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# HETEROGENEITY, MOLECULAR WEIGHT INTERRELATIONSHIPS AND DEVELOPMENTAL GENETICS OF THE ESTERASE ISOENZYMES OF THE RAINBOW TROUT

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SUMMARY

1. The developmental, physiochemical, molecular weight and polymorphism properties of the 14 soluble esterases in the tissues of rainbow trout have been studied.

2. Nine arylesterases, three carboxylesterases and two cholinesterases were resolved, with three distinct groups of arylesterases being differentiated. Consequently, it would appear that at least 5 structural genes are involved in the synthesis of these esterase forms.

3. The detection of polymorphism in one of the carboxylesterases and its distribution in the fish population, lends further support to the hypothesis that salmonid fish are tetraploid.

4. Ontogenic studies indicate that there is a marked increase in esterase activity and heterogeneity, at the time of hatching.

## INTRODUCTION

Early studies on salmonid fishes[1,2] indicated that the content of DNA per cell was unusually high, and the subsequent finding that at least two genes code for the production of heart-type lactate dehydrogenase in some salmonids[3], led to the hypothesis that a tetraploidy event (total genome duplication) occurred during the evolutionary development of the salmonid fishes. To date, haemoglobin[4–5], glucose-6-phosphate dehydrogenase[7], enolase[8], aldolase[9], muscle lactate dehydrogenase[3,10–11], triose phosphate dehydrogenase[13], phosphoglucomutase[14] and malate dehydrogenase[15], have been studied and all exhibit more electrophoretic forms than the comparable proteins of higher vertebrates.

The esterases constitute a further major system of multiple enzyme forms where precise classification has been rendered difficult because of the overlapping substrate specificity of individual heteromorphs, and the extensive heterogeneity of these enzymes. Precisely because of the complex nature of the multiple forms, the esterase isoenzymes are one of the best studied markers for investigating gene activation during tissue differentiation. Owing to recent extensive studies of the multiple forms of the

esterases of the tissues of a wide range of both vertebrate[16–21] and invertebrate species (N. E. KINGSBURY AND C. J. MASTERS, unpublished results), it is now possible to distinguish groups of esterases on the basis of differential substrate and inhibitor properties, and physical characteristics, including molecular weights[22,23]. Consequently, the use of these multiple forms as markers in the investigation of genetic variation has been enhanced. Studies have shown that polymorphisms exist in the plasma esterases of both the flounder and plaice[24], and in the serum esterases of Catostomid fish populations[25]. Similar polymorphisms have been shown to exist in the tissue esterases of the teleost *Fundulus heteroclitis*[26].

In order to gain a better understanding of both the interrelationships of the esterase multiple forms, and the genes coding for the esterases of the salmonid *Salmo gairdneri*, an extensive study of the esterases of this fish has been carried out in the present investigation. The distribution of the esterase isoenzymes in the adult tissues has been established, along with a classification of the activity types present. In addition, developmental studies have been coupled with population studies in order to determine both the ontogenetic timing of gene activity, and the possible significance of genetic variation.

## MATERIAL AND METHODS

Sexually mature trout (*Salmo gairdneri*) were caught from ponds at the New England Trout Hatchery, New South Wales, Australia. Tissues required were excised from freshly slaughtered trout, and stored at −10° until required.

Eggs were obtained by gently stripping the mature female trout, and the sperm suspension, obtained by stripping the male, was placed on the eggs. Eggs were allowed to develop under hatchery conditions, and samples were taken each day from a batch of eggs obtained from a single female. Staging of the embryos was based on the system of J. H. WALES[27]. Genetic studies were carried out on similarly developed 1-month-old fingerlings from a variety of parents.

Homogenates were prepared from freshly minced tissues in cold glass-distilled water in a ground glass and teflon Potter–Elvehjem homogenizer. 10% and 50% homogenates were used routinely. These homogenates were centrifuged in an International Centrifuge (105 000 × *g*, for 35 min), the supernatant fraction separated, and used in all subsequent analyses. Tissues with low esterase activity *e.g.* fingerling muscle, were concentrated by a factor of five by dialysis against dry Sephadex.

