CHOLINESTERASE (ChE) RESPONSE AND RELATED MORTALITY AMONG BIRDS FED ChE INHIBITORS

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Patterns of mortality and inhibition of brain and plasma ChE in birds treated with ChE inhibitors were studied in an attempt to determine the validity of using ChE activity as a monitoring and diagnostic technique. Analysis of brain ChE activity proved to be reliable for diagnosing and monitoring effects of selected ChE inhibitors in birds. Brain ChE inhibition exceeding 20% indicated exposure, and inhibition greater than 50% was sufficient for diagnosing cause of death. Individuals that died from dietary exposure to parathion[1] or carbofuran had brain ChE activities below 55% of normal; although individuals could survive with brain ChE activity lower than 50%. Problems associated with collection, storage, and analysis of tissues for ChE activity are discussed.

There has been an increase in the use of organophosphorus (OP) pesticides coincident with a decline in the use of organochlorine (OC) pesticides. OP's are rapidly metabolized by most organisms, thus limiting the usefulness of residue analyses for monitoring or diagnostic purposes. Reliable, standardized methods for monitoring the exposure of nontarget species of OP's are needed. Cholinesterase (ChE) activity has been used successfully in diagnosing OP exposure in human beings and is especially attractive as a diagnostic method because OP's are generally believed to owe their toxicity to acetylcholinesterase (AChE) inhibition of the nervous system. The potential use of ChE activity as monitor of OP exposure in nontarget animals has not been fully explored.

Several problems are associated with the application of ChE activity as a diagnostic method for detecting exposure of wild animals to OP compounds. There is a wide diversity of species and OP compounds which may yield varied responses depending upon the situation. Little information is available on normal ChE activities in tissues of wild animals, and valid control values may be difficult to obtain. No standardized methodology for the collection, processing, and analysis of samples exists. The susceptibility and response of many nontarget species to OP pesticides have been scantily studied, especially under natural conditions, which may include various types of natural stress and synergistic effects caused by other environmental pollutants.

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[1] Insecticides mentioned in text are chemically identified in Table VI.

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ChE activity has been used successfully on a limited basis to determine exposure of nontarget species to OP pesticides. Weiss (1958, 1959, 1961) using an adaptation of the colorimetric method of Hestrin (1949) monitored changes in fish brain ChE activity resulting from exposure to OP insecticides in the water. Brain ChE depression of 40 to 50% was lethal; and recovery of ChE activity after removing fish to uncontaminated water varied with the species, chemical, and extent of inhibition (Weiss 1958, Holland *et al.* 1967). Williams and Sova (1966) analyzed brain ChE of several species of fish from the Atlantic and Gulf Coasts and concluded that enzyme activities inhibited by 12 to 28% were indicative of possible OP pollution. Nicholson (1967) suggested that a 10% depression in ChE activity be used as a criterion for evaluating water pollution by organophosphates and carbamates. Gibson *et al.* (1969) studied possible sources of error in using fishbrain ChE as a monitoring method and reported variation depending on the methods of processing of samples prior to analysis. The same study presented evidence indicating death could occur at low levels of inhibition (25%) as a result of exposing fish to very high concentrations of parathion.

Bunyan *et al.* (1966, 1968a, 1968b) studied ChE activity and esterase patterns of the brain and blood of pigeons and pheasants treated with OP compounds. They concluded that blood was unsuitable as an enzyme source for diagnosing OP poisoning because it may be difficult to obtain and the variability of electrophoretic patterns of control and treated individuals was too great. They stated that a diagnosis of death was possible by measuring brain ChE activity and suggested the gradual development of normal ChE levels of "more common species" for use as control parameters.

Bunyan *et al.* (1968a) observed no significant change in brain ChE activity of untreated dead birds stored as long as 12 days post-mortem at ambient temperatures. Bunyan *et al.* (1968b) studied ChE activity of ring-necked pheasants (*Phasianus colchicus*) that were exposed to lethal and sublethal concentrations of chlorfenvinphos, demetonmethyl, dimethoate, azinphos methyl, diazinon, and ethion and found that brain ChE of dead birds was at least 90% inhibited by all the pesticides except diazinon. Following death, the ChE levels remained stable for 12 days with the exception of the diazinon- and ethion-treated birds, in which ChE underwent reactivation.

Other studies have demonstrated a close correlation between degree of exposure, toxicity, and ChE inhibition in birds. Mehrotra *et al.* (1967) tested the effect of malathion on house sparrow (*Passer domesticus*) brain ChE. Birds dying of malathion poisoning (dosed po) generally had brain ChE inhibited about 80%. The ChE of dead birds ranged from 36% to almost 100% inhibition. Shellenberger *et al.* (1970) found good correlation between whole blood or brain ChE inhibition and acute toxicity in feeding studies. Brain ChE activity was a good indicator of sensitivity of four species of birds to seven OP compounds. Shellenberger *et al.* (1966) studied the dietary toxicity of monocrotofos and dicrotofos in Japanese quail (*Coturnix c. japonica*) and bobwhite (*Colinus virginianus*). Gough *et al.* (1967) tested the dietary toxicity of azinphos methyl to Japanese quail in an effort to determine the usefulness of these species as indicators for testing sensitivity of birds to pesticides. Both studies demonstrated an excellent relationship between dietary exposure and brain ChE activity. ChE activity of whole blood was also a useful indicator

of exposure, but results were more erratic than brain ChE values (Gough *et al.* 1967). No attempt was made to determine ChE levels indicative of mortality. Brain ChE activity was depressed, with few exceptions, to a greater degree at equivalent exposures in species more susceptible to a given compound (Shellenberger *et al.* 1970).

Blood ChE was used to monitor the effects of OP pesticides on chickens (*Gallus gallus*) and ring-necked pheasants. Brust *et al.* (1971) measured a significant drop in whole blood ChE of young Leghorn cockerels fed 80 ppm of Dursban in their drinking water. Plasma ChE of pheasants dosed with 1.0 mg/kg of diazinon was inhibited by 83% within one hr.

The use of ChE activity to monitor exposure of birds in natural situations has had little application and given varied results. Elder and Henderson (1969) used caged fish and free-flying birds to determine the effects of ULV (ultralow volume) Baytex mosquito control applications. The study included assaying brain ChE activity of native populations before and after treatment. Investigators found marked ChE inhibition in brains of killdeer (*Charadrius vociferus*), red-wing blackbirds (*Agelaius phoeniceus*), and spotted sandpipers (*Actitis macularia*) collected from within the treatment area. There was no observable effect of fenthion on caged fish in the treated area, but there was an unmistakable increase in the number of individuals of several species with decreased brain ChE activity in the post-treatment groups contrasted with those from pretreatment samplings. Blue grouse (*Dendragapus obscurus*), apparently suffering from phosphamidon poisoning immediately following treatment for spruce budworm (Finley 1965), had blood ChE averaging 56% inhibition. Hill *et al.* (1971) found no evidence of significant exposure of house sparrow populations resulting from malathion applications (ULV). They stated that maximum negative variation of brain ChE for control individuals was never more than 20% below normal. Seabloom *et al.* (1973) attributed the death of 5,000 to 25,000 birds to possible fenthion poisoning. They based their conclusions on brain ChE analyses, which revealed low enzyme activity in four species of dead birds.

