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Avian Esterases as Indicators of Exposure to Insecticides—The Factor of Diurnal Variation

H. M. Thompson,[1] C. H. Walker,[1] and A. R. Hardy[2]

[1]Department of Physiology and Biochemistry, University of Reading, P.O. Box 228, Whiteknights, Reading RG6 2AJ, Berks., U.K. and [2]ADAS Worplesdon Laboratory, Ministry of Agriculture, Fisheries and Food, Tangley Place, Worplesdon, Guildford, Surrey GU3 3LQ, U.K.

Cholinesterases, especially brain acetylcholinesterase, have been more widely used than other "B" esterases for monitoring exposure to organophosphorus (OP) and carbamate pesticides (Ludke et al 1975). This reflects their greater sensitivity to inhibition and the fact that inhibition of brain acetylcholinesterase is a useful indicator of toxic effect (Hill and Fleming 1982). Avian erythrocytes contain little or no acetylcholinesterase activity (Stedman and Stedman 1935). The so called "carboxylesterases" also show some promise as indicator enzymes, particularly in serum, and, since they exist in a number of isoforms (Bunyan et al 1968; Csuka and Petrovsky 1968), it may be possible to observe patterns of inhibition characteristic of individual insecticides. In the following account the term "carboxylesterase" will refer to esterases that hydrolyse esters such as naphthyl acetate and N-methyl indoxyl acetate. Avian esterases are largely or entirely "B" esterases and, as such, are subject to inhibition by OP and carbamate insecticides. Since inhibition is only very slowly reversed, these esterases have considerable potential as biochemical indicators of exposure to low levels of OPs (Bunyan et al 1968,1969). They are less satisfactory indicators of carbamate exposure because inhibition is more readily reversed (O'Brien 1969).

Monitoring procedures based on an enzyme must take into account temporal variations in activity. As part of a laboratory and field study of pesticide exposure in passerine birds, the present study was undertaken to investigate diurnal variations in the levels of serum α-naphthyl acetate esterase (NAE) activity in captive starlings (Sturnus vulgaris): The aim was to provide a reliable baseline for measuring patterns of inhibition caused by the organophosphorus compounds chlorpyrifos and demeton-S-methyl.

Send reprint requests to H.M. Thompson at the above address.

MATERIALS AND METHODS

All chemicals were AR grade unless otherwise stated. Precast polyacrylamide analytical isoelectrofocusing plates pH 4-6.5 were purchased from LKB Produkter Ltd.Technical grade chlorpyrifos (Dursban) (O,O diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate) was a gift from Dow Chemical Company and technical grade demeton-S-methyl (Metasystox) (S-2-(ethylthio)ethyl) O,O dimethyl phosphorothioate) was a gift from the Ministry of Agriculture, Fisheries and Food, Tolworth, Surrey.

Starlings were trapped during the previous winter by the Ministry of Agriculture, Fisheries and Food, Worplesdon, Surrey and maintained on a diet of chick-starter crumbs (BOCM) (ad libitum) in a communal aviary. When required for experiment they were weighed and transferred to individual cages.

Diurnal variations were studied on several days during May 1987 by obtaining blood samples from the brachial veins of groups of 4- 6 birds at 07.00 hrs, 09.30 hrs, 12.30 hrs, 15.30 hrs and 19.00 hrs (British Summer Time (BST)) (sunrise 05.00hrs, sunset 21.00hrs). Each group was bled at 2 consecutive times; thus one group was bled at 19.00 hrs and 07.00 hrs and one group at 07.00 hrs and 09.30 hrs and so on.

Birds, in three groups of ten, were dosed during May at 09.30 hrs (BST) by oral intubation. Control birds received 1ml/kg of corn oil alone whilst the other 2 groups received either chlorpyrifos (10mg/kg) or demeton-S-methyl (2mg/kg) dissolved in corn oil. Blood samples were taken from the brachial vein of all birds before dosing and from 4 birds from each group at 3, 6 and 24 hours after dosing.

Serum was separated by centrifugation and assayed immediately for NAE activity by the method of Gomori (1953) as adapted by Bunyan et al (1968) but substituting 25mM tris/HCl buffer pH 7.6 containing 1mM calcium chloride.