Total esterase activity in the tissue homogenates was determined by a modification of GOMORI's[29,30] method, using fast blue RR salt, and the adsorption peak at 500 nm. These measurements were made with a Unicam SP 800 spectrophotometer at 37°. Protein concentrations in the tissue homogenates were measured from the absorbance at 280 nm and 260 nm, using the equation:

$$\text{Protein (mg/ml)} = (A_{280\text{ nm}})\ 1.55 - (A_{260\text{ nm}})\ 0.76.$$

Enzyme activity was calculated in I.U. per mg of protein.

Electrophoresis was carried out on vertical columns of polyacrylamide gel (7.5%) at pH 8.6 in 0.06 M Tris–glycine buffer over 2 h at 4°. Bromophenol blue was used as a reference dye, and separation was carried out at a constant current of 2.5 mA per gel[16–20]. Vertical starch gel electrophoresis (Buchler instruments, Fort

Lee, New Jersey) was accomplished at 4° with 12% starch gels in the pH 8.6 EDTA–borate–Tris buffer system of BOYER *et al.*[28]; aliquots of the supernatant were allowed to undergo electrophoresis at a voltage gradient of 400 V for 14–16 h. Following electrophoresis, the gels were sliced horizontally and stained for esterase activity under carefully controlled conditions[26]. The relative distribution of activity amongst the multiple enzyme forms was apportioned by scanning the gels in an integrating densitometer, as described previously[33].

Prior to staining for esterase activity, the starch slabs or the acrylamide columns were placed in 0.1 M Tris–HCl buffer (pH 7.2) for 30 min at room temperature. In inhibition studies, the inhibitor was incorporated into this buffer solution. Inhibitors used for this purpose include diisopropylphosphofluoridate $10^{-3}$ M), eserine sulphate ($10^{-5}$ M) and *p*-chloromercuribenzoate ($10^{-3}$ M). In this manner, four types of soluble esterase activity were characterized in these tissues; carboxylesterase (EC 3.1.1.1), arylesterase (EC 3.1.1.2), acetylesterase (EC 3.1.1.6) and cholinesterase (EC 3.1.1.8). The gels were subsequently stained for esterase activity by incubation at room temperature in a solution (50 mg/100 ml) of fast blue RR salt (diazotized product of 4-benzoyl-amino 2,5-dimethylanaline chloride) and α-napthyl acetate (100 mg/10 ml) as substrate.

Heat stabilities of the multiple forms of esterases were investigated by maintaining the polyacrylamide gels, following electrophoresis of the homogenates, in distilled water at 60° for varying lengths of time. Similarly, the urea lability of the trout esterases was studied by incubating the gels, following electrophoresis, in 10 M urea for varying periods of time after which the gels were equilibrated and stained as above.

The molecular weights of the multiple forms of esterases were determined by subjecting the homogenates to electrophoresis on polyacrylamide gels of three different concentrations[24]. The retardation coefficients were calculated from the ratio of the relative mobility with respect to bromophenol blue of each band on two separate gels of different concentrations. These values were then used to derive molecular weights by employing a standard curve of retardation coefficient against molecular weight for a series of standard proteins.

TABLE I

SPECIFIC ACTIVITY OF THE SOLUBLE ESTERASES OF THE RAINBOW TROUT (*Salmo gairdneri*)

Esterase activity was determined by a modification of GOMORI's method[29,30]. Protein concentrations were measured from the absorbance of 280 nm and 260 nm, using the equation:

$$\text{Protein (mg/ml)} = (A_{280\text{ nm}})\ 1.55 - (A_{260\text{ nm}})\ 0.76$$

Specific activity is expressed as I.U. per mg protein.

| *Tissues* | *Specific activity* |
|---|---|
| Brain | 0.018 |
| Gills | 0.0066 |
| Heart | 0.0075 |
| Kidney | 0.0075 |
| Intestine | 0.008 |
| Liver | 0.032 |
| Muscle | 0.0145 |
| Spleen | 0.01 |
| Testis | 0.5 |

RESULTS

The esterases from the tissues of rainbow trout have been separated by electrophoretic techniques into twelve separate forms. In Table I the specific activity of the esterases in each tissue is listed, and the separation of this total activity between the isoenzymes is represented diagrammatically in Fig. 1.

