The relationships between brain, serum, and whole blood ChE activity in the wood mouse (*Apodemus sylvaticus*) and the common shrew (*Sorex araneus*) after acute sublethal exposure to dimethoate

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Inhibition of cholinesterase (ChE) activity produced by a single acute intraperitoneal administration of dimethoate was studied in the wood mouse, *Apodemus sylvaticus*, and the common shrew, *Sorex araneus*, under laboratory conditions. ChE values from serum and whole blood were compared with those obtained from brain in order to obtain a non-destructive tool for predicting the severity of brain acetylcholinesterase (AChE) inhibition. In addition, serum and brain inhibition following oral exposure to dimethoate was also measured in the wood mouse. Normal ChE activity was higher in the brain and whole blood of the shrews than in wood mice. There was no difference between species in serum ChE activity. Exposure to dimethoate caused a dose-dependent reduction in ChE activity and there was a significant recovery in activity with increasing time after administration. In both species, serum and whole blood were more sensitive than brain for revealing organophosphate-induced ChE inhibition and serum was more sensitive than whole blood. Statistically significant relationships were defined between whole blood and brain ChE activity and between serum and brain ChE activity. Compared with serum, whole blood ChE activity was the more accurate predictor of brain AChE levels. The relationships between brain and serum ChE activity did not appear to be affected by the route of administration of the pesticide.

Keywords: *cholinesterase, organophosphate pesticides, dimethoate, wood mouse* (Apodemus sylvaticus), *common shrew* (Sorex araneus).

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Introduction

The inhibition of cholinesterases (ChEs) is a biomarker of exposure of vertebrates to organophosphate (OP) and carbamate pesticides (Bunyan *et al.* 1968, Thompson 1991). These enzymes are essential for the functioning of the cholinergic system and are abundant at postsynaptic membranes in nervous tissues, and in both erythrocytes and plasma. The cholinesterase activity measured in the brain of many mammal species is largely due to acetylcholinesterase (AChE), while in the whole blood and serum (or plasma), it can be the result of other esterases, such as butrylcholinesterase or non-specific carboxylesterases, as well as AChE (Walker and Thompson 1991).

Inhibition of AChE has traditionally been used as the principal, biochemical tool in studies assessing exposure to, and, more specifically, diagnosing the toxicity of, OPs on non-target vertebrates (Greig-Smith 1991). Inhibition of serum ChE has also been used for documenting exposure and sublethal effects, although its application in wildlife studies, apart from a limited number of studies on wood mice, *Apodemus sylvaticus* (e.g. see Westlake *et al.* 1982), has mainly involved birds (Thompson 1991). Measurement of whole blood ChE has received relatively little attention in either wildlife or laboratory studies (but see Padilla *et al.* 1994). However, quantification of ChE activity in serum and whole blood has important advantages over measurement of brain AChE, namely that it is a non-destructive means for monitoring sublethal exposure and can be assessed several times on the same individual. Thus, the time course of the effects of exposure can be studied and each animal acts as its own control, thereby eliminating problems caused by wide, inter-individual, variation in ChE activity. Obviously, a correct interpretation of the significance of ChE inhibition in serum or blood should be based on the knowledge of the relationship between these parameters and brain AChE inhibition. This knowledge may allow development of a non-destructive diagnostic tool to predict the impact of pesticides on free-living, wild species.

Several small mammal species inhabit agricultural and forestry areas and are potentially, albeit unintentionally, exposed to OPs. As far as we are aware, the relationships between inhibition of ChE activity in whole blood, serum, and brain following exposure to OPs have not yet been documented for these wild species. Provided that the relationships between brain AChE inhibition and effects and between whole blood/serum ChE and brain AChE inhibition are known, whole blood/serum ChE inhibition could be used to predict both exposure and the severity of impacts, including, perhaps, survival (Grue *et al.* 1991).

