BBA 65631

THE DEVELOPMENTAL MULTIPLICITY AND ISOENZYME STATUS OF RAT ESTERASES

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(Received March 24th, 1967)

SUMMARY

1. With the aim of clarifying the isoenzyme status of esterases, the developmental and physicochemical properties of these enzymes have been studied in an extensive range of rat tissues.

2. A total of 33 multiple forms of soluble esterase activity have been resolved, and the occurrence of these individual forms in the different tissues interrelated.

3. By means of substrate and inhibitor studies, these heteromorphs have been characterized into 4 main classes: carboxylesterases, arylesterases, acetylesterases and cholinesterases.

4. All of these classes are heterogeneous—the soluble carboxylesterases existing as 15 separate forms, arylesterases 5, cholinesterases 10, and acetylesterases 3.

5. Further differentiation of the multiple forms in these classes has been achieved on the basis of the physicochemical and developmental parameters utilized. This treatment would appear to implicate at least 13 structural genes in the biosynthesis of soluble rat esterases.

INTRODUCTION

Multiple molecular forms of an enzyme within the same species have been designated isoenzymes by the International Commission on Enzymes[1]. In recent years, considerable attention has been focussed on isoenzymes because of their broad biological significance and their usefulness as a tool for investigating fields of biological research such as protein structure, developmental biology, genetic expression and its control, and cell metabolism[2–7].

Association of multiple molecular forms of esterases with the term isoenzyme, however, requires caution because of the low order of substrate specificity of these enzymes. In the sub sub group 3.1.1. of the Enzyme Commission report[8] there are 20 carboxylic ester hydrolases listed including carboxylesterase (EC 3.1.1.1), aryles-

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terase (EC 3.1.1.2), lipase (EC 3.1.1.3), acetylesterase (EC 3.1.1.6), acetyl cholinesterase (EC 3.1.1.7) and cholinesterase (EC 3.1.1.8).

Identification of these different types of esterolytic activity has become possible on the basis of characteristic reactions with a variety of inhibitors and substrates[9–12]. By defining the type of esterase activity in this way, the extent of heterogeneity within a particular class may be examined by combining gel-electrophoresis techniques with histochemical identification[13–17].

Recently, the authors have published a paper on the developmental multiplicity and isoenzyme status of cavian esterases[16]. The present paper describes the developmental and physicochemical properties of the multiple forms of esterases from an extensive range of rat tissues, and is presented to illustrate the differences in esterase multiplicity, tissue distribution, and developmental behaviour which exist between these two rodents, and to further clarify the isoenzyme status of esterases.

METHODS

Tissue extracts

A number of rats (Wistar strain) were selected to cover a representative range of intervals during gestation, infancy and adulthood. The required tissues were excised from the freshly slaughtered animals and stored at −10° until required.

Electrophoresis

Homogenates were prepared in cold glass-distilled water and were centrifuged (100 000 × *g*, 30 min). Electrophoresis of the supernatant was carried out on vertical columns of polyacrylamide gel (7.5%; pH 8.6) which were subsequently histochemically treated (for details see our previous paper[16]).

Physicochemical properties of esterases

The urea (10 M) and heat (60°) lability of rat esterase multiple forms was investigated as previously described[16].

Esterase and protein assay

Total esterase activity was determined by a modification of GOMORI's method[18], using Fast Blue RR salt (Sigma Chemical Co.) and the absorption peak at 500 mμ. Measurements were made on a recording Unicam SP 800 spectrophotometer. Protein determination was performed by the biuret method[19] with bovine serum albumin for standards.

Enzyme activity was calculated as μmoles α-naphthol released (37°) per min per mg protein.

RESULTS

Total esterase specific activities of foetal, infant and adult rat tissues are given in Fig. 1. The highest concentrations of esterase activity in the adult occur in liver, epididymus, testis, lung and intestine, but activity is present in all tissues examined. Foetal esterase activity is low in all tissues. During the period following birth, general increases in activity are evident, but the subsequent post natal sequences show

considerable individual variation between tissues. The esterase activity of liver, lung, serum, testis and heart increases during post natal maturation, while intestine and kidney activity decrease during this period.

Zymograms of rat tissue esterases are represented diagramatically in Fig. 2. The distribution of the esterase multiple forms in adult rat tissues is tissue-specific. Fig. 3 represents a comparison between the esterase zymograms of liver, kidney, intestine and testis of the guinea pig and rat. The distribution of esterase multiple forms is species-specific also.

Fig.s 4–12 illustrate the developmental progression of multiple forms of esterases in different tissues of the rat.

