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Electrophoresis Studies on Blood Plasma Esterases

III. Conclusions

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A discussion is given of the results obtained in a comparative study of the esterases present in the plasmata from 27 various vertebrates. Each species has its own esterase pattern, and within each of the three groups of esterases, arylesterases (A-esterases), aliesterases (B-esterases) and cholinesterases (C-esterases), there are variations in the properties of individual enzymes. Arylesterase is the predominant esterase in mammalian plasmata, and is absent in the plasmata of birds, reptiles, amphibians and fish. Aliesterase (lipase) is the main esterase of lower vertebrate plasmata, and is not present in human, monkey, dog, ruminant, cock, or turtle plasmata. A propionyl-aliesterase is considered to be the most primitive type of esterase present in vertebrate plasmata, and other esterases including the various types of aryl- and cholinesterases are suggested to be phylogenetically evolved from such an esterase. The esterase present in turtle plasma is of an intermediate stage. The physiological function and origin of the plasma esterases are briefly discussed.

The plasma protein patterns of little piked whale, sea-scorpion and mackerel are illustrated.

A number of investigations have demonstrated that there are appreciable and characteristic variations in plasma (serum) protein patterns with animal species. Significant differences in the electrophoretic patterns for various species have been observed in respect of mobilities, number and relative proportion of protein components, and presence or absence of components [1–5]. Such electrophoresis studies were performed either with the Tiselius apparatus or with the paper electrophoresis technique. Only rarely were the biochemical properties of individual proteins investigated in these comparative studies. In the present investigation, the results of which were reported in the first two papers of this series [6] and in a preliminary paper [7], preparative electrophoresis on cellulose columns was used to separate esterase-active components for studies of the properties of individual enzymes with respect to specificity and sensitivity to various selective esterase inhibitors. Such investigations were regarded to be of importance because previous differentiation of these enzymes

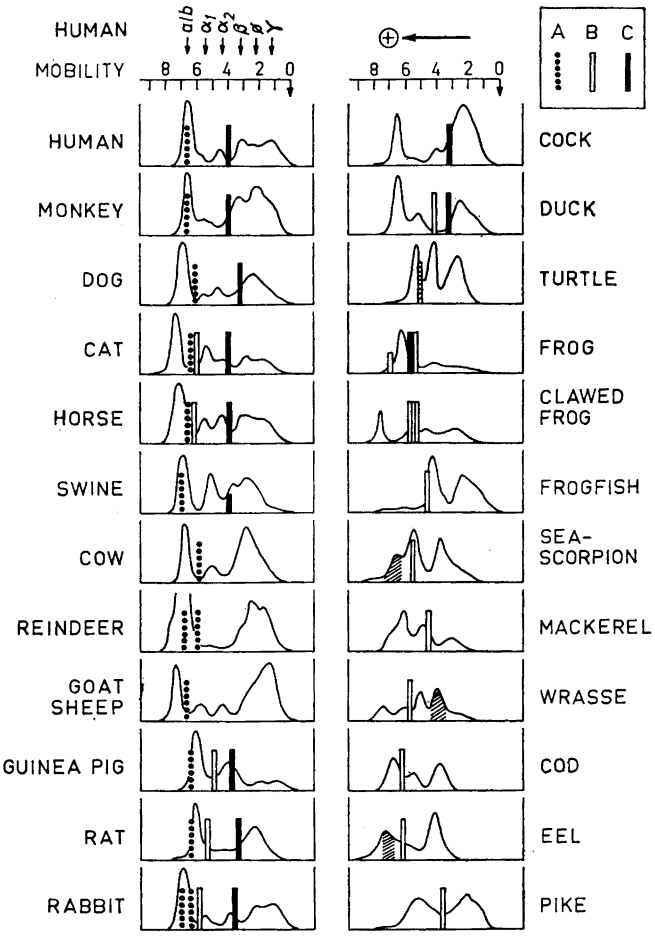

Fig. 1. Comparison of the electrophoretic mobilities of proteins and esterases of vertebrate plasmata relative to those of human plasma proteins (marked at the top of the graph). Experimental conditions: 5 ml plasma fractionated on cellulose columns (3 cm × 40 cm) for 30 h in veronal buffer (pH 8.4, $I = 0.1$) at an applied voltage of 260 V and 60 mA. The mobilities of human plasma proteins expressed in $cm^2V^{-1}s^{-1} \times 10^{-5}$ are those obtained with the Tiselius apparatus in the same buffer solution[8]. ————, protein; A, arylesterase; B, aliesterase and/or lipase; C, cholinesterase. The unique properties of the esterase of turtle plasma is specifically marked. Shaded areas refer to coloured proteins: blue for wrasse, green for sea-scorpion and eel.

was based mainly on work with original plasma (serum) or crude enzyme preparations. These previous studies have led to much difference of opinion as to the classification of plasma esterases.