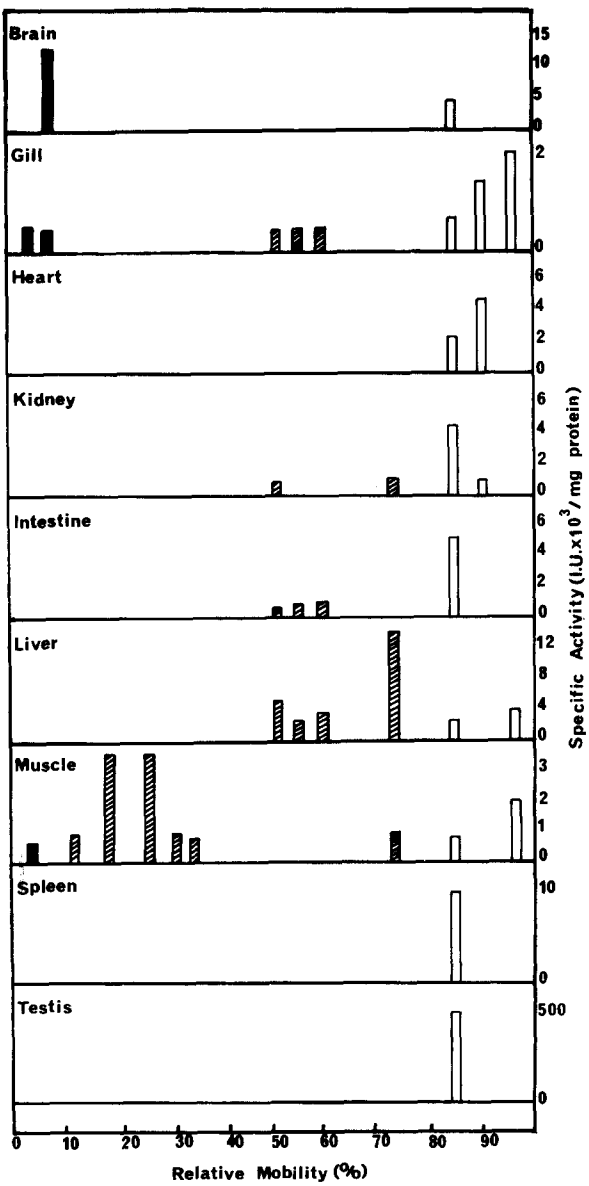


Fig. 1. The distribution of esterase multiple forms in adult trout tissues. Carboxylesterase activity is represented by open histograms, arylesterase by diagonal shading and cholinesterase by complete shading.

The twelve multiple forms of esterases found in the tissues may be classified in terms of their inhibitory properties as cholinesterases, arylesterases and carboxylesterases. In the trout brain two isoenzymes were resolved. The major band displayed the properties of a cholinesterase (relative mobility 3.5) and was accompanied by a fast moving carboxylesterase (relative mobility 85.0). In the gills, the slow moving cholinesterase is also present, but is accompanied by a second band (relative mobility 8.0) with similar characterisation. A triple banded group of arylesterases with relative mobilities 51.0, 54.0 and 60.0, were present behind the fast moving carboxylesterases (relative mobilities 85.0, 90.0 and 97.0).

