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A SIMPLE, QUANTITATIVE TEST FOR ERYTHROCYTIC PROTOPORPHYRIN IN LEAD-POISONED DUCKS [1]

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Abstract: Forty wild-type, pen-raised mallard *(Anas platyrhynchos)* hens were divided into 4 groups of 10. Each bird received 8, 2, 1 or 0 number four lead shot. Blood lead of treated birds exceeded normal concentrations (0.40 ppm) within 8 hours and attained maximum concentrations on the second day. Blood fluorescence spectra of these mallards, scanned on a fluorescence spectrophotometer, were characteristic of protoporphyrin IX (PP). Protoporphyrin IX is apparently synthesized and accumulated in the peripheral blood. Due to rapid leak of PP from erythrocytes and effective biliary clearance, PP concentrations were rarely elevated (>40 μg/dl) in freshly drawn blood from lead-poisoned ducks. However, when the same blood was oxygenated and refrigerated PP concentrations increased due to *in vitro* synthesis which terminated within two days. No such increase was manifest by controls. Blood PP exceeded normal concentrations (40 μg/dl) in blood which was drawn two days after shot ingestion and refrigerated for two days prior to testing. Maximum concentrations were attained by the eighth day. The PP concentrations had returned to normal in 90% of the lead-treated birds by the 36th day after shot administration. Sequential radiographs revealed shot clearance from the gizzards of 90% of the ducks by the 35th day. As the result of these observations a finding of a blood PP concentration above 40 μg/dl would suggest the ducks being examined ingested the equivalent of at least one lead shot approximately two days to one month prior to testing. Sixteen additional mallards (8 male, 8 female), administered various doses of lead shot, were examined daily for clinical signs of lead poisoning. Motor functions were impaired at blood PP concentrations exceeding 500 μg/dl. A commercially available fluorometer used to screen humans for lead intoxication by measurement of erythrocytic zinc protoporphyrin was modified to test ducks. This instrument is capable of measuring the PP concentration in a single drop of unprocessed blood.

INTRODUCTION

An estimated 1.6 to 2.4 million ducks die of lead poisoning in North America each year.[14] This mortality is due primarily to the ingestion of spent lead shot from marsh sediments. A calculated 25% of the mallard population ingests at least 1 lead shot each year.[2]

The U.S. Fish and Wildlife Service is implementing restrictions on lead shot for waterfowl hunting to diminish the level of sediment contamination and thus the number of lead poisoning mortalities. These regulations call for the use of relatively non-toxic steel shot in areas of heavy waterfowl harvest, high shot

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ingestion rates and documented mortalities due to lead poisoning.[14]

To aid in the delineation of these restricted areas this study was directed towards developing a simple, reliable test for lead poisoning of ducks by measuring erythrocyte fluorescence, since the erythrocytes of lead-intoxicated mallard ducks and Canada geese,[1] like those of rabbits[11] and humans,[15] fluoresce red when exposed to long-wave ultraviolet light (400 nm). Lead inhibits heme synthetase,[13] the enzyme responsible for the incorporation of iron in protoporphyrin IX (PP) to form heme. As the result PP accumulates in the erythrocytes. In human erythrocytes, PP bonds with zinc to form the fluorescent compound zinc protoporphyrin (ZPP),[7] which can be measured in a single drop of whole blood using the recently developed hematofluorometer[3] (front surface fluorometer). This study was designed to determine if ZPP was produced in erythrocytes of lead-poisoned ducks and if the hematofluorometer could be used to detect lead poisoning in ducks.

In addition this report describes some hematologic effects of lead on mallard ducks.

MATERIALS AND METHODS

Experiment 1

This experiment was designed to identify the fluorescent compound in the erythrocytes, establish the feasability of using the hematofluorometer to measure the fluorescent compound in lead poisoned ducks and record changes in various blood parameters.

Animals and Regimen: Forty individually caged, 14 month-old mallard hens were separated into 4 groups of 10. The birds of groups A, B and C were given 8, 2 and 1 number four lead shot, respectively. The shot weighed between 190 and 210 mg each, and were administered via esophageal catheter. Controls (group D) did not receive lead shot. All birds were provided with washed quartz grit prior to administration of lead shot; they also received continuously flowing water and a commercial duck ration (Duck Growena)[2] *ad libitum*.

Blood Collection: To follow the pattern of changes in various hematologic parameters a heparinized blood sample (¾ ml) was taken from the brachial vein of each duck prior to administration of shot. Blood samples also were taken at 8 h., 2 days, 8 days and at weekly intervals thereafter until the end of the five week study.

