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Environmental Toxicology and Chemistry, Vol. 17, No. 12, pp. 2521–2529, 1998
Printed in the USA
0730-7268/98 $6.00 + .00

BLOOD CHANGES IN MALLARDS EXPOSED TO WHITE PHOSPHORUS

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(*Received* 16 *October* 1997; *Accepted* 1 *May* 1998)

Abstract—White phosphorus (P_4) has been extensively used by the military for various purposes, including marking artillery impacts and as an obscurant. Target practice in an Alaskan tidal marsh during the last 4 decades has deposited large amounts of P_4 particles in sediments and water, which have resulted in die-offs of several waterfowl species. Because the toxicity of P_4 in birds has not been well documented and because it is quickly excreted or metabolized in living animals, we sought to determine the effects of experimental dosing on blood characteristics in game farm mallards (*Anas platyrhynchos*). In two experiments, one employing single doses of 5.4 mg P_4 per kilogram body weight in corn oil and the other using daily repeated doses of pelletized P_4 at either 0.5 or 1.0 mg/kg, there were significant changes in aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase (LDH), inorganic P, hematocrit, and hemoglobin. Other indications of exposure included changes in uric acid, creatinine, and total protein, which were consistent with reported liver and kidney damage due to this contaminant. Changes in white blood cells included a greater frequency of thrombocytes and fewer lymphocytes in dosed birds compared to controls. A biomarker of exposure based on LDH activity and hemoglobin is proposed.

Keywords—White phosphorus Hematology Bioindicators Mallards Alaska

INTRODUCTION

White phosphorus (elemental or P_4) has been used by the military for decades to mark the location of artillery impacts, in incendiary devices, and as an obscurant to conceal military maneuvers. In air, P_4 oxidizes to form a thick, white cloud. In water or wet sediments, however, P_4 lies inert as pellets, often for decades [1]. Particle size varies but often is similar to seeds and aquatic invertebrates that serve as food for water birds using contaminated wetlands. Thus, waterfowl can ingest pellets either by mistaking them for food or incidentally when ingesting sediments [2].

In 1982, extensive waterfowl mortality was observed at Eagle River Flats, part of the Fort Richardson U.S. Army base near Anchorage, Alaska, USA. This area is an 865-ha tidal marsh that has been used extensively for artillery practice since the 1940s. Since the first waterfowl mortalities were observed, more than 2,000 waterfowl, including mallards (*Anas platyrhynchos*), green-winged teal (*A. crecca*), northern pintails (*A. acuta*), trumpeter swans (*Cygnus buccinator*), and tundra swans (*C. columbianus*), have died each year. Residue determinations and overt signs of toxicity strongly support that P_4 is the primary cause of this mortality [3]. In addition, shorebirds, including long- and short-billed dowitchers (*Limnodromus scolopaceus, L. griseus*), red-necked phalaropes (*Phalaropus lobatus*), and greater and lesser yellowlegs (*Tringa flavipes, T. melanoleuca*) feed in the area and may be exposed to P_4. Other water birds that use the area but do not have apparent die-offs include northern shovelers (*Anas clypeata*), American widgeon (*A. americana*), Canada geese (*Branta canadensis*), and snow geese (*Chen caerulescens*); these birds may be less exposed to P_4 because of food habits or habitat preferences. Avian predators or scavengers including bald eagles (*Haliaeetus leucocephalus*), golden eagles (*Aquila chrysaetos*), ravens (*Corvus corax*), and herring gulls (*Larus argentatus*) feed on dead or dying waterfowl and may be poisoned secondarily [4–6].

Early work on the toxicity of P_4 in waterfowl found that it can cause liver and kidney damage and could be lethal in concentrations as low as 1.7 mg/kg body weight [7], although values of dose causing 50% lethality (LD50) were not determined. More recently, P_4 toxicity was further described, and two sets of toxicological signs were identified [8]. These sets can co-occur but are distinguished by the onset of signs and by target organs. A rapid (3–10 h) effect results in signs consistent with anoxia and include lethargy, ataxia, malaise, convulsions, and death. A slower acting (>24 h) effect results in liver and kidney damage with demonstrable lipid accumulation in liver, hepatomegaly, and cellular necrosis; this effect may or may not be lethal. The study also determined that the LD50 in mallards for P_4 dissolved in oil was 6.4 mg/kg, and for pelletized P_4 it was 3.9 mg/kg.

Studies on fish [9] and American kestrels (*Falco sparverius*) [5] have shown that P_4 may be detected in tissues of living organisms for only 1 or 2 d. Because P_4 is lipophilic, adipose tissue provides the most reliable source for residue determination in birds [8], but residue levels in dosed animals drop below detection limits within a few days. The usual method of analysis extracts P_4 from adipose tissue, which, if nonlethal sampling is desired, requires that samples of fat be taken through biopsies. Because biopsies require special training to minimize trauma to a bird, a less invasive indicator of P_4 exposure could be desirable. There is some practical value in

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finding a nonlethal indicator of P_4 exposure in that it occurs on 71 National Priority Listing sites in 29 states [10].