Numerous studies of the effects of OP compounds on wildlife include little or no ChE data (Mulla *et al.* 1966, Pillmore *et al.* 1971, Parsons and Davis 1971). Considering the sensitivity of many wildlife species to OP pesticides (Heath *et al.*, 1972, Tucker and Crabtree 1970, Hudson *et al.* 1972, McFarland and Lacy 1968, Hill 1971), studies which have not included ChE analyses provide little substantive evidence of OP toxicity to wildlife.

We studied the relative sensitivity of Japanese quail of different ages to parathion and the feasibility of using ChE activity of brain and blood as a means of determining exposure to, or mortality from, OP pesticides. Our objective was to investigate the methods and validity of using ChE activity of birds for monitoring exposure to OP compounds and to determine effects of storage conditions on ChE activity.

Materials and methods

Japanese quail, housed in identical battery cages (light regimen 14 hr light and 10 hr dark), were allowed unlimited feed and water. Assignment of birds and diets to pens was by random numbers. Diets were prepared by dissolving technical-grade parathion in corn oil. The concentrate was mixed with dry feed and constituted 2% of the diet. Corn oil was added to control diets at the same rate. Carbofuran was dissolved in minimal amounts of acetone before incorporation into corn oil concentrate.

Japanese quail were fed two different concentrations of parathion-treated food for five days beginning at 2, 4, or 8 weeks of age. (Dietary exposure was used since it is probably more representative of field exposure.) Eight replicates of 12 to 15 birds were used for each concentration. After 2, 6, 12, 24, 48, 72, 96 and 120 hr exposure and 72 additional hr on untreated food, a bird was randomly selected from each replicate and decapitated. The brain and 2 to 3 ml of blood were chilled on ice for ChE analysis.

Dietary concentrations of parathion were based on preliminary tests. Each age group received two concentrations: one was the expected LC_{50} and the other, the LC_{50} from the preceding age group. Comparisons between age groups and between dead and surviving birds within age groups were possible.

Supplemental study of age-related sensitivity was achieved by statistical comparison of LC_{50}'s for this study population at hatching and at 1, 2, 4, and 8 weeks of age. The test protocol and analysis were essentially those described by Heath *et al.* (1972). At each age, diets of six geometrically arranged concentrations of parathion were fed for five days, then untreated food was fed for the next three days. One group of 10 to 20 birds was used per concentration. Six groups of controls accompanied each experiment. LC_{50}'s and their 95% confidence limits, and sensitivity ratios between LC_{50}'s, were derived from probit analysis as described by Finney (1952) and programmed for computer by Daum and Killcreas (1966).

Food consumption was measured daily during each experiment and is reported as per bird-day based on pen averages. To compensate for an apparent 8 to 12 hr period of fasting prior to death, 0.5 day was subtracted from the total days of feeding attributed to each bird that died. Time of death was determined by periodic checks.

Dead birds were discarded during the final evening check to insure that birds retrieved in the morning for brain ChE analysis had been dead for less than 12 hr. To account for any time-related difference, four controls were killed on two evenings and their ChE activity compared with freshly killed controls the following morning. No deterioration in ChE activity was detected.

The postmortem stability of brain ChE under various storage conditions and durations was determined. Japanese quail, mallards (*Anas platyrhynchos*), and starlings (*Sturnus vulgaris*) were asphyxiated and immediately stored at 35°, 18-24°, 2°, or – 22°C. Stored specimens were randomly selected at 6, 12, 24, 48, 72, 168, or 336 hr and their brain

ChE activity determined. The storage response of inhibited ChE was determined from brains of Japanese quail exposed to 600 ppm of either parathion or carbofuran for four hr.

Brain ChE assay was achieved by homogenizing the brain in 0.1 *M* phosphate buffer (pH 8.0) at 20 mg/ml (wet weight) with a Talboys model 101 automatic homogenizer, followed by centrifugation for five min at about 1000 G using an Adams Dynac centrifuge equipped with a CT-1350 head. An aliquot of the supernatant was taken for protein analysis (Lowry *et al.* 1951), and brain ChE activity expressed as nanomoles (n*M*) of acetylthiocholine hydrolyzed per min per mg of protein. The brain supernatant was then analyzed for ChE activity (Ellman *et al.* 1961) using a Bausch and Lomb Spectronic 70 fitted with a TVC-15 Celvac cuvette (14 X 95 mm). Plasma ChE was analyzed using a modification of the Ellman procedure (Mannheim kit # 15984, Boehringer Mannheim Corp., Mannheim, Germany).

Statistical methods are indicated with the respective experiments described in "Results". Analysis of variance and t-test were used to determine significant differences among and between means. Unless specified otherwise, mean ChE activities of treated groups are based on normal values derived from control animals analyzed simultaneously.

Results

Normal brain and plasma ChE activities of birds were measured to determine differences attributable to species, age, and sex (Table I). There were significant differences ($P < 0.01$) among the three species tested. No difference was noted between brain ChE activity of male and female Japanese quail at either four or eight weeks of age. Plasma ChE activity of female quail was 84.1% that of the males at four weeks and 82.0% that of the males at eight weeks (Table I). Mean plasma ChE values of two-week-old birds were significantly higher than those of the four-week ($P < 0.05$) and eight-week-old ($P < 0.01$) birds. The coefficients of variation for brain ChE of control birds were 17.2% (two-week-old), 17.2% (four-week-old) and 13.1% (eight-week-old).

Age-related sensitivity of Japanese quail to parathion was determined through feeding experiments using hatchlings, 1-, 2-, 4-, and 8-week-old birds from the same hatch. LC_{50}'s and patterns of ChE inhibition subsequent to poisoning were compared.

Sensitivity to dietary parathion decreased with age as demonstrated by comparing LC_{50}'s derived by probit analysis (Table II). The relationship between LC_{50}'s was linear from hatching through four weeks of age and was accompanied by predictable mortality patterns. Relatively constant sensitivity ratios varied from only 1.3 to 1.5. A marked increase in the LC_{50} occurred between four and eight weeks of age (sensitivity ratio, 5.89), suggesting that adults are much more tolerant of parathion than are juveniles. This relationship was misleading, however, because eight-week-old birds avoid the toxic diets during the five-day test and survive. This phenomenon was verified when only 3 of 37 eight-week-old birds died after starvation for five days. Survivors regained vigor rapidly when fed.