Blood Lead: Blood lead determinations were made using the paper disc-in-Delves cup micro-technique.[6]

Protoporphyrin Measurement: Emission and excitation maxima of blood samples were determined to identify the fluorescent compound(s). These blood samples were oxygenated, refrigerated and tested at various intervals to detect any change in fluorescence.

Fluorescence excitation and emission maxima and intensity were determined using a fluorescence spectrophotometer (Perkin-Elmer-Hitachi MPF-2A) from 5 μl samples of blood. These samples were diluted 1000 fold in phosphate buffered detergent (PBD) and phosphate buffered saline (PBS).[7,8]

Blood samples were refrigerated for two days to allow for development of maximum fluorescence prior to measuring PP concentrations. These levels were determined by comparing the fluorescence intensity of PBD diluted blood samples with that of a standard.[12] The standard was prepared from 95% PP[3] diluted in PBD.

[2] Ralston Purina Co., St. Louis, Missouri 63136, USA.

[3] Porphyrin Products, Logan, Utah 84321, USA.

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Hematofluorometer Modification and Calibration: Since the hematofluorometer[1] is used to detect ZPP in human erythrocytes, it was necessary to modify it to measure PP by substituting an emission filter with a pass band of 620 nm for the factory installed filter (pass band 594 nm). It was calibrated with 40 mallard blood samples previously tested on the fluorescence spectrophotometer.

Radiography: Whole body radiographs of all birds were made prior to shot administration and at one week intervals for a period of five weeks after treatment to detect when the lead shot had been eliminated.

Hematology: The erythrocyte count (Coulter Counter, FN) packed cell volume, hemoglobin concentration (AO hemoglobinometer) and mean corpuscular hemoglobin concentration (MCHC) was determined for each sample. Erythrocyte counts and MCHC's of the ducks in each group were compared on the eighth day of the study using analysis of variance. Methanol fixed blood smears were stained with Giemsa and examined microscopically.

Erythrocyte zinc concentrations were determined by atomic absorption spectrophotometry on washed erythrocytes.[12]

Experiment 2

This experiment was intended to provide information on the relationship among erythrocytic PP, reticulocytes and clinical signs of lead poisoning in mallard ducks.

Animals and Regimen: Sixteen mallard ducks (8 male, 8 female) were divided into four groups of four and caged in an outdoor pen. The ducks of three groups were administered 1, 8 and 18 number four lead shot; the ducks of the fourth group did not receive lead shot. The ducks receiving 18 lead shot were fed whole corn. The remaining ducks were fed the same commercial duck ration as in experiment 1.

Clinical Examination and Blood Collection: Birds were examined daily for clinical signs of lead poisoning. The ducks were chased in the pen to precipitate signs of muscular weakness or ataxia. They also were tossed in the air to test flying ability. Heparinized blood samples (0.5 ml) were collected from the brachial vein of all birds at the outset and from individuals as clinical signs developed.

Protoporphyrin Measurement: All blood samples were tested for PP on the hematofluorometer. Blood samples, which had been oxygenated by briefly exposing the sample to the air followed by gentle mixing, were refrigerated for two days. A drop of this blood was placed on a glass cover slip (24×24 mm), oxygenated by stirring and inserted in the modified hematofluorometer. The measure button was pressed and the PP concentration was indicated on a digital meter within five seconds.

Reticulocyte Count: Blood smears were stained with brilliant cresyl blue and examined microscopically for reticulocytes. Only those cells with a basophilic reticulum completely surrounding the nucleus were considered reticulocytes. The percentage of reticulocytes was based on a sample of 500 erythrocytes.

RESULTS

Experiment 1

The highest blood concentration of lead observed in ducks not given lead shot was 0.39 ppm. All of the group A ducks, 80% of the group B ducks, and 50% of the group C ducks exceeded 0.40 ppm 8 h. after receiving shot. The highest blood concentrations of lead for all treatment groups were found on the second day

[1] AVIV Associates, Lakewood, New Jersey 08701, USA.

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(Figure 1). At this time the group A, B, C and D ducks had mean (range in parenthesis) blood lead concentrations (ppm) of 6.00 (1.28-11.99), 2.54 (0.81-5.67), 1.30 (0.52-3.44), and 0.14 (0.05-0.25), respectively. Lead concentrations remained above 0.40 ppm for some of the birds in the three treatment groups until day 29 of the study. On day 29, 10% of the group A ducks, 40% of the group B ducks and all of the group C ducks had dropped below 0.40 ppm. Ninety percent of the mallards had normal blood lead concentrations by day 36.

