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THE DEVELOPMENTAL MULTIPLICITY AND ISOENZYME STATUS OF CAVIAN ESTERASES

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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 cavian tissues.

2. A total of 24 multiple forms of esterolytic activity have been resolved, and the occurrence of these individual forms in the different tissues inter-related.

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

4. All of these classes are heterogeneous—the soluble carboxylesterases existing as 10 separate forms, arylesterases 4, cholinesterases 5, and acetylesterases 5. Each class multiplicity appears to be more extensive than has been previously reported.

5. Further differentiation of the multiple forms in some of these major classes has been achieved on the basis of the physicochemical and developmental parameters utilized. This treatment would appear to implicate at least 12 structural genes in the biosynthesis of the soluble cavian esterases; a multiplicity of control which is, again, considerably in excess of previous estimations for mammalian sources.

6. The significance of the localization and variation of individual esterases is discussed in relation to hormonal influences, cell metabolism and tissue individuality.

INTRODUCTION

Although esterases were amongst the first mammalian enzymes known to exhibit an extensive heterogeneity[1,2], the genetic control and metabolic significance of these multiple forms have remained comparatively ill-defined. Much of the reason for this lack of appreciable progress in defining the isoenzyme status of esterases may be attributed to the wide substrate specificity of these enzymes, and the consequent difficulty of precise classification. At the present time, however, it has become possible to distinguish between many different types of esterolytic activity on the basis of characteristic reactions with a variety of inhibitors and substrates. In mammalian

tissues, for example, four main types of esterases have been described[3-6]: Arylesterases (EC 3.1.1.2) which preferentially hydrolyze aromatic esters, are not inhibited by organophosphates, but are inhibited by sulphydryl reagents such as *p*-chloromercuribenzoate; carboxylesterases (E.C. 3.1.1.1) which preferentially hydrolyze aliphatic esters, and are inhibited by organophosphates but not by eserine; cholinester hydrolases (E.C. 3.1.1.7; 3.1.1.8) acting on choline esters at a higher rate than both aliphatic and aromatic esters, and being inhibited by eserine and organophosphates; and acetylesterases (E.C. 3.1.1.6) which hydrolyze aromatic esters, but are not inhibited by organophosphates, eserine, or sulphydryl reagents.

Once the type of esterase activity has been defined in this manner, the extent of heterogeneity within a particular class may be examined by the usual procedures of protein resolution. A number of workers have combined starch-gel electrophoresis with histochemical identification for this purpose[1,3,5,6], but more effective resolution is often apparent with polyacrylamide-gel systems[7-9], and the advantages of this latter technique have been utilized in the present study.

By means of this combined approach, then, developmental and physicochemical properties of the multiple forms of esterases have been examined in an extensive range of cavian tissues. The main aim of this investigation has been to contribute towards a fuller understanding of the isoenzyme status of esterases, and, as such, this study represents the continuation of a previous line of research into enzyme variformity and tissue differentiation[9,10].

METHODS

Tissue extracts

A number of guinea pigs 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 for analysis. Blood specimens were obtained by cardiac puncture, collected in heparinized tubes, centrifuged, and the sera analyzed within hours of separation.

Electrophoresis

Homogenates (approx. 10%) were prepared in cold, glass-distilled water, centrifuged (100 000 × *g*, 30 min), and disc electrophoresis of the supernatant carried out on vertical columns of polyacrylamide gel (7.5%)[11,12]. The buffer used was Tris–glycine (pH 8.6, *I* 0.03). Bromophenol blue was used as the reference dye, and separations were carried out at 4° with a constant current of 2.5 mA per gel for 2 h.

After electrophoresis, the gel columns were removed from the holders and allowed to stand in Tris buffer (pH 7.4; *I* 0.02) at room temperature for 10 min. If inhibition studies were intended, the inhibitor was incorporated into this buffer solution.

The gels were subsequently stained in a solution of fast blue RR salt (0.05%, Sigma Chemical Co.) and the appropriate substrate (0.16%) to demonstrate esterase activity. A variety of substrates and inhibitors were used for classification purposes and these are listed in Table I. Background staining was removed by washing gels with methanol–acetic acid–water (45:10:45, by vol.).

The resulting zymograms were recorded by photography and scanned in an integrating densitometer. Reproducibility of values was of the order of 1–2%.

TABLE I

CLASSIFICATION OF TYPES OF ESTERASE ACTIVITY

Activity and inhibition are denoted by +; (+) refers to a trace of activity. The terms 'fast' and 'slow carboxylesterases' refer to a division in the basis of the relative migration of these groups on gel electrophoresis (see RESULTS).

Substrates	*Slow carboxyl-esterase*	*Fast carboxyl-esterase*	*Cholin-esterase*	*Acetyl-cholin-esterase*	*Acetyl-esterase*	*Aryl-esterase*
α-Naphthyl acetate	+++	+++	+++	+++	+++	+++
β-Naphthyl acetate	+++	+++	+++	+++	+++	+++
α-Naphthyl butyrate	++	++	+++	++	(+)	+
β-Naphthyl laurate	−	−	−	−	−	−
Leucyl-β-naphthylamide	−	−	−	−	−	−
α-*N*-Benzoyl arginine naphthyl-amide	−	−	−	−		−
Indoxyl acetate	−	++	++	++	++	++
Inhibitors						
DFP (10^{-4} M)	+++	+++	+++	+++	−	−
Eserine (10^{-5} M)	−	−	+++	+++	−	−
PCMB* (10^{-3} M)	−	−	−	−		++
Acetylcholine iodide (10^{-3} M)	−	−	−	++	−	−

* PCMB, *p*-chloromercuribenzoate.

Physicochemical properties of esterases

The heat stabilities of the multiple molecular forms of cavian esterases were investigated by incubating the gel, following electrophoresis of the homogenate, in distilled water at 55° for varying lengths of time 0, 2, 4, 6, 8, 12, 16, 20 min. After this incubation, the gels were allowed to equilibrate in cold Tris–HCl buffer (pH 7.2; *I* 0.1) for 10 min, and subsequently stained for esterase activity. The urea lability of guinea-pig esterases was studied by incubating the gel, following electrophoresis, in 10 M urea for varying lengths of time *e.g.* 0, 5, 10, 15, 20, 25, 30, 40 min. After this time, the gels were equilibrated and histochemically treated as above. To study the effect of changes in gel concentration, separate columns were prepared, in which the polyacrylamide concentration was varied from 5% to 10% in steps of 0.5%. Electrophoretic separations of guinea-pig esterases were run, and the gels stained for esterase activity and the relative mobility of each band calculated at the different gel concentrations.

Enzyme assay

Total esterase activity was determined by a modification of GOMORI's method[13], using fast blue RR salt (Sigma Chemical Co.) and the absorption peak at 500 mμ. These measurements were made with a Unicam SP 500 spectrophotometer at pH 7.4 and 37°. Enzyme activity was calculated as international units per l. Protein determinations were performed by the method of LOWRY *et al.*[14], with crystallized bovine serum albumin for standards.

RESULTS

The specific activities of the total esterases of adult guinea-pig tissues are listed

TABLE II

ESTERASE ACTIVITY IN ADULT GUINEA-PIG TISSUES

Specific activity is expressed as μmoles α-naphthol released per min per mg of protein.