Brain ChE activity of Japanese quail followed a similar pattern of inhibition for birds of all ages exposed to dietary parathion approximating the LC_{50}'s (Figures 1-3). Birds of 2, 4, and 8 weeks of age exhibited significant ($P < 0.01$) ChE inhibition after 2, 12, and 6 hr exposure, respectively. Mean ChE activity, which steadily decreased throughout exposure, was indicative of toxic concentration and time. Partial recovery occurred after three days on untreated diet.

Mean ChE activities of two-week-old survivors reached their lowest levels after four days of exposure (Figure 1). Those dying from exposure to 170 ppm of parathion averaged ChE activity 26.1 (fourth day) and 32.1% (fifth day) of normal at four weeks of age. Survivors (four-week-old) had ChE levels averaging a low of 35.4% (170 ppm) on the fifth day and a low of 24.1% (350 ppm) on the 34th day (Figure 2). Birds dying from exposure to 350 ppm had mean brain ChE activities 24.4% and 38.6% of normal on days three and four. The 170 ppm treatment group recovered to 65.6% of normal after three days on a clean diet. There were too few survivors among four-week-old birds of the 350 ppm dietary treatment to determine enzyme recovery. Three birds, which died on the fifth day of treatment, averaged 31.2% (SE, 2.3) of normal (not shown in Figure 2).

Table I. *Brain and plasma ChE activity (mean and SD) of birds of different species, age, and sex*

			ChE Activity					
			Brain[a]			Plasma[b]		
Species	Age	Sex	No.	Mean	SD	No.	Mean	SD
Japanese[c] quail	2 wk	M+F	77	124.8	21.5	64	1735	535
	4 wk	M	34	132.6	23.0	20	1675	377
		F	37	129.1	22.0	35	1409[d]	340
	8 wk	M	38	119.1	15.7	39	1608	338
		F	27	117.2	15.7	28	1318[d]	412
Starling	adult	M+F	10	215.0	27.2	–	–	–
Mallard	10 days	M+F	10	169.7	17.9	–	–	–

[a] Brain ChE activity expressed as n*M* acetylthiocholine iodide hydrolyzed/min/mg protein.
[b] Plasma ChE activity expressed in milliunits.
[c] Differences between brain ChE of Japanese quail of different age and sex were not significant ($P > 0.05$; ANOVA).
[d] Female plasma ChE was significantly lower ($P < 0.01$; *t*-test) than males of the same age.

Eight-week-old quail exhibited greater variability in their response to dietary parathion than did the younger birds (Figure 3). Survivors of 350 and 1400 ppm had maximum mean depressions of 15.1% and 16.8% of normal ChE levels on the fourth day. Enzyme activity of birds dying on 1400 ppm averaged 14.0 (6 hr), 14.1 (1 day), 17.4 (2 day), 7.0 (4 day), and 31.4% (5 day) of controls (Figure 3). Mean brain ChE of dead birds from the 350 ppm treatment was 12.8% of normal (4 day). ChE activity of survivors given clean food for three days was about 60% of normal.

The mean brain ChE activity of dead birds did not exceed 40% of normal, and only two individuals exceeded 50%. The highest activity in the brain of a dead bird was 56.5% of normal. Ninety-one percent of the birds that died as a result of parathion poisoning had brain ChE activity less than 40% of normal, and 75% were less than 30% of normal.

ChE activities of all birds were pooled into categories according to age, sex, and treatment in order to determine the probability of reliably detecting toxic effects regardless of time after treatment as tested (Table III). It is apparent from data represented in Figures 1-3 that ChE of live birds falls below normal levels as exposure time increases and that the chances of diagnosing OP poisoning based on ChE activity would be greater after six hr post-exposure. Pooled mean ChE activities of birds on all treatment levels differed significantly ($P < 0.01$) from their control values. There were no marked differences between sexes fed the same dietary concentrations (Table III).

Feeding rate (Table IV) was reflected in the brain ChE inhibition and time to death (Figure 1-3) for two- and four-week-old groups. The two-week-old birds fed 85 ppm of parathion ate at a rate comparable to controls after two days exposure and marked ChE

Table II. *Comparative dietary toxicity of parathion to Japanese quail as related to age*

Age	LC_{50} [a](95% C.L.)	Sensitivity ratio[b]				
		1 day	1 wk	2 wk	4 wk	8 wk
1 day	109 (98-121)	–	1.38	1.81	2.71	15.95
1 wk	150 (135-167)	0.73	–	1.31	1.97	11.59
2 wk	197 (177-220)	0.55	0.76	–	1.50	8.83
4 wk	295 (262-336)	0.37	0.51	0.67	–	5.89
8 wk	1,739 (1,311-2,486)	0.06	0.09	0.11	0.17	–

[a] LC_{50} – The ppm of technical parathion in an ad libitum diet expected to kill 50% in 8 days. (Five days of toxic diet followed by 3 days on untreated feed.)

[b] Comparative sensitivity to parathion based on age (e.g., 1-day-old birds are 1.38 X more sensitive than 1-week-old birds).

inhibition ensued from that point. At 170 ppm, feeding was depressed throughout the five-day study, but the toxic level was such that ChE activity declined rapidly and was accompanied by death on the fourth day. The four-week-old birds fed 170 ppm ate at a rate comparable (based on controls) to the two-week-old groups, but they were not as markedly influenced. Brain ChE inhibition did not exceed 50% until the fifth day. At 350 ppm, food consumption decreased over the five-day exposure from 34% to 10% of the control rate, and ChE inhibition increased daily, and some birds died within three days.

