Hemoglobin Interference in the Direct Determination of Erythrocyte Protoporphyrin Concentrations for Monitoring Lead Intoxication of Waterfowl

Authors: Kerr, Kenneth D., and Hennes, Steven K.

Source: Journal of Wildlife Diseases, 21(3) : 338-340

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-21.3.338

Journal of Wildlife Diseases, 21(3), 1985, pp. 338–340

LETTER TO THE EDITOR . . .

Hemoglobin Interference in the Direct Determination of Erythrocyte Protoporphyrin Concentrations for Monitoring Lead Intoxication of Waterfowl

Kenneth D. Kerr, Forest Wildlife Populations and Research Group, Minnesota Department of Natural Resources, Grand Rapids, Minnesota 55744, USA; **and Steven K. Hennes,** Wetland Wildlife Populations and Research Group, Minnesota Department of Natural Resources, Bemidji, Minnesota 56601, USA

With the current concern regarding lead intoxication in waterfowl caused by ingested lead shot, researchers may be trying to test for the problem in their localities. To accomplish this, some will be looking to the pioneering work of Roscoe (1977, Ph.D. Thesis, University of Connecticut, Storrs, 130 pp.) and Roscoe et al. (1979, J. Wildl. Dis. 15: 127–136) for the determination of quantitative blood protoporphyrin concentrations. Because our laboratory is limited to the use of a filter fluorometer, we opted to use the double extraction technique of Peter et al. (1978, Clin. Chem. 24: 1515–1517) to assess concentrations in ducks from Minnesota. When comparing our results with those of Roscoe et al. (1979, op. cit.), we found that our normal values were several times higher. This report describes the methods used to elucidate the difference and presents the results obtained from these methods.

Blood samples were drawn from the brachial vein of 13 mallards (*Anas platyrhynchos*), five lesser scaup (*Aythya affinis*), one greater scaup (*Aythya marila*) and three ring-necked ducks (*Aythya collaris*) using a 22-ga. needle and 3-ml heparinized syringe. The blood was put into 2-ml heparinized vacutainers (Becton Dickinson #6498), aerated by shaking with the stoppers removed, and refrigerated at least 48 hr for maximum protoporphyrin production. Twenty additional whole blood samples were created using surplus portions of 10 of the mallard blood samples. These portions were centrifuged, the plasma and cells separated and recombined in different ratios to make the additional samples with hematocrits ranging from 9 to 85%. Microhematocrits were performed on each blood sample, and protoporphyrin levels were determined using the double extraction technique of Peter et al. (1978, op. cit.).

A direct method for determining protoporphyrin values was designed to duplicate the method of Roscoe et al. (1979, op. cit.) with the following exceptions. Roscoe et al. (1979, op. cit.) used a Perkin-Elmer-Hitachi MPF-2A spectrofluorometer with 20-nm slit widths, an excitation wavelength of 408 nm, an emission wavelength of 635 nm and a 3-mm cuvette (Roscoe, pers. comm.). They prepared and calibrated their own Protoporphyrin IX standard, and we estimated the buffer concentration as it was not provided. It should be mentioned that the formula in Roscoe (1977, op. cit.) for calculating the protoporphyrin (PP) standard concentration contained several typographical errors and should be:

$$\text{PPstd } (\mu g/dl) = \left(\frac{\text{O.D. acid PP}^a}{E^b} \times \text{M.W. PP}^c\right) \div 4{,}000^d \times 1{,}000{,}000^e$$

Received for publication 14 September 1984.

where

a = optical density of acidified PP
b = molar extinction coefficient of acidified PP = 2.41×10^5
c = molecular weight of PP = 561
d = 400-fold dilution factor and conversion of liter to deciliter = 4,000
e = correction factor to convert E from molar to micromolar extinction so answer will be in μg/dl

However, the need for this formula can be eliminated by using a pre-measured standard such as supplied by Porphyrin Products.

Five μl of each blood sample were diluted with 5 ml of a phosphate buffered detergent (PBD) (a 0.01 M phosphate buffer, pH 7.4, in 2% Ammonyx LO; Onyx Chemical Company, 190 Warren St., Jersey City, New Jersey 07302, USA) and mixed on a vortex mixer. A second set of these PBD-diluted samples was made and spiked with 5 μl of a 500-μg/dl aqueous protoporphyrin standard (Product #PFS-9, Porphyrin Products, P.O. Box 31, Logan, Utah 84321, USA) to be used as a recovery. Four blood-free standards were made by adding 5 μl of the 500-μg/dl standard to 5 ml of the PBD. Upon dilution of the blood samples with the PBD, a cloudiness began to develop causing the fluorometer reading to increase to a maximum after about 30 min. Most of this turbidity was removed by centrifugation before the fluorometer readings were taken (Fig. 1). These samples, recoveries, and standards were read against a PBD blank in a Turner Model 111 filter fluorometer with a high sensitivity sample holder using a 405-nm primary filter (band pass), a 620-nm secondary filter (sharp cut), and a 13-mm cuvette.