Tissue	*Specific activity*		*Tissue*	*Specific activity*	
	Male	*Female*		*Male*	*Female*
Liver	0.25	0.40	Serum	0.034	0.05
Kidney	0.11	0.10	Spleen	0.004	0.019
Intestine	0.052	0.13	Brain	0.01	0.01
Lung	0.06	0.064	Testis	0.18	
Heart	0.09	0.08	Epididymis	0.314	
Muscle	0.01	0.013	Uterus	0	0.04
			Ovary	0	0.09

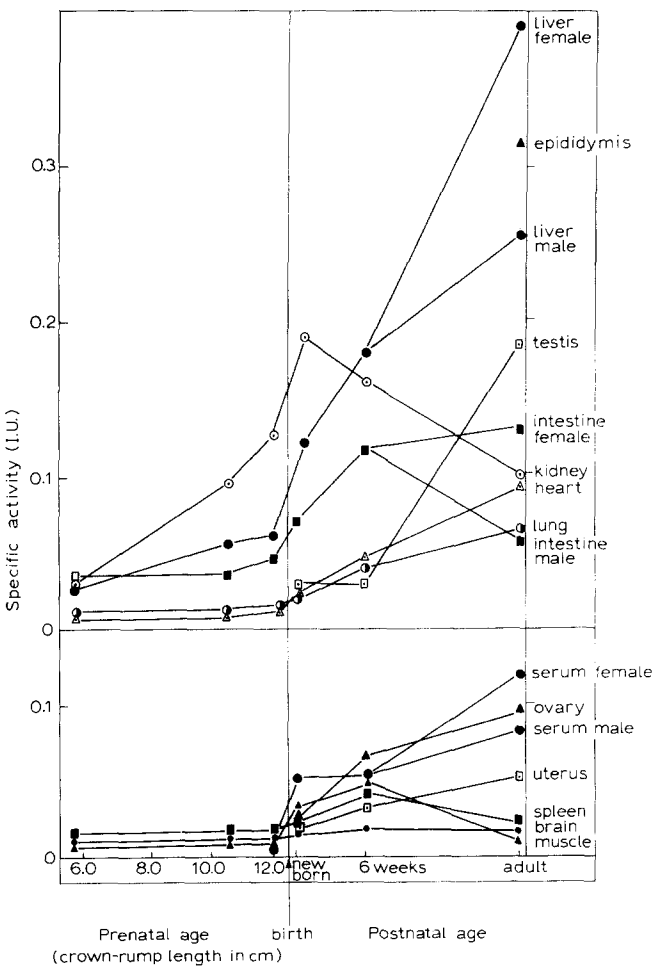


Fig. 1. Developmental alterations of total esterase activities in the different cavian tissues.

in Table II. It may be noted that there is general agreement between corresponding tissues of the male and female animal, although spleen, liver and intestine present somewhat higher values in the female. The highest concentrations of esterase activity occur in epididymis, testis and liver, but appreciable activity is present in all the tissues examined.

Developmental alterations of the total specific activities in the different cavian tissues are detailed in Fig. 1. In the period immediately following birth, general increases in activity are evident, but the subsequent post-natal sequences show considerable individual variation between tissues. The esterase activity increases during post-natal maturation of liver and ovary, but kidney esterase decreases in this period. The activity in testis exhibits an increase at the attainment of puberty.

In Fig. 2, a typical zymogram is illustrated, in this case the electrophoretic pattern of esterase activity in the liver of an immature guinea pig. Twelve regions of

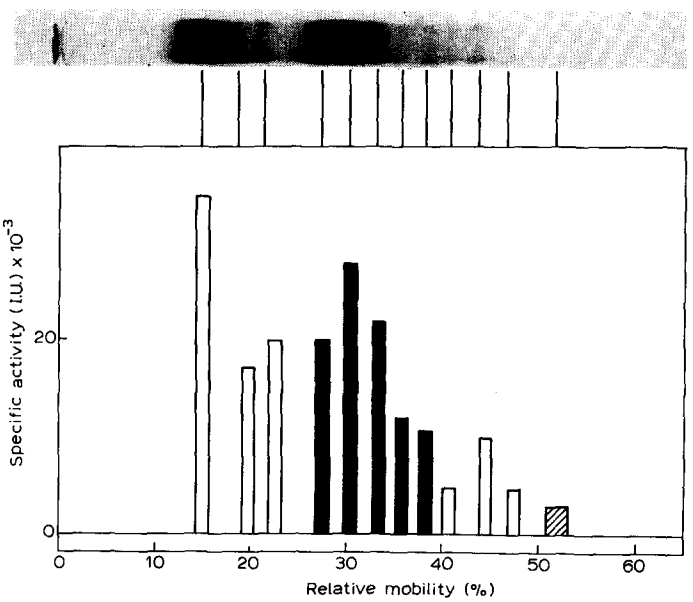


Fig. 2. The electrophoretic pattern of esterase activity in the liver of an immature guinea pig—zymogram and corresponding histogram representation. Carboxylesterase activity is represented by open histograms, arylesterase by diagonal shadings and cholinesterase by complete shading.

activity are discernible, and this same gel has been scanned, and the results represented diagrammatically in the lower portion of the figure. This figure is intended to illustrate the methodology of this investigation and the method of presentation of results. Histograms of specific activity have been drawn, showing the relative mobility of the bands and the type of esterase activity.

The contribution of each multiple form of esterase towards the total activity of adult tissues is detailed in Table III. This presentation emphasizes the individuality of the adult distributions and is meant to facilitate comparison of the multiplicity at this terminal stage of ontogeny.

TABLE III

DISTRIBUTION OF MULTIPLE FORMS OF ESTERASE ACTIVITY IN ADULT TISSUES OF THE GUINEA PIG

Activities are expressed as percentages of total esterase activity.

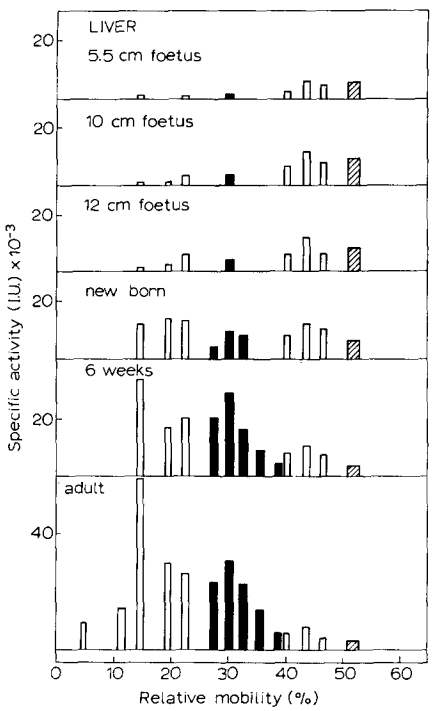
*Esterase classification**	*Rm***	*Liver*	*Kidney*	*Intestine*	*Lung*	*Heart*	*Muscle*	*Serum*	*Spleen*	*Brain*	*Testis*	*Epidydimis*	*Ovary*	*Uterus*
Carboxylesterase	4	3.7	—	—	3.8	—	—	10.1	—	—	—	—	3.5	2.9
Carboxylesterase	9	—	—	—	13.9	17.6	16.4	20.2	—	—	—	—	10.5	13.5
Acetylesterase	10	—	—	—	—	—	—	—	—	—	12.0	19.0	—	—
Carboxylesterase	11	5.6	5.1	—	11.0	11.6	13.1	22.2	—	—	—	—	10.5	11.4
Acetylesterase	13	—	—	—	—	—	—	—	—	—	13.8	19.9	—	—
Carboxylesterase	15	23.3	7.6	—	—	—	7.8	—	20	7.9	—	—	—	—
Acetylesterase	16	—	4.2	—	—	—	—	—	—	—	18.3	23.0	—	—
Acetylesterase	19	—	—	—	—	—	—	—	—	—	13.8	18.3	—	—
Carboxylesterase	20	11.8	5.1	—	—	—	5.9	—	—	—	—	—	—	—
Acetylesterase	22	—	—	—	—	—	—	—	—	—	10.0	15.1	—	—
Carboxylesterase	23	10.3	1.7	8.8	4.0	4.7	7.8	—	5	8	—	—	2.8	2.9
Cholinesterase	27	9.4	—	14.4	4.6	—	—	—	—	—	—	—	—	—
Cholinesterase	30	12.1	12.1	4.3	22.0	13.8	9.8	—	—	10.1	—	—	4.9	2.9
Cholinesterase	33	9.0	7.1	7.2	4.0	4.3	—	—	—	—	—	—	2.1	—
Cholinesterase	36	4.1	—	—	—	—	—	—	—	—	—	—	—	—
Cholinesterase	39	2.5	—	—	—	—	—	—	—	—	—	—	—	—
Carboxylesterase	40	2.2	10.2	12.0	28.0	36.5	26.2	43.6	20.5	19.7	15.5	3.2	36.8	27.0
Carboxylesterase	44	3.1	28.6	10.0	1.2	—	—	—	—	—	—	—	—	—
Carboxylesterase	47	1.6	12.7	5.6	1.2	—	—	—	—	—	—	—	—	—
Arylesterase	52	1.3	7.6	20	14.5	15.9	13.0	3.9	30	35.4	13.8	1.5	23.4	28.8
Arylesterase	63	—	6.0	—	—	—	—	—	24.5	18.9	2.8	—	5.5	9.6

