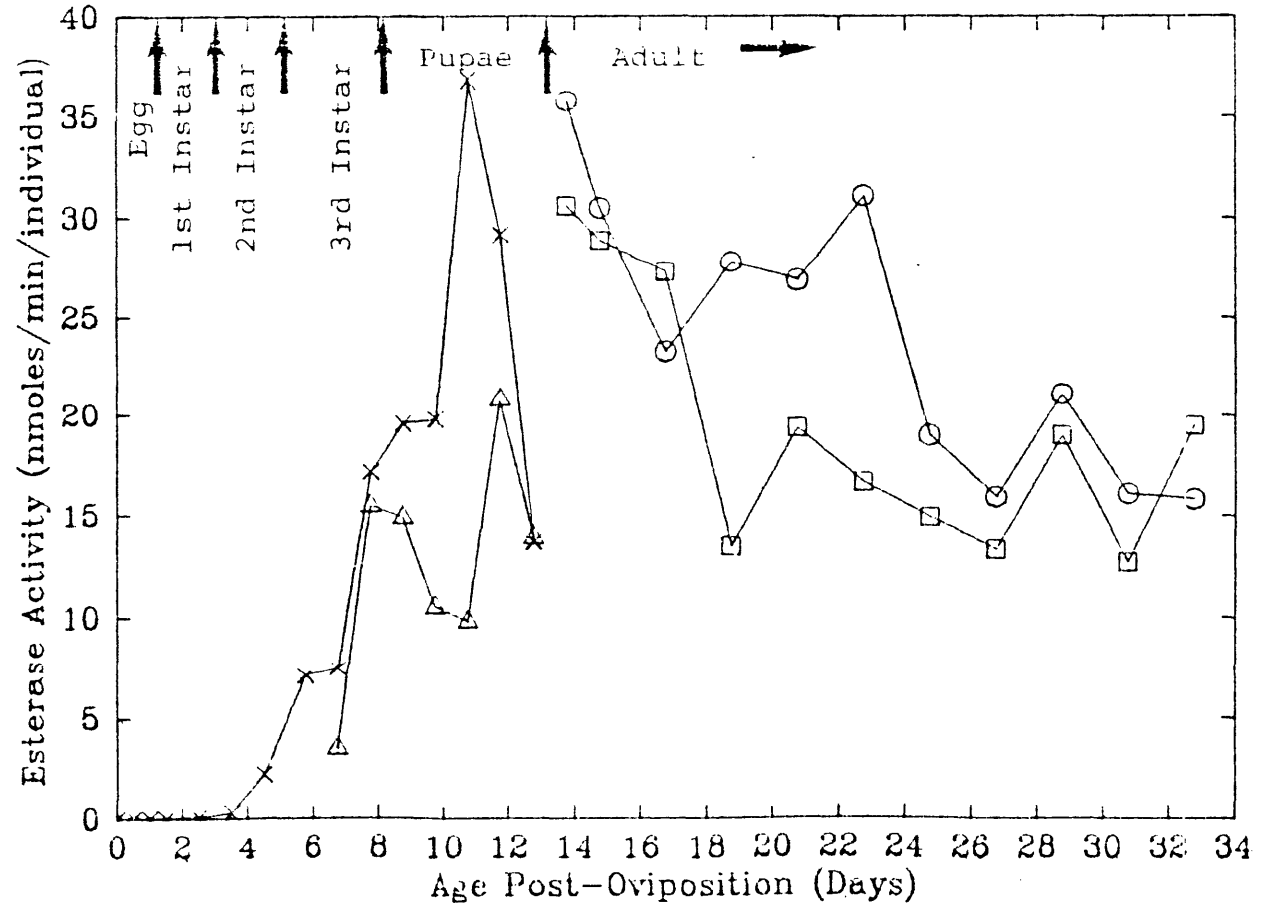


Figure 2.5 Ontogenic expression of the β-esterases in *D.buzzatii*. (△) EST-J; (X) EST-1 pre adult; (○) EST-1 adult female; (△) EST-1 adult male.

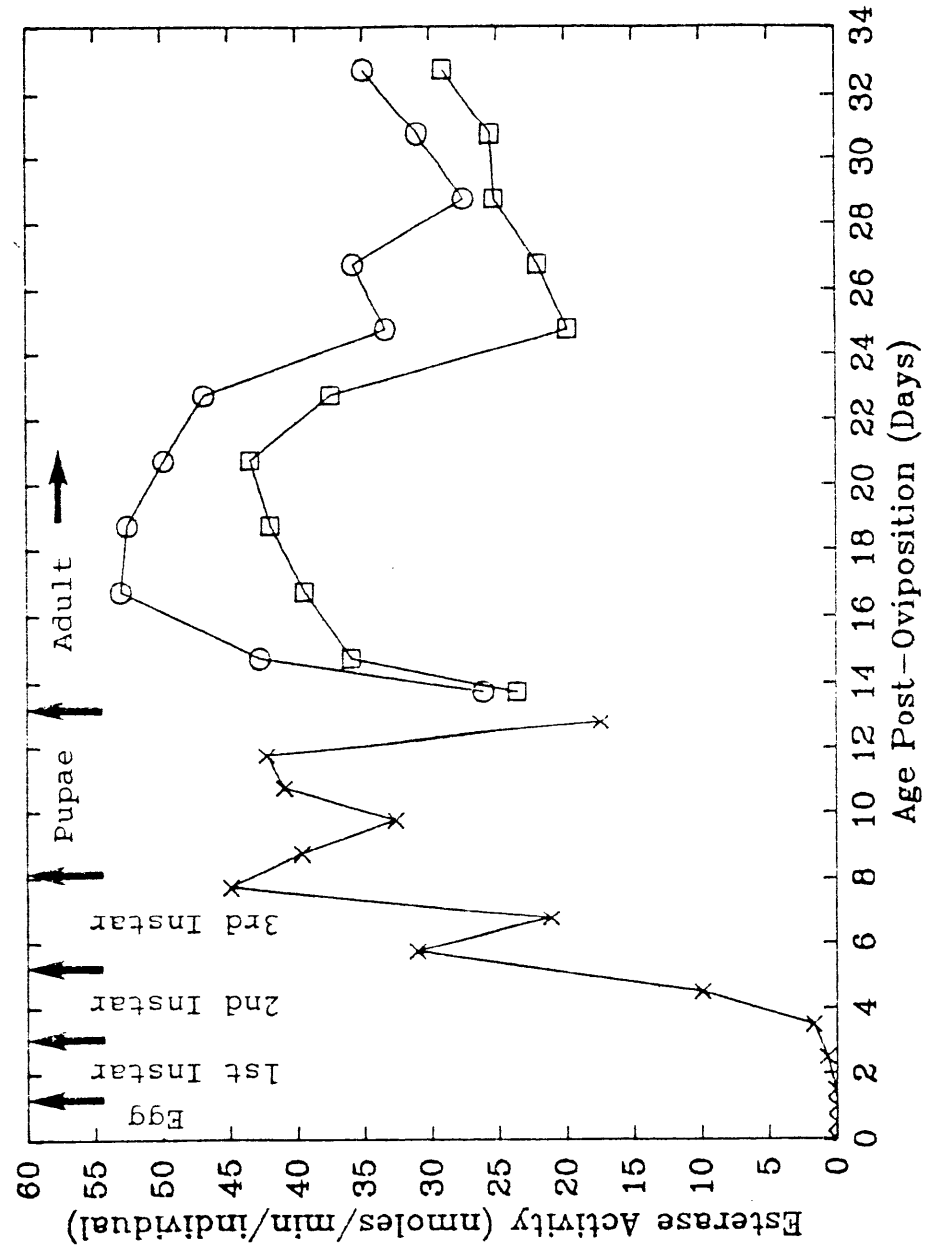


Figure 2.6 Ontogenic expression of the α-esterases in *D. buzzatii*. (**X**) EST-2 pre-adult; (○) EST-2 adult female; (□) EST-2 adult male.

2.4 DISCUSSION

The three major non-specific esterases of *D. buzzatii* are characterised by quite different distributions. The subcellular distribution was examined only for the two enzymes EST-1 and EST-2, and both were found primarily in the 100,000 g supernatant. It is not known whether the small amount of EST-2 activity found in other fractions was due to contamination by this very active enzyme, or whether there may be some degree of membrane association. However, the distribution suggests a predominantly cytosollic or extracellular location for these enzymes. The subcellular distribution of esterases in other species of *Drosophila* has not been reported, but in other insects they have been found predominantly in the cell free (100,000 g supernatant), and microsomal (100,000 g pellet) fractions.

The tissue distribution of the major esterase isozymes was examined in late third instar larvae, and each enzyme was found to possess essentially a unique pattern. ESTERASE-1 was found only in the haemolymph. ESTERASE-2 had a slightly wider distribution, but was located predominantly in the fore- and midgut regions of the alimentary tract, with a smaller amount also in the fat body.

Since the physiological function of none of these enzymes is known, it is very difficult to establish homology with esterases in other species of *Drosophila*. However, a comparison with other members of the *mulleri* sub-group of the *repleta* group suggests considerable similarity; Kambysellis *et al.* (1968) have reported a comparison of the adult esterases of two closely related cactophilic species *D. aldrichi* and *D. mulleri*. Both species show a major β-esterase (EST-D) which is located predominantly in the haemolymph, and on this basis is probably homologous to EST-1 in *D. buzzatii*. Similarly, both *D. aldrichi* and *D. mulleri* have a major α-esterase (EST-C) which is located primarily in the gut, and is likely to be homologous to the EST-2 enzyme of *D. buzzatii*.

