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Effects of Dietary Azinphos Methyl on Selected Plasma and Tissue Biomarkers of the Gray-Tailed Vole

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As important starting points for ecotoxicological investigations, laboratory toxicity tests often are focused on survival and reproduction within the test period, to the exclusion of sublethal effects that may have serious effects in the longer term. Yet, since all chemical effects at the population or ecosystem level ultimately start with biochemical and physiological reactions in individual organisms (Lenhardt 1992) it is desirable to include these more subtle phenomena in ecotoxicological studies (Amdur *et al* 1991; Mineau 1991). A single blood sample can be analyzed for characteristics and functionality of both red and white ceils, and for enzyme activity and products of various metabolic processes. Liver tissue provides additional information about activation of metabolizing enzymes while nervous tissue such as the brain identifies the primary neurotropic effects of many pesticides. By utilizing appropriate combinations of such biomarkers, it may be possible to determine whether an animal is healthy or to diagnose the nature and, in some cases, the cause of injury (Fairbrother 1994).

This study was designed to provide information on selected physiological effects of the organophosphorus insecticide azinphos methyl (AZM: O,O-dimethyl S-[(4-oxo-1,2,3-benzotriazin 3(4H)-yl)methyl] phosphorodithioate) on gray-tailed voles (*Microtus canicaudus*) under laboratory conditions. AZM is used world-wide for control of pests on a wide variety of crops (Hall *et al* 1992). Its acute oral LC_{sO} is 13 ppm for rats (U.S.EPA 1990) 3-4.5 ppm for mice (Benke and Murphy 1974) and 297 ppm for the gray-tailed vole (Meyers and Wolff 1994). The herbivorous vole was selected for testing because of its importance at the base of the terrestrial food chain (Cholakis *et al* 1981). The specific objectives of this study were to adapt biomarkers of exposure to the vole and to determine its responses to AZM intoxication in a laboratory situation with little variability in environment or stress.

MATERIALS AND METHODS

Gray-tailed voles were acquired from a laboratory colony at the U.S. EPA's Research Laboratory in Corvallis, Oregon. Only mature animals weighing 18-40 g were used. All animals were housed individually in 17 X 28 X 12 cm polypropylene cages, using sand as bedding material to facilitate collection of spilled food. Animals were kept on a 16:8 hr light:dark regimen with relative humidity at 30-65% and room temperature 20.5°C (±1.5°C). Voles were fed rabbit chow (Ralston-Purina, St. Louis, MO) *ad libitum* during a 5-day acclimation period, weighed, and then received amended feed for the next 9 days. Food was

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accessed through 3-cm diameter ports cut in the lids of 70-ml glass bottles; tap water was provided *ad libitum.* Food and water consumption were recorded daily.

Sixty animals (36 males, 24 females) were assigned by stratified random distribution to receive diets of 0, 161, 322, or 428 ppm AZM. Diets were prepared using technical grade Guthion 2S® (Bayer-Mobay Corp., Kansas City, MO) containing 22% active ingredient; all concentrations quoted below are as AZM. Guthion 2S® was dissolved in a carrier composed of acetone (1% by weight) and corn oil (2% by weight) and mixed into feed with a small electric drum-type mixer. Feed for the 0 ppm diet was amended with carrier only. The AZM concentration in each diet was verified via high resolution gas chromatography. Amended feed samples were shaken with ethyl acetate to produce liquid extracts that were analyzed without further cleanup. The gas chromatograph was operated in split mode, using a 25-m phenyl-methyl silicone (SE-54) capillary column and a nitrogen-phosphorus flame ionization detector.

Five animals, or all survivors if fewer than 5 remained, were euthanized from each treatment by carbon dioxide asphyxiation on days 3, 7, and 9. Immediately prior to euthanizing, blood was collected into heparinized 10 and 70-µl micro-capillary tubes via retroorbital sinus puncture (Stone 1954). After euthanasia, animals were weighed, brains were removed and frozen at -70° C pending analysis for cholinesterase activity, and livers were collected for hepatic P450 enzyme assays.

Blood samples from 70 µl tubes were centrifuged at 4° C at 1600 x *g* for 10 min and the plasma was collected for analysis. Hematocrit tubes were centrifuged in an Ames-Compur MI00 portable centrifuge and packed cell volumes (PCVs) were recorded as percent of blood volume. Two thin-film blood smears were prepared for each animal (Schalm *et al* 1975), stained with modified Wright's solution (Diff-Quik®, Baxter Corp., McGaw Park, IL) and used for leukocyte differentiation by light microscopy (Brown 1980).