The heart presented a very simple pattern, containing only two fast moving carboxylesterases (relative mobilities 85.0 and 90.0). These two carboxylesterases were also present in the kidney, but were accompanied by two slower moving arylesterases. One of these enzyme forms corresponded with that found in the gills (relative mobility 51), but the second was a separate band (relative mobility 73.0). Only four bands of esterase activity were found in the intestine. Three of these were arylesterases (relative mobilities 51.0, 54.0 and 60.0) and the fourth was a fast moving carboxylesterase (relative mobility 85.0). In the liver the main contributors to the total esterase activity were arylesterases. Those with relative mobilities 51.0, 54.0, 60.0 and 73.0 all being present. These were accompanied by two faster moving carboxylesterases (relative mobilities 85.0 and 97.0).

The muscle tissues presented a more complicated picture, and a new group of five arylesterases (relative mobilities 11.4, 18.5, 23.6, 30.0 and 32.4) were present. A small band of cholinesterase activity (relative mobility 3.0) preceded these. The fastest moving arylesterase (relative mobility 73.0) was also present, as were two bands of carboxylesterase activity (relative mobilities 85.0 and 97.0). The patterns of both spleen and testis are very simple, and in both tissues the total esterase activity was due to the carboxylesterase with relative mobility 85.0.

In Table II the physicochemical properties and the molecular weights of the esterase isoenzymes are tabulated. Both cholinesterases appeared to be large proteins

TABLE II

PROPERTIES OF THE RAINBOW TROUT ESTERASES

| *Relative mobility* | *Classification* | *Substrate specificity* | *Molecular weight* | *Half-life at 60° (min)* | *Half-life in 10 M urea (min)* |
|---|---|---|---|---|---|
| 3.5<br>8.0 | Cholinesterase | Acetate < butyrate | > 300 000 | 5 | 12 |
| 11.4<br>18.5<br>23.6<br>30.0<br>32.4 | Arylesterase | Acetate > butyrate | 65 000 | 5 | 10 |
| 51.0<br>54.0<br>60.0 | Arylesterase | Acetate > butyrate | 60 000 | < 2 | 10 |
| 73.0 | Arylesterase | Acetate > butyrate | 65 000 | 5 | 10 |
| 85.0<br>90.0<br>97.0 | Carboxylesterase | Acetate < butyrate | 75 000 | 6 | > 20 |

