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Formalin Preservation of Avian Blood for Metal and DDE Analysis

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Field investigators often take blood samples from wild birds for residue analysis to determine their exposure to various environmental contaminants, such as metals and organochlorines. Under field conditions immediate freezing of blood samples, one common technique of preservation, is not always possible or practical. If samples are not properly preserved, organochlorines may be metabolized (Ecobichon and Saschenbrecker 1967; Stafford and Stickel 1982), and some metals might be lost through volatilization. Therefore, specific techniques of blood preservation must be tested to determine which method is most suitable when blood is to be analyzed for contaminants. In addition, techniques of homogenization of formalin-preserved blood must be adequate if such samples are to be analyzed for both organochlorines and metals or for metals requiring different analytical techniques. The objectives of our study were to test the adequacy of a homogenization technique for formalin-preserved blood and determine which of two preservation techniques is better when samples are to be analyzed for metals and DDE.

MATERIALS AND METHODS

Black ducks (Anas rubripes) were obtained from a captive colony maintained at the Patuxent Wildlife Research Center. The birds, two per cage, were housed in elevated vinyl-coated wire cages 104 x 104 x 76 cm high. Water flowed constantly into a stainless steel water pan, 51 x 30 x 15 cm deep, in each cage. Duck developer pellets were provided ad libitum in a stainless steel feeder in each cage throughout the studies.

One duck was dosed with 10 mg cadmium/kg body weight and one number 4 lead shot to provide blood samples for a homogenization trial. Cadmium chloride was dissolved in dimethyl sulfoxide (DMSO) at the rate of 10 mg Cd/ml and was administered per os by syringe and tube. The lead shot was administered through a tube inserted into the esophagus to the area of the proventriculus. The bird was returned to its pen where it had free access to food

and water and was sacrificed by decapitation 24 h later. Blood was collected in a beaker and mixed well with a teflon rod. Glassware (beaker and sample jars), stirring rod, and syringes had been cleaned with nitric acid and distilled-deionized water. Jar lids were equipped with teflon liners. The beaker, rod, and blood transfer syringe were coated with heparin to prevent clotting. Heparin was also added to the blood. Four 5-ml blood samples were drawn from the beaker by syringe and placed into the clean sample jars. Each sample was preserved with 0.25 ml formalin (grade ACS; 37% formaldehyde) and swirled to promote mixing. These samples were used to determine if the homogenization technique (stirring the sample in its jar with a clean spatula before subdivision into subsamples) was adequate before proceeding with the major portions of the study. Each 5-ml sample was subdivided into 10 subsamples of 0.5 ml each and analyzed for cadmium and lead.

Six ducks (4 males, 2 females) were dosed with 50, 10, and 10 mg/kg of mercury, cadmium, and DDE respectively to provide blood samples containing biologically incorporated toxicants for a preservation test. Mercuric chloride, cadmium chloride and DDE were dissolved in one solution of DMSO at 50, 10, and 10 mg/ml, respectively, of mercury, cadmium, and DDE. Three of the six (all males) ducks were also given one number 4 lead shot. Dosage techniques were the same as in the blood homogenization trial. The birds received 1 ml DMSO per kg of body weight (range 1.0 - 1.4 ml). The average weight of the birds was 1115 g. Birds were immediately returned to their pens following dosage, where they had free access to food and water.

Five birds were sacrificed by decapitation 6 h following dosage. One (dosed with all four toxicants) died before sacrifice and many of the others were lethargic. Blood from sacrificed birds was collected in a single beaker and mixed well with a teflon rod. All items in contact with the blood were previously cleaned with nitric acid, distilled-deionized water, acetone, and hexane. The beaker, rod, and blood measuring syringe were pre-coated with heparin. Generous amounts of heparin were added to the blood to prevent clotting. The volume of blood collected from the five surviving birds was inadequate for the original design of the study (see below), therefore we added blood from two undosed birds (about 40% of the total pooled volume) making a total volume of about 180 ml.

Five-milliliter samples of blood were drawn from the pool by syringe and five were allocated to each of 7 of the 8 preservation-storage treatment groups (Table 1). Treatment G was omitted because of insufficient blood in the pool. Each 5-ml sample was placed in a separate jar. Samples preserved by freezing were placed in a freezer at -20°C within 1 h of blood collection. Formalin (0.25 ml) was added to those samples assigned to formalin-preservation groups, samples were swirled to promote mixing, and were held at room temperature until analysis.

Table 1. Blood preservation treatments for metal and DDE analysis.

Preservation method[a]	Time of analysis following collection[b]		
	Fresh	2 months	8 months
None	A	-	-
Formalin	B	C	D
Frozen	-	E	F
Formalin - frozen	-	G	H

[a] See text for details.
[b] Letters indicate treatment groups referred to in text.