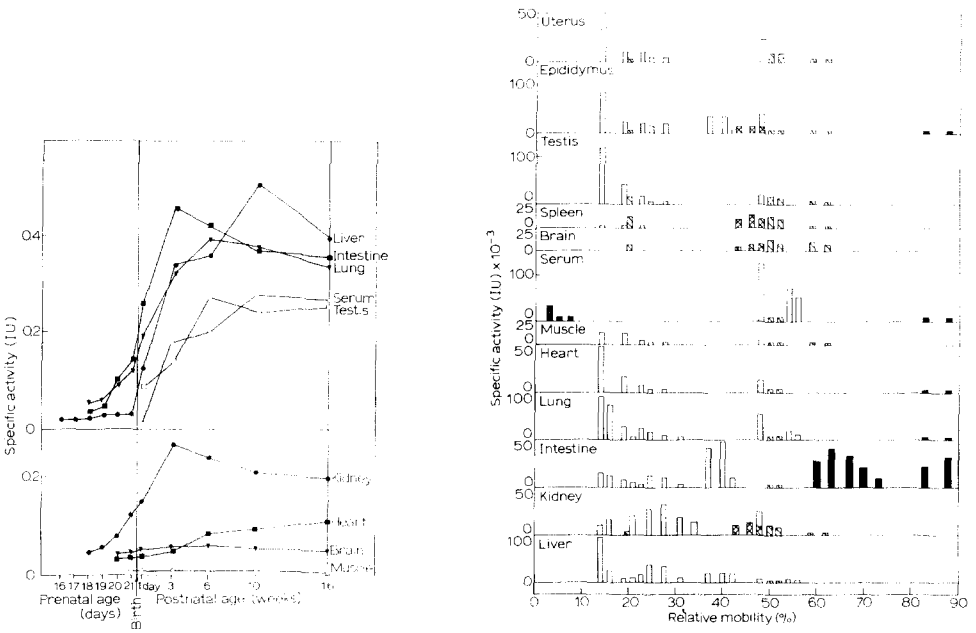


Fig. 1. Developmental alterations of total esterase activities in the different rat tissues.

Fig. 2. The distribution of esterase multiple forms in adult rat tissues. Carboxylesterase activity is represented by open histograms, arylesterase by diagonal shadings, acetylesterase by cross-hatching and cholinesterase by complete shading.

With liver (Fig. 4), the adult pattern has 14 forms of carboxylesterase and 4 forms of arylesterase. The main developmental tendencies may be summarized as post natal increase in all of the carboxylesterases except 3 forms (R_m 48, 54, 56)* and relatively little change in the arylesterases. Groups of carboxylesterases, however, may be distinguished on the basis of their differential rates of development.

In the case of kidney (Fig. 5), the adult pattern has 8 carboxylesterases, 3 acetylesterases and 5 arylesterases. Maximum activity occurs at 21 days where carboxylesterases (R_m 21, 24.5, 27.5, 31, 34) attain peak activity. During post natal maturation, carboxylesterases (R_m 14, 16, 48) and acetylesterases (R_m 43, 46, 48)

* R_m refers to relative mobility with respect to bromophenol blue at 7.5%.

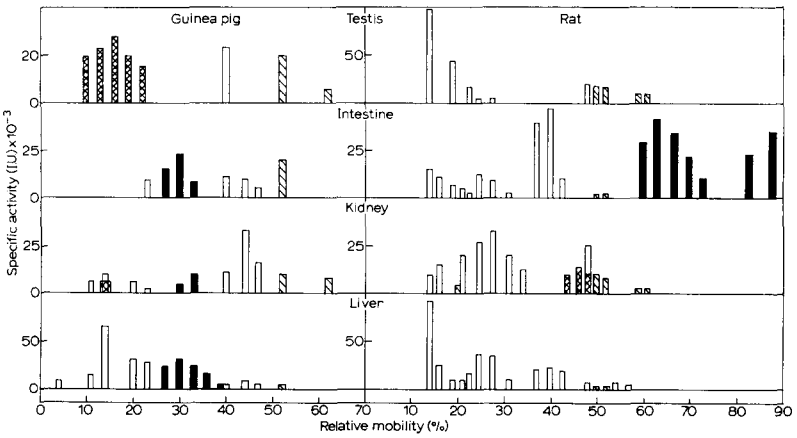
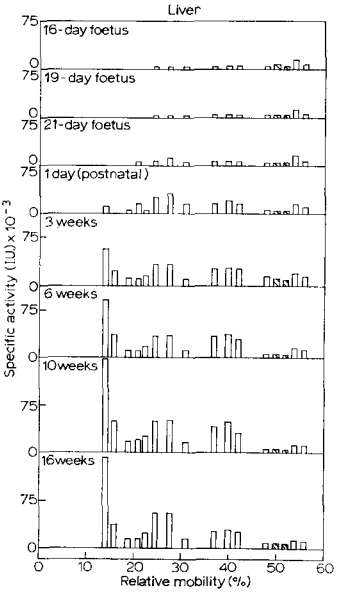


Fig. 3. A comparison of esterase multiplicity in some guinea pig and rat tissues. Representations of the type of esterase activity are the same as in Fig. 2.

increase in activity. The arylesterases show little change throughout development.