* The details of esterase classification are given in METHODS.

** The relative mobility of each band is defined by reference to bromophenol blue (see METHODS). A further carboxylesterase (*Rm* = 0) was present in adult tissues which migrated appreciably at gel concentrations below 5%.

Figs. 3–14 illustrate the developmental progression of multiple forms of esterases in different tissues of the guinea pig.

With liver (Fig. 3), the main developmental tendencies may be summarized as a decrease in the activities of the arylesterase components during maturation, and a post-natal increase in the contribution of the slower migrating carboxylesterases and cholinesterases. Eight separate carboxylesterase bands are present, a single diffuse arylesterase region and five forms of cholinesterase. This arylesterase band and another observable in other tissues were resolvable as a doublet at lower gel concentrations (see later RESULTS). The cholinesterase extracted by this methodology was entirely of the acylcholine acyl-hydrolase type (EC 3.1.1.8) in all the tissues studied; acetylcholinesterase being firmly bound in the cell and not solubilized by this procedure.



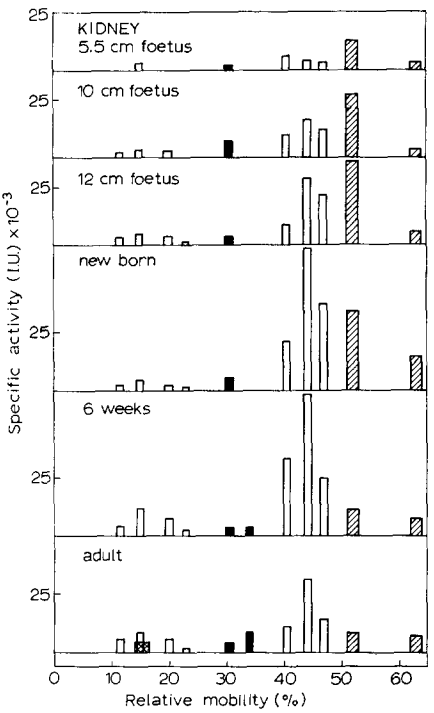
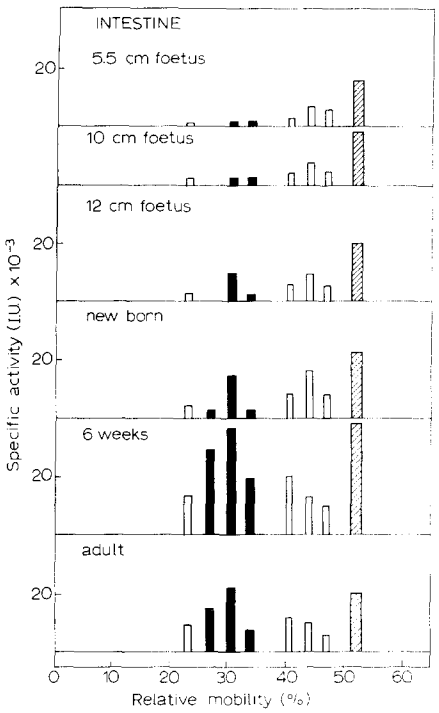


Fig. 3. The developmental progression of esterase forms in cavian liver. Carboxylesterase activity is represented by open histograms, arylesterase by diagonal shadings, acetylesterase by cross-hatching, and cholinesterase by complete shading.

Fig. 4. The developmental progression of esterase forms in cavian kidney. Representations of the type of esterase activity are the same as in Fig. 3.

In the case of kidney (Fig. 4), most of the maturative changes were concentrated in the arylesterase and fast carboxylesterase components. Both of these attain peak activities in the new-born animal, and tend to decrease in the later stages. In the adult animal, some acetylesterase activity is discernible.

Another rich source of esterase activity, intestine (Fig. 5), shows a very different distribution of enzyme type. Cholinesterase develops to become a major component,



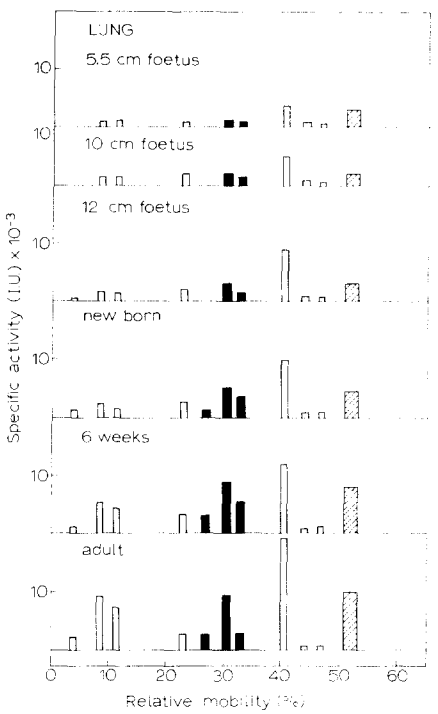


Fig. 5. The developmental progression of esterase forms in cavian intestine. Representations of the type of esterase activity are the same as in Fig. 3.

Fig. 6. The developmental progression of esterase forms in cavian lung. Representations of the type of esterase activity are the same as in Fig. 3.

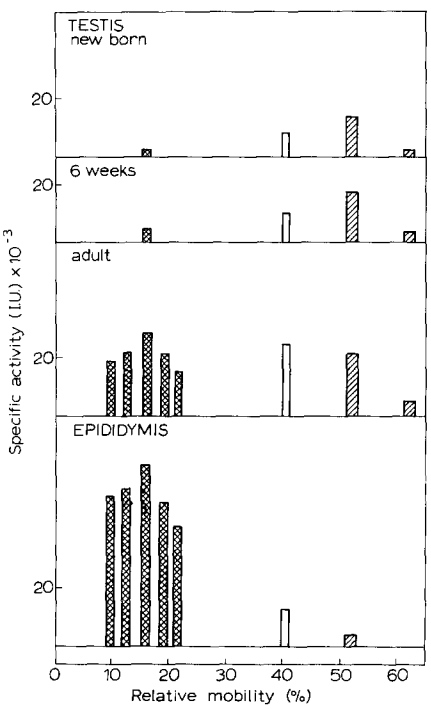
arylesterases are also prominent in the young animal, and there is a single band only of slow-migrating carboxylesterase (*Rm* 23)* throughout.

