BBA 66220

ON THE ONTOGENY, HETEROGENEITY, AND MOLECULAR WEIGHT INTERRELATIONSHIPS OF THE ESTERASE AND LACTATE DEHYDROGENASE ISOENZYMES IN THE GREEN TURTLE

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(Received July 28th, 1970)

SUMMARY

1. The developmental, physicochemical and molecular weight properties of 14 soluble esterases of green turtle tissue extracts have been studied.

2. Eight arylesterases and five carboxylesterases were resolved but cholinesterase existed as a single species. At least 5 structural genes would appear to be implicated in the synthesis of these esterase forms, as three distinct groups of arylesterases were differentiated on the basis of the physicochemical and developmental parameters utilized.

3. During early development, the A and B genes responsible for lactate dehydrogenase synthesis were both active. Subsequent developmental studies showed two periods of peak enzyme activity, preceding regular change to adult distribution.

4. The distribution and developmental aspects of multiplicity were not as complex as those of animals higher on the evolutionary scale, and apparently reflect a simpler form of genetic control of this stage of phylogeny.

INTRODUCTION

The developmental progressions of multiple enzyme forms have proved of considerable value in the elucidation of the isoenzyme status of enzyme heteromorphs, and as informative parameters contributing towards an increased understanding of tissue differentiation and the control of enzyme synthesis. In these regards, studies with lactate dehydrogenase[1–4] and the esterolytic enzymes have been especially useful.

Lactate dehydrogenase (EC 1.1.1.27) exists in most vertebrate tissues as five molecular forms. Each of these isoenzymes is a tetramer, and the multiplicity of the enzyme may be accounted for by the presence of two types of subunit (A and B) which are under the control of separate genetic loci. Hybridization between the different polypeptides allows for a sequence of heteromorphs whose subunit composition may be represented as A_4, A_3B, A_2B_2, AB_3, B_4.

The esterase activity of vertebrate tissues, by contrast, has been shown to con-

Abbreviation: PCMB, *p*-chloromercuribenzoate.

sist of complex groupings of multiple forms, whose interrelationships are clouded by the extensive heterogeneity and overlapping substrate specificity which are displayed by these enzymes. Furthermore, marked differences exist in regard to these characteristics between species which are closely related phylogenetically; a fact which emphasizes the need for additional comparative information on the esterases[5–9].

As an extension of previous studies[10–15,18] into the ontogeny of these enzymes, the developmental properties of the lactate dehydrogenase and esterase isoenzyme systems in the green turtle have been studied. This species was chosen because of its unique phylogenetic relationships, and unusual ability to shift from aerobic to anaerobic metabolism in response to lowered oxygen availability[19,20]. In addition to the developmental studies, molecular weights of the individual esterase forms in this species have been determined, as a further parameter directed towards clarification of the basic causation of vertebrate esterase heterogeneity.

METHODS

Tissues from green turtles (*Chelonia mydas*) were collected over a representative developmental range from 17 to 54 days after laying. For this purpose, eggs were gathered from a single female during oviposition on Heron Island, the Great Barrier Reef, Australia, and incubated at 84°F in moist sand. Adult tissues were obtained from a 3-year old turtle, reared in captivity from a hatchling. The required tissues were excised from the freshly slaughtered animals and stored at −10° until required.

Homogenates were prepared from finely minced tissues in cold, glass distilled water in a ground glass Potter–Elvehjem homogenizer. 10% and 50% homogenates were used routinely. These homogenates were centrifuged in an International Centrifuge (105000 × *g*, 35 min) and the supernatant fraction separated, and used for the analyses.

Electrophoresis was carried out on vertical columns of polyacrylamide gel (7.5%)[21,22] at pH 8.6 in 0.06 M Tris–glycine buffer for 2 h at +4°. Bromophenol blue was used as a reference dye, and separation was carried out with a constant current of 2.5 mA per gel. Vertical starch gel electrophoresis (Buchler Instruments, Fort Lee, N. J.) was accomplished at +4° with 12% starch gels in the EDTA–borate–Tris buffer (pH 8.6) system of BOYER *et al.*[23]; aliquots of the supernatants 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 and lactate dehydrogenase activity.

Prior to staining for esterase activity, the starch gel slabs and 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 included diisopropylphosphorofluoridate (DFP, $1 \cdot 10^{-4}$ M), eserine sulphate ($1 \cdot 10^{-5}$ M) and *p*-chloromercuribenzoate (PCMB, $1 \cdot 10^{-3}$ M). In this manner, four types of soluble esterase activity were characterized; 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-benzoylamino-2, 5-dimethoxyanaline chloride) and α-naphthyl acetate (100 mg/10 ml) as substrate.

