CHAPTER 4

COMPARATIVE BIOCHEMISTRY OF ALLOZYMIC VARIANTS PRODUCED BY THE *EST-1* AND *EST-2* LOCI

4.1 INTRODUCTION

In the preceding chapter, the biochemical properties of EST-1 and EST-2 were compared and discussed in terms of their potential evolution by gene duplication, and their homologies with the esterases of other *Drosophila* species. However, in the context of biochemical adaptation and processes of microevolution, it is the similarities and differences between allozyme variants which concern us, and it is these studies which are the subject of this and the following chapter.

Since the initial documentation of the extent and magnitude of genetic variation in natural populations (Lewontin and Hubby, 1966; Hubby and Lewontin, 1966; Harris, 1966), debate over the evolutionary significance of the observed variation has been both spirited and eloquent. At first, as theories and models were developed and elaborated, researchers became polarised into two groups, the neo-classical or neutralist group believing that electrophoretically detected variation is of no adaptive significance, (e.g. Kimura and Ohta, 1971), in contrast to the selectionist group which held the view that such variation is an integral part of the process of microevolution (e.g. Ayala, 1972; Richmond, 1970). Subsequently the emphasis has shifted somewhat, and the questions now perceived to be more relevant are: (a) what proportion of polymorphisms are neutral and what proportion selectively maintained, and (b) for those loci believed to be influenced by selection, what is the nature and mechanism of action of that selection

(see for example, Clarke, 1975)? Concomitant with this shift in emphasis came the development of a methodological approach for the study of allozyme polymorphism.

Clarke (1975) enumerated the following four points as a strategy for testing the adaptive significance of an enzyme polymorphism:

1. "We must make a detailed biochemical and physiological study of the enzymatic products of the alleles, noting any differences between them.
2. Knowing the nature of the differences, the function of the enzyme, and the ecology of the organism, we must postulate one or more selective factors and suggest a mechanistic connection between the selective factor and the gene product.
3. We must test our postulated mechanism by experimentally manipulating the environment to produce predictable responses.
4. In the light of the experiments, we must re-examine the natural populations, and seek a comprehensive explanation for the observed patterns of gene frequencies".

In a similar vein, Koehn (1978) defined a number of steps which he believed to be pre-requisites for establishing the adaptive significance of a given enzyme polymorphism. The first, and most basic point cited by both Koehn and Clarke was the establishment of diversity of function at the molecular level between different genotypes of a given locus.

Over the past ten years or so, many workers have initiated comparative studies of allozymes produced by a wide variety of enzyme coding loci, over a wide variety of species. Within the genus *Drosophila* the enzyme alcohol dehydrogenase of *D. melanogaster* has been by far the most exhaustively studied, not only at the biochemical level but also in laboratory and field population studies. Despite the enormous volume of work on the *Adh* locus, and quite good evidence of a selective basis for the maintenance of polymorphism at this locus (e.g. Oakeshott *et al.*, 1981), many inconsistencies

in the published data have led to an appreciation of the difficulties underlying studies of enzyme polymorphism (Gibson and Oakeshott, 1982). At the molecular/biochemical level the ADH allozymes have been extensively characterised, with the amino-acid sequence (Fletcher *et al.*,1978; Thatcher, 1980; Chambers *et al.*, 1981) and recently the nucleotide sequence (Benyajati *et al.*, 1981; Kreitman, 1983) of a number of common variants now known. The biochemical properties of the common variants ADH^f and ADH^s have been investigated by a number of workers and a variety of differences are known to exist (Day *et al.*, 1974 a,b; McDonald *et al.*, 1980). Despite the large amount known about this enzyme, attempts to relate *in vitro* properties of allozyme variants to a comprehensive description of the *Adh* polymorphism in *D. melanogaster* have thus far met with little success.

Comparisons of allozymic variants have now been made for a number of enzymes in *Drosophila* species, with mixed results (Table 4.1). At this stage the number of systems showing observed functional differences between allozymes outweighs those showing no difference. This is in agreement with the observation by Harris (1971) that 16 of 23 human enzymes examined showed biochemical differences between allozymic variants. A disturbing aspect of the *Drosophila* data is that for one enzyme (cytoplasmic malate dehydrogenase), results from two different laboratories gave completely conflicting conclusions. Since radically different procedures were employed for the preparation of enzyme used in these studies some discrepancies were to be expected. Nonetheless, in the context of studies such as these it is of some concern that different laboratories should report results which differ in every aspect.

With respect to studies of allozymic variants of esterase loci in *Drosophila* species, the results have been mixed in that some enzymes have shown differences while others have not. The first such study was made on products of the *Est-5* locus of *D. pseudoobscura* (Narise and Hubby, 1966). They examined the properties of allozymes produced by a common and a rare allele, and failed to resolve any appreciable differences between them.

Table 4.1 Biochemical comparisons of allozymes in *Drosophila* species

Enzyme	Species	Source(s)
a. Differences observed between allozymes		
Alcohol dehydrogenase	*D. melanogaster*	Day *et al.* (1974a,b)
Malate dehydrogenase (cytoplasmic isozyme)	*D. melanogaster*	Alahiotis (1979a)
Malate dehydrogenase (cytoplasmic isozyme)	*D. virilis*	Narise (1979)
α-glycerophosphate dehydrogenase	*D. melanogaster*	Miller *et al.* (1975)
Alkaline phosphatase	*D. melanogaster*	Harper and Armstrong (1973)
Esterase-6	*D. melanogaster*	Mane *et al.* (1983), Danford and Beardmore (1979)
α- and β-esterase	*D. virilis*	Narise (1973a,b)
Phosphoglucomutase	*D. melanogaster*	Fucci *et al.* (1979)
Glucose-6-phosphate	*D. melanogaster*	Bijlsma and van der Meulin-Bruijns (1979)
6-Phosphogluconate dehydrogenase	*D. melanogaster*	Bijlsma and van der Meulin-Bruijns (1979)
b. No differences observed between allozymes		
Malate dehydrogenase (cytoplasmic isozyme)	*D. melanogaster*	Hay and Armstrong (1976)
Esterase-5	*D. pseudoobscura*	Narise and Hubby (1966)

In a later study of a number of allozymic variants of the α- and β-esterase loci of *D. viridis*, Narise (1973a) observed differences in biochemical properties of enzymes at both loci, though these were not quantified. There have now been a number of biochemical studies on allozymes produced by the *Est-6* locus of *D. melanogaster* (Danford and Beardmore, 1979a; Mane *et al.*, 1983; Costa *et al.*, 1983). In general there appear to be differences between different electromorphs, but studies on highly purified enzymes (Mane *et al.*, 1983) suggest that the differences in catalytic properties at the molecular level may not be great.

In this chapter, a number of allozymic variants produced by the *Est-1* and *Est-2* loci are examined for a variety of biochemical properties, as a preliminary attempt to determine whether there are differences between allozymes which may be of physiological relevance in this species.

4.2 MATERIALS AND METHODS

The enzymes used for the studies reported in this chapter were prepared according to Protocol 1 (Section 3.2.1.1) described in the preceding chapter. The methodologies for the substrate specificity analysis and the pH-activity and thermostability studies were exactly as previously described (Sections 3.2.3, 3.2.6 and 3.2.7 respectively).

4.2.1 Genetic Material

The stocks used for the production of enzyme for these studies, and their genotypes at the *Est-1* and *Est-2* loci are given in Table 4.2.

Flies were cultured in vials containing 7 ml of the autoclaved sucrose-yeast-agar medium described in Section 2.2.1. Enzyme was extracted from young adult flies produced according to the following schedule:

a. Approximately 150 vials were set up, each containing 5 pairs of mature adults of the appropriate genotype.

Table 4.2 Genotypic constitution of stocks used for the production of partially purified ESTERASE enzymes for biochemical analysis

Stock	*Est-1* alleles	*Est-2* alleles	Allozyme(s) extracted	
M8	*Est-1*^{b}	*Est-2*^{a}	EST-1^{b},	EST-2^{a}
M19	*Est-1*^{a}, *Est-1*^{b}	*Est-2*^{b}		EST-2^{b}
M20	*Est-1*^{c}	*Est-2*^{c}	EST-1^{c},	EST-2^{c}
2.11	*Est-1*^{a}, *Est-1*^{b}	*Est-2*^{d}		EST-2^{d}

b. Flies were allowed to lay eggs for 3 days before being transferred without etherisation to fresh vials. This transfer was made three times, at three day intervals, to yield a set of approximately 600 vials of any given genotype.

c. Once flies began eclosing they were collected at three day intervals, so all material was 0-3 days old. Flies were held in a chilled container until collection was complete for that day. They were then weighed and stored frozen at -20^{o}C.

d. This procedure was continued with each stock until at least 50 grams of the desired genotypes had been collected.

4.2.2 Enzymatic Material

The flies produced as described in Section 4.2.1 were all treated according to the same purification protocol (Protocol 1, Section 3.2.1.1), and using the same columns and buffer preparations. This resulted in very similar yields and degrees of purification for the allozymic sets of each ESTERASE isozyme, with the exception of the EST-2^{C} enzyme, which had a somewhat lower yield. This may suggest that EST-2^{C} from this stock was significantly less stable than the other three EST-2 allozymes.

4.2.3 General Assay Procedures

For any given experiment all assays were done in one laboratory session, using a single batch of substrate reagent, buffers, temperatures *etc.* A single, homogeneous aliquot of thawed, partially purified enzyme was used for each experiment, and for any given test combination enzyme assays were performed either in duplicate or triplicate.

4.2.4 Statistical Analysis

In the substrate specificity studies reported in Section 4.3.3, the allozymes have been compared by simple factorial analysis of variance with a randomised complete block design. Since it was not technically possible to replicate each experiment, because of the amount of enzyme required, the enzyme assay replication was used as the source of error variance. For this reason, comparisons of allozymes were restricted to assays which were run in a single experimental session. No attempt has been made to compare substrates which were analysed at different times or using different thawed samples of enzyme.

4.3 RESULTS

All experiments were done on a set of allozymes of each of the *Est-1* and *Est-2* loci, and for each section below, the two isozymic systems EST-1 and EST-2 are considered separately.

4.3.1 Effect of pH on Esterase Activity

As described in Section 3.3.4, these experiments were conducted using only a single buffer system, and a limited range of pH values.

4.3.1.1 EST-1: - pH Activity Profiles

The effect of pH on the activity of the two EST-1 preparations is shown in Fig. 4.1. Although EST-1^C appears to show higher levels of activity in the lower end of the range of pH values tested, the two allozyme preparations show very similar profiles taken over the whole range. Both allozyme

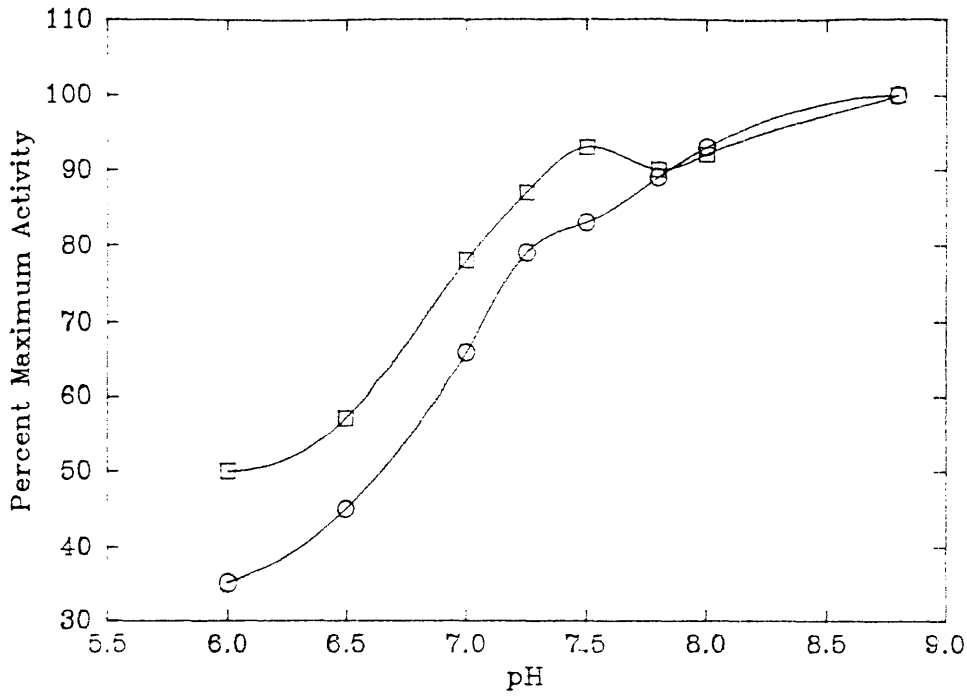


Figure 4.1 Effect of pH on EST-1 allozymes. All measurements made at constant ionic strength of 0.1M in phosphate buffers at 25°C using α-napthylacetate as substrate. (○) EST-1^{b}; (□) EST-1^{c}

profiles show a shoulder in the range 7.25 to 7.80, suggesting that this may reflect some intrinsic property of the EST-1 enzyme.

4.3.1.2 EST-2 : - pH Activity Profiles

The four EST-2 allozymes show very similar responses to changing pH values (Fig. 4.2). They all exhibit maximal activity in the vicinity of pH 7.0 - 7.25, and show a pronounced shoulder in the region 7.5 to 8.0. There is a tendency for EST-2^{d} to have higher relative activity at the upper end of the values tested, and for EST-2^{c} to show a slightly narrower optimal range. However, overall, the similarities between allozymes are far more striking than any differences between them.

4.3.2 ESTERASE Thermostability

The thermostability of the various enzyme preparations was determined at the physiologically lethal temperature of 50°C. While this may enable

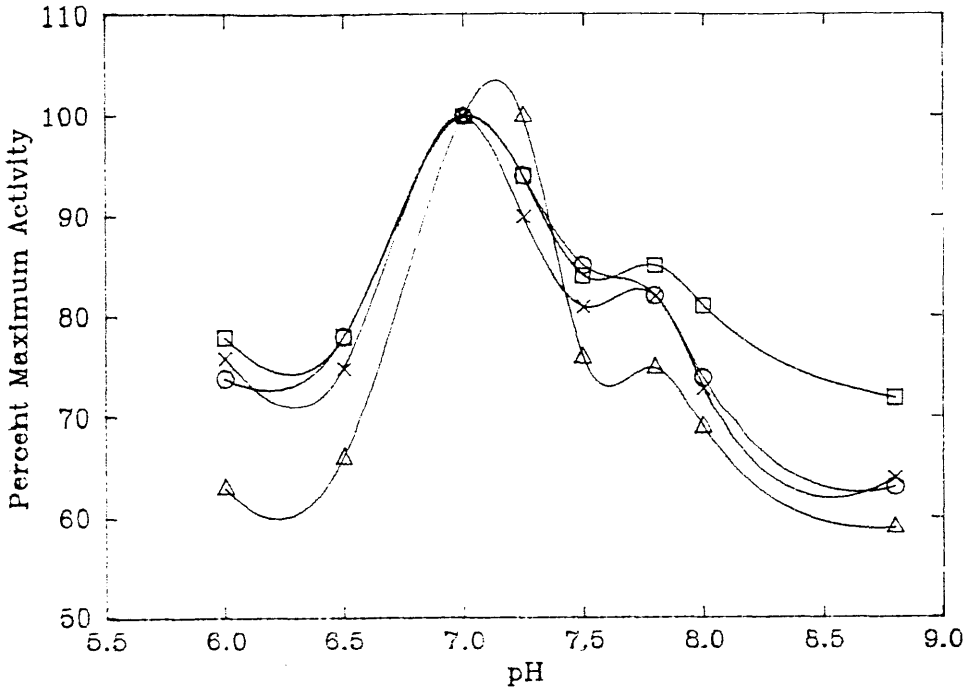


Figure 4.2 Effect of pH on EST-2 allozymes. All assays were performed at constant ionic strength of 0.1M in phosphate buffers at 25°C using α-naphthyl acetate as substrate.
(○) EST-2^a; (X) EST-2^b; (△) EST-2^c; (□) EST-2^d

detection of chemical differences between the various esterase preparations, the biological significance of any observed differences must necessarily remain open to question.

4.3.2.1 Thermostability of ESTERASE-1 Allozymes

Figure 4.3 shows the effect of denaturing temperature on the two EST-1 allozymes, and it is clear that they show very similar responses. If the data are re-plotted, with the logarithm of activity remaining as the ordinate, (Fig. 4.4) a satisfactory linearisation is obtained and the regression coefficient may be considered as a measure of the rate of denaturation. These rates and their standard errors are given in Table 4.3, and comparison between them reveals that they are not significantly different ($t_7 = 1.43$, $0.2 < P < 0.1$).