Recent studies of the esterases of *D. mojavensis* by Zouros and others (Zouros *et al.*, 1982; Zouros and van Delden, 1982) offer the possibility of close comparison with *D. buzzatii*. These authors discussed an α-esterase (EST-2) and two β-esterases (EST-4 and EST-5). One of the β-esterases (EST-4) was restricted in its developmental expression to third-instar larvae and pupae, and was located in the larval carcass. This pattern corresponds precisely to that of EST-J of *D. buzzatii*, and these enzymes may be presumed to be homologous. The other β-esterase of *D. mojavensis* (EST-5) was first detected in 10 hr eggs, and was present thereafter in all life-stages, with high levels of activity in late third-instar larvae and pupae, and again in newly eclosed adults. The larval tissue distribution showed EST-5 to be located in the haemolymph and fat body. Again, this pattern is identical to that of EST-1 in *D. buzzatii*, and these enzymes are likely to be homologous. Zouros *et al.* (1982) presented no data for the developmental expression of EST-2 in *D. mojavensis*, but the larval tissue distribution was the same as that for EST-2 of *D. buzzatii*, and these enzymes most likely are homologous.

Comparison of these data with those for esterases of other *Drosophila* species outside the *mulleri* subgroup is less straightforward. Within the *virilis/repleta* radiation of the sub-genus *Drosophila*, the only major physiological studies of esterases have been with *D. virilis* (Korochkin, 1980; Sasaki, 1974). Adults of *D. virilis* exhibit major α- and β-esterase activities, and a comparison of the developmental and tissue specific distributions of these two enzymes (Sasaki, 1974) strongly suggests homology with the major adult esterases of *D. buzzatii*. The α-esterase of *D. virilis* is mainly distributed in the gut, and shows peaks of activity in the second larval instar, and the young adult. This is precisely the pattern observed for *D. buzzatii*, and presumably indicates homology of the two enzymes. The patterns of expression of β-esterase do not match those of EST-1 quite so closely. The β-esterase is located in the haemolymph, but shows a peak of activity in second instar and high levels of activity over the first two weeks

of adult life. This deviates from the pattern of EST-1 in *D. buzzatii*, which shows peaks of activity in the pupa, and very young adult stages.

Within species of the sub-genus *Sophophora*, the α-esterases do not appear to have been studied in any detail. Among the β-esterases, the most detailed studies are those by Richmond and co-workers on the EST-6 enzyme of *D. melanogaster*. ESTERASE-6 is the major β-esterase in this species, and its tissue specificity in adults was reported by Sheehan *et al.* (1979). They found EST-6 activity in all body segments, but particularly high activity in male abdomens. A more detailed analysis revealed that the activity was located in the anterior ejaculatory duct. It seems most unlikely that this enzyme is homologous to the major β-esterases of *D. buzzatii* for several reasons. The tissue distribution of EST-6 is quite different from that of the β-esterase of *D. buzzatii*. ESTERASE-6 is a monomer of molecular weight 62,000 to 65,000 (Mane *et al.*, 1983), whereas EST-1 of *D. buzzatii* has an approximate molecular weight of 120,000 (Section 3.3), and individuals heterozygous at the Est-1 locus show a three banded phenotype typical of a dimeric molecule.

The developmental profiles of esterases in species of the sub-genus *Sophophora* have not been widely reported. Sheehan *et al.* (1979), investigated the EST-6 of *D. melanogaster*. In common with EST-1 and EST-2 of *D. buzzatii*, EST-6 is present throughout all developmental stages, but otherwise the pattern of expression is quite different to any of the *D. buzzatii* esterases. The only other species in which the ontogeny of esterases has been examined to any significant extent is *D. pseudoobscura* (Pasteur and Kastritsis, 1971; Berger and Canter, 1973). The predominant esterase in this species is ESTERASE-5, which is present throughout the whole of development (Berger and Canter, 1973) and is located primarily in the haemolymph (Pasteur and Kastritsis, 1971). The enzyme is apparently a dimer, with molecular weight 103,000 to 105,000, and shows a preference for β-naphthyl esters (Narise and Hubby, 1966). On this basis EST-5 may be homologous to EST-1 in *D. buzzatii*.

In summary, the available evidence suggests that enzymes homologous to EST-1, EST-2 and EST-J of *D. buzzatii* are widespread in species of the *mulleri* sub-group of the *repleta* species group. The presence of enzymes showing substantial homology to EST-1 and EST-2 in the much more distantly related species *D. virilis* suggests that the major α- and β-esterases evolved and have been conserved in function over a long period within this branch of the Drosophilidae. It has not been possible to establish any convincing homologies between the esterases of *D. buzzatii* and those of species belonging to the sub-genus *Sophophora*.

Despite extensive investigations in a large number of animals from insects to mammals, the physiological functions of non-specific esterases remain unknown. Amongst insects, non-specific esterases have been implicated in digestion/absorption (Colhoun, 1960), detoxification of xenobiotics (Dauterman, 1976; Robinson, 1979), regulation of hormone titre (Whitmore *et al.*, 1980; Gilbert and Richmond, 1982), and eclosion in holometabolous insects (Berger and Canter, 1973; Katzenellenbogen and Kafatos, 1971).

In *Drosophila* species, the work of Richmond's group has established a fairly convincing role for EST-6 in reproduction, possibly mediated through ejaculate transfer and sperm storage (Gilbert, 1981; Gilbert and Richmond, 1982) and pheromonal control of female remating (Richmond and Senior, 1981). However, even in this well documented case there may be some further, more general role of EST-6, since it is also present in larval and pupal stages, and has a widespread tissue distribution in adults (Sheehan *et al.*, 1979). The gut esterases of *Drosophila* do not seem to have been investigated to any significant degree, and although it has been suggested that EST-2 in *D. buzzatii* may have a role in digestion and, or detoxification (East, 1982), confirmation of this must await identification of the natural substrates.

The tissue distribution and developmental profile of EST-J suggest a degree of homology with pupal esterases described for a number of *Drosophila* species (Berger and Canter, 1973; Raushenbakh *et al.*, 1977). If so, this

enzyme may have some function in eclosion, and the substrate(s) for these enzymes are presumably wax or lipid components of the pupal case, though again no specific ester has ever been identified.

Hopefully the biochemical characterization of these enzymes, and the establishment of homologies with the esterases of other species will ultimately contribute to an understanding of the *in vivo* functions of this ubiquitous class of hydrolases.

CHAPTER 3

COMPARATIVE BIOCHEMISTRY OF THE ESTERASE-1 AND ESTERASE-2 ISOZYMES

3.1 INTRODUCTION

Some general observations concerning the roles of isozymes and allozymes in biochemical and physiological adaptation were considered in Chapter 1 (Sections 1.1.1 and 1.1.2). Hydrolytic enzymes in general, and the non-specific carboxylesterases in particular, pose considerable difficulties of definition for the biochemist. An esterase may be defined as any enzyme which effects the hydrolytic cleavage of an ester bond. However, esterases tend to have broad and overlapping substrate specificities, especially for the artificial substrates normally used for their characterisation. For this reason, esterases possibly should be considered as isozymes only in the broadest definition of the word, as their *in vivo* substrates may be chemically quite unrelated. Markert (1977) has stated' ...until the various esterases are distinguished from one another on the basis of their biological activity, it seems necessary to regard the entire group as a complex isozymic system. The evidence at hand suggests that several distinct isozymic systems compose this very complex group of enzymes."

In some mammalian species, evidence is gradually accumulating which suggests the existence of families of esterase isozymes derived by gene duplication. The most comprehensive biochemical genetic characterisation of mammalian esterases is for the house mouse *Mus musculus*, and has been the subject of a recent review (Peters, 1982). Of the 17 loci known to affect esterase expression in *M. musculus*, 15 have been mapped. Three of the loci

have been assigned to chromosome 9, and seven genes have been mapped to two clusters within 10 cM of each other on chromosome 8. Four of these genes are tightly linked in a single cluster, and no recombinants have been recovered in any crossing programme. Pooling data from a number of studies, Peters estimated that, at the 95% probability level, these 4 loci are within a distance of 0-0.73 cM and suggested this cluster may have arisen as a result of regional duplication of a common ancestral gene.

Womack and Sharp (1976) attempted to establish homologies between the esterases of *Mus musculus* and those of the rat, *Rattus norvegicus*. Peters (1982) extended this to include studies on the rabbit, *Oryctolagus cuniculus* (Fox and van Zutphen, 1979), and the prairie vole, *Microtus ochrogaster* Semeonoff, 1972), and concluded that the duplications which gave rise to the esterase gene clusters on mouse chromosome 8 were of ancient origin, and had been conserved over long periods of evolutionary time.

