BBA 65667

A COMPARATIVE STUDY OF THE MULTIPLICITY OF MAMMALIAN ESTERASES

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(Received June 8th, 1967)

SUMMARY

1 Multiple forms of soluble esterase activity have been resolved in horse, sheep, ox and possum tissue extracts and sera.

2. By comparing esterase zymograms from different tissues and from different species, it is apparent that the distribution and multiplicity of esterase activity is tissue and species specific.

3. By means of substrate and inhibitor studies, the esterase multiple forms have been characterized into four main classes: carboxylesterases, arylesterases, acetylesterases, and cholinesterases. Each of these can be considered as an isoenzymic group.

4. Evidence is presented for further differentiation of activity within these isoenzymic divisions giving 2 groups of arylesterases, 3 groups of cholinesterases, and 5 groups of carboxylesterases.

INTRODUCTION

In recent years, the esterase activity of mammalian tissues has been shown to consist of complex groupings of multiple enzyme forms[1–7]. For example, 24 different forms of esterolytic activity have been resolved from guinea-pig tissue extracts[7], and have been classified on the basis of their differential substrate and inhibitor specificity as arylesterases (EC 3.1.1.2), carboxylesterases (EC 3.1.1.1), acetylesterases (EC 3.1.1.6) and cholinesterases (EC 3.1.1.8).

Furthermore the heterogeneity of esterases displays many characteristics which set it apart from other intensively investigated isoenzyme systems such as lactate dehydrogenase[8,9] and aldolase[10,11]. In illustration, previous studies in this laboratory demonstrated that two species of rodent, the rat[12] and the guinea pig[7], showed considerable divergences in regard to the extent of tissue esterase multiplicity, the electrophoretic mobility and physicochemical properties of these forms, and the developmental behaviour of the heteromorphs.

These gross differences between animals which are closely related phylogene-

tically emphasized the need for further comparative studies, and prompted this communication which describes the distribution, characterization, and isoenzyme status of esterase activity from 4 further mammalian species—ox, sheep, horse and possum.

METHODS

Tissue extracts

Soluble tissue extracts from the following mammals were studied: horse, ox, sheep, and ring-tailed possum. The required tissues were excised from the freshly slaughtered animals and stored at −10° until required. Blood specimens were collected in heparinized tubes, centrifuged, and the sera analyzed within hours of separation.

Electrophoresis

Homogenates were prepared in cold glass-distilled water and were centrifuged (100 000 × *g*, 30 min). Separation and characterization of the esterase multiple forms was achieved by electrophoresis of the supernatant on vertical columns of polyacrylamide gel (7.5%; pH 8.6), and subsequent histochemical treatment (for details, see our previous papers)[7,12].

Physicochemical properties of esterases

The urea (10 M) and heat (60°) lability of the esterase multiple forms was investigated as previously described[7,12].

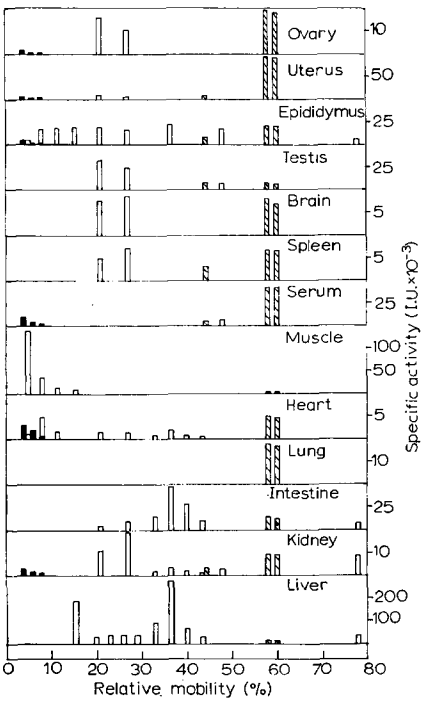
Esterase and protein assay

Total esterase activity was determined on a recording spectrophotometer (Unicam SP 800) by a modification of GOMORI's[13] method. Protein determination was performed by the biuret method[14] with bovine serum albumin for standards. Enzyme activity is expressed in I.U.B. units (μmoles α-naphthol released at 37° per min per mg protein).

