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CARBOFURAN POISONING IN HERONS: DIAGNOSIS USING CHOLINESTERASE REACTIVATION TECHNIQUES

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ABSTRACT: Exposure to the carbamate insecticide carbofuran was detected using brain cholinesterase (ChE) reactivation techniques in heron carcasses collected from a potential pesticide exposure incident. Great egrets (*Nycticorax nycticorax*), great blue herons (*Ardea herodias*), and black-crowned night herons (*Casmerodius albus*) were exposed to carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) either by dermal exposure while wading or through ingestion of contaminated food items. Carcasses may have been in the field up to 5 days prior to collection. Brain ChE, substantially inhibited in most samples, increased 7.9–208% in the reactivation assay after 4 to 96 hours at 37 C, providing evidence of exposure to a carbamate pesticide. Crayfish (*Procambarus clarkii*) identified in the crops of some herons contained carbofuran residues of up to 0.6 parts per million wet weight, providing additional evidence of exposure. Reactivated brain ChE in several samples approached the range of control values.

Key words: Cholinesterase, reactivation, carbofuran, herons, carbamate insecticides.

INTRODUCTION

Accurate diagnosis of carbamate-induced wildlife mortalities in pesticide exposure incidents using brain cholinesterase (ChE) inhibition often is difficult. It may be confounded by unknown postmortem carcass history prior to sample retrieval, during which inhibited brain ChE may spontaneously reactivate (Hill, 1989). Brain ChE values reported from carbamate kills routinely have widely variable activities, lending considerable uncertainty to the diagnosis of exposure (Hill and Fleming, 1982).

Techniques for stabilization and reactivation of carbamate-inhibited ChE's have been developed in our laboratory (Hunt and Hooper, 1993). Using these techniques, we successfully have characterized both the propensity for and magnitude of induced reactivation of brain and plasma ChE's inhibited to differing degrees in aldicarb-dosed northern bobwhite quail (*Colinus virginianus*) and deer mice (*Peromyscus maniculatus*) (Hunt et al., 1993). Application of the technique also provided a reliable means of obtaining an estimate of pre-exposure ChE activity, without the need to rely on concurrently collected control data. Based on this finding, we propose that in diagnosis of potential carbamate exposure, each individual sample can function as its own control. This would then eliminate the need to collect additional specimens to serve as controls, thus avoiding the uncertainty of possible exposure of controls to unknown anti-ChE substances.

The possibility of monitoring exposure to organophosphorus or carbamate pesticides in nestling colonial waterbirds has been investigated previously (Custer and Ohlendorf, 1989). Our objective was to assess the application of the reactivation technique to brain ChE activity determination in heron carcasses recovered under field conditions from a potential carbofuran exposure incident. The performance of the technique was assessed by comparison of the data to that previously collected in our laboratory in method validation trials of carbofuran-inhibited northern bobwhite quail brain ChE. Residue analyses of crop samples from some of the herons were performed to substantiate the diagnosis of carbofuran exposure resulting from brain reactivation data.

MATERIALS AND METHODS

Carcasses of six black-crowned night herons (*Nycticorax nycticorax*), including two juve-

niles, three adult great blue herons (*Ardea herodias*), and five adult great egrets (*Casmerodius albus*) were collected from an altered and natural creek in San Joaquin County (37°54′N, 121°2′W), California, (USA) on 11 November 1991. Furadan 4F® (FMC Corporation, Philadelphia, Pennsylvania, USA) flowable insecticide (active ingredient, carbofuran 44%) was applied for control of grape phylloxera (*Daktulosphaira vitifoliae*) through drip chemigation, a chemical application by means of irrigation. Chemigation was active on the night of 5 November 1991 when a discharge from a drain valve allowed the material to pass into the creek. Animal carcasses, water samples, and soil samples first were collected on 11 November. Birds in this incident were collected from the creek and adjacent banks, indicating rapid deaths. Only one scavenged bird was observed.