The situation in *Drosophila* is less clear, since little systematic work has been done to establish homologies, especially between species which are not closely related. It appears that esterases are frequently located on the same chromosome both within and between *Drosophila* species. In the most extensively genetically characterised species, *D. melanogaster*, *Est-6*, *Est-c*, *ali-est* and three acetylcholine esterase loci have all been mapped to chromosome III (O'Brien and MacIntyre, 1978). Ohba (1970, 1971) has mapped the major α- and β-esterase loci of *D. virilis* to chromosome II, and this is also the case for *D. montana*, another species of the *virilis* group (Roberts and Baker, 1973). Of the 10 esterase genes detected in *D. subobscura*, Loukas *et al.* (1979) have assigned two to the J chromosome, two to the O chromosome, and a complex esterase gene *Est-9* (see below) to the E chromosome. Within the *mulleri* subgroup of the *repleta* group, of which *D. buzzatii* is a member, Zouros (1976) has examined the chromosomal location of the polymorphic esterases of *D. mojavensis*, *D. arizonesis* and *D. mulleri*, and found that all were located on chromosome II. Evidence presented in Appendix A indicates that the

Est-1 and *Est-2* loci of *D. buzzatii* are also on chromosome II in this species, located approximately 24 ± 2 cM apart in the standard arrangement. Thus for members of the *virilis* and *repleta* groups of the subgenus *Drosophila*, and for the *obscura* and *melanogaster* groups of the subgenus *Sophophora* we find esterases located on the same chromosome. This may suggest the conservation of substantial tracts of DNA over long periods of evolution, but in the absence of nucleotide sequence data for individual esterase genes, it does not necessarily convey much information about the evolutionary origin of the various esterase isozymes. Despite this, however, there is at least some evidence for the evolution of *Drosophila* esterases by gene duplication.

The studies of Baker and co-workers have provided convincing evidence for an α-esterase gene cluster in *D. montana* (Roberts and Baker, 1973; Baker, 1975, 1980; Baker and Kaeding, 1981). They proposed the existence of four loci, located within a distance of less than 1 cM, and further suggested that these loci had arisen by an original duplication followed by a divergence of function, then a subsequent tandem duplication of the initial duplication to give the four genes.

Another complex α-esterase locus (*Est-9*) has been reported in *D. subobscura* (Loukas and Krimbas, 1975). Fourteen zones of activity were attributed to this locus, of which 1 to 4 zones were found in any single fly. A series of crosses suggested the existence of at least five, and probably more genes in the cluster. Fine structure mapping of the *Est-9* complex gave a distance between the "alleles" used for the cross of 0.00002, so the genes must be tightly linked.

It has been suggested that the two β-esterases, *Est-4* and *Est-5* of *D. mojavensis* may represent a gene duplication (Zouros *et al.*, 1982; Zouros and van Delden, 1982). It was argued in the previous chapter that *Est-4* and *Est-5* of *D. mojavensis* may be homologous to *Est-3* and *Est-1* respectively, of *D. buzzatii*. Assuming this to be the case, these two β-esterases in the cactophilic *Drosophila* represent an interesting example of duplication

followed by subsequent divergence of ontogenic and tissue specific expression. Whether there has been a concomitant divergence of function remains uncertain, since their physiological substrates are unknown, but the results presented in Chapter 2 suggest that they are probably not functionally equivalent.

These examples suggest that evolution of esterases by gene duplication may not be uncommon in *Drosophila*, and they appear to be good candidates for inclusion in a list of multiple locus isozymes. What is far less clear, however, is the evolutionary relationship between the α- and β-esterase isozymes. Reports in the literature suggest that for both α- and β-esterase there is frequently one predominant enzyme detectable in adult *Drosophila*, and based simply on molecular weight critera the α-esterase and β-esterase enzymes may represent two homologous series across a number of species in the genus (Sasaki and Narise, 1978). Since the function of these enzymes remains unknown, biochemical studies of their properties, in conjunction with the types of physiological studies reported in Chapter 2, are important tools for the establishment of homologies, both between species, and for esterase isozymes within a species (*i.e.*, orthologous and paralogous homologies respectively, in the terminology of Lundin, 1979). This chapter describes the comparative biochemistry of the EST-1 and EST-2 enzymes, to determine the nature and extent of the similarities and differences between them, and also to provide a biochemical basis for comparison with esterases of other *Drosophila* species.

3.2 MATERIALS AND METHODS

The chromatography gels; Sephadex G-100, G-150, DEAE-Sephadex A-50, and the proteins used for molecular weight standards were purchased from Pharmacia (South Seas) Pty. Ltd. (Sydney, Australia). Esterase substrates (other than volatile esters), enzyme inhibitors, and acrylamide were obtained from Sigma Chemical Company (St. Louis, Missouri). Volatile esters and all

other chemicals were reagent grade, and were purchased from Ajax Chemical Company (Sydney, Australia).

3.2.1 Partial Purification of Enzymes

3.2.1.1 Protocol Number 1:

Minimal purifications of large amounts of activity for general biochemical analysis of EST-1 and EST-2.

All steps were carried out at 0 to 4°C.

Preparation of Crude Homogenate

Forty grams of 0-3 day old adults were homogenised in 100 ml ice-cold phosphate buffer (0.1M, pH 6.8) in a Sorvall Omni-mixer with 4 x 30 second bursts at 16,000 rpm. The blender was rinsed with 20 ml of the same buffer and the pooled homogenate was passed through 4 layers of fine nylon gauze to remove large fragments of cuticle, wings etc. The resultant filtrate was further filtered through glass wool to reduce the lipid content, and this filtrate was subsequently centrifuged at 10,000 rpm for 30 minutes at 2°C. The supernatant was the crude homogenate (Fraction I).

Ammonium Sulphate Fractionation

Solid ammonium sulphate was added gradually to the cold homogenate (94 mls) to bring it to 45% saturation. After stirring for 30 minutes at 4°C the suspension was centrifuged at 10,000 rpm for 30 mins at 2°C. The pellet was discarded and the supernatant brought to 75% saturation with the addition of solid ammonium sulphate. This was stirred for 30 minutes and the precipitate collected by centrifugation at 10,000 rpm for 30 minutes at 2°C. The supernatant was discarded and the pellet redissolved in 20 ml of ice-cold phosphate buffer (0.1M, pH 6.8). The resultant solution was extensively dialysed (16.5 hours) against 4 litres 0.1M phosphate, pH 6.8. The dialysate (36 mls) was concentrated 2-fold with lyphogel (Gelman Sciences) to yield 18 mls (Fraction II).

Sephadex G-150 Chromatography

The 45-75% ammonium sulphate cut (Fraction II) was applied to a 2.5 x 90 cm column of Sephadex G-150, prepared and packed according to the manufacturers' instructions (Pharmacia Technical Bulletin), and equilibrated with 0.1M phosphate pH 6.8. Sample application and elution were carried out with an ascending flow rate of 30 ml/hr, and after 120 mls had been eluted a fraction collector was connected and 2 ml fractions were collected. Alternate fractions were spot-tested for esterase activity, and fractions of interest were then analysed electrophoretically and by enzyme assay. Peak fractions of EST-2 were pooled (Fraction III), as were those for EST-1 (Fraction IV).

3.2.1.2 Protocol Number 2:

For the preparation of a more highly purified fraction of EST-2 for enzyme kinetic analyses.

All steps were carried out at 0 to 4°C.

The preparation of crude homogenate and ammonium sulphate fractionation were the same as for Protocol 1, except that 0.05M Tris-HCl pH 7.5 was used for all steps.

Sephadex G-100 Chromatography

The ammonium sulphate dialysate (15 mls) was applied to a column (2.5 x 70 cm) of Sephadex G-100 prepared according to the manufacturers' instructions and equilibrated with 0.05M Tris-HCl pH 7.5. Sample was applied and eluted with an ascending flow rate of 40 mls/hr, and after 80 mls had passed through the column, a fraction collector was connected and 5 ml fractions were collected. Spot-tests for esterase activity were used to localise peaks, and fractions of interest were further analysed electrophoretically and by enzyme assay. Peak fractions of EST-2 were pooled (Fraction III).

DEAE Sephadex A-50 Chromatography

Fraction III was loaded onto a column (2.5 x 40 cm) of DEAE Sephadex A-50 prepared according to manufacturers' instructions (Pharmacia Technical Bulletin) and equilibrated with 2 column volumes of 0.05M Tris-HCl pH 8.0,

containing 0.02M NaCl. Sample was applied and washed on with 1 column volume of the equilibration buffer. Enzyme was eluted in a linear gradient of 0.02 to 0.50M NaCl in 0.05M Tris-HCl pH 8.0, with a flow rate of 30 mls per hour, and 5 ml fractions were collected. As before, enzyme was localised with spot-tests and further characterised by electrophoresis and enzyme assay. Peak fractions were pooled (Fraction IV), and this was the EST-2 preparation used for further analyses.

3.2.2 Molecular Weight Determination

Approximate molecular weight estimations were made by gel filtration on an 87 x 2.5 cm column of Sephadex G-150 using bovine ribonuclease-A (M.W. 13,700), bovine chymotrypsinogen-A (M.W. 25,000), chicken ovalbumin (M.W. 43,000), bovine serum albumin (M.W. 67,000), collagenase (M.W. 100,000) and rabbit muscle aldolase (M.W. 158,000). Molecular weight estimation was carried out according to the Pharmacia Gel Filtration Calibration Kit Instruction Manual (Pharmacia Fine Chemicals, Uppsala, Sweden). Data were analysed according to the methodology of Rodbard (1975) using standard statistical techniques (Snedecor and Cochran, 1967).

3.2.3 Enzyme Assays

Standard assays used during enzyme purification, molecular weight estimation, determination of pH profiles and thermostability studies employed either p-nitrophenylacetate (p-NPA) following the method of Townson (1972), or α- naphthylacetate (α-NA) or butyrate (α-NB) using the method of Mastropaolo and Yourno (1981). In all cases, assays were adjusted for spontaneous hydrolysis of the substrate by using heat denatured enzyme blanks.