The purpose of this study is to determine the effects of P_4 on blood characteristics in birds. Plasma chemistry and cell counts are valuable in diagnosing pathologies [11–13] and are often used to indicate exposure to contaminants with only minor invasion of a subject [14–16]. Specific goals include using changes in blood chemistry to learn more about the toxic effects of P_4 and to examine the possibility of using plasma constituents in developing a bioindicator of exposure that might be useful in identifying exposed birds in areas contaminated with P_4.

MATERIALS AND METHODS

Two experiments are reported in this paper. The first was conducted in March 1994 and was designed specifically to test the effects of exposure to P_4 on nonbreeding birds. Five birds of each sex were randomly assigned to each of three groups: controls, single dose, and two doses of P_4. White phosphorus was dissolved in corn oil, and birds were dosed via oral gavage with 5.4 mg P_4 per kilogram body weight, which was shown in earlier tests to be below the LD50 but high enough to cause toxic effects [8]. Controls were given 3 ml oil per kilogram without P_4. A pretreatment blood sample was drawn 6 d prior to testing. Following dosing we waited 2 d before drawing the first postdose sample to allow for effects to occur. Half of the birds were then immediately dosed a second time. After an additional 2 d, we drew a final blood sample. Its not known how often birds may be exposed in the field, but we conjectured that a 2-d interval might be appropriate if birds became slightly ill after an initial exposure. In each sample period we drew 3–5 ml blood from each bird via jugular venipuncture into nonheparinized syringes and recovered the serum via centrifugation at 1,200 *g* for 10 min. Serum was sent to a commercial veterinary laboratory that conducted analyses for blood chemistry factors. Separate aliquots of whole blood in lithium-heparinized caraway tubes were used to measure hemoglobin via the cyanonmethemoglobin method using Drabkin's reagent (Sigma Chemical, St. Louis, MO, USA). Hematocrit was read from heparinized capillary tubes that were spun for 10 min at 1,200 *g* and then compared to a hematocrit card.

The second experiment was part of a study on the reproductive effects of P_4 in mallards and occurred in the spring of 1995. The study design called for different dose regimens for males and females. Females were randomly assigned to control, 0.5-, 1.0-, or 2.0-mg/kg treatments, whereas males were assigned only to control or 1.0-mg/kg groups; both sexes were dosed daily for 7 d. Blood samples were taken from birds 1 week prior to dosing, on the first day following dosing, and 8 d following the last dose. Blood was collected in lithium-heparinized syringes via venipuncture and spun at 1,200 *g* for 10 min. Plasma was used instead of serum because smaller quantities of blood can be drawn for the same volume of sample when plasma is used. Plasma was stored in cryovials and frozen at −4°C until analyzed. Blood constituents were analyzed on a Centrifichem 400 centrifugal analyzer (Union Carbide, Rye, NY, USA) with Trace America (Miami, FL, USA) reagents and protocols. Hematocrit and hemoglobin were analyzed as in the first experiment. Specific tests included alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), alkaline phosphatase (ALK), creatinine, globulins, glucose, inorganic phosphorus (P), potassium (K), total protein, uric acid, triglycerides, albumin, calcium, sodium, chloride, carbon dioxide, and relevant ratios of selected constituents such as BUN/creatinine (BUCR). However, only those constituents or ratios that showed a significant difference due to dose or its interactions are reported.

On the 1st d postdose, blood smears were made and stained with Wright's stain. Differential white blood cell counts and examination for Heinz body formations were made with oil immersion at 1,000×. Heinz bodies are inclusions of methemoglobin and have been associated with phosphine toxicity in humans [17], and we wished to determine if they could be produced by P_4.

To determine if differential white blood cell counts differed among treatment groups, we used one-way analysis of variance (ANOVA) on the arcsin transformed proportions with SAS [18]. All other tests on chemistry among treatments, sexes, or time (before or after dosing) were conducted with repeated measures ANOVA on the log-transformed value of concentrations; values are reported as geometric means with 25 and 75% quartiles. Hematocrit values were normally distributed, analyzed without transformations, and reported as means ± SEM. Because of an unbalanced design in the number of repetitions of dosing in the first experiment and the dose regimens between males and females in the second experiment, we had to run more than one ANOVA per experiment. In either case, the interaction terms between treatment and condition provided the clearest tests for a true treatment effect in repeated measures ANOVA. A posteriori tests were conducted with Tukey's HSD test; all significant differences were determined at $\alpha \leq 0.05$.

RESULTS

Experiment 1, nonbreeding birds

Blood constituents for mallards administered P_4 in oil solution are listed in Tables 1 and 2. For each of the factors in Table 2 there are two sets of probabilities. The top set compares controls ($n = 10$) with birds that had been dosed once ($n = 19$). The bottom set of probabilities refers to the tests using controls and birds that had been dosed twice ($n = 9$). One bird died after being given a single dose, and another died after two doses. Significant time-by-treatment effects for birds given single doses were observed for BUCR, hematocrit, hemoglobin, and uric acid; each of these retained significant interaction effects for birds given two doses compared with controls. Time-by-treatment interactions that appeared after the second dose but were not seen after a single dose included ALT, AST, globulins, LDH, K, and total protein. Compared with controls, AST, BUCR, LDH, K, and uric acid increased in dosed birds with number of doses, whereas globulins, hematocrit, hemoglobin, and total protein decreased.

