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Green-blood pigmentation in lizards

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The green pigment in the plasma of the scincid lizard genus *Prasinohaema* is identified as the bile pigment biliverdin. Concentrations of biliverdin in the plasma of *P. flavipes*, *P. prehensicauda* and *P. virens* are 714 ± 179 μmol/l (mean ± one standard deviation), 1020 ± 624 μmol/l and 819 ± 89 μmol/l, respectively. These values represent the highest concentration of plasma biliverdin measured for any organism and are the first report of non-pathological biliverdin accumulation in amniotes. We review the literature for fish species with high concentrations of plasma biliverdin and pathological biliverdin accumulation in humans; we find that *Prasinohaema* species have plasma biliverdin concentrations approximately 1.5–30 times greater than fish species with green blood plasma and 40 times greater than humans with green jaundice.

Key words: Bile pigments; Bilirubin; Biliverdin; Jaundice; Lizard; New Guinea; *Prasinohaema*; Scincidae.

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Introduction

The blood plasma of most vertebrates is light yellow in coloration. In contrast, the scincid lizard genus *Prasinohaema* is characterized by bright lime-green colored plasma that results in a green coloration of the muscles, bones, tongue, and mucosal tissues (Greer and Raizes, 1969). The genus includes five described species that occur in New Guinea and associated archipelagos. These species are the only known amniotes with green-blood pigmentation.

Green-colored plasma, however, has been documented in several different families of fish (Bada, 1970; Low and Bada, 1974; Mudge and Davenport, 1986; Makos and Youson, 1988; Fang and Bada, 1988; see Fang and Bada, 1990 for a review), some species of frogs (David Cannatella and Bob Drewes, *pers. comm.*), and one family of insects (Law and Wells, 1989; Goodman *et al.*, 1985). The green coloration of the blood plasma in these fish and the hemolymph in these insects results from the accumulation of the bile pigment biliverdin. The green blood pigment in frogs has not been identified but it is presumed to be biliverdin.

The bile pigments biliverdin and bilirubin are the toxic products of heme catabolism and, therefore, are ubiquitous waste products (Gray, 1958; With, 1968; Cowger, 1974; Bissel, 1986). In most vertebrates, the accumulation of biliverdin and/or bilirubin in the circulatory system and tissues causes the pathological condition known as jaundice (Greenberg *et al.*, 1971; Schenker and Hoyumpa, 1986). Studies of bile pigment metabolism have primarily been restricted to humans and laboratory mammals

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commonly used in medical research such as rats and dogs. Phylogenetic comparisons in bile pigment metabolism of normal and diseased organisms are of critical importance for understanding the role that bile pigments play in the pathogenesis of human jaundice (Colleran and O'Carra, 1977; Cornelius, 1986; Fang and Bada, 1990).

In this paper, we identify the green pigment in the blood plasma of three species of *Prasinohaema* as the bile pigment biliverdin. For a comparative approach to the taxonomic distribution and concentration of this pigment in lizards, we quantify levels of biliverdin in the plasma of three species of *Prasinohaema* and seven species, from four families, with normal blood coloration. The concentrations of biliverdin in the plasma of *Prasinohaema* represent the highest concentration of plasma biliverdin measured for any organism and are the first report of non-pathological biliverdin accumulation in amniotes.

Materials and Methods

Collection of animals and serum

Three species of *Prasinohaema* and seven other lizard species from four families were examined in this study. Species examined from the family Scincidae were: *Prasinohaema flavipes*, *Prasinohaema prehensicauda*, *Prasinohaema virens*, *Papuascincus stanleyanus*, *Lobulia elegans*, *Sphenomorphus leptofaciatus*, and *Lamprolepis smaragdina*. Species examined from the families Agamidae, Varanidae, and Polychrotidae were: *Gonocephalus nigrensis*, *Varanus indicus*, and *Anolis carolinensis*, respectively. The scincid genera *Lobulia* and *Papuascincus* are closely related to *Prasinohaema* (Greer, 1974; Allison and Greer, 1986). The inclusion of *Lobulia* and *Papuascincus*, other more distantly related scincid lizards, and representative species from three other families of squamates provides a measure by which to compare the degree of physiological deviation in *Prasinohaema* from other lizard taxa.

The first nine species listed above occur in New Guinea. Specimens from New Guinea were collected by hand in Madang Province and Morobe Province, Papua New Guinea (PNG) from August 1991 to January 1992. The last species listed, *A. carolinensis*, occurs in North America and was collected near Austin, Texas.