The eight-week-old birds withstood brief periods of starvation more readily than younger groups. This factor was important because they avoided the toxic diets initially (Table IV) causing erratic ChE activity and mortality patterns (Figure 3). When fed

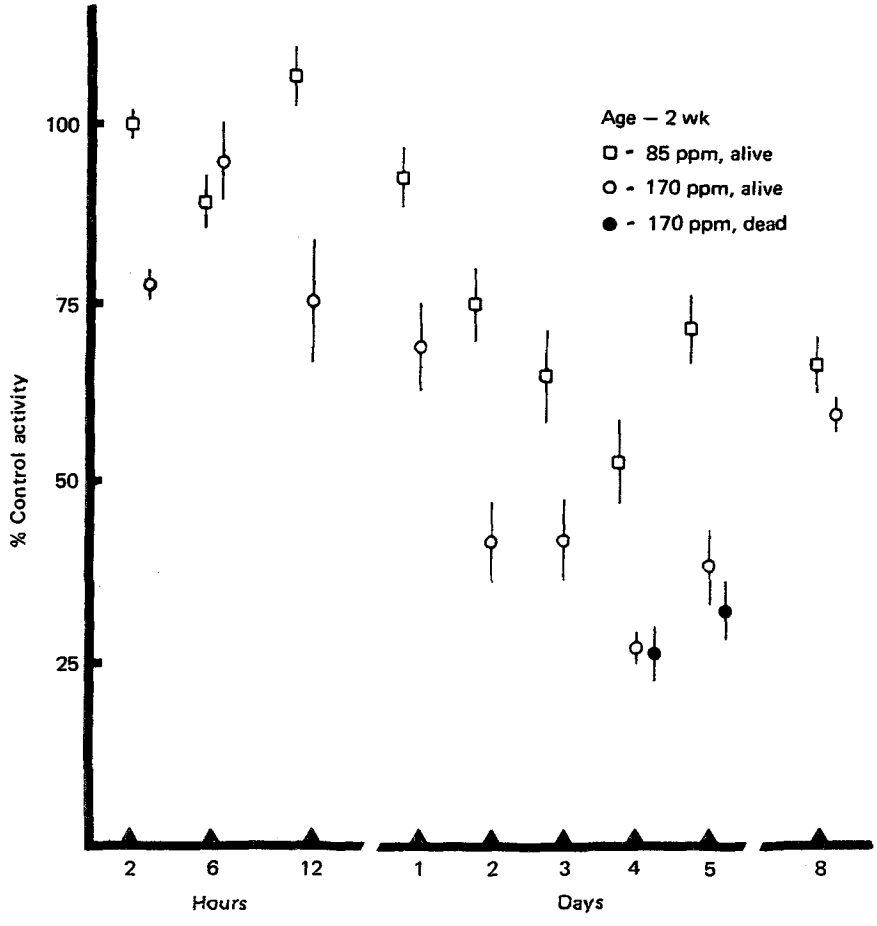

Fig. 1. Brain ChE activity of 2-week-old Japanese quail fed diets containing parathion for 5 days, followed for 3 days with untreated food. (Circles and squares = means; lines = SE).

350 ppm, food consumption of two-week-old birds was less than 20% that of control birds for the first three days; it then increased to over 40% by the fifth day. This pattern contrasts with that for four-week-old birds. When fed 1,400 ppm, average consumption was severely depressed from the onset of the study. Some birds died within six hr although survivors still had normal ChE activity. This indicates that certain birds were acutely dosed after consuming little food; others apparently avoided the toxic diet initially.

Mean plasma ChE activity of quail exposed to parathion in their diet dropped markedly after two hr (Figure 4). Treated groups of all ages had a mean ChE activity of less than 50% of normal after six hr of exposure. Birds exposed to the higher concentrations had generally lower enzyme activity at each sampling. Mean ChE activity decreased steadily for 12 to 24 hr and plateaued at about 30% of normal. After being placed on clean food for three days mean plasma ChE activities approached normal in all groups.

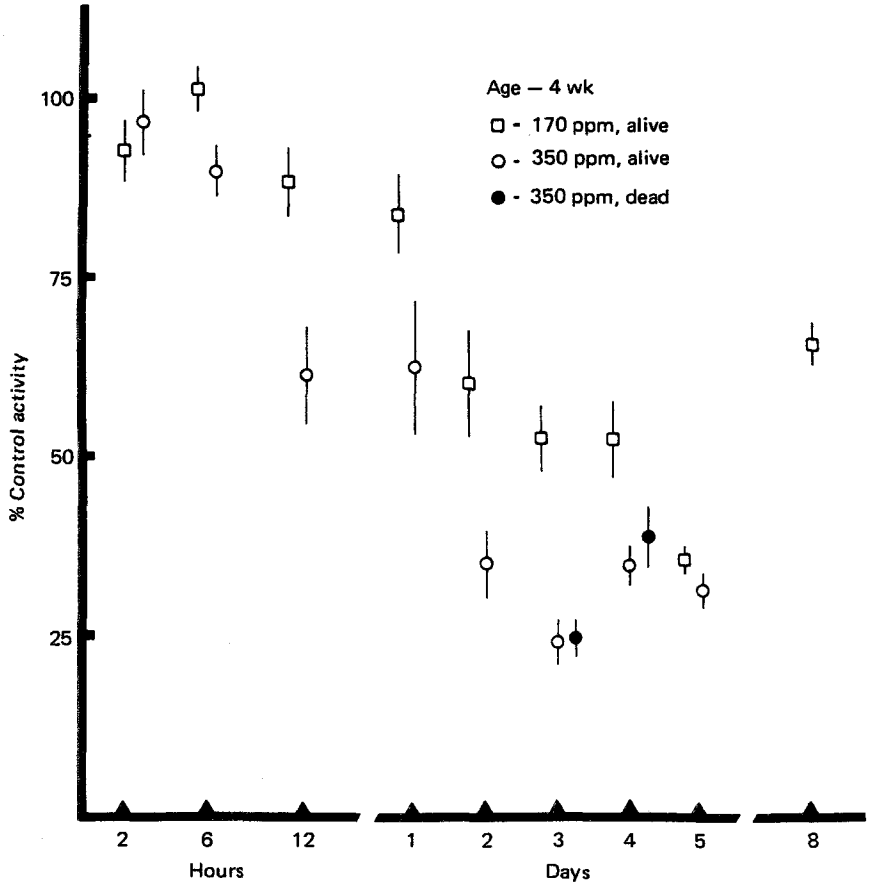

Fig. 2. Brain ChE activity of 4-week-old Japanese quail fed diets containing parathion for 5 days, followed for 3 days with untreated food. (Circles and squares = means; lines = SE).

The relationship of various storage conditions to the length of time brain ChE of dead birds would continue to be a reliable index for OP monitoring purposes is summarized in Figure 5. Brains stored at 35° and 18°-24°C (ambient) were suitable for dissection and analysis for only 24 and 48 hr, respectively. These brains had mean activities slightly elevated above the mean specific activity for 216 control quail of different ages, two- to eight-week-old) and sex. Refrigerated (2°C) brains could be handled and analyzed up to two weeks with no significant loss of activity. Freezing at −22°C depressed brain ChE by 25% within 24 hr after which time it stabilized. Storage at −22°C yielded significantly lower enzyme activity ($P < 0.01$) at all sampling periods after 12 hr.