The heat stabilities of the multiple forms of turtle esterases were investigated by incubating the polyacrylamide gels, following electrophoresis of the homogenates, in distilled water at 60° for varying lengths of time. Similarly, the urea lability of turtle esterases were studied by incubating the gels following electrophoresis in 10 M urea for different 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 electrophoresing the homogenates on polyacrylamide gels of three different concentrations. The retardation coefficients were calculated from the ratio of the relative mobility with respect to bromophenol blue of each esterase 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[24].

Visualization of lactate dehydrogenase activity after electrophoresis on starch gel was achieved by incubating the slabs in a staining mixture containing, 45 ml of Tris–HCl buffer (0.2 M, pH 8.0), 9 ml of sodium lactate (0.5 M, pH 7.0), 5 ml of nitro blue tetrazolium (1 mg/ml), 5 ml of phenazine methosulphate (1.6 mg/ml) and 2 ml of NAD (10 mg/ml)[25]. All resulting zymograms were recorded by scanning in an integrating densitometer.

Total esterase activity was determined by a modification of the method of GOMORI[26], using fast blue RR salt, and the adsorption peak at 500 nm. Total lactate dehydrogenase activity was determined by observing the formation of NAD^+ from NADH at 340 nm in a reaction mixture containing sodium pyruvate as substrate[27]. These measurements were made with a Unicam S.P. 800 spectrophotometer at 37°. Protein concentrations were measured from the absorbance at 280 nm, and 260 nm using the equation: {Protein} mg/ml = $(A_{280\ nm})$ 1.55× $(A_{260\ nm})$ 0.76. Enzyme activity was calculated in I.U./mg of protein.

RESULTS

The specific activities of the soluble esterases and lactate dehydrogenase of adult green turtle tissues are listed in Table I in terms of I.U./mg of protein. From

TABLE I

LACTATE DEHYDROGENASE AND SOLUBLE ESTERASE ACTIVITY IN ADULT GREEN-TURTLE TISSUES

Tissues	*Specific activity (I.U./mg protein)*	
	Esterase	*Lactate dehydrogenase*
Liver	0.650	2.9
Kidney	0.083	3.0
Heart	0.028	19.0
Intestine	0.023	0.9
Muscle	0.013	10.3
Brain	0.038	1.5
Lung	0.014	2.0
Spleen	0.011	1.0

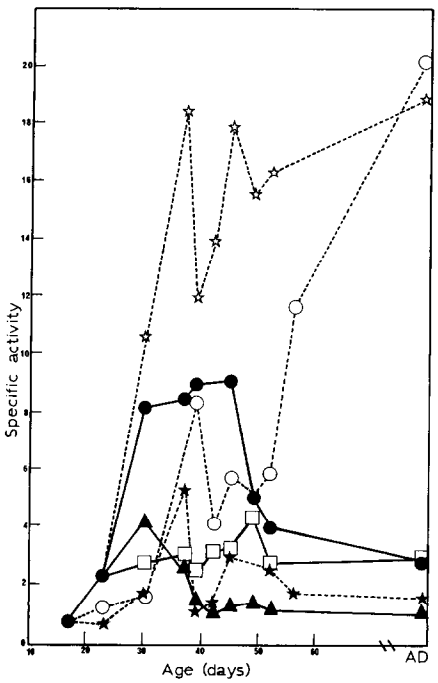


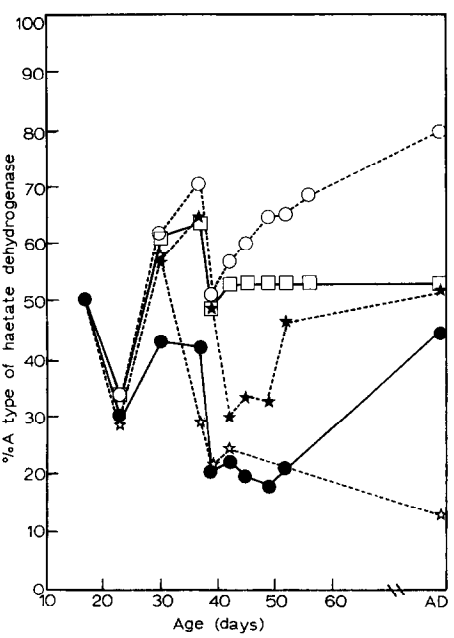
Fig. 1. The developmental changes in lactate dehydrogenase specific activity with age in days: ○, muscle; □, kidney; ●, liver; ★, brain; ▲, intestine; ☆, heart.

these results it is evident that lactate dehydrogenase activity is greatest in the heart and muscle, while significant amounts are present in the liver, kidney, gut and brains. By far the highest concentration of esterase activity occurs in the liver. All other tissues which were examined contained smaller, but still appreciable amounts of esterolytic activity.