RESULTS

Zymograms of horse tissue esterases are represented diagramatically in Fig. 1. Esterase activity is observable in all tissues examined, but the highest concentrations occur in liver, muscle, intestine, and epididymus. There are 16 multiple forms of carboxylesterases and 3 forms of arylesterase occurring in soluble horse tissue extracts. The relative distribution of these esterases, however, is tissue specific. Carboxylesterase represents the major contribution of esterase activity in most horse tissues examined, but arylesterase is predominant in lung, serum, and uterus. Table I summarizes the observed properties of horse tissue esterases. Several groups of carboxylesterases may be distinguished on the basis of their tissue distribution, electrophoretic mobility, substrate specificity, and heat and urea lability. For example, carboxylesterases (*Rm* 21, 27)* are distinguished by their preferential hydrolysis of acetyl esters and their stability to urea treatment, while the faster-

* *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%.



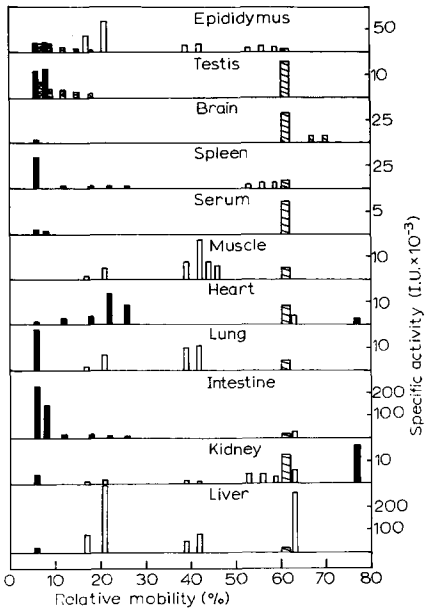


Fig. 1. Horse tissue esterase zymograms. Carboxylesterase activity is represented by open histograms, arylesterase by diagonal shadings, acetylesterase by cross hatching, and cholinesterase by complete shading.

Fig. 2. Sheep tissue esterase zymograms. Representations of the type of esterase activity are the same as in Fig. 1.

migrating carboxylesterases (*Rm* 48, 78) are distinguished not only by their electrophoretic mobility but also by their heat and urea lability.

Fig. 2 illustrates diagramatically sheep tissue esterase zymograms. The

TABLE I

PROPERTIES OF HORSE ESTERASES

Relative mobility	*Classification*	*Substrate specificity**	*Half life (min) (60°)*	*Half life (min) (10 M urea)*
4, 6, 8	Cholinesterase	Ac < Bu	< 2	< 5
5, 8, 12	Carboxylesterase	Ac < Bu	6	< 5
16	Carboxylesterase	Ac < Bu	10	5
20, 23, 26, 29	Carboxylesterase	Ac < Bu	3	5
21, 27	Carboxylesterase	Ac ≫ Bu	< 2	20
33, 37, 40, 43	Carboxylesterase	Ac < Bu	< 2	< 5
48	Carboxylesterase	Ac < Bu	< 2	< 5
44, 58, 60	Arylesterase	Ac < Bu	2	5
78	Carboxylesterase	Ac < Bu	< 2	< 5

* Ac, α-naphthyl acetate: Bu, α-naphthyl butyrate.

TABLE II

PROPERTIES OF SHEEP ESTERASES

Relative mobility	*Classification*	*Substrate specificity**	*Half life (min) (60°)*	*Half life (min) (10 M urea)*
6, 8	Cholinesterase	Ac <Bu	20	12.5
7, 9, 12, 15, 18	Acetylesterase	Ac ≫Bu	>20	>40
12, 18, 22, 26	Cholinesterase	Ac <Bu	<2	5
17, 21	Carboxylesterase	Ac <Bu	4	35
39, 42, 44, 46	Carboxylesterase	Ac <Bu	<2	30
53, 56, 59	Carboxylesterase	Ac ≫Bu	10	35
61, 67, 70	Arylesterase	Ac >Bu	<2	10
63	Carboxylesterase	Ac <Bu	<2	9
77	Cholinesterase	Ac <Bu	<2	7.5

* Ac, α-naphthyl acetate; Bu, α-naphthyl butyrate.