Serum samples were collected from the postorbital sinus of the eye using heparinized microhematocrit capillary tubes (Fisher). Samples were centrifuged for 5 min at 2000 rpm. In almost all cases, blood from a single individual was used in the analysis. In four cases, however, serum from two to four small animals was pooled to provide a large enough sample to be used in the analysis. Plasma samples were then transferred to 1.5 ml Eppendorf tubes, covered with aluminium foil and stored in the dark in liquid nitrogen for 1–5 months. Upon return to the United States, plasma samples were stored in the dark at −80°C.

Pigment identification

Five biochemical tests described by Fang (1982) were used to identify the green pigment as biliverdin. The first test was a spectrophotometric analysis of crude blood plasma to determine if a diagnostic peak for biliverdin occurs at 650 nm. Crude plasma was scanned from 300 to 800 nm on a Beckman Spectrophotometer (Model DU 64).

The second test was a spectrophotometric analysis of the purified green pigment compared with that of known biliverdin (Sigma). The pigment was isolated by the liquid column chromatography methods described by Fang (1982). Five volumes of methanol–HCl (3N) were added to 20–30 μl aliquots of crude plasma samples and stirred at 4°C in the dark for 1 hr to acidify the prosthetic group. One volume of chloroform (30 μl) was added and stirred for an additional 10 min. The total chloroform volume was then brought to 500 μl by adding 470 μl of chloroform. Serum proteins were removed by washing with an equal volume of water three times. The chloroform extracts were loaded onto a silicic acid (Sigma) micro-column and equilibrated with chloroform. The column was washed with chloroform until the elution of a yellow band occurred. The pigment at the top was then eluted with a 2:1:3 volume to volume ethanol:methanol:chloroform mixture (plus several drops of 3N HCl). The green fractions were analysed using a spectrophotometer to determine if the absorbance spectrum matched that of known biliverdin.

The third test involved thin layer chromatography (TLC) analysis of the isolated green pigment to determine if the chromatographic migration pattern matched that of known biliverdin (Fang, 1982). Fractions containing the green band were dried by evaporating the chloroform from the suspension obtained from the previous purification step. The pellets were then resuspended in 20 μl chloroform and applied to a silica thin layer chromatography plate that had previously been washed in methanol and dried to remove water. The plate was developed with a 2:1:1.5 volume-to-volume mixture of butanol: methanol:H_2O.

The fourth test used the reaction of biliverdin with barbituric acid to form a characteristic chromagen. This reaction indicates the presence of biliverdin and constitutes the basis for the quantification of biliverdin in blood plasma (Gutteridge and Tickner, 1978; Tickner and Gutteridge, 1978).

The fifth test used the reaction of biliverdin with concentrated sulfuric acid to distinguish biliverdin from its isomer mesobiliverdin. Biliverdin when mixed with concentrated sulfuric acid and heated is rapidly destroyed, while mesobiliverdin is not (Noir *et al.*, 1965; Fang, 1982).

Serum biliverdin quantification

Levels of biliverdin in the blood sera were determined using a modification of the methods described by Eng and Youson (1991). The technique is based on the reaction of biliverdin with barbituric acid to form a specific chromagen. The chromagen has a maximum absorption of 570 nm under alkaline conditions. This colorimetric method is superior to direct spectrophotometric measures. It provides a more direct measure of biliverdin concentration in body fluids and tissues by removing interference from pigments other than biliverdin by allowing each sample to be blanked against itself (see below; Tickner and Gutteridge, 1978).

A biliverdin stock solution was made by adding 2.0 ml of 17.5 M glacial acetic acid and 20 μl of a 4 mM ferric chloride solution to 0.60 mg of bilirubin in bovine albumin base (Sigma). This solution was heated at 95°C for 2 hr, allowed to cool, and then brought to a final volume of 20.0 ml with 17.5 M glacial acetic acid to produce a 50 μmol/l standard biliverdin stock solution. Six standards ranging from 0 to 50 μmol/l were made using serial dilutions of the stock solution with 17.5 M glacial acetic acid. Five hundred microlitres of each standard were added to 500 μl of double-distilled water, 400 μl 40 mM ascorbic acid, and 100 μl 200 mM barbituric acid in 1 M sodium hydroxide. Standard blanks were prepared as above, except that the 100 μl of 200 mM barbituric acid in 1 M sodium hydroxide was replaced with 100 μl of 1 M sodium hydroxide.

