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Acetylcholinesterase Activity and the Effects of Chlorfenvinphos in Regions of the Starling Brain

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Inhibition of brain acetylcholinesterase (AChE) activity is generally regarded as a useful indicator of poisoning by organophosphorous or carbamate pesticides (Martin et al. 1981, Grue et al. 1983). However, the enzyme is not uniformly distributed in the brain, nor is the distribution necessarily similar in different species. Overall differences between grossly defined regions of the pigeon brain were reported for AChE and 3 other enzymes by Aprison et al. (1964). More recent studies of other bird species have reported variation within the regions defined by Aprison et al., between areas which can be distinguished on the basis of involvement in visual, auditory and other functions (Vischer et al. 1982, Ryan and Arnold 1981). Detailed distribution of AChE may differ between species (eg. Goldberg and McCaman 1967), and both overall levels and distribution of activity change during avian development (Grue and Hunter 1984, Pilo and Iyengar 1981). In some circumstances biochemical changes in the brain following drug administration can differ from area to area (Weiss and Heller 1969). Results of this nature are of potential practical importance to the design of procedures for investigating wildlife deaths. More comparative data might help to predict which non-target species are most likely to be susceptible to the increasing use of anticholinesterase agents in agriculture.

In this paper we present measurements of baseline AChE activity in different regions of the brain of the European starling (Sturnus vulgaris) for comparison with the data already available for the pigeon (Aprison et al. 1964). Secondly we compare the effects in these brain regions of dosing starlings at three sublethal levels with the organophosphate chlorfenvinphos.

MATERIALS AND METHODS

The results of Aprison et al (1964) suggested that 4 subjects should be sufficient to detect differences in baseline AChE activity of different brain regions. Four male starlings were taken from a group which were caught from the wild 6 months previously and maintained on unlimited water and Turkey Starter Crumbs. Each bird in turn was killed by decapitation and the brain removed, dissected and stored on ice. Brains were divided into sagittal halves, each of which was further divided to separate roughly the regions distinguished by Aprison et al. (1964): telencephalon, diencephalon with optic lobes, cerebellum, medulla oblongata with pons (for convenience we shall refer to these regions as forebrain, midbrain, cerebellum and hindbrain respectively). This division was achieved by transverse cuts immediately before and behind the optic lobes, and a third cut at the base of the cerebellum. To improve distinction between the mid- and fore-brain a sliver of tissue about 1mm. thick was discarded from the rear of the fore-brain section. The samples were frozen at -20° C pending analysis. Finally the sex of the subjects was confirmed by dissection.

For analysis each sample was thawed, then immediately weighed and homogenised in 4 volumes of ice cold 1% Triton X-100 (9 volumes for the smaller hind-brain sections). The post-mitochondrial supernatant (PMS) was prepared by centrifuging the homogenate at 17000 G for 45 minutes at 4° C. The AChE activity of the PMS was assayed by the method of Ellman et al. (1961) adapted for use on a Gilford 203 analyser with a final assay volume of 1.21 ml (0.8ml of 1.01mM 5,5'- dithiobis-2-nitrobenzoic acid in 150mM phosphate buffer pH 7.4, 0.4ml of 3.11mM acetyl thiocholine iodide and 10ul of brain PMS). Optical density change was measured at 412nm and 25° C.

To investigate effects of chlorfenvinphos twelve starlings taken from the same stock as before were used in groups of 4 on three successive days. The birds were weighed and transferred to individual aviaries (3m x 2m x 2m high) in the afternoon and dosed with chlorfenvinphos the following morning. The four birds in each group were assigned to treatments at random; one to each of the 3 treatment levels 6.4, 4.0, 2.4 mg chlorfenvinphos/kg and one to the control treatment (2µl acetone carrier only). Solutions of technical grade chlorfenvinphos (82.9% z-isomer; donated by Shell Research PLC) in acetone were made up each day such that the dose required for each bird was contained in 1.2 - 2.5 µl. The calculated doses were injected into freshly boiled maggots (blowfly larvae) with a

micro-syringe calibrated to 0.2 µl. Maggots were used for comparability with future behavioural experiments in which we intend the subjects to take dosed maggots voluntarily without handling; but in the present experiment we caught the birds, placed the maggots in the back of the mouth with blunt forceps, and encouraged swallowing with 3-5 drops of distilled water. The birds were left undisturbed in their aviaries until being recaptured and killed by decapitation 210 minutes after dosing (range 208 to 212 minutes). (In a previous experiment (unpublished) we had demonstrated that peak inhibition of starling AChE occurs at about three and a half hours after dosing with chlorfenvinphos in maggots at 1.6 mg/kg.) Dissection and analysis of the treated birds were carried out as described above, except that only the left sagittal half of each brain was used; the forebrain samples were homogenised in 4 volumes and other samples in 9 volumes of 1% Triton X-100; and 200 ul of the PMS from each sample was set aside for chemical reactivation. Reactivation was initiated by mixing with 50 ul of 125 mM aqueous pyridine 2-aldoxime methiodide (2-PAM) solution and 10 µl samples were taken for AChE assay after 30 minutes (Martin et al. 1981).