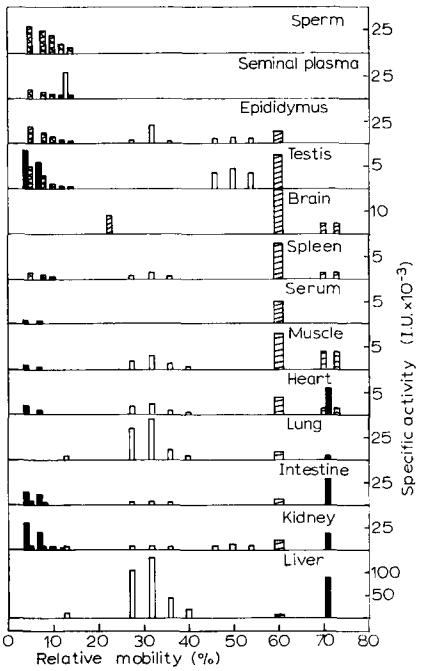
highest concentrations of esterase activity occur in liver, intestine, and epididymus, but activity was present in every tissue examined. There are 10 multiple forms of carboxylesterase, 7 forms of cholinesterase, 3 forms of arylesterase, and 5 forms of acetylesterase occurring in soluble sheep tissue extracts. Their relative distribution, however, is tissue specific. Carboxylesterase is the major esterase component in liver, muscle, and epididymus, while cholinesterase predominates in intestine, heart and spleen. Arylesterase is ubiquitous in sheep tissues and serum with brain being the best source for this type of activity; while acetylesterase activity is limited to sheep testis and epididymus extracts, Table II summarizes the properties of sheep tissue esterases. Carboxylesterase activity can be divided into various groups according to their differential properties: carboxylesterases (*Rm* 17, 21) represent the major liver carboxylesterases; (*Rm* 39, 42, 44, 46) is a heat-labile group with a specific tissue distribution; (*Rm* 13, 56, 59) is a urea-stable group hydrolysing preferentially acetyl-esters; and (*Rm* 63) is a heat-labile carboxylesterase. The 7 cholinesterases can be subdivided into 3 groups from a consideration of their properties: (*Rm* 4, 6, 8) being a heat- and urea-stable group, (*Rm* 12, 18, 22, 26) being a labile group, and (*Rm* 77),

TABLE III

PROPERTIES OF OX ESTERASES

Relative mobility	*Classification*	*Substrate specificity**	*Half life (min) (60°)*	*Half life (min) (10 M urea)*
4, 7	Cholinesterase	Ac <Bu	<2	8
5, 8, 10, 12, 14	Acetylesterase	Ac ≫Bu	>20	40
13	Carboxylesterase	Ac <Bu	4	30
22	Arylesterase	Ac ≫Bu	<2	5
27, 32, 36, 40	Carboxylesterase	Ac <Bu	3	30
46, 50, 54	Carboxylesterase	Ac ≫Bu	16	40
60, 70, 73	Arylesterase	Ac ≫Bu	<2	5
71	Cholinesterase	Ac <Bu	<2	6

* Ac, α-naphthyl acetate; Bu, α-naphthyl butyrate.



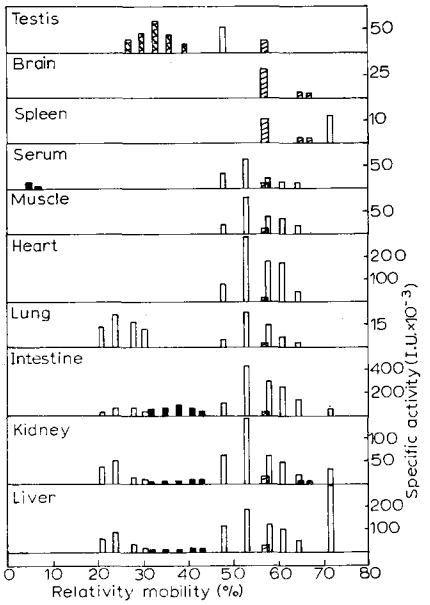


Fig. 3. Ox tissue esterase zymograms. Representations of the type of esterase activity are the same as in Fig. 1.

Fig. 4. Possum tissue esterase zymograms. Representations of the type of esterase activity are the same as in Fig. 1.

a labile and probably low molecular weight cholinesterase. Arylesterases are labile to heat and urea incubation, however, acetylesterase activity is distinguished by its extreme stability to these treatments.