Adult intestine zymogram (Fig. 6) shows a characteristic distribution of enzyme types. Choline esterase develops to become a major component, arylesterases are low in activity, and 10 forms of carboxylesterase occur. Maximum activity occurs at



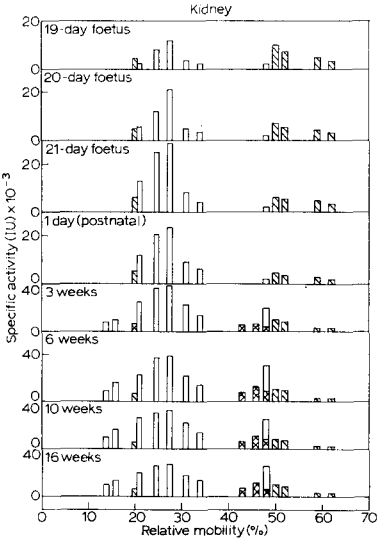
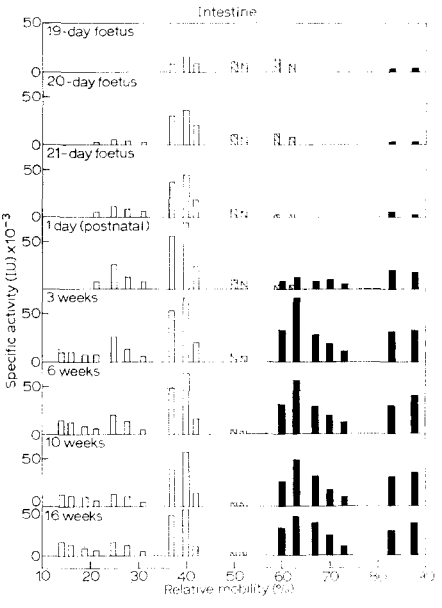


Fig. 4. The developmental progression of esterase forms in rat liver. Representations of the type of esterase activity are the same as in Fig. 2.

Fig. 5. The developmental progression of esterase forms in rat kidney. Representations of the type of esterase activity are the same as in Fig. 2.



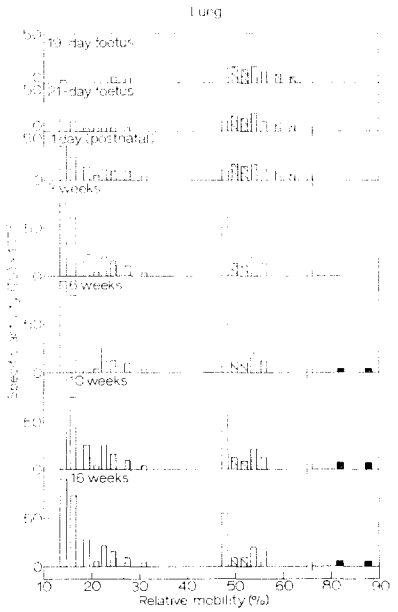
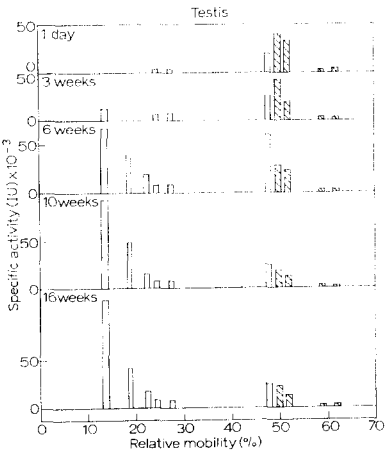


Fig. 6. The developmental progression of esterase forms in rat intestine. Representations of the type of esterase activity are the same as in Fig. 2.

Fig. 7. The developmental progression of esterase forms in rat lung. Representations of the type of esterase activity are the same as in Fig. 2.



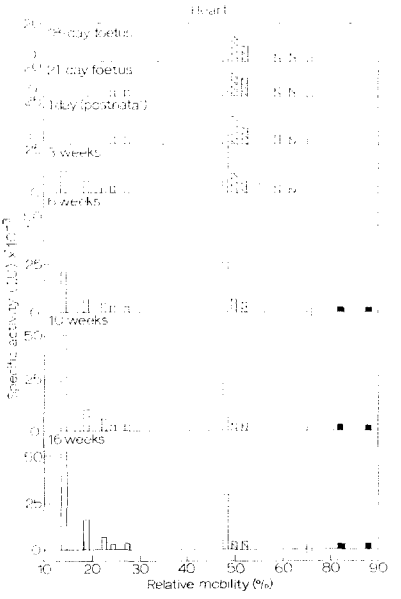


Fig. 8. The developmental progression of esterase forms in rat testis. Representations of the type of esterase activity are the same as in Fig. 2.

Fig. 9. The developmental progression of esterase forms in rat heart. Representations of the type of esterase activity are the same as in Fig. 2.

21 days where carboxylesterases (R_m 21, 24.5, 27.5, 31, 37, 49, 42) and cholinesterases (R_m 60, 63, 67, 70, 73, 83 and 88) attain maximum activity. Arylesterases seem to decrease in activity with development.