Mallard duck and starling brains stored under conditions identical to those of quail produced similar results. There was no change in brain ChE activity of mallards stored one day at 35°C or one week at 2°C. When frozen (−22°C) mallard brains were 19%

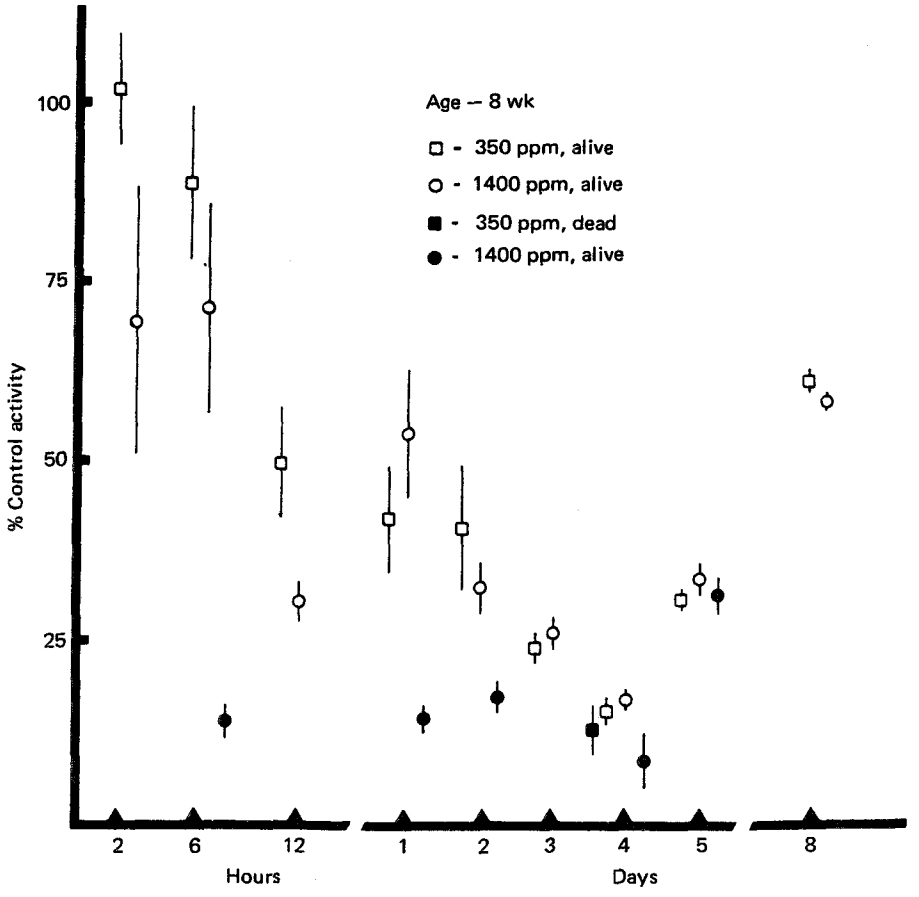

Fig. 3. Brain ChE activity of 8-week-old Japanese quail fed diets containing parathion for 5 days, followed for 3 days with untreated food. (Circles and squares = means; lines = SE).

below normal after one week. Starlings treated the same way showed a slight (6%) depression in ChE activity after one week.

Japanese quail were fed 600 ppm of either parathion (organophosphate) or carbofuran (carbamate). After four hr, birds were randomly selected and stored as described. Brain ChE was analyzed at various intervals to determine the response of brain ChE of poisoned birds using the respective storage methods (Table V). ChE activity of birds poisoned with parathion was not different after storage for two days at 35°, 22° or 2°C. Frozen brains had significantly depressed activity after two days ($P < 0.01$) at –22°C.

Brain ChE activity of birds poisoned with carbofuran reacted differently from that of parathion-treated birds during storage (Table V). ChE of surviving and dead quail reactivated to a significantly higher level ($P < 0.01$) after 24-hr storage at 35°C. Brain ChE activity of frozen birds that survived or died after exposure to carbofuran did not differ

Table III. *Brain cholinesterase (ChE) activity of Japanese quail exposed to dietary concentrations of parathion. Each treatment mean is calculated from all individuals from the 5-day test period*

Age (weeks)	Treatment	Brain ChE Activity[a]			
		No.	*Mean*	*SD*	*% Inhib.*
2	Control	77	124.8	21.5	–
	85 ppm (alive)	63	99.5	23.7	20.3
	170 ppm (alive)	63	69.1	29.0	44.6
	Dead	16	34.4	14.6	72.4

		Male				Female			
		No.	*Mean*	*SD*	*% Inhib.*	*No.*	*Mean*	*SD*	*% Inhib.*
4	Control	34	132.6	23.0	–	37	129.1	22.0	–
	170 ppm (alive)	29	86.7	31.3	34.6	34	95.2	26.8	26.3
	350 ppm (alive)	28	87.3	35.2	34.2	28	59.7	25.8	53.8
	Dead	9	45.8	14.0	65.5	10	38.9	8.9	69.9
8	Control	38	119.2	15.7	–	27	117.2	15.7	–
	350 ppm (alive)	35	61.8	43.2	48.2	25	47.9	32.6	59.1
	1400 ppm (alive)	28	61.3	40.9	48.6	33	37.6	29.2	67.9
	Dead	19	14.5	8.1	87.8	13	18.1	6.2	84.6

[a]ChE activity expressed as n*M* acetylthiocholine iodide hydrolyzed/min/mg protein.

Table IV. *Mean food consumption for Japanese quail fed potentially lethal concentrations of parathion for 5 days*

Age (weeks)	Dietary[a] conc.	Variate	Feed consumption (g/bird/day) 1	2	3	4	5
2	Control	Mean (SD)	10.2 (1.17)	9.6 (1.08)	10.6 (1.00)	10.9 (1.53)	12.1 (3.46)
		Extremes	8.6–12.3	7.7–11.0	9.2–12.4	8.3–13.1	7.0–14.4
		Birds/pen[b]	8	6	5	4	2
	85 ppm	Mean (SD)	7.3 (0.59)	8.0 (1.49)	10.0 (1.26)	11.2 (2.85)	11.7 (2.07)
		Extremes	6.8– 8.2	5.7– 9.7	7.4–11.4	9.0–17.2	9.0–14.7
		Birds/pen	8	6	5	4	3
		% Control	72	83	94	103	97
	170 ppm	Mean (SD)	5.8 (1.11)	5.6 (0.60)	5.6 (0.47)	6.1 (0.73)	8.5 (3.10)
		Extremes	4.1– 7.8	4.4– 5.9	5.1– 6.5	4.6– 7.0	4.6–14.2
		Birds/pen	13	11	10	8	6
		% Control	57	58	53	56	70
4	Control	Mean (SD)	18.5 (1.14)	14.4 (0.91)	15.1 (0.95)	15.6 (1.55)	15.5 (2.03)
		Extremes	16.9–20.3	13.2–15.7	13.0–16.0	12.3–17.1	12.5–18.0
		Birds/pen	8	6	5	4	3
	170 ppm	Mean (SD)	10.6 (0.95)	8.3 (0.99)	9.2 (1.14)	10.7 (1.30)	9.5 (1.80)
		Extremes	9.4–12.3	7.3– 9.2	7.6–11.4	8.6–11.8	6.5–11.8
		Birds/pen	8	6	5	4	3
		% Control	57	58	61	69	61
	350 ppm	Mean (SD)	6.2 (0.89)	3.1 (0.78)	2.6 (0.73)	1.3 (1.01)	1.6 (1.37)
		Extremes	4.6– 7.3	1.8– 3.7	1.5– 2.7	0.0– 3.3	0.0– 3.1
		Birds/pen	11	9	7	4	2
		%Control	34	22	17	8	10