Developmental changes in the total lactate dehydrogenase activity (Fig. 1) indicate that the turtle has established adult levels of this enzyme activity in each specific tissue before hatching. Heart and muscle contain high activities compared to all other tissues studied. Each tissue shows a first peak of activity around 37 days development, and a second peak around 47 days, after which the adult level was approached. During ontogeny the percentage of A-type activity (Fig. 2) also reaches adult levels before hatching. After 37 days of maturation, each tissue (with the exception of heart) shows a peak of A-type activity, after which individual tissues being to develop adult levels of the A subunit.

Developmental alterations of the specific esterase activities in the separated turtle tissues are detailed in Fig. 3. General increases in activity are evident in most tissues before hatching. Liver shows the most dramatic increase in activity with age; the augmentation in this case being several times that in other tissues. All other sources studied showed a peak of activity between 30 and 37 days, and the gut and kidney showed a further peak of activity around 47 days.

The contribution of each multiple form of esterase towards the total activity of adult tissues is represented in Fig. 4. Although some of these isoenzymes have a



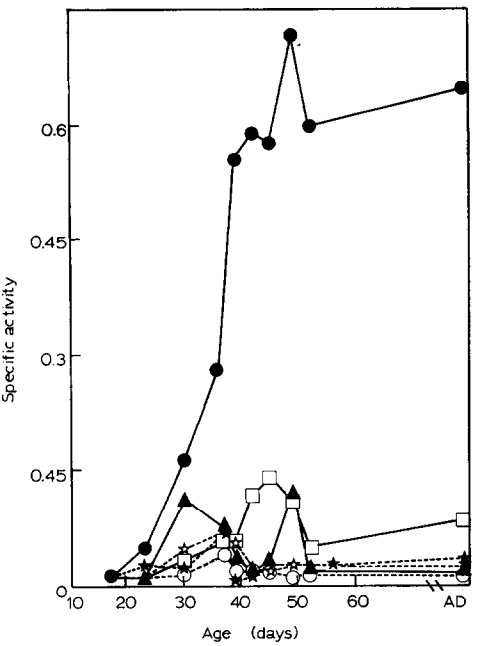


Fig. 2. The developmental changes in percentage A type of lactate dehydrogenase with age in days: ○, muscle; □, kidney; ★, brain; ●, liver; ☆, heart.

Fig. 3. The developmental changes in esterase specific activity with age in days: ●, liver; □, kidney; ★, brain; ☆, heart; ▲, intestine; ○, muscle.

common occurrence in different tissues, the distribution varies between individual sources and provides a tissue specific pattern in each case. Figs. 5–11 represent the developmental progression of multiple forms of esterases in different tissues of the turtle. With liver (Fig. 5), the main developmental tendency may be summarized as a decrease in the activities of the arylesterases during early development, and an increase in the contribution of the fast moving carboxylesterases at a stage well before hatching of the egg. Five separate carboxylesterases were present, as well as three slow moving arylesterases, and an intermediate arylesterase doublet, present only in the 30 day specimens.

In the case of kidney (Fig. 6), the carboxylesterase and arylesterase multiple forms which were present in the liver are again in evidence, and increase in activity through to the adult stages. Once again a doublet of arylesterase activity with intermediate mobility appears transiently during maturation. The esterases of the intestine (Fig. 7) show similar maturative changes to the kidney, but the intermediate arylesterases (R.M. 53, 58.5) gain dominance. The relative mobility, R.M., is measured as the percentage mobility with respect to the mobility of bromophenol blue. The slow migrating arylesterases (R.M. 22, 29, 33) are also proportionally much higher in activity. In lung, the arylesterases (Fig. 8) all disappear before the adult stage is reached. The five carboxylesterases present in other tissues are again present but with tissue-specific distribution. With heart (Fig. 9) the ontogenetic changes show dramatic alterations in the activity of arylesterases, while the carboxylesterases show