Significant three-way (sex, treatment, and time) effects occurred for ALT, hemoglobin, inorganic P, and uric acid. Alananine aminotransferase decreased with dose in females but increased in males. Hemoglobin dropped more precipitously in males, whereas there was a sharper increase in P and uric acid than in females.

Experiment 2, reproductive males and females

Three of the 10 females on 2.0 mg P_4 per kilogram body weight died within the first 3 d of treatment, and we ceased dosing at this concentration because we were interested in sublethal effects. One hen died at the 1.0 mg/kg, but we continued dosing for the duration of 7 d.

Table 1. Geometric means (GM) with first (Q1) and third (Q3) quartiles of blood constituents in female mallards dosed once or twice with 5.4 mg/kg white phosphorus in oil solution

Treatment		Control			Dosed		
Number of doses		0	1	2	0	1	2
Sample size		5	5	5	10	10	4
Alanine aminotransferase (U/L)	GM	16	18	21	25	19	18
	Q1–Q3	14–18	17–25	18–30	15–44	14–24	16–25
Aspartate aminotransferase (U/L)	GM	15	15	13	13	17	37
	Q1–Q3	15–19	9–17	9–17	10–29	15–26	28–55
Blood urea nitrogen/creatinine	GM	3.3	2.9	3.3	3.3	6.7	6.7
	Q1–Q3	3.3–3.3	2.5–4.7	2.5–3.3	3.3–3.3	3.3–6.7	4.7–6.7
Globulin (mg/dl)	GM	2.6	2.9	2.8	2.7	2.6	2.6
	Q1–Q3	2.5–2.7	2.6–3.0	2.7–3.0	2.5–2.8	2.4–3.0	2.3–3.1
Glucose (mg/dl)	GM	219	184	186	235	190	188
	Q1–Q3	219–236	165–200	180–207	212–258	149–210	156–201
Hematocrit (%)	GM	48	48	44	45	42	36
	Q1–Q3	48–49	45–49	43–47	40–48	39–44	34–39
Hemoglobin (mg/dl)	GM	16.4	16.8	14.4	14.6	15.6	11.2
	Q1–Q3	16.0–17.3	16.7–16.8	14.3–15.3	12.8–16.7	12.8–15.8	10.1–11.7
Lactate dehydrogenase (U/L)	GM	678	557	631	538	916	1,032
	Q1–Q3	393–684	514–918	489–832	484–789	751–1,068	919–1,342
Inorganic phosphorus (mg/dl)	GM	2.7	4.3	5.4	4.9	4.9	5.3
	Q1–Q3	2.3–2.8	2.9–5.1	3.8–5.7	2.6–6.9	3.4–6.3	3.1–9.3
Potassium (meq/L)	GM	1.8	2.7	2.1	2.5	4.8	7.2
	Q1–Q3	1.8–1.9	2.6–2.9	2.0–2.5	2.0–2.7	4.5–7.4	4.9–7.8
Total protein (mg/dl)	GM	4.5	5.0	5.3	4.7	4.5	4.5
	Q1–Q3	4.3–4.8	4.7–5.2	5.0–5.3	4.5–5.0	4.2–5.0	4.2–5.2
Uric acid (mg/dl)	GM	3.3	3.2	3.2	3.5	3.2	3.9
	Q1–Q3	3.0–3.6	3.0–3.9	2.7–4.2	2.6–4.2	1.8–4.3	3.7–4.0

In females at 0.5 or 1.0 mg/kg (Table 3), the first row of probabilities corresponds to controls and 0.5- and 1.0-mg/kg birds over all three time periods (predose, 1 d after the last dose and 1 week postdose). The second row of probabilities corresponds to controls and birds given 2 mg/kg 10 d following the last dose (following 3 additional days of dosing and one week post dosing for the other birds). Significant interactions (time-by-treatment) effects for controls through 1.0 mg/kg were seen for AST, BUN, creatinine, hemoglobin, hematocrit, inorganic P, and LDH. Mallards at both 0.5 and 1.0 mg/kg had higher plasma levels of AST after dosing than before, but concentrations 1 week postdose were less than those of pretreatment. Blood urea nitrogen tended to increase throughout the time period in dosed birds but not in controls, whereas creatinine increased in all three treatment groups but at a steeper rate in dosed birds than in controls. Hemoglobin and hematocrit markedly declined from pretreatment to immediately after dosing but regained their original levels 1 week following dose. Birds that were dosed at either 0.5 or 1.0 mg/kg demonstrated an increase in LDH activity, which returned to near normal levels 1 week following dose. There were significant main effects for uric acid, although the interaction term was not significant. This factor tended to decrease through time and was generally higher in birds on 0.5 mg P_4 per kilogram than in controls. In comparison with controls, mallards dosed at 2 mg/kg demonstrated elevated ALT and depressed hemoglobin and hematocrit concentrations 10 d following the last dose.