Analytical isoelectric focusing of serum samples was performed on thin layer polyacrylamide gels pH 4.0-6.5 according to LKB application note 1804. The gels were stained according to the method of Martin et al (1983) and quantified using an LKB laser densitometer linked to an Apple II computer using the Gelscan program (LKB Gelscan System GLSC CP2 (Non-Arithmetical)).

RESULTS AND DISCUSSION

Diurnal variation of NAE activity is shown in Figure 1 and is expressed as a percentage of the mean of all the activities measured at 09.30 hours (hrs) (all birds were bled at 09.30 hrs before dosing). NAE activity rose continuously after 07.00 hrs and reached a maximum at 19.00 hrs, this representing a 2.5 fold increase over a period of 12 hours. By 09.30 hrs on the following day the activity had fallen to the same level as that found at 09.30 hrs on day 1. The mean activities determined at 07.00 hrs and 19.00 hrs were significantly different ($p<0.01$ and $p<0.001$ respectively) from the mean activity at 09.30 hrs on the same day.

The esterase profiles shown by isoelectric focusing also appear to show diurnal variation not only in the overall intensity of the profiles but also in the percentage contribution of each band to the total intensity (the 5 bands under discussion are those which are present in all profiles) (Figure 2). The diurnal variation in overall intensity is to be expected since N-methyl indoxyl acetate is a substrate for the same carboxylesterases as naphthyl acetate; the variation observed between 07.00hrs and 19.00hrs was 1.9 fold in overall intensity compared to 2.5 fold in NAE activity.

Table 1 Diurnal variation in band intensity[1]

Band	Mean Intensity at 19.00 hrs / Intensity at 07.00 hrs
1	6.38 (± 2.81) **
2	1.24 (± 0.56)
3	1.96 (± 0.22) **
4	1.64 (± 0.06) *
5	1.23 (± 0.21)
Total	1.88 (± 0.26)

[1] Intensity = absolute intensity of band as calculated by the LKB Gelscan program.
* Intensity at 19.00 hrs significantly greater than that at 07.00 hrs $p<0.2$.
**Intensity at 19.00 hrs significantly greater than that at 07.00 hrs $p<0.01$.

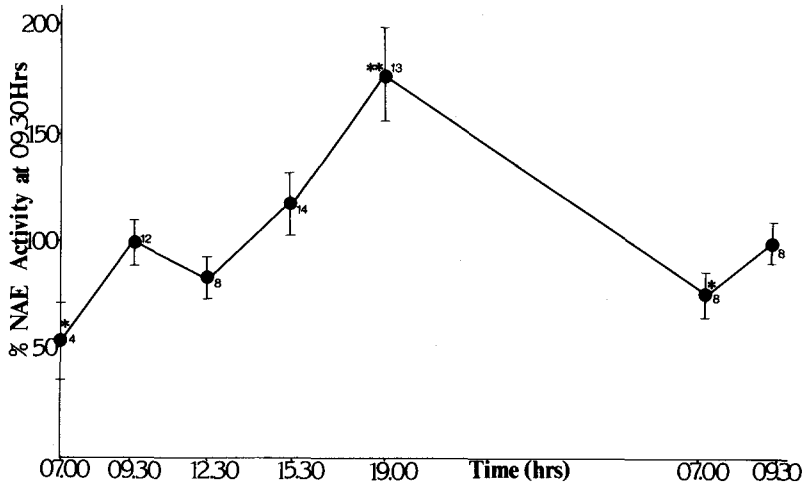

Figure 1 Diurnal variation in NAE activity between 07.00 hrs and 09.30 hrs (data from several experiments on different days). Activity is expressed as a percentage of the mean activity at 09.30 hrs on the first day (0.83 ± 0.09 µmol/min/ml). Numbers of birds are shown at each point with points representing means and vertical lines standard errors of means. Activity significantly different from that at 09.30 hrs on the first day * $p<0.01$, ** $p<0.001$ (student's t test)

All the profiles under discussion were run on the same isoelectric focusing gel and are, therefore, directly comparable with one another with respect to intensity. Bands 3 and 4 differ in intensity between 07.00 hrs and 19.00 hrs by factors of 1.96 and 1.64 respectively which are comparable with the overall diurnal variation in intensity of 1.88 (Table 1).

