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# Application of Brain Cholinesterase Reactivation to Differentiate Between Organophosphorus and Carbamate Pesticide Exposure in Wild Birds

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ABSTRACT: Brain cholinesterase activity was measured to evaluate pesticide exposure in wild birds. Thermal reactivation of brain cholinesterase was used to differentiate between carbamate and organophosphorus pesticide exposure. Brain cholinesterase activity was compared with gas chromatography and mass spectrometry of stomach contents. Pesticides were identified and confirmed in 86 of 102 incidents of mortality from 29 states within the USA from 1986 through 1991. Thermal reactivation of cholinesterase activity was used to correctly predict carbamates in 22 incidents and organophosphates in 59 incidents. Agreement ($P < 0.001$) between predictions based on cholinesterase activities and GC/MS results was significant.

*Key words:* Avian brain cholinesterase, carbamate, organophosphate, famphur, carbofuran.

Deliberate abuse, misuse, or approved agricultural application of organophosphorus and carbamate pesticides exposes wildlife to hazardous chemicals. The pesticides inhibit brain cholinesterase (ChE) activity and can cause death. Analysis of brain ChE activity is a diagnostic technique that can be used with necropsy findings and other laboratory results to identify organophosphorus and carbamate pesticide exposure and lethality (Bunyan et al., 1968; Ludke et al., 1975). Moreover, because many carbamates are reversible inhibitors and many organophosphorus pesticides are non-reversible inhibitors (Martin et al., 1981), ChE depression or inhibition followed by thermal reactivation can be employed to discriminate between organophosphorus and carbamate poisonings (Stansley, 1993). Birds are particularly suitable subjects for study because their brain ChE activity is stable at ambient temperatures and little affected by post mortem decomposition (Prijono and Leighton, 1991). Previous investigations of wildlife pesticide poisonings were principally feeding or dosing studies. Our objective was to evaluate the effectiveness of applying thermal reactivation of ChE to distinguish between carbamate and organophosphate exposure of wild birds in 102 incidents of mortality. The gastrointestinal contents of each carcass were analyzed by gas chromatography and mass spectrometry to identify and confirm the presence of pesticide.

From 1986 through 1991, the carcasses of suspected pesticide poisoned birds were collected from 29 states within the USA. These states included: Alabama, California, Colorado, Delaware, Florida, Idaho, Illinois, Iowa, Kansas, Louisiana, Maryland, Massachusetts, Minnesota, Missouri, Montana, Nebraska, New Mexico, North Dakota, Ohio, Oklahoma, Oregon, South Dakota, Texas, Utah, Virginia, Washington, West Virginia, Wisconsin, and Wyoming. The carcasses were chilled or frozen and shipped to the National Wildlife Health Center (NWHC), Madison, Wisconsin. Brains were partially or completely thawed, removed, and evaluated following Ellman et al. (1961) as described by Hill and Fleming (1982). Cholinesterase activities were determined at 20 to 23 C on a UV visible spectrophotometer model 100-20 (Hitachi Instruments, Danbury, Connecticut, USA) with a R100A recorder (Perkin Elmer, Norwalk, Connecticut) from 1986 through 1989, and on a model DU-65 UV visible spectrophotometer (Beckman Instruments, Inc., Fullerton, California) from 1990 to 1991. Brain ChE activities were expressed as micromoles

acetylthiocholine hydrolyzed/minute/gram of wet weight brain tissue (μmoles/min/g).

Cholinesterase activities were compared with the conspecific control ChE activities determined by NWHC or with the extensive list of brain ChE values of apparently healthy wild birds provided by Hill (1988). Whenever possible, control brains from the same species were tested simultaneously with brains from birds suspected to have died of pesticide poisoning. If the ChE activity of a sample was inhibited by more than two standard deviations (diagnostic threshold) from the control brain ChE activity for that species, the brain homogenate was incubated 16 to 18 hr at 36 to 37 C and retested for ChE activity. When the activity of an initially inhibited sample was equal to or greater than the diagnostic threshold after incubation, carbamate exposure was suspected. If the activity remained below the diagnostic threshold after incubation, organophosphate exposure was suspected. The activity of incubated control brain homogenates remained constant or rose 2 to 3 μmoles/min/g.