The major contribution to adult rat-lung esterase activity (Fig. 7), is carboxylesterase which exists in 11 multiple forms. Arylesterase and cholinesterase are present in small amounts. Maximal activity occurs at 21 days for carboxylesterases (R_m 21, 24.5, 27.5, 31 and 48) while with others (R_m 14, 16, 19, 22.5), it occurs with postnatal maturation.

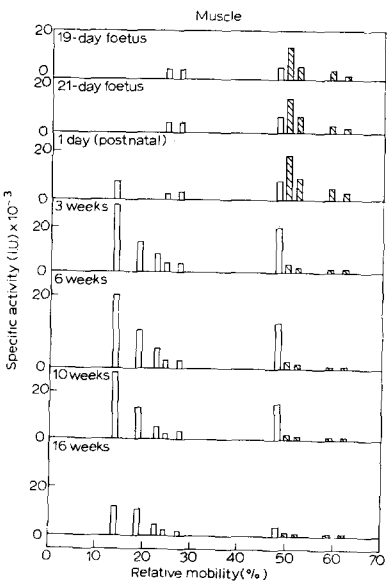
The most striking feature of the developmental changes in testis (Fig. 8) is the post-pubertal increase in carboxylesterase (R_m 14, 19, 22.5) which becomes the major component of the adult. Other carboxylesterases (R_m 24.5, 27.5) undergo very little change while arylesterase seems to decrease with the onset of maturity.

Adult rat-heart esterase pattern (Fig. 9) reveals 6 carboxylesterases, 2 arylesterases and 2 cholinesterases. On development, the carboxyl esterases (R_m 14, 19, 22.5, 48) and cholinesterases (83, 88) increase to a maximum at maturity while the arylesterases undergo a decrease in activity.

The carboxyl and arylesterases of the developing rat skeletal muscle (Fig. 10) undergo similar changes to that of heart.

Rat-brain esterase patterns (Fig. 11), which consist of 3 forms of acetylesterase and 5 forms of arylesterase, undergo no visible change in development.

Adult rat-serum esterase pattern (Fig. 12) reveals 3 carboxyl esterases, 2 arylesterases and 2 groups of cholinesterases; a slow-moving group (R_m 3, 5, 7) and a fast-moving group (R_m 82, 88). During post natal growth rat serum develops all of



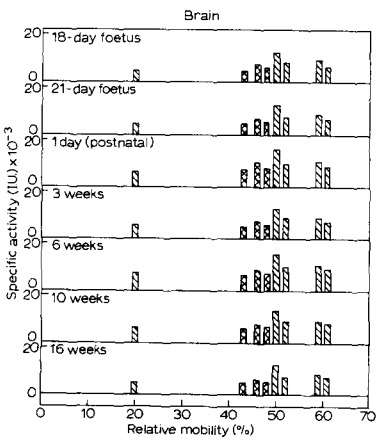


Fig. 10. The developmental progression of esterase forms in rat muscle. Representations of the type of esterase activity are the same as in Fig. 2.

Fig. 11. The developmental progression of esterase forms in rat brain. Representations of the type of esterase activity are the same as in Fig. 2.

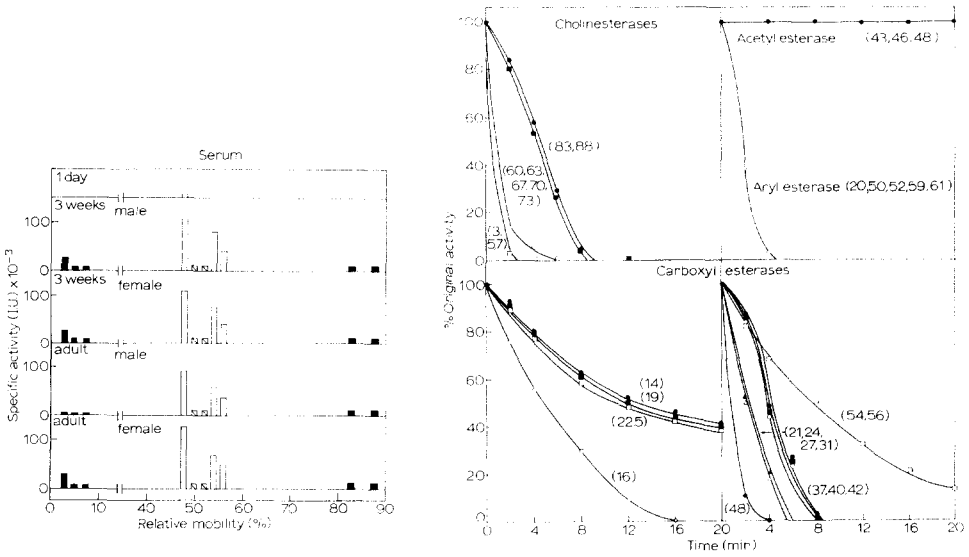


Fig. 12. The developmental progression of esterase forms in rat serum. Representations of the type of esterase activities are the same as in Fig. 2.