The mean intensities of bands 2 and 5 do not differ significantly between 07.00 hrs and 19.00 hrs (Table 1). At 19.00 hrs the intensity of band 1 differs from that at 07.00 hrs by a factor of 6.4 which indicates considerable diurnal regulation in the level of activity of this enzyme. As can be seen in Figure 2 this is the only band which makes a significantly different contribution to the intensity of the profile at 07.00hrs in comparison with 19.00hrs.

Effects of exposure to chlorpyrifos and demeton-S-methyl on total NAE activity are shown as a percentage of the value at 09.30 hrs (before dosing) (Figure 3).

In Figure 4 the levels of inhibition caused by the administration of chlorpyrifos and demeton-S-methyl are expressed after taking into account diurnal variation of activity. For the purposes of this calculation,

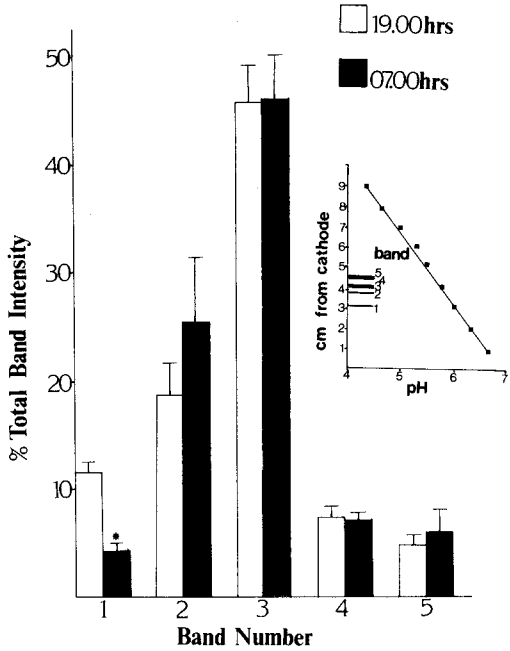

Figure 2 Diurnal variation in intensity of bands separated by IEF. Intensity of individual bands is represented as a percentage of the total intensity of bands 1-5. Inset - tracing of typical starling IEF banding pattern to show band designation.* - % of total intensity at 07.00 hrs significantly different from that at 19.00 hrs $p<0.02$ (student's paired t test).

levels of activity in control birds at 12.30 hrs and 15.30 hrs were estimated to be 112% and 124% of the value at 09.30 hrs respectively (based on Figure 1). These values are not significantly different from those found for the undosed controls at 12.30 hrs and 15.30 hrs.

The influence of diurnal variation on the estimation of the percentage inhibition of NAE activity is shown in Table 2. Taking data for 15.30 hrs, the percentage inhibition of NAE activity calculated from activities at 09.30 hrs are 41% and 53% for demeton-S-methyl and chlorpyrifos respectively. If, on the other hand, the calculation is based on controls sampled at the same time of day the corresponding figures are 52% and 62% inhibition.

Thus the first method of calculation,which assumes no diurnal variation, underestimates levels of inhibition by 11% and 9% respectively. A considerably larger

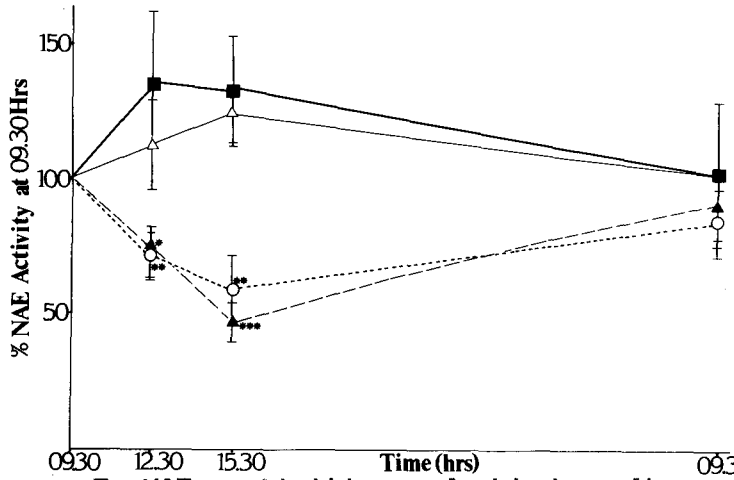

Figure 3 NAE activities of birds after dosing. Activities are expressed as a percentage of the activity of the same bird at 09.30 hrs.Birds were dosed with corn oil (■), demeton-S-methyl (O) or chlorpyrifos (▲) (diurnal variation is also shown (△)). Points represent means with vertical lines representing standard error of means. Activity significantly different from value at 09.30 hrs * $p<0.2$, ** $p<0.1$, *** $p<0.05$ (student's paired t test).