References for esterase assays used in the substrate specificity study are given below, but details of the techniques are given in Appendix B. The series of p-nitrophenyl-esters were assayed according to Townson (1972). Esters used ranged from acetate (C=2) to caprate (C=8). Extension of the acyl C-chain length beyond 8 C-atoms resulted in difficulties with solubility

in the aqueous system employed. Naphthyl esters were assayed either by a modification of the colourimetric method of van Asperen (1962) or the continuous spectrophotometric method of Mastropaolo and Yourno (1981). The series of volatile esters were assayed in the pH-stat (Radiometer-Copenhagen, TTT 80 titrator coupled to ABU-80 autoburette and REC-80 recorder, equipped with an REA 160 titrigraph) using a modification of the method of Dudman and Zerner (1975). The cholesterol esters of acetic acid, hexanoic acid and oleic acid were tested using a method adapted from Gallo (1981). A pH-stat esterase-lipase assay was used to test the lipids tri-caproin and tri-olein (Hipps and Nelson, 1974). An assay for 4-methylumbelliferyl acetate was developed in this laboratory. Finally, the assay system for the ethyl esters of the aromatic amino-acids L-phenylalanine and L-tyrosine was developed in this laboratory as an extension of the L-amino acid oxidase assay of Nicholson and Kim (1975).

3.2.4 Protein Assays

During the enzyme purification steps, protein was monitored either by continuous recording of the absorbance at 280 nm. (Isco dual channel U.V. recorder), or it was assayed using the protein-dye binding assay of Bradford (1976).

3.2.5 Inhibitor Studies

Eserine and diethyl-p-nitrophenyl phosphate (Paraoxon, E600) were prepared as stock solutions in propan-2-ol, all other inhibitors were made up as stocks in aqueous solution. A 0.1 ml aliquot of enzyme was pre-incubated with 2.8 ml of inhibitor solution, and the enzyme reaction was initiated by the addition of 0.1 ml of 3×10^{-3}M α-naphthyl acetate in acetone, for a final concentration of 10^{-4}M substrate. The reaction was allowed to proceed for 20 mins, after which it was terminated and colour developed with 5% w/v SDS and 1% w/v diazoblue-B. Controls indicated that none of the inhibitors interferred with colour development at the concentrations used.

3.2.6 pH-Activity Profiles

The activity of partially purified esterases was determined at a number of pH values in the range 6.0 to 8.8 using only one buffer type, *viz.* 0.1M phosphate. The substrate used was α-naphthyl acetate, in preference to the p-nitrophenyl esters, which undergo substantial acid-base catalysed hydrolysis at pH values more than 0.5 units removed from 7.00. All assays were corrected for spontaneous hydrolysis.

3.2.7 Thermostability

Aliquots of partially purified enzyme were incubated at 50 ± 0.5°C in a water bath, and samples were taken at 2 minute intervals for EST-2 and 5 minute intervals for EST-1. All samples were cooled immediately and held in ice until assay. The substrate was 10^{-4} M p-NPA in 0.1M phosphate pH 7.00. Assays were carried out for 2 to 5 minutes, according to the amount of residual activity.

3.3 RESULTS

3.3.1 Partial Purification of Enzymes

For the biochemical studies reported in this thesis two large scale purification runs were carried out. The first of these, (Protocol 1) was designed to yield a large amount of minimally purified ESTERASE-1 and ESTERASE-2 enzyme, so that as many biochemical tests as possible could be carried out to characterise these enzymes. All experiments of Chapters 3 and 4 were done using this material. For these experiments my objective was simply to produce EST-1 and EST-2 preparations which were essentially free of other electrophoretically detectable esterase activity. This was achieved by taking a large number of small volume samples and then pooling conservatively around the peak fractions of interest. The results of a typical fractionation run are given in Table 3.1 and Fig. 3.1 for a stock homozygous for the *Est-1*^{b} and *Est-2*^{b} alleles.

Table 3.1 Preparation of partially purified EST-1 and EST-2, using protocol 1

	Fraction	Volume (mls)	Total protein (mg)	Enzyme activity (µmoles/ min)	Specific activity (µmoles/ min/mg)	Purification (fold)	Yield %
I.	Crude homogenate	94	1186.1	133.9	0.113	1	100
II.	45-75% $(NH_4)_2SO_4$ dialysate	17	255.6	34.4	0.135	1.19	25.7
III.	Pooled EST-2 fraction	45	45.9	15.8	0.343	3.04	11.8
IV.	Pooled EST-1 fraction	36	39.4	12.7	0.323	2.86	9.5

EST-1 activity was determined using β-naphthyl acetate as substrate and EST-2 activity was determined using α naphthyl acetate.

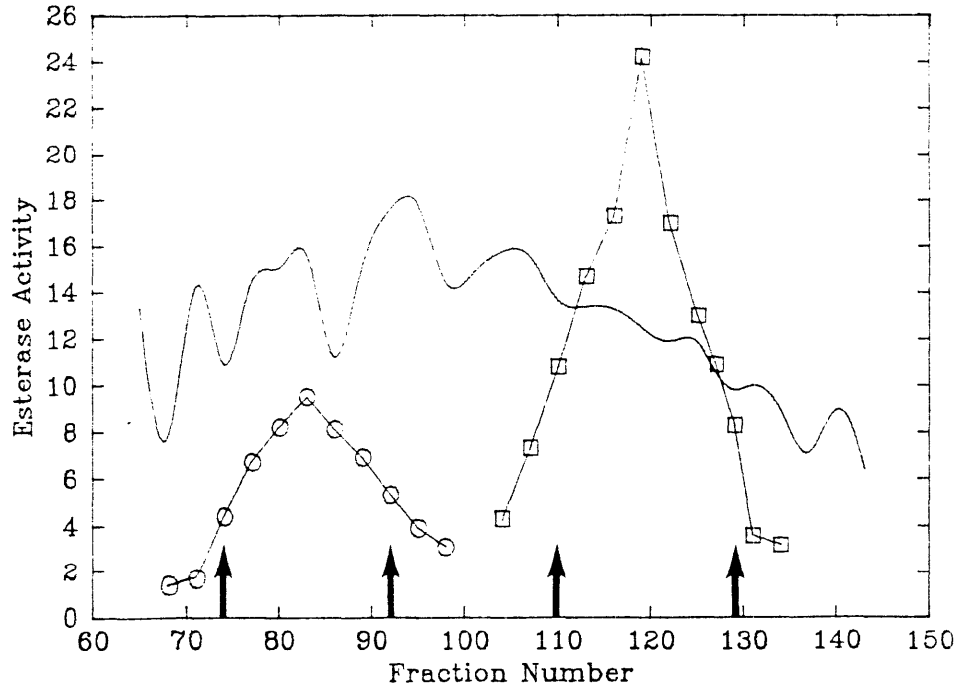


Figure 3.1 Chromatography of ESTERASES on Sephadex G-150. The concentrated $(NH_4)_2SO_4$ cut (Fraction II) was applied to a column (2.5 x 90 cm) of Sephadex G-150 and eluted with an ascending flow rate of 30 ml/hr. Fractions between the vertical arrows were pooled and frozen. Protein (—) was monitored as $O.D._{280}$; (○) EST-1 activity; (□) EST-2 activity.

For the preparation of more highly purified EST-2 for enzyme kinetic analysis Protocol 2 was followed, and the results of a typical run for a stock homozygous for *Est-1*^{b} and *Est-2*^{a} alleles are shown in Table 3.2 and Figs. 3.2 and 3.3.

3.3.2 Molecular Weight Estimation

The relevant elution data for the series of proteins used to calibrate the Sephadex G-150 column, and other information required to construct the calibration curve are summarized in Table 3.3. These data were treated according to the recommendations of Rodbard (1975). Two models were fitted for K_{av} *vs* log. (M.W.).

$$\log (M.W.) = a + bK_{av} \qquad (1)$$

$$\log (M.W.) + a + bK_{av} + c\ (K_{av})^2 \qquad (2)$$

The choice of M.W. rather than K_{av} as the dependent variable in these regressions was based on Rodbard's detailed discussion of the relative magnitudes of the errors associated with K_{av} and M.W., and also of the observed deviation of the points in terms of K_{av} around the calibration curve.

The results of fitting the two curves specified by equations (1) and (2) respectively (Table 3.4) indicated that including the second degree factor $(K_{av})^2$ did not significantly reduce the residual variance, and so equation (1) was treated as the appropriate model for the estimation of molecular weight of EST-1 and EST-2 from the calibration curve (Fig. 3.4). Applying the simple linear regression equation (Table 3.4) to the estimated values of K_{av} for EST-1 and EST-2 (Table 3.3), gave molecular weight estimates of 129,800 (92,700-181,700) for EST-1 and 54,000 (39,700-73,600) for EST-2 respectively. The figures given in parentheses are the lower and upper 95% confidence limits for the estimated values of molecular weight, based on the statistics given in Table 3.4. It should be noted that this estimate for EST-1 is rather unreliable, since the K_{av} value lies towards one end of the regression line, and hence is subject to a large error.