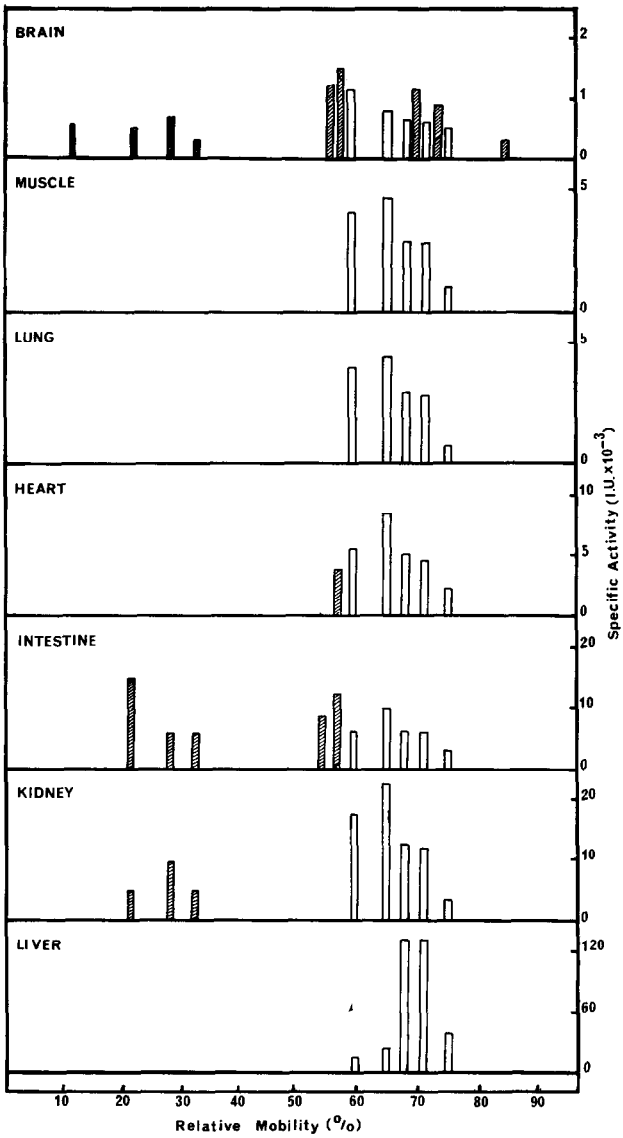


Fig. 4. The distribution of esterase multiple forms in adult turtle tissues. Carboxylesterase activity is represented by open histograms, arylesterase by diagonal shading and cholinesterase by complete shading.

only modest increases in activity to the adult stage. All five arylesterases are strongly evident until the 37-day stage, but then disappear leaving only one form (R.M. 58.5) present in the adult.

The distribution of esterases in skeletal muscle (Fig. 10) also shows considerable changes in arylesterase banding with the carboxylesterases being relatively stable, but showing an increase during maturation. Cholinesterase (R.M. 12.5) is present in the early developmental stages, but then disappears, as do the three slow arylestera-

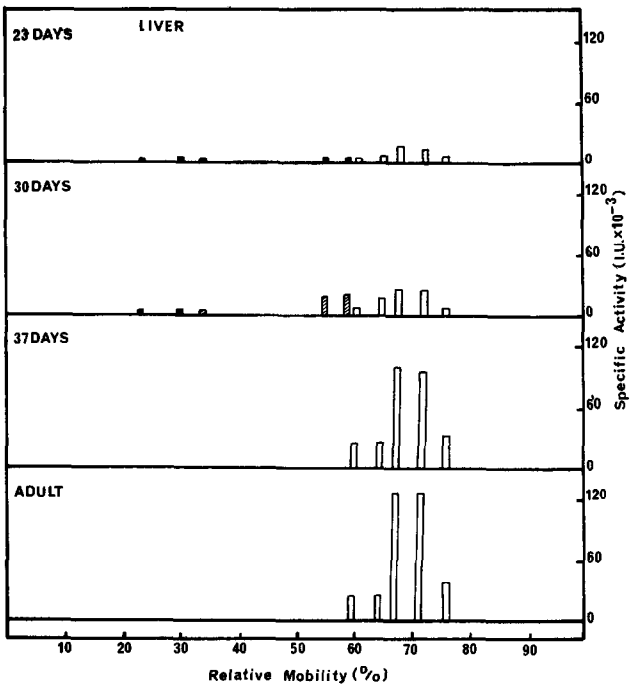


Fig. 5. The developmental progression of esterase forms in turtle liver. Representations of the type of esterase activity are the same as in Fig. 4.

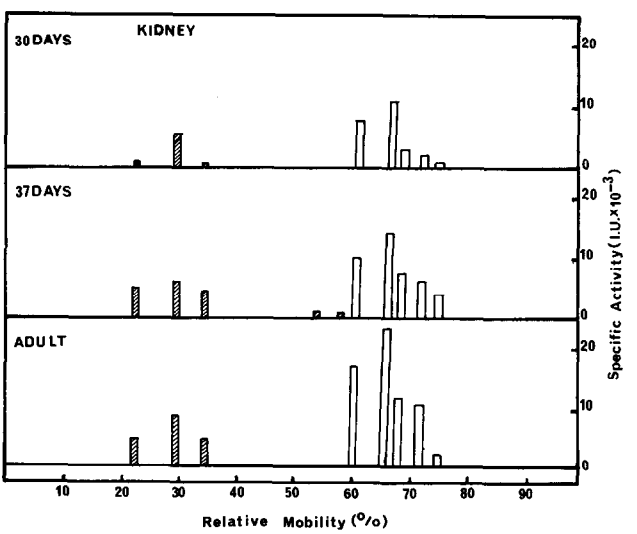


Fig. 6. The developmental progression of esterase forms in turtle kidney. Representations of the type of esterase activity are the same as in Fig. 4.