Male mallards demonstrated significant interaction terms for ALT, AST, creatinine, hemoglobin, hematocrit, P, LDH, triglycerides, and uric acid (Table 4). For most of these factors, concentrations tended to return to near normal 1 week following dosing but triglycerides and uric acid showed a rebound. Dosed birds tended to have elevated ALT, AST, and LDH activities immediately after dosing compared to controls. Creatinine concentrations were depressed in dosed birds but not until 1 week following dosing. Blood urea nitrogen levels were generally higher in dosed birds, regardless of time, compared with controls.

When males and females in the control and 1.0-mg/kg groups were pooled, there were significant sex-by-time-by-treatment effects for AST ($p = 0.037$), creatinine ($p = 0.011$), and LDH ($p = 0.011$). Aspartate aminotransferase activities were higher in females than in males and declined during the dosing period in control males, whereas they increased in control females and both treated groups during the same period. Creatinine decreased in dosed males 1 week following treatment, whereas it remained elevated in females. The increase in LDH concentrations was much steeper in treated males immediately after dosing than it was in treated females. Overall sex differences were observed in AST, ALT, BUN, P, triglycerides, uric acid, and in other factors not showing treatment effects (ALK, albumin, glucose, and total protein).

To identify a potential bioindicator of exposure to P_4, we examined relationships among combinations of factors that were indicative of different physiological systems being compromised. A ratio of hemoglobin divided by LDH provided a clear separation between dosed and undosed birds (Fig. 1). Based on discriminant analysis on the reproductively active mallards after the dosing period, discriminant functions for unexposed and exposed birds took the form:

Unexposed: $-2.759 + 30.949 \cdot$hemoglobin/LDH
0.5 mg/kg: $-3.283 + 13.071 \cdot$hemoglobin/LDH
1.0 mg/kg: $-2.178 + 1.943 \cdot$hemoglobin/LDH

Ninety-four percent of birds (16 of 17) given 1.0 mg/kg and sampled the day following dose were correctly identified by these functions as being exposed. However, birds that were given 0.5 mg P_4/kg body weight and those given 1.0 mg/kg

Table 2. Geometric means (GM) with first (Q1) and third (Q3) quartiles of blood constituents in male mallards dosed once or twice with 5.4 mg/kg white phosphorus in oil solution. ANOVA results are for Tables 1 and 2; the first line of probabilities for each constituent is for controls compared to birds dosed once and the second line of probabilities is for controls and those dosed twice

Treatment		Males						Results of ANOVA[a]				
		Control			Dosed							
No. of doses		0	1	2	0	1	2	Dose	Sex	Time	Time × dose	Three-way interaction
Sample size		5	5	5	10	10	5					
Alanine aminotransferase (U/L)	GM	24	31	30	22	23	32	NS[b]	NS	NS	NS	NS
	Q1–Q3	21–25	25–31	28–30	18–24	16–47	25–40	NS	NS	0.018	0.030	0.009
Aspartate aminotransferase (U/L)	GM	12	18	14	13	35	49	NS	NS	0.020	NS	NS
	Q1–Q3	9–20	11–19	7–15	12–25	11–67	37–57	0.014	NS	0.011	0.0003	NS
Blood urea nitrogen/creatinine	GM	3.3	3.3	3.3	3.3	6.7	6.7	NS	NS	0.002	0.014	NS
	Q1–Q3	3.3–5.0	3.3–3.3	3.3–3.3	2.5–3.3	3.3–6.7	4.7–8.2	0.0008	NS	0.013	0.002	NS
Globulin (mg/dl)	GM	2.4	2.4	2.4	2.3	2.3	1.9	NS	0.002	NS	NS	NS
	Q1–Q3	2.1–2.9	2.2–3.1	2.4–2.4	2.3–2.6	2.2–2.4	1.9–2.2	NS	0.019	NS	0.053	NS
Glucose (mg/dl)	GM	223	194	185	258	192	196	NS	NS	0.0001	0.058	NS
	Q1–Q3	182–226	192–204	179–188	233–268	163–226	171–204	NS	NS	0.002	NS	NS
Hematocrit (%)	GM	45	45	42	48	42	33	NS	NS	0.0002	0.008	NS
	Q1–Q3	42–45	45–45	41–45	47–50	41–45	30–38	0.005	NS	0.0001	0.0003	0.059
Hemoglobin (mg/dl)	GM	15.6	16.4	14.6	16.5	14.6	9.5	0.05	NS	NS	0.022	NS
	Q1–Q3	15.5–16.6	15.4–17.0	14.3–14.7	14.8–17.2	13.8–15.7	8.6–11.0	0.003	NS	0.0001	0.0001	0.053
Lactate dehydrogenase (U/L)	GM	352	692	460	374	1,063	2,770	NS	NS	0.0001	NS	NS
	Q1–Q3	248–446	580–800	260–673	272–510	562–1,528	1,952–3,485	0.007	NS	0.0001	0.005	NS
Inorganic phosphorus (mg/dl)	GM	3.1	1.2	2.2	2.0	2.9	3.8	NS	0.003	NS	NS	0.027
	Q1–Q3	2.3–3.2	1.2–2.5	1.4–2.3	1.9–3.1	2.2–4.0	2.6–5.1	NS	NS	0.042	NS	0.028
Potassium (meq/L)	GM	2.5	3.2	1.8	2.2	3.5	6.7	0.006	NS	0.0001	NS	NS
	Q1–Q3	2.3–2.8	1.9–3.8	1.7–2.9	1.8–3.6	3.0–4.4	6.0–7.2	0.0003	NS	0.0001	0.0009	NS
Total protein (mg/dl)	GM	4.2	3.8	4.0	4.0	4.0	3.4	NS	0.0001	NS	NS	NS
	Q1–Q3	3.9–4.4	3.7–4.0	3.9–4.0	3.8–4.2	3.8–4.2	3.2–3.7	NS	0.0001	NS	0.023	NS
Uric acid (mg/dl)	GM	3.5	2.7	3.1	4.0	4.6	4.9	NS	NS	NS	0.026	0.031
	Q1–Q3	3.3–3.6	2.7–3.4	2.8–3.4	2.7–4.7	3.2–5.7	3.6–7.4	NS	NS	0.034	0.042	NS