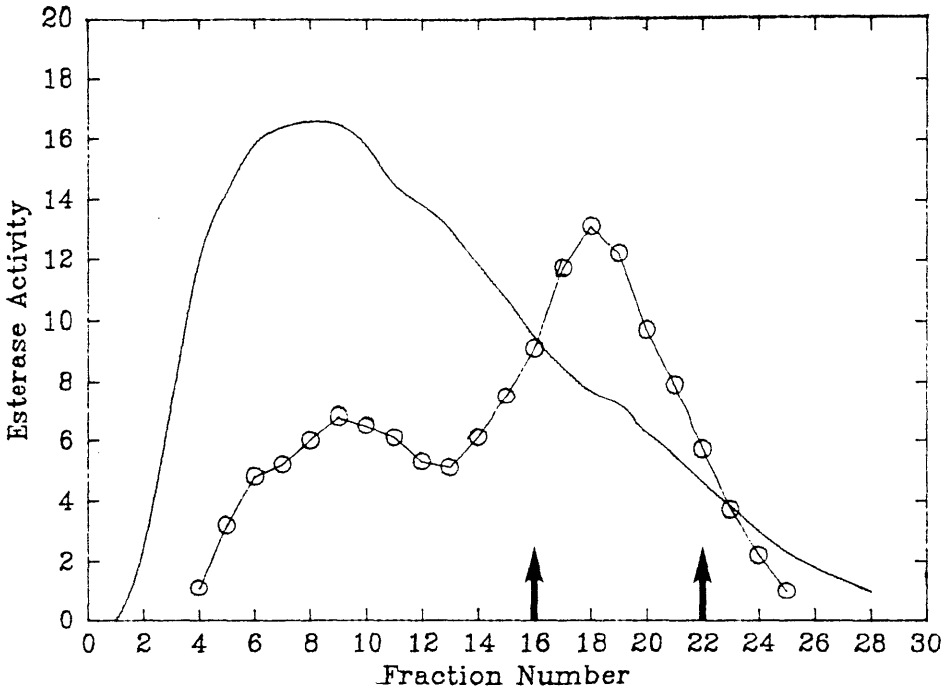


Figure 3.2 Chromatography of ESTERASES on Sephadex G-100. Concentrated $(NH_4)_2SO_4$ dialysate was applied to a column (2.5 x 70 cm) of Sephadex G-100, and eluted with an ascending flow rate of 40 mls/hr. Fractions between the vertical arrows were pooled for DEAE Sephadex chromatography. Protein (—) was monitored as $O.D._{280}$; (○) Total ESTERASE activity.

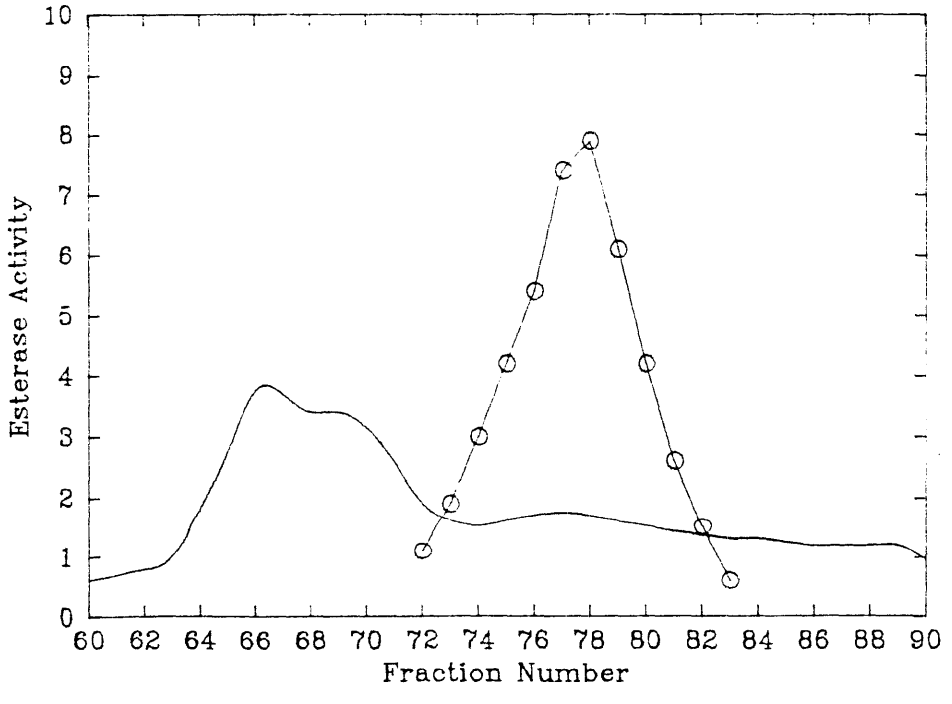


Figure 3.3 Chromatography of ESTERASES on DEAE Sephadex A-50. The pooled fractions from the G-100 column shown in Fig. 3.2 were applied to a column (2.5 x 40 cm) of DEAE Sephadex and after equilibration, were eluted in a linear gradient (0.02-0.50M) of NaCl. Protein was monitored as $O.D._{280}$; (○) EST-2 activity.

Table 3.2 Preparation of partially purified EST-2 for enzyme kinetic analyses using protocol 2

	Fraction	Volume (mls)	Total protein (mg)	Enzyme activity (µmoles/ min)	Specific activity (µmoles/ min/mg)	Purific- ation (fold)	Yield %
I.	Crude homogenate	98	1580	243.32	0.154	1	100
II.	45-75% $(NH_4)_2SO_4$ dialysate	11	390	78.08	0.200	1.30	32.1
III.	Sephadex G-100	35	30.8	20.68	0.671	4.36	8.6
IV.	DEAE- sephadex A-50	25	2.7	12.76	4.67	30.30	5.2

ESTERASE-2 activity was monitored using α-naphthyl acetate as substrate.

Table 3.3 Statistics for the construction of the calibration curve shown in Fig. 3.4, used to estimate the molecular weights of EST-1 and EST-2

Protein	Molecular weight	Log (M.W.)	Elution volume (mls)	$^{*}K_{av}$
Ribonuclease-A	13,700	4.137	38.0	0.730
Chymotrypsinogen-A	25,000	4.398	327.0	0.562
Ovalbumin	43,000	4.634	304.0	0.487
Albumin	67,000	4.826	269.2	0.373
Collagenase	100,000	5.037	249.5	0.309
Aldolase	158,000	5.199	220.8	0.215
EST-1	-	-	232.5	0.254
EST-2	-	-	287.5	0.433

$^{*}K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution volume, V_t is the total volume and V_o is the void volume of the column.

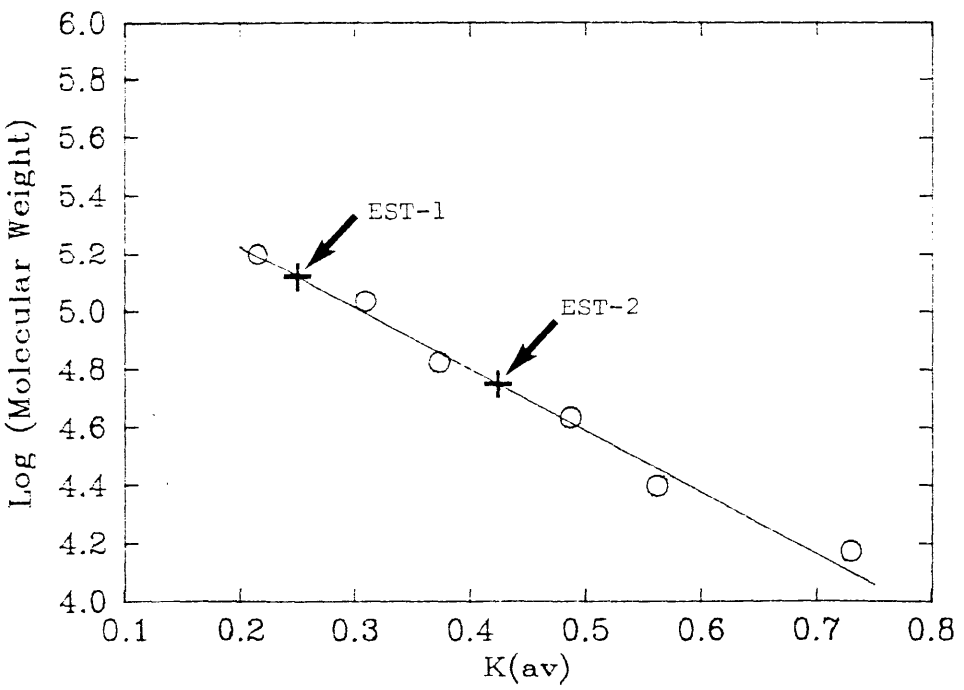


Figure 3.4 Molecular weight of EST-1 and EST-2 using molecular sieving chromatography. Standard proteins and ESTERASE samples were applied to a column (2.5 x 90 cm) of Sephadex G-150, and eluted with an ascending flow rate of 30 ml/hr. Standards were monitored at 280nm and ESTERASES were determined by enzyme assay.

Table 3.4 Results of fitting linear and quadratic models to the data in Table 3.3

Model 1. $\log (M.W.) = a + b(K_{av})$

Regression equation : $\log (M.W.) = 5.653 - 2.126 (K_{av})$

Standard error of intercept = 0.051

Standard error of regression coefficient = 0.108

Standard error of log (M.W.) about the regression line = 0.045

Analysis of Variance

Source	d.f.	S.S.	M.S.
Regression	1	0.7828	0.7828
Residual	4	0.0080	0.0020
Total	5	0.7908	

Model 2 $\text{Log } (M.W.) = a + b(K_{av}) + c(K_{av})^2$

Regression equation : $\log (M.W.) + 5.776 - 2.726(K_{av}) + 0.635 (K_{av})^2$

Standard error of intercept = 0.143

Standard error of first order coefficient = 0.658

Standard error of second order coefficient = 0.686

Standard error of log (M.W.) about the regression line = 0.046

Analysis of Variance

Source	d.f.	S.S.	M.S.
Regression			
due to K_{av}	1	0.7828	0.7828
due to $(K_{av})^2$	1	0.0018	0.0018
Residual	3	0.0062	0.0021
Total	5	0.7908	

3.3.3 Inhibitor Studies

A variety of chemicals known to inhibit esterases in other organisms were tested (Table 3.5), and the compounds used were selected on the basis of their ability to discriminate between the various classes of esterases described by Aldridge (1953). Since the synthetic substrates employed in studies of non-specific esterases can also be hydrolysed by the lyase, carbonic anhydrase, acetazolimide, a known inhibitor of carbonic anhydrases was also included in the study.

