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SPECIES DIFFERENCES IN AVIAN SERUM B ESTERASES REVEALED BY CHROMATOFOCUSING AND POSSIBLE RELATIONSHIPS OF ESTERASE ACTIVITY TO PESTICIDE TOXICITY

H. M. THOMPSON,* M. I. MACKNESS,† C. H. WALKER‡ and A. R. HARDY§

* Central Science Laboratory, MAFF, Hook Rise South, Tolworth, Surrey, U.K.; † Department of Medicine, University of Manchester, Stopford Building, Manchester, U.K.; ‡ Department Biochemistry and Physiology, AMS Building, University of Reading, PO Box 228, Whiteknights, Reading, Berkshire, U.K.; and § Central Science Laboratory, MAFF, London Road, Slough, Berkshire, U.K.

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Abstract—Serum cholinesterase (BChE) and carboxylesterase (CbE) activities were investigated in ten species of birds. Multiple forms of serum BChE and CbE were also separated by chromatofocusing. Higher CbE activity and a wider range of CbE and BChE forms were present in the sera of omnivorous/herbivorous birds than carnivores. Omnivores/herbivores studied were the starling, house sparrow, tree sparrow, pigeon, partridge and magpie. Serum CbE activities of these species ranged from 0.46 to 2.93 μmol/min/mL with 2–6 forms separated by chromatofocusing. 0–6 forms of BChE were separated by the same method. The serum CbE activities of the little owl, tawny owl, barn owl and razorbill ranged from 0.19 to 0.58 μmoles/min/mL with 0–2 forms separated by chromatofocusing. No ChE forms were present within the pH gradient. These results may be significant in contributing to the understanding of the selective toxicity of organophosphorus and carbamate pesticides.

B esterases, such as brain acetylcholinesterase (AChE), are inhibited by the active oxon forms of organophosphorus (OP) and by carbamate insecticides [1, 2]. For the detection of sublethal exposure of birds to pesticides the serum and plasma B esterases, i.e. carboxylesterases (CbE) and cholinesterases, may be better indicators of poisoning than brain AChE due to their more rapid and greater inhibition by insecticides [3]. Inhibition of serum B esterases has been used to monitor the sub-lethal effects of anticholinesterase pesticides in a range of avian species [4–6].

Serum esterases may be divided into A and B types depending on their interaction with organophosphate compounds. A esterases that hydrolyse organophosphates such as paraoxon and pirimiphos methyl oxon are absent or only have very low activity in the serum of birds [7]. However, high activities are found in mammalian sera. There may also be variations in forms of B esterases present in different species. Plasma CbE activity is generally low in mammalian species but is higher in fish, amphibia and birds. This may be important in determining susceptibility to compounds such as soman [8]. Inhibition of plasma CbEs by 2-(2-methylphenoxy)-4H-1,3,2-benzodioxaphosphorin-2-oxide (CBDP) resulted in similar toxicity data in a range of mammalian species [8].

Serum cholinesterases are also present in a range of forms [9]. These individual forms of esterase may have differing affinities for inhibitors similar to the species differences found in the inhibition of brain AChE e.g. chicken brain AChE is inhibited 20 times more rapidly than rat or mouse brain AChE by organophosphates [10].

The wide range of esterases present in pheasant (*Phasianus colchicus*) tissues may give protection against anticholinesterase pesticides and so contribute to the success of this species in regions of the U.S.A. where other avian species are adversely affected by pesticides [11]. Few studies have attempted to relate species differences in serum esterases to the selective toxicity of OPs and carbamates to wild birds.

Here we report species differences in avian serum B esterases in activity and forms that can be separated by chromatofocusing. The results are discussed in relation to the functions of these esterases and their possible significance in modulating the toxicity of organophosphorus and carbamate insecticides.

METHODS

Chemicals. All chemicals were of Analar grade and were purchased from the Sigma Chemical Co. (St Louis, MO) unless otherwise stated. Polybuffer 74, Polybuffer exchanger PBE94 and analytical polyacrylamide isoelectric focusing (IEF) gels were purchased from Pharmacia-LKB (Uppsala, Sweden).

Sources of sera. Blood samples were taken by puncture of the brachial vein and samples from several birds were bulked. All samples were taken at the same time of day. Starlings (*Sturnus vulgaris*) (N = 6), house sparrows (*Passer domesticus*) (N = 6), tree sparrows (*Passer montanus*) (N = 4), magpies (*Pica pica*) (N = 3), barn owl (*Tyto alba*) (N = 1), tawny owl (*Strix aluco*) (N = 2) and little owl (*Athene noctua*) (N = 2) were all obtained from the Ministry of Agriculture, Fisheries and Food, Worplesdon, Surrey, U.K. Red-legged partridges (*Alectoris rufa*)

(N = 4) were obtained from the Game Conservancy, Fordingbridge, Hants, U.K., and feral pigeons (*Columba livia*) (N = 4) from Lincolnshire Pheasantries, Lincs, U.K. Razorbills (*Alca torda*) (N = 3) were obtained under licence from the Saltee Islands, Eire.