Brain acetylcholinesterase (AChE), and plasma lactate dehydrogenase (LDH), isocitrate dehydrogenase (ICDH), and creatine phosphokinase (CPK) activities and plasma creatinine and blood urea nitrogen (BUN) concentrations were determined using a microplate reader (Molecular Devices Corp., Menlo Park, CA) for spectrophotometric techniques (Redinbaugh and Turley 1985; Ashour *et al* 1987; Humphries 1988). The AChE assay method of Ellman *et al (1961)* was modified for the microplate reader as described by Fairbrother *et al (1991).* Plasma LDH and BUN assays were conducted using Ciba Corning Express reagent kits (Ciba Corning Diagnostics, Oberlin, OH). CK, ICDH and creatinine assays used Sigma reagent kits. Samples were diluted for LDH, CK and BUN assays with 0.85% NaCl solution; ICDH and creatinine analyses were conducted using undiluted samples. Analyses followed manufacturer's specifications and all assays were conducted at 30° C. Precision of all assays was controlled by using normal vole brain and plasma samples prepared from colony animals. Commercial standards were used to verify accuracy of measurements

Liver tissues were weighed and homogenized with 5-6 ml ice-cold homogenization buffer (0.1 M KH2PO4 + 0.15 M KCl + 1 mM EDTA + 1 mM DTT, pH 7.4 with KOH) using an electric glass-teflon homogenizer. Homogenized tissues were centrifuged at 14000 x *g* for 20 min at 4 °C. Microsomal supernatant was centrifuged at 100,000 x *g* for 1 h at 2-4 °C. Microsomal pellets were resuspended in homogenization buffer and the tube was filled with the same buffer solution and recentrifuged. Final microsomal pellets were removed from the centrifugaton tubes and rehomogenized in storage buffer (homogenization

buffer with 20% glycerol) at 1 ml per g liver tissue. Total protein concentrations were determined by the method of Smith *et al* (1985) using a total protein assay kit designed for use with a microplate reader system. Total cytochrome P450 activity was determined according to Estabrook *et a*/ (1972) using a double-beam spectrophotometer. Total P450 induction was assayed on samples diluted 40-fold. Pentoxyresorufin O-deethylase (PROD) activity was determined by the rate of formation of resorufin from 7-pentoxyresorufin according to the method of Fowles *et al* (1994). Each sample was scanned 7 times on 8-9 sec intervals to read the rate of product formation.

Biomarkers were not measured in animals that died in the test, due to the difficulty of collecting sufficient blood from dead animals, and concern that nonuniform delays between death and sample collection might lead to variable degradation in analyte levels. Results from both genders were analyzed together, because neither gender alone existed in sufficient numbers to confer reasonable analytical confidence. Statistical analyses were performed using Statgraphics Plus for Windows software (Manugistics, Inc. Rockville, MD). Analysis of variance (ANOVA) was used to determine whether significant differences occurred among test groups. Fisher's Protected Least Significant Differences test was run *a posteriori* identity which groups differed from the controls. A value of $P \leq 0.05$ was considered significant for all tests.

RESULTS AND DISCUSSION

Animals on AZM-amended diets generally ate less than control animals, either avoiding the amended food, or suffering loss of appetite as an effect of exposure to the chemical. Treated voles also lost more weight, on average, than controls. Weight loss was inversely proportional to trends in water consumption. Animals that died on test generally had consumed less water than those that survived until they were euthanized. In a subacute LC_{50} test of AZM with gray-tailed voles, Meyers and Wolff (1994) observed reduced food consumption and loss of weight among exposed animals, with no animals surviving weight loss of 29% or more. In our test, 6 of 15 mortalities and 3 of 45 survivors suffered at least that much weight loss.

Differences in food consumption notwithstanding, AZM exposure tended to be higher in groups receiving higher dietary concentrations. After 7 days, differences consistent with this trend between the three groups were statistically significant. However, accumulated mean exposures of animals that succumbed to the 322 and 428 ppm diets were 12% less than those of the survivors. It is possible that peak serum levels before death were higher among those that died, if they compressed their intake into short periods of feeding. Illustrative of this point, in an oral gavage LD_{50} test, Meyers and Wolff (1994) arrived at an AZM LD_{50} of 32 mg/kg for the gray-tailed vole. While this exposure was lower than most mean multi-day exposures among survivors in our test, it undoubtedly resulted in much higher serum levels by virtue of its being administered all at once.