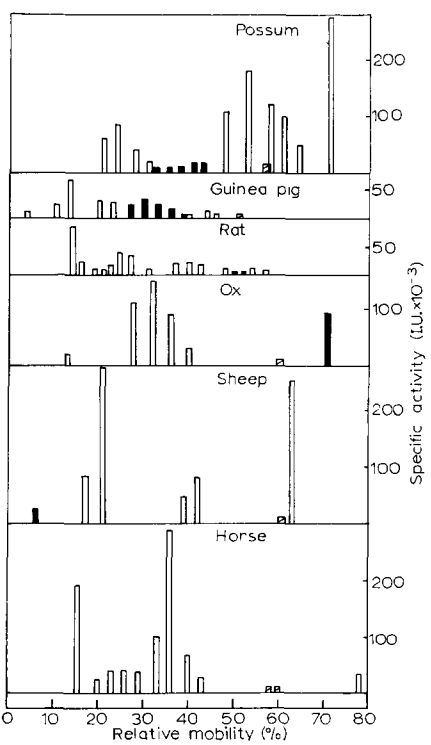
Ox tissue esterase zymograms (Fig. 3) illustrate that highest esterase concentrations occur in liver, epididymus, lung and kidney, and that significant amounts of esterase occur in other tissues also. Eight multiple forms of carboxylesterase, 3 cholinesterases, 4 arylesterases, and 5 acetylesterases occur in soluble ox tissue extracts, and their relative distribution is, again, tissue specific. High concentrations of carboxylesterase occur in liver and lung, cholinesterase predominates in intestine, arylesterase occurs in most tissues with brain being the best source, and male sex tissue extracts reveal high acetylesterase activity. Analysis of the properties of the ox tissue esterases (Table III) confirms the differentiation of activity into 4 groups and reveals further subdivision within these groups. The liver carboxylesterases (*Rm* 27, 32, 36, 40) are distinguished from another group of similar activity (*Rm* 46, 50, 54) on the basis of substrate specificity and heat lability—the latter group preferentially hydrolysing acetyl esters and being relatively heat stable. Differences in electrophoretic mobility and tissue distribution would suggest that cholinesterase activity can be subdivided into 2 groups, (*Rm* 4, 7) and (*Rm* 47), and that arylesterase activity consists of 3 groups—(*Rm* 22), (*Rm* 60), and (*Rm* 70, 73). Acetylesterase properties confirm the proposition of a closely related group of enzymes which is very heat and urea stable.

TABLE IV

PROPERTIES OF POSSUM ESTERASES

Relative mobility	*Classification*	*Substrate specificity**	*Half life (min) (60°)*	*Half life (min) (10 M urea)*
5, 7	Cholinesterase	Ac $<$ Bu	<2	5
21, 24, 28, 31	Carboxylesterase	Ac $<$ Bu	3	10
27, 30, 33, 36, 39	Acetylesterase	Ac $\gg$ Bu	>20	>40
32, 35, 37, 41, 43	Cholinesterase	Ac $<$ Bu	<2	15
48	Carboxylesterase	Ac $<$ Bu	2	<5
53, 58, 61, 64	Carboxylesterase	Ac $<$ Bu	12	25
57, 65, 67	Arylesterase	Ac $\gg$ Bu	<2	7.5
72	Carboxylesterase	Ac $<$ Bu	3	10

* Ac, α-naphthyl acetate; Bu, α-naphthyl butyrate.



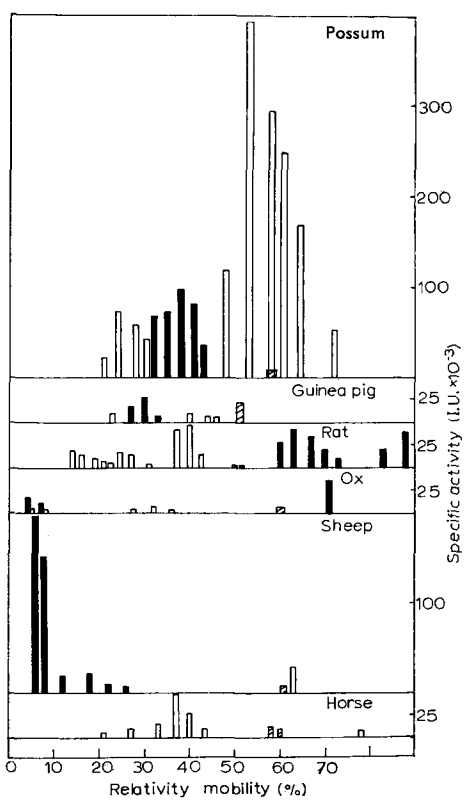


Fig. 5. Mammalian liver esterase zymograms. Representations of the type of esterase activity are the same as in Fig. 1.