Serum was prepared by centrifugation of blood samples at 11,000 *g* for 10 min and stored at −20° until required.

Separation of multiple forms of serum esterases. Separation of multiple forms of serum esterases was achieved by chromatofocusing. All procedures were conducted at 4°.

Serum samples (0.25–1.0 mL) were diluted with an equal volume of 25 mM bis-Tris/HCl buffer pH 6.3 and pumped on to a 1.5 by 15 cm column (Amicon-Wright) containing Polybuffer exchanger PBE94. (The column had been equilibrated with three column volumes of 25 mM bis-Tris/HCl buffer pH 6.3 followed by 5 mL of degassed Polybuffer 74 pH 4.0 (1:10 dilution in water).)

The column was eluted with 300 mL of Polybuffer 75 pH 4.0 (1:10 dilution in water) at 20 mL/hr and 2.7 mL fractions were collected. When Polybuffer elution was complete the column was washed with 1 M sodium chloride and 2.7 mL fractions collected. For each fraction pH, protein content (A280), CbE and butyryl-cholinesterase (BChE) activity were assayed.

Analytical isoelectric focusing (IEF). Analytical IEF of serum samples was performed on thin layer polyacrylamide gels pH 4.0–6.5 according to LKB-Pharmacia application note 1804. The gels were stained according to method of Martin *et al.* [12] using *N*-methyl indoxyl acetate as the substrate.

Enzyme assays. All assays were conducted at 37°.

Butyrylcholinesterase activity. BChE activity was assayed by the method of Ellman *et al.* [13] as adapted by Westlake *et al.* [14] using 25 mM Tris/HCl buffer pH 7.6. Butyrylthiocholine iodide was used as the substrate (0.5 mM) and the subsequent detection of released thiocholine by reaction with 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) was monitored over a 2–3 min period with a recording spectrophotometer set at 410 nm.

Carboxylesterase activity. CbE activity towards α-naphthyl acetate was measured by the method of Gomori [15] as adapted by Bunyan *et al.* [16] but substituting 25 mM Tris/HCl buffer pH 7.6 for the buffer used by Bunyan *et al.* Naphthol released by hydrolysis of the substrate (0.03 mM) was assayed by reaction with Fast Red ITR following a 10 min incubation and the absorbance of the complex measured at 530 nm.

RESULTS

In general the sera of the starling, house sparrow and tree sparrow have higher CbE activities in association with a greater diversity of forms of CbE with isoelectric points between 4.3 and 5.2 (Table 1) than do other species. In contrast, the little owl and razorbill have serum CbE activities 6–15 fold lower than that of the starling. The profiles from individual starlings, house sparrows and tree sparrows were comparable with those obtained from bulked samples.

The chromatofocusing profiles of starling and tawny owl sera are contrasted in Figs 1 and 2. One major peak of CbE activity and several major peaks of BChE activity were eluted from starling serum during the course of the pH gradient whilst a major peak of BChE activity was eluted by the sodium chloride wash. By contrast, no BChE or CbE activity was eluted from tawny owl serum by the pH gradient, although major peaks of both activities were found in the sodium chloride wash. This indicates a major difference in the surface charge and hence pI of esterase forms in the serum for these two species.

As summarized in Table 1 the little owl, tawny owl and razorbill showed no CbE activity eluted in the pH gradient and the barn owl and magpie only two forms with CbE activity in the gradient, the remaining species showed a number of forms of CbE activity.

The highest serum BChE activity was found in the barn owl and the lowest in the magpie (Table 1). The BChE activity of the pigeon, razorbill, tawny owl, little owl and barn owl eluted with the sodium chloride wash (pI 4.2). Elution of serum from other species showed a range of proteins of isoelectric points between 4.28 and 5.30 with BChE activity.

Figure 3 shows the analytical IEF banding patterns of serum esterases from the starling, pigeon and partridge. These patterns appear to be comparable with those obtained by chromatofocusing, given that there were differences in the substrates used for detecting esterase activity; there may be differences in substrate specificity. CbEs hydrolyse *N*-methyl indoxyl acetate more readily than do BChE. Sera from razorbills showed no bands of esterase activity when separated by analytical IEF and this confirms the results of the chromatofocusing profile where no CbE or BChE activity was found in the pH 6–4 gradient.