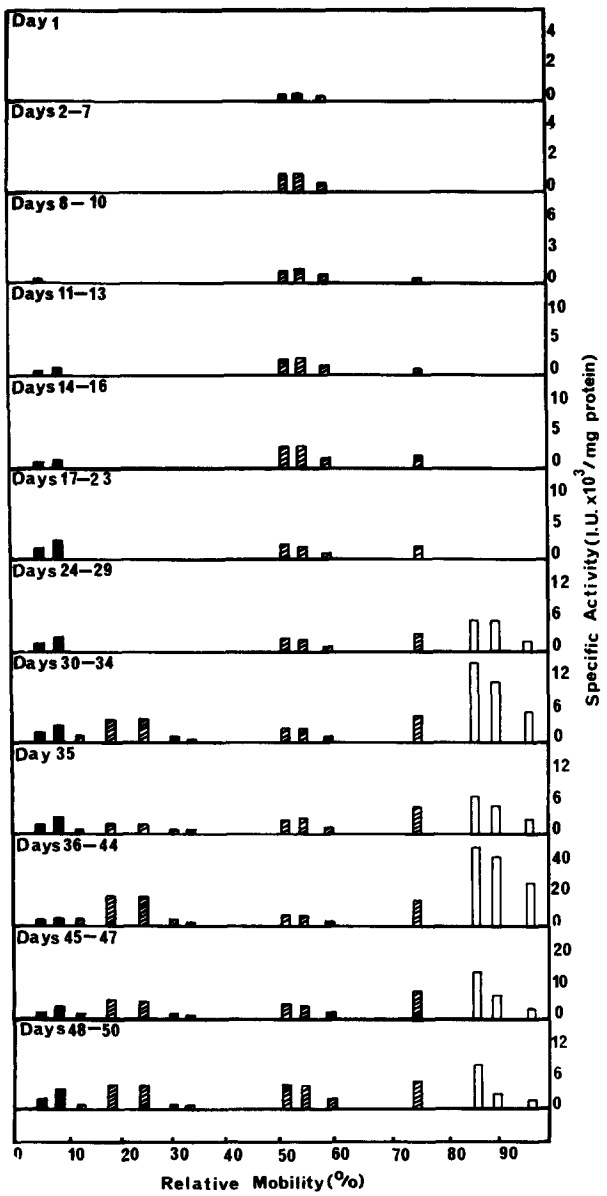


Fig. 2. The developmental progression of esterase forms in whole trout. Representations of the type of esterase activity are the same as in Fig. 1.

with molecular weights greater than 300 000; α-napthyl butyrate was a better substrate in terms of staining on polyacrylamide gels than α-napthyl acetate. All of the arylesterase species preferred the acetate esters to the butyrate esters. Also in contrast to the cholinesterases, the arylesterases appeared to be much smaller proteins, having a molecular weight in the range from 60 000–70 000. The carboxylesterases were similar to the cholinesterases in preferring butyryl esters, but were smaller proteins

with molecular weights of approximately 70 000. All of these esterases had short half-lives at 60° and were stable in 10 M urea.

Developmental studies carried out on whole specimens are represented in Fig. 2. During the first week after fertilization, only arylesterase activity is present with relative mobilities 51.0, 54.0 and 60.0. Between the 7th and the 23rd the fastest moving arylesterase (relative mobility 73.0) is expressed, as are both of the cholinesterase forms. Between the 24th and the 29th day the three fast moving carboxylesterases (relative mobilities 85.0, 90.0 and 97.0) appear, and between 30 and 34 days, just prior to the hatching, there is a peak of total esterase activity coinciding with the appearance of the five slow moving arylesterases (Fig. 3).

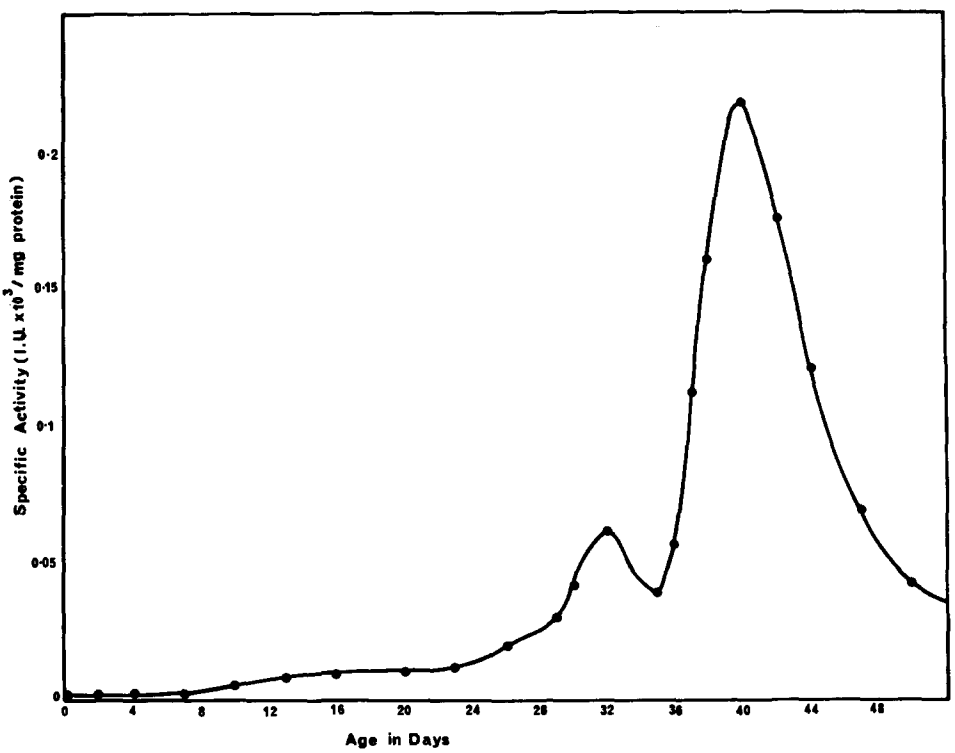


Fig. 3. The developmental changes in esterase specific activity with age in days.