Protein patterns. The absolute electrophoretic mobilities of various protein components were difficult to evaluate with the technique used. However, all

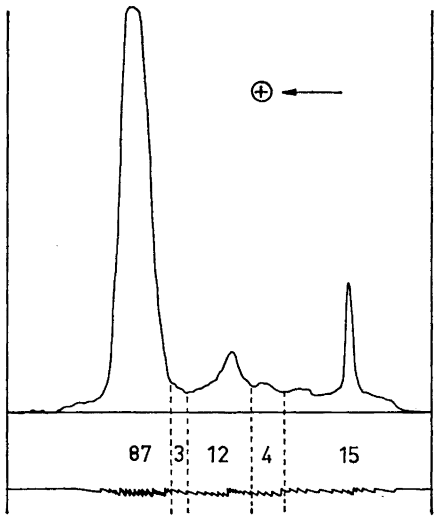

Fig. 2. Electrophoretic pattern of a plasma sample from *Balaenoptera acutorostrata* (little piked whale). Integrated curve from a paper electropherogram; veronal buffer (pH 8.6, $I = 0.1$), 150 V, 5 mA, 8 h. Numbers refer to relative protein concentrations.

electrophoresis runs and elutions were carried out under identical conditions, and therefore the relative mobilities of the components of various plasmata could be compared relative to those of human plasma proteins. Such a comparison of the results obtained with most of the plasmata studied is shown in Fig. 1. The results presented for individual species are based on a relatively low number of animals, but the patterns obtained are reproducible enough to demonstrate the species variation of protein patterns of the plasmata investigated. It will be remembered in this connection that individual variations in these patterns have been observed [1] in certain species. In the experience of the present author, such variations are particulary frequent for the esterase patterns of rabbit and swine plasmata. In addition, sex differences for certain vertebrate plasmata, *e. g.*, fowl plasma [2], have been reported, and alterations in anatomy and ecology during metamorphosis and growth has been shown to be accompanied by radical biochemical changes, also in the blood plasma proteins [9,10]. These individual variations, however, were not further investigated in the present investigation, since the electrophoretic plasma patterns of each species were generally reproducible with a few exceptions.

The electrophoretic patterns of all mammalian plasmata studied were characterized by a sharp albumin peak, the mobility of which varied relatively little from animal to animal. Albumin was also the predominant protein in a plasma sample from a whale, *Balaenoptera acutorostrata*, the electrophoretic pattern of which is shown in Fig. 2; the esterase activity of this sample was too low to be detected after electrophoresis. Proteins with similar mobilities to those of mammalian albumins were present in avian and amphibian plasmata, but not in the plasma of turtle. The absence of albumin-like serum proteins

in turtles was reported previously [11,12]. The influence of genetic and physiologic factors on plasma protein composition are obvious for amphibian and reptilian plasmata [13], and is particularly striking for teleostian plasmata [14,15]. Considerable differences were thus observed in the present investigation for two *Rana* species, the clawed frog *(Xenopus laevis)* which is a member of the peculiar family Aglossa, and seven teleostian fishes. Frogfish plasma was characterized by a sharp protein peak with a mobility similar to that of human plasma α_2-globulin. Other teleostian plasmata contained albumin-like proteins in varying amounts. It is of possible significance that the phylogenetically lowest teleostian studied, pike, had a very low amount of albumin-like protein, which was absent in the plasmata of both the spiny dogfish shark (an Elasmobranch), confirming a previous observation [16], and the hagfish (a Cyclostome). The electrophoretic pattern of pike plasma was also in other main characteristics similar to that of the elasmobranch plasma.

In certain species (cat, reindeer, rat, cock, duck, frog, eel) a small amount of protein, migrating somewhat faster than albumin, was present. These components had no relation to the esterases studied, and their relation to other plasma proteins is unknown. A fast-moving protein was present in a relatively high amount in the plasma of clawed frog.