DISCUSSION

The species distribution in CbE activity appears to bear little relation to species weight (Table 1). However, it may relate to the considerably higher metabolic rate of the small passerines, the metabolic rate of the razorbill being approximately one quarter that of the tree sparrow [17] (Table 1).

The CbE activity may also be related to diet. Generally the omnivorous, herbivorous, insectivorous and granivorous species (i.e. omnivores and non-carnivores) have higher CbE activity than the carnivores investigated. The possibility should be considered of a requirement for this enzyme in the metabolism of constituents in the diet. The omnivorous and non-carnivorous species also showed a wider range of surface charges of the proteins with CbE activity than do the carnivores. The possibility that this is an artefact due to bulking samples from individuals is counteracted by the comparability of these profiles with those from single birds. Not only were more forms with CbE activity eluted from the serum of omnivorous and non-carnivorous species but the isoelectric points of the proteins with activity were generally higher than those of the carnivores.

Table 1. Physiological and biochemical data for the ten species of birds studied (pooled samples)

Species	Body wt (g)	Metabolic rate [17] (kCal/24 hr/g body wt)	Diet*	CbE		BChE	
				Activity (μmol/min/mL)	Peaks eluted within pH gradient (by NaCl)	Activity (μmol/min/mL)	Peaks eluted within pH gradient (by NaCl)
Passeriformes							
Starling	80	.26	O/H	2.93	5 (0)	3.24	4 (1)
House sparrow	28	.35	O	2.50	3 (0)	0.96	3 (0)
Tree sparrow	22	.37	I/H	2.28	3 (0)	1.11	6 (1)
Magpie	220	.20	O	0.57	2 (1)	0.53	2 (1)
Columbiformes							
Pigeon	390	.10	G	0.78	4 (1)	7.37	0 (1)
Galliformes							
Partridge	450	.09	I/H	0.46	6 (1)	4.43	5 (1)
Strigiformes							
Little owl	164	.13	I/C	0.19	0 (1)	1.82	0 (1)
Tawny owl	480	.09	C	0.38	0 (1)	1.11	0 (1)
Barn owl	315	.11	C	0.58	2 (1)	8.65	0 (1)
Charadriiformes							
Razorbill	720	.09	C	0.22	0 (1)	1.26	0 (1)

Species variations in the isoelectric points of serum CbE and BChE are shown by the number of peaks eluted in the course of the pH gradient, and the presence or absence of activity in the sodium chloride (NaCl) wash on chromatofocusing.

* O—omnivore, H—herbivore, I—insectivore, G—granivore, C—carnivore.

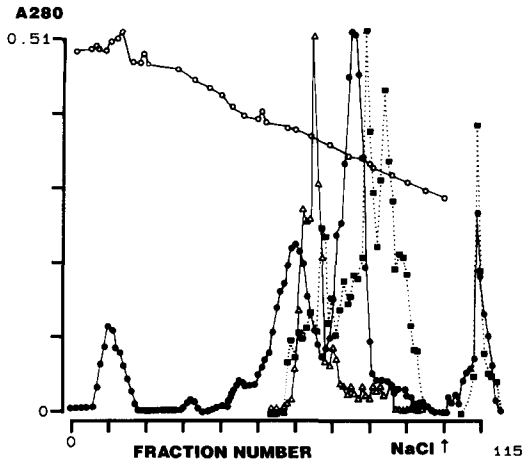

Fig. 1. Chromatofocusing profile of starling serum from one individual (pH 6 to 4). Elution was with Polybuffer 74 pH 4.0 and at the end of the gradient 1 M sodium chloride at 20 mL/hr. The pH gradient is shown (○) (max 6.4) together with the protein (*y*-axis) (●) (max .505 at 280 nm), BChE (■) (max 100 nmol/min/fraction) and CbE (△) (max 138 nmol/min/fraction) activities of all fractions eluted (*x*-axis).

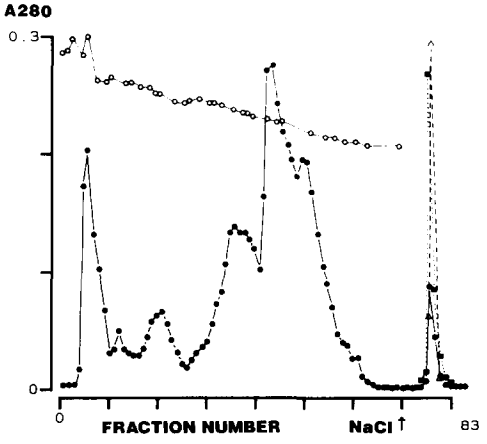

Fig. 2. Chromatofocusing profile of tawny owl serum (pooled sample) (pH 6 to 4). Elution conditions as for figure 1. The pH gradient is shown (○) (max 6.05) together with the protein (*y*-axis) (●) (max .272 at 280 nm), BChE (■) (max 260 nmol/min/fraction) and CbE (△) (max 81 nmol/min/fraction) activities of all fractions eluted (*x*-axis).