The fluorescence spectrum of PBD diluted whole blood from mallard ducks had an excitation maximum of 408±1 nm and an emission maximum of 635±1 nm. These maxima are identical to those of metal free PP in the detergent solution.[9] The maxima of PBS diluted blood (excitation 398-405 nm, emission 624±1 nm) were also characteristic of metal free PP bound to hemoglobin.[9]

On the average, 97% of the PP was present in the erythrocytes of mallard ducks given lead shot. This probably represents a lower limit since the values obtained for the plasma fractions were not corrected for hemolysis.

PP concentrations of controls was below 40 μg/dl after two days of refrigeration. The highest blood PP concentration from 110 samples collected from 40 mallards free of lead shot at various intervals during the study was 36 μg/dl.

The PP concentrations in blood samples collected from four ducks given one lead shot were below 40 μg/dl two days after they received the lead if samples were measured immediately after collection. However, when these samples were oxygenated and refrigerated for two days, PP concentrations of three rose above 64 μg/dl, which is indicative of *in vitro* PP synthesis. The concentrations remained relatively constant (±10 percent) for at least seven additional days.

Blood PP of 40% of the group A ducks equaled or exceeded 40 μg/dl 8 h. after receiving shot (Table 1). By the second day PP concentrations exceeded 40 μg/dl

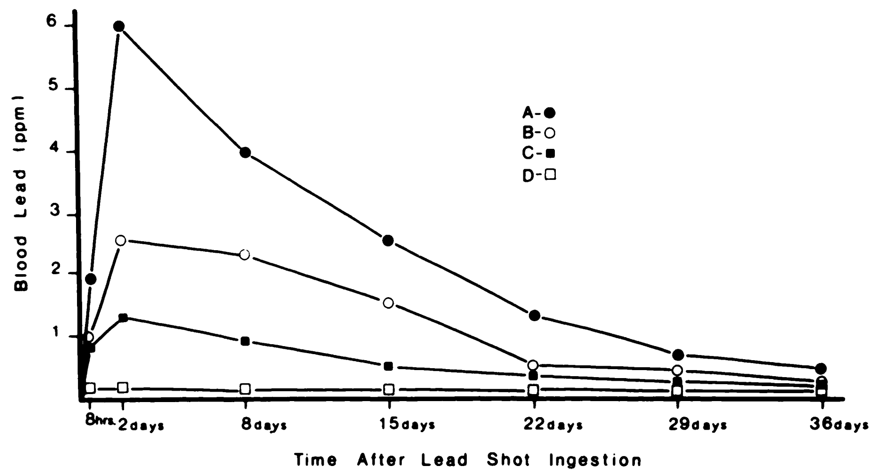

FIGURE 1. Mean blood lead concentrations of mallard ducks (ten per treatment group) at various intervals after ingesting 8(A), 2(B), 1(C), and 0(D) number four lead shot pellets.

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TABLE 1. Percentage of mallards (10 birds/treatment group) with blood protoporphyrin IX concentrations equal to or exceeding 40 μg/dl at various intervals after ingestion of 8(A), 2(B), 1(C), and 0(D) number 4 lead shot.

Treatment Group	Time after shot ingestion							
	0	8 hrs.	2 days	8 days	15 days	22 days	29 days	36 days
A	-	40	100	100	100	90	60	30
B	-	-	100	100	90	20	-	-
C	-	-	100	100	60	10	-	-
D	-	-	-	-	-	-	-	-

in ducks receiving lead. By the eighth day of the study, PP concentrations were at their highest for all groups receiving lead (Figure 2). At this time the mean (range in parenthesis) PP concentrations (μg/dl) of the group A, B, C and D ducks was 1070 (332-2284), 242 (97-729), 129 (61-235), and 15 (9-32), respectively. The mean PP concentrations remained above 40 μg/dl for 22 days in some of the birds of each of the three lead-treatment groups. On the 29th day the PP concentrations had dropped below 40 μg/dl in 40% of the group A ducks, and all of those in group B and C. By the 36th day only 30% of the group A ducks had values over 40 μg/dl.