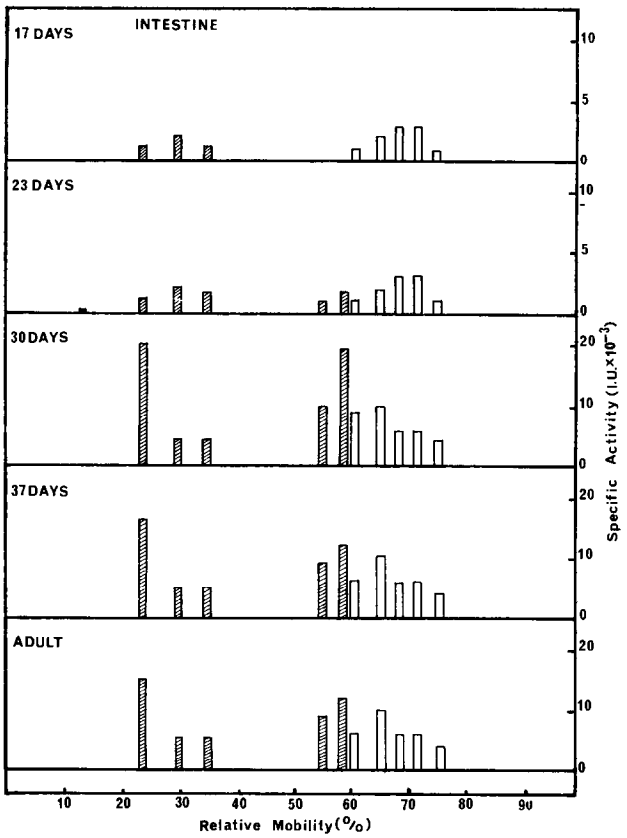


Fig. 7. The developmental progression of esterase forms in turtle intestine. Representations of the type of esterase activity are the same as in Fig. 4.

ses. Both arylesterase R.M. 53 and 58.5 are present during early maturation, the latter reaching high activity at 37 days, but then regressing.

The ontogenetic changes in the esterase multiplicity in brain (Fig. 11) are the most extensive of all these tissues. Cholinesterase (R.M. 12.5) reaches a high level of activity in the embryo but falls to a somewhat lower level in the adult. The same arylesterases found in other tissues of this amphibian are also found in the brain, and as in heart exhibit high activity at the 37-day developmental stages. Carboxylesterases are also present and the activity of these isoenzymes varies quite markedly throughout development. A unique group of arylesterases (R.M. 68, 72.5, 84.5) finds expression in this tissue around the 37-day period and is present in the adult, but with reduced activity.

In Table II are listed the physicochemical characteristics of the individual multiple forms of these esterases. The cholinesterase and arylesterases showed marked heat lability, but in contrast only the slower arylesterases (R.M. 22, 29, 33 and 58, 58.5) were urea sensitive. All the arylesterases which preferentially hydrolysed acetyl esters could be distinguished from the cholinesterase which preferred butyryl esters. The group of carboxylesterases shows greater heat stability.

TABLE II

PROPERTIES OF TURTLE ESTERASES

R.M.	*Classification*	*Substrate specificity*	*Mol. wt.*			*Half life at 60° (min)*	*Half life in 10 M urea (min)*
			6.5/5	*8/5*	*8/6.5*		
12.5	Cholinesterase	Ac < Bu	>300000			<2	15
22	Arylesterase	Ac > Bu	60000	62000	63000		
29			59000	63000	60000	2.5	<5
33			60000	60500	62000		
53	Arylesterase	Ac > Bu	62500	63000	62000	<2	<5
58.5			63500	64000	60000		
60	Carboxylesterase	Ac < Bu	70000	74000	73000		
64			70000	74000	73000		
67			70000	74000	73000	7.5	5
71			73000	72000	71000		
74			74000	76000	71000		
68	Arylesterase	Ac > Bu	61000	60000	61500		
72.5			60000	59000	61000	<2	9
84.5			61000	64000	63000		