Loss of body weight seemed to be tied closer to AZM exposure than to food or water consumption. AZM-induced dehydration could have been responsible; AZM has been seen to promote diarrhea, urination, salivation, and lacrimation in mammals. Yet, none of these symptoms were observed, and the existence of dehydration severe enough to account for the weight loss would be inconsistent with the normal PCV and BUN levels observed.

It is conceivable that the weight loss was due at least partially to a metabolic burden imposed by AZM intoxication. In some cases, intoxicated animals clearly consumed more, or nearly as much, food as control animals, and still lost considerably more weight than controls. Dietary AZM-induced weight loss in the absence of a concomitant decrease in food consumption has also been observed in pine voles (*Microtus pinetorum*) exhibiting no obvious behavioral signs of intoxicantion (Durda *et al* 1989). This is consistent with observations of increased metabolic rates in rats exposed to anticholinesterase agents (Gordon *et al* 1997).

There was no mortality among control animals. For groups receiving AZM, mortality, calculated as percent of animals available at the start of a period that subsequently died during the period, was similar for all three treatments by day 3, since little mortality had occurred by then. Between days 3 and 7, the least mortality occurred in the 161 ppm group. During this period, one more animal died in the 322 ppm group than in the 428 ppm group, but most deaths in the latter group occurred earlier. Both animals that died in the 161 ppm group experienced higher exposure than their surviving counterparts in the same time frames. After sampling on the 7th day, no voles remained from the 322 or 428 ppm groups. Three animals remained in the 161 ppm group, and all survived to be sampled on day 9.

Several researchers have reported that AZM inhibits brain AChE in rodents and other vertebrates (Worden *et al* 1973; Benke and Murphy 1974; Short *et al 1980).* Among intoxicated gray-tailed voles surviving an AZM LC_{50} test, Meyers and Wolff (1994) observed 30-44% depression of brain AChE relative to controls. In our test, survivors of the lower dietary concentrations of AZM had treatment group averaged suppression of 13-27% relative to controls, confirming sufficient exposure to elicit a physiological response (Table 1).

That exposure, however, did not result in statistically significant impact on the measured plasma constituents among survivors (Table 1). Mean LDH was statistically significantly elevated relative to control in the 322 ppm group only on day 7, while CPK differed from the control only in the 161 ppm group on day 9. Mean ICDH, BUN, and creatinine in exposed groups were never significantly different from the control group. AZM exposure had no effect on relative percentages of leukocytes, or on PCV. It is likely, therefore, that little, if any, damage had occurred to organ systems that influence those constituents. This interpretation is consistent with lack of visible abnormalities on necropsy. Likewise, intact ratios and morphology of leukocytes indicate no effect on leukocytogenesis that might have implications for immune function.

There was no significant change in liver cytochrome P450 content or PROD activity in any treatment group after 3, 7, or 9 days of dietary exposure to AZM (Table 2).

Although most organophosphorus compounds are relatively poor inducers of MFO, repeated exposures to low doses may induce P450 and MFO activity (Khan 1980). It appears that the exposures of the voles in this study to AZM were insufficient to induce any P450 activity. It may be that greater than 9 days of exposure are required for induction or it may be that voles are refractory to induction of hepatic cytochromes by AZM. Thus, it is unlikely that insecticide exposures will interfere with the use of PROD activity or cytochrome P450 content assays as biomarkers of exposure for environmental contaminants such as PCBs or PAHs.

Table 1. Summary of physiological data.

