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Development of an Immunoassay for Avian Serum Butyrylcholinesterase and Its Use in Assessing Exposure to Organophosphorus and Carbamate Pesticides

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Inhibition of serum cholinesterase (ChE) activity levels is being increasingly used in the assessment of exposure of animals to organophosphorus and carbamate pesticides (Thompson 1991, Hooper *et al.* 1989, Wilson *et al.* 1991). Increases in "B" esterase activity have been observed following exposure of starlings to low levels (1% LD_{50}) of demeton-S-methyl (Thompson *et al.* 1991a), as a diurnal variation in starlings (Thompson *et al.* 1988) and raptors (Garcia-Rodriguez *et al.* 1987) and during the development of nestling tree sparrows (Thompson 1991). Increases in "B" esterase activity following pesticide exposure may be due to induction, release of the esterase from the liver as a defense mechanism or leakage following damage of the hepatocytes.

Present methods for monitoring inhibition, which involve assay of the activity of whole serum or plasma towards acetyl- or butyryl-thiocholine iodide (Ellman *et al.* 1961, Westlake *et al.* 1980) do not take into account changes in the amount of the enzyme. Therefore, a method is required which takes into account not only the activity of the enzyme but also the amount of the enzyme present. In combination these parameters can be used to assess the specific activity of the enzyme (activity per mg protein), allowing assessment of inhibition even when induction or release has occurred. Antibodies raised to serum cholinesterase can be used to assess the amount of antigenic enzyme present by enzyme linked immunosorbant assay (ELISA) and its associated activity by immunoprecipitation. This paper describes the development of such an antibody based method for measuring serum butyrylcholinesterase (BChE) activity together with initial studies on its use to monitor exposure of pigeons (*Columba livia*) to organophosphorus and carbamate pesticides.

MATERIALS AND METHODS

All materials were obtained from Sigma Chemical Company, Dorset, UK, unless otherwise stated. Rabbits were obtained from Hylyne Rabbits, Cheshire, UK and pigeons were supplied by Abbot Brothers, Norwich, UK.

Activity towards butyrylthiocholine iodide was assayed at 37°C by the method of Ellman *et al.* (1961) as adapted by Westlake *et al.* (1980) but scaling down the method for use in the wells of a microtitre plate.

Butyrylcholinesterase was purified from bulked chicken serum by the method of Ralston *et al.* (1983) with adaptations from Khattab *et al.* (1993). All steps were performed at 4°C. Protein concentration in fractions eluted from the column were monitored at 280nm.

Procainamide was linked to CH-Sepharose 4B by the method of Ralston *et al.* (1983). The column was equilibrated with 0.02M phosphate buffer pH 7.0 and chicken serum was applied, the column was washed and activity eluted with a 0-0.8M sodium chloride gradient. The fractions eluted with highest activity were bulked and dialysed overnight against 0.02M phosphate buffer. The dialysate was applied to a DEAE cellulose ion-exchange column and the column was washed with 0.02M phosphate buffer. BChE activity was eluted using a 0-0.2M sodium chloride gradient.

Purified fractions with highest activity from the ion-exchange column were pooled, lypholised, redissolved in 2ml buffer and dialysed against 0.02M phosphate buffer. The protein content of the dialysed purified BChE was determined using Bio-Rad protein assay reagent and bovine serum albumin (Fraction V 98-99% albumin) as standard. The purified BChE was stored at -20°C.

The purity of the BChE prepared by the above method was assessed by SDS and non-SDS PAGE. Protein bands were visualised using a Bio-Rad silver stain kit and prestained molecular weight markers were used to establish the molecular weight of the protein. Activity of the enzyme in the non-SDS PAGE was visualised by the method of Martin *et al.* (1981) using N-methyl indoxyl acetate as the substrate.

Antibodies to the purified BChE were raised in Simone-Noir half lop rabbits. The rabbits were control bled prior to immunisation in order to confirm no cross reactivity was present. The purified BChE (200µg BSA equivalents) was mixed with 1ml Freunds complete adjuvant and injected sub-cutaneously at 4 sites. Additional booster injections were administered in Freunds incomplete adjuvant. The rabbits were bled after 6 weeks and immediately before each subsequent boost. The blood was

allowed to clot and antiserum was isolated. IgG was purified from the antiserum using protein A-sepharose (Catty and Raykundalia 1988).

Cross reactivity of the antibodies raised with avian serum components was assessed by Western blotting of SDS-PAGE gels on which a variety of passerine and non-passerine sera (mallard (*Anas platyrhynchos*), pheasant (*Phasianus colchicus*), pigeon, starling (*Sturnus vulgaris*), greenfinch (*Carduelis chloris*) and house sparrow (*Passer domesticus*)) had been run (Towbin *et al.* 1979).