Among all plasmata studied much variation was observed in the globulins, both those migrating between albumins and fibrinogen and the γ-globulins with lower mobility relative to that of fibrinogen. No distinct separation of β- and γ-globulins and fibrinogen was obtained in most cases. The α-globulins of several plasmata were more complex than those of human plasma. Turbidity, probably associated with lipoid, appeared to be caused in most cases by these components, especially the fast-migrating α-globulins. The amount of γ-globulins in mammalian plasmata varied considerably with species, and a component comparable to this protein was absent, or present in low amount, in most teleostian plasmata. Great variation in fibrinogen concentration was also observed. The amount of this component was particularly low in animals belonging to lower phylogenetic orders. In the whale plasma sample analysed, fibrinogen was apparently absent (Fig. 2).

Esterase patterns. Electrophoretic separation of plasma esterases has been reported only in a few cases. Human plasma *cholinesterase*, which precipitates together with albumin on ammonium sulphate fractionation [17,18], migrates on electrophoresis between the α_2- and β-globulin components [19–21]. Horse plasma cholinesterase behaved similarly on paper electrophoresis [22,23]. It was demonstrated in the present series of investigations that there was very little variation from animal to animal in the electrophoretic mobility of this type of esterase, when present. Frog plasma cholinesterase was an exception in this respect, with an unusually high electrophoretic mobility. Although the biochemical properties of these esterases varied considerably with species, the cholinesterases present in mammalian and avian plasmata behaved similarly on electrophoresis, with mobilities close to those of α_2 and/or β-globulins of human plasma. The low cholinesterase activity of ruminant and fish plasmata could not be detected after electrophoresis, but by indirect methods it was found that the cholinesterase present had an electrophoretic mobility close to those of other plasma cholinesterases. The unique single esterase of turtle

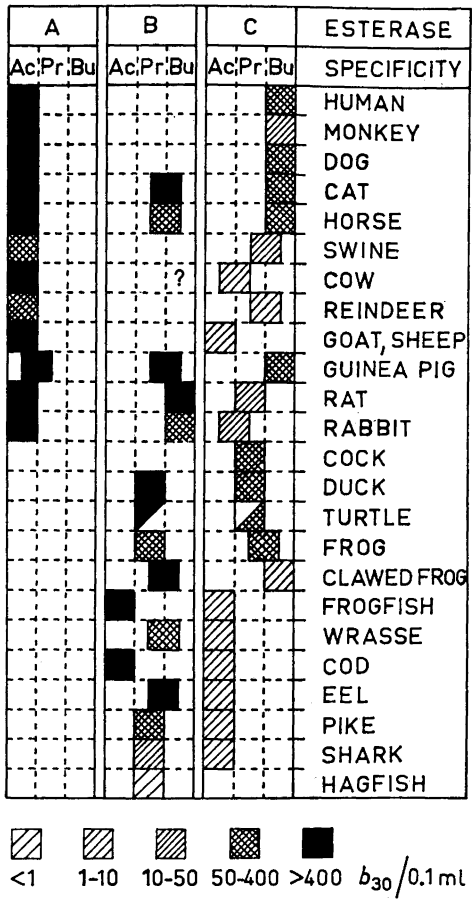

Fig. 3. Plasma-esterase patterns of vertebrates. Groups of esterases: A, arylesterase; B, aliesterase, lipase; C, cholinesterase. Relative esterase specificity based on the acyl radical of the substrate, selectively hydrolysed by the enzyme in each group: Ac, acetyl; Pr, propionyl; Bu, butyryl. Activity values in b_{30} per 0.1 ml plasma (μl CO_2 evolved from a bicarbonate — CO_2 buffer in 30 min at 25°C corrections made for the spontaneous substrate hydrolysis) refer to the hydrolysis rates observed with the substrate which is hydrolysed at the highest rate by each esterase. Monkey plasma is a sample from a macaque monkey. The properties of the esterase of turtle plasma are those of a propionyl-B- *and* propionyl-C-esterase. This histogram includes revisions in addition to that previously reported [7].

plasma moved at a higher rate than other plasma cholinesterases, but more slowly than the aliesterases of mammalian plasmata. The electrophoretic behaviour, as well as other properties of turtle-plasma esterase, are intermediate between the C- and B-esterases.

