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Characterization of Blue and Yellow Pigments in Eggs of the Mexican Leaf Frog†

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ABSTRACT: Blue and yellow pigments were isolated from eggs of the Mexican leaf frog, *Agalychnis dacnicolor*. The major yellow pigment has chemical and spectral properties similar to the xanthophyll lutein. It is readily extracted from yolk platelets with acetone or methanol and has major absorption peaks in hexane at 441 and 470 nm. The dimethyl ester of the blue pigment has absorption maxima in methanol at 374 and 640–680 nm. The blue pigment is protein bound and requires acetone–HCl for extraction. The dimethyl ester of the major blue pigment has chemical, chromatographic, ultraviolet–visible, infrared, and proton magnetic resonance spectra identical with those of the dimethyl ester of biliverdin IXα. Although biliverdin IXα is the major component of the blue pigment, two other minor blue pigments occur which are believed to be biliverdin isomers. Analysis of eggs varying in color from brilliant blue to pale yellow-green show that the color is dependent on the ratio of the two pigments present. These pigments may provide protective coloration to the eggs which are laid on leaves of trees.

During our study on the lipid composition of the eggs and embryos of the Mexican leaf frog, *Agalychnis dacnicolor* (Marinetti et al., 1981), we observed that their green coloration was due to the presence of two pigments, one blue and one yellow. In the present study we have identified the major blue pigment as biliverdin IXα and the major yellow pigment as lutein. The presence of the latter pigment is not surprising since lutein is widely distributed among plants and animals (Fox, 1979). On the other hand, biliverdin is more restrictive as a pigment, although it has been found in the dog placenta, in the shells of bird eggs, bones and skin of some fishes and amphibians (Rudiger, 1970), the eggs and larvae of the tobacco hornworm (Cherbas, 1973), and the serum and eggs of *Xenopus* (Redshaw et al., 1971). It seems likely that the utilization of these two diverse pigments by *A. dacnicolor* evolved as a mechanism for producing green eggs. The green coloration of eggs of leaf frogs which are laid on green vegetation may afford camouflage in protecting eggs and embryos from predation. The biochemical, physiological, ecological, and evolutionary significance of these pigments seem far reaching and have provided the impetus for our elucidating their chemical structures.

Materials and Methods

Eggs of *A. dacnicolor* (900 eggs) were obtained from greenhouse reared frogs. The eggs varied in color from yellow-green to brilliant blue. Silica gel 60 TLC glass plates 20 × 20 cm (0.25 mm) were obtained from VWR. Chloroform, ethyl acetate, hexane, methanol, acetone, and glacial acetic acid (ACS grade) were obtained from Fisher Scientific. Chloroform, methanol, and acetone were redistilled before use. Unisil silicic acid (100–200 mesh) was purchased from Clarkson Chemical Co.

^{1}H NMR spectra and infrared spectra were run in $CDCl_3$ at ambient temperature on a Brüker WHFT 400 mHz spectrometer and Perkin-Elmer Model 220 spectrometer, respectively. Electron microscopy was done on a Zeiss 10-Å electron microscope. Ultraviolet–visible spectra were performed on a Cary Z19 spectrophotometer. Lutein and zeaxanthin were supplied by Hoffmann-La Roche, Switzerland. α- and β-carotene and biliverdin were obtained from Sigma Chemical Co. Commercial biliverdin is primarily biliverdin IXα but does contain other biliverdin isomers (Lehner et al., 1978; McDonagh & Palma, 1980).

The blue pigment and Sigma biliverdin were converted to their dimethyl esters by heating in methanol–3 N HCl (Supelco) for 1 h at 80 °C in a sealed tube under nitrogen. The dimethyl esters were purified by column chromatography on Unisil silicic acid and then by TLC in chloroform–acetone, 95:5.