When normal brain ChE activities of a species were not available, the distinction between carbamate and organophosphate exposure was not as clear. Organophosphate exposure was suspected when the ChE activity fell below 4 to 5 μmoles/min/g initially and rose less than 3 μmoles/min/g after incubation. Carbamate exposure was suspected if the ChE activity fell below 5 to 6 μmoles/min/g initially and rose substantially more than 4 μmoles/min/g after incubation.

To identify and confirm the presence of a pesticide, the gastrointestinal contents were homogenized, extracted three times with 1:1 acetone:methylene chloride, filtered, and adjusted to a 50 ml volume for analysis by gas chromatography (GC) (Belisle and Swineford, 1988). The quantitative analysis for organophosphorus pesticides was made on a model 5840 gas chromatograph (Hewlett Packard, Avondale, Pennsylvania, USA) equipped with a J&W Megabore 14% cyanopropylphenyl–86% methyl silicone capillary column and a flame photometric detector. For carbamate analysis, a Hewlett-Packard 5890 gas chromatograph equipped with a J&W Megabore 5% phenyl–95% methyl silicone capillary column and nitrogen phosphorus detector was used. The presence of pesticides was confirmed on a Hewlett-Packard 5890 gas chromatograph/5970 MSD mass spectrometer (MS) equipped with a 59970 ChemStation computer data system. The GC column was a 50 m cross-linked methyl silicone gum column with 0.2 mm i.d. and 0.32 micron film thickness. Detection limits were 1.0 μg/g wet weight for carbamate and 0.5 μg/g wet weight for organophosphorus pesticides, except for the carbamate methomyl, which had a detection limit of 5.0 μg/g wet weight.

The following carbamate pesticides were within the analytical capability of GC/MS: aldicarb, carbaryl, carbofuran, methiocarb, methomyl and oxamyl. Organophosphorus pesticides within the analytical capabilities of GC/MS included: phenylphosphonothioic acid O-ethyl O-p-nitrophenyl ester (EPN), acephate, azinphos-methyl, chlorpyrifos, coumaphos, demeton, diazinon, dichlorvos, dicrotophos, dimethoate, disulfoton, ethoprop, famphur, fensulfothion, fenthion, fonofos, malathion, methamidophos, methyl parathion, mevinphos, monocrotophos, parathion, phorate, phosphamidon, terbufos, and trichlorfon.

We evaluated one or more bird carcasses in 102 incidents of mortality (Table 1). Based on ChE activity, carbamates occurred in 22 incidents, organophosphates in 76 incidents, and no pesticides were found in four incidents. However, using GC/MS analysis we found carbamates in 27 incidents, organophosphates in 59 incidents, and no carbamate or organophosphorus pesticide in 16 incidents.

Two situations resulted in the five carbamate discrepancies between the results of brain ChE and GC/MS. In one, aldicarb exposure followed by spontaneous reacti-

TABLE 1. Brain cholinesterase (ChE) versus gas chromatographic and mass spectrometric (GC/MS) results from 102 incidents of mortality in wild birds in the United States from 1986 through 1991.