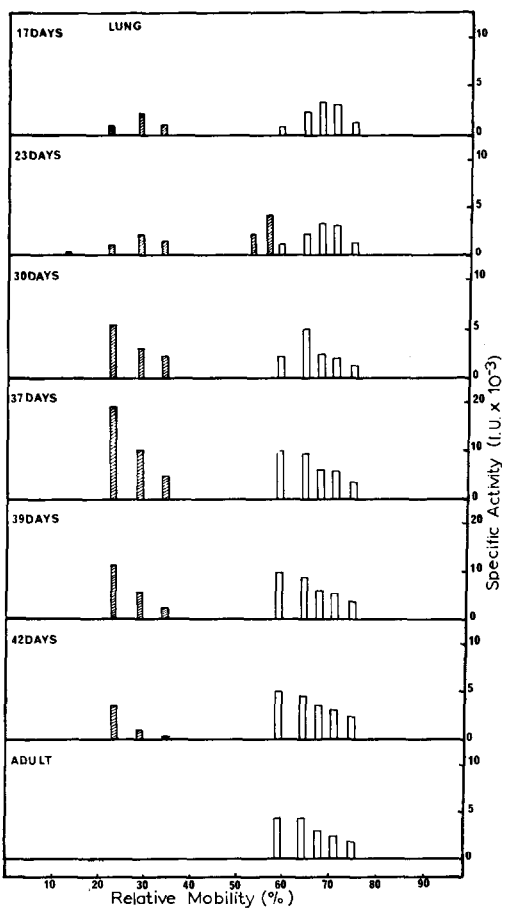


Fig. 8. The developmental progression of esterase forms in turtle lung. Representations of the type of esterase activity are the same as in Fig. 4.

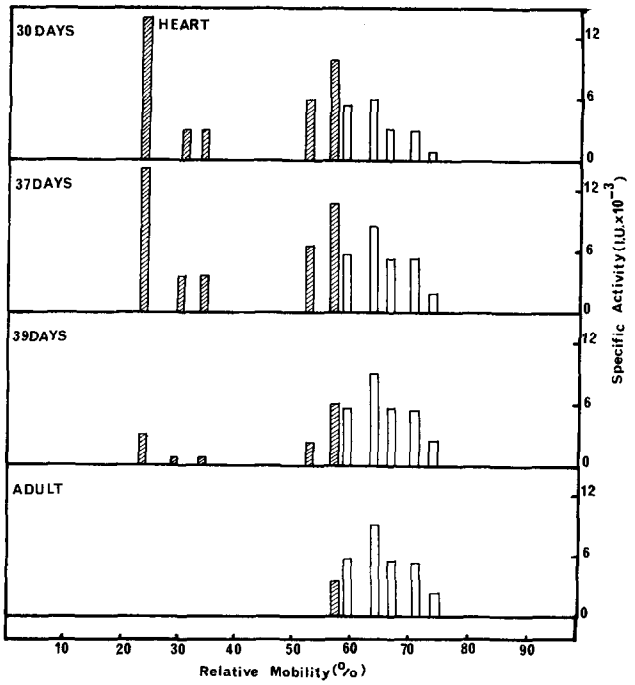


Fig. 9. The developmental progression of esterase forms in turtle heart. Representations of the type of esterase activity are the same as in Fig. 4.

Following electrophoresis on 5%, 6.5% and 8% polyacrylamide gels, the retardation coefficient for each esterase form was calculated from the respective relative mobility ratios (*e.g.* R.M. 6.5%/R.M. 5%, R.M. 8%/R.M. 5% and R.M.8%/R.M. 6.5%). The molecular weights were estimated from a standard curve (Table II)[24]. The cholinesterases (R.M. 12.5) exhibited the mobility characteristics of a very large protein. The arylesterases included in three groups all exhibited similar molecular weights, around 60000. The group of carboxylesterases also appeared to occur in a restricted molecular weight range, in this case around 72000.

DISCUSSION

Among the main reasons governing the choice of the green turtle (*Chelonia mydas*) for these studies, were the significant evolutionary status of this species (these amphibians evolved 80 million years before all other reptiles except the lizards), and the possession by this species of a basic mechanism permitting the animal to extract energy from anaerobic sources for prolonged periods of time during submersion[19,20]. These unusual characteristics were considered to afford a valuable opportunity for the study of functional and distributional relationships of isoenzyme systems.

In regard to the latter characteristic, turtles differ from the great majority of other reptiles and vertebrates in that they exhibit the ability to shift from aerobic to anaerobic metabolism, in response to depressed level of intracellular oxygen dependent metabolism. During strict anaerobiosis, a marked accumulation of lactate and