Great variation in the properties of cholinesterases present in various vertebrate plasmata was observed (Fig. 3). The cholinesterases of human, monkey,

dog, cat, horse and guinea-pig plasmata had the specificity of butyrylcholinesterases. A similar esterase was also present in low amounts in clawed-frog plasma. The ruminant plasmata had low cholinesterase activity, and the specificity of these esterases varied considerably from animal to animal, but in no case was a butyrylcholinesterase detected. Swine plasma cholinesterase had unique properties, and was present in high concentration in sow's colostum and milk [24,25]. An acetylcholinesterase was present in low concentration in the plasmata of goat and sheep and in those of all teleostians studied. Rat and rabbit plasmata were characterized by propionylcholinesterases, the properties of which were different with respect to sensitivity to quaternary ammonium compounds and organophosphorus compounds, respectively [6]. The plasmata of cock and duck also contained propionylcholinesterase, differing from both the rat and rabbit cholinesterases, *e. g.*, in the activity-substrate concentration relationship for choline esters. The esterase in turtle plasma had the properties of a cholinesterase in being highly sensitive to physostigmine, but differed from all other esterases of this type by hydrolysing choline esters at a lower rate than non-choline esters. It is suggested that this esterase is of a phylogenetically intermediate stage between the B-esterases present in high concentration in the plasmata of lower vertebrates, and the C-esterases of vertebrates belonging to higher orders. It is also of probable significance that the plasma esterase of turtle is a propionylesterase, since such an esterase type may be the most primitive one from a phylogenetic point of wiew. During the biochemical evolution, the propionyl- and butyrylcholinesterases might have originated from the B-esterases, the former being more specialized forms of the latter. The acetylcholinesterases of goat, sheep and fish plasmata and the propionylcholinesterase of rabbit plasma, the properties of which differed from those of most propionylcholinesterases of other vertebrates, might have a different source of origin. The similarity of these esterases to the acetylcholinesterases present in nervous and muscle tissues and in erythrocytes is suggestive evidence for this hypothesis.

Plasma cholinesterases therefore are regarded as a group of esterases with much divergent properties, with regard to specificity against various choline and non-choline esters, sensitivity to inhibitors, and activity-substrate concentration relationships. No clearly cut classification can be made, and it is advisable to state the enzyme source in any work with these esterases. It is not possible to apply results obtained with the plasma of one species to any other species. Intermediate types between "specific" cholinesterases, as butyryl-, propionyl- and acetylcholinesterases, also exist, and in most vertebrate plasmata the cholinesterase present is characteristic of the species.

Aliesterase seems to be the main esterase of lower vertebrate plasmata. It was absent in human and monkey plasmata, and its presence in ruminant plasmata is questionable. This esterase was not detected in cock (and chicken) plasma, but was present in high concentration in fish plasmata, and was the only detectable esterase in cyclostome plasma. The electrophoretic mobility of aliesterase varied considerably with species, but the enzyme migrated in most cases together with α-globulins, at a higher rate than cholinesterases but more slowly than arylesterases. The electrophoretic separation of the A- and B-esterases of rabbit and guinea-pig plasmata was reported previously [26].

The aliesterases of vertebrate plasmata are represented by various types as far as the specificity is concerned. Acetyl-, propionyl-and butyryl-aliesterases as well as intermediate types were found in the present investigation. Although "aliesterase" is not always an adequate term for these esterases, since phenyl esters are also hydrolysed, in some cases at a higher rate than aliphatic esters, this nomenclature is still recommended. Aliesterases are in most cases the predominant esterases for the hydrolysis of aliphatic esters, including triglycerides. When these esterases are absent, the cholinesterases are the only esterases able to hydrolyse these substrates. In no case were the arylesterases found to split aliphatic esters. Most aliesterases are highly sensitive to organophosphorus compounds and, when aromatic esters are used as substrates for these esterases, certain organophosphorus compounds are the best reagents for differentiating them from plasma arylesterases, all of which are resistant to these agents. However, animal tissues contain other esterases differing from those discussed in the present series of papers [27,28]. Aliesterases differ from the cholinesterases in being unable to hydrolyse choline esters, and being in most cases resistant to 10^{-5} M physostigmine. The latter agent, however, is not an absolute selective inhibitor of cholinesterase, because the aliesterases of duck, frog, clawed frog, frogfish and pike are sensitive to physostigmine in comparatively low concentrations (pI_{50} 5.5—4.5), although they do not hydrolyse choline esters. This is indicative of a close relation between the B- and C-esterases mentioned above. The turtle-plasma esterase displays the characteristics of both a B- and C-esterase including high sensitivity to physostigmine (pI_{50} 6.3). The plasma aliesterases of the lowest vertebrates studied in this series were propionylesterases and this type of esterase is therefore suggested to represent a more primitive stage in the phylogenetic evolution of plasma esterases, of which both cholinesterases and arylesterases may be regarded as specialized forms.