All of the recovery samples showed less fluorescence than the blood-free standards, indicating considerable quenching caused by the blood. The net decrease in fluorescence of each recovery when compared to the blood-free standard is expressed as percent quenching (% Q) as shown by the following formula:

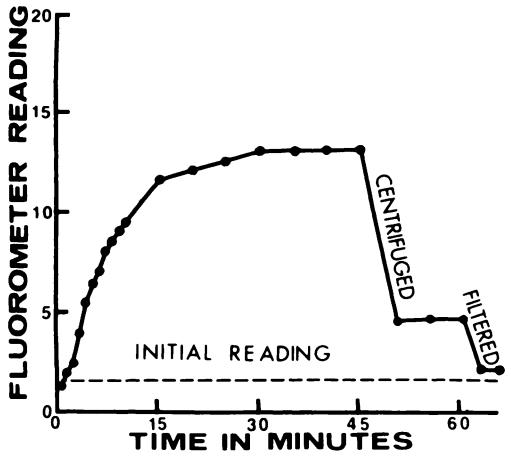

FIGURE 1. Development of fluorescence resulting from turbidity that formed after dilution of blood sample with phosphate buffered detergent, and results of attempts at its elimination.

$$\% Q = \left(1 - \frac{F_r - F_b}{F_s}\right) \times 100$$

where

F_b = fluorometer reading of the diluted blood sample
F_r = fluorometer reading of the blood sample's respective recovery
F_s = fluorometer reading of the blood-free standard

Since hematocrit is a function of erythrocyte mass, the correlation between % Q and hematocrit (Fig. 2) indicates that the quenching is caused by an intracellular component of the erythrocytes, presumably hemoglobin. The amount of quenching is the same for protoporphyrin levels of 156 to 5,000 μg/dl (n = 6, $\bar{x}$ = 67 $\pm$ 2.0%; hematocrit = 44).

Theoretically, the recoveries could have been used as internal standards for calculating the native protoporphyrin in their respective unspiked samples, but in practice the turbidity problem prevented this

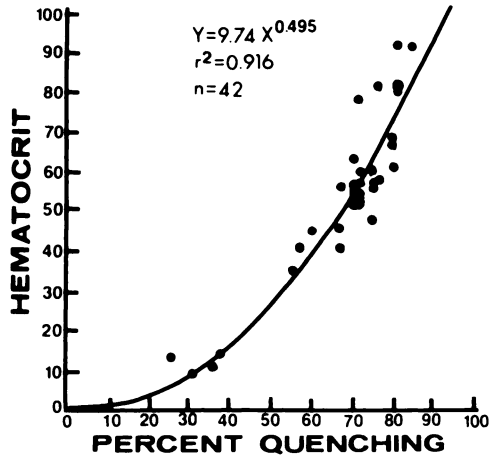

FIGURE 2. Scatter diagram of duck hematocrit values vs. quenching of protoporphyrin fluorescence including power regression line.

because even after centrifugation a residual turbidity persisted. The residual turbidity could be reduced further after centrifugation by filtering through a 0.2-μm filter (Schleicher and Schüll catalog #FP 030/3), but not eliminated (Fig. 1). The residual turbidity after centrifugation caused the protoporphyrin values calculated from the internal standards to be about thee times higher than those obtained by the extraction technique. The high level of dilution in this technique coupled with the turbidity problem made reasonable accuracy impossible using our fluorometer. Still, there was a significant correlation ($n = 42$, $r = 0.59$, $P < 0.001$) between the direct protoporphyrin values calculated with internal standardization and the values obtained by the double extraction technique.

Using our direct method, a blood sample with a hematocrit of 50% will cause an approximate 67% quenching of any protoporphyrin present when compared with a blood-free standard. We have shown that direct measurements of protoporphyrin in detergent-diluted blood can cause significant quenching. The reading of fluorescent emissions of protoporphyrins at wavelengths of 620 nm and above will exclude the zinc-bound protoporphyrin which has been shown to be present in significant amounts in mute swans (*Cygnus olor*) by Birkhead (1983, J. Zool. (Lond.) 199: 59–73), and is likely present in other waterfowl, also. This quenching coupled with the exclusion of at least one significant species of protoporphyrin may result in inaccurate low values. We believe this may be the reason that the blood protoporphyrin values reported by Roscoe et al. (1979, op. cit.) are lower than ours. In initiating a system to test for protoporphyrin levels, one should be aware of these pitfalls and design the techniques accordingly. Those considering a direct dilution technique should use protoporphyrin standards containing blood at the same level as the unknown samples, use a diluent which will not cause an interfering turbidity to form, and read at wavelengths which will not exclude any of the significant forms of protoporphyrin. The better alternative would be to quantitate blood samples to be used as hematofluorometer standards using an extraction technique to avoid interfering substances. This would also convert all forms of protoporphyrin to the free form which could be detected at one wavelength.

The advice and assistance of Todd Fuller, Bill Snow, Pat Karns, and Mark Sperry, all of the Wildlife Research Unit, Minnesota Department of Natural Resources, are gratefully acknowledged.