The blood PP readings on the fluorescence spectrophotometer (s) were compared with those on the modified hematofluorometer (H) not yet set to final calibration (Figure 3). The correlation coefficient (r=0.91) indicated that these data are described by the linear regression equation of H=0.54 + 0.91 S with a standard deviation of 12 μg PP/dl. The offset and gain of the instrument were adjusted using the intercept and slope

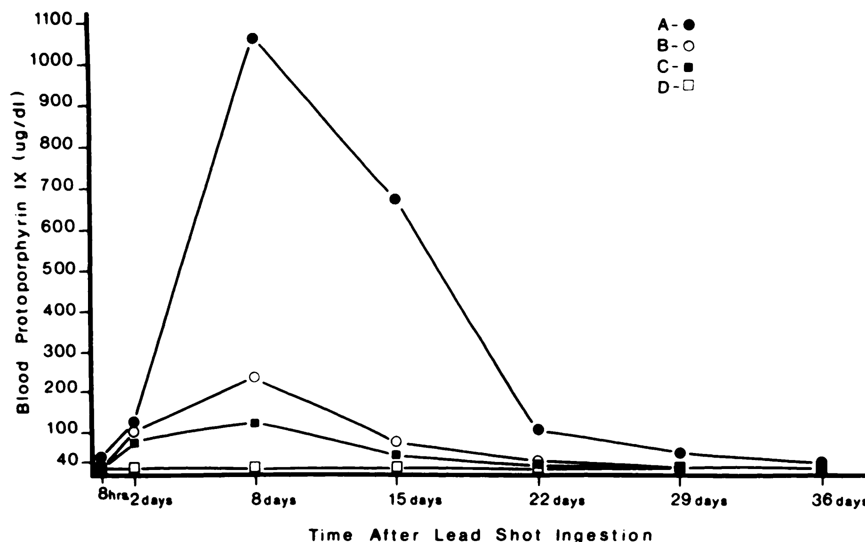

FIGURE 2. Mean blood protoporphyrin IX concentrations of mallard ducks (ten per treatment group) at various intervals after ingesting 8(A), 2(B), 1(C) and 0(D) number four lead shot pellets.

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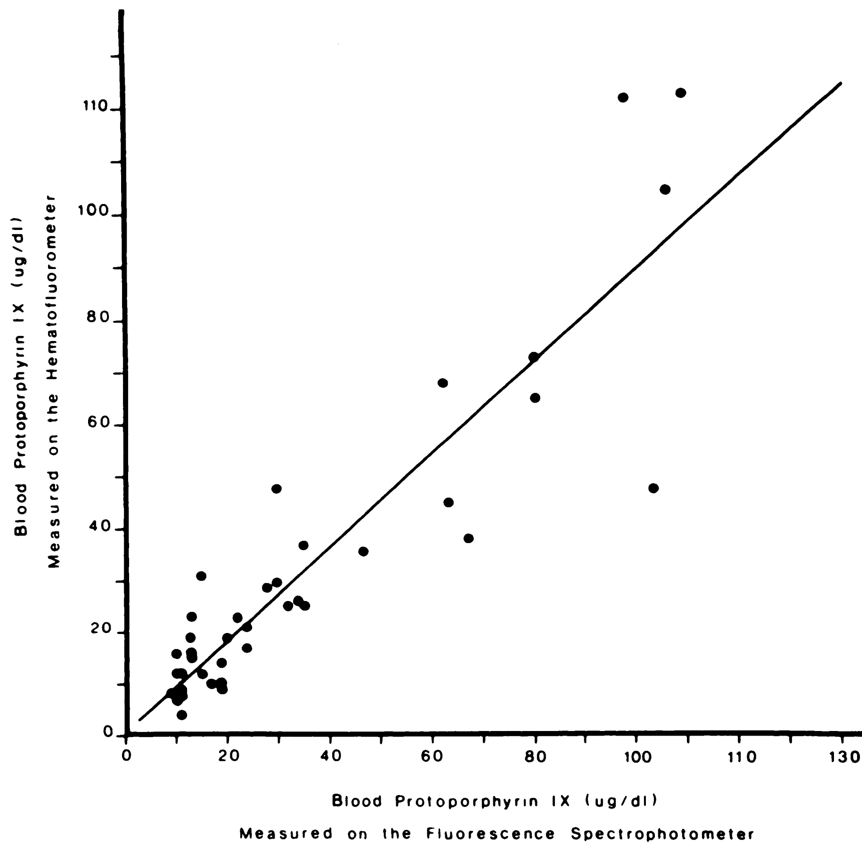

FIGURE 3. Blood protoporphyrin IX values of 40 mallard ducks measured on the fluorescence spectrophotometer compared to the values obtained on the modified hematofluorometer. $y=0.54 + 0.91x$.

values to bring subsequent instrument readings into agreement with spectrophotometer readings.