Arylesterase activity in guinea-pig lung (Fig. 6), appears to be concentrated in one fast moving component, *Rm* 52, and the contribution of this activity increases during development. The activity of the fast migrating carboxylesterases converges towards a single band (*Rm* 40) in the adult, and slow carboxylesterase activity is concentrated in four components.

The most striking feature of the developmental changes in testis (Fig. 7) is the post-pubertal increase in acetylesterase which becomes the major component of the adult. Five multiple forms are evident at this stage, and are also present in the epididymis of the adult. Two arylesterase bands are present in the growing animal, but only one diminished peak is observable in the fully grown guinea pig. Carboxylesterase occurs as one region of high activity (*Rm* 40) throughout.

There are fewer major ontogenetic alterations in the heart patterns (Fig. 8), although arylesterase and a single fast-moving carboxylesterase (*Rm* 40) increase in activity during maturation.

* *Rm* refers to the electrophoretic mobility of the band expressed as a percentage of the relative bromophenol blue mobility at a gel concentration of 7.5%.



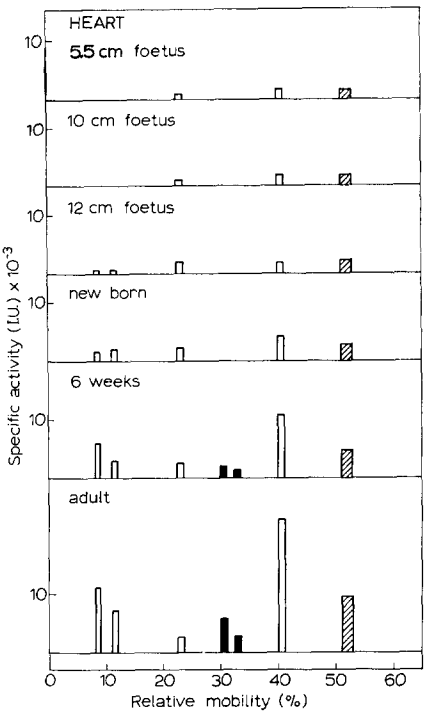


Fig. 7. The developmental progression of esterase forms in cavian testis, and the adult pattern of epididymus. Representations of the type of esterase activity are the same as in Fig. 3.

Fig. 8. The developmental progression of esterase forms in cavian heart. Representations of the type of esterase activity are the same as in Fig. 3.

The distribution of esterases in skeletal muscle (Fig. 9), brain (Fig. 10) and spleen (Fig. 11) again, do not change markedly throughout the period studied. Brain and spleen esterases show simple patterns of distribution, with only three carboxylesterase bands occurring in each of these tissues, along with major areas of arylesterase activity.

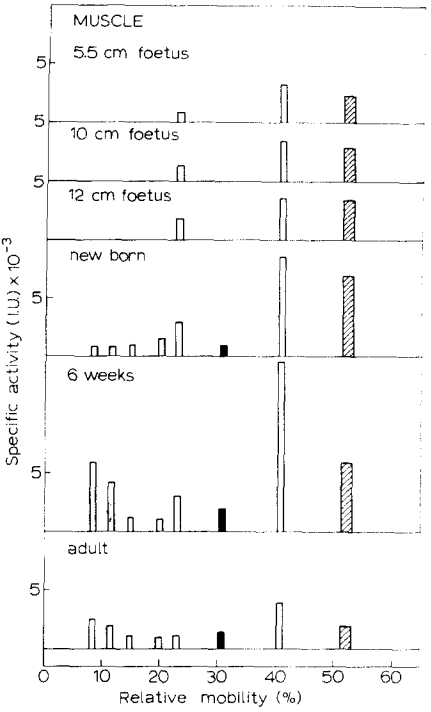
As the cavian uterus matures (Fig. 12) both bands of arylesterase increase in activity, as does carboxylesterase. The latter class of enzyme exhibits only one fast-moving component (*Rm* 40) throughout. Initially no slow-moving carboxylesterase or cholinesterase was detected, but three bands of the slow carboxylesterase appear in the adult.

The main developmental alterations in the esterase patterns of ovary (Fig. 13) occur in the slower arylesterase band (*Rm* 52), and the single fast carboxylesterase (*Rm* 40). The small quantity of cholinesterase activity, and the bands of slow carboxylesterase activity show little change during development.

Serum (Fig. 14) develops increased multiplicity of the slow carboxylesterases, and increased activity of the single fast-moving carboxylesterase (*Rm* 40) with maturation.

The results of the physicochemical studies are illustrated in Figs. 15–17.

The effects of heating (Fig. 15) may be summarized as follows:



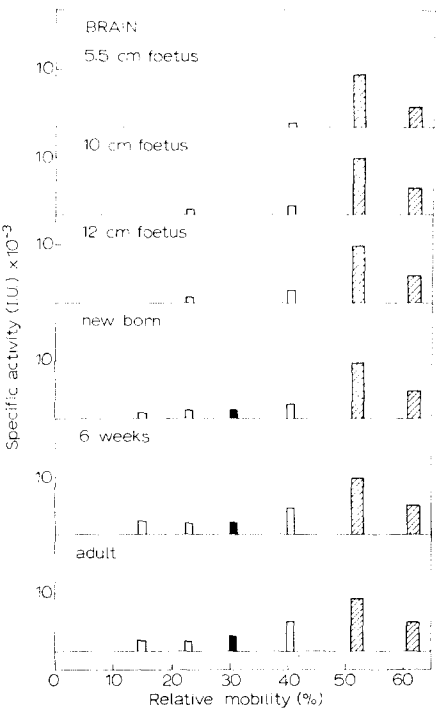


Fig. 9. The developmental progression of esterase forms in cavian skeletal muscle. Representations of the type of esterase activity are the same as in Fig. 3.

Fig. 10. The developmental progression of esterase forms in cavian brain. Representations of the type of esterase activity are the same as in Fig. 3.

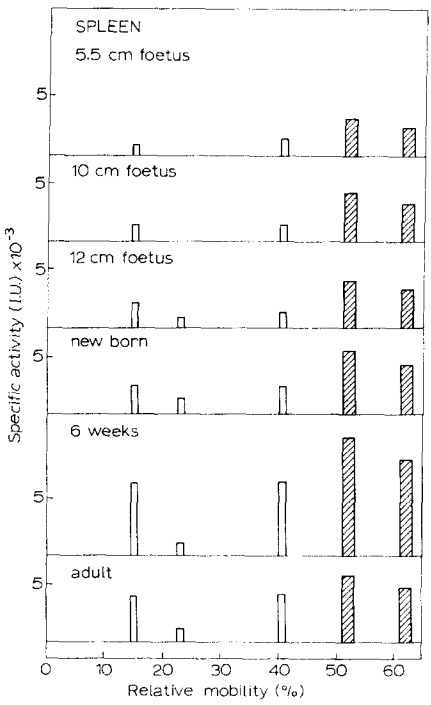
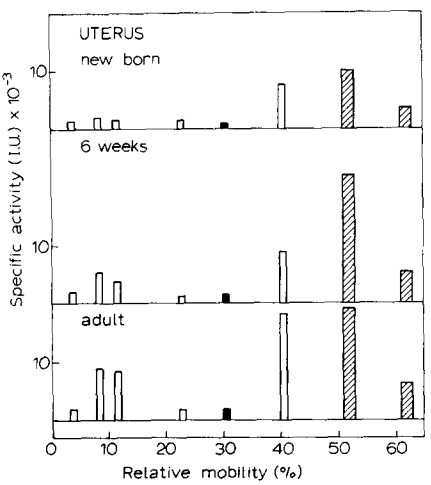


Fig. 11. The developmental progression of esterase forms in cavian spleen. Representations of the type of esterase activity are the same as in Fig. 3.