Birds were collected, then frozen at −20 C on 11 November 1991 by the investigating game warden. The following day frozen carcasses were decapitated in the California Department of Fish and Game Pesticide Investigations Unit laboratory, Rancho Cordova, California, (USA), to preserve brains for cholinesterase determination. Whole heads were shipped on dry ice to The Institute of Wildlife and Environmental Toxicology, Clemson University, Pendleton, South Carolina, (USA) and stored at −80 C for 4 wk until analysis. Carcasses were thawed at 22 ± 2 C and necropsied. Stomach contents, if any, were removed for pesticide residue analysis.

Stomach contents were blended with 200 ml of methylene chloride. All solvents used were glass distilled, manufactured by Burdick and Jackson, and supplied by Baxter Scientific Products (Hayward, California, USA). The mixture was vacuum filtered and separated. The methylene chloride layer was passed through a layer of sodium sulfate and concentrated to 10 ml in a Kuderna-Danish flask with a 10 ml concentrator tube and Snyder column (Kontes, Hayward, California). Solvents were exchanged with petroleum ether by adding 50 ml of petroleum ether into Kuderna-Danish flask through the Snyder column, and evaporating to 10 ml. This was repeated with another 50 ml portion of petroleum ether and evaporated to 10 ml. The 10 ml extract was placed on a 22 × 300 mm chromatographic column (VWR, San Francisco, California) packed with 10 cm of florisil, eluted with 200 ml of petroleum ether, and the ether phase discarded. The florisil column was again eluted, this time with 200 ml of 35% ethyl acetate in petroleum ether. One ml of iso-octane was added, and the solution evaporated to 1 ml using a rotary evaporator (Haake Buchler Instruments, Saddlebrook, New Jersey, USA). This was transferred to a 10 ml concentrator tube and diluted to 10 ml with iso-octane. The solution was injected into a Varian 3600 gas chromatograph equipped with a thermionic specific detector and a capillary column (Varian Associates, Sugarland, Texas, USA). The minimum limit of detection for carbofuran was 0.05 parts per million (ppm).

Brain ChE was analyzed with the method of Ellman et al. (1961), as modified by Gard and Hooper (1993). Reactivation of carbamate-inhibited ChE was determined according to the method of Hunt and Hooper (1993). The reactivation method consisted of measuring ChE activity immediately after dilution with ice cold (2 C) buffer, termed the absolute activity, followed by an incubation period during which equal aliquots of the sample were maintained at 4 C and 37 C under conditions which maximized spontaneous reactivation of the inhibited enzymes. Cholinesterase activity was assessed at 4, 24, 48, 72, and 96 hr in order to characterize the progression of reactivation. Data analysis was accomplished by comparing the two post-incubation solutions and evaluating them for a statistically distinguishable difference. An activity obtained at 37 C which was 5% or more greater than that obtained at 4 C and was statistically significant ($P < 0.05$), as shown by a one-tailed Student's t-test (Zar, 1974), was considered reactivatable.

Whole heads were partially thawed and brains were removed while still frozen; therefore, the condition of the brain was not thoroughly assessed. Based on their odor, many of the samples had begun to putrefy. The incubation period for reactivation assays was 24 hr for great egret samples, 48 hr for great blue heron samples and 96 hr for black-crowned night heron samples. In a separate experiment, previously analyzed great egret and great blue heron samples were analyzed over the course of 96 contiguous hr, at 24 hr intervals, to assess whether they could be of further utility in diagnosing carbamate inhibition. These samples were stored as five-fold diluted whole brain homogenates at −80 C for the 4-wk period between assays.

Brain samples were collected from laboratory-reared captive northern bobwhite quail fed by oral gavage with 5.9 mg/kg analytical grade carbofuran (99% pure, Chem Service, Inc., West Chester, Pennsylvania, USA), or approximately 1.5 times the estimated dose lethal to 50% of the test population (LD_{50}) as determined in our laboratory. Controls were euthanized 1 hr after intubation by CO_2 asphyxiation. Carcasses were stored at 19 to 22 C and 29 to 76% relative humidity in the laboratory for up to 8 days post-mortem to simulate a lethal exposure under field conditions, where carcass discovery may require

TABLE 1. Reactivation of cholinesterase (ChE) activity in whole brain homogenates from *Ardeidae* species collected from a possible carbofuran exposure incident. Samples were diluted 200-fold and incubated with 1×10^{-4}M EDTA at 4 C and 37 C for the indicated time periods.