The aim of the present study was to analyse the relationships between brain, whole blood and serum ChE inhibition in two species of small mammals, the granivorous/omnivorous wood mouse, *Apodemus sylvaticus*, and the common shrew, *Sorex araneus*, which predates on invertebrates. Both species inhabit cereal fields and/or their margins (Green 1979) and so are likely to be exposed to OPs (Bunyan *et al.* 1981). There are no data at all on ChE levels in shrews but, for wood mice, control

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ChE activity levels in brain and serum have been defined (Westlake *et al.* 1983, Fishwick *et al.* 1996) and the degree of sublethal brain AChE inhibition has been related to impairment of behaviour in this species (Dell'Omo and Shore 1996a, b). Thus, definition of the relationship between ChE in the brain and in the blood would provide a non-destructive means for assessing the ecotoxicological impact of exposure.

METHODS

Animals

Adult, laboratory-bred male wood mice were housed in Plexiglas boxes in groups of 3–10 under constant photoperiod (14L:10D) and temperature (19 °C). Food (Rat and Mouse number 1 pellets, SDS, Witham, Essex) and water were available *ad libitum*. Shrews of both sexes were kept singly in boxes with a 4 cm deep peat base following capture, were maintained under the same environmental conditions as the wood mice, and were given water and mealworms (*Tenebrio* larvae) *ad libitum*.

Chemical treatment and dosing

Dimethoate (*O,O*-dimethyl *S*-2-(methylamino)-2-oxoethyl phosphorodithioate, Cheminova, Lemvig, Denmark) is a widely used systemic and/or contact insecticide (McEwen and Stephenson 1979) which, based on its acute oral toxicity in rats, is in the moderately toxic group of OPs (Morgan 1989); it was used as the anticholinesterase agent in the present study. Dimethoate was diluted in saline (0.9% NaCl) and administered at various doses and time intervals, within a 24 h period, to produce various degrees of ChE inhibition in the experimental animals. It was administered by intraperitoneal (IP) injection at 5, 15, or 50 mg kg^{-1} body weight to 69 male wood mice (21 controls, 48 dimethoate) and at 50 mg kg^{-1} (IP) to shrews. Control mice and shrews received saline only. Data obtained for wood mice were compared with those from an earlier study (Fishwick 1994) in which 66 female wood mice (23 controls, 43 dosed) were orally (bulb catheter to the mouth) given dimethoate formulated in vegetable oil at doses which ranged between 15 and 90 mg kg^{-1}. Control mice were given oil only. In both species, the doses were chosen on the basis that they were lower than the LD_{50} values reported for laboratory mice (Hayes and Laws 1991).

Measurements of ChE activity

ChE activity was measured in the serum and brain of the 69 male wood mice treated IP. Corresponding measurements had been made on the 66 female mice treated orally by Fishwick (1994). ChE activity from the whole blood was measured in only 21 of the IP-treated mice (9 controls) selected at random. In shrews, brain, serum, and whole blood ChE activities were measured in all animals (12 controls, 15 dimethoate). The shrews were all immature animals and could not be sexed from external morphology; this was done by post-mortem examination.

Trunk blood was collected in both species at the time the brain was excised. It was allowed to clot and then separated by centrifugation (2000 rpm at 4 °C for 10 min). Whole blood samples were taken from trunk blood and, in some cases, by tail puncture; 5 µl were immediately added to 2.875 ml of Tris–HCl buffer which was subsequently used in the ChE assay; dilution of the blood prevented it clotting. The brain was homogenized in 25 mM Tris–HCl buffer (containing 0.1% Triton X-100) using a glass homogenizer; the concentration of the resultant homogenate was 0.06 g ml^{-1}. Serum, brain homogenate and diluted blood were all kept on ice until analysed. Cholinesterase assays were performed as soon as possible after the collection of samples and always within 5 h.