The lack of inhibition of both enzymes by acetazolimide and eserine suggests that neither is a carbonic anhydrase or a cholinesterase. Their high sensitivity to low concentrations of diethyl-p-nitrophenyl phosphate implies that they are serine hydrolases and could be classified as carboxyl-esterases (E.C. 3.1.1.1). However, the original classification system specified that carboxylesterases were resistant to sulphydryl inhibitors such as PCMB and $HgCl_2$ (see Section 1.2.4). On this basis, EST-1 might reasonably be classified as a carboxylesterase, but EST-2 shares features of both carboxylesterases (E.C. 3.1.1.1) and arylesterases (E.C. 3.1.1.2).

3.3.4 pH-activity Profiles

The pH profiles of EST-1 and EST-2 were not rigorously examined, only one buffer system of a limited pH range being used for their characterisation. Despite this limitation, however, it is clear that the two enzymes have very different pH optima, with a clear optimum around pH 7.0 for EST-2 and an optimum greater than pH 8.0 for EST-1 (Fig. 3.5).

3.3.5 Thermostability

The time course of denaturation of these enzymes at pH 7.0 and 50^{o}C suggested that there were substantial differences in the stability of EST-1 and EST-2 at this temperature (Fig. 3.6). To investigate these differences further, the data were re-plotted as the logarithm of activity remaining versus time (Fig. 3.7), and the result suggested a linear relationship.

Table 3.5 Effect of inhibitors on ESTERASE-1 and ESTERASE-2

Inhibitor	Concentration [M]	ESTERASE-2	ESTERASE-1
Eserine-sulphate	10^{-5}	-*	-
	10^{-4}	-	-
diethyl-p-nitrophenyl-phosphate	10^{-8}	+	+
	10^{-7}	++	++
	10^{-6}	+++	+++
p-chloromercuri benzoate	10^{-5}	++	-
	10^{-4}	+++	-
mercuric chloride	10^{-5}	++	-
	10^{-4}	+++	-
potassium cyanide	10^{-3}	-	-
acetazolimide	10^{-3}	-	-

* - indicates no inhibition, + weak, ++ strong, +++ total, inhibition respectively.

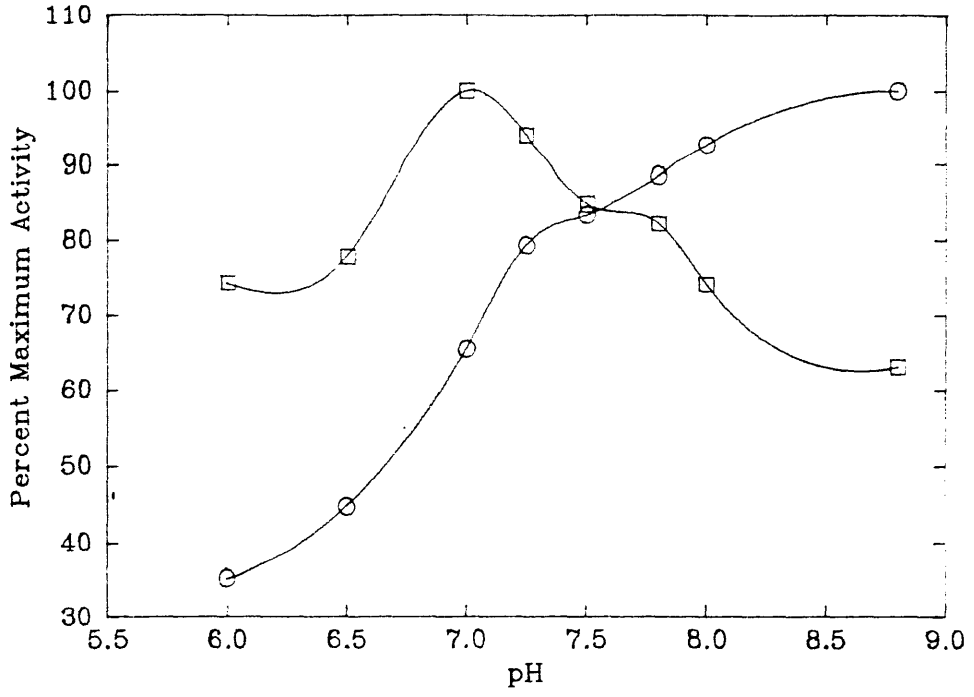


Figure 3.5 ESTERASE activity as a function of pH. All estimates were made at constant ionic strength (0.1M) in phosphate buffers, using α-naphthyl acetate as substrate. (○) EST-1; (□) EST-2.

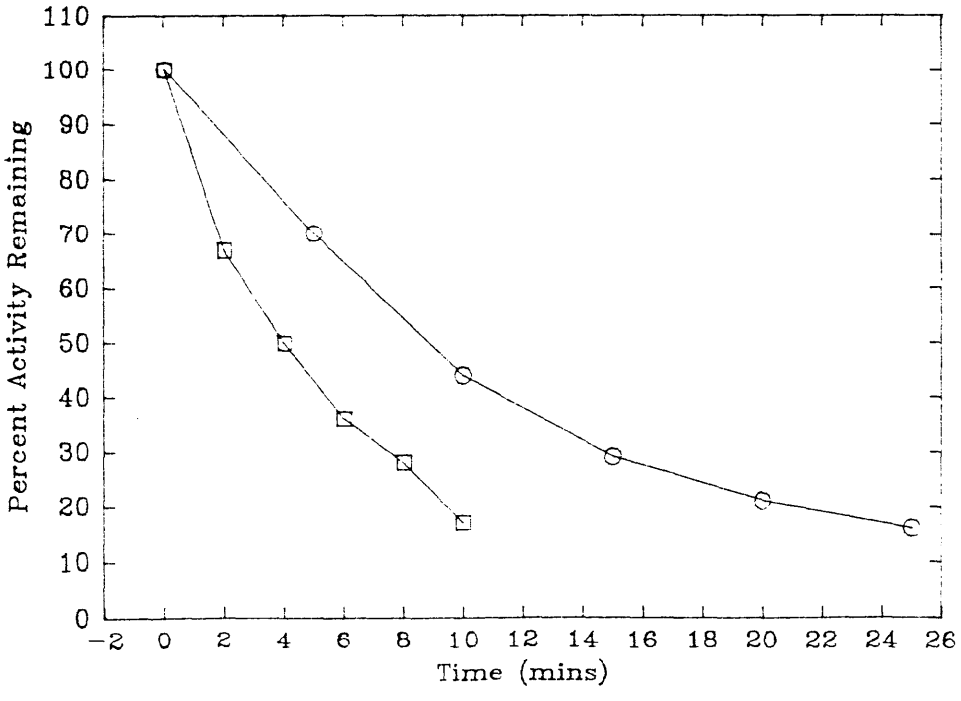


Figure 3.6 Effects of denaturing temperature on ESTERASE activities. Samples of enzyme were incubated at 50°C ± 0.5°C in a circulating waterbath. (○) EST-1; (□) EST-2.

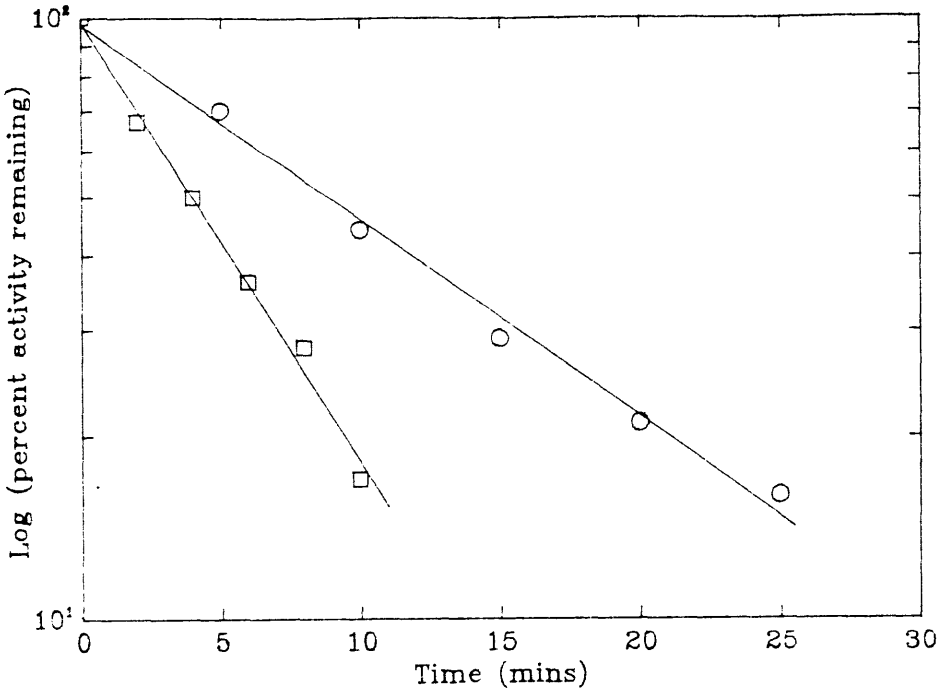


Figure 3.7 Regression of ESTERASE activity on time of exposure to denaturing temperatures. Enzyme activities were log transformed prior to analysis. Solid lines are the least-squares regression of activity on time; (○) EST-1 data points; (□) EST-2 data points.