Sample number	Absolute activity[b]	Percent change[a]				
		4 hr	24 hr	48 hr	72 hr	96 hr
Black-crowned night heron						
1	16.5	0.9	1.4	−1.4	−2.4	−8.9
2	0.893	−7.1	2.2	20[c]	44[c]	106[c]
3[d]	0.891	−1.3	20[c]	59[c]	110[c]	202[c]
4[d]	1.38	13	30[c]	76[c]	136[c]	209[c]
5	6.62	−7.0	50[c]	94[c]	126[c]	138[c]
6	0.901	−6.9	4.9	27[c]	59[c]	143[c]
Great blue heron						
1	4.05	43[c]	40[c]	33[c]		
2	16.9	0.3	−1.0	−8.8		
3	8.20	25[c]	34[c]	25[c]		
Great egret						
1	11.9	26[c]	1.3			
2	14.2	7.9[c]	−7.4			
3	1.49	14[c]	11[c]			
4	16.0	−16	−42			
5	2.58	−6.2	17[c]			

[a] The two post-incubation subsamples, 4 C and 37 C, were compared.
[b] Expressed as μmoles acetylthiocholine hydrolyzed/min/g wet weight.
[c] The ChE activity value obtained at 37 C was both 5% or more greater than the value obtained at 4 C and was statistically significant as shown by a one-tailed Student's *t*-test ($P < 0.05$), and was therefore considered reactivatable.
[d] Identified as juveniles by plumage characteristics.

several days. All samples collected were stored at −80 C for at least 7 days prior to analysis. Brain samples were analyzed for absolute ChE activity and reactivation of carbamate-inhibited ChE after a 4 hr incubation period as described previously.

RESULTS

Brain ChE, substantially inhibited in most samples, increased 7.9 to 208% in the reactivation assay after 4 to 96 hr at 37 C, providing evidence for exposure to a carbamate pesticide. Crayfish (*Procambarus clarkii*) identified in the crops of some herons contained carbofuran residues of up to 0.6 ppm (wet weight), providing further evidence for exposure. Reactivation of brain ChE was detected in all but three of the 14 herons analyzed (Table 1). Incubation activity at 37 C was near or below the 4 C incubation activity throughout the assay for each of these three individuals, black-crowned night heron 1, great blue heron 2, and great egret 4. Of the six inhibited great egret and great blue heron samples, five had induced reactivation at the earliest measured timepoint, 4 hr (Fig. 1a). However, inhibited ChE activity in black-crowned night heron samples had a much slower pattern of recovery (Fig. 1b) taking as long as 48 hr before the first significant reactivation was evident (Table 1). All inhibited black-crowned night heron ChE had similarly slow patterns of reactivation. No differences were noted between ChE recovery patterns of adult and juvenile black-crowned night herons. Black-crowned night heron ChE reactivated to levels with a mean (±SE) of 704 (±159)% over absolute ChE values after 96 hr. Great blue heron ChE reactivated to levels 136% over absolute ChE values after 48 hr, and great egret ChE reactivated to levels with a mean (±SE) of 36 (±16)% over absolute ChE after only a 24-hr incubation.

Carbofuran-inhibited northern bobwhite quail brain ChE had absolute activities at similar levels of inhibition as herons, yet we observed dramatic increases in activity of 116 to 198% after only 4 hr as a result of induced reactivation techniques (Table 2). At the highest levels achieved by induced reactivation, ChE activity reached 85% of the mean absolute control activity of 17.2 units, as determined in the same experiment. Spontaneous reactivation occurred swiftly in carcasses remaining under ambient conditions for increasing lengths of time prior to collection. Cholinesterase activities in only two of the six samples collected at 8 days achieved apparently normal levels. One of these samples had no inducible reactivation, while the other reactivated by less than 6%.