Samples for the formalin-freezing group (H) were preserved as above with formalin, held at room temperature for 2 weeks, and then frozen as above until analysis. Those samples assigned to the formalin only preservation groups (B, C, D) were removed from the pooled sample first, followed by fresh (A), frozen (E, F), and formalin-freezing group (H), respectively, because we were uncertain as to the exact blood volume present and wished to insure that each treatment to be included received its full allotment of five samples.

Shot were retained by the two sacrificed birds that had been dosed with lead, but erosion of lead from the shot was not visible. Lead residues in the blood were inadequate, thus necessitating a separate lead dosing trial.

Four female black ducks were each dosed with one number 4 lead shot as previously described to provide blood samples containing biologically incorporated lead for a preservation test. Birds were sacrificed by decapitation 24 h later. The average weight of the birds was 1030 g. An eroded shot was recovered from the gizzard of each bird. Blood collection and mixing was as previously described. Two-ml samples were drawn from the pooled sample and five samples allocated to each of the eight treatments (Table 1). Formalin (0.1 ml) was added to appropriate jars. Allocation of blood to treatments was by filling each replicate in numeric order across treatments.

Samples for lead and cadmium analysis were prepared and analyzed by a method similar to that described by Hinderberger et al. (1981). One-half g of blood was weighed into a 12-ml graduated polypropylene centrifuge tube, 1 ml of concentrated nitric acid was added, and the tube was placed in a boiling water bath for 1 h. Three-quarter ml of 30% hydrogen peroxide was added and the tube replaced in the boiling water bath for 1 h. The digestate was diluted to 10 ml with 1% ammonium phosphate for analysis by graphite furnace atomic absorption spectrophotometry.

Determinations were made on either a Perkin-Elmer 460 atomic absorption spectrophotometer with a HGA 2200 graphite furnace by using deuterium arc background correction or a Perkin-Elmer Zeeman 5000 with a HGA 500 furnace utilizing the Zeeman effect for background correction. Operating conditions were as follows:

	Lead	Cadmium
Wavelength (nm)	217.0	228.8
Slit (nm)	0.7 low	0.7 low
Mode	Peak height	Peak height
Dry (Temp/time/ramp)	250°C/40s/30s	250°C/40s/30s
Char (Temp/time/ramp)	950°C/30s/20s	750°C/30s/20s
Atomize (Temp/time)	2200°C/5s	2200°C/5s

We ran samples using maximum power heating, a pyrolytically-coated graphite tube, a L'vov platform, argon flow of 50ml/min during atomization and a 5s integration time. Quantitation was performed by comparison of sample peak heights with those of standards containing 10% nitric acid and 1% ammonium phosphate. The lower limits of reportable residue were 0.05 ppm for lead and 0.005 ppm for cadmium.

For mercury analysis, a 2-g blood sample was digested for 4 h with 20 ml of 3:1 sulfuric : nitric acid, with additions of nitric acid to prevent charring, in the digestion apparatus described by Monk (1961). After digestion, the sample was transferred to a 100-ml volumetric flask. Determination was made by comparison of sample peak heights with those of aqueous standards after reduction to mercury vapor by addition of 10% stannous chloride as described by Hatch and Ott (1968). Measurement was made with a Coleman MAS-50 mercury analyzer. The lower limit of reportable residue was 0.02 ppm.

For DDE analysis, a 2-g blood sample was ground with anhydrous sodium sulfate and extracted with hexane on a soxhlet apparatus for 7 h. Extracts were cleaned by either Florisil column chromatogaphy as described by Cromartie et al. (1975) or by passing the extract through a Florisil Sep-pak as described by Clark et al. (1983). The cleaned extract was concentrated and transferred to a graduated centrifuge tube for gas-liquid chromatographic analysis. We performed quantitation by comparison of sample peak area with that of the laboratory standard using a Hewlett-Packard 5840 gas-liquid chromatograph equipped with a nickel 63 electron capture detector and with a 1.5/1.95% SP-2250/2401 column. Operating temperatures were oven 200°C, detector 300°C, and injection port 250°C. Carrier gas was argon-methane (95+5) at 60 ml/min. The lower limit of reportable residue was 0.01 ppm.

Clean, formalin-preserved mallard (Anas platyrhynchos) blood was fortified at the 0.1 ppm and 0.5 ppm concentrations with DDE, and at the 0.1 and 1.0 ppm concentrations with lead, cadmium, and

mercury. Three samples at each fortification level were analyzed to verify the methodology and to determine recovery percentages. Percent recovery (Table 2) did not appear to be related to the concentration of contaminant present. Residue concentrations were not corrected on the basis of recovery percentages nor were they corrected for the dilution caused by the addition of formalin.

We used a one way analysis of variance (ANOVA) to determine if there were significant differences among treatment means. When significant differences in ANOVA were found we used the Bonferroni method (Kirk 1968) to evaluate changes with respect to time of preservation. Finally, we used Dunnett's method (Kirk 1968) to compare mean values in treated samples with that present in fresh non-preserved (without formalin) blood.