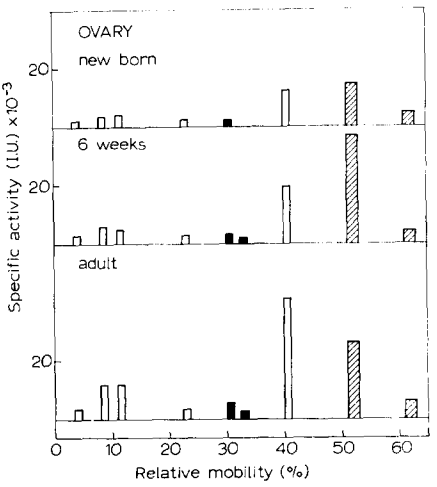


Fig. 12. The developmental progression of esterase forms in cavian uterus. Representations of the type of esterase activity are the same as in Fig. 3.

Fig. 13. The developmental progression of esterase forms in cavian ovary. Representations of the type of esterase activity are the same as in Fig. 3.

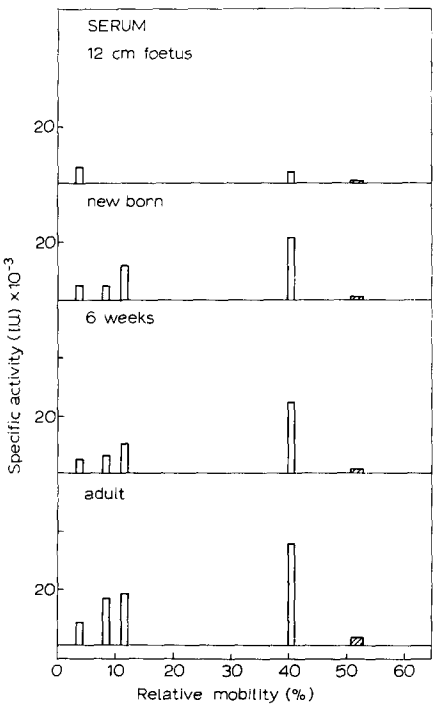


Fig. 14. The developmental progression of esterase forms in cavian serum. Representations of the type of esterase activity are the same as in Fig. 3.

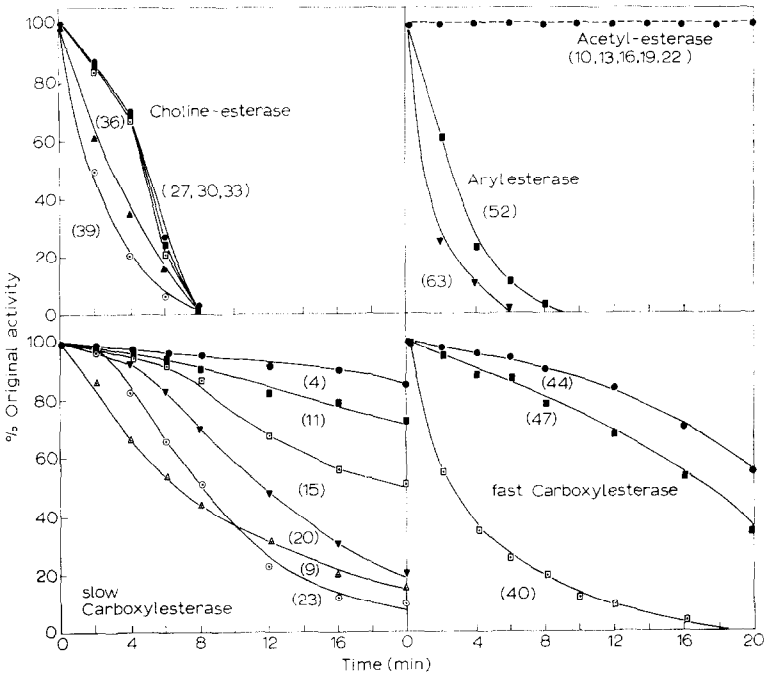


Fig. 15. The effect on esterase activity of incubation at 55° for varying lengths of time. Sequential activities are represented as a percentage of the original activity.

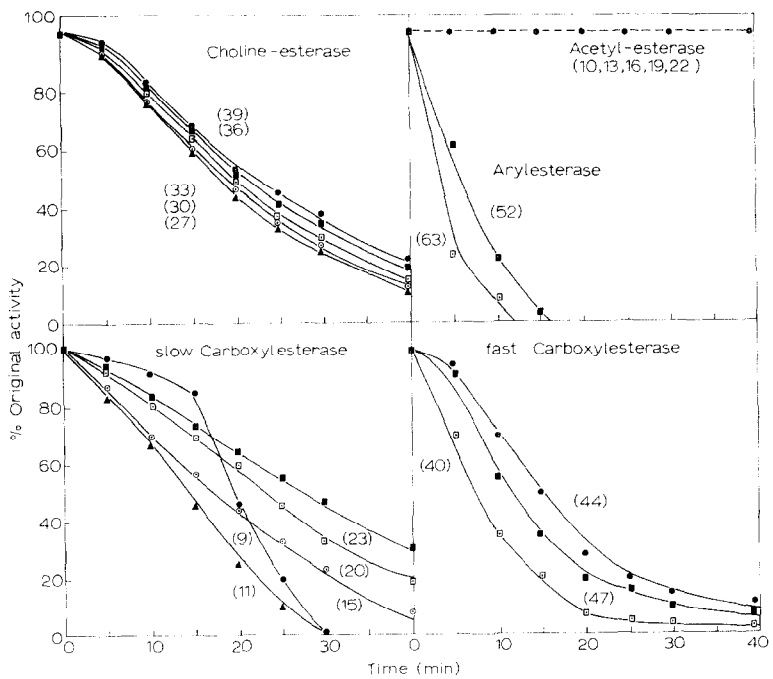


Fig. 16. The effect on esterase activity of incubation with 10 M urea for varying lengths of time. Sequential activities are represented as a percentage of the original activity.

(a) Carboxylesterases. (i) Slow-moving group. All five bands appear to be relatively heat stable, but there were quite marked differences between the individual curves. In particular, the band with *Rm* 9 lost activity more quickly than the adjacent gel band (*Rm* 11), and band *Rm* 15 was distinguished from bands *Rm* 20 and 23. (ii) Fast-moving group. The carboxylesterase which appeared as the predominant component in serum (*Rm* 40) was appreciably more heat labile than the 2 faster-moving carboxylesterase components (*Rm* 44, 47).

(b) Arylesterases. These 2 bands of esterases were very heat labile and disappeared almost completely after only 2 min incubation at 55°.

(c) Cholinesterases. All bands of cholinesterase activity were heat labile and disappeared completely after 8 min incubation.

(d) Acetylesterases. The five forms of this esterase were very stable at high temperature with no visible loss in activity occurring over the 20-min period.

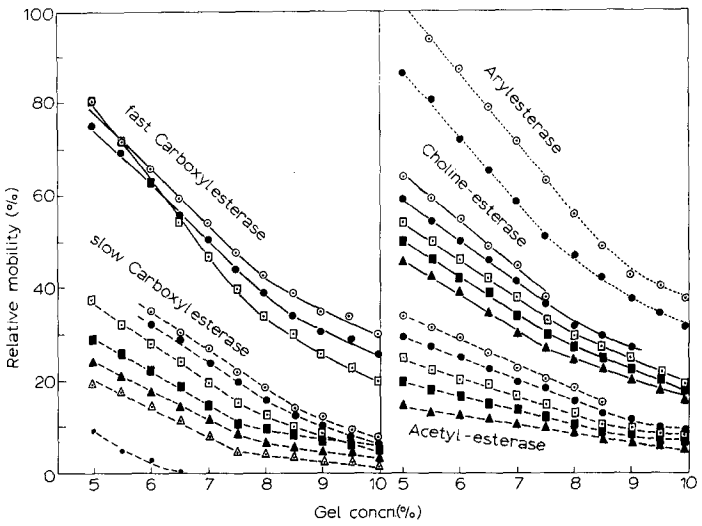


Fig. 17. The variations in the relative mobility of the different types of esterases with alteration of polyacrylamide-gel concentration. Bromophenol blue mobility is taken as 100%.