Results

Isolation and Microscopic Analysis of the Yolk Platelets. Fifteen eggs were homogenized in 3 mL of Krebs–Ringer's bicarbonate (KRB) buffer, pH 7.4, by using 50 strokes of a Dounce homogenizer. The sample was filtered through two layers of 86 mesh nylon (Southern Sign Supply Co., Glen Burne, MD). The filtrate was treated with 1 mg of collagenase (ICN Pharmaceuticals, 183 U/mg) for 18 h at 21 °C. The sample was centrifuged at 2400*g* for 5 min, and the pellet (yolk platelets) was washed twice with 3 mL of KRB buffer. The

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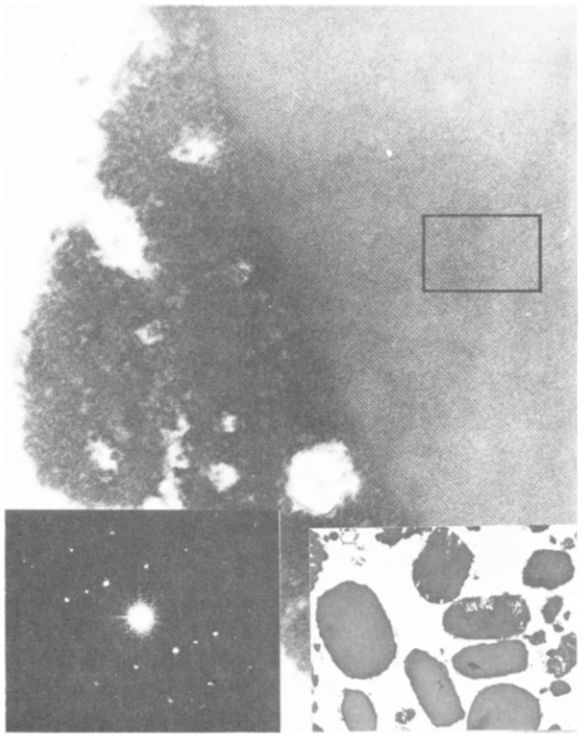

FIGURE 1: Electron microscopy and optical diffraction pattern of egg yolk platelets. Yolk platelets were examined by electron microscopy. The upper photograph represents a portion of one platelet at a magnification of 51840×. Several platelets are shown in the lower right inset at a 2160× magnification. The body of the platelet contains a crystalline array as shown by the optical diffractogram (lower left inset) taken from the region defined by the rectangle. Amorphous material occurs on the surface of the platelets. Details of fixing and staining the platelets are given in the text.

yolk platelets were fixed in 3% glutaraldehyde and stained with toluidine blue and azure II for light microscopy and were fixed with 3% glutaraldehyde and stained with osmium tetroxide, uranyl nitrate, and lead citrate for electron microscopy. The yolk platelets readily took up osmium tetroxide and fat soluble dyes (oil red O, toluidine blue, and azure II). The unstained and untreated yolk platelets had a pale blue-green color when isolated from green eggs.

Electron microscopic analysis (Figure 1) indicated that the platelets were rounded rectangular plates (lower right inset) which on higher magnification revealed closely stacked lamellar arrays approximately 70 Å thick not unlike those observed in other amphibians (Wallace, 1978; Karasaki, 1963; Hope et al., 1963; Honjin & Nakamura, 1967; Leonard et al., 1972). These lamellar arrays represent a crystalline structure as indicated by the optical diffractogram shown in the lower left inset. Crystallinity of the platelets is well maintained. Measurements of the optical diffractogram indicate Bragg spacings up to 29 Å. Preliminary observations of reciprocal lattice are in agreement with orthorhombic crystals observed with other yolk platelets (Lange, 1982). A three-dimensional structure for the lipovitellin–phosvitin complex has been proposed by Ohlendorf et al. (1978) and Leonard et al. (1972).