Forty-three percent of the ducks in groups A, B and C lost all shot, through erosion, absorption and defecation within the first three weeks. By day 28 all of group B and C birds, and 60% of the group A birds had lost their shot. On day 35, 90% of the lead treated mallards were free of shot.

The erythrocytes of mallard ducks contain an average Zn concentration of 4.7 ppm (dry weight) or 3.7 ppm Zn (wet weight), which is less than half the concentration (11 ppm wet weight) in human eythrocytes.[5]

Changes in the blood profile of ducks were most evident on day 8 after receiving lead shot. The group A, B, C and D ducks had mean (range in parenthesis) erythrocyte counts ($1 \times 10^6/mm^3$) of 2.52 (2.20-2.87), 2.74 (2.48-2.92), 2.82 (2.70-2.98) and 2.88 (2.62-3.14), respectively. There was a highly significant difference ($P<.01$) between the erythrocyte counts of group A and the other ducks and no significant difference among the B, C and D ducks.

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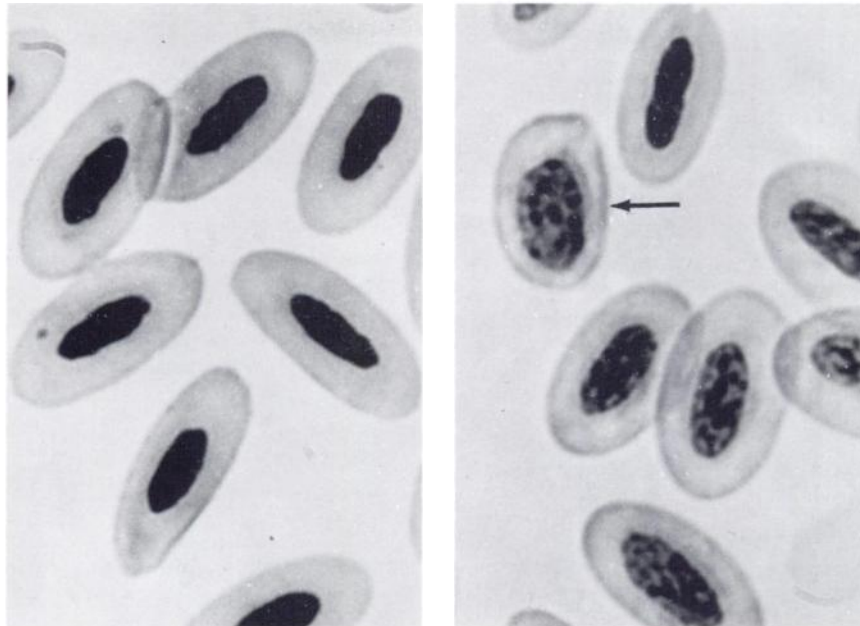

FIGURE 4. Comparison of peripheral blood smears of a control mallard (left) and a group A mallard (right) eight days after ingestion of eight number four lead shot. The arrow indicates one of several hypochromic immature erythrocytes displaying a lack of nuclear condensation with margination of the depleted hemoglobin. Giemsa 1000×.

The average (range in parenthesis) MCHC (g%) of the group A, B, C and D ducks, eight days after receiving shot, were 25 (20-30), 29 (24-32), 30 (27-32), and 31 (26-33), respectively. The difference between the MCHC of the group A ducks and the group D ducks was highly significant ($P<.01$). A significant difference ($P<.05$) occurred between the MCHC of the groups B and D, but no significant difference was evident between groups C and D.

Immature and hypochromic erythrocytes were evident in the smears of peripheral blood from the group A ducks on the eighth day of the study (Figure 4).

Experiment 2

The clinical signs of lead poisoning at PP concentrations below 500 μg/dl are mild and dominated by bile stained feces. The green color is imparted to the feces by biliverdin, which increases in the bile due to the hemolytic effect of lead. At PP concentrations above 500 μg/dl various states of motor function impairment occur (Table 2).

Control ducks with PP concentrations below 40 μg/dl had reticulocyte counts ranging from 0 to 15 percent. Mallards with PP concentrations above 500 μg/dl had a marked reticulocytosis (25-49%). The reticulocyte counts and PP concentrations diminished as the ducks recovered.