The ChE activity in brain and serum was measured by the colorimetric method of Ellman *et al.* (1961), as adapted by Fishwick *et al.* (1996). Acetylthiocholine iodide (0.287 mM) was used as a substrate in the reaction cuvette; although used specifically to detect AChE activity, this substrate can also react with other unspecific esterases found in the blood. Activity was determined in 5 µl of both serum and whole blood and 20 µl of brain homogenate, the final assay volumes being 3.0 ml and 3.015 ml respectively. Absorbance was measured at 412 nm and 37 °C using a Unicam SP100 Ultraviolet Dual Beam Spectrophotometer (Unicam Ltd, Cambridge, UK). In the case of whole blood, however, the absorbance of the assay mixture without substrate was high at 412 nm and assays could not be run; therefore, they were carried out at 480 nm. Because no extinction coefficient was available at 480 nm, the activity of the enzyme was then calculated by multiplying the values obtained by a previously determined regression coefficient which related the measures obtained at 412 nm (using dual beam spectrophotometry) to those at 480 nm (see Figure 1). Dual beam spectrophotometry was not carried out routinely for whole blood because this requires twice as much blood as analysis by single beam mode and a methodology which minimized the amount of blood taken was needed, because of the small size of species such as shrews. Preliminary studies using substrate and sample blanks determined that there was no spurious activity due to background substances or non-specific hydrolysis of other compounds in the assays. Each assay was run for 3–4 min to ensure that the linear phase of the reaction was measured. Replicate assays of a commercial reference material (Precinorm U, Boehringer Mannheim UK, Lewes, E. Sussex, UK) were analysed before and after the unknowns to ensure that the assay gave consistent results. The activities detected in the reference material were always within the manufacturer's specified range. ChE activity was expressed in international units (IU): µmol min^{-1} g^{-1} for brain, µmol min^{-1} ml^{-1} for serum and whole blood.

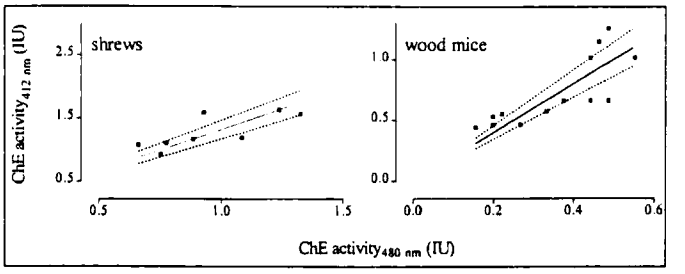

Figure 1. Relationship between ChE activities measured in the whole blood at 480 nm in single beam mode and those measured for the same sample at 412 nm in dual beam mode in common shrews (*Sorex araneus*) and wood mice (*Apodemus sylvaticus*). The regression models were: common shrews, $A_{412} = 1.32A_{480}$, $r^2 = 0.98$, $p < 0.01$; wood mice, $A_{412} = 2A_{480}$, $r^2 = 0.95$, $p < 0.01$.

Statistical analysis

Regression analyses were carried out to describe the relationships between ChE activity and dose/time after administration and between ChE activities in different tissues (brain, serum, whole blood). In the latter case, a visual inspection of data suggested that non-linear analyses were more suitable to describe the relationships between the three parameters and these relationships also gave higher r^2 values than linear models.

Results

Overall, ChE activity in brain and whole blood of non-exposed shrews was significantly higher than the corresponding values for wood mice (Table 1), but there was no difference between species in their serum ChE activity. In both species, ChE activity in whole blood was lower than in serum. In shrews, administration of 50 mg kg^{-1} dimethoate produced a rapid depression of ChE activity in brain, serum, and in the whole blood. This was maximal 1.5 h after injection and was

followed by a recovery phase which was more marked in brain than in serum or whole blood. The time course profile of these effects is presented in Figure 2. Comparison of data for males and females by ANOVA which had treatment, time of sampling after injection of dimethoate and sex as factors, did not indicate any significant effect of sex on ChE activity in brain, serum, or whole blood ($F_{1,9} < 3.32$, $p < 0.05$ in all cases). In wood mice, dimethoate induced a dose-dependent decrease of ChE activity as assessed at 3 h after injection in brain, serum and whole blood (Figure 3, lower graph). Moreover, there were