error is to be expected at 19.00 hrs when control activities are 1.75-fold higher than at 09.30hrs.

In the absence of data on diurnal variation it is unwise to attempt the estimation of percentage inhibition of NAE activity by comparison with controls taken at a different time of day from the inhibited samples. In earlier studies of changes in avian NAE activity levels after exposure to organophosphorus pesticides under laboratory conditions, it was concluded that the levels of NAE activity were too variable to be useful in monitoring exposure (Westlake

Table 2 Comparison of methods for estimating inhibition of NAE shown by birds exposed to organophosphorus insecticides.

	Mean % of activity at 09.30 hrs		Mean % of control at same time point	
TIME	12.30 hrs	15.30 hrs	12.30 hrs	15.30 hrs
COMPOUND				
DEMETON-S-METHYL	71	59	63	48
CHLORPYRIFOS	73	47	65	38

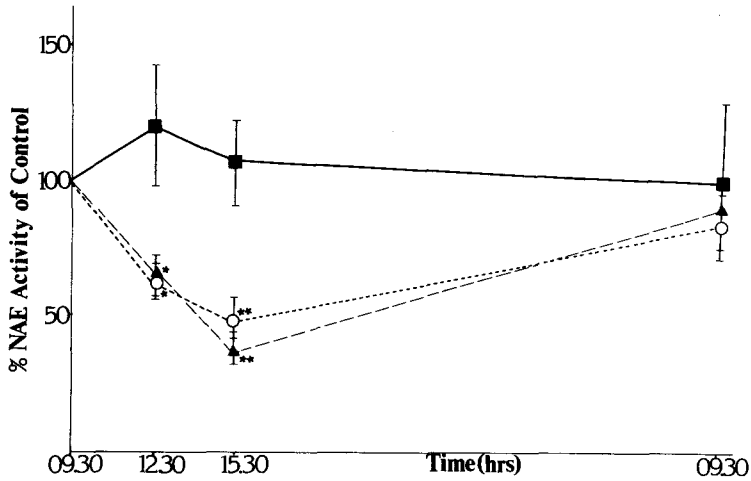

Figure 4 NAE activities of birds after dosing. Activities are expressed as a % of the value expected for the time point based on diurnal variation (see text). Birds were dosed with corn oil (■), demeton-S-methyl (○) or chlorpyrifos (▲) (undosed = 100%). Points represent means with vertical lines representing standard error of means. Activity significantly different from control (100%) * $p<0.05$, ** $p<0.02$ (student's paired t test).

et al 1981; Bunyan and Taylor 1966). However, these papers did not consider the possibility of diurnal variations when samples are taken up to 18 hours apart (Bunyan and Taylor 1966). The safest procedure in the absence of such data is to ensure that controls are sampled at the same time of day as individuals exposed to the insecticide.

Serum butyrylcholinesterase activity did not show a clear pattern of diurnal variation in the starling during the course of this study although it does show seasonal variations (Hill and Murray 1987). The value of studying carboxylesterase activity in combination with serum cholinesterase activity when monitoring higher levels of exposure to insecticides is evident here since levels of only 20-30% inhibition of NAE activity were found in association with 90% inhibition of serum cholinesterase during the course of these experiments.

Further work needs to be done to establish the extent of diurnal variations in NAE activity, and in the specific activity of individual isozymes (e.g. by use of specific antibodies), in different species of birds and its relationship, if any, to daylength and feeding behaviour. Knowledge of such diurnal variation is important if the measurement of carboxylesterase

activity is to be used to monitor exposure of birds to organophosphorus and carbamate pesticides in the field.

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