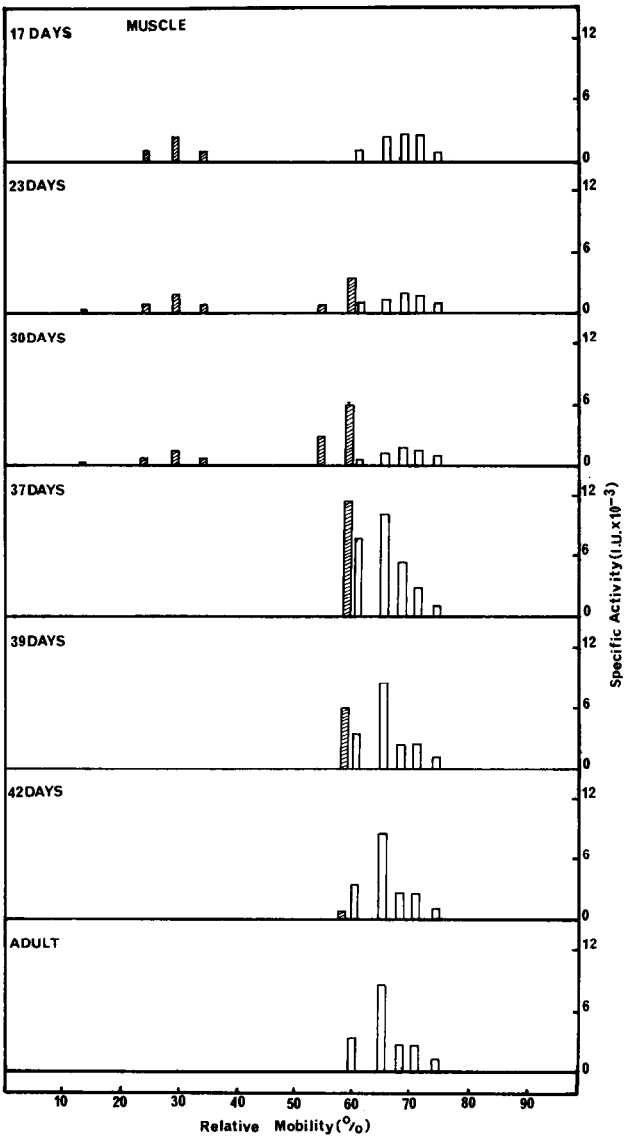


Fig. 10. The developmental progression of esterase forms in turtle muscle. Representations of the type of esterase activity are the same as in Fig. 4.

pyruvate occurs, generating H^+. The fresh water turtle possesses a large volume of coelonic fluid with a high bicarbonate concentration and a pH more alkaline than plasma[28]. It has been shown that exogenously administered lactic acid is capable of penetrating this compartment, which thus may serve as a buffer site for minimizing pH changes produced by H^+ accumulation during anaerobiosis. At least two possibilities may be conceived, then, which would enable the turtle to adapt to an extended anaerobic mode of metabolism. The type and levels of enzymes producing harmful metabolic intermediates (*e.g.* H^+) may be regulated so that under anaerobic condi-

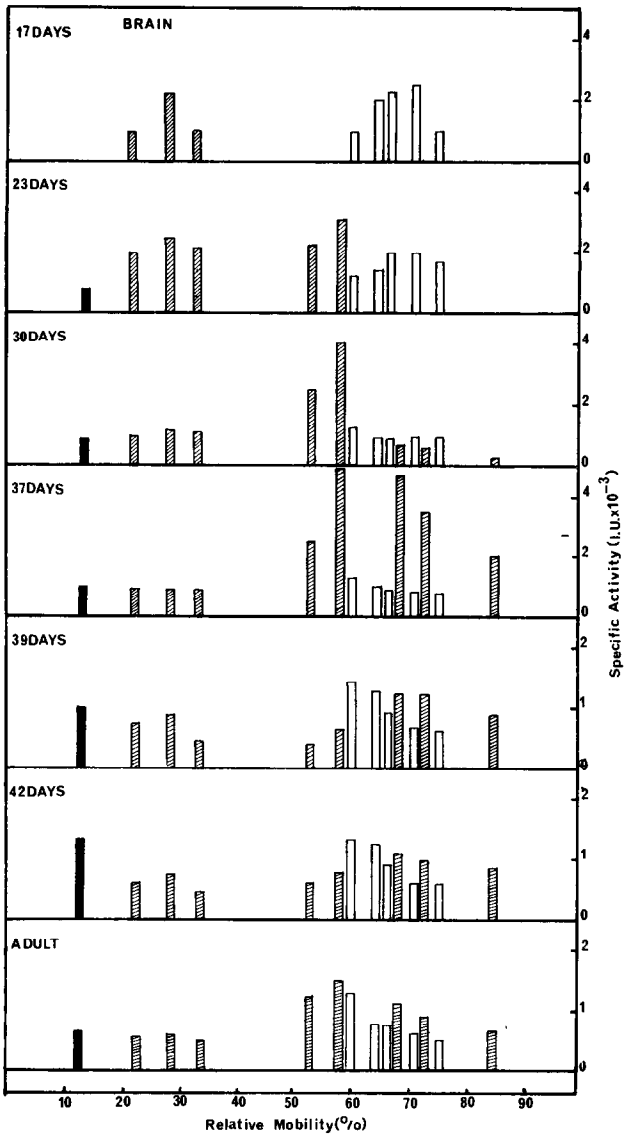


Fig. 11. The developmental progression of esterase forms in turtle brain. Representations of the type of esterase activity are the same as in Fig. 4.

tions, production is diminished; or, alternative, a physiological "sink" may develop which renders such products harmless. Since the levels and types of lactate dehydrogenase activity found in the turtle tissues are similar to those found for corresponding mammalian tissues, it would appear that the green turtle has synthesized lactate dehydrogenase in a manner designed to satisfy its terresterial aerobic mode of life, and that during anaerobiosis, the increased levels of lactate and pyruvate are probably accomodated by the available coelonic buffer system.