	Brain (μmol min^{-1} g^{-1})	Serum (μmol min^{-1} ml^{-1})	Whole blood (μmol min^{-1} ml^{-1})
Shrews (12)	20.2 ± 0.41**	2.17 ± 0.18	1.51 ± 0.16**
Wood mice (28)	12.8 ± 0.44	2.17 ± 0.07	0.78 ± 0.04 (n = 8)

Table 1. Mean (±SE) ChE control activity of adult male wood mice (*Apodemus sylvaticus*) and of common shrews (*Sorex araneus*) of both sexes in brain, serum and the whole blood.

**p<0.01 in between-species comparisons, as determined by ANOVA.

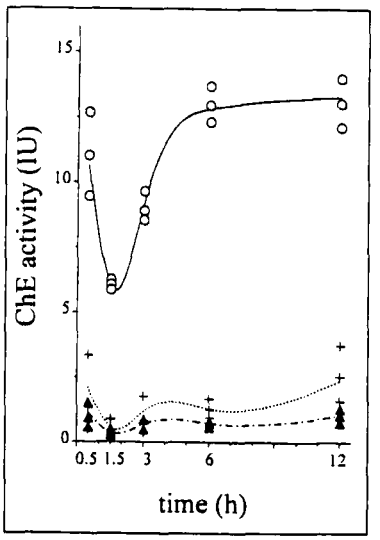

Figure 2. Time course profile of the effects of a 50 mg kg^{-1} IP dose of dimethoate on ChE activity in brain (—○—), serum (·····+·····) and whole blood (–·–·–▲–·–·–) of common shrews (*Sorex araneus*). Cubic spline curves were fitted to the data.