DISCUSSION

The fluorescence maxima (excitation 408± nm, emission 635± nm in PBD; excitation 398 - 405 nm, emission 624± nm in PBS) of blood from ducks ingesting

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TABLE 2. Blood protoporphyrin IX concentrations measured on the hematofluorometer and corresponding clinical signs of lead poisoning in mallard ducks.

Blood protoporphyrin IX ($\mu g/dl$)	Clinical signs
≥ 801	Death Inability to stand, walk, or fly Marked tail and wing droop Loss of voice Green watery diarrhea
501-800	Muscular weakness Easily fatigued Unsteady gait Slight tail droop Green watery diarrhea
201-500	Hyperexcitability Green watery diarrhea
40-200	Green watery diarrhea
0-39	No evidence of lead poisoning

lead shot identified the fluorescing compound as metal-free protoporphyrin IX.[9] The reason for chelation of zinc by PP in human erythrocytes and not duck erythrocytes may be due in part to the fact that duck erythrocytes contain only about $^1/_3$ (3.7 ppm) the concentration of zinc found in human erythrocytes (11 ppm).[5]

Metal-free PP differs from ZPP in that the former apparently can diffuse from the erythrocytes *in vivo* and be cleared from the plasma by the liver.[10] This could explain why the PP concentrations were frequently normal (<40 $\mu g/dl$) when measured in freshly drawn blood of lead poisoned ducks.

Since the hematofluorometer, as originally purchased, is equipped with filters best suited for measurement of ZPP, it was necessary to replace the factory installed emission filter (pass band 594) with an emission filter (pass band 620 nm) appropriate for PP measurement in whole blood. The high linear correlation of the PP readings from the diluted blood samples using the fluorescence spectrophotometer and from whole blood using the modified hematofluorometer supports the feasibility of using the latter for testing waterfowl.

The hematofluorometer measures the PP/hemoglobin ratio directly, which is independent of the PCV.[3] Ideally, the hematofluorometer should be calibrated to read μg PP/g hemoglobin, which would be free of errors induced by anemia. The decision to calibrate the modified hematofluorometer to read μg PP/dl blood was prompted by federal standards applied to the ZPP hematofluorometer. Anemia, as seen in the ducks receiving eight lead shot, would tend to increase the level of PP, expressed in $\mu g/dl$. However, this would have no significant effect on the practical diagnostic and screening application of the instrument.

A lag period of about six days occurred between the peak blood lead concentration and the peak blood PP concentration. During this period an increased erythropoiesis probably occurred in response to anemia. This hypothesis is consistent with the increase in immature erythrocytes in the peripheral blood. Roscoe[14] reported erythroid hyperplasia in the femoral marrow of lead-poisoned ducks which also had elevated

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erythrocytic PP concentrations and erythroblasts in the peripheral blood. The bulk of the PP observed in the present study was associated with the erythrocytes and was synthesized *in vitro*. Since mature erythrocytes have lost the mitochondria and ribosomes essential for such synthetic activity and reticulocytosis accompanied increased PP levels, it may be deduced that the immature erythrocytes were synthesizing the PP.

Synthesis of PP with reduced hemoglobin concentration in these cells is indicative of heme synthetase inhibition. Another heme biosynthetic enzyme inhibited by lead in mallards is delta-aminolevulinic acid dehydratase.[4] The net effect of the inhibition of these two enzymes is the reduction in hemoglobin as manifested by the appearance of hypochromic erythrocytes.

Elevated blood concentrations of PP in mallard ducks reflects undue lead absorption and more importantly, are a metabolic lesion of lead poisoning. PP concentrations are related to the clinical signs of lead poisoning in mallard ducks and those with concentrations above 500 μg/dl begin to show impaired motor functions, which could seriously affect their survival.

Assuming that shot retention time in the gizzards of free flying wild birds is similar to that of experimental birds, the interval of PP elevation should be approximately the same. As the result, a wild bird with a PP concentration above 40 μg/dl probably ingested at least one lead shot from approximately two days to one month prior to testing.

The PP test using the hematofluorometer is inexpensive (<\$0.10 U.S./sample) and does not require a trained technician, elaborate sample preparation or repeated standardizations as is the case with blood and other tissue lead determinations. In addition, it can be used as a quantitative measure of lead poisoning. The hematofluorometer enables a biologist to test living birds, thus providing a means of screening waterfowl during the critical post-hunting period when the birds are resident and banding operations are being conducted.

Detailed biochemical studies of porphyrins in lead-poisoned ducks and results of field surveys using the hematofluorometer will be reported elsewhere.

Acknowledgements

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