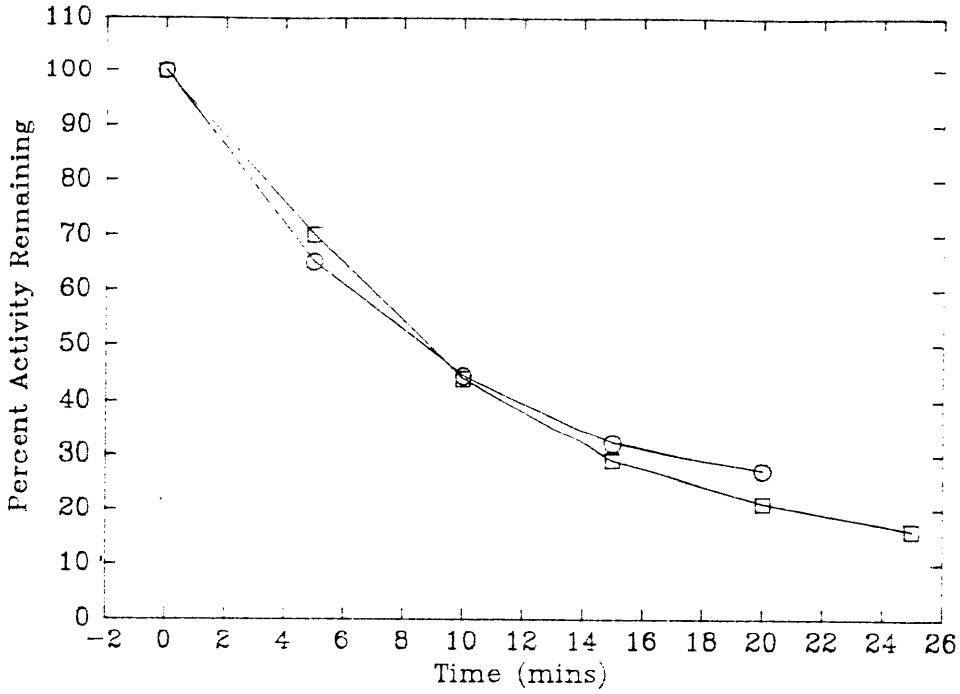


Figure 4.3 Thermostability of EST-1 allozymes at 50°C. Assays were done in 0.1 M phosphate at pH 7.0.
(○) EST-1^{b}; (□) EST-1^{c}

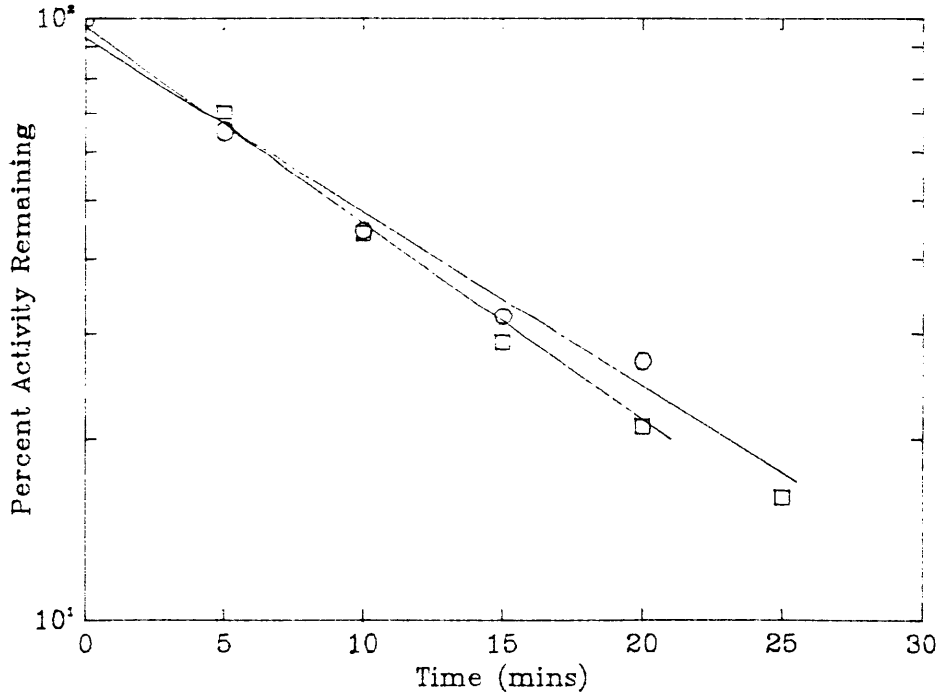


Figure 4.4 Regression EST-1 allozyme activity after exposure to heat on time. Solid lines are the least-squares regressions.
(○) EST-1^{b}; (□) EST-1^{c} data points.

Table 4.3 Rates of denaturation for EST-1 allozymes at 50°C, based on the regressions shown in Figure 4.4

Enzyme	Rate*	s.e.(rate)
EST-1[b]	0.029	0.002
EST-1[c]	0.033	0.001

* Rate is decrease in $\log_{10}$ (% activity remaining) per minute.

4.3.2.2 Thermostability of ESTERASE-2 Allozymes

In contrast to the results obtained for EST-1, the four EST-2 allozymes appear to be quite different in their thermostability properties (Fig. 4.5). The regression of $\log_{10}$ (% activity remaining) against time reveals a fairly linear response over the early stages (Fig 4.6), and the estimated denaturation rates are given in Table 4.4. It is clear from Figs. 4.5 and 4.6 that EST-2[a] and EST-2[d] are very similar to each other, but that EST-2[b] appears to be much more stable, and EST-2[c] much less stable than this pair. Pairwise comparisons of the regression coefficients indicate that these differences are statistically significant, even on this limited set of data (Table 4.5).

4.3.3 Substrate Specificity Studies

Enzyme assays were developed for esters of a large variety of chemical structures and physico-chemical properties. The substrates were grouped together according to some common chemical property, and all assays for that group were run in the same experimental session.

4.3.3.1 Effect of Acyl Carbon Chain Length

For this experiment, assays were developed for a series of esters of the chromogen p-nitrophenol. Under the standard assay conditions of 25°C and pH 7.00, it was possible to vary the acyl carbon chain length from C=2 (acetate) to C=6 (caproate). It was also possible to assay the caprylic

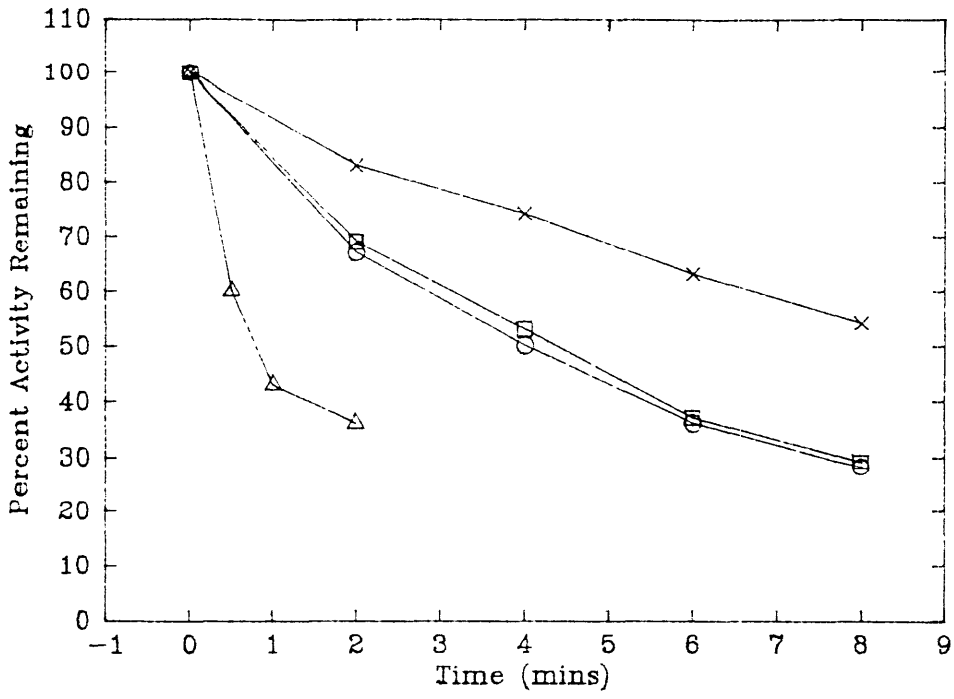


Figure 4.5 Thermostability of EST-2 allozyme at 50°C. Assays were done in 0.1M phosphate at pH 7.0
(○) EST-2^a; (X) EST-2^b; (△) EST-2^c; (□) EST-2^d.

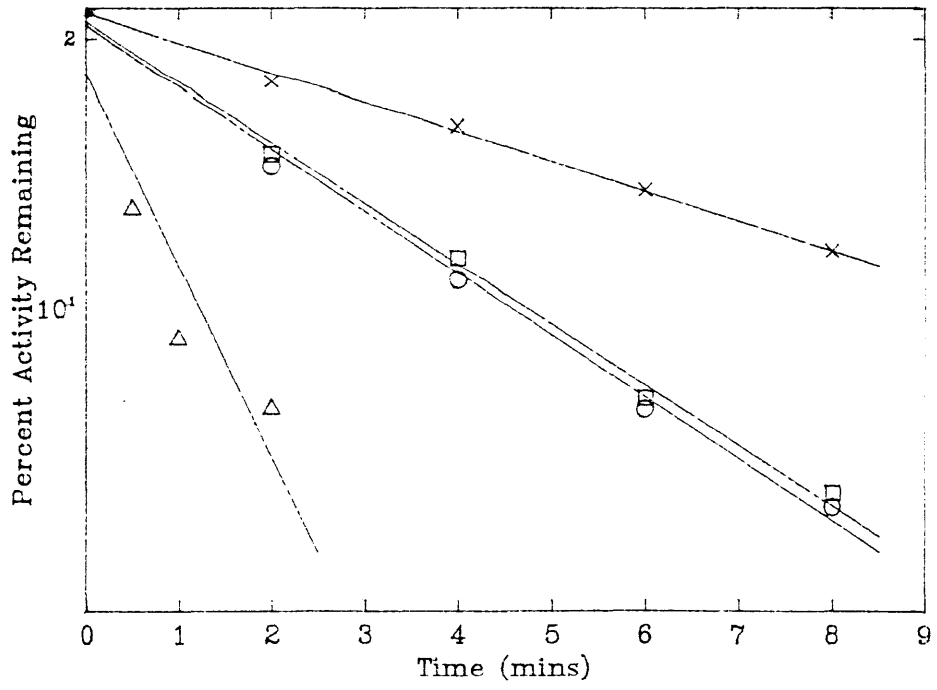


Figure 4.6 Regression of EST-2 allozyme activity after exposure to heat on time. Solid lines are the least-squares regressions.
(○) EST-2^a; (X) EST-2^b; (△) EST-2^c; (□) EST-2^d.

Table 4.4 Rates of denaturation of EST-2 allozymes at 50°C, based on the regressions shown in Figure 4.6

Enzyme	Rate*	s.e.(rate)
EST-2[a]	0.069	0.003
EST-2[b]	0.033	0.001
EST-2[c]	0.211	0.062
EST-2[d]	0.067	0.003

* Rate is the decrease in $\log_{10}$ (% activity remaining) per minute.

Table 4.5 Comparison of rates of denaturation of EST-2 allozymes at 50°C

Allozyme pair	d.f.	M.S.	Probability
EST-2[a] vs EST-2[b]	6	11.01	0.01
EST-2[a] vs EST-2[c]	5	3.44	0.05
EST-2[a] vs EST-2[d]	6	0.26	n.s.†
EST-2[b] vs EST-2[c]	5	4.43	0.01
EST-2[b] vs EST-2[d]	6	12.17	0.001
EST-2[c] vs EST-2[d]	5	3.51	0.05

† n.s. = not significant at the 5% level.

acid ester (C=8), but the results must be treated with some caution, since p-nitrophenyl caprylate was not completely soluble under these conditions, and this may drastically affect the reaction rate.

4.3.3.1.1 Effect of Acyl Carbon Chain Length on ESTERASE-1 Activity

Increasing the chain length of the acyl moiety had a spectacular effect

on enzyme activity. Both allozymes showed very similar responses, with generally higher activity on the short chain fatty acid esters (C=2 to C=4), and a clear optimum for the propionate ester (Fig. 4.7). Despite the apparent similarity in the reactions of the two EST-1 allozymes with this group of esters, there is a suggestion of a difference in that EST-1^{C} shows higher activity on C2 to C4 fatty acids, but lower activity for C5 to C8. The data summarised in Fig. 4.7 were subjected to a simple two-way analysis of variance (Table 4.6), and the main effects for allozyme type and chemical were both highly significant ($P < 0.001$). However, of greater interest is the first-order interaction of allozyme with chemical, which is significant at 5% level. This interaction term presumably reflects a differential response of the two EST-1 allozymes to changing the acyl chain length, as was noted above.

4.3.3.1.2 Effect of Acyl Carbon Chain Length on ESTERASE-2 Activity

The four EST-2 allozymes showed clear, and ostensibly different responses to increasing acyl carbon chain length (Fig. 4.8). All enzymes showed quite high activity on fatty acid esters from acetate to caproate. Changing the fatty acid moiety from acetate to butyrate resulted in an approximate threefold increase in activity for all enzymes. However, further increases to the valeric and caproic esters resulted in quite different effects depending on the allozyme. The most obvious difference is that EST-2^{C} shows essentially the same activity on fatty acids of C4, C5 and C6, whereas the other three allozymes show large decreases in activity from C4 to C6. Another major difference is that, although EST-2^{a}, EST-2^{b} and EST-2^{d} show qualitatively similar responses, there are some differences, notably the abrupt decrease in activity of EST-2^{a} in changing from the valerate to the caproate ester. This heterogeneity in the responses of the four EST-2 allozymes is shown clearly in an analysis of variance done on this data set (Table 4.7).

Table 4.6 Analysis of variance of the effect of acyl carbon chain length on ESTERASE-1 activity †

Source	d.f.	Mean square	F
Allozyme (A)	1	49.61	161.36***
Chemical (C)	4	982.02	3193.97***
A x C	4	51.94	168.93***
Error	9	0.31	

† Only esters up to C=6 (caproate) were included in the analysis of variance.

*** $P < 0.001$.

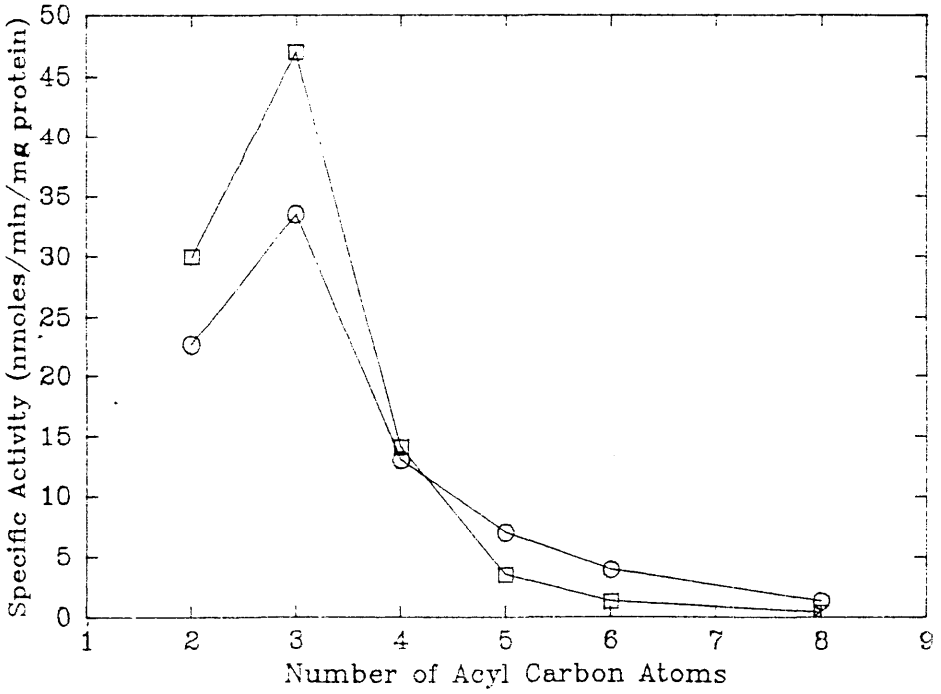


Figure 4.7 The effect of increasing acyl carbon chain length on ESTERASE-1 activity. All substrates were the saturated fatty acids of p-nitrophenol.
(○) EST-1^b; (□) EST-1^c.

Table 4.7 Analysis of variance of the effect of acyl carbon chain length on ESTERASE-2 activity [±]

Source	d.f.	Mean square	F
Allozyme (A)	1	55865.24	179.42***
Chemical (C)	4	83774.64	269.06***
A x C	4	7120.69	22.87***
Error	9	311.36	

[±] Only esters up to C=6 (caproate) were included in the analysis of variance.