Fig. 13. The effect on esterase activity of incubation at 60° for varying lengths of time. Sequential activities are represented as a percentage of the original activity. (R_m values as indicated).

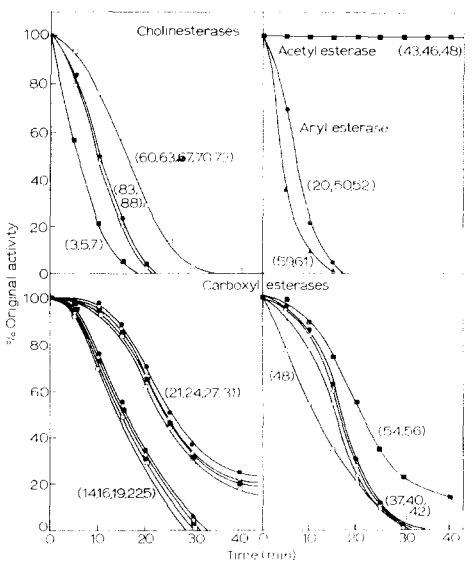


Fig. 14. The effect on esterase activity of incubation with 10 M urea for varying lengths of time. Sequential activities are represented as a percentage of the original activity. (R_m values as indicated).

these esterases, attaining maximum activity in the adult female. Adult male serum has lowered levels of slow-moving choline esterases and carboxylesterases.

The results of the physicochemical studies are given in Figs. 13 and 14.

The effects of heating at 60° (Fig. 13) may be summarized as follows: The 15 carboxylesterases are divided into groups according to their heat lability. Two groups of carboxylesterases—(R_m 14, 19, 22.5) and (R_m 54, 56)—are relatively heat stable with a half life of 20 min, while the others are less stable. Carboxylesterase (R_m 16) has a half life of 5 min, carboxylesterases (R_m 21, 24.5, 27.5, 31, 34) have a half life of 2.5 min, carboxylesterases (R_m 37, 40, 42) have a half life of 4 min, while R_m 48 is very heat labile with a half life of 1 min. The 7 multiple forms of cholinesterase are divided into 2 groups on the basis of their heat lability. Five cholinesterases (R_m 60, 63, 67, 70, 73) are very heat labile (half life, 1 min), while the other 2 (R_m 83, 88) are more stable (half life, 4 min). The 5 multiple forms of arylesterase are heat labile (half life, 2 min) while the 3 multiple forms of acetylesterase are very heat stable with no visible loss in activity during the 20-min period of heating.

The effects of 10 M urea (Fig. 14) may be summarized as follows: Carboxylesterases (R_m 14, 16, 19, 22.5, 37, 40, 42) are urea stable with a half life of 15 min, while another group (R_m 21, 24.5, 27.5, 31, 33) are more urea stable with a half life of 25 min. Carboxylesterase (R_m 48) is relatively labile (half life, 10 min) while carboxylesterases (R_m 52, 56) are more stable (half life, 20 min). Five cholinesterases (R_m 60, 63, 67, 70, 73) have a half life of 17 min, while the other group (R_m 83, 88) are less stable (half life, 9 min). The arylesterases are urea labile, while the acetylesterases are very stable to this treatment.

DISCUSSION

The distribution of total esterase activity in rat tissues has been investigated by NACHLAS AND SELIGMAN[20] using histochemical techniques. Tissues of high esterase activity include the hepatic parenchymal cells of liver, the seminiferous tubules and the interstitial cells of testis, the epithelium of the epididymus, the bronchial epithelial cells of lung, the surface epithelium of the small intestine and the cortex of kidney. Activity data presented in this paper (Fig. 1) records high activity for each of the tissues mentioned.

Further histochemical investigations have been carried out on rat-tissue esterases by other workers using differential inhibition and a variety of substrates as a means of classifying esterases. By this method, ALDRIDGE[9,10] investigated intestinal and pancreatic esterases, NIEMI[21,22] discovered carboxyl and acetylesterases in rat testis, and BERNSOHN[23,24] classified rat-brain esterases.

This kind of histochemical analysis achieved the precise cellular or subcellular localization of esterases but failed to identify the individual molecular types in a family of closely related enzymes. This was achieved by HUNTER AND MARKERT[13,14] who coupled starch-gel electrophoresis with histochemical techniques and investigated the esterase composition of mouse liver.

A number of workers have since investigated the multiplicity of rat esterases using this method. These workers include AUGUSTINSSON[17,25] (serum esterases), BERNSOHN[23,24] (brain esterases), FRIEDMANN, STRACHAN AND DEWEY[26], LEWIS AND HUNTER[27] (intestine esterases), and READ, MIDDLETON AND MCKINELY[28] (liver and kidney esterases).

These papers illustrate the high degree of multiplicity of rat esterase activity in some tissues but lack in specific activity data and in some cases no attempt is made to classify the multiple esterase forms. This paper presents an intensive investigation into the multiplicity of rat esterases with the aim of clarifying their isoenzyme status.