Absolute ChE activity in assays of brain samples stored as homogenates and reanalyzed increased 3.5 to 16% from absolute ChE values determined initially (prior to −80 C homogenate storage) in three of the eight heron samples. Activity in four of the other samples, initially all less than 10 units, decreased by a mean (±SE) of 43 (±5.3)% between the two assays. The final sample, one of two which demonstrated no induced reactivation, had very little change. Reactivation profiles for the corresponding time periods of paired analyses of initial whole brains and stored homogenates in great egret and great blue heron samples closely resembled each other, though 37 C incubation activity generally was greater in the second analysis when compared to the first (Fig. 2a). At 24 hr, the 37 C activity from homogenated great egret samples had a mean (±SE) of 18 (±10)% higher than initially analyzed 37 C activity. The two most severely inhibited samples among the great egrets, numbers 3 and 5, had increases of 26 and 52%, respectively, in their 37 C activities. In great blue heron samples, homogenates had 37 C activity after 48 hr which averaged only 0.7% higher than that obtained initially from whole brains.

Induced reactivation, as measured by the percent increase between 4 C and 37 C incubation activities, increased by a mean (±SE) of 5 (±3)% in great egret samples, but was more than 7 (±4)% lower in great blue heron samples the second time they were analyzed. Based on data from reanalyzed samples, reactivation was detected sooner (4 hr instead of 24 hr) in

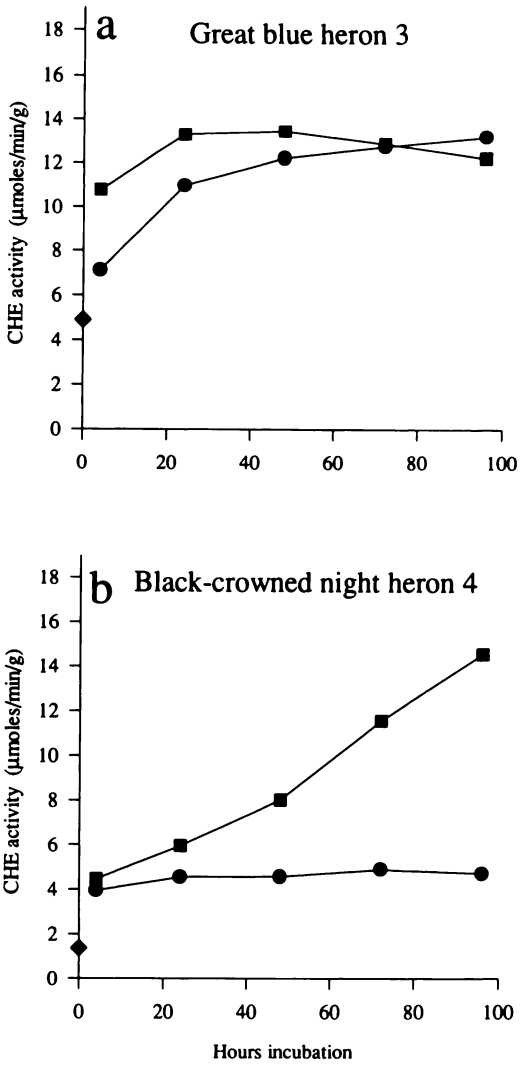

FIGURE 1. Absolute ◆, 37 C ■, and 4 C ● incubation brain cholinesterase (ChE) activities from a representative a) great blue heron and b) black-crowned night heron collected from a wildlife exposure incident showing different patterns of induced reactivation. Samples were diluted 200-fold and incubated with 1 × 10^{-4} M EDTA for the indicated time periods.

TABLE 2. Reactivation of cholinesterase (ChE) activity in whole brain homogenates of northern bobwhite quail given 5.9 mg/kg of carbofuran. Samples were diluted 200-fold and incubated 4 hr with 1×10^{-4} M EDTA at 4 C and 37 C.