[a] Eight pens per treatment

[b] Birds remaining following mortality or removal for ChE analysis.

from their respective prestorage means. Though there was a tendency toward reactivation of brain ChE in birds that had survived treatment, when refrigerated (2°C, seven days) or at ambient temperatures (18°-24°C, two days) the increases were not significant. Brain ChE of lethally poisoned birds seemed also to be reactivating under storage at 2° and 18°-24°C, but their relative increases were insignificant (Table V).

Discussion

Reliable monitoring and diagnostic methodology for determining exposure of non-target animals to ChE-inhibiting chemicals in the environment is a prerequisite for assessing the impact of such substances on the ecosystem. Though the techniques for assessing acute toxic effects exist, they have been neither fully utilized nor adapted for monitoring wildlife. Problems associated with collecting and processing samples, quality control, sources of valid control values, and data interpretation need more study.

Table V. *Effects of storage time and temperature on brain ChE activity of surviving (S) and dead (D) 3-week-old Japanese quail after 4-hr diets containing 600 ppm of either parathion or carbofuran*

Storage		% ChE Inhibition					
		Parathion			Carbofuran		
Temp. (°C)	Time (days)	No.	Mean	SD	No.	Mean	SD
Fresh		8 (S)	60.5	20.1	4 (S)	42.1	10.6
					4 (D)	32.4	12.7
35	1	5 (S)	41.0	15.9	4 (S)	89.3[b]	9.0
					4 (D)	65.6[b]	10.2
18-24	2	5 (S)	48.7	12.0	4 (S)	56.8	8.9
					4 (D)	51.9	19.1
2	7	5 (S)	48.5	19.4	4 (S)	57.7	22.7
					4 (D)	38.6	5.0
−22	1	5 (S)	54.5	25.8	4 (S)	44.1	3.0
					4 (D)	40.3	10.1
	2	5 (S)	31.5[a]	10.9	4 (S)	40.7	4.6
					4 (D)	37.5	10.2
	7	5 (S)	25.6[b]	8.7	3 (S)	45.7	17.6
					4 (D)	22.6	3.5

[a] Difference from fresh samples significant ($P < 0.05$; *t*-test).
[b] Difference from fresh samples highly significant ($P < 0.01$; *t*-test). Stored samples were compared to fresh samples from the same treatment.

Our findings confirm reports of others concerning differences in normal brain ChE levels among species (Table I). Dieter (unpublished data 1972) has found differences in plasma ChE levels among several species of birds. We also found a sex-related difference in plasma ChE of Japanese quail. Bunyan and Taylor (1966) recommended gradually obtaining mean ChE values of "more common species" to serve as a base-line for normal enzyme activities with which birds suspected of being poisoned could be compared. Such norms would be a helpful guideline for control enzyme activities with which birds suspected of being poisoned could be compared, but only of limited use without standardization of methods of collection, storage and analysis.

Although there are a number of acceptable methods presently used for ChE analysis (Aldrich 1969), we believe kinetic methods such as the "pH-stat" (Nabb and Whitfield 1967) or colorimetric (Ellman *et al.* 1961) analyses are desirable for use with wild birds. Both methods are relatively simple, quick, and inexpensive, and results may be expressed as micromoles of substrate hydrolyzed per min per unit of tissue (weight or volume). Though the Michel method (Michel 1949) is widely accepted, interpretation of results of analyses from animals poisoned by chemicals that do not form a stable enzyme-inhibitor complex (such as carbamates and some OP compounds) might be questionable. A positive control such as commercially purified ChE of known activity, as used by Hawkins and Knittle (1972), should be employed for purposes of quality control within and between laboratories.

Different species and individuals of the same species, but of different ages, showed similiarities in the pattern of inhibition of ChE by parathion but varied markedly in their LC_{50} (Table II; Figure 1-3). There is an apparent decrease in susceptibility to parathion with age in Japanese quail. This phenomenon is probably related to food consumption since the younger birds eat considerably more food relative to their body weight than do the older birds (Table IV). A more accurate term for describing the apparent susceptibility of the younger birds might be "vulnerability". The increase in tolerance with age in birds treated via the diet is not reflected in the acute oral toxicity (LD_{50}) as determined with mallards of different ages (Hudson *et al.* 1972). Differential food consumption and apparent sensitivity has been discussed by Hill (1971) in comparing house sparrows to bobwhites, demonstrating that marked differences in LC_{50}'s between species may not be as pronounced when associated with actual amounts of toxicant eaten in proportion to body size. The possibility cannot be ruled out that increased tolerance may also be associated with an increased ability to detoxify parathion in older birds.

Plasma appears to have limited value as a ChE source for purposes of monitoring, because it would not be obtainable from individuals killed by ChE inhibitors unless the sample could be freshly collected at the time of death. Should the lower normal ChE activity in plasma of adult female quail occur in other species, it could lead to misinterpretation of results from groups of samples comprising both sexes. The rapid return of plasma ChE to normal activity when birds are placed on uncontaminated diet would limit the value of plasma enzyme activity except under conditions of continued exposure, which are unlikely with OP pesticides because of their instability. Plasma ChE activity of Japanese quail was indicative of exposure, however, and decreased rapidly immediately

following parathion exposure (Figure 4). Plasma ChE activity can be useful in measuring effects of ChE inhibitors under controlled laboratory conditions. O'Brien (1967) has stated that erythrocyte ChE is a better index of mortality than serum ChE and probably agrees more closely with brain ChE inhibition by most OP compounds. For these and similar reasons expressed by others (Bunyan *et al.* 1968; Bunyan and Taylor 1966) plasma ChE appears to have only limited value as a ChE source of monitoring effects of OP compounds in wildlife.