*** $P < 0.001$.

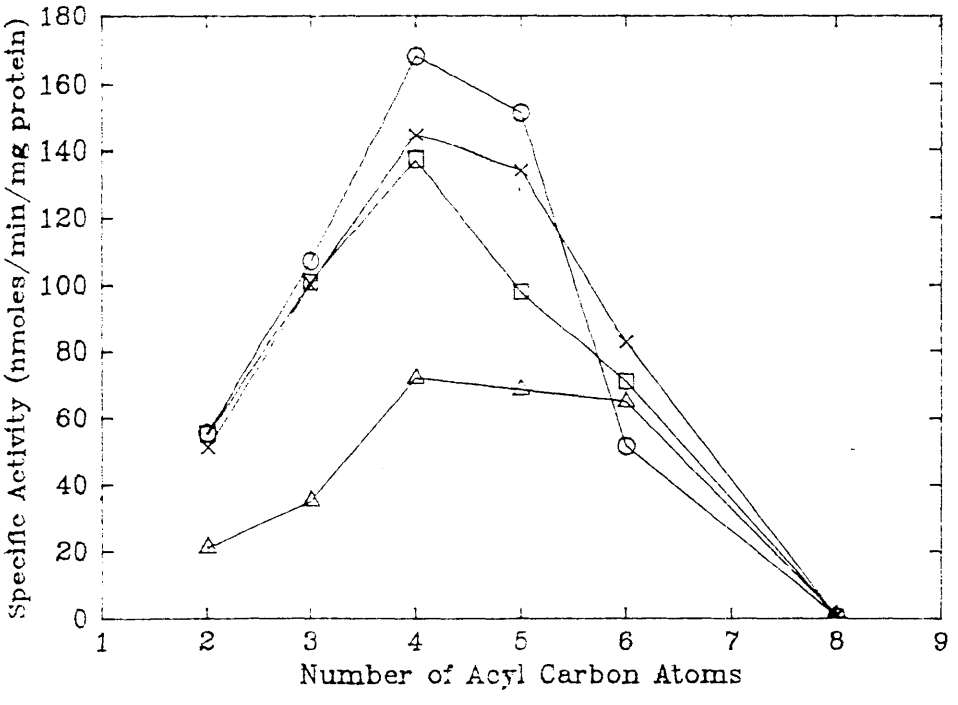


Figure 4.8 The effect of increasing acyl carbon chain length on ESTERASE-2 activity. All substrates were the saturated fatty acids of p-nitrophenol.
(○) EST-2[a]; (×) EST-2[b]; (△) EST-2[c]; (□) EST-2[d].

The significant main effects of allozyme and chemical simply reflect differences in susceptibility of the various chemicals to hydrolytic attack by EST-2. However, the highly significant ($P < 0.001$) first order interaction between allozyme type and substrate indicates that the various allozymes have not all responded in the same way to substrate structure.

4.3.3.2 Volatile Ester Assays

A fairly comprehensive group of low molecular weight, low boiling point esters were assayed for their activity as substrates for EST-1 and EST-2 using a pH-Stat technique. As with many esterase assay systems, the problem of dissolving the relatively non-polar esters in an aqueous phase increases rapidly with increasing molecular weight, thereby setting an upper limit to the molecular weight of the compounds to be tested. In this system, the solubility limit was reached with the amyl acetates (M.W. 130.19) and ethyl butyrate (M.W. 116.16). Nonetheless, within these limitations it was possible to assay a group of esters, and some interesting patterns were revealed.

4.3.3.2.1 ESTERASE-1 Activity on Volatile Esters

The results of these assays are presented in Table 4.8, and it is clear that there are substantial activity differences, both for the chemicals and between the two allozymes. The results were subjected to analysis of variance (Table 4.9) and highly significant effects of allozyme and chemical were observed. The interaction between allozyme species and chemical also was highly significant, and this will be discussed later (Section 4.4). In an attempt to further investigate the effect of substrate type on EST-1 activity, the total set of activity measures was regressed on substrate molecular weight. This gave a regression coefficient of $b = -19.2$, and a test of H_o: $b = 0$ gave $t_{72} = 2.48$, $P < 0.01$, so it appears that there was a significant decrease in EST-1 activity with increasing molecular weight.

Table 4.8 ESTERASE-1 activity on volatile esters

Ester	M.W.	EST-1[b] ±	EST-1[c]
ethyl formate	74.08	687.2	1394.8
n-propyl formate	88.80	1524.5	2565.0
n-butyl formate	103.13	1311.2	2198.6
ethyl acetate	88.10	158.0	295.5
n-propyl acetate	102.14	134.3	331.0
i-propyl acetate	102.14	134.3	295.5
n-butyl acetate	116.16	134.3	283.7
i-butyl acetate	116.16	178.8	271.9
n-amyl acetate	130.19	158.0	390.1
i-amyl acetate	130.19	244.9	307.3
ethyl-n-propionate	102.13	371.3	555.6
ethyl-n-butyrate	116.16	150.1	236.4

± Specific activity in nmoles acid released/min/mg protein, expressed as the mean of two replicate assays.

Table 4.9 Analysis of variance of EST-1 allozyme activities on the volatile esters shown in Table 4.9

Source	d.f.	Mean square	F
Allozyme (A)	1	1296021.5	3187.8***
Chemical (C)	11	1719421.1	4229.2***
A x C	11	117228.8	288.3***
Error	24	406.6	

*** $P < 0.001$

However, an inspection of the standardised residuals after fitting this regression indicated a highly non-random distribution of positive and negative deviations. The reason for this is apparent from the data given in Table 4.8. The EST-1 enzymes are clearly recognizing certain structural features of the ester substrates. For example, the formate esters show much higher activity than any other group. It seems more informative therefore, to consider the various structural groupings, *viz.* formates, acetates and ethyl esters separately, (Figs. 4.9 to 4.11, Tables 4.10 to 4.12). Analyses of variance for each group indicate significant effects of allozyme, chemical and allozyme x chemical interaction. For each of these three structural groupings EST-1 activity was regressed on molecular weight, but in no instance were the regression coefficients significantly different from zero.

4.3.3.2.2 ESTERASE-2 Activity on Volatile Esters

The activities of the four EST-2 allozymes are summarised in Table 4.13, and an analysis of variance based on the total data set is presented in Table 4.14. There were highly significant effects of allozyme and chemical on EST-2 activity as measured by analysis of variance, and the allozyme x chemical interaction also was highly significant. As for EST-1, to further investigate the effect of substrate structure, EST-2 activity was regressed on substrate M.W. (Table 4.15). The regression coefficient was positive and significant ($P < 0.05$), but as was found with EST-1, inspection of residuals after fitting this regression indicated a highly non-random distribution of positive and negative deviations, and the regression accounted for only 10% of the variation. Again it was more informative to consider the various chemical structural groupings separately, Figs. 4.12 to 4.14, Table 4.15). For each ester group a highly significant ($P < 0.001$) positive regression of activity on M.W. was observed, and a much greater proportion of the variation in the data was explained (Table 4.15). It appears that EST-2

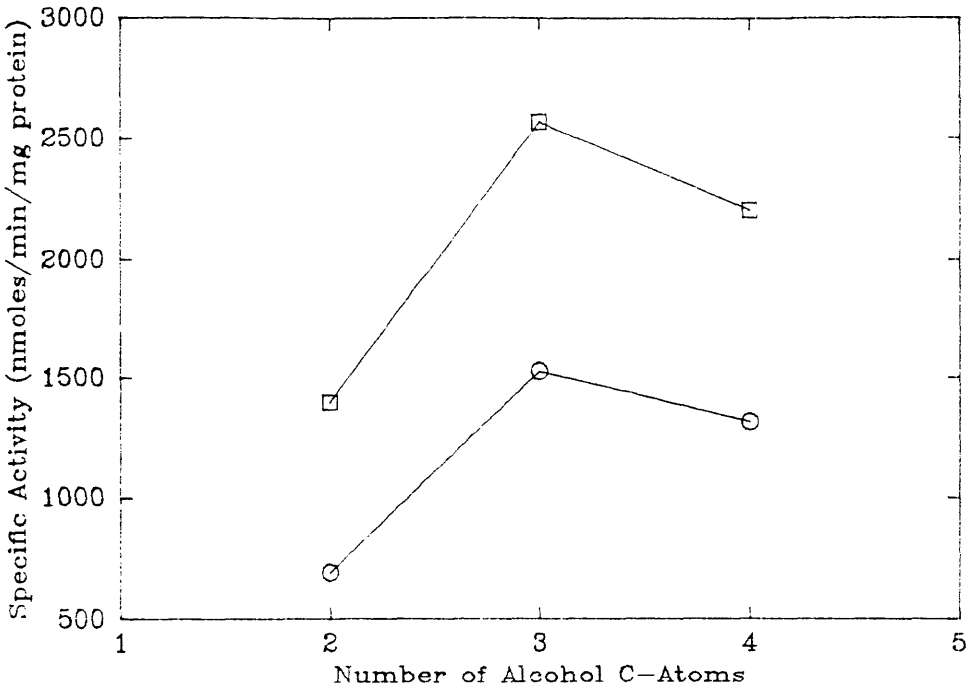


Figure 4.9 Effect of increasing alcohol carbon chain length on EST-1 allozyme activities for a group of formate esters.
(○) EST-1^{b}; (□) EST-1^{c}.

Table 4.10 Analysis of variance for the effect of varying alcohol carbon chain length on EST-1 allozyme activities for a group of formate esters

Source	d.f.	Mean square	F
Allozyme (A)	1	2315268.7	6856.7***
Chemical (C)	2	1067447.5	3163.2***
A x C	2	27768.8	82.2***
Error	6	337.7	

*** $P < 0.001$.

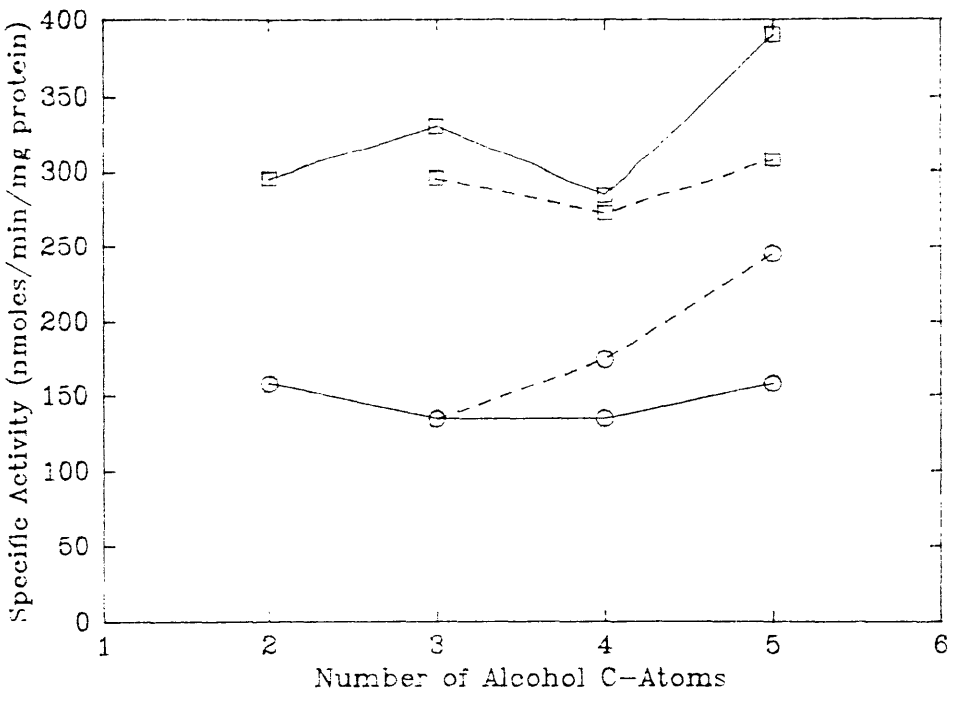


Figure 4.10 Effect of alcohol carbon chain length and structure on EST-1 allozyme activities for a group of acetate esters. Solid lines represent esters of primary alcohols, dashed lines are esters of secondary alcohols. (○) EST-1^b; (□) EST-1^c.

Table 4.11 Analysis of variance for the effect of varying alcohol carbon chain length and structure on EST-1 allozyme activities for a group of acetate esters

Source	d.f.	Mean square	F
Allozyme (A)	1	134992.5	233.9***
Chemical (C)	2	8257.3	14.3***
Structure (S)	1	2.2	0.0
A x C	2	1535.1	2.7***
A x S	1	10957.2	19.0***
C x S	2	509.1	0.9
A x C x S	2	2687.3	4.7*
Error	12	577.2	

* $P < 0.05$
*** $P < 0.001$

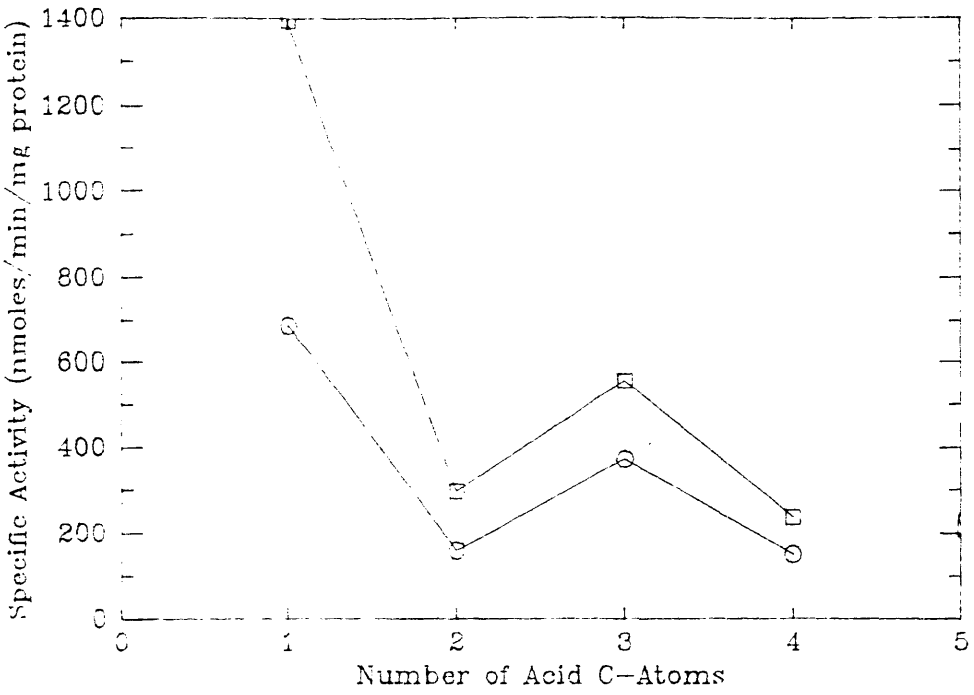


Figure 4.11 Effect of acid carbon chain length on EST-1 allozyme activity for a group of ethyl esters.
(○) EST-1^{b}; (□) EST-1^{c}.

Table 4.12 Analysis of variance for the effect of varying acid carbon chain length on EST-1 allozyme activity for a group of ethyl esters

Allozyme (A)	1	311235.7	2667.6***
Chemical (C)	3	615144.1	5272.4***
A x C	3	83266.5	713.7***
Error	8	116.7	

*** $P < 0.001$

Table 4.13 ESTERASE-2 activity on volatile esters

Chemical	M.W.	EST-2[a] [+]	EST-2[b]	EST-2[c]	EST-2[d]
ethyl formate	74.08	0	0	17.2	30.9
n-propyl formate	88.80	265.4	379.9	498.3	216.1
n-butyl formate	102.13	530.8	1029.4	1030.9	848.8
ethyl acetate	88.10	0	0	0	0
n-propyl acetate	102.14	0	0	0	0
i-propyl acetate	102.14	0	0	0	0
n-butyl acetate	116.16	222.9	122.6	103.1	154.3
i-butyl acetate	116.16	318.5	245.1	223.4	277.8
n-amyl acetate	130.19	329.1	330.9	257.7	354.9
i-amyl acetate	130.19	371.6	416.7	292.1	401.2
ethyl-n-propionate	102.13	212.3	159.3	274.9	262.4
ethyl-n-butyrate	116.16	1082.8	1004.9	584.2	740.7

[+] Specific activity : nmoles acid released/min/mg protein.

Table 4.14 Analysis of variance of EST-2 allozyme activities on the volatile esters shown in Table 4.13

Source	d.f.	Mean square	F
Allozyme (A)	3	7207.1	9.7***
Chemical (C)	11	732701.7	989.0***
A X C	33	24268.2	32.8***
Error	48	740.8	

*** $P < 0.001$

Table 4.15 Regression of ESTERASE-2 activity on substrate molecular weight

Independent variable	r^2	b
Molecular weight (all esters)	0.10	6.37*
Molecular weight (formate esters	0.84	30.10***
Molecular weight (acetate esters)	0.82	9.59***
Molecular weight (ethyl esters)	0.71	19.67***

* $P < 0.05$
*** $P < 0.001$

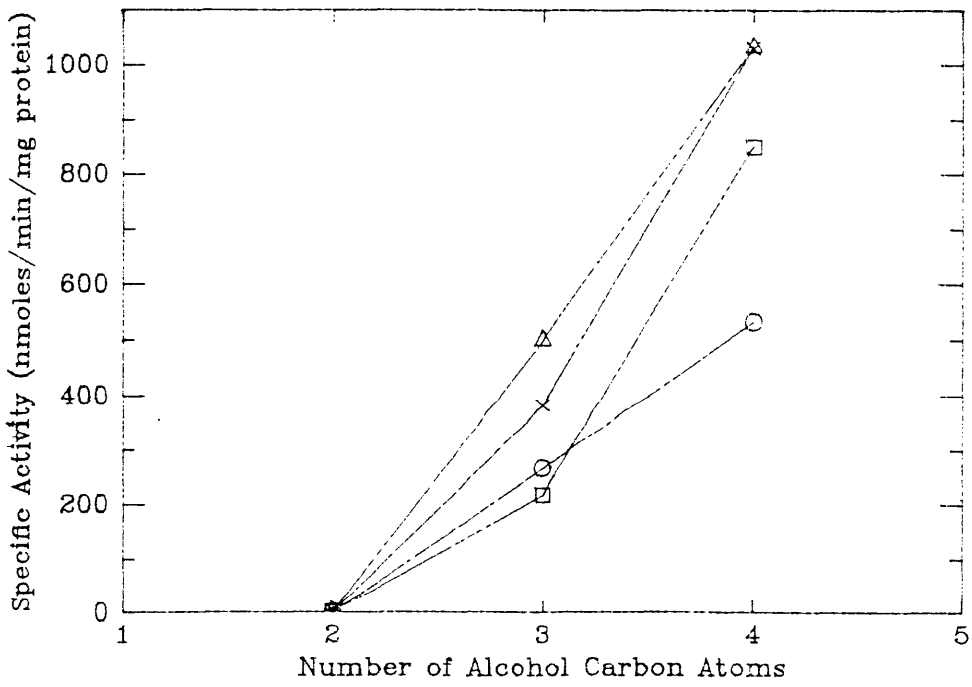


Figure 4.12 Effect of increasing alcohol carbon chain length on EST-2 allozyme activities for a group of formate esters. (○) EST-2^a; (x) EST-2^b; (△) EST-2^c; (□) EST-2^d.