Arylesterases are typical for mammalian plasmata and are absent in all other vertebrate plasmata studied. They have the highest electrophoretic mobility, in most cases migrating close to the albumins. In certain cases (human, swine, rabbit) it was not possible to separate the albumin and arylesterase peaks by the technique used. For swine plasma, the biosyntheses of albumin and arylesterase during the suckling period for piglets were found to parallel each other [25]. This does not mean, however, that the two proteins have the same origin (*e. g.*, the liver; see further below). In addition, albumin itself may have esterase activity, which was actually shown for the reaction between certain α-naphthyl esters (*e. g.*, acetate, N-methyl carbamate) and the albumin of various plasmata [29].

Arylesterases are, with few exceptions, acetylesterases. In all cases aromatic butyrates (phenyl and naphthyl) were found to be hydrolysed at a much lower rate than the corresponding acetates. These esterases are probably also responsible for the hydrolysis of aromatic phosphates, e.g., *p*-nitrophenyl phosphate and its diethyl derivative. In addition, the hydrolysis by blood plasma of other organophosphorus compounds, e. g., di*iso*propoxy-phosphoryl fluoride (DFP), dimethylamido-ethoxy-phosphoryl cyanide (tabun) and tetraethyl pyrophosphate [26,30,31] is in some cases at least partly due to arylesterase. The hydrolysis of dimethylcarbamoyl fluoride was also found to parallel the

hydrolysis of DFP [32] and tabun in separated rabbit plasma (unpublished results from this laboratory), and arylesterase may therefore be one of the esterases responsible for this reaction.

Arylesterases differed from the aliesterases and cholinesterases in their resistance to all enzyme inhibitors tested in this investigation. Introductory experiments demonstrated that *p*-hydroxymercuribenzoate (and *p*-chloromercuribenzoate) and certain metallic ions (*e. g.*, Ag^+, Hg^{2+}, Cu^{2+}) are powerful inhibitors of arylesterases. These agents are useful selective inhibitors of these esterases, because both B- and C-esterases were resistant to those concentrations of the SH-reagents, which gave at least 50 % inhibition of the arylesterase activity. It was found that the reaction between the active SH-group(s) of those enzymes and the SH-reagents (including the metallic ions) was time-dependent, and that the inhibited esterase could be reversed by cystein. A full account of these studies will be reported elsewhere.

Physiological function and origin of plasma esterases. As long as the natural substrates of the plasma esterases are unknown, the physiological function of these enzymes are not easily understood. They may be of importance in the metabolism of various types of compounds containing ester linkages. Such compounds may be natural metabolites or administered medical products. Aryl- and aliesterases are present in high amounts in liver, kidney and pancreas. Preliminary *in-vivo* experiments with swine (performed in collaboration with B. Olsson) have shown that acetylsalicylic acid is hydrolysed to salicylic and acetic acids more readily in an animal with high plasma arylesterase activity compared with one with low activity. Aliesterase (lipase) may be the predominant enzyme for the hydrolysis of triglycerides, including fats, and the high aliesterase of eel plasma may be related to the toxic properties of this plasma [33]. Plasma cholinesterase may be of importance in hydrolysing acetylcholine or other naturally occurring choline esters (*e.g.*, propionylcholine) which come out into the blood stream. Finally, it is the opinion of the author that the great variation in the properties of plasma esterases with species reflects random species-differentiation of specific properties, rather than being the result of enzyme adaptation in relation to specific natural substrates.

The distributation of plasma esterases in animal tissues and the origin of these enzymes have not been investigated in the present study, a knowledge of which should be helpful in approaching the problem with regard to the physiological function of these enzymes. It is known that various tissues, *e. g.*, liver, kidney, pancreas and intestinal mucosa, contain esterases which sometimes are identical with those present in blood plasma, sometimes have fundamentally different properties compared with the plasma esterases. The latter types of esterases may occur in the plasma under pathological conditions. Human plasma cholinesterase is produced in the liver, according to a number of previous investigations. However, the origin of plasma cholinesterases of other vertebrates may not be the same. For instance, it has been demonstrated recently in this laboratory that swine plasma cholinesterase is not produced in the liver, but in the pancreas. The arylesterase of the same species, being closely related to albumin, is probably produced in the kidney. Any of these organs (liver, kidney, pancreas) may be the site of biosynthesis of plasma

esterases. The acetylcholinesterase present in the plasmata of lower vertebrates originate probably from still other sources.

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