Chromatographic Purification of the Blue and Yellow Pigments. The pigments were dissolved in 1 mL of chloroform and eluted through a 10-g Whatman cellulose column with chloroform and then put on a 10-g silicic acid column (Clarkson Unisil, 100–200 mesh). The blue pigment and yellow pigment were eluted from cellulose with chloroform. When this eluate was run through a silicic acid column, the yellow pigment was eluted with chloroform, and the blue pigment was eluted with acetone. Both pigments were further purified by preparative TLC using chloroform–acetone, 95:5. The yellow pigment and authentic lutein had identical mobilities on silica gel coated glass plates when run in ethyl acetate–hexane, 1:1 (R_f 0.38), and in chloroform–acetone, 95:5 (R_f 0.21). The yellow pigment and lutein gave a green color reaction when the TLC plates were exposed to iodine vapors. Zeaxanthin (R_f 0.36) and α- and β-carotenes were separated from lutein and the yellow pigment in ethyl acetate–hexane, 1:1. In chloroform–acetone, 95:5, zeaxanthin and β-carotene had R_f values of 0.19 and 0.95, respectively, whereas lutein and the yellow pigment had an R_f value of 0.21.

Table I: Correlation of Egg Color with Relative Absorption at 441 and 645 nm

egg sample	egg color	absorbance ratio 441/645 nm
H-H	brilliant blue	1.15
G-G	blue-green	1.72
G-I	blue	1.85
R	blue	2.26
G-2	green	3.43
F-2	green	3.57
C	yellow-green	10.4

Approximately 100 eggs each of seven different batches of eggs varying in color from yellow-green to brilliant blue were extracted, and the blue and yellow pigments were separated by column chromatography on silicic acid as given above. The absorbance ratio of the yellow and blue pigments were determined at 441 and 645 nm, respectively. As seen in Table I, the color of the egg is related to the relative amounts of blue and yellow pigments since the yellow-green eggs had the highest ratio and the brilliant blue eggs had the lowest ratio.

Purification and Spectral Properties of the Dimethyl Esters of the Blue Pigment and Sigma Biliverdin. Treatment of the blue pigment and Sigma biliverdin with methanol–3 N HCl for 1 h at 80 °C converted these to their dimethyl esters. The major fraction in each had an R_f value of 0.62 in chloroform–methanol, 9:1, and 0.90 in chloroform–methanol–water, 65:25:4, on Merck–Darmstadt silica gel 60 plates. Two other biliverdin isomers occurred in each. In the case of Sigma biliverdin, these are probably the IIIα and XIIIα isomers (McDonagh & Palma, 1980; Lehner et al., 1978) although some IXβ, IXγ, and XIδ isomers cannot be excluded. All isomers give a purple color reaction when exposed to I_2 vapors. The major dimethyl esters (R_f 0.62) were purified by preparative thin-layer chromatography on silica gel 60 plates using chloroform–methanol, 9:1, as solvent. The UV–visible spectra of the dimethyl ester of the purified blue pigment and the dimethyl ester of biliverdin are given in Figure 2. The blue pigment and biliverdin have major bands at 374 and 640–680 nm. The ^{1}H NMR spectra (in $CDCl_3$) of the dimethyl esters of the TLC purified blue pigment and purified biliverdin IXα were identical (Figures 3 and 4). The chemical shifts (ppm relative to Me_4Si at 0) and coupling constants (J) in hertz are given in Figure 5. The ^{1}H NMR spectra are in agreement with the ^{1}H NMR spectra of biliverdin IXα reported previously (Lehner et al., 1978; Bonnett & McDonagh, 1970; Stoll & Gray, 1980; Rasmussen et al., 1980) and distinguish the IXα isomer from the IIIα, XIIIα, IXβ, IXγ, and IXδ isomers. The infrared spectra of the purified dimethyl ester of Sigma biliverdin IXα and our blue pigment were identical and gave the expected absorption bands for this compound (McDonagh & Palma, 1980). On the basis of the chemical, spectral, and chromatographic properties of the TLC purified blue pigment it is identified as biliverdin IXα. Small amounts of two other isomers of biliverdin were also present in the yolk platelets.