Fig. 6. Mammalian intestine esterase zymograms. Representations of the type of esterase activity are the same as in Fig. 1.

The distribution of esterase multiple forms in various possum tissues is given in Fig. 4. Esterase activity was observed in all tissues examined, with intestine, liver, heart, kidney, and testis being the more active sources. Ten carboxylesterase forms, 7 cholinesterases, 3 arylesterases, and 5 acetylesterases were separated from these sources, and their distribution found to be tissue specific. Carboxylesterase was the most active esterase type in all possum tissues except brain and spleen, which contained high arylesterase activity, and testis, which revealed a predominant acetylesterase activity. The properties of the possum esterases (Table IV) generally support the overall division of esterase activity into 4 classes, and provides evidence for further subdivision in the carboxylesterase group, from which 4 subgroups can be distinguished: (*Rm* 21, 24, 28, 31), (*Rm* 53, 58, 61, 64) and (*Rm* 72).

Figs. 5 and 6 present a comparison of esterase zymograms from mammalian liver and intestine extracts, respectively, and demonstrate the species specificity of carboxylesterase and cholinesterase activity, relative mobility and degree of multiplicity.

DISCUSSION

The distribution of total esterase activity in mammalian tissues, was investigated by HUGGINS AND MOULTON[15] in 1948, using esters of *p*-nitrophenol as substrates, and by NACHLAS AND SELIGMAN[16] in 1949, using histochemical techniques. These workers observed that some tissues such as liver, small intestine, renal cortex, and testis, contained large amounts of esterase activity irrespective of the animal source, whereas the activity of other tissues varied widely from species to species. Activity data presented in these studies (Figs. 1–6) generally support these observations, and clearly indicate species specificity in the distribution of esterase activity.

In 1953, ALDRIDGE[17,18] characterized serum esterases from a variety of animals on the basis of their substrate and inhibitor specificity. He showed that the relative activities of the E 600 (*O,O*-diethyl *O*-(*p*-nitrophenyl) phosphate)-resistant arylesterase and the E 600-sensitive carboxyl- and cholinesterases varied with the species, and reported predominant arylesterase activity in horse, ox, and sheep serum, while guinea-pig and rat serum contained mostly carboxylesterase. Also an extensive survey on the multiplicity of esterases in vertebrate blood plasma was reported by AUGUSTINSSON[3] in 1961. He observed that aryl, choline and carboxylesterases exist in multiple forms, and that each animal species has its own typical set of plasma esterases. Ruminant plasmas were found to have low cholinesterase activity, no detectable carboxylesterase, and significant amounts of arylesterase. Horse serum, however, contained all three types of activity. The more detailed results in this present investigation (Figs. 1–4) are in general agreement with these previous findings.

In regard to the distribution of esterase multiple forms, tissue specific patterns have been found previously in the mouse[2], human[4,6], and guinea pig[7]. By comparing the zymograms from these animals, together with other available results[5,19,20], it becomes apparent that the distribution and multiplicity of esterase activity is species specific. In the 4 mammals investigated in this study, for example, the individual tissues present very different distributions of activity, yet underlying species characteristics can be recognized in each case.

Liver (Fig. 5) is always a rich source of esterase activity, and contains pre-

dominant amounts of carboxylesterase, some arylesterase, and sometimes cholinesterase. The degree of multiplicity, the electrophoretic mobilities, and physicochemical properties of the major liver carboxylesterases vary from species to species. Intestine (Fig. 6) is also a good source of activity, and usually contains high percentages of carboxyl- and cholinesterases, as well as some arylesterases. The relative activities of these two predominant esterase types, as well as their degree of multiplicity and electrophoretic mobilities, however, vary with the species. Horse intestine is distinguished by its apparent lack of cholinesterase activity.