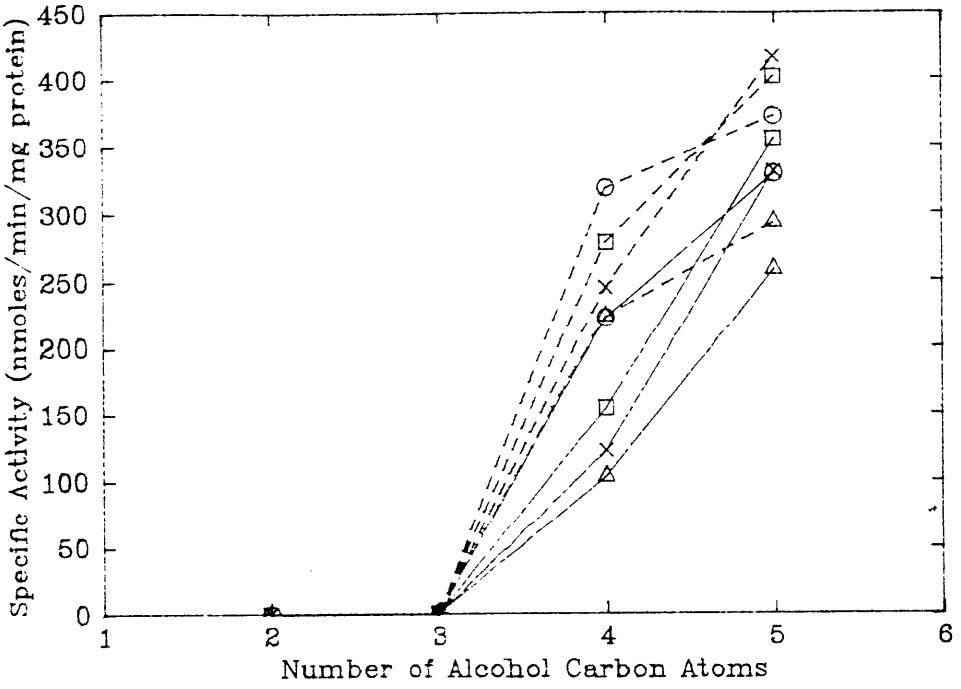


Figure 4.13 Effect of alcohol carbon chain length and structure on EST-2 allozyme activities for a group of acetate esters. Solid lines represent esters of primary alcohols, dashed lines are esters of secondary alcohols. (○) EST-2^a; (x) EST-2^b; (△) EST-2^c; (□) EST-2^d.

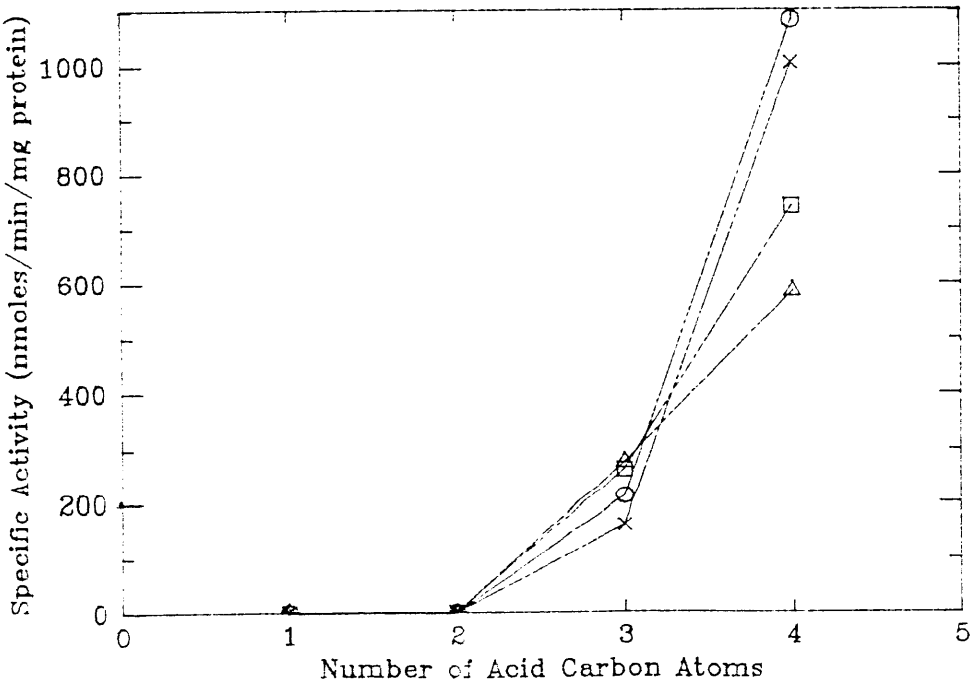


Figure 4.14 Effect of acid carbon chain length on EST-2 allozyme activity for a group of ethyl esters. (○) EST-2^a; (x) EST-2^b; (△) EST-2^c; (□) EST-2^d.

activity increases with increasing molecular weight for every class examined in this set of volatile esters. Analyses of variance on each subset of the data reveal significant effects of allozyme, chemical and allozyme x chemical interaction (Tables 4.16 to 4.18). The set of acetate esters can be further subdivided according to the structure of the alcohol moiety, *i.e.* whether it was a primary or secondary alcohol derivative (Table 4.17). The main effect, structure (S) in this analysis is highly significant, and inspection of the data indicates that it is the result of all enzymes having higher activity on acetates of alcohols with secondary structure. The first order interaction of allozyme with substrate structure was non-significant, presumably reflecting the fact that all allozymes had higher activity on esters of secondary alcohols as compared with primary alcohols of the same carbon number (Table 4.8, Fig. 4.13). A wealth of diversity among allozyme species can be seen in these data, and this will be discussed more fully later.

4.3.3.3 Assays on Esters of Acetic Acid

The objective of these experiments was to compare the effects of alcohol group structure on esterase activity for a very diverse group of esters in which the acid moiety was maintained as acetate. Since it was not technically feasible to conduct this study as a single experiment, the data have not been analysed by any statistical procedure, but are simply presented in tabular form.

4.3.3.3.1 Effect of Alcohol Structure on EST-1 Allozyme Activity

The results of comparing the activities of two EST-1 allozymes on a group of 13 acetate esters are presented in Table 4.19. There did not appear to be any simple effect of substrate molecular weight on activity. The group of volatile esters had much higher activity than the non-volatiles, and among the volatiles there was a suggestion of some differentiation between the two allozymic variants. The EST-1^{b} allozyme had similar or

Table 4.16 Analysis of variance for the effect of varying alcohol carbon chain length on EST-2 allozyme activities for a group of formate esters

Source	d.f.	Mean square	F
Allozyme (A)	3	74120.2	70.4***
Chemical (C)	2	1500742.6	1426.4***
A x C	6	34273.8	32.6***
Error	12	1052.1	

*** P 0.001

Table 4.17 Analysis of variance for the effect of varying alcohol carbon chain length and structure on EST-2 allozyme activities for a group of acetate esters

Source	d.f.	Mean square	F
Allozyme (A)	3	9057.5	14.9***
Chemical (C)	2	484075.1	798.3***
Structure (S)	1	31542.8	52.0***
A x C	6	3908.5	6.5***
A x S	3	56.2	0.1
C x S	2	10321.5	17.0***
A x C x S	6	577.4	1.0
Error	24	606.4	

*** P 0.001

Table 4.18 Analysis of variance for the effect of varying acid carbon chain length on EST-2 allozyme activity for a group of ethyl esters

Source	d.f.	Mean square	F
Allozyme (a)	1	18012.8	20.9***
Chemical (C)	3	1300540.2	1511.8***
A x C	3	31570.1	36.7***
Error	8	860.3	

*** P 0.001

Table 4.19 Effect of alcohol structure on ESTERASE-1 activity for a group of acetate esters. All assays were conducted at 25°C at pH 7.00

Substrate	M.W.	ESTERASE-1[b]	ESTERASE-1[c]
(a) Volatile esters			
ethyl acetate	88.10	158.0	295.5
n-propyl acetate	102.14	134.3	331.0
i-propyl acetate	102.14	134.3	295.5
n-butyl acetate	116.16	134.3	283.7
i-butyl acetate	116.16	178.8	271.9
n-amyl acetate	130.19	158.0	390.1
i-amyl acetate	130.19	244.9	307.3
(b) Non-volatile esters			
p-nitrophenyl acetate	181.2	12.8	18.4
o-nitrophenyl acetate	181.2	1.6	2.0
α-naphthyl acetate	186.2	23.2	34.6
β-naphthyl acetate	186.2	43.5	65.0
4-methylumbelliferyl acetate	218.2	75.3	112.7
cholesteryl acetate	428.7	N.D.[a] N.D.	N.D.

[a] N.D. = Not detectable.

lower activity on acetates of primary alcohols compared to secondary, whereas the reverse situation applied for the EST-1[c] allozyme. Some of these compounds were shown to be quite toxic in a closed atmospheric system (Le, 1983), and it is conceivable that differences such as those observed here (Table 4.11, Fig. 4.10) could be of some physiological significance. The group of synthetic esters showed considerably less activity, but do clearly illustrate one important fact, viz. for closely related compounds, the stereochemistry of the ester bond can have a very large influence on enzyme activity. Thus, with the nitrophenyl acetates, placing the $-NO_2$ group in the ortho configuration, adjacent to the ester bond, reduces activity almost to zero. Similarly with the naphthyl esters, where placing the ester

linkage in the β position on the naphthol ring results in greater activity than when it is in the α position. With this system, no activity could be detected on the acetate ester of cholesterol.

4.3.3.3.2 Effect of Alcohol Structure on EST-2 Allozyme Activity

The activities of the four EST-2 allozymes on the group of acetate esters (Table 4.20) show a number of interesting features. Perhaps the most striking result is for the group of volatile esters where the lower molecular weight ethyl and propyl esters appear to be ineffective as substrates, whereas, the butyl and amyl acetates are hydrolysed quite rapidly. These data were analysed in more detail previously (Section 4.3.3.2.2; Fig. 4.13, Table 4.17). As was found for the EST-1 allozymes, the stereochemistry of the ester bond exerted a profound influence on activity. The ortho-nitrophenyl acetate again was a much poorer substrate than the para-nitrophenyl isomer, and α-naphthyl ester was a much more active substrate than the β-naphthyl form. However, the data for the EST-2 enzymes differ from the results for EST-1 among the group of non-volatile esters, where alterations in substrate structure appeared to affect the activity of both allozymes equally. The results in Table 4.20 are strongly suggestive of a differential effect of substrate structure on the various EST-2 allozymes, although as was found with most of the other studies, it is the EST-2^C allozyme which stands out as being different from the other three, which tend to be fairly similar in their responses.

4.4 DISCUSSION

A number of allozymes produced by the *Est-1* and *Est-2* loci were characterised with respect to their pH-activity profiles, thermostability and substrate specificity. If allozyme polymorphism is maintained by balancing selection, it is necessary that there be physiologically relevant differences between the allozyme variants. The results presented indicate a wealth of diversity between allozymes of the *Est-2* locus, and some

Table 4.20 Effect of alcohol structure on ESTERASE-2 activity for a group of acetate esters. All assays were conducted at 25°C and pH 7.00

Substrate	M.W.	EST-2[a]	EST-2[b]	EST-2[c]	EST-2[d]
(a) Volatile esters					
ethyl acetate	88.10	N.D.*	N.D.	N.D.	N.D.
n-propyl acetate	102.14	N.D.	N.D.	N.D.	N.D.
i-propyl acetate	102.14	N.D.	N.D.	N.D.	N.D.
n-butyl acetate	116.16	222.9	122.6	103.1	154.3
i-butyl acetate	116.16	318.5	245.1	223.4	277.8
n-amyl acetate	130.19	329.1	330.9	257.7	354.9
i-amyl acetate	130.19	371.6	416.7	292.1	401.2
(b) Non-volatile esters					
p-nitrophenyl acetate	181.2	51.4	42.4	14.9	38.2
o-nitrophenyl acetate	181.2	15.1	13.8	5.3	12.3
β-naphthyl acetate	186.2	261.9	238.1	170.6	290.4
α-naphthyl acetate	186.2	129.9	123.5	122.7	157.3
4-methylumbelliferyl acetate	218.2	262.8	215.4	106.4	229.3
cholesteryl acetate	428.7	N.D.	N.D.	N.D.	N.D.

* N.D. = not detectable.

differences between the two allozymes of *Est-1* locus which were examined. Since these studies were carried out with only partially purified enzyme, the differences in specific activity between the various enzyme preparations must be viewed with some caution. They may well be due to differences in the amount of enzyme protein which survived purification, rather than differences in the catalytic capacities of the respective allozymes. In this context, significant interactions between allozyme type and the various experimental treatments are far more sensitive indicators of differences between allozymes than are simple differences in activity.

With respect to the pH and thermostability studies a number of comparisons can be made with enzymes in other species. In general, for allozymic variants of *Drosophila* species, differences in pH optima (Narise, 1973a, 1979; Harper and Armstrong, 1973; Alahiotis, 1979a) and thermostability (Day *et al.*, 1974a,b; Narise, 1973a; Alahiotis, 1979a) have been found for a number of loci. In one instance, for α-glycerophosphate dehydrogenase in *D. melanogaster*, no difference in thermostability of allozymic variants was detected, but temperature related differences in such enzymic properties as specific activity, K_m and reaction rate constancy were found when measurements were made within the normal physiological range (Miller *et al.*, 1975).

For the studies on the *D. buzzatii* esterases reported above, there was no convincing evidence for differences in thermostability or pH optimum for the two EST-1 allozymes, and no difference in pH optimum for the four EST-2 allozymes. There were however, substantial differences in thermostability for the EST-2 enzymes. On the basis of the studies reported in Chapters 2 and 3, I believe it has been possible to make a convincing case for homology of the *Est-1* and *Est-2* loci of *D. buzzatii* with the *Est-β* and *Est-α* loci respectively of *D. virilis*. Narise (1973a) compared the pH optima and thermostabilities of a large number of allozymic variants at the *Est-α* and *Est-β* loci of *D. virilis*. The enzyme materials which she used were more highly purified than those employed in the present study, and the experimental conditions were quite different, which may account for some of the differences between the two sets of data. For the *D. virilis* enzymes, Narise reported differences of pH optima and thermostability for allozymic variants at both loci. She suggested four different pH profiles for the total set of allozymes produced by both esterase loci, with optima in the ranges 7.0 - 7.5, 7.5 - 8.0, 8.0 - 8.5 and 8.5 - 9.0. For allozymes of the *Est-α* locus, 8 of 9 tested had a pH optimum in the range 7.0 - 8.0, and for *Est-β* allozymes 3 of 4 had their optima in the range 8.0 - 9.0. In general this agrees with the results observed for *D. buzzatii* esterases, with the

four EST-2 allozymes showing optima around 7.0 - 7.5 and the two EST-1 allozymes with optima in the range 8.0 - 9.0.

The thermostability results provide the only significant inconsistency between the data of Narise (1973a,b) and those reported here. For thermal denaturation at 60°C, Narise reported that the β-esterase allozymes were much less stable on average than the α-esterase enzymes, though for the group of 8 *Est-α* allozymes tested there was a very large range of thermostabilities. The studies with *D. buzzatii* enzymes were made at 50°C, and under those conditions the EST-2 (α-EST) allozymes were generally less stable than the EST-1 (β-EST) allozymes. However, I would argue that thermostability determined at non-physiological temperatures is probably not a very reliable parameter of evolutionary divergence, and it may well be that observed differences are of no physiological relevance. There are two pieces of data which I would adduce in support of this contention. First is the great heterogeneity of stability observed for the four EST-2 allozymes reported here. The EST-2^b allozyme was not significantly different in thermostability from the two EST-1 allozymes, though it was significantly more stable than the other three EST-2 enzymes (Table 4.5). In the preceding chapter, I argued that the EST-1 and EST-2 enzymes were different in essentially every property examined, and that if the *Est-1* and *Est-2* loci had arisen as the result of a gene duplication event, it would appear from the little data available that the duplication had occurred prior to the divergence of *Sophophora* and *Drosophila* sub-genera, by late Eocene (Throckmorton, 1975). Taken at face value, these data suggest that the extent of thermostability differences between allelic forms of a given enzyme is as great as the difference between isozymes which have been evolving at least partly independently for 30-40 million years. The results of Narise (1973a) indicate that a similar situation occurs for the esterases of *D. virilis*.