The results of urea treatment (Fig. 16) were:

(a) Carboxylesterases. (i) In the slow-moving group all five bands lost activity slowly but the faster-moving band at *Rm* 23 did so at a slower rate than the others, and the response of *Rm* 11 is distinctive. (ii) In the fast-moving group the serum carboxylesterase (*Rm* 40) lost activity at a faster rate than the other two (*Rm* 44, 47).

(b) Arylesterases. These two bands were labile to this treatment, as well, and disappeared almost completely after 10 min incubation.

(c) Cholinesterases. All bands gradually lost activity but it was noted that the faster bands were slightly more stable than the slower.

(d) Acetylesterases. These five bands are very stable to 10 M urea and no visible loss of activity occurred.

The variations of mobility with polyacrylamide-gel concentration are shown in Fig. 17. It is noticeable with the slow carboxylesterases that the curves are nearly

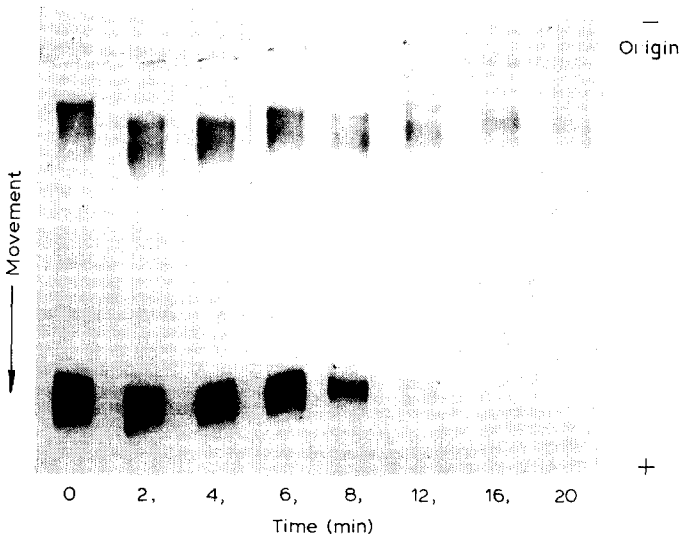


Fig. 18. Sequential zymograms illustrating the effect of incubation at 55° for varying lengths of time on serum carboxylesterase activity.

parallel. One slow carboxylesterase is accommodated on the gel at low concentrations but not above 7%.

In the fast carboxylesterases, the band with *Rm* 40 at 7.5% concentration gives a curve which differs in slope from the other two components.

In the other esterase types, a general similarity of response is evident within the individual groups.

In order to illustrate the methodology of the physicochemical studies more clearly, a photograph is included (Fig. 18) which follows the change of esterase activity during increasing periods of incubation at 55°. The labile band at the bottom of this photograph is a carboxylesterase (*Rm* 40).

DISCUSSION

In recent years, the developmental progressions of isoenzymes have afforded considerable insight into the genetic and structural inter-relationships of multiple enzyme forms, the physiological significance of this multiplicity, and the control of enzyme synthesis during tissue differentiation[15–18]. Even with well-defined systems such as mammalian lactate dehydrogenase, however, it has become increasingly evident that comprehensive detail is necessary in this type of study in order to avoid incorrect interpretations of the data[19]. This principle applies with particular force to the esterases, because of the extensive heterogeneity of these enzymes, their overlapping biochemical characteristics, and the difficulty of assessing isoenzyme status from the limited information which has been available in the literature. It is with this background in mind that this intensive treatment of cavian esterases has been presented as a unified study, and the desirability and advantages of this procedure are indicated further during the following discussion.

One particularly noticeable deficiency in the available information on esterases

in development has been the lack of specific activity data for these enzymes. Most previous reports have been confined to histochemical indications of alterations[20–22], and, on this basis, general increases in activity during maturation have been noted. The present results, however, make evident a more graduated response. While some tissues (*e.g.* liver, and intestine) exhibit a large increase of total esterase activity during maturation, others (*e.g.* brain, spleen, lung) show relatively minor changes. Also, post-natal and post-pubertal decreases of specific activity are evident in some tissues, as well as the more widely recognized increases at these stages. In most tissues, birth is accompanied by quite a sharp increase in total esterase activity.

The causation 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[23–25]. Having localized the alterations in this case, however, more specific causalities may now be investigated. It is intended, for example, that the relationship between the post-pubertal increase in testis esterase and the response to specific androgens be further examined. Suggestive evidence for a general relationship of this type is already available with the rat[26]. Also, the decrease of intestinal esterases in the later stages of maturation may be an additional reflection of an increased production of androgenic hormones. These steroids are known to depress the synthesis of cholinesterases[27], and in these investigations, it has been observed that the intestine and liver of adult males contain consistently lower levels of this class of esterase activity than the corresponding females. Again, an explanation for the considerable prenatal increase in the renal esterase of this animal may lie in the comparative anatomy of guinea-pig kidney, which displays an unusually mature development at birth[21]. Another enzyme which exists in multiple forms, lactate dehydrogenase, has also been shown to exhibit a similar, early, developmental increase in cavian kidney*.

In regard to the multiformity of esterases, the present studies have revealed the existence of a more extensive heterogeneity than has been entertained for these enzymes previously. A total of twenty four 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 esterolytic proteins.

The distribution of these multiple forms in the tissues of the adult guinea pig is clearly tissue specific: no two tissues amongst those sampled possessing patterns of activity which are closely similar. Adult liver (Fig. 3) presents the most complex pattern of these cavian tissues, and it is of interest to note that the banding is broadly similar to that previously reported for this organ by PAUL AND FOTTRELL[6]. In the present study, however, additional regions of activity are discernible, and the total activity may be seen to be constituted from several bands each of carboxylesterase, cholinesterase, and arylesterase type. Similarly, the unique distribution of esterases in adult testis of this species (Fig. 7) is characterized not only by the number of bands and their relative mobilities and activities, but also by the acetylesterase content of this organ; and the intestinal esterase pattern (Fig. 5) is distinguished by the major contribution of cholinesterase, relative to the slow carboxylesterases.

* C. J. MASTERS, unpublished results.

The many facets of esterase characterization, illustrated by these examples point to a noteworthy difference between the tissue specificity of esterolytic enzymes, and the more familiar, but less complex patterns of the dehydrogenase isoenzymes[18]. 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[6,28,*].

It has been reported by some workers that no differences occur in the electrophoretic patterns of esterases between prenatal and adult tissues[6,29], but in these studies a distinct ontogenetic variation is manifest, with clear indications of epigenetic control (Figs. 3–14). In general, these developmental alterations of pattern are not synchronous between the individual tissues, but differ markedly in the nature of the progression with the different enzyme sources. The patterns 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.

It is noticeable that the individuality of these esterase patterns on the different cavian tissues is discernible at an earlier stage of development than is the case with the multiple forms of lactate dehydrogenase, even though the latter system has been widely used as an index for tissue differentiation. This is probably a further reflection of the comparative sensitivity of the esterase parameter; but it may also indicate that metabolic differences which involve the physiological roles of esterases, are implicated at an earlier stage of maturation than variation of the type of carbohydrate metabolism[16,18]. At the present time, however, further evaluation of the functional significance of these developmental changes appears to be dependent on the provision of additional ontogenetic and metabolic detail.