[a] ANOVA = analysis of variance.
[b] NS = nonsignificant.

Table 3. Geometric means with first (Q1) and third (Q3) quartiles for blood chemistry factors in reproductively active female mallards prior to, 1 d post, and 1 week postdosing with white phosphorus[a]

Time		Alanine aminotransferase	Alkaline phosphatase	Aspartate aminotransferase	Blood urea nitrogen/creatinine	Creatinine	Hematocrit[b]	Hemoglobin	Inorganic phosphorus	Lactate dehydrogenase	Triglyceride	Uric acid
						Control (n = 11)						
Pre	GM	716	139	38	2.8	0.20	42.2	13.2	7.6	105	205	5.1
	Q1–Q3	249–1,111	83–201	25–47	1.1–3.7	0.08–0.28	0.89	11.7–13.7	6.6–8.5	58–158	177–236	4.8–5.8
1 day	GM	769	126	89	6.3	0.34	40.9	14.5	8.4	158	374	3.3
	Q1–Q3	139–2,100	77–195	66–310	1.6–7.5	0.30–0.42	0.74	13.1–15.3	7.8–9.6	103–229	217–407	1.9–3.5
1 week	GM	807	128	61	3.6	0.40	39.5	15.3	7.0	112	496	3.2
	Q1–Q3	403–1,086	50–235	22–156	2.2–9.6	0.32–0.52	0.54	12.9–15.5	6.2–8.1	62–197	193–230	6.1–7.8
						0.5 mg/kg (n = 6)						
Pre	GM	788	189	88	1.4	0.19	40.0	12.8	7.5	93	204	7.4
	Q1–Q3	573–1,327	86–246	48–202	0.7–1.9	0.10–0.35	1.1	10.2–14.6	6.3–8.2	55–152	193–230	6.1–7.8
1 day	GM	992	166	136	6.6	0.39	35.9	12.1	8.3	185	307	5.9
	Q1–Q3	120–2,061	71–348	65–183	4.0–8.6	0.23–0.45	1.3	10.6–12.2	7.8–8.2	130–244	242–426	2.4–6.5
1 week	GM	877	103	34	7.6	0.55	39.8	13.4	6.9	143	395	3.5
	Q1–Q3	180–1,162	55–234	17–53	4.8–38.3	0.41–0.68	1.3	11.7–14.9	7.8–9.2	97–185	328–443	2.7–4.9
						1.0 mg/kg (n = 7)						
Pre	GM	673	82	57	2.1	0.22	40.2	12.0	6.0	141	221	6.8
	Q1–Q3	401–1,507	38–114	37–154	0.8–4.2	0.07–0.33	0.71	10.9–13.3	5.3–7.4	61–270	180–228	5.0–7.3
1 day	GM	1,414	60	279	3.1	0.35	24.2	7.5	9.7	519	292	2.5
	Q1–Q3	411–1,834	51–76	146–417	1.3–4.3	0.19–0.72	1.5	6.3–8.4	5.2–7.3	387–2,013	205–351	1.9–3.5
1 week	GM	631	21	30	3.8	0.49	39.3	13.2	7.3	148	258	3.4
	Q1–Q3	446–882	17–98	14–33	5.6–35.6	0.46–0.62	1.2	12.8–15.0	6.8–8.6	109–183	227–427	1.1–5.7
						2.0 mg/kg (n = 6)						
Pre	GM	386	122	77	0.9	0.16	41.4	13.1	8.2	122	224	5.4
	Q1–Q3	130–1,737	72–161	23–85	0.5–1.5	0.13–0.18	1.1	12.7–13.7	6.4–10.2	49–135	170–359	5.1–6.0
10 day	GM	795	43	13	7.6	0.40	42.0	13.7	8.3	172	207	4.8
	Q1–Q3	409–1,355	16–221	12–688	2.7–35.2	0.33–0.42	1.4	10.7–16.9	7.1–8.6	60–174	100–353	3.5–5.3
						Results of ANOVA[c]						
Dose A		NS	0.018	NS	0.050	NS	0.001	0.001	0.047	0.026	0.021	0.001
Dose B		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Time A		NS	NS	0.0002	0.0001	0.029	0.001	0.0001	0.052	0.0001	0.0002	0.0001
Time B		NS	0.048	NS	0.005	0.003	0.01	0.012	NS	NS	NS	NS
Interaction A		NS	NS	0.050	0.030	0.005	0.0001	0.0001	0.014	0.042	0.070	NS
Interaction B		0.024	NS	NS	NS	NS	NS	0.043	NS	NS	NS	NS