The relationship between brain ChE activity and OP exposure is quite good regardless of species, age, and sex. Though the degree of inhibition considered to be lethal has varied, there is general agreement as to the direct relationship between brain ChE inhibition and mortality. Bunyan (1967) stated that death occurred in birds with brain ChE inhibited 100%, but then revised it to 90% inhibition (1968b). Our findings did not concur with Bunyan's statement, "The previously postulated diagnostic rule that death due to organophosphorus poisoning is accompanied by > 90% brain cholinesterase inhibition, while nonlethal exposure leads to significant but lesser inhibition would seem to hold" (Bunyan *et al.* 1969). Weiss (1958) concluded that 40% to 70% inhibition was indicative of mortality in three species of fish, whereas Gibson *et al.* (1969) found that fish exposed to very high concentrations of parathion died with brain ChE inhibited by only 25%. Mehrotra *et al.* (1967), working with house sparrows, generally considered 80% inhibition to be lethal. However, they observed brain ChE inhibition of birds dying from malathion to range from 31% to greater than 90%. We found mortality generally occurred in birds with brain ChE below 40% of normal (Figure 1-3). Only two birds in our experiments died with brain ChE exceeding 50% of normal. We consider mortality accompanied by inhibition of brain ChE greater than 50% suitable for diagnostic purposes and a 20% inhibition of brain ChE activity indicative of exposure.

Sacrificed birds stored for two weeks under refrigeration (2°C) showed no loss in brain ChE activity (Figure 5). Individuals held at ambient (18°-24°C) and incubator (35°C) temperatures had a slight rise in ChE activity and were suitable for analysis for 48 hr. ChE activity of brains of frozen birds dropped significantly ($P < 0.01$) by 24 hr and remained about the same for two weeks (Figure 5). The effect of storage at lower temperatures (e.g., dry ice at −78.5°C) should be studied since freezing at −22°C is apparently unsuitable or would require treating control samples in the same manner.

Storage of birds treated with parathion or carbofuran prior to ChE analysis revealed that chemicals which are reversibly bound may be released from ChE (Table V). Some ChE-inhibited brains could be stored for extended periods at various temperatures as indicated by the parathion-inhibited brains. But those inhibited by other chemicals such as carbofuran, and possibly diazinon (Bunyan *et al.* 1968b), would have to be stored under conditions suitable to prevent reactivation of the inhibited enzyme. ChE from brains of birds frozen after parathion treatment decreased significantly (2 days, $P < 0.05$; 7 days, $P < 0.01$), as also occurred in stored brains of untreated birds (Figure 5). This trend was not apparent, however, in the frozen brains of birds treated with carbofuran. Additional research should be undertaken to better determine optimum storage conditions for brains of carbamate- and OP-inhibited birds.

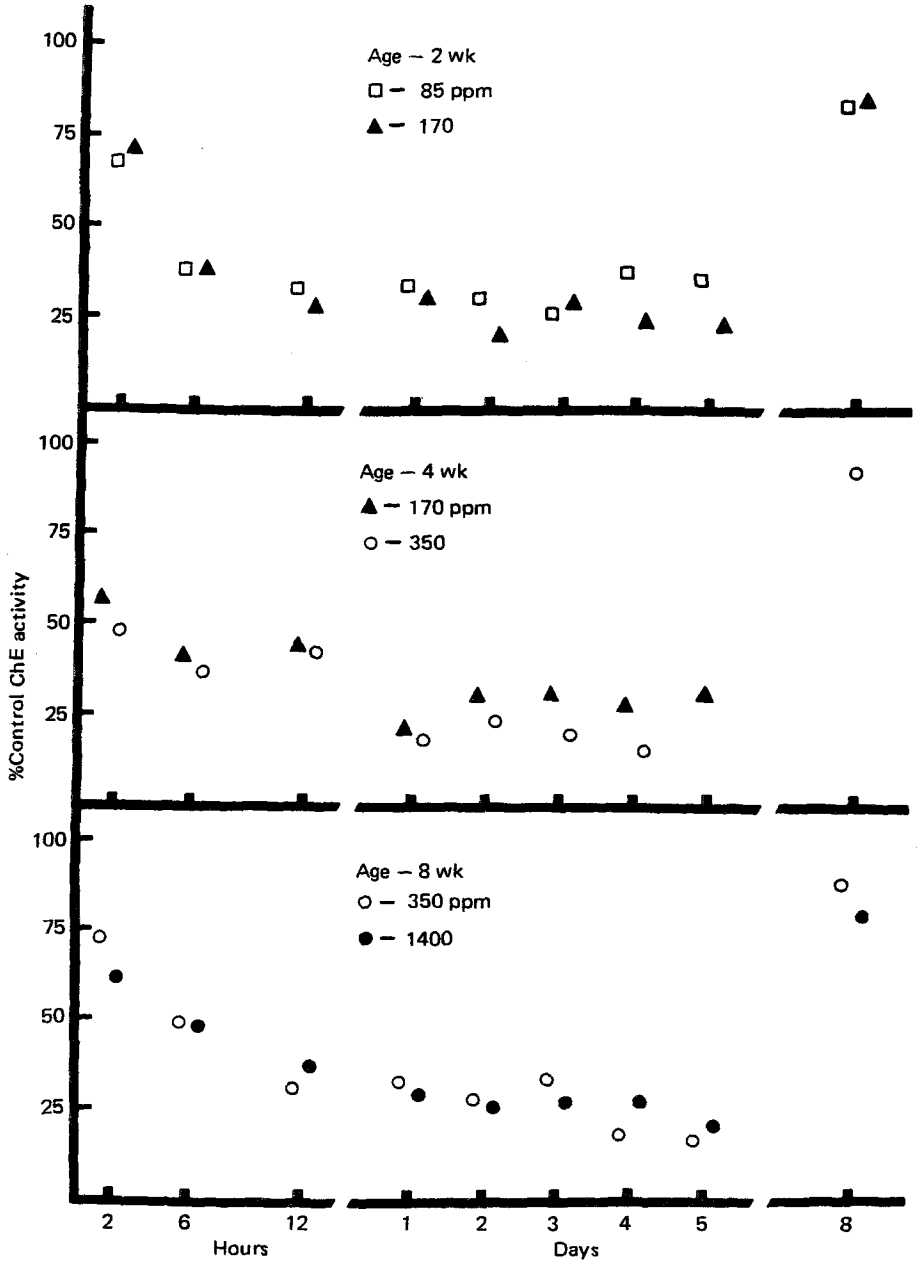

Fig. 4. Mean plasma ChE activity of surviving Japanese quail fed parathion in their diet for 5 days, followed for 3 days with untreated food.