Ontogenic studies of isoenzymes have facilitated our understanding of the

genetic and structural interrelationships of multiple enzyme forms, the physiological significance of the multiplicity, and the control of enzyme synthesis during tissue differentiation. In this case, studies on the developing turtle show that A-type and B-type lactate dehydrogenase activity are present to an almost equal extent in the early embryo prior to functional differentiation, and this finding indicates that both A and B genes are operational at this stage of development. This finding is in agreement with the previous data of HINKS AND MASTERS[16,17] on a wide variety of mammalian species, and provides an extension of their findings to include primitive oviparous vertebrates. Whereas earlier investigators had assumed that the embryonic lactate dehydrogenase was a parental type in vertebrates, and that the synthetic control of lactate dehydrogenase synthesis was analogous to the complementary polypeptide production observed with hemoglobin[29,30], the present results point to the wide range of species specific values in the isoenzyme pattern of the early embryo, and to the independent control of synthesis of the lactate dehydrogenase subunits.

Around 37 days after incubation the individual tissues begin to show their own characteristic lactate dehydrogenase type and a peak of A-subunit activity occurs. This variation may be related to the dramatic change in rigidity of the egg shell which is the most notable development feature at this time, and which may decrease, the availability of oxygen to the embryo, or influence other epigenetic directive mechanisms in response to the change in metabolites produced in the turtle at this time.

Comparative studies of the vertebrate esterases have confirmed the wide distribution of these enzymes in animal tissues, and established the species specificity of esterase multiplicity, activity and tissue distribution. In the tissues studied in this investigation, a prominent feature was the occurrence of carboxylesterases with high electrophoretic mobility, and molecular weights in the range 70000–75000 (ref. 30). This type of enzyme has also been observed in a number of herbivores, ruminants and marsupials, but not in rodents, fish or reptiles. The predominance of carboxylesterase as the main form of esterase in this species is in agreement with the results found in most of the animals studied by HOLMES AND MASTERS[11–14] and HOLMES *et al.*[15]; exceptions being the frog and lizard, where cholinesterase was the major contributor to total esterase activity. In contrast, only one form of cholinesterase was found in turtle tissues, and this occurred only in the brain of the adult. This cholinesterase behaved as a protein of very high molecular weight (>300000) on electrophoresis, as did many cholinesterases of the mammalian species. In the lizard, frog and catfish tissues, cholinesterases of much lower molecular weight (approx. 55000) have been identified. In respect of these data, then, the presence of the low molecular weight cholinesterase, and the absence of high molecular weight species, do not appear to be the prerogative of species lower on the evolutionary scale than mammals. It should also be noted in this regard that the difference in molecular weights of cholinesterases between species may signify a concentration effect on polymerization, rather than a significant alteration in the relevant area of the genome[32,33], and it is of further interest to note that marked similarities in active site sequences and other properties exist between the carboxylesterases and the cholinesterases[34,35]. In the light of these resemblances and in view of the characteristic distributions of these esterase types between different stages of phylogeny, the possibility that these different classes of esterase are capable of fulfilling a similar metabolic role in vertebrates seems worthy of consideration.

All eight bands of arylesterase activity had molecular weights around 60000, and in this and their other properties, appear very similar to all such esterases classified in other species. No enzymes exhibiting acetylesterase activity were found in the turtle. Previous investigations have shown the presence of acetylesterases in mammalian species, but this activity appears to be totally absent, in lower vertebrates, and hence acetylesterase would appear to represent a more recently evolved esterase species.

With regard to the ontogeny of the esterases in this species, little change in the pattern of isoenzymes occurred until 30–37 days of development. This lack of tissue specificity in the early stages is unusual in that the esterase of mammals and higher vertebrates display a marked early sensitivity in regard to this parameter. Again, in contrast to the extensive available data in regard to Fundulis heteroclitus, no dramatic changes in esterase expression occurred following hatching[36]. Instead, it would appear that each tissue in the turtle has attained maximum expression of the genes coding for esterase activity by the 40-day stage, and the most distinctive feature of the later stages of development is the phasing out of the arylesterases in all tissues but brain.

Overall, the ontogeny of the multiple forms of turtle esterase may be said to be characterized by gradual alterations in distribution of activity amongst the heteromorphs and this, when considered in relation to the properties and interrelationships of the individual esterase forms would appear to signify a far less complicated gene implication than is evident in more highly evolved species.

ACKNOWLEDGEMENTS

These investigations were supported in part by grants from the Australian Research Grants Committee. We gratefully acknowledge the gift of a 3-year old turtle, one of the few reared in captivity, by Mr L. Tanis, Brisbane.

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