Male sex tissue extracts from ox, sheep, possum and guinea pig are characterized by their high acetylesterase activity, however, species differences occur since horse and rat testis extracts contain predominant amounts of carboxylesterase. Soluble brain extracts from all mammals studied in this laboratory contain arylesterase as the major esterase component. Kidney and epididymus tissue extracts from ox, sheep, and horse are characterized by the activity of a specific group of carboxylesterases which preferentially hydrolyse acetyl esters. Skeletal muscle extracts are usually distinguished by their low esterase activity, however, horse muscle (Fig. 1) and possum muscle (Fig. 4) can be seen to contain high activity which is associated with specific groups of carboxylesterases. MACRAE AND RANDALL[21] have recently investigated esterases from bovine skeletal muscle and have shown a higher degree of esterase multiplicity than is reported here (Fig. 3). Extraction procedures used by these workers, however, involved freezing and thawing which may solubilize particulate bound esterase and possibly give a different electrophoretic pattern to that of the soluble enzymes.

In these studies, plasma esterase activity has been resolved into several components. Many plasma enzymes have previously been found to consist of families of closely related enzymes derived from multiple tissue sources[22–26], and in view of the considerable clinical significance of this phenomenon, it is of interest to consider the possible tissue origin of the observed plasma esterase forms. Although the mechanisms by which tissue enzymes enter the plasma are complex and little understood, liver, intestinal mucosa, heart kidney, lung and genital organs are generally considered as the major sources on the basis of cell turnover and generous blood supply[27,28]. In the past, most workers have been of the opinion that plasma cholinesterase was derived from liver[27,29], but these investigations into the tissue distribution of the high molecular weight plasma type cholinesterases provide evidence for multiple tissue sources for this enzyme. Horse heart, kidney and genital tissue extracts (Fig. 1), sheep intestine, lung, and genital tissue extracts (Fig. 2) and ox kidney and intestine (Fig. 3) contained significant amounts of plasma cholinesterase activity, while liver extracts from these mammals exhibited little or undetected activity. Unless liver has an extraordinarily efficient and selective mechanism for secreting cholinesterases into the plasma, therefore, it would appear that other tissues are more likely contributory sources. Using similar lines of reasoning, the present date suggests multiple tissue contributions to plasma arylesterase and carboxylesterase as well.

Previous publications on the multiplicity of mammalian arylesterase have reported two forms[3,30], ArI and ArII, occurring in human, reindeer, dog, rabbit, and rat plasma. These forms show similar substrate specificity, but were distinguished on the basis of their electrophoretic mobility, metal ion inhibition, and their ability to

hydrolyse certain organophosphorus compounds. More recently, we have investigated the multiplicity of guinea-pig arylesterases[7], and reported two main groups of activity. The developmental behaviour and individual tissue distribution reported for each group, appeared to implicate separate genetic control.

In the present studies, two main groups of arylesterase activity are observable also, but further multiplicity exists in ox brain and in a variety of horse tissues. The major arylesterase component has an electrophoretic mobility of 50–60% of the bromophenol blue under these conditions, and migrates closely to the albumin. It exists in all mammalian tissues studied as a broad band of activity which, at low gel concentrations, reveals two sub bands. The second arylesterase component has a higher electrophoretic mobility (about 70%) and a more limited distribution in mammalian tissue extracts. It was not detected in horse tissues at all, and generally is only observed in other mammalian tissues of high arylesterase activity.

Acetylesterase activity exists in 5 multiple forms in the adult male genital tissue extracts of sheep, ox, and possum, and in ox seminal plasma and sperm. Some acetylesterase activity was noticeable also in ox kidney, intestine and spleen. Recently the authors observed 5 forms of acetylesterase activity in guinea-pig testis and epididymus[7] and established that an association existed between the appearance of acetylesterase activity and testis maturation, but the specific function of this enzyme is not yet clear. The physicochemical properties of the acetylesterases illustrate the similarity of the multiple forms, not only within a single tissue or species, but also between species. These enzymes have a low electrophoretic mobility under these conditions, and considerable stability to heat and urea treatments and are distinguished from the other esterases by their insensitivity to normal esterase inhibitors and their preferential hydrolysis of acetyl esters.

In considering the multiplicity of mammalian cholinesterase activity, it is necessary to emphasize the distinction between cholinesterase of sera and those of tissues. Human[31] and equine[32] serum cholinesterases have been investigated, and their molecular properties summarized in a recent review by SVENSMARK[29]. They have an estimated molecular weight of over 300 000 which would explain their low electrophoretic mobility on polyacrylamide gel, and exist in 4 multiple forms, each as a sialo protein. The nature of this multiplicity does not seem to be dependent upon the number of bound sialic acid residues since neuraminidase incubation reduces the mobility of each multiple form to the same extent[33]. They appear then to be 4 different sialo proteins, and it has been proposed that there are four allelic genes governing their synthesis[29]. Tissue cholinesterases also exist in multiple forms. SVENSMARK[34] gave evidence that human liver cholinesterase activity contains a "plasma like" enzyme, a high molecular weight sialic acid free enzyme, and a low molecular weight enzyme. More recently, the present authors observed multiple forms of cholinesterase activity in guinea-pig tissues.