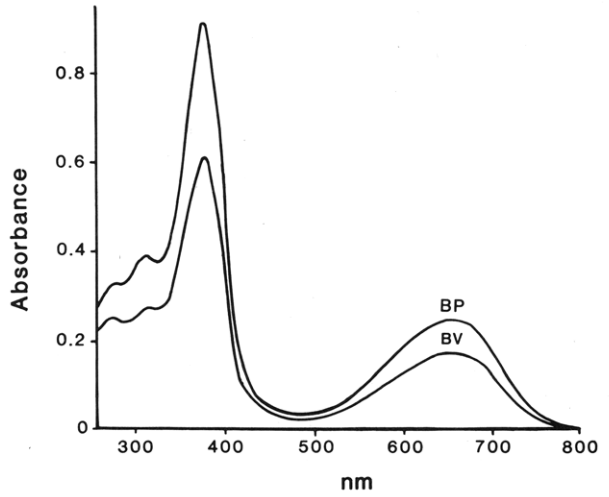

FIGURE 2: Ultraviolet–visible spectra of the dimethyl esters of the frog egg blue pigment and biliverdin IXα. The spectral curves of the dimethyl ester of the TLC-purified blue pigment and the dimethyl ester of biliverdin were run in $CHCl_3$ on a Cary 219 spectrophotometer. BP, frog blue pigment; BV, Sigma biliverdin IXα.

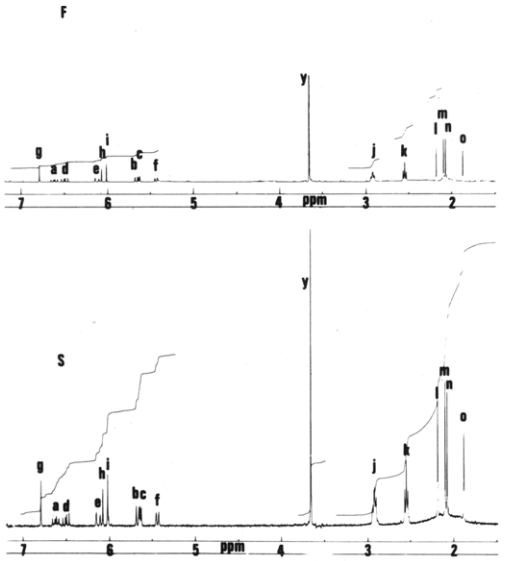

FIGURE 3: ^1H NMR spectra of biliverdin IXα and the frog egg blue pigment. The spectra were run in $CDCl_3$. S, Sigma purified biliverdin IXα; F, frog blue pigment.

These were detected by TLC and may be the natural β, γ, and/or δ isomers of biliverdin IXα or possibly the IIIα and XIIα isomers produced by acid-catalyzed isomerization.

We have isolated biliverdin from yolk platelets of *Xenopus laevis* and the salamander *Pleurodeles* and find both have chromatographic properties identical with those of biliverdin from *A. danicolor*.

Characterization of the Yellow Pigment. The yellow pigment was compared to authentic lutein obtained from Hoffman-La Roche (Switzerland). Both had identical R_f values when chromatographed on silica gel 60 plates in ethyl acetate–hexane, 1:1 (R_f 0.38), and in chloroform–acetone, 95:5 (R_f 0.21). Both compounds gave a green color reaction when exposed to iodine vapors and were separated from zeaxanthin (Hoffman-La Roche) and α- and β-carotene (Sigma Chemical Co.) by thin-layer chromatography in ethyl acetate–hexane, 1:1. The yellow pigment had bands in hexane at 441 and 470 nm which are identical with those of lutein (Figure 6). Zeaxanthin had bands at 444 and 473 nm, and β-carotene had bands at 447 and 475 nm. The partition test between hexane–95% methanol, 2:1 v/v, showed that most of the yellow