[a] Only factors showing significant differences are presented. Enzymes are measured in U/L, hematocrit is measured in percent, all others are measured in milligrams per deciliter.
[b] Hematocrit was normally distributed and values are mean and standard error of mean.
[c] For the analyses of variance (ANOVAs) each term is given as A = test across controls, 0.5- and 1.0-mg/kg treatments; B = test between controls and 2.0-mg/kg treatment. NS = nonsignificant.

Table 4. Geometric means and first (Q1) and third (Q3) quartiles for blood factors in reproductively active male mallards prior to, immediately following, and 2 weeks post repeated dosing with either 0 or 1.0 mg white phosphorus/kg body weight (only those factors showing significant differences are presented)

		Control			1.0 mg/kg			Repeated measures ANOVA[a]		
Factor		Predose	Postdose	1 week	Predose	Postdose	1 week	Dose	Time	Interaction
n		10	10	10	10	10	10			
Alanine aminotransferase (U/L)	GM	22	26	11	22	39	12	NS[b]	0.0001	0.009
	Q1–Q3	22–25	24–32	11–14	21–25	30–45	10–14			
Alkaline phosphatase (U/L)	GM	53	59	45	62	66	47	NS	0.001	NS
	Q1–Q3	51–63	46–68	31–61	51–76	50–116	35–56			
Aspartate aminotransferase (U/L)	GM	12	9	12	12	61	13	0.0001	0.0001	0.0001
	Q1–Q3	9–15	8–12	9–16	12–14	56–102	10–15			
Blood urea nitrogen (mg/dl)	GM	0.5	0.9	0.5	1.5	1.6	1.7	0.0001	0.0001	NS
	Q1–Q3	0.3–1.2	0.5–1.6	0.3–1.1	1.2–1.7	0.9–1.9	1.5–2.4			
Creatinine (mg/dl)	GM	0.48	0.51	0.42	0.47	0.46	0.17	0.017	0.0001	0.0008
	Q1–Q3	0.44–0.50	0.36–0.55	0.40–0.45	0.40–0.52	0.39–0.49	0.04–0.32			
Hematocrit (%)	$\bar{x}$	48.2	46.2	44.3	46.5	31.9	45.6	NS	0.001	0.0001
	SEM	0.57	0.53	0.60	46.5	2.3	0.86			
Hemoglobin (mg/dl)	GM	17.9	16.3	15.3	16.2	9.9	16.9	0.005	0.0001	0.0001
	Q1–Q3	17.6–18.4	15.4–16.8	14.7–15.8	15.6–16.5	7.3–11.0	15.5–18.5			
Inorganic phosphorus (mg/dl)	GM	3.8	4.5	5.5	3.4	4.1	5.8	NS	0.0004	0.013
	Q1–Q3	3.4–4.5	3.3–5.0	4.9–5.7	3.3–4.2	3.3–4.6	4.8–6.0			
Lactate dehydrogenase (U/L)	GM	94	163	83	83	1,950	92	0.0001	0.0001	0.0001
	Q1–Q3	76–104	150–245	80–108	73–102	1,725–1,950	84–104			
Triglycerides (mg/dl)	GM	61	33	63	63	15	94	NS	0.0008	0.037
	Q1–Q3	53–73	26–44	51–79	49–78	11–69	49–270			
Uric acid (mg/dl)	GM	3.4	2.0	2.7	3.3	2.5	4.2	0.035	0.019	0.045
	Q1–Q3	2.4–4.4	1.6–2.6	1.7–3.3	2.6–3.4	2.0–3.5	3.7–4.7			

[a] ANOVA = analysis of variance.
[b] NS = nonsignificant.