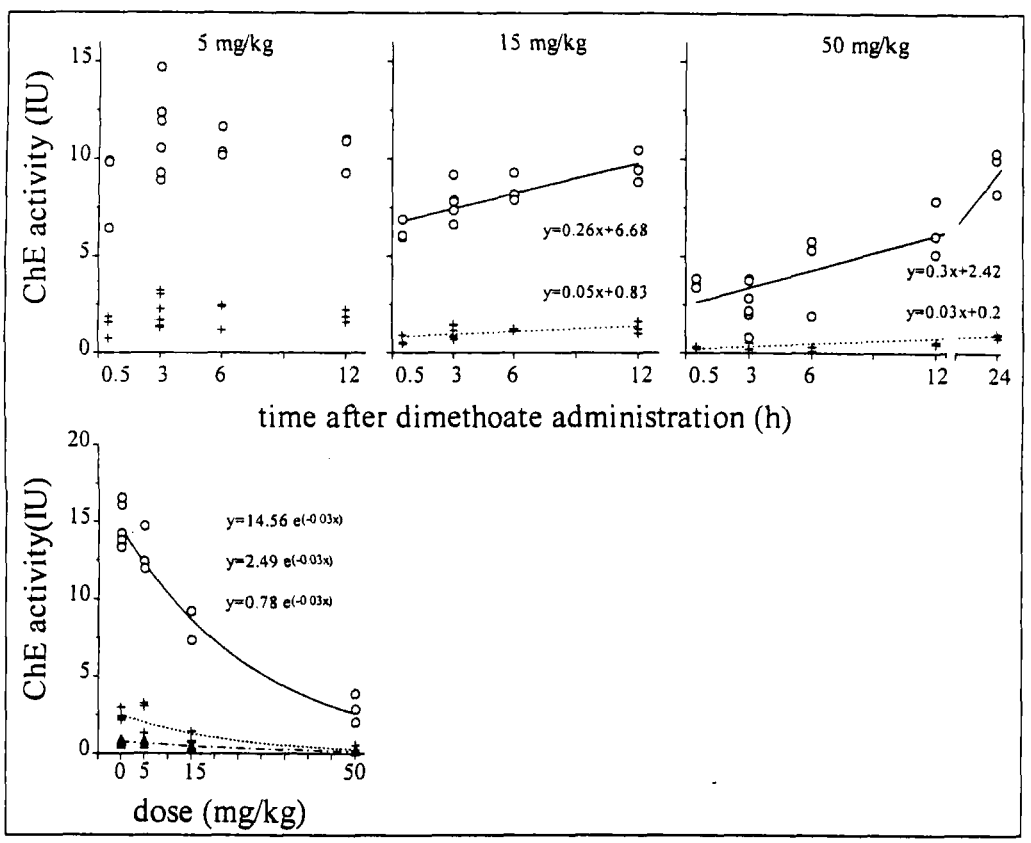

Figure 3. Upper graphs: relationships between ChE activity (—○— brain, ·····+····· serum) and time after IP administration of dimethoate to male wood mice (*Apodemus sylvaticus*). Doses used were 5, 15, and 50 mg kg^{-1}. There were significant linear relationships between time after dimethoate administration and both brain and serum ChE activity in animals given 15 mg kg^{-1} ($F_{1,13} > 6.2$, $p < 0.01$ in all cases) and 50 mg kg^{-1} ($F_{1,16} > 44.6$, $p < 0.01$ in all cases). There were no significant relationships between ChE activity and time in animals given 5 mg kg^{-1}. Lower graph: relationship between ChE activity (brain, serum, and –·–·–▲–·–·– whole blood) and dose of dimethoate administered to male wood mice 3 h after IP injection. The relationships were described by exponential growth curves and were all significant ($F_{1,15} > 40.8$, $p < 0.01$ in all cases).

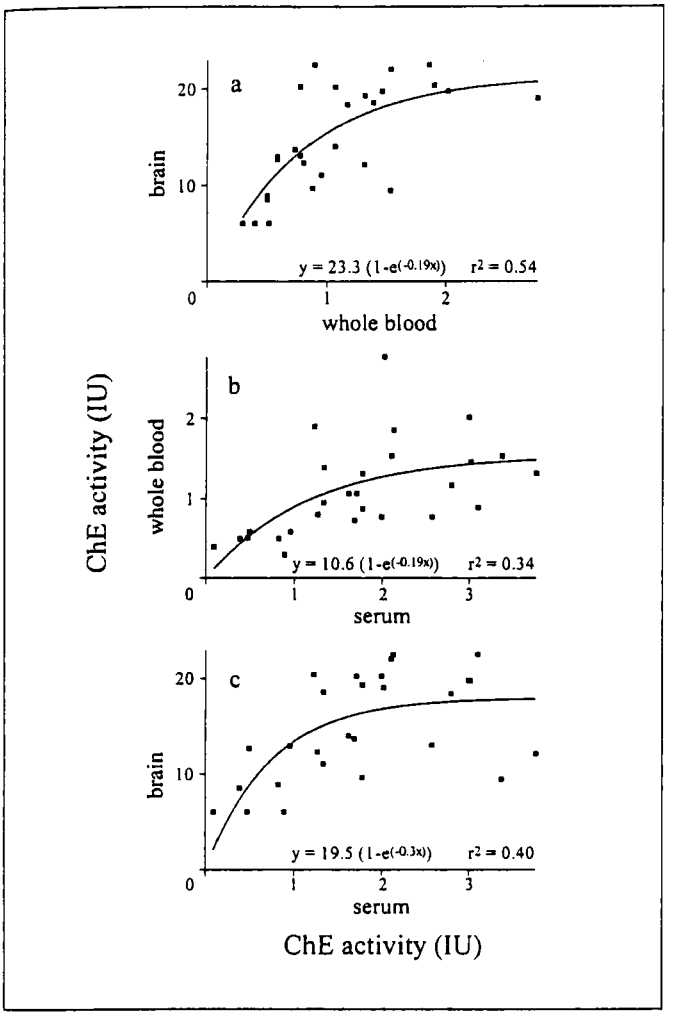

Figure 4. Relationships between the inhibitory effects of dimethoate administered IP at various time intervals on brain, serum, and whole blood ChE activities of common shrews (*Sorex araneus*) of both sexes. In all the graphs the relationships are represented by highly significant ($p < 0.01$), second order, non-linear curves.

linear relationships between the inhibition of ChE activity in brain and serum (whole blood ChE activity was not measured) and time after injection of dimethoate. These relationships were significant in the case of 15 and 50 mg kg^{-1} doses but not for the 5 mg kg^{-1} dose (Figure 3, upper graphs).