In regard to the multiplicity of rat esterases, this study has revealed the existence of a more extensive heterogeneity than that previously published. A total of 33 different forms of the soluble esterases have been identified, and this high degree of resolution is considered to provide an important advantage towards defining the inter-relationships of the different esterases.

The distribution of these multiple forms in the tissues of the adult is clearly tissue-specific (Fig. 2) but underlying species characteristics can be recognized. Adult liver presents a complex pattern of carboxylesterases while adult intestine reveals an equally complex yet characteristically different distribution pattern. Intestine is distinguished, also, by the major contribution of cholinesterase activity. Adult kidney presents another complex pattern of carboxylesterases with a different distribution to that of liver and intestine. Contributing also to adult kidney esterase activity are significant amounts of aryl and acetyl esterases. Brain and spleen patterns are characterized by high percentages of aryl and acetyl esterase activity. Tissue specific patterns of esterase activity have also been found in mouse[14], human[15] and guinea pig[16].

These examples in the rat, illustrate a difference between the tissue specificity of esterolytic enzymes, and the more familiar but less complex patterns of the dehydrogenase isoenzymes[5]. There is a far greater depth of variation possible in the distributions of esterase forms, and this renders them considerably more effective in the characterization of tissue individuality; not only within a single species but also between species, in cell culture, and ontogenetically[17,29,*].

Guinea pig[16] and rat-tissue esterase zymograms can be compared to illustrate this tissue and species specificity (Fig. 3). Carboxylesterases show a higher degree of multiplicity in the rat than the guinea pig in all tissues. Some similarities exist, however, since the major liver carboxylesterase and the major serum carboxylesterase in each species have similar relative mobilities, and physicochemical and developmental properties. Cholinesterases contribute a major portion of rat and guinea pig intestine activity yet the degree of multiplicity and the relative mobilities differ. Five multiple forms of cholinesterase exist in guinea pig liver yet none was detected in rat liver. The distribution, relative mobility, physicochemical and developmental properties of guinea pig and rat arylesterases appear to be very similar. One very distinct difference between the esterase compositions of these animals is the acetylesterase levels in testis. In guinea pig, 5 multiple forms of acetylesterase account for over 90% of esterase activity in the mature testis while in rat, carboxyl esterase is the major component.

It has been reported that no differences occur in the electrophoretic patterns between pre natal and adult tissues[17,30]. Recently some workers have found developmental changes in multiple esterase forms. MARKERT AND HUNTER[13,31] have noted a small increase of complexity in the esterase patterns of mice with increasing maturity, BLANCO AND ZINKHAM[29] have reported similar conclusions in regard to human esterases, and BERNSOHN *et al.*[23] have observed small changes in the development

* R. S. HOLMES, unpublished results.

of rat-brain esterases. More recently, we have investigated the developmental multiplicity of guinea pig esterase[16] and shown a distinct ontogenetic variation with clear indications of epigenetic control. This paper reports a similar ontogenetic variation of esterases in developing rat tissues.

The patterns, in this study, tend to diverge during differentiation, and display a more extensive heterogeneity in the later stages of maturation; on occasion, however, some types of activity tend to diminish or disappear with ageing. In most tissues, birth is accompanied by a sharp increase in total esterase activity.

The cause of these developmental changes is probably complex and may include such factors as induction or stabilization by substrate, developmental changes in tissue function and cell type, altered sources of nutrients, the influence of hormones, and other environmental factors[27,32,33]. Some of these factors are being investigated at the present time.

The developmental progression of rat and guinea-pig esterases[16] provide interesting comparisons. The major liver carboxylesterase (R_m 14) in both species has low activity in the foetal animal and develops maximum activity with maturity, however, the guinea pig exhibits a more mature pattern at birth than does the rat. In kidney, there is a considerable pre natal increase in esterase activity in the guinea pig, while the rat does not obtain maximum activity until 21 days after birth. An explanation may lie in the comparative anatomy of guinea pig and rat tissues[34]. The former has a much longer gestation period and its tissues would be expected to be more mature at birth.

The arylesterase activity of rat tissues has been resolved into 5 separate bands. A similar degree of multiplicity has been recently reported in rat brain[23]. The develop mental behaviour of these arylesterase forms, their physicochemical properties, their individual occurrence and variation of activity in different tissues, appears to implicate separate genetic control for each doublet and shows very similar properties to that reported for guinea pig arylesterases[16].

The genetic control[25,35] and the endocrinological influence[32,35] on the synthesis of arylesterases has been recently investigated by AUGUSTINSSON and co-workers. They concluded that the post natal biosynthesis of pig-plasma arylesterase is genetically controlled by a set of multiple alleles and may be influenced by sex hormones. Also they have investigated the genetic control of a 'pre-albumin arylesterase' in rat plasma[25] but have not given evidence for its identity as an arylesterase. The present paper gives evidence for a pre-albumin esterase which is inhibited by organophosphates and eserine and probably would be a low molecular weight cholinesterase. Rat-plasma arylesterase activity is low and does not vary significantly between the adult male and female.