However, a larger number of individuals and variety of species should be investigated in order to confirm this hypothesis.

A greater diversity of forms of BChE but not overall higher BChE activity, were also shown by omnivores/non-carnivores.

It is possible that the observed variation in total enzyme activity may be due in part to differences between species in optimal assay conditions. However, it is unlikely that this would account for the difference in both total activity and diversity of forms observed.

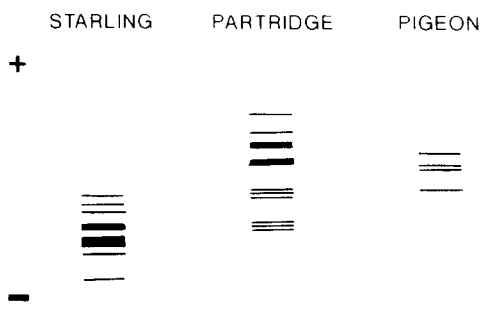

Fig. 3. Analytical IEF profiles (pH 6.5 to 4.0) of serum esterases from the starling, partridge and pigeon. Areas of esterase activity were shown by reaction with *N*-methyl indoxylacetate linked to Fast Blue RR.

These results are consistent with those of Westlake *et al.* [18] who reported differences between the general serum esterase activity (nitrophenyl acetate esterase) of herbivorous/omnivorous and carnivorous avian and mammalian species. In other studies the levels of detoxifying enzymes of the liver of a variety of fish-eating sea birds and raptors were lower than those of birds further down the food chain [19, 20]. Low levels of hepatic microsomal monooxygenases were related to a marked ability of certain predators to bioaccumulate organochlorine compounds. Thus, on present evidence the omnivorous and herbivorous species of birds have a greater range of esterase forms with correspondingly higher CbE activity than carnivorous species. This suggests that any difference may be primarily related to dietary factors rather than metabolic factors. Carnivorous species may be reliant on the enzyme systems of their prey to detoxify substances such as toxic plant esters which are constituents of the diet of species further down the food chain. However, investigation of a greater number of species with a variety of dietary habits is required to confirm this apparent division in serum esterase activity.

The forms and activities of esterase present in the serum may influence the susceptibility of particular species to the toxic effects of organophosphorus and carbamate compounds. There is evidence that the relatively high susceptibility of birds, compared to mammals, to poisoning with certain organophosphates e.g. pirimiphos methyl and diazinon is due to the generally low activity of A esterases in the serum of birds [7, 21]. Similarly, a low level of some B esterases e.g. CbE may influence the susceptibility of certain species to the toxic effects of organophosphates or carbamates.

Different forms of esterase may also vary in their binding capacity for pesticides. If less pesticide is irreversibly bound in the serum i.e. by phosphorylation of esterases, a greater amount is available to bind to the site of toxic action i.e. AChE of the nervous system. Certain OPs, e.g. monocrotophos and fenthion, are considerably more toxic to predatory species such as the golden eagle (*Aquila chrysaetos*) or American kestrel (*Falco sparverius*)

than to omnivorous species such as the house sparrow [22, 23]. This may be due, at least in part, to the apparently higher activity and greater diversity of forms of serum B esterase in omnivorous species.

Diurnal variations in esterase activity may also affect the binding capacity of serum and the esterase forms present. Serum CbE activity levels in the starling significantly increased between 0700 and 1900 hr during May [4]. Garcia-Rodriguez *et al.* [24] reported a diurnal variation in the serum BChE activity of the buzzard (*Buteo buteo*). In the present study all samples were taken at approximately the same time of day. However, the investigation of variations in serum esterases of nocturnal species may aid understanding of the reasons behind these variations.

A study of CbEs of rat liver microsomes revealed four major forms which hydrolyse a variety of endogenous and xenobiotic substrates including lipid esters and substituted amides [25–27] and human serum CbE is capable of hydrolysing triglycerides [28]. It is therefore possible that avian serum B esterases also have a dual role: (i) In the hydrolysis of dietary plant esters many of which are toxic and (ii) Acting as a 'sink' for organophosphate and carbamate pesticides to inhibit, thus reducing the concentration available to the site of action. This 'sink' effect is illustrated by a CbE isolated from the peach potato aphid (*Myzus persicae*) which confers resistance by increasing binding capacity [29]. Omnivorous/non-carnivorous birds have apparently developed a greater multiplicity of esterases to detoxify dietary poisons than have specialised predators; this may be important in determining the differential susceptibility to certain pesticides shown by these two groups.

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