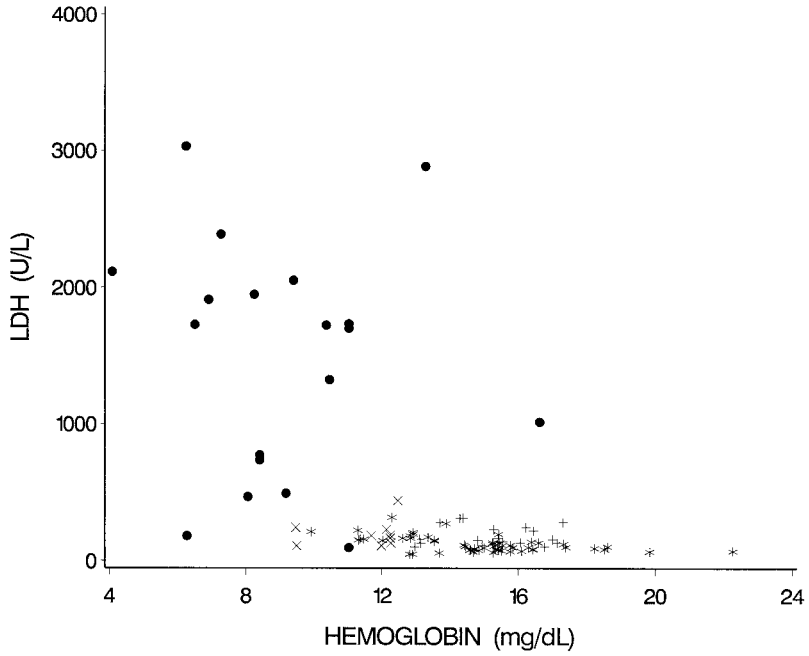

Fig. 1. Plot of lactate dehydrogenase values against hemoglobin taken 1 d after dosing regimen for mallards experimentally dosed for seven days with 0 (+), 0.5 mg/kg (*), or 1.0 mg pelletized P_4/kg body weight (dots) and for the same dosed birds at 7 d after the last dose (×). Note that birds given 1.0 mg/kg could be easily discriminated from other birds 1 d postdose but that other treatments and times could not be distinguished.

and examined 1 week after dosing could not be distinguished from controls.

Lactate dehydrogenase activity was related to hepatomegaly in birds exposed to 1.0 mg/kg P_4 1 d postexposure in that there was significant correlation between LDH and both liver weight ($r = -0.542$, $p = 0.037$, $n = 15$) and the ratio of liver weight/body weight ($r = -0.557$, $p = 0.030$). However, hemoglobin concentration did not correlate with either liver measurement ($r = -0.368$, $p = 0.195$, $n = 14$; $r = -0.333$, $p = 0.245$, respectively). Nor did hemoglobin concentration and LDH correlate significantly for these birds ($r = -0.101$, $p = 0.670$, $n = 23$). Hemoglobin was highly correlated with hematocrit ($r = 0.894$, $p = 0.0001$, $n = 26$).

Significant differences were observed in the percent lymphocytes and thrombocytes between control and dosed birds (Table 5). Lymphocytes decreased after 1.0 mg/kg and thrombocytes increased after 0.5 mg/kg. Heinz bodylike formations were found in several erythrocytes, but their occurrence was not consistent with exposure to P_4.

Table 5. Differential white blood cell count (% of cells examined) in adult mallards repeatedly dosed with white phosphorus

		Dose (mg P4/kg body weight)				
		0	0.5	1.0	2.0	*p*
n		22	8	16	5	
Heterocytes	$\bar{x}$	13.9	25.1	18.9	18.6	0.069
	SD	8.1	12.6	10.9	6.8	
Lymphocytes	$\bar{x}$	72.6[a]	57.0[ab]	53.4[b]	37.1[b]	0.0001
	SD	10.9	19.6	17.9	17.9	
Monocytes	$\bar{x}$	7.8	6.5	6.6	5.4	NS[c]
	SD	3.6	4.1	4.6	3.2	
Eosinophils	$\bar{x}$	1.1	1.2	1.3	1.0	NS
	SD	1.2	1.4	1.5	1.4	
Thrombocytes	$\bar{x}$	4.6[a]	10.1[b]	19.8[bd]	37.9[d]	0.0001
	SD	7.3	9.9	12.2	21.7	

[abd] Dose levels with the same superscript could not be distinguished at $p = 0.05$.
[c] NS = nonsignificant.

DISCUSSION

In the first experiment, 5.4 mg/kg P_4 resulted in the death of only 2 of 30 individuals. Sublethal signs of exposure were similar to those found in other mallards and included lethargy, thirst, ataxia, and inappetance. In the second experiment, only one animal died at 1.0 mg/kg. Thus, we believe that both experiments measured the sublethal effects of P_4 rather than lethal effects.

The most sensitive blood changes to P_4 exposure, as indicated by significant differences after a single dose, included the BUCR ratio, depressed hematocrit and hemoglobin, and elevated uric acid. The BUCR ratio is difficult to interpret but may be related to elevated urea concentrations that are diagnostic of renal problems and would be consistent with the elevated uric acid concentrations [13]. Hematocrit and hemoglobin depression were caused by hemolysis, which was frequently seen in mallards and other species intoxicated with P_4. Other investigators [19,20] have shown compromises in the cardiovascular systems of mammals, but extensive hemolysis in birds due to P_4 has not been previously reported. In acutely lethal exposures, mallards and other waterfowl display intense convulsions that are similar in form to those occurring during euthanasia with CO_2 but last for 30 min to 1 h instead of a few seconds [8]. These convulsions may be due to anoxia caused by hemolysis. The mechanism behind hemolysis is unknown. Other changes that were not observed until the second dose could have been stimulated by the first exposure but required more time to become apparent or may have required a larger dose of P_4. These included elevated concentrations of AST, LDH, ALT, K, and depressed total protein levels.