RESULTS AND DISCUSSION

Baseline AChE activities for the different starling brain regions are shown in Table 1. Each value in the table is the mean activity measured for two sub-samples of a single homogenised brain section. The coefficient of variation for differences between sub-samples was 5.1%, and would have been much lower but for two instances where the differences were about 20% of the mean. The esterase activities measured in the cerebellum of subject 2 correspond closely to those obtained for the hindbrain. It is possible that this resulted from cutting too close to the ventral surface of the brain in separating these samples in subject 2, though we made every effort to be consistent.

The values in Table 1 were used to test the significance of differences between parts and sides of the brain by analysis of variance (ANOVA). The ANOVA was performed by the statistical computer package GENSTAT (Genstat Manual 1977) which estimated replacements for the two missing values (bird 1 hindbrain samples). The values in Table 1 were converted to logarithms for ANOVA, since this transformation removes the correlation between mean and variance in the raw data (Sokal and Rohlf, 1969). The analysis confirms the differences apparent in the tabulated values.

Table 1. AChE activities (μmol substrate changed/min/g tissue (I.U.)) in different regions of the starling brain.

	Subject	1	2	3	4	Mean
Forebrain	right	45.9[a]	49.8	45.5	44.7	48.0
	left	50.8	49.3	51.1	46.8	
Midbrain	right	81.9	102.1	87.1	86.0	89.3
	left	90.9	83.8	96.1	86.7	
Cerebellum	right	14.6	26.2	13.4	13.5	17.5
	left	15.1	27.5	15.3	14.6	
Hindbrain	right	-[b]	27.0	26.4	21.9	25.7
	left	-	28.0	27.6	23.3	

[a] each value is the mean of two measurements for a single tissue homogenate. Brain regions are defined in the text.
[b] sample not analysed

There are highly significant differences between the four parts of the brain (variance ratio $F_{3,19} = 200$, $P<0.01$), but no differences between the left and right sides ($F_{1,19} = 0.8$, $P>0.05$; interaction with brain region also not significant). Differences between birds are significant, though small ($F_{3,19} = 4.44$, $P<0.05$; coefficient of variation 3%) relative to those between brain regions. These natural differences between subjects account for some of the variability we encounter below in the relationship between dose rate of chlorfenvinphos and the resulting inhibited levels of AChE.

The results of the second part of our study in which starlings were dosed with chlorfenvinphos are summarised in Table 2. The degree of inhibition of enzyme activity increases with increasing dose rate in each region of the brain but the data are rather variable. ANOVA was used to test the significance of the treatment effect on activity, and to test whether the effect was the same in each brain region. The treatment effect was divided into linear and non-linear components (Sokal and Rohlf 1969); in every analysis performed treatment effects were exclusively linear (i.e. directly proportional to dose rate). ANOVA of the measured AChE activities indicated a significant interaction between treatment and brain region ($F_{3,30} = 3.2$, $P<0.05$), i.e. the absolute amount of inhibition for a given dosage differs between regions. A second ANOVA was performed on the data for the treated birds expressed as a percentage reduction from the mean control AChE for the corresponding brain region, as

Table 2. Inhibition of starling brain AChE following dosing with chlorfenvinphos.

	Control	Dose mg/kg		
		2.4	4.0	6.4
Forebrain	42.9[a] (0.7)	11.1[b] (5.4)	19.1 (4.4)	35.1 (6.1)
Midbrain	82.6 (3.9)	8.0 (6.4)	20.1 (8.3)	25.3 (4.8)
Cerebellum	14.8 (0.5)	22.6 (0.9)	22.2 (11.0)	30.5 (7.6)
Hindbrain	29.9 (1.4)	15.1 (9.9)	18.9 (6.4)	31.2 (12.2)
	(I.U.)	(percent reduction from control level)		

[a] mean AChE activity for 3 subjects expressed in I.U.
[b] activities expressed as percentage reduction from the control levels given in the first column (mean of 3 subjects at each dose rate). Standard errors are given in brackets.