	Control			161 ppm			322 ppm			428 ppm		
Day	n	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.	n	Mean	S.E.
Mean Brain Cholinesterase (U/g)												
3	5	2.63	0.10	5	2.06[a]	0.11	5	1.93[a]	0.04	5	2.06[a]	0.06
7	5	2.68	0.11	5	2.10[a]	0.15	2	2.34	0.17	4	2.04	0.10
9	5	2.76	0.11	5	2.15[a]	0.10						
Mean Plasma LDH (U/l)												
3	5	1219	303	4	1465	409	5	1343	235	5	1930	327
7	5	1229	280	5	1465	313	3	2396	408	4	1445	442
9	5	1781	274	3	2419	478						
Mean Plasma CPK (U/l)												
3	5	1749	366	4	2281	1057	5	1932	310	5	3156	128
7	5	2469	703	5	2216	686	3	2521	816	4	2054	555
9	5	1370	333	3	2876[a]	192						
Mean Plasma ICDH (U/l)												
3	5	52.9	4.3	4	76.3	30.1	5	93.6	7.8	5	112.0	39.7
7	5	60.2	6.2	5	34.5[a]	8.7	3	112.7[b]	47.8	4	60.6	0.6
9	5	67.7	17.9	3	127.4	39.8						
Mean Plasma BUN (mg/dl)												
3	5	33.4	2.4	4	31.6	2.5	5	27.0	2.5	5	30.6	4.5
7	5	39.9	4.0	5	37.3	4.4	3	33.7	4.2	4	38.8	5.0
9	5	36.6	0.9	3	28.5	3.9						
Mean Plasma Creatinine (mg/dl)												
3	5	0.61	0.08	4	0.83	0.13	5	0.61	0.07	5	0.65	0.12
7	5	0.57	0.05	5	0.80	0.10	3	0.85	0.04	4	1.01	0.29
9	5	0.78[c]	0.05	3	0.74	0.17						

[a] Significantly different from control value ($p < 0.05$)
[b] Significantly different from 161 ppm value ($p < 0.05$)
[c] Significantly different from day 7 value ($p < 0.05$)

Table 2. Microsomal total protein, cytochrome P450 content, and specific PROD activity of male and female gray-tailed voles with 3 to 9 days of dietary exposures to azinphos methyl (mean ± standard error).

Day	control						161 ppm						322 ppm						428 ppm					
	male			female			male			female			male			female			male			female		
	n	mean	±SE	n	mean	±SE	n	mean	±SE	n	mean	±SE	n	mean	±SE	n	mean	±SE	n	mean	±SE	n	mean	±SE
Microsomal total protein content (mg/ml)																								
3	3	22.15	4.27	2	14.71	1.58	3	22.49	3.78	2	20.35	0.36	3	16.03	1.73	2	12.70	8.65	3	18.63	3.11	2	17.55	0.40
7	3	17.29	3.00	2	11.28	0.00	3	15.55	2.36	1	9.61	0.00	2	12.28	2.86	1	10.06	0.00	1	11.90	0.00	3	16.83	4.72
9	3	9.01	0.71	2	13.16	0.59	1	13.31	0.00	2	9.70	1.61	-	--	--	-	--	--	-	--	--	-	--	--
Microsomal cytochrome P450 content (nMol/mg total protein)																								
3	3	0.742	0.04	2	0.726	0.04	3	0.879	0.29	2	0.987	0.18	3	0.830	0.05	2	2.338	2.79	3	0.658	0.09	2	0.748	0.17
7	3	0.813	0.06	2	0.868	0.00	3	0.844	0.41	1	0.744	0.00	2	0.827	0.26	1	1.347	0.00	1	0.734	0.00	3	0.739	0.15
9	3	1.630	0.30	2	1.090	0.02	1	1.261	0.00	2	1.155	0.15	-	--	--	-	--	--	-	--	--	-	--	--
Specific PROD activity (mMol/min/ml total protein)																								
3	3	0.006	0.002	2	0.008	0.000	3	0.006	0.000	2	0.005	0.001	3	0.006	0.001	2	0.014	0.009	3	0.005	0.001	2	0.007	0.001
7	3	0.005	0.001	2	0.001	0.000	1	0.006	0.001	1	0.015	0.000	2	0.007	0.002	1	0.009	0.000	1	0.007	0.000	3	0.006	0.002
9	3	0.009	0.001	2	0.009	0.002	1	0.006	0.000	1	0.011	0.003	-	--	--	-	--	--	-	--	--	-	--	--

It is possible that, had the resources been available to continuously monitor the test so blood samples could be taken from morbid animals, evidence of organ damage would have appeared. While the biomarkers utilized in this laboratory study revealed no evidence of physiological or organic deterioration in animals sublethally intoxicated with AZM other than the expected cholinesterase depression, Fairbrother *et al* (1997) found a significant depression in the neutrophil:lymphocyte (N:L) ratio of gray-tailed voles exposed to AZM in a field situation. Given that changes in the N:L ratio appears to be a generalized stress response, it may be that the combined stresses of pesticide exposure and natural environmental variables result in the expression of effects that would not be evident in a benign laboratory environment (Porter *et al 1984).*

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