| ChE results | GC/MS | | | Totals for ChE inhibition and reactivation |
|---|---|---|---|---|
| | Carbamate present | Organophosphate present | No carbamate or organophosphates detected | |
| Inhibition and reactivation | 22 | 0 | 0 | 22 |
| Inhibition with no reactivation | 4 | 59 | 13 | 76 |
| No inhibition | 1 | 0 | 3 | 4 |
| Total for carbamates and organophosphates confirmed by GC/MS | 27 | 59 | 16 | |

vation (no inhibition) of ChE probably accounted for a false negative. In the other four, carbofuran, a carbamate, caused continued inhibition of brain ChE activity after incubation. The mean (±SD) initial ChE activity in these four incidents was 3.0 (±1.5) μmoles/min/g and the mean incubated ChE activity was 4.1 (±2.0) μmoles/min/g. This finding led to false predictions of organophosphate exposure or poisoning. The reason for this result may be especially high exposure levels, which would cause excessive concentrations of carbamate in the brain. In one of these incidents, 1,600 ppm of carbofuran were in the gizzard contents of one bird. Sustained inhibition is expected under such conditions because high amounts of free carbamate prevent reactivation of ChE (Aldridge and Reiner, 1972).

Cholinesterase reactivation did not occur in carcasses of birds exposed to organophosphate. However, there was evidence for organophosphate exposure or poisoning in 13 incidents, but no pesticide was found on GC/MS. False positives, involving sustained ChE inhibition, but no detected pesticide, may occur because of pesticide concentrations below detection limits, dermal exposure, autolysis, or unknown ChE inhibitors. In this study, there were no false positives for carbamates and no false negatives as defined by Kelsey et al. (1986), for organophosphate using GC/MS. In three incidents, no ChE inhibition occurred and no pesticides were found by GC/MS.

To assess the statistical agreement between the ChE results and the GC/MS procedures, a log-linear model (Tanner and Young, 1985) was applied. This method of analysis was used to calculate the chance agreement and test the statistical significance of a chance-corrected agreement between the two procedures. Based on the log-linear analysis there was significant ($\chi^2 = 84.1$, df = 1, $P < 0.001$) overall agreement between the ChE and GC/MS procedures. However, the level of agreement was not homogeneous across the three outcome categories (carbamate, organophosphate, and no inhibition) of the two procedures ($\chi^2 = 7.2$, df = 2, $P < 0.05$). From further log-linear model analysis among categories, similar strong agreement occurred between the ChE and GC/MS procedures for the carbamate and organophosphate categories. However, for samples with no pesticides (no inhibition) chance-corrected agreements between the two procedures were not significant.

Pesticides identified by GC/MS in 86 confirmed exposures were: famphur (35%), carbofuran (29%), parathion (8%), diazinon (6%), fenthion (6%), monocrotophos (3%), aldicarb (2%), phorate (2%), chlorpyrifos (2%), fonofos (2%), coumaphos (1%), dimethoate (1%), disulfoton (1%), and phosphamidon (1%). In one case, fonofos and chlorpyrifos were in the intestinal contents of two birds. Using GC/MS analysis, we observed fonofos and chlorpyrifos concentrations of 420 and 410 μg/g, respectively in one bird, and 62 and 48 μg/g

TABLE 2. Comparison of brain cholinesterase (ChE) activity of unexposed birds and initial and incubated brain ChE activity of birds exposed to famphur in the United States, 1986 through 1991.

| | ChE activity (μmoles/min/g) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Initial | | | Incubated | | | Unexposed | | |
| Species | Number sampled | Mean | SD | Number sampled | Mean | SD | Number sampled | Mean | SD |
| Red-tailed hawk (*Buteo jamaicensis*) | 6 | 3.3 | 1.4 | 6 | 4.1 | 1.6 | 6 | 17.3 | 1.4 |
| Bald eagle (*Haliaeetus leucocephalus*) | 16 | 2.2 | 0.9 | 16 | 2.8 | 1.4 | 76 | 16.1 | 2.1 |
| European starling (*Sturnus vulgaris*) | 6 | 5.3 | 1.1 | 6 | 3.9 | 0.8 | 12[a] | 22 | 2.0 |
| Red-winged blackbird (*Agelaius phoeniceus*) | 6 | 8.3 | 2.8 | 6 | 8.2 | 3.5 | 5 | 24.5 | 1.1 |
| Common grackle (*Quiscalus quiscula*) | 5 | 4.9 | 1.0 | 5 | 4.6 | 1.4 | 20[a] | 20 | 3.0 |
| Northern cardinal (*Cardinalis cardinalis*) | 6 | 6.5 | 1.2 | 6 | 8.4 | 1.1 | —[b] | — | — |
| American tree sparrow (*Spizella arborea*) | 7 | 5.7 | 1.6 | 7 | 6.1 | 1.5 | 4[a] | 20 | 4.2 |