Whether the observed differences in thermostability of allelic variants are of any physiological relevance also must be questioned in the light of

results obtained for the alcohol dehydrogenase enzyme in *D. melanogaster*. Gibson and co-workers (Gibson *et al.*, 1981; Wilks *et al.*, 1980) analysed an electrophoretically cryptic variant of the ADH^{f} allozyme which was twice as thermostable as the common ADH^{f} and ADH^{s} forms. This thermostability varient ($ADH^{fch.d}$) was found at low but polymorphic frequencies in natural populations of *D. melanogaster* in Australia, and a thermostability variant with similar properties was known in North American populations (Sampsell, 1977). These data provided the opportunity to seek correlations between the frequencies of alleles encoding thermally stable enzyme variants and various environmental parameters related to temperature. The results were summarised by Gibson and Oakeshott (1982) as; "...these data provide no evidence that the frequency of *FCh.D* is consistently correlated with environmental temperatures, and the differentials in ADH activity are only manifest in extracts subjected to temperatures which are lethal to larvae or adult flies...".

Although differences in thermostability between allozymes are quite common, in no instance has any physiological relevance yet been ascribed to the differences. In fact the only convincing evidence that heat stability is related to the environmental temperatures which organisms encounter comes from the study of orthologous enzyme homologues of species adapted to different thermal regimes (Alexandrov, 1977; Somero, 1978). Even under these conditions Somero (1978) added a cautionary rider: "...the correlation between adaptation temperature and enzyme thermal stability has many facets, and must be viewed not only as a reflection of the selective advantage of adequate heat stability, but also as an outcome of the co-evolution of functional and structural enzymatic traits."

In general it is probably fair to say that differences in pH optima and thermostability are reasonable indicators of molecular diversity when making comparisons of allozymic variants, but it is not immediately apparent how such differences may be manifest in any physiologically meaningful sense.

In contrast to this, any differences in substrate specificity between allozymes may be of considerable physiological import to the organism if it encounters a diversity of these substrates, and they are not distributed constantly over time and space.

The results of the substrate specificity study (Section 4.3.3) suggested that there are differences between the allozymes produced by the *Est-1* and *Est-2* loci. The differences noted for the two EST-1 allozymes were not particularly great and for the enzymes used in these studies, the EST-1^{C} preparation almost invariably showed a higher specific activity than the EST-1^{b} preparation. Despite the apparent catalytic superiority of the EST-1^{C} enzyme, allozyme by substrate interactions were significant, indicating different relative effects of varying substrate structure.

The differences between allozyme preparations were much greater for products of the *Est-2* locus. Among the data presented in Section 4.3.3, it is possible to find instances in which each of the four allozymes shows greatest specific activity.

The most striking observations are the high activity shown by both EST-1 and EST-2 on formate esters, and volatile esters generally, and for EST-2 the trend of increasing activity with increasing substrate molecular weight within different structural groups (Table 4.20). Narise (1973b) briefly reported some substrate specificity results for the α- and β-esterase allozymes of *D. pseudoobscura*, and while these agree very satisfactorily with the results reported here, she concluded that there were no differences in substrate specificity between the allozymes which she examined. Since only a small number of substrates were common to both studies and she reported data only for a representative allozyme of each locus, it is not possible to make detailed comparisons.

Danford and Beardmore (1979) reported an extensive substrate specificity study of the two common allozymes produced by the *Est-6* locus in *D. melanogaster*. They found significant differences between allozyme preparations with respect to substrate specificity, though the differences were not as

great as those reported here, and one allozyme was almost always more active than the other, especially for those compounds which were hydrolysed rapidly. It is worth noting that, of the 26 esters for which data were reported, as a group the formates were all hydrolysed extremely rapidly. Although I argued previously (Section 2.4) that there was no convincing evidence of homology between EST-6 in *D. melanogaster* and the esterases of *D. buzzatii*, it is interesting that formates should be hydrolysed so rapidly in both species. In a closed system in which adult *D. buzzatii* were exposed to atmospheric esters, Le (1983) found that, as a group, formates were extremely toxic. It is not known whether formate esters occur in appreciable quantities in the feeding and breeding habitats of *Drosophila*, but if they do, then it may be necessary to hydrolyse them rapidly as a detoxification mechanism.

Although substrate specificity studies for non-specific enzymes have not been widely reported in the literature, especially for multi-allelic enzyme systems, they are important in the context of models which seek to relate levels of enzyme polymorphism to enzyme function. The idea that enzymes which act on a multiplicity of substates are more polymorphic than those which have only a single substrate (Gillespie and Kojima, 1968; Kojima *et al.*, 1970) has never been adequately investigated. In its original conception the "substrate specificity hypothesis" as presented by Gillepsie and Kojima (1968) and subsequently elaborated (Kojima *et al.*, 1970; Gillespie and Langley, 1974), suggested that enzymes involved in pathways of glucose metabolism (Group I) which have only a single physiological substrate are less genetically variable than enzymes which have multiple substrates derived from the external environment (Group II). The reason for the higher level of heterozygosity of Group II enzymes which was implicit in this hypothesis is the action of selection operating in heterogeneous environments, in this case, variable substrates. Johnson (1973, 1974) subsequently extended this model to subdivide the Group I enzymes into those

which have a regulatory function vs those which do not, arguing that allozymic variation for regulatory enzymes provided greater physiological flexibility. These models have been the subject of some criticism in the ensuing period (Selander, 1976; Zouros, 1975; Singh, 1976). A major difficulty as noted by Selander (1976) is that it is not always a straightforward matter to assign enzymes to one or other of the groups, and this is especially true of the Group II enzymes where their assignment is based solely on *in vitro* properties. Zouros (1975) noted that a high proportion of Group II enzymes were monomorphic, and it may well be that if their physiological substrates were known, some of these enzymes should be more properly categorised as belonging to Group I. The most thorough analysis of the "substrate specificity model" is that of Singh (1976). He concludes from a survey of the literature and experimental work on alcohol oxidising enzymes of *D. pseudoobscura* that environmental variability has promoted the evolution of multiple isozyme systems rather than multiple allozymes at a single locus. While I believe that Singh's criticisms are valid if one is seeking a global explanation of polymorphism among Group II enzymes, nonetheless I feel that it is premature to discount the model entirely, for the simple reason that we have so little empirical information which is directly relevant. There are few, if any precedents which indicate that natural selection has favoured single solutions to the challenges of adaptation. Myers (1978) reconsidered the available data on isozymic and allozymic variation, and concluded that they represented alternative forms of adaptation.

The polymorphism at the Est-2 locus in *D. buzzatii* may represent an excellent opportunity to further assess the "substrate specificity hypothesis". The enzyme is located primarily in the alimentary tract, and consequently will be in direct contact with ingested materials (Chapter 2). It has a very broad substrate specificity (Sections 3.3 and 4.3.3) and allozymic variants show different specificities (Section 4.3.3). Singh (1976) noted that:

> "...(1) most polymorphic loci have only 2-4 alleles and very few have more than 4; (2) with each group of these loci... the distribution of allelic frequencies is very uneven. Most loci have one allele which is the most common allele in most populations, and all the other alleles are quite rare".

The *Est-2* locus is an exception to both these generalisations. Singh went on to note that:

> "The number of alleles at a single locus can be taken to reflect the qualitative aspect of the substrate specificity hypothesis and the average heterozygosity, its quantitative aspect."

In natural populations of *D. buzzatii* we currently know of 7 alleles at the *Est-2* locus (Barker, East and Sene, unpublished) and in Australia there are 4 common alleles, with an average level of heterozygosity of 0.635 ± 0.006, (Barker, 1981). The *Est-2* locus and the allozymes produced by it appear to have all of the properties suggested by Singh (1976) which are pre-requisites for a valid test of the "substrate specificity hypothesis".

CHAPTER 5

COMPARATIVE KINETIC STUDIES OF ESTERASE-2 ALLOZYMES

5.1 INTRODUCTION

From the population genetics viewpoint, the *Est-2* locus provides us with a particularly interesting case study. As noted previously, polymorphism at this locus is atypical in that the very high level of average heterozygosity, as measured by conventional electrophoretic techniques, results from the ubiquitous presence of four alleles, with the frequency of the rarest of these being seldom less than 0.1 in natural populations (Barker and Mulley, 1976; Barker, 1981). A compilation of data for about 400 single locus polymorphisms by Singh (1976) suggests that less than 10% of the loci have a pattern similar to that shown by the *Est-2* locus. Apart from this unusual allelic distribution, a considerable number of studies (reviewed in Section 1.2.5) suggest that some form of balancing selection is involved in the maintenance of the *Est-2* polymorphism. Of course, such data can only provide circumstantial evidence of selection, since it may not be possible to exclude the possibility that selection is acting on a linked locus with which the *Est-2* locus is in disequilibrium.

The difference in molecular properties between the four allozymic variants of the *Est-2* locus which were examined in the previous chapter seemed sufficiently great as to warrant further investigation. Detailed description of the differences between allozymes, especially in the context of enzyme function, and the interaction between environmental factors and the physiology of the organism appear to provide our best hope for breaking the nexus between the action of selection on a particular locus as distinct from loci linked to it.

If allozymes are not neutral with respect to natural selection, but rather, represent an evolutionary solution to the problems of adaptation to unpredictable environmental conditions, then it is reasonable to expect this to be manifest in the catalytic properties of the enzyme variants. The most useful descriptors we have of enzymes as biological catalysts are their kinetic parameters, particularly the Michaelis constant (K_m) and the catalytic turnover number (k_{cat}). For many enzymically catalysed reactions the velocity of the reaction (v), as a function of substrate concentration (S), can be represented as a section of a rectangular hyperbola described by the relationship:

$$v = V_{max}.[S]/([S] + K_m) \qquad 5.1$$

where $V_{max} = k_{cat}.E_o$, and E_o is the total concentration of active enzyme.

The two parameters, V_{max} and K_m, which define equation 5.1 are complex functions of individual rate constants (see Section 5.2), and as a consequence they may vary with experimental conditions such as temperature, pH, ionic strength etc., and in the case of enzymes which can act on more than one substrate, they may also vary with substrate type. Excellent examples of this are provided by the serine proteases. This family of enzymes has been extensively characterised with respect to primary, secondary and tertiary protein structure, and also with respect to factors affecting substrate specificity. Hartley (1979) in a discussion of the evolution of enzyme structure observed that: "Remarkable conservation of tertiary structure is the outstanding lesson that we learn from this divergent enzyme family. Differences in specificity are readily grafted on to a common pocket ... by only one or two amino-acid changes, and we see isoenzymes .... with considerable surface sequence differences but identical substrate specificities". The elegant kinetic studies of these enzymes by Bender and his colleagues (e.g. Bender and Kezdy, 1965; Brot and Bender, 1969) provide a model for the study of enzymes of broad substrate specificity.

There has recently been some theoretical interest in the effects of natural selection on enzyme kinetic parameters (Fersht, 1974; Crowley, 1975; Cornish-Bowden, 1976). The results suggest that natural selection is likely to have favoured different strategies for different classes of enzyme. For enzymes involved in the integrated pathways of intermediary metabolism, natural selection appears to have favoured a system in which k_{cat} is large, and K_m is approximately equal to the physiological concentration of the substrate. This has the effect of maximising flux through the pathway concerned (Crowley, 1975). For enzymes involved in digestion, such as pepsin, trypsin and chymotrypsin, the values of K_m tend to be small with respect to likely substrate concentration. This ensures that the enzyme is normally saturated *in vivo* and consequently provides a constant rate of product release. For enzymes involved in detoxification, evolution is likely to have promoted high values of k_{cat}, and low values of K_m (Cornish-Bowden, 1976). This strategy ensures both the efficient binding of low levels of substrate, and a high sensitivity of the enzyme to changes in substrate concentration (Tipton, 1980).

From these theoretical considerations and the extensive empirical studies of the serine proteases it seems clear that the kinetic parameters V_{max} ($= k_{cat.} E_o$) and K_m should be sensitive indicators of differences between allozymic variants produced by a single locus. This is especially true of enzymes such as esterases, which have broad and ill-defined substrate specificity, and also of enzymes of ectothermic organisms, which are subject to fluctuations in environmental variables such as temperature and pressure.

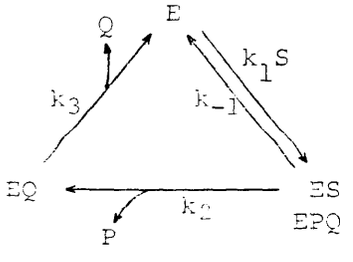
The results of the comparative studies reported in Chapter 4 suggested that a kinetic analysis involving temperature and substrate variables might provide some useful clues to the nature of the polymorphism at the Est-1 locus in *D. buzzatii* and mechanisms which might be involved in its maintenance.

5.2 THEORY

Although equation 5.1 is an adequate descriptor of the variation in rate of catalysis with varying substrate concentration for many enzymes including the EST-2 allozymes reported below, it is important to recognize that the parameters K_m and V_{max} are functions of a number of rate constants which are unknown, and cannot normally be readily estimated. The precise form of the functions which describe K_m and V_{max} is dependent on the mechanism of the reaction. Literally, the esterase catalysed hydrolysis of esters follows a crypto ping-pong uni-bi mechanism in which the second substrate is water (Roberts, 1977). However, since water is normally present at extremely high concentrations, the enzyme is usually considered to be saturated with this substrate at all times. Under these conditions the catalytic reaction can be written as:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{(EPQ)}{(ES)} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EQ + P \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + Q \qquad \text{V.1}$$

where: E is free enzyme, S is substrate, P and Q are products, and ES, EPQ and EQ are enzyme bound intermediates of the reaction process. For initial reaction conditions the concentration of products P and Q are effectively zero, and so k_{-2} and k_{-3} will be negligible compared to k_2 and k_3. In this instance the reaction can be re-written (in folded form) as:



V.2

For any enzyme species E_i, the proportional concentration of that species is given by (King and Altman, 1956):

$$\frac{(E_i)}{(E)_o} = \frac{\Delta_i}{\sum_{i=1}^{n}(\Delta_i)} \qquad 5.2$$

King and Altman (1956) provided a schematic method for the calculation of the determinants Δ_i and these are summarised in Table 5.1

From this we deduce the following expressions:

$$\begin{aligned} \Delta_E &= k_2k_3 + k_{-1}k_3 \\ \Delta_{ES} &= k_1k_3S \\ \Delta_{EQ} &= k_1k_2S \end{aligned} \qquad 5.3$$

The rate of formation of product P is:

$$\frac{dP}{dt} = k_2.[ES] \qquad 5.4$$

Application of equations 5.2 and 5.3 gives:

$$\frac{dP}{dt} = k_2.[ES] = \frac{k_2\ k_1\ k_3\ S\ E_o}{k_2k_3+k_{-1}k_3+k_1k_3S+k_1k_2S} \qquad 5.5$$

Similarly, the rate of formation of product Q is:

$$\frac{dQ}{dt} = k_3.[EQ] = \frac{k_3\ k_1k_2SE_o}{k_2k_3+k_{-1}k_3+k_1k_3S+k_1k_2S} \qquad 5.6$$

From equations 5.5 and 5.6, the rate of reaction, v is:

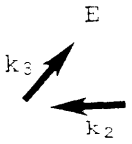
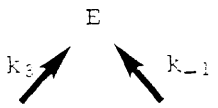
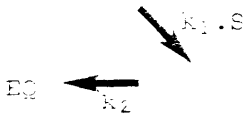
$$v = \frac{dP}{dt} = \frac{dQ}{dt} = \frac{k_1k_2k_3SE_o}{k_2k_3+k_{-1}k_3+k_1k_2S+k_1k_3S} \qquad 5.7$$

Dividing numerator and denominator through by $k_1(k_2+k_3)$ gives:

$$v = \frac{\frac{k_2k_3}{k_2+k_3}\ E_o\ S}{\frac{k_3(k_{-1}+k_2)}{k_1(k_2+k_3)} + S} \qquad 5.8$$

Equation 5.8 is of the form of equation 5.1, in which:

Table 5.1. Estimation of the uncancellable, non-cyclic determinant terms for reaction mechanism V.2

Enzyme Species	Vector Diagram	Determinant (Δ)
E		$k_2.k_3$
E		$k_{-1}.k_3$
ES		$k_1.k_3.S$
ES		$k_1.k_2.S$

$$k_{cat} = \frac{k_2 k_3}{(k_2+k_3)} \qquad 5.9$$

$$k_m = \frac{k_3(k_{-1}+k_2)}{k_1(k_2+k_3)} \qquad 5.10$$

Since normal techniques of kinetic analysis using initial rate methods provide estimates of V_{max} ($=k_{cat.}E_o$) and K_m, these must be regarded as "apparent" values only, valid for the particular set of conditions used. They provide us with no information as to the values of the true rate constants k_1, k_{-1}, k_2, k_3. The greatest difficulty arises in interpretation of K_m effects, since it is common practice to treat K_m as a measure of enzyme-substrate affinity. The valid measure of affinity of an enzyme for a substrate is the dissociation constant K_s ($=k_{-1}/k_1$). Clearly there is no simple relationship between K_s and K_m as defined by equation 5.10, and any use of K_m as an estimator of affinity must be treated with caution in the absence of extra information concerning the rate constants for the reaction.