Serum samples were prepared by diluting 10–50 μl of serum in 17.5 M glacial acetic acid and brought to a final volume of 500 μl. Five hundred microlitres of double-distilled water, 400 μl of 40 mM ascorbic acid, and 100 μl of 200 mM barbituric acid in 1 M sodium hydroxide were then added to the serum samples. Serum blanks were prepared as above except that 100 μl of 1 M sodium hydroxide were added to the blanks instead of 100 μl of 200 mM barbituric acid in 1 M sodium hydroxide.

Standards, standard blanks, serum samples, and serum blanks were heated in the dark for 5 min in a 95°C water bath. After the samples had cooled to room temperature, 2.5 ml of *n*-butanol was added followed by 1 ml of 10 M sodium hydroxide. Samples were vortexed thoroughly and then centrifuged for 5 min at 2000 rpm. Two phases resulted; the top phase was discarded and the bottom phase, containing the chromagen, was used in the analysis. Absorbance values were recorded at 570 nm. The calibration curve was based on five replicates ($r^2 = 0.952$) and all concentration values were interpolated from the calibration curve. The error associated with the regression line of the calibration curve was much less than the variation found within species. Serum concentrations of biliverdin are presented as a mean ± one standard deviation for each species if more than one individual was measured. Concentration values predicted from the calibration curve may be negative. Negative concentration values, however, are not physiologically possible but rather reflect difficulties in determining small concentration values from absorbance measurements using the colorimetric method out-lined by Tickner and Gutteridge (1978).

Results

Pigment identification

All five biochemical tests demonstrated that the green pigment in the blood plasma of *Prasinohaema* is biliverdin. Figure 1 shows the absorption spectra of crude blood plasma from *Prasinohaema flavipes*, *Prasinohaema prehensicauda*, *Prasinohaema virens*, and *Papuascincus stanleyanus*. An absorbance peak at 650 nm is diagnostic for biliverdin and occurs for all three species of *Prasinohaema*. The absorption spectrum for *Papuascincus stanleyanus* lacks a peak at 650 nm and represents a typical absorbance curve for all species examined with normal blood coloration (see Fig. 1d).

The absorbance curves for the green pigment isolated from three species of *Prasinohaema* closely match that of known biliverdin. Figure 2 shows absorption spectra for the isolated pigment from *P. flavipes*, *P. prehensicauda*, and *P. virens* compared with the absorbance curve of known biliverdin.

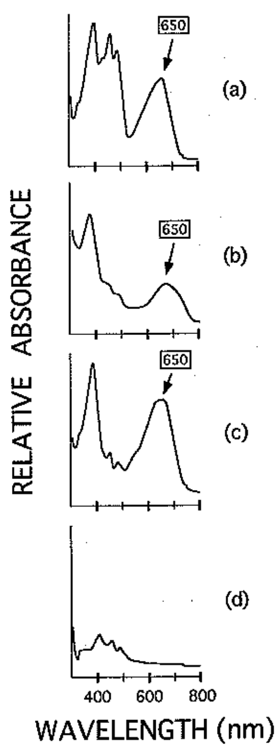

Fig. 1. Absorption spectra of crude blood plasma for *Prasinohaema flavipes* (a), *Prasinohaema prehensicauda* (b), *Prasinohaema virens* (c), and *Papuascincus stanleyanus* (d).

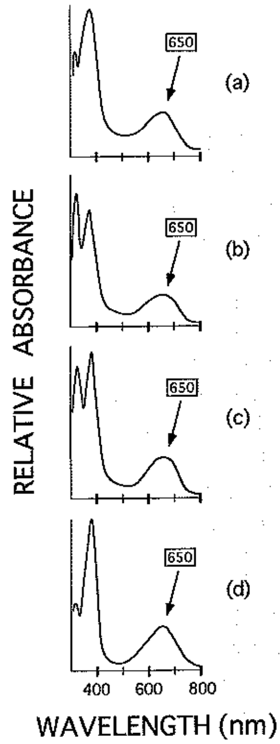

Fig. 2. Absorption spectra showing similarity in the green pigment isolated from *Prasinohaema flavipes* (a), *Prasinohaema prehensicauda* (b), and *Prasinohaema virens* (c) compared with known biliverdin (d).

The TLC analysis demonstrated that the green pigment isolated from all three species of *Prasinohaema* had a mobility similar to known biliverdin (data not shown). Plasma samples from all three species of *Prasinohaema* underwent a color change from green to orange–red when mixed with barbituric acid demonstrating a positive biliverdin reaction. The green coloration rapidly disappeared when plasma samples from all three species of *Prasinohaema* were mixed with concentrated sulfuric acid and heated. This result indicates that the green pigment is biliverdin, not mesobiliverdin.