[a] Number of observations, mean, and standard deviation from Hill (1988).
[b] No normal data available.

respectively in the other. We infer that these organophosphorus pesticides were applied in a 1:1 ratio.

Famphur and carbofuran were the most commonly identified pesticides in this study. Following exposure to famphur, an organophosphorus pesticide, the brain ChE activity remained below levels of the unexposed birds even after incubation (Table 2). After exposure to carbofuran, a carbamate, the incubated ChE activity rose substantially to within two standard deviations of the levels of the unexposed birds (Table 3).

Bunyan et al. (1968) reported spontaneous reactivation of esterases after diazinon was administered orally to pheasants (*Phasianus colchicus*). However, in five incidents when 14 birds were exposed to diazinon, we observed that inhibition of ChE persisted after incubation. The mean (±SD) initial ChE activity was 2.8 (±1.0) μmoles/min/g and the mean incubated ChE activity was 3.7 (±1.0) μmoles/min/g.

We conclude from our results that the thermal reactivation technique for bird brain ChE analysis is a suitable screening tool for identifying and differentiating field

TABLE 3. Comparison of brain cholinesterase (ChE) activity of unexposed birds and initial and incubated brain ChE activity of birds exposed to carbofuran in the United States, 1986 through 1991.

| | ChE Activity (μmoles/min/g) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Initial | | | Incubated | | | Unexposed | | |
| Species | Number sampled | Mean | SD | Number sampled | Mean | SD | Number sampled | Mean | SD |
| Canada goose (*Branta canadensis*) | 5 | 5.0 | 2.9 | 5 | 8.8 | 2.9 | 14 | 12.4 | 1.3 |
| Snow goose (*Chen caerulescens*) | 5 | 5.6 | 1.4 | 5 | 15.1 | 1.5 | 13 | 13.0 | 1.2 |
| Mallard duck (*Anas platyrhynchos*) | 3 | 5.4 | 4.2 | 3 | 14.4 | 2.4 | 18 | 10.5 | 1.4 |
| Northern pintail (*Anas acuta*) | 11 | 6.3 | 1.7 | 11 | 14.7 | 1.7 | 11 | 12.2 | 1.5 |
| Green-winged teal (*Anas crecca*) | 2 | 4.0 | 1.3 | 2 | 11.9 | 1.3 | 2 | 13.7 | 0.9 |
| Bald eagle (*Haliaeetus leucocephalus*) | 10 | 7.5 | 3.8 | 10 | 16.8 | 1.9 | 76 | 16.1 | 2.1 |
| Golden eagle (*Aquila chrysaetos*) | 5 | 4.9 | 3.2 | 5 | 18.6 | 1.8 | 29 | 16.2 | 2.2 |
| Dark-eyed junco (*Junco hyemalis*) | 7 | 10.1 | 8.6 | 7 | 31.0 | 3.2 | —[a] | — | — |

[a] No normal data available.

exposure to carbamate and organophosphorus pesticides. The procedure was efficacious even though carcasses remained in the field for different periods of time, were collected and stored under dissimilar conditions, and shipped at various temperatures. Application of thermal reactivation of brain cholinesterase on a routine basis, when organophosphate or carbamate exposure is suspected, would save time and reduce analytical costs. Although GC/MS analysis is not completely avoided, the analyst at least knows whether to first test for a carbamate or an organophosphorus pesticide.

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