Table 2. Average percent recovery of contaminants in avian blood fortified at different concentrations.

Contaminant	Fortification concentration - ppm		
	0.1	0.5	1.0
Lead	92	-[a]	100
Cadmium	104	-	97
Mercury	100	-	98
DDE	103	111	-

[a] Percent recovery not determined.

RESULTS AND DISCUSSION

The average coefficients of variation for the cadmium and lead concentrations in the samples analyzed to test the homogenization technique were 10.2 and 7.8% with only one of four values for each metal exceeding 9%. Therefore, the homogenization technique was considered adequate.

There were no significant ($P>0.05$) differences among treatments in concentrations of lead, mercury, or cadmium, but there were significant ($P<0.0001$) differences among treatments in concentrations of DDE (Table 3). Samples frozen for 2 and 8 months had significantly lower DDE concentrations than that found in fresh unpreserved blood, whereas there were no significant differences between DDE concentrations in blood of other treatments and that found in fresh blood (Dunnett's method; $P>0.05$). DDE concentrations decreased significantly more between 0 and 2 months and between 0 and 8 months for frozen samples than for formalin-preserved samples (Bonferroni method; $P<0.05$). The amount of change in DDE concentrations was the same between 2 and 8 months regardless of preservation method (Bonferroni method; $P>0.05$).

Table 3. Residue concentrations of cadmium, mercury, lead, and DDE (mean ± SD; ppm wet weight) in blood of dosed black ducks that was preserved by different methods and for different periods of time.

Preservation method[a]	Time of analysis following collection		
	Fresh	2 months	8 months
	Cadmium[a]		
None	0.070 ± 0.006	-	-
Formalin	0.072 ± 0.007	0.082 ± 0.005	0.082 ± 0.017
Frozen	-	0.084 ± 0.002	0.079 ± 0.012
Formalin - frozen	-	-	0.082 ± 0.005
	Mercury[a]		
None	1.8 ± 0.05	-	-
Formalin	1.7 ± 0.09	1.7 ± 0.04	2.0 ± 0.23
Frozen	-	1.8 ± 0.10	1.9 ± 0.05
Formalin - frozen	-	-	1.9 ± 0.26
	Lead[a]		
None	0.68 ± 0.03	-	-
Formalin	0.70 ± 0.06	0.69 ± 0.05	0.69 ± 0.04
Frozen	-	0.68 ± 0.05	0.65 ± 0.10
Formalin - frozen	-	0.72 ± 0.03	0.60 ± 0.13
	DDE[b]		
None	0.26 ± 0.02	-	-
Formalin	0.24 ± 0.01	0.28 ± 0.02	0.28 ± 0.02
Frozen	-	0.17 ± 0.02*	0.17 ± 0.01*
Formalin - frozen	-	-	0.26 ± 0.01

[a] No significant differences among treatments ($P>0.05$).
[b] Significant differences among treatments ($P<0.0001$); see text for details.
* Starred means were significantly different ($P<0.05$; Dunnett's method) from that of fresh unpreserved blood.

Our results regarding loss of DDE residues in frozen samples support those of Stafford and Stickel (1982) who found losses of both DDT and DDE in frozen samples compared with formalin-preserved samples. In our study DDE concentrations in frozen samples were 35% below those in the fresh sample.

Concerns regarding possible volatilization of metals from samples preserved by freezing or formalin now appear unfounded. We highly

recommend the use of formalin for preservation of blood, especially if samples are to be analyzed for an organochlorine. The ease with which this method can be used in the field is a major advantage even if samples are to be analyzed only for metals. Stafford and Stickel (1982) provided detailed instructions for the collection of blood samples and their preservation with formalin. To prevent contamination of samples by metals, we suggest that collection syringes and sample jars also be cleaned with nitric acid and distilled-deionized water.

Blood plasma has been used for monitoring DDE in a number of species of wild birds (Henny 1977; Capen and Leiker 1979: Henny et al. 1981;1982). Ecobichon and Saschenbrecker (1967) added DDT to avian blood and plasma and preserved the samples by freezing; DDT was degraded in whole blood but not in plasma. If biologically incorporated DDE is not lost from frozen plasma, it would be superior to whole blood, for monitoring residues in wild birds, however specific evidence is unavailable. If DDE loss from whole blood occurs soon after collection, then the timing of plasma separation from whole blood might be important for the proper interpretation of DDE concentrations in plasma. If the use of plasma for monitoring DDE concentrations in wild birds is continued, we suggest that additional studies be performed to determine whether DDE is lost from plasma samples preserved by freezing or formalin, and to determine if the timing of plasma separation from whole blood affects DDE levels in plasma. Data on the timing of DDE loss from whole blood samples preserved by freezing is also needed.

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