Levels of AST, ALK, and ALT in our control birds of both experiments were generally within the range found by other

investigators for mallards or black ducks (*Anas rubripes*) [13,15]. However, in the first experiment, our control LDH values were higher than those reported previously, and in the second experiment, LDH values were lower. These differences in LDH might be related to breeding condition in that reproductively active females had higher values than reproductively active males in the second experiment, and neither sex was in reproductive condition during the first experiment. Reproductive or gender influences could be seen in the second experiment in AST, BUN, inorganic P, ALT, ALK, and triglycerides. There may be gender-specific responses to P_4, as seen by changes in plasma levels of AST, creatinine, and LDH.

Plasma constituents can vary in concentration after exposure to contaminants due to inhibition of metabolism, injury to organs that store or produce the constituent and subsequent leakage into the circulatory system, or release from erythrocytes [21]. Lactate dehydrogenase is found in numerous tissues, including liver, kidney, heart, and erythrocytes. Elevated levels of this enzyme can indicate damage to one of these organs [12]. Aspartate aminotransferase is found in comparatively high concentrations in liver and kidney of mallards [13], and elevated levels can be diagnostic of damage to these organs and would be consistent with known liver and kidney damage in mallards and rats exposed to P_4 [7,8,22,23]. Kidney concentrations of ALT are four times higher than liver concentrations in mallards [13], and elevated concentrations of this enzyme might be more diagnostic of renal damage. Lower total protein is not typically diagnostic of pathology [24] but may be related to the inappetance that often accompanies P_4 toxicity. Elevated K, creatinine, P, and uric acid are characteristic of renal damage, which has also been reported in birds [7,8] and mammals [25]. However, elevated levels of P may also be related to the composition of P_4. After an insult to tissue or organs that results in altered levels of plasma constituents, physiological mechanisms may be activated to return the body to homeostatic conditions; these mechanisms may overcompensate or have lag responses [21] that may have caused the observed rebound effects.

In the second experiment, females dosed with 1.0 mg pelletized P_4 per kilogram showed elevated AST, BUN, and LDH concentrations. In males, AST, LDH, ALT, and inorganic P also increased following exposure. Uric acid initially decreased and then increased. Hemoglobin and hematocrit decreased in both sexes after dosing. The diagnostic features of each of these elements were discussed above and tend to represent renal or hepatic impairment and hemolysis. Few of these changes persisted in the 2.0-mg/kg females, and none were significantly different from controls in the 1.0- or 0.5-mg/kg regimens at 1 week postdose. Hemoglobin levels appeared to rebound at 10 d postdose compared with 1 d after dosing in females given 2.0 mg/kg P_4. Because only changes in ALT were persistent, it appears that repeated exposure to 1.0 mg/kg per day for several days will not produce long-lasting plasma effects in mallards.

None of the observed blood chemistry changes were unique to P_4. Diseases, heavy metals, and a host of other compounds can damage liver or kidney and result in similar responses in plasma [15]. Elevated LDH seemed to be related to hepatomegaly in birds dosed with 1.0 mg/kg but in a negative direction—the greater the evidence for hepatomegaly in affected birds, the lower the value of LDH. It is possible that P_4 elicits elevated plasma LDH through a incompletely understood mechanism but that severe liver damage associated with necrosis and hepatomegaly blocks the full expression of that elevation but actual physiological processes are not known. The hemolytic features of P_4, however, are closely related to decreased hemoglobin concentration and independent of hepatomegaly. Thus, the two plasma constituents are reflecting different toxicological phenomena and, when combined with other factors related to liver or kidney impairment, can be useful in screening for birds that have been exposed to P_4. Although elevated ratios of hemoglobin and LDH do not appear to be substantially more persistent than the presence of P_4 in adipose of living organisms, blood samples are much easier to obtain, require less training, and are easier and less expensive to analyze than adipose biopsies. Because blood samples are less invasive than biopsies, plasma analysis would be preferred when the survival of subject animals is of concern. However, these laboratory findings must be confirmed by field measurements from exposed birds before they can be used with certainty.

Avian lymphocytes are involved in the immune response. Significant decreases in this type of leukocyte may affect immunosuppression in mallards, but more work is needed to confirm that hypothesis. Thrombocytes are important in clotting, and significant increases may be a response to kidney or liver damage caused by P_4, but they are not considered as direct indicators of P_4 toxicity. An increase in monocyte count among guinea pigs experimentally dosed with yellow phosphorous was attributed to liver damage [26].

In conclusion, the results of these experiments support histological and pathological effects of P_4 in birds and mammals. One of the earliest and most dramatic effects of P_4 toxicity is the destruction of erythrocytes that can severely reduce oxygen transport and result in the convulsions and sterotypic behaviors associated with acutely lethal doses of P_4. Other plasma changes such as elevated LDH, ALT, AST, uric acid, and K reflect the more chronic effects on liver and kidney. Although the ratio of hemoglobin and LDH provides a good indicator for recent exposure, it is not much more persistent than the presence of P_4 in living tissues. However, the facility of obtaining blood and determining these values can make the ratio preferable to biopsies in many situations once field validations have been conducted.

Acknowledgement—Partial funding for this paper came from the U.S. Army Environmental Health Agency through Fort Richardson, U.S. Army. Several volunteers from the Student Conservation Association including Karen Mah and Colleen O'Rourke helped collect data and analyze plasma. David Hoffman and Barnett Rattner reviewed an early draft of the manuscript.

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