Biliverdin quantification

Biliverdin levels in the three species of *Prasinohaema* examined are: *P. flavipes*, $714 \pm 179\ \mu$mol/l ($n = 10$) [mean $\pm$ one standard deviation (number of individuals examined)]; *P. prehensicauda*, 1020 ± 624

μmol/l ($n = 3$); *P. virens*, 819 ± 89 μmol/l ($n = 2$). Biliverdin levels for species with normal blood and plasma coloration examined in this study are: *P. stanleyanus*, 80 ± 149 μmol/l ($n = 2$); *L. elegans*, 59 μmol/l ($n = 1$); *S. leptofaciatus*, 101 μmol/l ($n = 1$); *L. smaragdina*, −5 ± 61 μmol/l ($n = 2$); *V. indicus*, 28 ± 82 μmol/l ($n = 3$); *G. nigrensis*, 17 ± 59 μmol/l ($n = 2$); *A. carolinensis*, 123 μmol/l ($n = 1$). The levels for red-blooded species all spanned the zero concentration level and represent levels of biliverdin indistinguishable from zero in these taxa (see Fig. 3).

Discussion

Brown *et al.* (1984) proposed a method for the biosynthesis of biliverdin; it involves the cleaving of heme by heme oxygenase to yield biliverdin. Biliverdin is the final product of hemoglobin catabolism in lizards (Bissell, 1986). Heme catabolism, as opposed to a dietary source rich in biliverdin or a biliverdin precursor, is the probable source of biliverdin in *Prasinohaema* since animals of both sexes of *P. prehensicauda* and *P. flavipes* have been maintained in the laboratory for over one year with no discernible change in plasma coloration.

Accumulation of biliverdin and/or bilirubin in the tissues and circulatory system produces the pathological condition known as jaundice in most vertebrates (Greenberg *et al.*, 1971; With, 1968; Gray, 1958). The highest concentration of biliverdin reported for humans is 46 μmol/l (Greenberg *et al.*, 1971). Accumulation of biliverdin to produce green jaundice can result from various pathological conditions such as carcinomatous obstruction of the common bile duct, cirrhosis, bile duct stenosis and other pathological conditions associated with the liver (Larson *et al.*, 1947; Eng and Youson, 1991). The exact mechanism of bile pigment toxicity is unclear. Bilirubin apparently has greater toxic effects on tissue cell cultures than biliverdin (Cowger, 1974). Results from toxicity experiments by Cowger (1974)

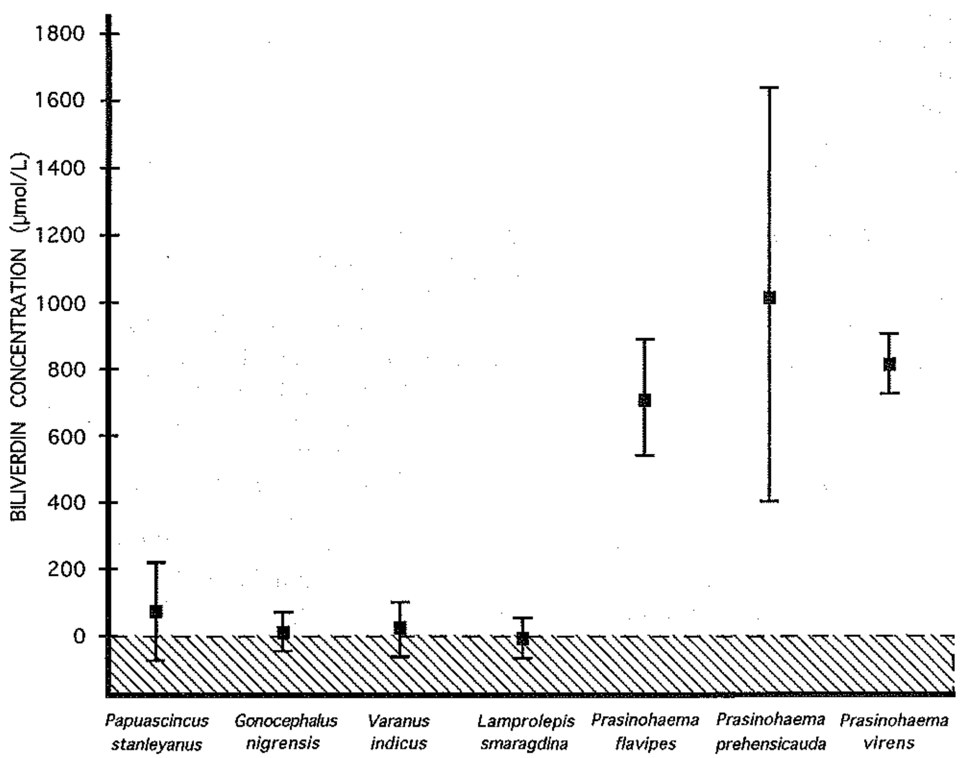

Fig. 3. Biliverdin concentration in μmol/l (mean ± one standard deviation) for seven species of lizards. Hatched area represents physiologically unrealistic portions of the graph space which can result from determining small concentrations using the colorimetric method outlined by Tickner and Gutteridge (1978).