The complex nature of V_{max} and K_m make it difficult to compare the kinetic results obtained for different substrates. Brot and Bender (1969) showed for the protease α-chymotrypsin, that the ratio k_{cat}/K_m, which they called the specificity constant, was the best estimator of substrate effectiveness for that enzyme. In the present case, for the reaction mechanism V.2, a consideration of equations 5.9 and 5.10 indicates that the specificity constant (k_{cat}/K_m) is equal to $k_1k_2/(k_1+ k_2)$. For the comparative studies reported in Section 5.4, it has not been possible to estimate k_{cat}, since only partially purified material was used. However, since the same homogeneous batch of each allozyme preparation was used for all the studies, comparisons will be made in terms of the pseudo-first-order rate constant V_{max}/K_m. When comparing between allozymes, the absolute value of this parameter may not be very meaningful, but alterations in relative values should be a sensitive indicator of any differences between them.

5.3 MATERIALS AND METHODS

The genetic material used for these studies was that described previously (Section 4.2.1), and the enzymes were purified according to Protocol 2 as outlined in Section 3.2.1.2. As before, at the final step of purification for any given allozyme, fractions were pooled, thoroughly mixed, and stored at -20°C in 0.4 ml aliquots. For the experiments reported below, sufficient of each allozyme was thawed for one day of assays.

5.3.1 Kinetic Procedures

For a particular set of environmental conditions (*i.e.* temperature, pH, substrate type) all assays were done in a single experimental session. For any given value of temperature and pH (Section 5.4.1) or temperature and substrate (Section 5.4.2) all assays were completed within 8 hours.

Assays were performed in 0.1M phosphate buffers at pH 6.5, 7.0, 7.5 and 8.0, and temperatures of 10, 25 and 40°C. Temperature was maintained by a circulating water bath at T ± 0.5°C. For the substrate study, the acetate, butyrate and caproate esters of α-naphthol were used. All reagents were prepared freshly on the morning of use from stock solutions of substrates in acetone stored at -20°C. Buffer of any particular pH was prepared as a single large batch sufficient for the whole experiment.

For the determination of kinetic constants, each assay was conducted in triplicate at each of seven different substrate concentrations. Thus, the velocity *vs* substrate curve in each case was based on 21 points.

5.3.2 Estimation of Kinetic Parameters and Thermodynamic Constants

Data were analysed according to the procedures of Cleland (1979) using a weighted, non-linear least squares regression procedure. The data were fitted to the rectangular hyperbola described by equation 5.1 using the two programmes HYPER and HYPERL of Cleland (1979). Listings of these Fortran programmes are given in Appendix C, since the initialisation of some variables had been omitted in the listing of the HYPER programme given in

Cleland (1979).

Enzyme thermodynamic parameters were calculated from the following set of equations taken from Robert and Gray (1972).

$$Ea = 2.303 \log \frac{k_2}{k_1}.R. \frac{(1}{T_1} - \frac{1)}{T_2}$$

$$\Delta H^{\ddagger} = E_a - RT$$

$$\Delta S^{\ddagger} = 2.303R (\log k - 10.753 - \log T + \frac{Ea}{4.576T})$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$$

where: Ea is the Arrhenius energy of activation, $\Delta H^{\ddagger}$ is the enthalpy of activation, $\Delta S^{\ddagger}$ is the entropy of activation, $\Delta G^{\ddagger}$ is the Gibbs free energy of activation, k is a velocity constant (given by V_{max}), R is the gas constant (1.987 cal/deg per mole), and T is absolute temperature (^{O}K).

5.3.3 Graphical and Statistical Procedures

The three-dimensional surfaces shown in Figs. 5.1 and 5.3 were drawn by computer using Akima's (1974) regular rectangular grid bi-variate cubic interpolation algorithm, using Fortran plotting and hidden-line drawing subroutines provided by the PLOT-79 graphics package.

Kinetic parameters were analysed by analysis of variance using a randomised complete block design. In theory the error sum of squares for these analyses could have been provided by a pooled estimate of the residual variance after fitting the non-linear regressions. However, the wide range of environmental test conditions used in these studies resulted in a significant heterogeneity of the residual variances, preventing the valid calculation of a pooled estimate of variance. Accordingly, a much more conservative test of main effect and first-order interactions was done using the second-order interaction as the source of error mean-square.

5.4 RESULTS

The results of the kinetic analyses are presented in terms of the apparent K_m and V_{max} parameters, and the pseudo-first-order rate constant V_{max}/K_m. The standard errors accompanying each estimate were provided by the computer programme used for the analysis, and are based on the residual variance after fitting the non-linear regression (Cleland, 1979).

5.4.1 Effect of Variable Temperature and pH on EST-2 Allozyme Kinetic Parameters

In broad outline, the effect of changing the assay environment was similar for the four allozymes, though a more detailed examination revealed significant differences in many instances.

5.4.1.1 Effects on Apparent K_m

The apparent K_m's and their respective standard errors are presented in Table 5.2. In general terms, averaging effects over the different allozymes, there is a tendency for K_m to increase as the pH moves away from the observed optimum of 7.0 - 7.5 (Section 4.3.1.2). Similarly, on average, temperature has little effect on K_m, though values tend to be higher at the very low temperature of 10°C. To analyse the effects in more detail, the data given in Table 5.2 were subjected to analysis of variance, and the results are summarised in Table 5.3.

There are no significant differences in K_m for the various enzymes, and none of the interaction terms involving allozymes is significant. The main effect of pH is highly significant, and presumably reflects the trend noted earlier of increasing K_m values with departure from the enzyme pH optima. Temperature has no significant effect on K_m in this analysis, and this will be considered further in the discussion. There is a significant interaction between pH and temperature on substrate affinity as measured by the apparent K_m. Inspection of the data suggests that this significant

Table 5.2 Effects of variable pH and temperature on K_m (app.) for four EST-2 allozymes. Parameter estimates were provided by the HYPERL programme of Cleland (1979)

Enzyme	Temp. (°C)	pH			
		6.5	7.0	7.5	8.0
Est-2[a]	10	22.32 (0.75)	15.14 (1.28)	13.33 (0.55)	16.87 (1.16)
	25	11.82 (0.95)	14.23 (1.25)	13.65 (1.08)	18.74 (0.82)
	40	19.22 (1.97)	12.84 (0.54)	9.64 (0.63)	6.19 (0.54)
Est-2[b]	10	19.61 (1.29)	13.74 (1.24)	12.24 (0.43)	8.72 (0.52)
	25	11.51 (0.89)	11.02 (0.68)	9.46 (0.39)	20.89 (1.22)
	40	23.79 (5.22)	11.71 (0.73)	10.52 (0.40)	5.70 (0.64)
Est-2[c]	10	26.01 (1.38)	9.70 (0.74)	14.74 (0.61)	17.11 (1.68)
	25	11.80 (0.82)	18.30 (1.55)	7.70 (0.33)	13.84 (0.50)
	40	22.38 (3.19)	14.22 (0.77)	6.63 (0.59)	13.82 (1.32)
Est-2[d]	10	34.02 (4.96)	14.83 (1.72)	17.55 (0.62)	12.58 (1.10)
	25	11.72 (1.18)	16.29 (2.09)	11.60 (1.07)	36.03 (3.46)
	40	46.86 (14.08)	13.12 (1.10)	4.86 (0.81)	13.03 (1.00)

Table 5.3 Analysis of variance of the effect of variable pH and temperature on K_m (app.) for EST-2 allozymes, based on the data of Table 5.2. Data were log. transformed prior to analysis

Source	d.f.	Mean square	F
Allozyme (A)	3	0.160	1.66
pH (P)	3	0.834	8.62***
A x P	9	0.069	0.72
Temperature (T)	2	0.252	2.61
A x T	6	0.028	0.29
P x T	6	0.577	5.97**
Error	12	0.097	

** $P < 0.01$

*** $P < 0.001$

interaction results from a general reduction of K_m with increasing pH at 40°C, but generally little consistent effect of pH at the other two assay temperatures.

5.4.1.2 Effects on V_{max}

For the purpose of this analysis it is important to bear in mind the fact that differences between allozymes in the absolute values of V_{max} have relatively little meaning. The reason for this is that we are dealing with only partially purified material, and since V_{max} is a function of both catalytic efficiency (k_{cat}) and enzyme concentration (E_o), we cannot attribute differences between allozymes with respect to V_{max}, to differences in catalytic efficiency alone. The effects of variable environmental conditions on V_{max} are summarised in Table 5.4. There clearly have been large effects of the environmental factors, and an analysis of variance on these data (Table 5.5) illustrates this. The highly significant allozyme effect appears to be mainly attributable to the generally low values for *Est-2*^{C} under all conditions. However, as noted above, the main effect of allozyme is probably not very informative. The main effect, pH is highly significant, and taken as an average, reflects generally lower values of V_{max} at pH 8.0 relative to the other pH's tested. Naturally temperature has the largest single effect on V_{max}, as is typical of all enzyme catalysed reactions. The first-order interaction term between pH and temperature also is significant and probably is due to the rapid decline in V_{max} with increasing pH at 40°C, whereas there is little systematic effect at the other two temperatures.

Of greatest interest in the genetic context, however, are the two first-order interaction terms involving allozymes, since it is these which will reveal whether there is significant heterogeneity between the allozymes in response to environmental changes. In the present study neither of these interaction terms was significant, and we must conclude that all four enzymes responded in a similar fashion to changes in temperature and pH.

Table 5.4 The effects of variable pH and temperature on V_{max} for the four EST-2 allozymes. Parameter estimates were provided by the HYPERL programme of Cleland (1979)

Enzyme	Temp. (°C)	pH			
		6.5	7.0	7.5	8.0
Est-2[a]	10	54.95 (1.07)	55.07 (2.47)	43.99 (0.85)	46.85 (1.60)
	25	69.23 (2.44)	71.21 (3.03)	117.50 (4.37)	41.39 (0.96)
	40	154.15 (8.34)	144.72 (2.67)	133.56 (3.30)	64.15 (1.74)
Est-2[b]	10	34.85 (1.26)	35.36 (1.60)	26.10 (0.41)	29.88 (0.64)
	25	72.02 (2.43)	45.03 (1.21)	72.64 (1.17)	31.23 (0.88)
	40	169.35 (21.37)	136.98 (3.59)	109.16 (1.64)	73.57 (2.43)
Est-2[c]	10	16.60 (0.54)	13.40 (0.44)	15.01 (0.30)	13.76 (0.59)
	25	30.12 (0.92)	26.37 (1.38)	31.66 (0.47)	22.58 (0.37)
	40	55.36 (4.43)	50.90 (1.27)	36.27 (0.96)	38.10 (1.77)
Est-2[d]	10	41.52 (4.00)	38.07 (2.30)	29.57 (0.54)	26.48 (1.03)
	25	45.94 (2.02)	62.07 (4.65)	88.58 (2.91)	40.01 (2.63)
	40	190.20 (40.82)	98.67 (3.65)	80.06 (3.24)	115.06 (4.19)

Table 5.5 Analysis of variance of the effect of variable pH and temperature on V_{max} for the EST-2 allozymes. The data of Table 5.4 were log. transformed prior to analysis

Source	d.f.	Mean square	F
Allozyme (A)	3	2.532	70.42***
pH (P)	3	0.424	11.79***
A x P	9	0.029	0.82
Temperature (T)	2	5.107	142.04***
A x T	6	0.053	1.49
P x T	6	0.166	4.62**
Error	18	0.036	

** $P < 0.01$

*** $P < 0.001$

5.4.1.3 Effects on V_{max}/K_m

The pseudo-first-order rate constant V_{max}/K_m should be a sensitive measure of the effects which environmental changes have on enzyme catalysis, since it simultaneously reflects changes in catalytic efficiency (*via* k_{cat} effects) and substrate binding efficiency (*via* K_m effects). The values of this parameter and their associated errors are summarised in Table 5.6. As for V_{max} alone, differences between allozymes in the absolute value of V_{max}/K_m cannot tell us much about the differences in inherent catalytic efficiency of the enzymes, since they may result from differences in enzyme concentration. An analysis of variance on this set of data (Table 5.7) yielded essentially the same result as the analysis on V_{max} alone, and this probably indicates that under these experimental conditions V_{max} effects dominate the V_{max}/K_m parameter. In terms of average effects, the significant allozyme effect probably results primarily from the low values of the EST-2^c allozyme, since the other three are fairly similar. As before, pH effects seem to indicate that efficiency declines as pH is moved away from the optimum of 7.0 to 7.5. As might be expected from simple thermodynamic considerations, the value of V_{max}/K_m shows a substantial increase with increasing temperature.

The two interaction terms involving allozyme effects (A x P and A x T) were both non-significant, and again we must conclude that, by this measure of enzyme efficiency, all four allozymes responded in a similar fashion to alterations in the environment. In an attempt to visualise more clearly the response of enzyme catalytic efficiency to changes in environmental conditons, three dimensional surfaces were fitted to the data in Table 5.6 and these are shown in Fig. 5.1. There are some striking features of these response surfaces. The most obvious of these is the fact that EST-2^c shows relatively little change with the environmental variables, and the surface is almost planar. Three dimensional contour surfaces were constructed for these data, and the EST-2^a, EST-2^c and EST-2^d allozymes all gave surfaces which were topographically quite similar. This also can be seen in Fig. 5.1 where

Table 5.6 Effects of variable pH and temperature on the pseudo-first order rate constant V_{max}/K_m for EST-2 allozymes. Parameter estimates were provided by the programme HYPERL of Cleland (1979)

Enzyme	Temp. (°C)	pH			
		6.5	7.0	7.5	8.0
Est-2[a]	10	2.46 (0.04)	3.64 (0.15)	3.30 (0.08)	2.78 (0.10)
	25	5.85 (0.28)	5.00 (0.24)	8.61 (0.39)	2.21 (0.05)
	40	8.02 (0.42)	11.27 (0.28)	13.85 (0.60)	10.37 (0.65)
Est-2[b]	10	1.78 (0.06)	2.57 (0.12)	2.13 (0.04)	3.43 (0.14)
	25	6.26 (0.30)	4.09 (0.15)	7.68 (0.20)	1.50 (0.05)
	40	7.12 (0.72)	11.70 (0.45)	10.38 (0.25)	12.90 (1.06)
Est-2[c]	10	0.64 (0.01)	1.38 (0.06)	1.02 (0.02)	0.80 (0.05)
	25	2.55 (0.11)	1.44 (0.36)	4.11 (0.12)	1.63 (0.03)
	40	2.47 (0.17)	3.58 (0.11)	5.47 (0.35)	2.76 (0.14)
Est-2[d]	10	1.22 (0.07)	2.57 (0.15)	1.68 (0.03)	2.11 (0.11)
	25	3.92 (0.24)	3.61 (0.22)	7.64 (0.47)	1.11 (0.04)
	40	4.06 (0.38)	7.52 (0.37)	16.47 (2.13)	8.83 (0.38)

Table 5.7 Analysis of variance of effect of variable pH and temperature on the pseudo-first order rate constant V_{max}/K_m, based on the data of Table 5.6. The analysis was done on log. transformed data

Source	d.f.	Mean square	F
Allozyme (A)	3	2.527	41.25***
pH (P)	3	0.825	13.47***
A x P	9	0.037	0.60
Temperature (T)	2	7.641	124.74***
A x T	6	0.063	1.03
P x T	6	0.661	10.78***
Error	18	0.061	

*** $P < 0.001$

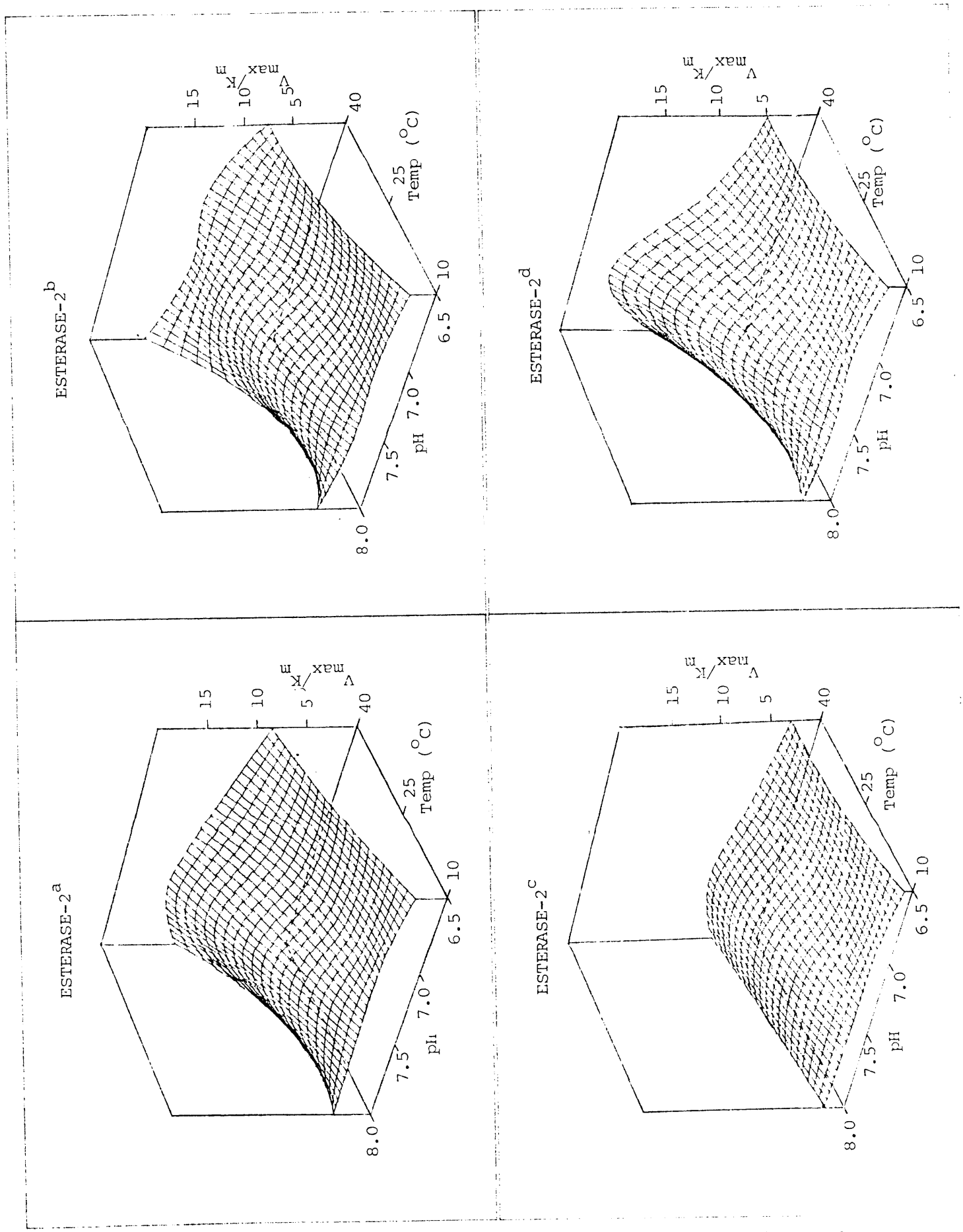


Figure 5.1 Three dimensional surfaces showing the pseudo-first order rate constant (V_{max}/K_m) as a function of temperature and pH for four EST-2 allozymes.

the differences between the surface for these three enzymes appear to be quantitative rather than qualitative. The fourth enzyme, EST-2^b gave a qualitatively different surface, with apparently superior efficiency at higher temperatures and pH values as compared with the other enzymes.