On the day of hatching there is a drop in total esterase activity, and this is reflected in a net decrease in both the slow moving arylesterase, and the fast carboxylesterases. During the following post-hatch period from 36–40 days, however, there is a dramatic rise in total activity. All of the isoenzymes appear to increase in activity, but once again, it is the slow moving arylesterases and the fast moving carboxylesterases, as well as arylesterase 73.0 which alter most. By the 47th day the total activity has dropped almost to the adult level, and the pattern of the whole fish is a composite of the individual tissues of the adult fish. The overall changes in the development are tabulated in Table III, as are the morphological changes occurring in conjunction.

TABLE III
ONTOGENY OF THE ESTERASE ISOENZYMES OF THE RAINBOW TROUT (*Salmo gairdnei*)

| *Sampling day* | *Morphological change* | *Developmental progression of esterases* | | | | |
|---|---|---|---|---|---|---|
| | | *Es-1* | *Es-2* | *Er-1* | *Er-2* | *Er-3* |
| 1 | Germinal disc | − | − | − | + | − |
| 2 | Blastodisc | − | − | − | + | − |
| 3–5 | Embryo formation | − | − | − | + | − |
| 6–7 | Lip of blastopore (tender stage) | − | − | − | + | − |
| 8–10 | Notocord continuous with brain | + | − | − | + | + |
| 11–13 | Myomeres in all somites | ++ | − | − | + | + |
| 14–16 | Gills and eyes (end of tender stage) | ++ | − | − | + | + |
| 17–23 | Active organodifferentiation | +++ | − | − | + | + |
| 24–29 | Inc. brain size | +++ | + | − | + | + |
| 30–33 | Fins visible; heart differentiated | +++ | +++ | ++ | + | + |
| 34 | Prehatch | +++ | ++ | ++ | + | + |
| 35 | Posthatch (early) | +++ | + | + | + | + |
| 36–40 | Posthatch | +++ | +++ | +++ | ++ | ++ |
| 41–50 | Growing | +++ | +++ | + | + | + |

In a population of 120 fish fingerlings, three phenotypes of the carboxylesterases with relative mobility 97.0 were observed, as is represented in Fig. 4. Of these, 114 of Variant 3 were observed, two of Variant 1 and 14 with both esterase bands.

## DISCUSSION

Many workers investigating the multiplicity of enzymes of known subunit structure in the tissues of salmonids (*e.g.* lactate dehydrogenase[3,12,30,31] and malate dehydrogenase[15]) have concluded that the salmonid genome arose by tetraploidization from a primitive ancestor. OHNO *et al.*[2], investigating the DNA content and chromosome number of these fish, came to a similar conclusion.

In these studies of the multiplicity of esterases in the tissues of the rainbow trout, only one of the 14 enzyme forms (the carboxylesterase with relative mobility 97),

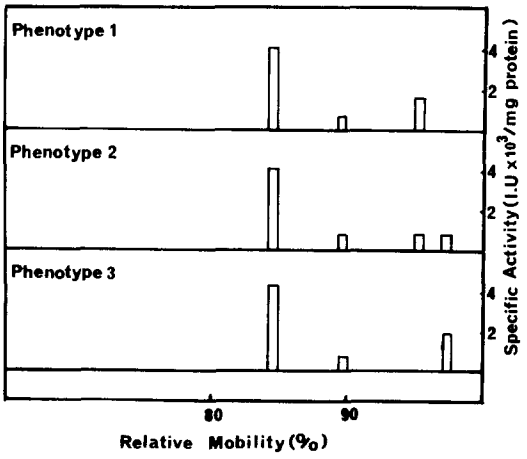


Fig. 4. Diagrammatic representation of variant esterase phenotypes of the carboxylesterases with relative mobility 97.