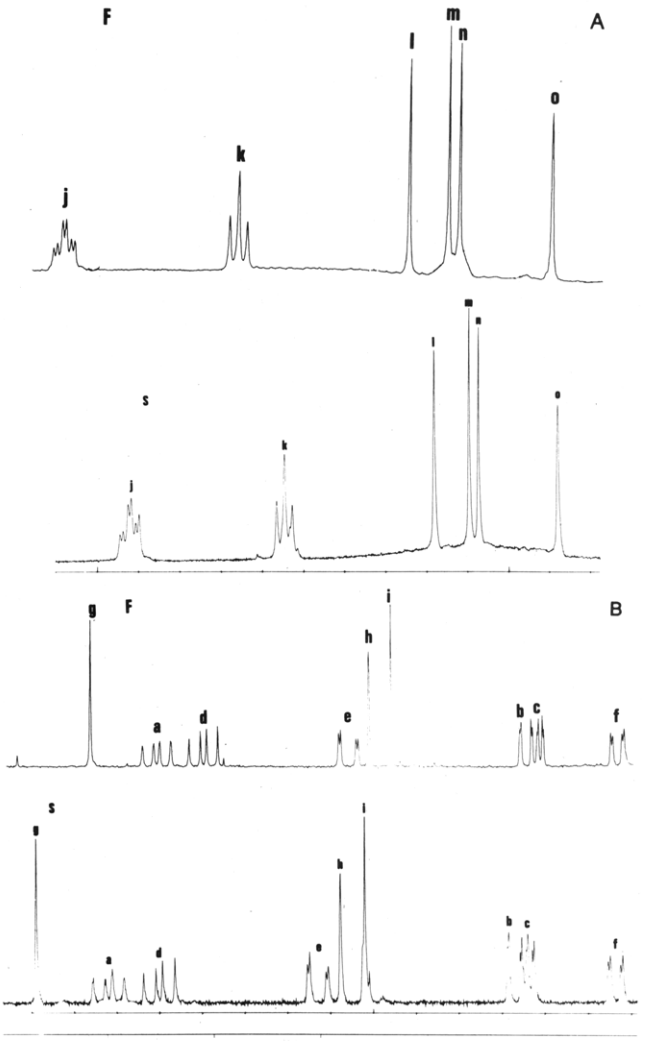

FIGURE 4: (A, B) Expanded ^1H NMR spectra of biliverdin IXα and the frog egg blue pigment. There are expanded spectra of those shown in Figure 3. The spectra were recorded at different speeds so that spectra for S were photographically reduced to attempt to match the scale of spectra F.

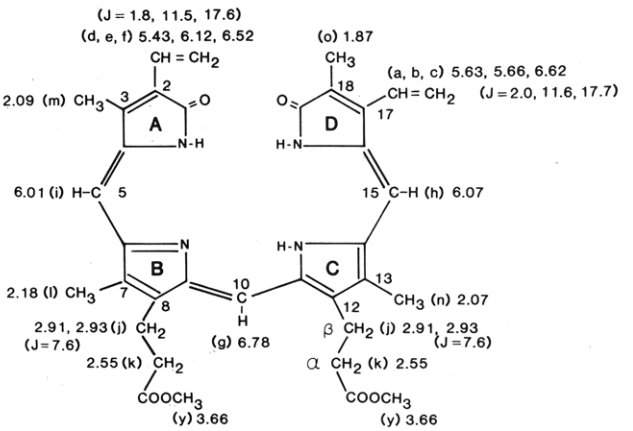

FIGURE 5: Assignment of the ^1H NMR bands to the protons of the frog egg blue pigment and of biliverdin IXα. The structure and numbering system of biliverdin IXα are taken after Lehner et al. (1978). The ^1H NMR band assignments are shown for the protons and are relative to Me_4Si set at 0 ppm.