In shrews, there were significant relationships (one phase exponential association, $p < 0.001$ in all comparisons) between ChE activity in serum, whole blood, and brain (Figure 4). Serum appeared to be more sensitive than whole blood as a biomarker of exposure when using ACTI as a substrate in the assay, serum activity having already fallen by approximately 50% before there was any marked decrease in whole blood ChE activity. Serum and whole blood ChE activities were both more sensitive biomarkers of exposure than brain AChE, depression of activity in serum and blood being more prolonged than in the brain (Figure 2). This was reflected in the comparisons of ChE activity in different tissues (Figure 4), ChE activity in serum and blood being decreased in magnitude appreciably before there was any depression in brain AChE activity. The relationships between ChE activity in the brain and in the whole blood was less variable (higher r^2) than that between brain and serum, suggesting that ChE activity in whole blood, rather than serum, may be the better predictor of brain AChE activity.

In wood mice, there was also significant ($p < 0.01$ in all cases) relationships (one-phase exponential association) between brain, serum, and whole blood ChE activities (Figure 5). As in shrews, there was slightly more variability in the relationship between serum and brain ChE activity than between that of whole blood and brain, suggesting that activity in whole blood might be the more accurate predictor of brain AChE activity. However, again as in shrews, serum ChE levels appeared to be a more sensitive measure of exposure than blood ChE, the degree of inhibition being more marked in serum than in whole blood (Figure 3). Overall, the