Euthanasia or collection time[b]	Sample size	Mortality or survivor	Total CHE activity[a] (mean ± SD)			Mean percent change[c]
			Absolute	4 C	37 C	
Control[d]	8	S[e]	17.2 ± 1.24	16.3 ± 1.33	16.1 ± 1.34	−0.9
0 day	6	M	0.647 ± 0.187	2.11 ± 0.502	6.42 ± 2.70	194[f]
1 day	6	M	1.56 ± 1.06	3.42 ± 1.27	10.1 ± 3.53	198[f]
2 day	6	M	4.10 ± 3.58	5.35 ± 3.08	11.5 ± 3.04	155[f]
4 day	6	M	7.69 ± 5.12	8.67 ± 4.32	14.6 ± 1.84	116[f]
8 day	6	M	7.93 ± 7.68	7.98 ± 7.18	8.93 ± 6.75	40[f]

[a] Expressed as μmoles acetylthiocholine hydrolyzed/min/g wet weight.
[b] Carcasses were stored at 19 to 22 C and 29 to 76% relative humidity in the laboratory and collected at the indicated times postmortem.
[c] The two post-incubation subsamples, 4 C and 37 C, were compared.
[d] Controls were euthanized 1 hr after given corn oil carrier.
[e] S, survived; M, died.
[f] The ChE activity value obtained at 37 C was both 5% or more greater than the value obtained at 4 C and was statistically significant as shown by a one-tailed Student's t-test ($P < 0.05$), and was therefore considered reactivatable.

only one of six inhibited samples (great egret 5). Time to detection of diagnostically significant reactivation did not differ between any of the other paired analyses. Interestingly, the two most severely inhibited great egret samples had similar patterns of reactivation, when reanalyzed over the 96 hr period, as seen in initial analyses of black-crowned night herons (Fig. 2b). Evidently, the shortened incubation period followed in the initial analyses of great egrets was not sufficiently long enough to allow full expression of the pattern. Cholinesterase activity from these two samples achieved a mean of 512% over initial absolute activities at 96 hr, compared to only 47% after the initial 24 hr analyses.

DISCUSSION

In aldicarb-dosed northern bobwhite quail, we previously found that brain ChE reactivation techniques were able to restore an average of 90% of control activity, independent of the initial level of inhibition (Hunt et al., 1993). Although no controls were collected, the same probably was not true for carbofuran-inhibited ChE activity in the heron species tested in the present study, especially within the given incubation periods. Reactivated brain ChE in several samples approached the range of control values reported in the literature by Smith et al. (1986) and Hill (1988). In many of the samples where initially inhibited ChE activity had not yet reached levels comparable to published values by the end of the assay, activity was still increasing, often aggressively, implying that it might have achieved near pre-exposure levels had the incubation period been lengthened. This also was evident by the large percent increases reported between 37 C and 4 C activity over time, which were often greater than 100%. In those samples which displayed no reactivation, inhibited ChE most likely had undergone spontaneous hydrolysis back to normal levels as observed in trials with northern bobwhite quail. Nevertheless, induced reactivation produced significant results in all samples tested with inhibited ChE, identifying carbamate poisoning.

Induced reactivation of carbofuran-inhibited ChE in herons was considerably slower than than observed for northern bobwhite quail. Black-crowned night heron ChE, especially, had a unique pattern of reactivation not previously reported. Hill (1989) proposed that the magnitude of in-

hibition, as measured by absolute ChE activity, may influence the extent of reactivation. Excess carbofuran present in heron samples with very low absolute activities therefore may have continued to re-inhibit ChE as it was spontaneously reactivating, thereby resulting in slower apparent increases in activity. The same slow pattern of ChE recovery, though, also was observed in one black-crowned night heron sample, which had a higher absolute activity (6.62 units); but it was noticeably absent in both inhibited great blue heron samples; thus there was a possible species-specific response. The observation that northern bobwhite quail brain ChE samples, with comparable levels of initial inhibition, had maximum reactivation in only 4 hr is further evidence that species differences may be an important consideration of interpreting reactivation data. Species differences in the rates of spontaneous decarbamylation of aldicarb-inhibited plasma and brain ChE in northern bobwhite quail and deer mice previously have been identified in our laboratory (Hunt et al., 1993).