exhibited polymorphism; and in the 130 fingerlings investigated, 114 contained the most negatively charged species two the least negatively charged, and 14 contained both species (Fig. 4). If one interprets the two band combination as a heterozygous combination of two alleles, that are represented homozygously in the single species, the results are found to fit the Hardy–Weinberg equation, and thus the detection of these apparently duplicate genes for the expression of this esterase provides additional support for the hypothesis that salmonic fish are tetraploid.

The other 13 esterases would appear to exist in five distinguishable groups. This conclusion is reached both from a study of their properties listed in Table II, and from the results obtained during ontogenetic studies (Fig. 2). The 9 arylesterases which were characterized, for example, were divisible into three groups, largely on the basis of mobility and developmental studies. As each had a molecular weight in the range from 60 000–70 000, these enzymes would appear to be broadly similar to the corresponding arylesterases characterized in mammalian and amphibian species[21,22].

Arylesterases are the major contributors to the total esterase activity in liver and muscle (Fig. 1), although only that protein with relative mobility 73 is common to both. It is interesting to note that the properties of the arylesterases in this species allow them to be classified in a similar manner to the vertebrate esterases studies by HOLMES AND MASTERS[20]. Subgroups I and III are present in muscle and I and II in the liver. Subgroup II esterases are the first esterases to appear during development, and are the only esterolytic enzymes present in the developing fish up until the tender stage begins at about seven days. At this time both the cholinesterases and the arylesterase with relative mobilities 73 appear; but until active organo differentiation begins at about 24 days, arylesterase activity remains as the main contributor to total esterase activity. The fact that each group of arylesterases is expressed at a time of developmental change would appear to indicate that they are under individual genetic control.

At a comparable stage of maturation, all three of the apparently closely related carboxylesterases are expressed. In all properties, excepting that of genetic variation, these carboxylesterases apparently correspond to Subgroup V of the carboxylesterases as previously defined by HOLMES AND MASTERS[20]. The apparent molecular weights of all three carboxylesterases were close to the values for the Subgroup V carboxylesterase occuring in the sheep, but larger than that calculated for the type of enzyme in the possum and rat[23].

At pre-hatch and late post-hatch stages of development two peaks of total activity are observed (Fig. 3). These peaks reflect an increase in the individual activities of the carboxylesterases and of the slowest group of arylesterases which only appear at the pre-hatch stage. The pre-hatch surge follows a period of rapid organo differentiation in the trout and thus seems likely to represent a nett increase in the synthesis of esterases during this period. The increase during the post-hatch period is many times greater than the former, and may either reflect further synthesis, or the unmasking of originally inactive molecules under the stimulus of hatching and the need to become dependent on the environment[32].

Carboxylesterases were found in each of the tissues studied, but in the heart, spleen and testis this form of enzyme was the sole contributor to the total esterase activity. In the latter tissue in particular the total esterase activity was nearly 50 times that of its nearest rival, the liver. The two cholinesterases characterized in

trout tissues behaved in a similar fashion to those of high molecular weight found in the tissues of sheep and turtle. In muscle and brain only a single form of this type of activity was found, and consequently the double banded system of the gills may either represent the same protein altered in mobility by the attachment of a moiety which gives greater mobility, or a new gene product. As the two forms appear at the same stage during development they may well be products of the same gene.

In each of the tissues of the adult trout, then, a pattern of esterase isoenzymes was found which was relatively simple by comparison with higher animals[16–20]. Muscle and liver exhibited high levels of arylesterases, while in all other tissues but the brain carboxylesterases were the major form. From the combined relative mobility, molecular weight, physicochemical, polymorphism and ontogenetic studies, a number of structural genes (probably between five and seven) would appear to be responsible for the synthesis of the proteins exhibiting esterolytic activity in the rainbow trout.

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