An ELISA for BChE was developed using purified BChE as the standard and the antibodies raised and purified as described above. Purified BChE standards (50-0.8ng BSA equivalents/ml) or diluted serum (1:10,000) were diluted in 0.1M sodium carbonate buffer pH 9.6 and 100µl was added to the wells of a microtitre plate. The plate was incubated overnight at 4°C, washed with PBS-Tween and blocked for 1 hour at 37°C with PBS containing 5% skimmed milk (essentially fat free). The plate was washed again with PBS-Tween and incubated with a 1:10,000 dilution of antibody (2mg IgG/ml) at 37°C for 1 hour. After washing, the plate was incubated with goat anti-rabbit antibody/horse-radish peroxidase conjugate at 37°C for 1 hour. The plate was then washed with PBS-Tween and incubated at room temperature in the presence of substrate (O-phenylenediamine hydrochloride/H_2O_2) in 0.15M citrate buffer pH5.0. The reaction was stopped with 12% sulphuric acid. The absorbance was read at 490nm using a Bio-Rad plate reader.

The ELISA was used to assess the changes in levels of BChE in sera from pigeons following exposure to pesticides. Six groups of five pigeons, randomly grouped but including at least one of each sex, were orally dosed with 0.5 or 5mg/kg chlorfenvinphos, methiocarb or demeton-S-methyl in corn oil. Seven control pigeons were dosed with corn oil. Blood samples were taken from the brachial vein prior to dosing and at 3, 6, and 24 hours. Blood samples were allowed to clot at 4°C and serum aspirated into labelled eppendorf tubes. Serum was held at 4°C and assayed for BChE activity within 8 hours of collection. Subsamples of serum were stored at -20°C for later ELISA analysis.

RESULTS AND DISCUSSION

The purification of chicken serum BChE yielded a single protein band following both SDS- and non-SDS-PAGE with the molecular weight on SDS-PAGE of approximately 90,000 and following non-SDS-PAGE of approximately 360,000. These molecular weights are similar to those reported for pigeon serum BChE (Khattab *et al.* 1993). The protein and esterase activity stains coincided on non-SDS-PAGE. The overall

purification factor in the fraction with highest activity following ion-exchange chromatography was approximately 1500 when compared to the specific activity of BChE in the serum.

Investigation of the cross-reactivity of the antibodies with avian sera by Western blotting of denaturing- and non-denaturing-PAGE gels showed single bands coinciding with that of the purified protein. Additional experiments also showed that 100% of the BChE activity of starling, chicken and pigeon sera could be immunoprecipitated following incubation with the antibody and therefore separate analysis of antigenic as compared to total BChE activity was not required in subsequent serum samples.

An ELISA was developed using purified BChE and the antibodies raised. Log transformed plots of amount purified BChE against absorbance following ELISA detection, using a 1:10,000 dilution of the antibody, gave a linear standard curve ($r=0.994$) and minimum detection limits of 1.6ng/ml BSA equivalents. The ELISA was used to establish the effects of pesticides on the levels of BChE present in avian sera. Figure 1 shows the activity of BChE in the sera of pigeons dosed with organophosphorus and carbamate insecticides expressed in terms of per ml serum and in terms of the levels of BChE protein present. Control birds showed no diurnal variations in the amount of BChE present. Dose related decreases in serum BChE activity were observed in all cases with serum BChE activity being lower following dosing with either 0.5mg/kg or 5mg/kg demeton-S-methyl than either methiocarb or chlorfenvinphos at the same dose levels.

The results show that in all but one case exposure to pesticides did not appear to result in changes in the amount of the enzyme present, i.e. parallel and equal changes were observed in both BChE activity per ml serum and per mg enzymic protein. Only in the pigeons dosed with low levels of methiocarb were differences observed between the two methods of analysis, lower levels of activity per mg protein were observed at 6 hours due to higher levels of enzyme present ($p<0.05$, Students paired t-test). Higher levels of exposure may result in inhibition of protein synthesis by these pesticides (Varshenya *et al.* 1986).