We observed both increases and decreases in absolute ChE activity between initial whole brain and stored homogenate analyses. Only the decreases, however, were consistent with Hill (1989), who compared ChE activities of fresh vs. intact frozen (−25 C) half brains in controls and carbamate-inhibited Japanese quail (*Coturnix japonica*) samples. Controls in that study decreased in activity by an average of 27%, while five of six carbamate-inhibited samples, with similarly severe levels of inhibition as observed in many heron samples in the present study, decreased by an average of 9%. Absolute levels of inhibited ChE from herons decreased by a much greater average, 43%; we propose that additional carbamate released by the homogenization process may have contributed to further increase observed levels of inhibition. In the three samples that increased in activity, initial absolute ChE was much less inhibited; thus spontaneous hydrolysis may already have begun and little free carbamate remained in the sample.

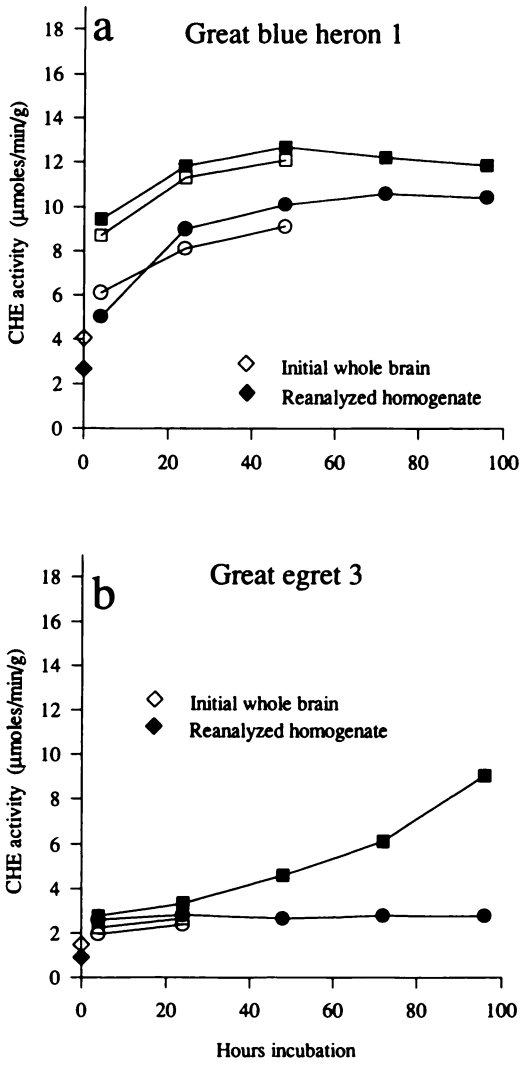

FIGURE 2. Comparison of absolute ◆, 37 C ■, and 4 C ● incubation brain cholinesterase (ChE) activities in initial whole brain (open symbols) vs. 5-fold diluted homogenates stored at −80 C for 4 wk (closed symbols) in a carbofuran-inhibited a) great blue heron and b) great egret sample collected from an exposure incident. Initial assay lasted 48 and 24 hr respectively; samples were reanalyzed over 96 hr.

The above results are evidence that carbamate pesticide-induced mortalities can be correctly detected based on the measurement of brain ChE activity, coupled with the additional technique of induced

reactivation. Based on our data, diagnostic results can be obtained even after carcasses have been exposed to unknown ambient conditions prior to collection, when carcass retrieval does not take place promptly, and when samples are not maintained under optimal conditions for ChE analysis for carbamate exposure. We also showed that previously analyzed brain ChE, stored at −80 C as minimally diluted homogenates, still can provide diagnostic results which may implicate carbamate pesticide exposure, though absolute activity and the magnitude of induced reactivation may not necessarily be accurately reflected.

This research was possible due to the abundance of tissue from a number of individuals from a variety of species, allowing for assay optimization. The different induced reactivation patterns observed between carbofuran-inhibited ChE from black-crowned night herons, great egrets, great blue herons, and northern bobwhite quail, as well as the variability which was observed between the different carbamate inhibitors, are evidence for the importance of optimizing ChE reactivation techniques for each new species/pesticide combination. Until such characterizations have been made, single sample forensic evaluations should proceed conservatively, allowing for sufficient duration of incubation to ensure adequate reactivation of carbamate-inhibited ChE.

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