Accordingly, simple linear regressions were fitted to the data (Table 3.6), and as expected both enzymes showed a highly significant negative association of activity with time. Tests of the null hypothesis H_o: b = 0 gave t = 23.57 for EST-1 and t = 22.41 for EST-2, with 4 d.f. in each instance. Of greater interest however, is the comparison of the slopes for the two enzymes (H_o: $b_1 = b_2$), and in this case there is a highly significant difference, $F_{1,8} = 124.34$, with the rate of denaturation of EST-2 more than twice that of EST-1. Regression coefficients were compared according to the method of Sokal and Rohlf (1969, p.455), using the statistics given in Table 3.6.

3.3.6 Substrate Specificity Studies

Assays were developed for as many esters as possible, the main objective being to determine the patterns of activity as the size and shape of the acid

Table 3.6 Analysis of thermal denaturation data for ESTERASES by simple linear regression of Ln(% activity remaining) on time

Statistic	ESTERASE-1	ESTERASE-2
Intercept (a)	4.579	4.588
Regression coefficient (b)	-0.075	-0.169
s.e.(y.x)	0.067	0.067
s.e.(b)	0.003	0.008

s.e.(y.x) = standard error of y about the regression line $y = a + bx$.
s.e.(b) = standard error of regression coefficient (b).

moieties were altered in a systematic fashion. Because the differences in activity patterns for the two esterases are large, they will be treated in only a qualitative, descriptive way here. A more quantitative analysis of the effects of changing substrates will be undertaken in subsequent chapters, comparing the allozymes produced by the *Est-1* and *Est-2* loci.

3.3.6.1 Effect of Changing Acid Carbon Chain Length

For a series of esters of the chromogen p-nitrophenol, the acid carbon chain length was varied from 2 (acetate) to 8 (caprate). Only esters of straight chain, saturated fatty acids were tested. Both enzymes showed a dramatic effect of carbon chain length on activity, but they differed in their patterns of activity (Fig. 3.8). ESTERASE-1 showed maximal activity on the propionate ester (C=3), and very little activity on esters with a carbon chain length of 4 or greater. ESTERASE-2 showed much higher specific activity on these esters, and also sustained a much broader peak of activity with a maximum for the butyrate ester, but with substantial activity for esters from C=2 to C=6 (caproate).

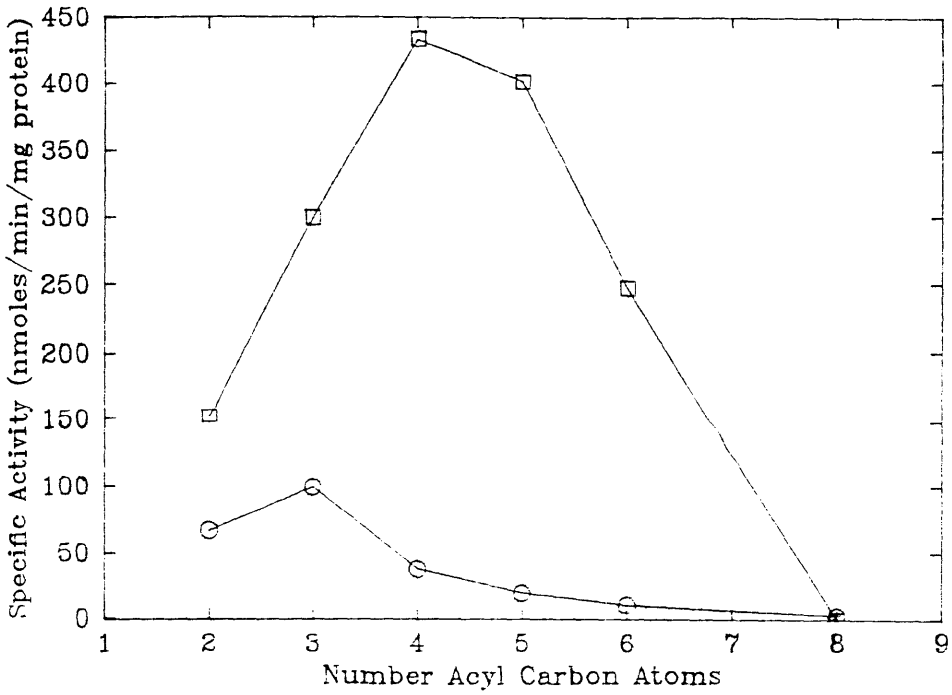


Figure 3.8 The effect of increasing acyl carbon chain length on ESTERASE activity. All substrates were the saturated fatty acid esters of the chromogen p-nitrophenol, assayed in 0.1 M phosphate buffer at pH 7.0. (○) EST-1; (□) EST-2.

3.3.6.2 Effect of Changing Alcohol Carbon Chain Structure

To determine if changes in the structure of the alcohol moiety of the ester produced any systematic changes in activity, a series of acetate esters were examined (Table 3.7). A number of observations may be made from these data. Firstly, as with the series of p-nitrophenyl esters, EST-1 and EST-2 differ markedly in their apparent substrate specificity. This is particularly striking among the series of volatile esters, where EST-1 shows appreciable activity, but relatively little difference between substrates, whereas EST-2 shows a clear pattern of little or no activity on the low molecular weight volatiles, but substantial activity on the higher molecular weight volatile esters.

Amongst the other esters, consistent with their classification as α- and β-esterases, EST-2 has twice the activity on α- naphthyl acetate than it shows on β-naphthyl acetate, and conversely EST-1 shows twice the activity on β-naphthyl acetate than it does on α-naphthyl acetate. A comparison of the two isomers of nitrophenol indicates that both esterases are sensitive to changes in the stereochemistry of the substrate molecule. In this case, moving the bulky, hydrophobic $-NO_2$ group adjacent to the ester linkage (i.e. the ortho-configuration) drastically reduces the rate of hydrolysis of the ester bond.

3.3.6.3 Further Substrate Studies

In addition to the series of p-nitrophenyl- and acetate esters, a further group of compounds not sharing any systematic structural features were examined (Table 3.8). Again EST-1 and EST-2 behave quite differently, and some trends are evident, especially for EST-2. Most striking is the very high level of activity of EST-1 against the group of volatile formate esters. The most interesting feature of the EST-2 data is the consistent trend among the volatile esters of increasing activity with increasing molecular weight, as was observed also with the acetate series.

Table 3.7 Effect of alcohol structure on esterase activity for acetate esters

Ester	ESTERASE-1*	ESTERASE-2
(a) Volatile esters		
ethyl acetate	158.0	N.D.+
n-propyl acetate	134.3	N.D.
i-propyl acetate	134.3	N.D.
n-butyl acetate	134.3	222.9
i-butyl acetate	173.8	318.5
n-amyl acetate	158.0	329.1
i-amyl acetate	244.9	371.6
(b) Non-volatile esters		
p-nitrophenyl acetate	22.6	55.4
o-nitrophenyl acetate	1.7	15.1
4-methyl-umbelliferyl acetate	75.3	262.8
α-naphthyl acetate	23.2	261.9
β-naphthyl acetate	43.5	129.9
cholesteryl acetate	N.D.	N.D.

* Specific activities are expressed as nmoles product released/min/mg protein.

+ N.D. = not detectable.

Table 3.8 Substrate specificity of esterases

Ester	ESTERASE-1	ESTERASE-2
ethyl formate	687.2	N.D.*
n-propyl formate	1524.5	265.4
n-butyl formate	1311.2	530.8
ethyl acetate	158.0	N.D.
ethyl propionate	371.3	212.3
ethyl butyrate	150.1	1082.8
triacetin	130.3	297.2
tricaproin	N.D.	53.1
triolein	N.D.	N.D.
cholesteryl acetate	N.D.	N.D.
cholesteryl hexanoate	N.D.	N.D.
cholesteryl oleate	N.D.	N.D.
L-phenylalanine ethyl ester	10.2	13.4
L-tyrosine ethyl ester	15.1	23.7

* N.D. = not detectable

ESTERASE-1 showed no activity against the lipids tricaproin, triolein or the sterol esters, and EST-2 displayed only very low activity against tricaproin. Activity against the amino-acid esters was detectable but very low.

3.4 DISCUSSION

It is quite obvious from these studies that EST-1 and EST-2 differ in every biochemical property examined. On the basis of gel electrophoretic analysis, and gel chromatographic estimation of molecular weight, it appears that EST-1 is a dimer with an approximate M.W. of 130,000 daltons. By contrast EST-2 is apparently a monomer with a M.W. of about 54,000 daltons. The enzymes differ in their response to inhibitors. Though both can be classified as carboxylesterases on the basis of sensitivity to low levels of the organophosphate diethyl-p-nitrophenyl phosphate, EST-2 is further distinguished by its sensitivity to sulphydryl blocking agents such as PCMB and Hg^{++} ions. The failure of EST-2 to fall neatly into one of the classes of non-specific esterase elaborated for mammalian systems highlights the problems of classification of these enzymes which were discussed previously (Section 1.2.4). It seems that wherever the properties of insect esterases are examined in sufficient detail they fail to correspond closely to their mammalian counterparts. In terms of classification, in the absence of specific information regarding their function, assigning insect esterases to classes according to the IUB/IUPAC system does not appear to be justified beyond the broad grouping E.C. 3.1.1.