It has been suggested that the specific activity of BChE would be more constant between individuals than activity per ml serum (Khattab *et al.* 1993). This would remove the need to obtain control values for individuals by pre-exposure sampling. The population distribution of control serum BChE activity in the pigeons used in these experiments is shown in Figure 2 (expressed as per ml serum and per mg protein) together with the distribution of amounts of BChE protein. The specific activity shows similar breadth of population distribution to the activity when expressed

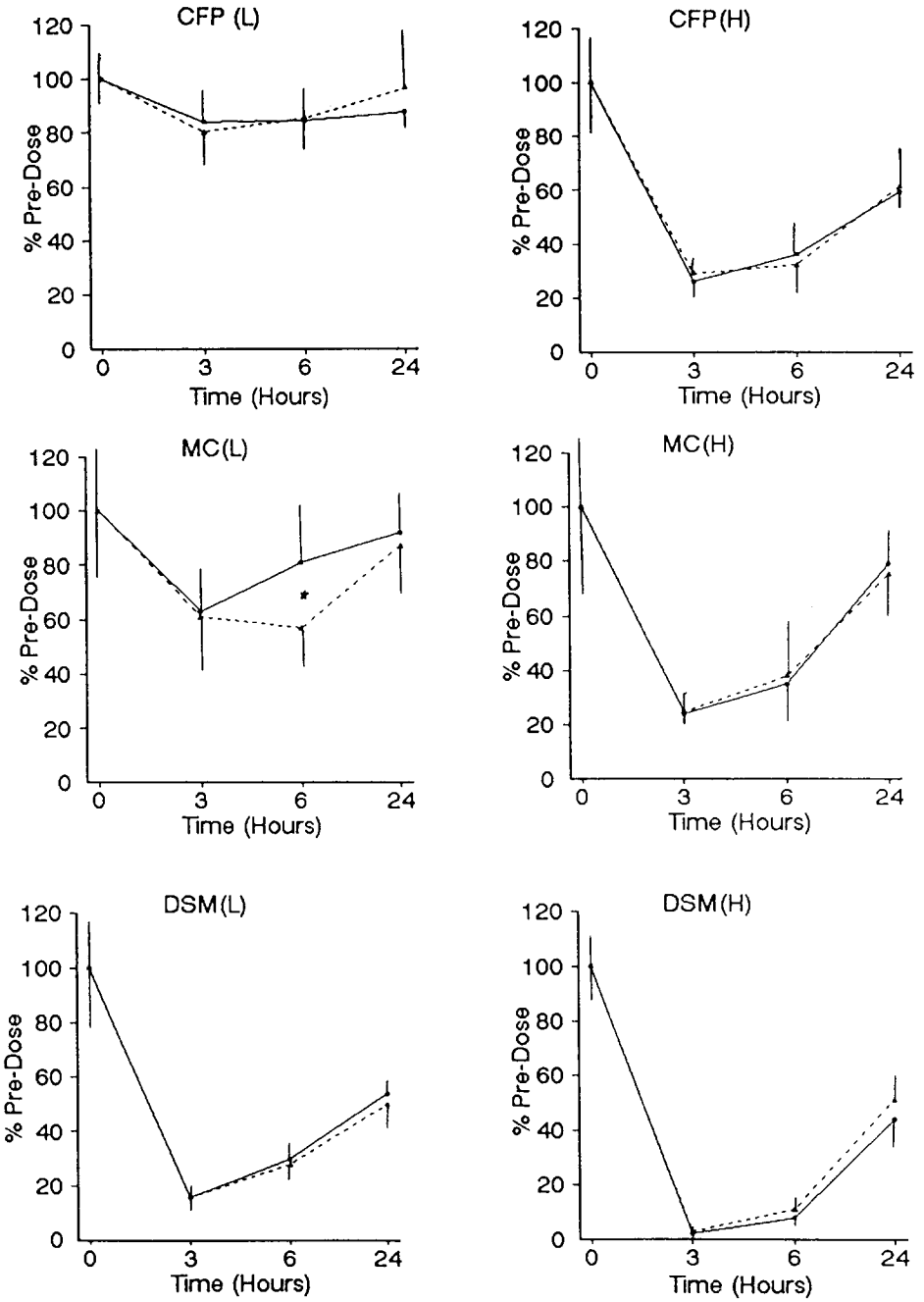

Figure 1. Activity of pigeon serum BChE following dosing with chlorfenvinphos (CFP), methiocarb (MC) and demeton-S-methyl (DSM) at 0.5 (L) and 5.0mg/kg (H). Activity is shown as —— µmol/min/ml serum and -----µmol/min/mg protein as a percentage of the pre-dose activity. Standard errors for all points are shown as bars. * Significant difference in level of inhibition observed ($p<0.05$, Students paired t-test).