summarised in Table 2. This transformation provides an estimate of percentage inhibition, and the analysis shows that there is a positive linear effect of dose rate ($F_{1,22}=9.3$, $P<0.001$) but no difference at all between brain regions ($F_{3,22}=0.5$, $P>0.05$), nor any interaction between dose rate and region ($F_{6,22}=0.3$, $P>0.05$). This is evidence that the percentage inhibition of AChE is similar in different brain regions. AChE was reduced by 4.1% (standard error 1.3) relative to control values for every 1mg/kg of chlorfenvinphos administered. This is inconsistent with the LD50 of 3.2mg/kg reported by Schafer (1972) for starlings dosed by gavage, since esterase inhibition in excess of 50% would usually be expected to result from dosing at lethal levels (Grue et al. 1983). None of the birds in the present study showed any overt signs of intoxication. The milder response (and some of its variability) in our study may be partly attributable to the method of dosing, though our unpublished results obtained by dosing with chlorfenvinphos and corn oil in gelatin capsules are again consistent with an LD50 in excess of 10mg/kg.

The linear relationship with dose rate accounts for only 26% of the variation in the response to dosing measured as reduction from control values. Some of the remaining variation is due to individual differences, but because actual pre-treatment AChE activities are not obtainable this cannot be separated into components due to differences in baseline levels (as seen in the first part of our study) and differences in response to treatment. We had hoped that chemical reactivation (Martin et al. 1981) might provide an estimate of what AChE activities had been in the starlings prior to dosing, and thus enable us to control for the individual differences in baseline levels. In fact ANOVA of the AChE activities before reactivation expressed as a percentage reduction from activities in the same homogenates after reactivation again indicated proportionately similar inhibition in the different brain regions ($F_{1,21}=27.1$, $P<0.001$, slope 4.1, standard error 0.8; region/dose rate interaction not significant). There was however additional variation between regions ($F_{3,21}=18.8$, $P<0.001$) which was unrelated to dose rate, and appears to depend on reactivation conditions. This limits the usefulness of reactivation for this purpose. The application of the technique would be limited in any case if the degree of reactivation obtainable is variable from one AChE inhibitor to another, as is indicated by the results reported for a range of organophosphate and carbamate pesticides by Martin et al (1981).

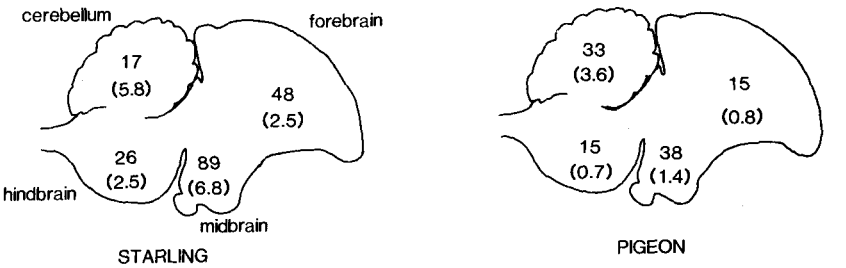

Figure 1. Diagram illustrating differences in patterns of acetylcholine esterase activity between the brains of the starling and pigeon. Values given are means, with standard errors in brackets. Data for the pigeon are from Aprison et al. (1964; values converted to micromoles per minute per gram of tissue).

Major differences in AChE activity clearly do exist between different brain regions in the European starling, as Aprison et al. (1964) have already shown for the pigeon. Though we cannot be sure that our dissection of the brain was fully comparable to that by Aprison et al., the data suggest that the pattern of variation between brain regions is not the same in the two species (Figure 1). A functional explanation for species differences in detailed AChE distribution might be suggested by comparable measurements for a wider range of species with varying ecology and behaviour (Bullock 1984). Comparative measurements for areas of the brain implicated in specific functions might also suggest reasons why some species are much more susceptible than others to organophosphate poisoning (Stanley and Bunyan 1979), and ultimately provide an indicator of non-target species at risk from the use of anticholinesterase agents in agriculture.

Whether our results are expressed relative to controls or reactivated levels, they indicate that inhibition of AChE three and a half hours after oral administration of sublethal doses of chlorfenvinphos is proportionally similar in different regions of the starling brain. This suggests that measures of AChE inhibition representative of the whole brain may be obtained from analysis of samples from any brain region, provided they are expressed relative to control values for comparable control tissues. This may sometimes be useful since it obviates the need to collect and homogenise whole brains where this is impracticable. Since whole-brain control values are already available for a wide range of species (Westlake et al. 1983) it will usually be more convenient to obtain whole brains for the investigation of wildlife deaths.

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