No previous studies of the ontogeny of the multiple forms of cavian esterases appear in the available literature, and there are few developmental studies on mammals for comparison. MARKERT AND HUNTER[1,7] have noted a small increase of complexity in the esterase patterns of mice with increasing maturity, and, more recently, BLANCO AND ZINKHAM[28] have reported similar conclusions in regard to human esterases. None of the previous developmental studies have reported results in the form of specific activities of individual multiple forms, however, and little attempt has been made to correlate the identity of individual bands in different tissues. Also, the reported heterogeneity has, in general, been considerably less than that detailed in this communication. Overall, then, this limited developmental background serves to emphasize the desirability of further comprehensive investigations in this field, so as to enable a more complete understanding of the gene expression of the esterase multiple forms.

In these studies, the arylesterase activity of cavian tissues has been resolved into four separate bands. Only one or two forms have been reported in mammalian tissues previously[3,10,31], but it will be of interest to see whether some of these activities are further divisible under the special conditions defined in this present study. The nature of the fine sub-banding observable at low gel concentrations (see RESULTS) is currently under further investigation.

* R. S. HOLMES AND C. J. MASTERS, unpublished results.

The developmental behaviour of the major cavian arylesterase forms in the guinea pig with individual occurrence of each broad band (*Rm* 52, 62) and individual variation of activity evident in different tissues, appears to implicate separate genetic control for each doublet; and the physicochemical studies confirm the group characteristics of these bands.

The technique of studying the size or shape of proteins by means of the relative retardation of electrophoretic migration that is induced by increasing gel concentrations, has been employed successfully by a number of investigators[41-44]; and the similarity of the graphic analyses in this case (Fig. 17) rules out the possibility that polymerization is the determinant of the arylesterase band differences. Instead the enzymes appear to resemble each other closely in size or shape, but show distinct differences from the other multiple forms of esterases.

Further confirmation of the similarities in protein structure between the main arylesterase forms is provided by the close resemblance of the responses to heat and urea treatment (Figs. 15 and 16); and the retention of identity of these forms under conditions favouring alteration of the tertiary or quaternary structure along with the individual developmental occurrence of the bands, weighs against the possibility of the multiplicity resulting from configurational variations of a single native structure. Another possible interpretation, the production of artifacts during manipulation, would seem to be obviated as far as possible in these studies by means of the mild procedures used during the extraction and separation of the enzymes, and the minimizing of handling. Also, the reproducibility of the zymograms, the developmental characteristics, and the lack of artifact formation in the denaturation studies, are at variance with this interpretation.

When all this developmental and physicochemical data is considered in relation to the substrate and inhibitor studies, the collective information would seem to indicate that the different arylesterase forms possess similar active sites but differ primarily in the electrical charge of the carrier protein. The data presented to date is consistent with an interpretation of divergent genes governing the synthesis of the cavian esterases, but confirmation of this concept will require more extensive information on the primary structure and genetics of these enzymes.

Earlier workers have provided evidence suggesting an endocrinological influence on the synthesis of arylesterases[3,24], and increases in arylesterase activity are observable during the development of sexual organs (testis, ovary and uterus) in this species, whereas many other tissues which are not the direct target of these hormones (*e.g.* liver, kidney, intestine, skeletal muscle and brain) exhibit post-natal decreases of arylesterases in the development patterns. The changes of activity in the former set of tissues involves the doublet at *Rm* 52 more than that at *Rm* 62, so that hormonal effects may be differential in respect of the multiple forms.

This communication would appear to provide the first report of heterogeneity occurring in the acetylesterase of mammalian testis and epididymis. Five distinct bands may be recognized (Fig. 7) and the relative activity of these forms follows an approximately binomial distribution. These facts raise the interesting possibility of tetramer structure, dual gene control and hybridization for these enzymes, but the clarification of this inter-relationship requires additional data. At present, the work of SCHWARTZ[31] with maize provides the only recognized instance of hybrid forms of esterases in a higher organism.

The physicochemical parameters again confirm the similarity of group characteristics with these five multiple forms of acetylesterase, and contraindicate polymerization or configurational variants as causes of heterogeneity. All forms of the enzyme are noticeable for their considerable stability to heat and urea treatments, and the individual molecular types are indicated as possessing a regular and graduated relationship of conformation within a narrow size range.

Some acetylesterase activity was noticeable also, in the adult kidney of the guinea pig, and in this regard it should be noted that BERGMANN, SEGAL AND RIMON[4] have previously isolated two acetylesterases from pig kidney.

As many as five bands of cholinesterase activity are observable in these studies. In considering the significance of this finding a clear distinction should be made between the cholinesterases of sera and those of tissues. Most previous studies of mammalian cholinesterase activity have been concerned with the activity of sera, and SVENSMARK[32] in a recent review has considered the possibility of four allelic genes controlling this enzyme formation. The molecular weights of these serum enzymes in mammals have been estimated to be over 300 000 (ref. 6), however, and it is not to be expected that such a large molecule would be accommodated on the polyacrylamide-gel concentration used in these investigations. Instead it is suggested that the bands observable in these results represent the activity of proteins of lower molecular weight. This interpretation is supported by the lack of cholinesterase bands in these studies of cavian sera (although cholinesterase activity has been previously reported for this source)[3], the mobility–gel concentration studies (Fig. 17) and an earlier finding that human tissue cholinesterase contained low molecular weight enzyme[32]. SVENSMARK considers that tissue cholinesterases may bear a precursor relationship to the serum activity[6].

The situation in this animal also allows the exclusion of the possibility that the presence of cholinesterase in any of the cavian tissues is due to serum contamination, although this suggestion has been made to cover the observation of this activity in some other mammalian tissues[33].

The developmental expression of these multiple forms of tissue cholinesterase displays little variation of pattern; most of the activities occurring in the intermediate bands with no evidence of terminal dominance. These observations would appear to rule against the possibility of either independent genetic control or these five forms, or of two independent genes governing the synthesis of these forms through polypeptide hybridization. Similarly polymerization appears to be ruled out as an explanation of this multiplicity on the basis of the gel concentration studies (Fig. 17). Instead the physicochemical properties indicate that the five forms possess closely related shapes. 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. In earlier investigations, sialoprotein cholinesterase has been identified in mammalian tissues and sera, with more than five sialic acid residues having been estimated in some cases, and the loss of the sialic acid moiety has been shown to affect mobility without altering the activity[32,34,35].

The carboxylesterases present the most complicated heterogeneity of all the esterase classes examined in the guinea pig. As a first step, the ten recognizable bands may be divided into two groups on the basis of their mobility at various gel concentrations and their substrate specificity; the individual components of these groups

may then be further differentiated on the basis of other characteristic properties.

In regard to the group of slow-migrating carboxylesterases, there is evidence available in the literature to support the concept of a tetramer structure for this type of enzyme in other species[36], and, hence, the possibility of interacting subunits under the control of separate alleles must be considered for this cavian subgroup. Perhaps the best indication of parental forms in these studies would be the occurrence of single bands of activity in some tissues, and this is observable with two enzyme forms; a band (*Rm* 15) in foetal spleen and kidney (Figs. 4 and 11), and one (*Rm* 23) in adult intestine, and foetal brain and heart (Figs. 5, 8 and 10). The electrophoretic migration of these particular forms in relation to the other slow carboxylesterase bands, and the apparent lack of binomial-type distributions in this subgroup, would appear to weigh against the possibility of an inter-relationship of the mammalian lactate dehydrogenase type amongst these forms, however. Similarly, the phenotypic expression of these esterases in tissues such as brain, spleen and skeletal muscle (Figs. 9–11) seem to contraindicate band *Rm* 20 as representing an intermediate hybrid between bands *Rm* 15 and *Rm* 23. Furthermore, although bands *Rm* 9 and *Rm* 11 migrate in propinquitous electrophoretic positions, they differ markedly in their developmental behaviour and physicochemical properties. The remaining bands, *Rm* 0 and *Rm* 4, also possess individual characteristics, so that the slow carboxylesterases as a group show little indication of close genetic inter-relationships.