Arylesterase activity occurs in all rat tissues investigated and throughout development does not undergo large changes, although decreases can be seen with the onset of maturity in some tissues.

The acetylesterase activity of rat tissues has been resolved into 3 components. Previous evidence for acetylesterase multiplicity has been given by the authors who demonstrated 5 multiple forms in guinea pig testis[16].

All forms of the enzyme in guinea pig and rat are noticeable for their considerable stability to heat and urea treatments.

The developmental behaviour of acetylesterases (R_m 43, 46, 48) differs between

tissues. In brain these forms are stable throughout development while in kidney they are induced with the onset of maturity.

As many as ten bands of cholinesterase activity are observable in these studies. They can be divided into 3 groups on the basis of tissue distribution, physicochemical properties, developmental behaviour, and electrophoretic mobility. Most previous studies of mammalian cholinesterase activity have been concerned with the activity of sera, and SVENSMARK[36] in a recent review, has considered the possibility of four allelic genes controlling this enzyme formation. Serum cholinesterases have been reported to have a molecular weight of over 300 000 (ref. 36) and such a molecule would have a very low mobility in gel concentrations used in this investigation. Three such multiple forms have been found in rat serum but have not been detected in other rat tissues. These cholinesterases have distinctive physicochemical properties in being very labile to urea and heat treatment. The level of activity of these enzymes varies with age (no activity at birth increasing to a maximum in the adult female animal) and sex (decreased levels in the adult male). Similar results have recently been reported by LEEUWIN[37] in rat serum and liver.

The other 2 groups of cholinesterase activity have a high mobility even at high concentrations of polyacrylamide gel* which indicates a low molecular weight. Tissue cholinesterases of this type have been reported by SVENSMARK[36] and by ourselves[16], and has been considered by the former to bear a precursor relationship to the serum activity.

The developmental expression of the multiple forms of tissue cholinesterase displays variation between the 2 groups of activities. The slower group (R_m 60, 63, 67, 70, 73) appears in the post natal period and reaches a maximum at 21 days while the faster group (R_m 83, 88) is present in the prenatal period but reaches a maximum with maturity. The slower group has most of its activity occurring in the intermediate bands with no evidence of terminal dominance, and throughout development it displays little variation in pattern. These observations would appear to rule against the possibility of either independent genetic control of these 5 forms, or of two independent genes governing the synthesis of these forms through polypeptide hybridization. An explanation which is consistent with these facts is that a single gene governs the synthesis of these tissue cholinesterases, and the multiple forms differ by the attachment of small molecules.

The faster group of cholinesterases has only 2 bands and the relative contribution of each does not vary significantly throughout development. This group is under an independent genetic control different to that of the slower tissue cholinesterase group because of different tissue distribution and developmental behaviour.

The carboxylesterases present the most complicated heterogeneity of all of the esterase classes examined in the rat and probably represent the greatest number of closely related enzymes ever examined in one animal. The 15 bands of carboxylesterase activity may be divided into various groups on the basis of their electrophoretic mobility, physicochemical properties, tissue distribution and developmental behaviour.

Carboxylesterases (R_m 14, 19, 22.5) occur in liver, intestine, heart, muscle, testis, epididymus and uterus. They have similar physicochemical properties and

* R. S. HOLMES, unpublished results.

undergo similar developmental changes. This seems to indicate that this group of closely related enzymes is probably under the same genetic control.

Carboxylesterase (R_m 16) has its own distinctive properties and appears to be under independent genetic control.

A group of 5 carboxylesterases (R_m 21, 24.5, 27.5, 31, 34) have similar properties. Because there is evidence available in recent literature to support the concept of a tetramer structure for this type of enzyme in other species[38], the possibility of interacting subunits under the control of separate alleles must be considered for this rat sub group. This does not seem to be the case, however, since most of the activity occurs in the intermediate bands and there is no evidence of terminal dominance in any tissue during development.

Another group of carboxylesterases (R_m 37, 40, 43) has similar properties. They occur in liver, intestine and epididymus and probably represent another isoenzymic group of esterases.

There are 3 faster-moving carboxylesterases which can be divided into 2 groups. They represent the major portion of activity in rat serum and are distinguished from the slow carboxylesterases by their lack of change of activity throughout tissue development. This would indicate that it is not only an increased synthesis of tissue carboxylesterase that produces an increased serum carboxylesterase level after birth but also an increased release of enzyme from the tissues.

Overall then, the 33 forms of soluble esterase activity which have been resolved in rat tissues, may be divided into 4 main groups on the basis of substrate and inhibitor studies: arylesterases, acetylesterases, cholinesterases and carboxylesterases. Each of these groups may be considered as an isoenzyme system in terms of the definition recommended by the Standing Committee on Enzymes[1]. The developmental, tissue distribution and physicochemical studies have confirmed these broad groupings of esterase types, but also allow further differentiation within each of these groups.