5.4.2 Effect of Variable Temperature and Substrate Structure on EST-2 Allozyme Kinetic Parameters

The differences in substrate specificity observed in previous experiments (Section 4.3.3) seemed sufficient to warrant investigation at the kinetic level since differences between allozymes for this property might have important implications for polymorphism at the *Est-2* locus. Temperature was included as an extra variable because of the association it had shown with *Est-2* polymorphism in previous analyses (Section 1.2.5) and also because of certain perceived shortcomings in the experiments reported in Section 5.4.1 above (see Discussion, Section 5.5). Unfortunately it was possible to analyse only three of the four allozymes in this study because the extensive preliminary analyses required in all these experiments had been done using EST-2^a preparation, and this had depleted the stock of frozen enzyme to the extent that insufficient material remained for this analysis.

The three substates used were the acetate, butyrate and caproate esters of α-naphthol. They were chosen for their ease and sensitivity of assay, and because similar derivatives of the chromogen p-nitrophenol had shown a significant allozyme by substrate interaction with respect to activity at saturating concentrations of substrate (Section 4.3.3.1.2). A similar experiment was conducted with the esters of α-naphthol (Fig. 5.2) and the results were very similar to those which had been obtained previously with the p-nitrophenol esters (Fig. 4.8). The rank order of activity was: butyrate $\gg$ caproate $>$ acetate, and as before EST-2^c allozyme showed a much higher relative activity on the caproic acid ester. Having confirmed that there was a difference between substrates, and a possible allozyme by substrate interaction, a detailed kinetic analysis was undertaken.

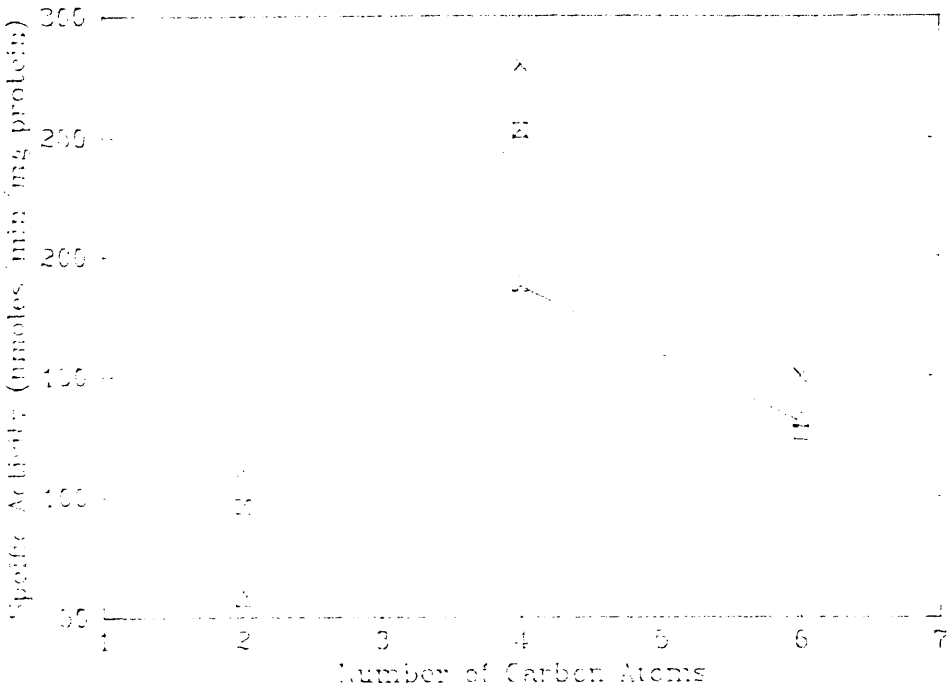


Figure 5.2 Effect of acyl carbon chain length on enzyme activity for a group of esters of α-naphthol.
(X) EST-2^{b}; (△) EST-2^{c}; (□) EST-2^{d}.

5.4.2.1 Effects on Apparent K_m

The apparent K_m values and their associated errors are presented in Table 5.8. Alterations in substrate structure had a large effect, and this is shown clearly in the analysis of variance based on these data (Table 5.9). The main effect of allozyme was significant and probably reflects the fact that, on average EST-2^d had a higher K_m (app.) than the other two allozymes. The main effect of substrate structure was highly significant, and there was no allozyme by substrate interaction with respect to K_m. It seems fairly clear that the significant K_m effect results from large differences between the esters. The ranking of substrates is; butyrate > caproate > acetate. This is precisely the opposite to what might have been expected simply from the data presented in Fig. 5.2 if one assumes that better binding means better catalysis.

There was a significant effect of temperature on apparent K_m as measured in this experiment, and it appears to result from a trend of decreasing K_m with increasing temperature. There was also a significant allozyme by temperature interaction, and the reason for this may be that the pattern of change in K_m with temperature was different for EST-2^d than for EST-2^b and EST-2^c which showed similar patterns.

5.4.2.2 Effects on V_{max}

As expected from the preliminary study (Fig. 5.2) there were very large effects of substrate structure on V_{max} (Table 5.10). As before, differences between allozyme preparations with respect to V_{max} are not particularly informative since absolute enzyme concentrations were unknown. An analysis of variance on these data showed that all three main effects were highly significant (Table 5.11). The allozyme effect is likely to reflect the lower values of EST-2^c relative to the other two enzymes. The substrate difference results from the large differences between substrates which, in general terms are the same for all three enzymes, with the activity ranking butyrate

Table 5.8 Effects of variable temperature and substrate on K_m (app.) for EST-2 allozymes. Parameter estimates were provided by the HYPERL programme of Cleland (1979)

Enzyme	Temp. (°C)	Substrate		
		α-NA	α-NB	α-NC
Est-2[b]	10	5.89 (0.62)†	18.76 (1.11)	8.45 (0.77)
	25	7.25 (1.01)	26.28 (2.85)	10.49 (0.73)
	40	4.87 (0.49)	20.95 (2.22)	12.60 (0.74)
Est-2[c]	10	9.59 (0.70)	22.30 (2.12)	17.19 (3.17)
	25	11.29 (2.17)	26.91 (2.46)	8.52 (0.96)
	40	4.47 (0.42)	14.20 (0.99)	8.44 (0.65)
Est-2[d]	10	13.04 (1.22)	28.17 (3.09)	24.22 (3.38)
	25	7.03 (0.85)	19.05 (0.89)	15.62 (1.45)
	40	5.34 (0.34)	30.84 (3.13)	20.62 (1.91)

† Figures in parentheses are the standard errors of the estimates.

Table 5.9 Analysis of variance of effect of variable temperature and substrate on K_m (app.) for EST-2 allozymes. The data of Table 5.8 were log. transformed prior to analysis

Source	d.f.	Mean square	F
Allozyme (A)	2	0.33	9.42**
Substrate (S)	2	2.95	85.11***
A x S	4	0.10	2.78
Temperature (T)	2	0.19	5.36*
A x T	4	0.20	5.70*
S x T	4	0.12	3.37†
Error	8	0.03	

† $0.05 < P < 0.10$

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

Table 5.10 Effects of variable temperature and substrate on V_{max} for EST-2 allozymes. Parameter estimates were provided by the HYPERL programme of Cleland (1979)

Enzyme	Temp. (°C)	Substrate		
		α-NA	α-NB	α-NC
Est-2[b]	10	32.73 (0.98)	155.70 (5.41)	69.33 (2.64)
	25	90.57 (4.16)	413.77 (29.23)	182.99 (5.88)
	40	153.95 (4.80)	677.21 (43.66)	341.31 (9.93)
Est-2[c]	10	13.32 (0.41)	88.36 (5.21)	66.12 (6.91)
	25	29.95 (2.25)	166.20 (9.98)	114.18 (5.38)
	40	59.02 (1.62)	260.04 (9.59)	191.54 (6.05)
Est-2[d]	10	36.30 (1.55)	145.87 (8.99)	81.29 (5.85)
	25	70.07 (3.25)	305.08 (8.40)	174.30 (8.87)
	40	140.55 (2.94)	595.69 (41.21)	305.84 (14.05)

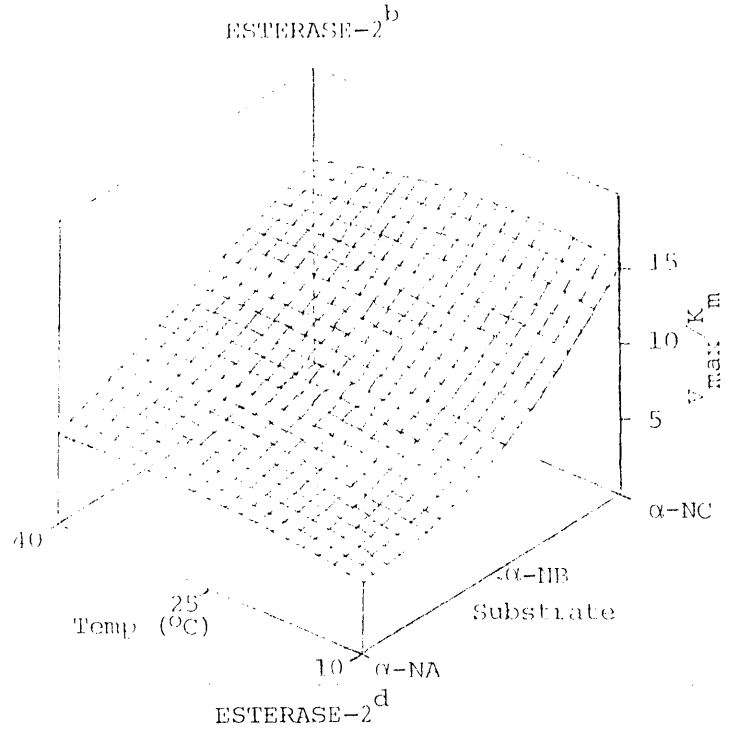
[a] Figures in parentheses are the standard errors of the estimates.

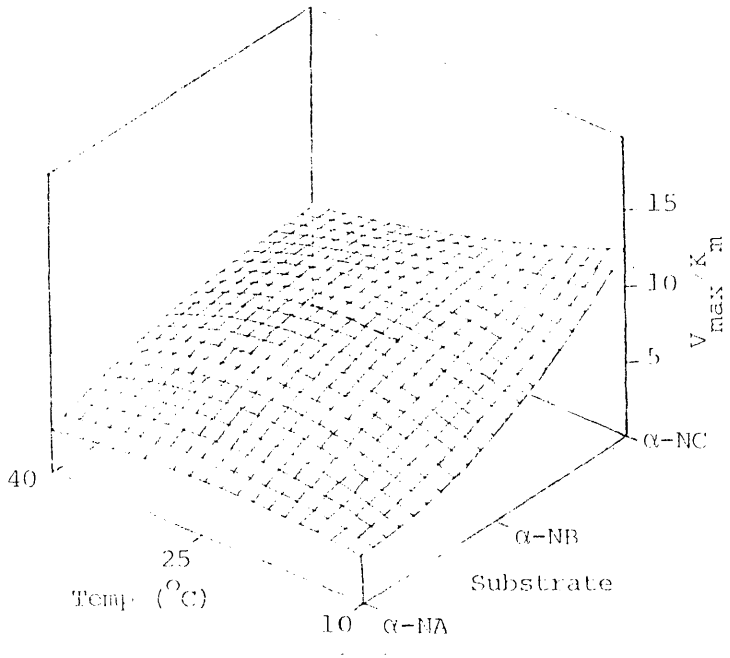
Table 5.11 Analysis of variance of effect of variable temperature and substrate on V_{max} for EST-2 allozymes. The data of Table 5.10 were log. transformed prior to analysis

Source	d.f.	Mean square	F
Allozyme (A)	2	1.454	219.72***
Substrate (S)	2	5.631	851.15***
A x S	4	0.101	15.32***
Temperature (T)	2	4.249	642.29***
A x T	4	0.032	4.85*
S x T	4	0.006	0.89
Error	8	0.007	

* $P < 0.05$

*** $P < 0.001$





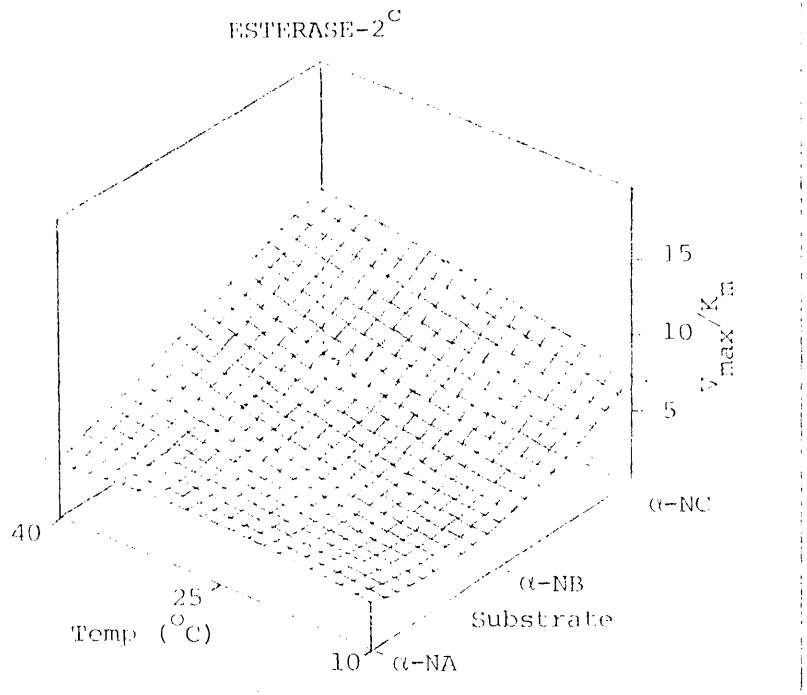


Figure 5.3 Three dimensional surfaces showing the pseudo-first order rate constant (V_{max}/K_m) as a function of substrate structure and temperature for three EST-2 allozymes.

> caproate > acetate. The temperature effect was to be expected of course, with V_{max} increasing with temperature. The effects of temperature are analysed in greater detail below (Section 5.4.2.4).

In terms of meaningful differences between allozymes, there was a highly significant allozyme by substrate interaction, and also a significant allozyme by temperature interaction. The significant allozyme by substrate interaction is probably explained as before (Section 4.3.3.1.2) by the relatively much higher activity of EST-2^C on the caproic acid ester. The allozyme by temperature interaction does not appear to be a large effect and probably results from the relative performance of EST-2^b and EST-2^d.

5.4.2.3 Effects on V_{max}/K_m

As before, I have chosen to represent the data for the pseudo-first-order rate constant as three-dimensional response surfaces, with dimensions of temperature, and acyl carbon chain length in the horizontal plane, and V_{max}/K_m in the vertical dimension (Fig. 5.3). This is a somewhat contrived presentation since acyl carbon chain length cannot strictly be treated as a continuously distributed variable. Nonetheless it seems worthwhile, since the graphical representation greatly facilitates a visual interpretation of the effects of these environmental factors on catalytic efficiency.