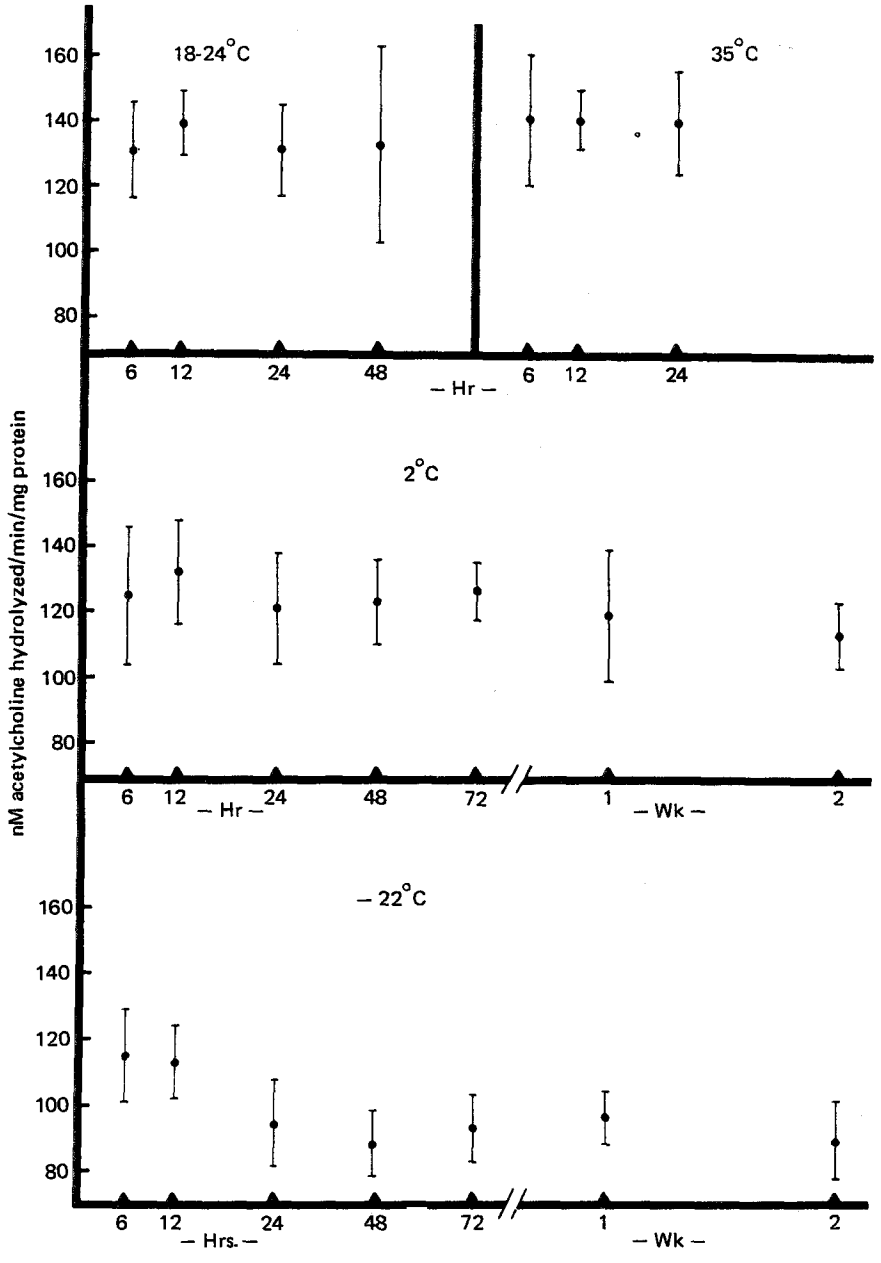

Fig. 5. Mean ChE activities of brains of 3-week-old Japanese quail analyzed at intervals under different storage temperatures. (Dots = means; lines = SD) (Mean control activity = 125.4; SD = 21.3).

It should be noted that not all ChE inhibitors readily penetrate the blood-brain barrier, in which cases mortality may occur without an accompanying decrease in brain ChE activity. However, insects as a group are noted for a more effective blood-brain barrier than vertebrates (O'Brien 1967); thus brain ChE activity should be useful in determining effects of insecticidal ChE inhibitors on non-target species. A wide variety of

Table VI. *Chemical designations of insecticides mentioned in text*

Common name	Chemical name
azinphos-methyl (Guthion)	*O,O*-dimethyl *S*-(4-oxo-1,2,3-benzotriazin-3(4*H*)-ylmethyl) phosphorodithioate
carbofuran (Furadan)	2,3-dihydro-2,2-dimethylbenzofuranyl-7-*N*-methylcarbamate
chlorfenvinphos	2-chloro-1-(2,4-dichlorophenyl)-vinyl diethylphosphate
demeton methyl	*O,O*-dimethyl *O*(and *S*)-2(ethylthio) phosphorothioates
diazinon	*O,O*-diethyl O-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate
dicrotofos (Bidrin)	3-(dimethoxyphosphinyloxy)-*N,N*-dimethyl-*cis*-crotonamide
dimethoate	*O,O*-dimethyl *S*-(*N*-methylcarbamoylmethyl) phosphorodithioate
Dursban	*O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate
ethion	*O,O,O',O'*-tetraethyl *S,S'*-methylene *bis*-phosphorodithioate
fenthion (Baytex)	*O,O*-dimethyl O-[4-(methylthio)-*m*-tolyl] phosphorothioate
malathion	*O,O*-dimethyl phosphorodithioate of diethylmercaptosuccinate
monocrotofos (Azodrin)	3-hydroxy-*N*-methyl-*cis*-crotonamide dimethyl phosphate
parathion	*O,O*-diethyl *O*-*p*-nitrophenyl phosphorothioate
phosphamidon	1-chloro-diethylcarbamoyl-1-propen-2-yl dimethylphosphate

the more popular ChE inhibitors require study to determine possible exceptions which do not penetrate the central nervous system. Erythrocytes may serve as a suitable ChE source for monitoring the effects of poorly penetrating polar inhibitors.

ChE activity can be used to monitor exposure of wildlife species to ChE inhibitors if proper care is exercised. Further research should be directed toward determining normal values of brain ChE activity in a number of widely occurring indicator species using a standardized protocol for the collection, storage, and analysis of samples. Additional studies should be undertaken to determine the validity for using 50% inhibition as suitable for diagnosis of mortality from exposure to a wide variety of ChE inhibitors. Birds suspected of poisoning should be handled uniformly, and a quality control technique, such as simultaneous analysis of ChE of known activity, should be employed. A thorough knowledge of the history of the poisoning accompanied by residue analysis for qualitative confirmation is desirable.

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