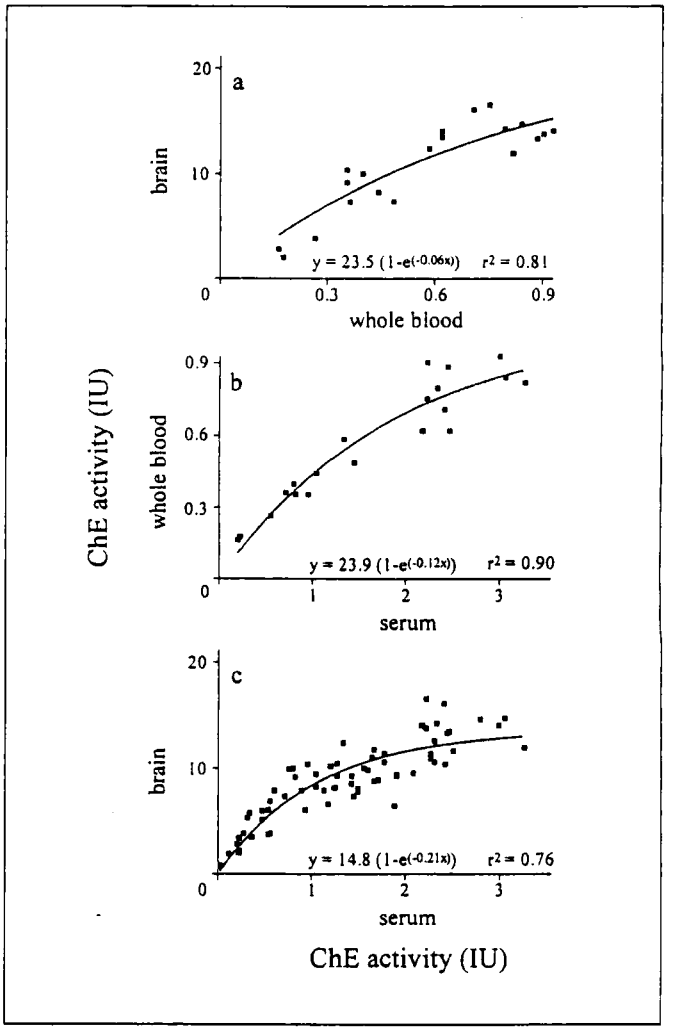

Figure 5. Relationship between the inhibitory effects of dimethoate administered IP at various doses and time intervals on brain, serum, and whole blood ChE activities of male wood mice (*Apodemus sylvaticus*). In all graphs the relationships are represented by highly significant ($p < 0.01$), second order, non-linear curve.

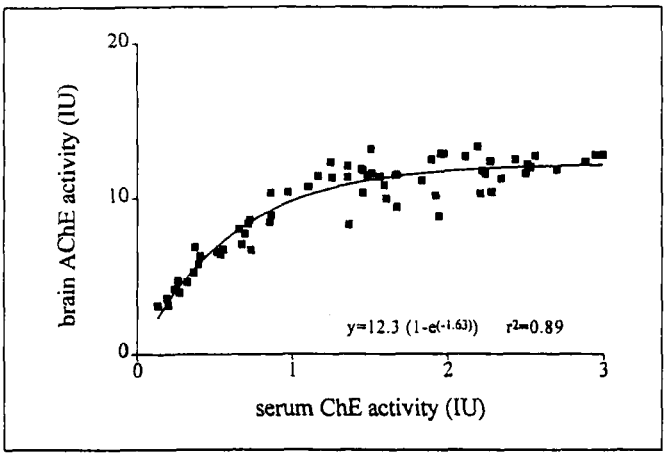

Figure 6. Relationship between the inhibitory effects of dimethoate administered orally at various doses on brain and serum ChE activities of female wood mice (*Apodemus sylvaticus*). The relationship is represented by a highly significant ($p < 0.01$), second order, non-linear curve.

relationships between ChE activity in blood, serum, and brain were more evident (higher r^2 values) in mice than in shrews (Figures 4 and 5).

The relationship (one-phase exponential association) between serum and brain ChE activity following oral administration of dimethoate (Figure 6) was highly significant ($p < 0.01$) and was similar to that following IP injection (Figure 5c). The similarity in the shapes of these two curves suggests that the relationship between ChE inhibition in serum and brain is unaffected by the route of exposure to OP pesticide.

Discussion

It is clear from the results of the present study that there are significant relationships between ChE activity in brain, serum, and whole blood in both wood mice and shrews. The existence of these relationships demonstrates the feasibility of using measures of ChE activity in either whole blood or serum as a non-destructive predictor of brain AChE inhibition which can, in some cases, be related to functional effects, such as behavioural impairment (Hart 1993, Dell'Omo and Shore 1996a, b). Prediction of brain AChE activity from measures in blood, as opposed to actual measurements on brain tissue, allows the monitoring of brain AChE activity in field studies without the need to remove animals for analysis; such removals are likely to affect demographic processes and behaviour in free-living animals. Analysis of blood rather than brain will also result in a reduction in the number of animals which would normally be killed in experimental protocols in which it is necessary to monitor brain AChE activity.

Dose and time data indicated that, in both species, brain AChE activity was related to dose of pesticide and time after exposure. This was also the case for serum and whole blood ChE, although the level of activity in these tissues was low compared with that in the brain. There were, however, some differences in the time course profile of inhibition and recovery of activity between the different tissues, recovery of brain ChE activity being more rapid than that in serum and whole blood (Figure 2, and Figure 3 upper graph on the right). The relatively prolonged depression of blood and serum ChE activity means that they are more sensitive biomarkers than brain AChE activity in that they will be detectable for longer after exposure. These differences in rate of recovery are the reason why the relationships between ChE activity in the different tissues are curvilinear rather than linear (Figure 4–6).