The pseudo-first-order rate constant V_{max}/K_m is very sensitive to changes of substrate structure and temperature (Table 5.12). Temperature appears to have the largest effect, probably reflecting the very large changes in V_{max} associated with increased temperature. However, analysis of variance on these data (Table 5.13) reveals many other less obvious trends. As before, the highly significant main effect of allozyme reflects the generally lower values of the EST-2^C allozyme. The main effect of substrate type was highly significant, and apparently results from the generally lower values for the acetate ester as compared to the other two. The two first-order interactions involving allozymes are both significant and serve

Table 5.12 Effects of variable temperature and substate on the pseudo-first order rate constant V_{max}/K_m for EST-2 allozymes. Parameter estimates were provided by the HYPERL programme of Cleland (1979)

Enzyme	Temp. (°C)	Substrate		
		α-NA	α-NB	α-NC
Est-2[b]	10	5.55 (0.45)[a]	8.30 (0.23)	8.20 (0.48)
	25	12.49 (1.22)	15.75 (0.68)	17.45 (0.72)
	40	31.59 (2.35)	32.33 (1.53)	27.10 (0.88)
Est-2[c]	10	1.39 (0.06)	3.96 (0.16)	3.85 (0.35)
	25	2.39 (0.26)	6.18 (0.22)	13.39 (0.95)
	40	13.22 (0.93)	18.32 (0.68)	22.69 (1.11)
Est-2[d]	10	2.78 (0.15)	4.68 (0.35)	3.36 (0.24)
	25	9.97 (0.79)	16.02 (0.35)	11.16 (0.53)
	40	26.34 (1.22)	19.32 (0.72)	14.83 (0.74)

[a] Figures in parentheses are the standard errors of the estimates.

Table 5.13 Analysis of variance of effect of temperature and substrate on the pseudo-first order rate constant V_{max}/K_m. The data of Table 5.12 were log. transformed prior to analysis.

Source	d.f.	Mean square	F
Allozyme (A)	2	1.508	64.37***
Substrate (S)	2	0.487	20.76***
A x S	4	0.293	12.52**
Temperature (T)	2	6.268	267.53***
A x T	4	0.115	4.90*
S x T	4	0.156	6.67*
Error	8	0.023	

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

to indicate that the three allelic enzymes differ in their responses to the environmental factors being tested. The allozyme by substrate interaction appears to result from the fact that the caproic acid ester is a much better substrate for the EST-2^C allozyme than for the other two enzymes. The reason for the significant allozyme by temperature interaction is not immediately apparent, and the effect is not particularly strong. It probably is the result of different patterns of response of V_{max}/K_m to temperature changes for the three enzymes. EST-2^b shows a relatively uniform increase of V_{max}/K_m with temperature, whereas EST-2^c shows much greater effects in the range 25-40°C, and EST-2^d tends to show the greatest response in the range 10-25°C (Fig. 5.3). There is also a significant substrate by temperature interaction, which is not of great interest in the genetic context, but is worth mentioning for what it may tell us about substrate binding. The interaction possibly results from the fact that the acetate appears to be a much more effective substrate relative to the other two at 40°C than it is at 10 and 25°C.

5.4.2.4 Comparison of Thermodynamic Activation Parameters

Arrhenius plots were constructed for each enzyme and substrate based on the data in Table 5.10. In some instances there was appreciable non-linearity of the plots, and so the Arrhenius energy of activation (E_a) was calculated for the temperature interval 10 to 25°C. For each enzyme and substrate the four thermodynamic parameters, E_o, $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ and $\Delta G^{\ddagger}$, were calculated according to the equations in Section 5.3.2. Simple one-way analyses of variance on these data revealed no effect of substrate type, and values were pooled to provide a better estimate of each parameter, for each enzyme (Table 5.14). A number of pairwise t-tests were made using these data to compare the three allozymes (Table 5.15). There were significant differences between the enzymes for the energy of activation and the enthalpy and entropy of activation. However, there were no differences for the Gibbs free energy of activation. The pairwise comparisons revealed that EST-2^b

Table 5.14 Estimation of thermodynamic activation parameters (25°C)†

Enzyme	E_a	$\Delta H^{\ddagger}$	$S^{\ddagger}$	$\Delta G^{\ddagger}$
Est-2[b]	11046 (±165)	10453 (±165)	-12.21 (±0.58)	14092 (±260)
Est-2[c]	7012 (±512)	6420 (±512)	-27.46 (±1.20)	14605 (±329)
Est-2[d]	8038 (±354)	7556 (±354)	-22.71 (±1.95)	14213 (±254)

† Estimates based on pooled substrate data.

Table 5.15 Pairwise comparisons of allozymes for the thermodynamic parameters given in Table 5.14

Parameter	Est-2[b] vs Est-2[c]	Est-2[b] vs Est-2[d]	Est-2[c] vs Est-2[d]
E_a	$t_2 = 7.51^{*}$	$t_2 = 7.70^{*}$	$t_3 = -1.66^{n.s.}$
$\Delta H^{\ddagger}$	$t_2 = 7.51^{*}$	$t_2 = 7.70^{*}$	$t_3 = -1.65^{n.s.}$
$\Delta S^{\ddagger}$	$t_2 = 11.46^{**}$	$t_2 = 5.17^{*}$	$t_3 = -2.08^{n.s.}$
$\Delta G^{\ddagger}$	$t_3 = -1.23^{n.s.}$	$t_3 = -0.33^{n.s.}$	$t_3 = 0.95^{n.s.}$

n.s. not significant, $p > 0.05$

* $p < 0.05$

** $p < 0.01$

differed from EST-2^{c} and EST-2^{d} which did not differ significantly from each other. The data reveal an interesting positive covariation between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, such that an increase in enthalpy is accompanied by an increase in entropy. This covariation will be considered in greater detail in the discussion which follows.

5.5 DISCUSSION

The results of the experiments reported above confirm the conclusions of Chapter 4, *viz.* that the four common allozyme variants produced by the *Est-2* locus cannot be considered to be biochemically equivalent. Before discussing the implications of these data, however, it is necessary to address a problem which emerges from them.

One set of conditions was common to the series of experiments reported in Section 5.4.1 and 5.4.2; where the substrate was α-naphthyl acetate and pH = 7.0. Both experiments utilised the same enzyme preparation, and yet the values obtained in each case differed markedly, with the V_{max} estimates being considerably lower for the series reported in Section 5.4.1. It is not possible *a posteriori* to give a reason for these differences, but a number of plausible explanations can be offered, and some criticisms need to be made. The most obvious cause of differences is that the two series of experiments were conducted quite independently. They were separated by many months, which means that the enzymes used for the second series had been stored for much longer, and this could have had an effect on the biochemical properties of the enzyme. In addition, different batches of buffer reagents and substrate reagents were employed in the two studies, and this may well have contributed to the differences in results. However, the potentially most important difference is that two different methods of enzyme assay were used. The first series of experiments (Section 5.4.1) utilised the assay procedure of van Asperen (1962) in which the rate of reaction is estimated by the colourimetric determination of the amount of product

(α-naphthol) produced after a fixed period of incubation. The efficacy of this assay depends on the linearity of colour production with length of incubation, concentration of enzyme, and stability of the solubilised diazo-naphthol complex. Although preliminary experiments were undertaken to define assay procedures which fulfilled these pre-requisites, because of the large scale of the experiment (4 enzymes x 4 pH values x 3 temperatures x 7 substrate concentrations x 3 replicate assays = 1008 independent determinations), not every combination of enzyme, pH, temperature and substrate concentration was tested for linearity of colour production. Before the second series of experiments was undertaken a new assay procedure became available (Mastropaolo and Yourno, 1981). This technique allows the continuous monitoring of product release over a short assay period, and one consequently can be sure that the rate of product release is constant with time. For this reason I believe that the estimations for the series of experiments reported in Section 5.4.2 must be considered to be more reliable than those made in the first series (Section 5.4.1). Accordingly, a more detailed analysis was made of experiments in Section 5.4.2, and most of the discussion will be based on them.

Factorially designed kinetic studies of allelic enzyme variants of the type reported above are extremely rare in the literature. Indeed, the only detailed study of which I am aware is that of Place and Powers (1979), on a polymorphic muscle lactate dehydrogenase in the fish, *Fundulus heteroclitus*. The studies reported above were modelled on those of Place and Powers, and I believe that their work serves as a paradigm for the molecular enzymatic dissection of genotype-environment associations. To facilitate discussion of my results I shall consider the effects of pH, temperature and substrate type separately.

5.5.1 pH Effects

The effects of pH on the various enzymic parameters allow for a fairly

simple interpretation. For none of the parameters K_m, V_{max} or V_{max}/K_m was there significant interaction involving allozymes. In other words, within the limitations of this study the four common variants of the *Est-2* locus showed similar responses to pH changes. However, pH had a highly significant effect on all three enzyme descriptors; as pH is moved away from the apparent optimum of 7.0 - 7.5, K_m increases, V_{max} decreases dramatically at high pH, and V_{max}/K_m decreases at values away from the optimum. For all enzymes, catalytic activity and efficiency is optimal in the range 7.0 to 7.5.

5.5.2 Temperature Effects

The effects of temperature on the catalytic activity of EST-2 are manifold. Enzymic adaptation to temperature in ectothermic organisms has been the object of intense study, and the subject of many review papers and monographs (e.g. Hazel and Prosser, 1974; Hochachka and Somero, 1973; Somero 1978; Alexandrov, 1977). Since temperature has been implicated as a factor involved in the maintenance of polymorphism at the *Est-2* locus (Section 1.2.5), it was particularly interesting to find significant allozyme by temperature interactions with respect to K_m, V_{max} and V_{max}/K_m for the experiments reported in Section 5.4.2. The role of allozymic variation as an evolutionary solution to the problems of temperature compensation in ectotherms has not been extensively analysed. The widespread existence of allele frequency clines in natural populations of insects (Oakeshott *et al.*, 1981; Johnson, 1976) and fish (Merritt 1972, Place and Powers, 1979) suggest that temperature may be an important selective factor in the maintenance of some polymorphisms in ectotherms. Of course, the existence of a cline does not prove a selective effect of temperature *per se*, since temperature variation influences many components of the environment, and it may be these which are responsible for any selection pressures.

The effects of temperature on enzyme catalysis have been analysed at the level of both K_m and K_{cat}. Temperature adaptive differences in K_m's

of enzymes important in energy production have been observed in a number of interspecific studies. Graves and Somero (1982) analysed the muscle LDH's of four congeneric species of barracudas adapted to different thermal regimes, and found a strong conservation of apparent K_m at the physiological temperature of the species. Studies of tropical *versus* temperate species of *Drosophila* revealed adaptive differences in K_m (app.) for α-*Gpdh* (Alahiotis *et al.*, 1977), acetylcholinesterase and NADP-Idh (Alahiotis and Berger, 1978) and supernatant Mdh (Alahiotis, 1979b). At the intraspecific level, temperature adaptive differences in K_m between allozyme variants were reported for muscle LDH in the fathead minnow, *Pimephales promelas* (Merritt, 1972). In this species the frequency of one allele at the polymorphic LDH locus decreased dramatically with increasing temperature over only a 3°C change in mean temperature. The enzyme encoded by that allele showed a dramatic increase in K_m over the same temperature range *in vitro*, and Merritt concluded that substrate affinity, as measured by K_m (app.) was an adaptively significant enzymatic property for muscle LDH in that species. Miller *et al.* (1975) analysed three enzyme phenotypes of the α-*Gpdh* locus in *D. melanogaster*. They concluded that the patterns of change of K_m (app.) of the various enzymes with temperature were basically in agreement with the observed cline in allele frequencies at the α-*Gpdh* locus.

Among marine ectotherms, energetically important enzymes frequently show a pattern of increasing K_m with increasing temperature (Hazel and Prosser, 1974). Hochachka and Somero (1973) termed this phenomenon "positive thermal modulation", since it provides a built-in mechanism of immediate temperature compensation. This pattern has been seen less frequently in insects (e.g. Alahiotis, *et al.*, 1977; Alahiotis and Berger, 1978). The results for the EST-2 allozymes reported above provide no evidence of any systematic effect of temperature on K_m (Table 5.8). There was a significant allozyme by temperature interaction (Table 5.9) but no allozyme by substrate interaction with respect to K_m. Averaging over substrates, it appears that $EST\text{-}2^b$ had

a lower K_m at 10°C than the other two enzymes; there were no differences between enzymes at 25°C, and EST-2^c had a lower K_m at 40°C. Because of the complex nature of K_m, these differences do not necessarily reflect changes in enzyme-substrate affinity. Nonetheless, by definition K_m is the concentration of substrate at which the enzyme achieves one half V_{max}, and consequently EST-2^b should be saturated at lower substrate concentrations than EST-2^c and EST-2^d at 10°C, and EST-2^c at lower concentrations than EST-2^b and EST-2^d at 40°C, and this may reflect an important adaptive difference between the EST-2 allozymes.

An alternative adaptive strategy to K_m modifications involves changes in substrate turnover numbers (k_{cat}) and enzyme concentration. Alterations in k_{cat} have not been extensively studied, since they require highly purified enzyme material. In general it has been found in studies of thermal acclimation that, as temperature is decreased metabolic rate is conserved by increasing the levels of enzyme activity in the major pathways of energy metabolism (Somero, 1978). These changes are usually effected by alterations in enzyme concentration. The reverse pattern appears to obtain with detoxification and digestive enzymes, where acclimation to warm temperatures is accompanied by increases in enzyme levels (Hazel and Prosser, 1974). The alternative mechanism of compensation involves alterations in k_{cat} values, which in turn will amost certainly require an alteration in the primary structure of the protein. Comparisons of orthologous homologues of enzymes from endo- and ectotherms indicates that k_{cat} is inversely proportional to adaptation temperature. There appears to be no evidence currently available which indicates adaptive differences between allozymic variants in this regard. However, Graves and Somero (1982) found temperature compensatory adaptive differences in k_{cat} of muscle LDH's in their study of four congeneric species of barracudas.

In the present study, since only partially purified material was used it is not possible to determine whether differences in k_{cat} exist between

the allozymic variants. However, there was a significant allozyme by temperature interaction for V_{max} (Table 5.11), and this must indicate that k_{cat} responded differently to changes in temperature for the allozymes being tested, since enzyme concentration was not being altered. The nature of this interaction is unfortunately difficult to assess because there was a highly significant interaction between allozyme and substrate type also, which is confounded with temperature effects (Table 5.11). At least a partial explanation is provided by the thermodynamic activation parameters (Tables 5.14 and 5.15). The EST-2^b enzyme has a much higher enthalpy of activation at 25°C than either EST-2^c of EST-2^d, which do not differ significantly. Whether these differences have any temperature adaptive significance however is debatable, since it is the free energy of activation ($\Delta G^{\ddagger}$) which determines the rate of catalysis, and these data do not permit any discrimination between $\Delta G^{\ddagger}$ for the three allozymes. These data do, however, reveal an important phenomenon which has been described in studies of orthologous homologues of a number of enzymes, viz. a significant covariation between enthalpy and entropy of activation (Somero, 1978). In the case of EST-2 allozymes, the amino acid changes which resulted in increasing enthalpy also caused an increase in entropy of activation such that the free energy of activation remained essentially unchanged. The results reflect the very tight constraints placed on permissible alterations in enzyme primary structure.

Finally, I should like to consider the effects of temperature on the pseudo-first-order rate constant V_{max}/K_m. This parameter should be the best estimator of temperature mediated effects on catalytic efficiency, since it simultaneously reflects alterations in k_{cat} and K_m. Again we find a marginally significant allozyme by temperature interaction ($P < 0.05$), but the interpretation is obscured by the presence of many other significant interaction terms. In general terms it appears that EST-2^d is relatively

inefficient at extreme temperatures by comparison with EST-2^b and EST-2^c, and EST-2^c may be relatively more efficient at 40°C than the other two enzymes.

In conclusion, these data suggest that the EST-2 allozymes are probably not functionally equivalent over a range of temperatures. However, the lack of any significant allozyme by temperature interactions in the experiments of Section 5.4.1, coupled with only moderate significance levels for experiments of Section 5.4.2, suggests that temperature related differences between allozymes are not great.

5.5.3 Substrate Effects

The experiments of Section 5.4.2 showed highly significant effects of substrate structure on all three of the enzyme parameters tested. There appeared to be no difference between allozymes with respect to K_m as substrate was changed. However, there were highly significant allozyme by substrate interactions for both V_{max} and V_{max}/K_m effects, and in both instances the major contributing factor appeared to be the relatively superior performance of EST-2^c on the caproic acid ester, especially at higher temperatures (Fig. 5.3). Since V_{max}/K_m reflects the effectiveness of a given substrate for an enzyme (Brot and Bender, 1969) the significant allozyme by substrate interaction for this parameter again suggests that the EST-2 allozymes are not functionally equivalent if substrate heterogeneity is a feature of the environment.

5.5.4 Concluding Comments

The kinetic analyses reported in this chapter confirm and extend the wealth of molecular diversity between allozymic variants produced by the EST-2 locus. The results suggest that, if allozymic variation represents an adaptive solution to the problem of environmental unpredictability, then EST-2 in *D. bussardi* probably affords an excellent opportunity to analyse the molecular bases of that adaptation. However, for these kinetic data to

be of any predictive value, we need more information concerning the natural substrates and the physiological function of the EST-2 enzyme.