Apart from the common substrate and inhibitor specificities, there is little uniformity in the physicochemical properties of this group, either. Indeed these parameters exhibit a wider range of values in this subgroup than in any of the other esterase classes. Altogether, these indications of considerable conformational variation between the individual enzymes add support to an interpretation of the status of these multiple forms as a series of proteins of widely varying size and configuration, catalysing the same general type of reaction, but probably under independent genetic control.

The inter-relationships of the three enzyme forms in the faster-migrating group of carboxylesterases are more readily apparent. The band with *Rm* 40 possesses a number of characteristics which distinguish it from the other bands; for example, the ubiquitous distribution and individualistic gene expression (Figs. 3–14), the indications of a markedly different size and conformation (Fig. 17), and the responses of the additional physical parameters (Figs. 15 and 16).

The other two bands (*Rm* 43 and *Rm* 47) are usually observed to be both present at the same time in the developmental studies, with the latter band as the subordinate activity (Figs. 3–14). They also resemble each other in many of their other properties (Figs. 15–17), so that the available facts would suggest a responsibility of at least two structural genes for this subgroup.

Overall, then, the twenty four forms of soluble esterase activity which have been resolved in cavian tissues may be divided into four 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[45], although there is a case for further subdivision of the carboxylesterases on the basis of differences in substrate specificity. The developmental and physicochemical studies have confirmed these broad groupings of esterase types, but also allow further

differentiation and understanding of the individual inter-relationships within each of these groups.

A number of esterase polymorphisms under simple genetic control at a single locus have been described by previous investigators (one gene controlling the synthesis of a group of isoenzymes), and this has led other workers to suggest that similar relationships may hold in each of the separate mammalian esterase groups[37-40]. Such a proposal would seem inadequate in this instance, however, in view of the further evidence of differential properties within the cavian esterase groups. Instead of four genes controlling the synthesis of esterases as would follow from the application of this suggestion, the characteristics of the soluble esterases detailed in this investigation would appear to implicate at least twelve structural genes governing the synthesis of these enzymes.

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REFERENCES

1 R. L. HUNTER AND C. L. MARKERT, *Science*, 125 (1957) 1294.
2 C. L. MARKERT AND R. L. HUNTER, *J. Histochem. Cytochem.*, 7 (1957) 42.
3 K. B. AUGUSTINSSON, *Ann. N.Y. Acad. Sci.*, 94 (1961) 844.
4 F. BERGMANN, R. SEGAL AND S. RIMON, *Biochem. J.*, 67 (1957) 481.
5 D. J. ECOBICHON AND W. KALOW, *Can. J. Biochem. Physiol.*, 43 (1965) 73.
6 J. PAUL AND P. FOTTRELL, *Biochem. J.*, 78 (1961) 418.
7 R. L. HUNTER, J. T. ROCHA, A. R. PFRENDER AND D. C. DEJONG, *Ann. N. Y. Acad. Sci.*, 121 (1964) 532.
8 R. C. ALLEN, R. A. POPP AND D. J. MOORE, *J. Histochem. Cytochem.*, 13 (1965) 249.
9 M. HINKS AND C. J. MASTERS, *Biochemistry*, 3 (1964) 1789.
10 M. HINKS AND C. J. MASTERS, *Biochim. Biophys. Acta*, 113 (1966) 611.
11 L. ORNSTEIN AND B. DAVIS, (1962) *Disc Electrophoresis*, preprinted by Distillation Products Industries, Div. Eastman Kodak Company, Rochester, N.Y.
12 S. RAYMOND, *Ann. N.Y. Acad. Sci.*, 121 (1964) 350.
13 G. GOMORI, *J. Lab. Clin. Med.*, 42 (1953) 445.
14 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
15 C. L. MARKERT AND F. MØLLER, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 1210.
16 R. R. CAHN, N. O. KAPLAN, L. LEVINE AND E. ZWILLING, *Science*, 136 (1962) 962.
17 I. H. FINE, N. O. KAPLAN AND D. KUFTINEC, *Biochemistry*, 2 (1963) 116.
18 C. L. MARKERT, in M. LOCKE, *Cytodifferentiation and Macromolecular Synthesis*, Academic, New York, 1963, p. 65.
19 B. FIELDHOUSE AND C. J. MASTERS, *Biochim. Biophys. Acta*, 118 (1966) 538.
20 W. BUNO, *Acta Anat.*, 60 (1965) 285.
21 M. WACHSTEIN AND M. BRADSHAW, *J. Histochem. Cytochem.*, 13 (1965) 44.
22 J. M. ALLEN, *Anat. Record.*, 132 (1958) 195.
23 M. J. R. DAWKINS, *Brit. Med. Bull.*, 22 (1966) 31.
24 K. B. AUGUSTINSSON AND B. HENRICSON, *Acta Physiol. Scand.*, 64 (1965) 418.
25 A. A. M. LEWIS AND R. L. HUNTER, *J. Histochem. Cytochem.*, 14 (1966) 33.
26 M. NIEMI AND M. KORMANO, *J. Reprod. Fertility*, 10 (1965) 49.
27 P. L. RISLEY AND C. N. SKREPTETOS, *Anat. Record*, 150 (1964) 195.
28 A. BLANCO AND W. H. ZINKHAM, *Bull. Johns Hopkins Hospital*, 118 (1966) 27.
29 Y. CROISILLE, *Compt. Rend.*, 254 (1962) 2103.

30 K. B. AUGUSTINSSON AND S. BRODY, *Clin. Chim. Acta*, 7 (1962) 560.
31 D. SCHWARTZ, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 1210.
32 O. SVENSMARK, *Acta Physiol. Scand.*, 59 (1963) 378.
33 D. J. ECOBICHON AND W. KALOW, *Can. J. Biochem. Physiol.*, 42 (1964) 277.
34 H. HARRIS, D. A. HOPKINSON AND E. B. ROBSON, *Nature*, 196 (1962) 1296.
35 D. J. ECOBICHON AND W. KALOW, *Can. J. Biochem. Physiol.*, 41 (1963) 969.
36 K. B. AUGUSTINSSON AND B. OLLSEN, *Biochem. J.*, 71 (1959) 484.
37 S. L. ALLEN, *Genetics*, 45 (1960) 1051.
38 C. R. SHAW, F. N. SYNER AND R. E. TASHIAN, *Science*, 138 (1962) 31.
39 R. A. POPP AND D. M. POPP, *J. Heredity*, 53 (1962) 114.
40 M. L. PETRAS, *Proc. Natl. Acad. Sci. U.S.*, 50 (1963) 112.
41 A. L. KOEN AND C. R. SHAW, *Biochem. Biophys. Res. Commun.*, 15 (1964) 92.
42 S. RAYMOND AND M. NAKAMICHI, *Anal. Biochem.*, 7 (1964) 225.
43 D. J. ECOBICHON ,*Can. J. Biochem.*, 43 (1965) 595.
44 S. HJERTEN, *J. Chromatog.*, 11 (1963) 66.
45 E. C. WEBB, *Experientia*, 20 (1964) 592.