The two esterases also differ with respect to their pH optima, which is possibly not surprising given their different tissue distributions. The observed optimum around pH 7.0 for EST-2 is quite consistent with its localisation in the alimentary tract. House (1974), in a review of insect digestion tabulated data on the pH of the alimentary canal and, or its contents for several orders of insecta. No data appear to be available for

Drosophila, but the values for other diptera range widely, especially in the mid-gut where values may be as low as 3.0. However, average values, especially taking into account fore- and hind-gut regions tend to fall in the range 6.5 to 8.0. ESTERASE-2 has quite a broad optimum over this range (Fig. 3.5). Unfortunately the pH optimum has not been accurately determined for EST-1, but it appears to be greater than 8.0. This is not particularly compatible with its location in the haemolymph, since insect haemolymph tends to be slightly acidic and in *D. melanogaster* one estimate put it around 6.6 - 6.7, (Begg and Cruikshank, 1963). The reason for the lack of correspondence between the apparent optimum for the enzyme and the pH of its physiological environment is not clear. The choice of buffer system can affect the measurement of pH optima (e.g. O'Brien, 1973), and the phosphate buffer employed in this study can in no way be considered a good model for insect haemolymph, which in addition to bicarbonate is buffered primarily by amino acids, organic acids and proteins, and has only low buffering capacity in the vicinity of normal physiological pH (Wigglesworth, 1972).

The two enzymes differ dramatically in their thermostability at 50°C, at least under the *in vitro* conditions used. From the regressions of log (% activity remaining) on time (Table 3.6), the estimated times to 50% denaturation (T_{50}) are T_{50} = 8.9 minutes for EST-1 and T_{50} = 4.0 minutes for EST-2. Differences of this magnitude might seem to imply rather large differences in physico-chemical properties of the enzymes, but this may not be a very sensitive measure of evolutionary divergence. It will be demonstrated later (Section 4.2.6) that the magnitude of thermostability differences between allozymes produced by different alleles of the *Est-2* locus is as great as the difference between EST-1 and EST-2 isozymes.

The extent of the differences between the two esterases is particularly obvious when their substrate specificities are examined. Despite their capacity to hydrolyse a broad array of esters, they differ markedly in their rates and patterns of hydrolysis of the groups of chemicals tested. For

both the series of p-nitrophenol esters, and the group of ethyl esters, EST-1 hydrolysed the propionate ester more rapidly than either the acetate or butyrate ester. By contrast, EST-2 preferentially hydrolysed the butyrate ester in these groups.

Among the group of volatile acetate esters (Table 3.7), EST-1 showed very little specificity, hydrolysing all compounds quite rapidly. ESTERASE-2 showed no detectable activity on the ethyl- or propyl-esters, but considerable activity on the butyl- and amyl- acetates. Both enzymes showed greater activity on the esters of secondary alcohols as compared with primary alcohols of the same carbon number. Among other volatile esters (Table 3.8), patterns of hydrolysis within chemically related groups again differ markedly for the two esterases. For a series of ethyl esters EST-2 displays a remarkable amount of activity against the butyrate ester. ESTERASE-1 is distinguished by very high activity on the group of formate esters, and EST-2 has quite high activity against n-propyl and n-butyl formate. Whether or not this high activity has any biological significance is not known, but it is interesting that ESTERASE-6 of *D. melanogaster* showed very high levels of activity against formates as compared with other volatile esters (Danford and Beardmore, 1979).

Dixon (1966) suggested that the mammalian serine hydrolases, i.e. those enzymes with a serine residue in the active site, evolved by gene duplication and subsequent divergence of function. This group of enzymes includes the endopeptidases; elastase, chymotrypsin-A and B, trypsin and thrombin, and also the non-proteolytic esterases; ali-esterases, pseudo-cholinesterase and acetylcholinesterase. If EST-1 and EST-2 of *D. buzzatii* arose by gene duplication, the results presented in this chapter indicate that they have diverged greatly in their biochemical properties. The differences are so great that I feel these two enzymes can be classified as isozymes only in the loose sense, i.e. they are both capable of the hydrolytic cleavage of ester bonds.

Further evidence of the ancient origin of any such duplication is provided by the fact that major α- and β-esterase enzymes are found in the *Sophophora* subgenus (Sasaki and Narise, 1978) as well as the *Drosophila* subgenus. Of course it is not possible to try to entend the homology too far at this stage, since the non-specific nature of esterases requires a more thorough investigation to establish a potential homology, and such studies have not been made in any members of the *Sophophora* subgenus to my knowledge. Within the *virilis - repleta* radiation, however, we have an interesting opportunity to investigate possible homologies.

Narise (1973a,b) has reported some biochemical properties of the adult α- and β-esterases of *D. virilis*. A comparison of her results with those reported in this chapter for *D. buzzatii* show some remarkable similarities (Tables 3.9 and 3.10) which strongly suggest homology between α-*est* locus of *D. virilis* and the *Est-2* locus of *D. buzzatii*. Similarly the β-*est* gene of *D. virilis* appears to be homologous to *Est-1* of *D. buzzatii*. The only significant discrepancy in properties is between the molecular weights of the β-esterases (but see Section 3.3.2 for criticism of the *D. buzzatii* data), and the thermostabilities. Narise (1973a) reported that the α-EST was more thermostable than the β-EST in *D. virilis* whereas the reverse is true for *D. buzzatii*, though it should be noted that the experiments used different temperatures for denaturation.

The extent of the similarities in biochemical properties of these enzymes in two species which have been separated for a substantial period of evolution strongly suggests that whatever the physiological function of these enzymes may be, that function has been conserved in evolution. For enzymes which are infamous for their lack of specificity, and their unknown function, the degree of homology noted above is quite remarkable. If, as has often been suggested, the natural substrates of these enzymes are extracellular and derived from the external environment, then these data suggest that there is some chemical similarity in the feeding sites of species which

Table 3.9 Comparison of the β-esterases of *D. virilis* and *D. buzzatii*

Character	*D. buzzatii** EST-1	*D. virilis* β-EST	Source for *D. virilis* data
Presumptive quarternary protein structure	Dimer	Dimer	Narise, 1973a
Molecular weight	130,000	102,000	Sasaki and Narise, 1978
Organophosphate inhibited	Yes	Yes	Narise, 1973a
Sulphydryl reagent inhibited	No	No	Narise, 1973a
pH optimum	8.0	8.0-9.0	Narise, 1973a
Optimal acyl C-chain length	propionate	propionate	Narise, 1973b
Chromosome location+	2	2	Ohba, 1970, 1971

* Data for *D. buzzatii* derived from this Chapter.

+ The second chromosomes of *virilis* and *repleta* group species are deemed to be homologous on cytological criteria (Wasserman, 1982, p.72).

Table 3.10 Comparison of the α-esterases of *D. buzzatii* and *D. virilis*

Character	*D. buzzatii* EST-2	*D. virilis* α-EST	Source for *D. virilis* data
Presumptive quarternary protein structure	Monomer	Monomer	Narise, 1973a
Molecular weight	54,000	51,000	Sasaki and Narise, 1978
Organophosphate inhibited	Yes	Yes	Narise, 1973a
Sulphydryl reagent inhibited	Yes	Yes	Narise, 1973a
pH optimum	6.5-7.5	7.0-8.0	Narise, 1973a
Optimal acyl c-chain length	butyrate, but substantial activity C2-C6	valerate, but substantial activity C2-C6	Narise, 1973b
Chromosome location	2	2	Ohba, 1970, 1971

share very different ecological environments.

The natural breeding substrates of *Drosophila* species have not been chemically analysed to any great extent, especially with respect to volatile esters. A small pilot study of the volatile fraction of four naturally occurring *Opuntia* rots by combined gas chromatography-mass spectrometry was undertaken. For comparison, four cactophilic yeasts grown in monoculture on sterilised, autoclaved cactus were analysed at the same time. The results showed qualitatively very similar chromatograms among the four rots, and among the four yeast cultures, but quite different chromatograms for rots as compared with yeast cultures. The difference was due to alcohols plus their respective ketones in the rots, and alcohols plus their respective acetate esters in the yeast cultures (East, 1982). This small study would most likely have only detected the lower boiling point esters such as the acetates, since sample preparation was by headspace collection at 40^{o}C, and could not have detected formate esters, since these cannot be identified with a flame ionisation detector (Drucker, 1981). It is known that micro-organisms can produce a variety of short chain carboxylic acids (C1-C7) and alcohols (C2-C5) by utilising a number of different fermentation pathways (Drucker, 1981). It is also known that yeast esterases can synthesise esters in a fermentation medium from the alcohols and fatty acids present, and an equilibrium is established between the ester product and the acid and alcohol precursors (Parkkinen and Suomalainen, 1982). It was found that most esters appeared only in the fermentation medium, but that some, notably of higher molecular weight, were found in the medium and also in the yeast cell (Nordstrom, 1964; Nykanen *et al.*, 1977). Although the small study mentioned above suggested that low molecular weight, low boiling point esters are not routinely found in *Opuntia* rots, the high activity of EST-1 and EST-2 on the volatile esters examined, and especially the trend shown by EST-2 of increasing activity with increasing molecular weight of the substrate, suggests that further analyses, particularly of the higher boiling

point esters may be of interest. The possibility that the cactophilic yeasts might contain esters within the cell also warrants investigation, since the *Drosophila* feed on the yeasts, and store them in the crop prior to digestion, which would have the effect of presenting any sequestered chemicals in a packaged, concentrated form. The synthetic capacity of the cactophilic yeasts, combined with the diversity of fermentation products found in cactus rots certainly provides the potential for the production of a very heterogeneous collection of esters to confront any Drosophilid feeding in that milieu.