In both wood mice and shrews, ChE activity in whole blood was lower than in serum, as has also been reported for rats (Padilla *et al.* 1994). This suggests that the activity of various non-specific ChEs in the serum was greater than that of AChE, and possibly other ChEs, associated with erythrocytes. Despite ChE activity in whole blood being lower than that in serum, it is still a relatively sensitive biomarker of exposure to OPs in wood mice and shrews. There are, in fact, at least two advantages in determining ChE activity in whole blood rather than in serum. The first is that a smaller volume of blood is needed to carry out the ChE assay. In the present study, we were able to modify our methods to analyse successfully blood samples as small as 5 µl and this is of great advantage if repeated sampling from very small mammals, such as shrews, is required; such species are relatively difficult to bleed and only small blood samples can be taken. The second advantage is that the relationships between whole blood and brain activity are tighter than those between serum and brain. This suggests that intra-specific variation in ChE activity is lower in whole blood than in serum, when using ATCI as an assay substrate, and is consistent with measures in humans in which the coefficients of variation in serum and whole blood activity were mostly 15–25% and < 15% respectively (Gage 1967, Sidell and Kaminskis 1975). Therefore, although not as sensitive a biomarker of exposure as serum ChE activity, whole blood ChE activity is likely to be the more accurate predictor of brain activity in both wood mice and shrews, as is also the case for laboratory rats (Padilla *et al.* 1994).

The normal ChE activity levels in common shrews presented in this study are, as far as we are aware, the first reported values for this species, incidental exposure to OPs not having been investigated in shrews even though they are found in and around arable fields (Johnson *et al.* 1992, Tew and MacDonald 1993). Control ChE activity in whole blood and brain in shrews was higher than in wood mice and this is consistent with the view that, in mammals in general, brain AChE values are inversely related to size (Westlake *et al.* 1983). In birds, high brain AChE activity is also associated with higher metabolic rate and blood supply to the tissues (Westlake *et al.* 1983). It has been suggested that a high level of ChEs may give some protection against the toxicity of OPs (Walker and Thompson 1991), and so it might be expected that shrews would be more resistant than mice to the same exposure to OPs. However, there are many factors which can affect species sensitivity to OPs, including differences in affinity and rate of phosphorylation of the enzyme (Wang and Murphy 1982), in the reactivity of brain AChE (Murphy *et al.* 1968, Johnson and Wallace 1987), and in hepatic activation of phosphohydrolases hydrolysis (Kennedy 1993). In the case of dimethoate, in particular, its degradation in mammalian liver tissues is

crucial in determining toxicity and the contribution of liver amidases (the enzymes which hydrolyse the pesticide) can vary widely with species (Uchida *et al.* 1964).

Most of the data presented in this paper are derived from studies in which animals were injected with dimethoate. In the natural environment, oral exposure is thought to be the main route of exposure of vertebrates to OPs (Hardy 1990). However, the similarity in the curves relating serum and brain ChE activity in wood mice dosed by IP injection and orally suggests that route of exposure is unlikely to have a significant impact on the relationships between blood and brain ChE activity. Hence we would expect the results presented in this paper to be applicable to free-living wood mice and shrews which are exposed when fields are treated with OPs.

In conclusion, the data from the present study have defined for the first time normal ChE activity levels in whole blood, serum and brain of common shrews and in whole blood of wood mice. It has also been established that there are clear and significant relationships between ChE activity in blood (whole blood and serum) and in the brain and measures of activity in the blood can be used as a non-destructive predictor of AChE activity in the brain. However, all the studies reported here involved dosing animals with dimethoate and, although there is no *a priori* reason to suppose that relationships between blood and brain ChE activity will vary with active ingredient, this remains to be established and merits further investigation.

Acknowledgements

This work was supported by a European Environmental Research Organisation (EERO) fellowship to Giacomo Dell'Omo. The authors are grateful to Daniel Osborn for reading a previous draft of this manuscript, to Tim Sparks for expert statistical advice, and to Anthony Turk for his assistance during the experimental work.

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Received 6 December 1995, revised form accepted 16 February 1996