Considering these differential properties of esterase activity, it would appear that the evidence provided us in this study may be considered to provide additional support to the concept of a complex genetic control for these enzymes in mammalian tissues. We have suggested previously that at least twelve structural genes are involved in the synthesis of cavian esterases[16], and the data for the rat appear to implicate a control which is even more diverse.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Prof. E. C. WEBB for his advice and encouragement. These investigations were supported in part by grants from the National Health and Medical Research Council of Australia, the Queensland Cancer Fund, and the National Heart Foundation of Australia.

REFERENCES

1 E. C. WEBB, *Experentia*, 20 (1964) 592.
2 C. L. MARKERT AND F. MØLLER, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 1210.

3 R. CAHN, N. O. KAPLAN, L. LEVINE AND E. ZWILLING, *Science*, 136 (1962) 962.
4 I. H. FINE, N. O. KAPLAN AND D. KUFTINEC, *Biochemistry*, 2 (1963) 116.
5 C. L. MARKERT, in M. LOCKE, *Cytodifferentiation and Macromolecular Synthesis*, Academic Press, New York, 1963, p. 65.
6 M. HINKS AND C. J. MASTERS, *Biochemistry*, 3 (1964) 1789.
7 M. HINKS AND C. J. MASTERS, *Biochim. Biophys. Acta*, 113 (1966) 611.
8 *Enzyme Nomenclature*, Elsevier, Amsterdam, 1965.
9 W. N. ALDRIDGE, *Biochem. J.*, 53 (1953) 110.
10 W. N. ALDRIDGE, *Biochem. J.*, 53 (1953) 117.
11 K. B. AUGUSTINSSON, *Ann. N.Y. Acad. Sci.*, 94 (1961) 844.
12 F. BERGMANN, R. SEGAL AND S. RIMON, *Biochem. J.*, 67 (1957) 481.
13 R. L. HUNTER AND C. L. MARKERT, *Science*, 125 (1957) 1294.
14 C. L. MARKERT AND R. L. HUNTER, *J. Histochem. Cytochem.*, 7 (1959) 42.
15 D. J. ECOBICHON AND W. KALOW, *Can. J. Biochem. Physiol.*, 43 (1965) 73.
16 R. S. HOLMES AND C. J. MASTERS, *Biochim. Biophys. Acta*, 132 (1967) 379.
17 J. PAUL AND P. FOTTRELL, *Biochem. J.*, 78 (1961) 418.
18 G. GOMORI, *J. Lab. Clin. Med.*, 42 (1953) 445.
19 A. G. GORNALL, C. S. BARDWELL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
20 M. M. NACHLAS AND A. M. SELIGMAN, *Anat. Record*, 105 (1949) 677.
21 M. NIEMI AND M. KORMANO, *J. Reprod. Fertility*, 10 (1965) 49.
22 M. NIEMI, M. HÄRKÖNEN AND M. IKONEN, *Endocrinology*, 79 (1966) 294.
23 J. BERNSOHN, K. D. BARRON, A. R. HESS AND M. T. HEDRICK, *J. Neurochem.*, 10 (1963) 783.
24 J. BERNSOHN, K. D. BARRON, P. F. DOOLIN, A. R. HESS AND M. T. HEDRICK, *J. Histochem. Cytochem.*, 14 (1966) 455.
25 K. B. AUGUSTINSSON AND B. HENRICSON, *Biochim. Biophys. Acta*, 124 (1966) 323.
26 B. FRIEDMAN, D. S. STRACHAN AND M. M. DEWEY, *J. Histochem. Cytochem.*, 14 (1966) 560.
27 A. LEWIS AND R. L. HUNTER, *J. Histochem. Cytochem.*, 14 (1966) 33.
28 S. J. READ, F. S. MIDDLETON AND W. P. MCKINELY, *Can. J. Biochem.*, 44 (1966) 809.
29 A. BLANCO AND W. H. ZINKHAM, *Bull. Johns Hopkins Hosp.*, 118 (1966) 27.
30 Y. CROISSILLE, *Compt. Rend.*, 254 (1962) 2103.
31 R. L. HUNTER, J. T. ROCHA, A. R. PFRENDER AND D. C. DEJONG, *Ann. N.Y. Acad. Sci.*, 121 (1964) 532.
32 K. B. AUGUSTINSSON AND B. HENRICSON, *Acta Physiol. Scand.*, 64 (1965) 418.
33 M. J. R. DAWKINS, *Brit. Med. Bull.*, 22 (1966) 31.
34 M. WACHSTEIN AND M. BRADSHAW, *J. Histochem. Cytochem.*, 13 (1965) 44.
35 K. B. AUGUSTINSSON AND B. OLSSON, *Hereditas*, 47 (1961) 1.
36 O. SVENSMARK, *Acta Physiol. Scand.*, 59 (1963) 378.
37 R. S. LEEUWIN, *Acta Endocrinol.*, 50 (1965) 391.
38 W. BOGUTH, K. KRISCH AND H. NIEMANN, *Biochem. Z.*, 341 (1965) 149.