Table 1. Serum levels of biliverdin in lizards, fish, and humans

Taxa	Serum biliverdin level ± SD	Reference
Lizards		
Scincidae		
Prasinohaema flavipes	714 ± 179 μmol/l	This study
Prasinohaema prehensicauda	1020 ± 624 μmol/l	This study
Prasinohaema virens	819 ± 89 μmol/l	This study
Fish		
Petromyzontidae		
Lampetra lamottenii	231 ± 27 μmol/l	Eng and Youson, 1991
Petromyzon marinus	2 ± 3 μmol/l	Makos and Youson, 1987
Anguillidae		
Angullia rostrata	314 ± 79 μmol/l*	Ellis and Poluhowich, 1981
	660 ± 272 μmol/l†	Ellis and Poluhowich, 1981
Cottidae		
Clinocottus analis	33 ± 3 μmol/l	Fang and Bada, 1983
Clinocottus analis	92 μmol/l‡	Low and Bada, 1974
Leiocottus hirundo	92 μmol/l‡	Low and Bada, 1974
Scorpaenichthys marmoratus	92 μmol/l‡	Low and Bada, 1974
Primates		
Homo sapiens	46 μmol/l§	Greenberg *et al.*, 1971

*For eels acclimated to freshwater.
†For eels acclimated to brackish water.
‡Reported as "concentration of biliverdin in the serum was at least" 92 μmol/l for the three species. Discrepancies in values reported for *Clinocottus analis* by Low and Bada (1974) and Fang and Bada (1983) are probably the result of different and less accurate techniques used for biliverdin quantification by Low and Bada (1974).
§Highest biliverdin concentration reported for patient with biliverdinemia; non-pathological concentrations typically undetectable.

who worked with rat-liver mitochondria and biliverdin concentrations ranging from 20 to 400 μmol/l found negative effects on cell respiratory control and O_2 uptake at concentrations near 400 μmol/l.

Biliverdin accumulation in the blood plasma occurs as a non-pathological condition in several fish families, at least two insect species, and lizards in the genus *Prasinohaema* (see Table 1). The levels of biliverdin in the hemolymph of Lepidoptera have not been quantified. Biliverdin has been measured in fish at concentrations ranging from 2.4 to 660 μmol/l. Our results demonstrate that levels of biliverdin in *Prasinohaema* are the highest recorded to date in any organism (see Table 1).

This result is important because it is the only documentation of this sort of deviant physiology in an amniote. Second, it is now apparent that biliverdin accumulation occurs in several distantly related taxonomic groups.

The physiological and/or ecological importance of biliverdin accumulation is unknown. The three families of fishes with high concentrations of biliverdin do not appear to have any ecological similarities (Fang and Bada, 1990). Although all five described species of *Prasinohaema* occur on the island of New Guinea, they occupy a wide range of habitats. *P. virens* occurs in low elevation tropical forests while *P. flavipes* and *P. prehensicauda* occur in tropical montane habitats; *P. prehensicauda* has been collected as high as 8000 feet on Mt. Wilhelm in PNG (Loveridge, 1948). The evolutionary relationships of species now assigned to the genus *Prasinohaema*, and other related species, are not well understood, and indeed the monophyly of the genus is questionable (Greer and Raizes, 1969; Greer, 1974, 1986). One of us (CCA) is currently investigating the systematics of this group. Cryptic coloration (Low and Bada, 1974), lipid transport (Yamaguchi and Hashimoto, 1968), protection against UV light (Yamaguchi *et al.*, 1976), thermoregulatory advantages (Schwalm *et al.*, 1977; Emerson *et al.*, 1990), and distastefulness have all been suggested as hypotheses for the biological significance of biliverdin accumulation. Preliminary data from feeding experiments conducted in PNG using native bird and snake species, as well as personally (CCA) tasting specimens, suggest that these lizards are edible. None of the other possibilities, however, has been seriously examined.

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