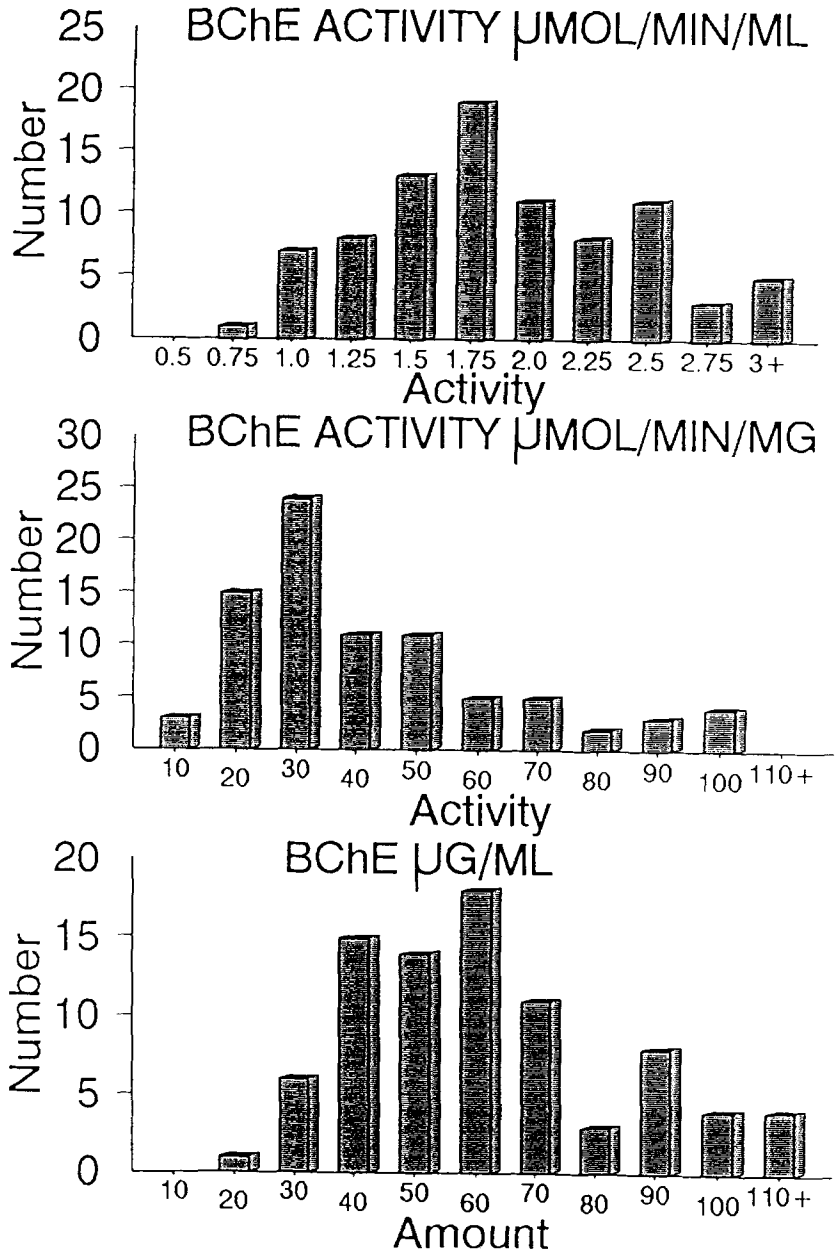

Figure 2. Distribution of serum BChE activity (µmol/min ml serum (mean 1.85, coefficient of variation (cv) 0.324) and µmol/min/mg protein (mean 39.3, cv 0.577)) and of serum BChE (µg protein (mean 55.6, cv 0.415)) in a population of undosed pigeons (84 birds). The activity or amount is shown by groupings between the stated values e.g. 0.75 represents data falling between 0.51 and 0.75.

per ml serum. Such inter-individual variability may be due to the wide range of BChE isozymes present in serum of birds (Thompson *et al.* 1991b) which have varying affinity for the substrate. Therefore, the use of specific activity in monitoring levels of inhibition does not remove the need for pre-exposure sampling to assess control levels.

This study has shown that serum BChE specific activity can be assessed by the development of an ELISA using antibodies raised to the enzyme. The exposure of pigeons and starlings to a range of organophosphorus and carbamate pesticides showed only one occasion on which levels of enzymic protein were altered sufficiently by the pesticide to show higher levels of inhibition of specific activity than of activity per ml serum. However, on this occasion the difference between the two methods was over 20% illustrating the substantial underestimation of inhibition caused by increases in enzyme levels. This method of assessing specific activity will be of greatest utility when assessing inhibition against background variations which were not observed in this experiment, e.g. changes in the amount of BChE due to diurnal responses (Garcia-Rodriguez *et al.* 1987) or development (Thompson 1991).

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