The present communication illustrates the species specificity of cholinesterase activity, distribution, and multiplicity in a variety of mammalian tissues and sera. Considering the results overall, it would appear that there are three distinguishable types of cholinesterases in mammalian tissues:

(1) A high molecular weight group of enzymes which are identical to those in plasma. They exist in 2 or more multiple forms and are present in all mammals studied. This group has a low electrophoretic mobility ($Rm < 10$).

(2) An intermediate group of cholinesterases which occurs in a variety of possum, guinea pig, and sheep tissues, and also in rat intestine. It exists in 4–5 multiple forms and has an intermediate electrophoretic mobility (*Rm* 10–70).

(3) A group of low molecular weight cholinesterases of high electrophoretic mobility (*Rm* > 70). It exists in 1 or 2 forms and occurs in a variety of sheep, ox, and rat tissue extracts.

Carboxylesterase activity exists in a large number of multiple forms and presents the most complex heterogeneity of all of the mammalian esterase classes examined. This type of activity shows large species variations in the degree of multiplicity and also the electrophoretic mobilities, tissue distribution, and activities of its multiple forms. By examining the properties of each multiple form in a particular species, however, the carboxylesterase activity can be differentiated into a number of groups, and by comparing the properties of each group from that species with similar groups from other species, it would appear that mammalian carboxylesterase activity can be tentatively subdivided into at least 5 groups:

(1) The first group, which migrates slowly under the electrophoretic conditions used in these studies (*Rm* 0–45), usually accounts for most of the esterase activity in mammalian liver extracts, and occurs in a wide variety of mammalian tissues. This type of carboxylesterase has been purified from pig[35,36], ox* and goat[37] tissue extracts, and a molecular weight of about 150 000 is reported in each case Horse displays a high degree of multiplicity for this group, while others (sheep, possum, and ox) show much simpler patterns of activity.

(2) Another group of carboxylesterases are distinguished by their preferential hydrolysis of acetyl esters. This type of activity exists in 2 or 3 multiple forms in ox, sheep, and horse tissue extracts but was undetected in the other animals.

(3) A heat- and urea-labile serum carboxylesterase constitutes a third type of activity. It appears to be of low molecular weight and has an intermediate electrophoretic mobility under these conditions (*Rm* 40–50). It occurs in rat, guinea-pig, possum and horse serum and tissue extracts, but was undetected in the ruminants.

(4) This group is heat and urea stable, appears to have a lower molecular weight than Group 1, and is usually present in high concentrations in serum. It has a high electrophoretic mobility (*Rm* 40–70) and exists in 2–4 multiple forms in possum, rat, and guinea-pig tissue extracts, but was undetected in horse, ox, and sheep.

(5) A carboxylesterase exists in some mammals which has a very high electrophoretic mobility. This enzyme has a low molecular weight and is distinguished from the 2 previous groups of activity by its physicochemical properties and its tissue distribution. It occurs in a variety of sheep, horse, and possum tissue extracts but was undetected in the other mammals investigated.

At this point it would seem appropriate to re-emphasize that the purpose of presenting these groupings has been to facilitate comparisons between these complex systems of multiple enzyme forms, and to provide a basis for further detailed investigation. It is not an intention to imply that common physicochemical properties within groupings signify a close structural similarity of the individual proteins; indeed, the data available at present** suggest that many individual characteristics

* M. RUNNEGAR, E. C. WEBB AND B. ZERNER, private communication.

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

and many different modes of heterogeneity may be recognizable within some of these groupings.

In summary, then, the multiple forms of soluble esterase activity, which have been resolved in mammalian 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 isoenzymic system in terms of the definition recommended by the Standing Committee on Enzymes[38]. These studies, however, present evidence for further differentation of activity within these isoenzyme divisions, giving 2 groups of arylesterases, 3 groups of cholinesterases, and 5 groups of carboxylesterases.

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.

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