pigment and lutein remain in the hypophasic methanol layer but a small amount of each is epiphasic. On the basis of chemical, spectral, and chromatographic analyses the major yellow pigment is identified provisionally as lutein. Two other minor yellow pigments were also detected by TLC. We were

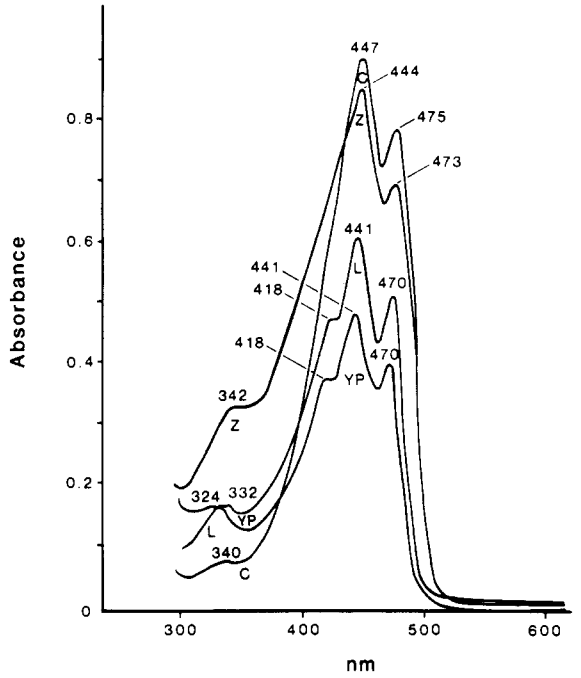

FIGURE 6: Ultraviolet–visible spectra of the frog egg yellow pigment, lutein, zeaxanthin, and β-carotene. The spectral curves of the yellow pigment lutein, zeaxanthin, and β-carotene were done in hexane. YP, frog yellow pigment; L, lutein; Z, zeaxanthin; C, β-carotene.

not able to isolate sufficient yellow pigment from the frog eggs for NMR analysis.

Spectral Properties of the Triton-Solubilized Pigment–Protein Complex. The yolk platelets were dissolved in 1% Tritox X-100 and passed through a Sephadex G-200 column. The UV–visible spectrum of the eluate which was blue-green in color is given in Figure 7. Major bands were observed at 375, 454, 642, and 662 nm. The bands at 375 and 642–662 nm are due to the blue pigment, and the band at 454 nm is due to the yellow pigment. When compared to the yellow pigment in hexane, the yellow band in the Triton-solubilized protein complex is shifted from 441 to 454 nm. The bands for biliverdin in the solubilized protein complex are at 375 and 642–662 nm as compared to 374 and 640–660 nm for the isolated biliverdin in $CHCl_3$. We have isolated the lipovitellin and phosvitin fractions from green eggs by the method of Wallace et al. (1966). Only lipovitellin contained the blue and yellow pigments. Incubating lipovitellin isolated from yellow eggs with added biliverdin IXα showed that this lipoprotein was able to bind the biliverdin.

Discussion

Green coloration is common among amphibians, reptiles, and birds, where it is most often accomplished by blending of blue and yellow colors (Fox, 1979; Bagnara & Hadley, 1973). Blues are usually structural colors arising from light scatter, diffraction, and interference while yellows stem from specific yellow pigments such as pteridines and carotenoids. The eggs from the leaf frog used in this study also are green due to a mixture of blue and yellow elements. However, unlike the situation in frog skin, where the blue color is due to light scatter (Bagnara & Hadley, 1973), the blue component of the egg is the true pigment, biliverdin. The presence of biliverdin in *Xenopus* egg has been reported previously (Redshaw et al., 1971), but the specific type of biliverdin was not characterized. Our study using ^1H NMR spectroscopy demonstrates unequivocally the presence of biliverdin IXα in eggs of *A. dacnicolor*. As with *Xenopus*, biliverdin in the eggs of *A. dacnicolor* is protein bound. In the former it is associated with

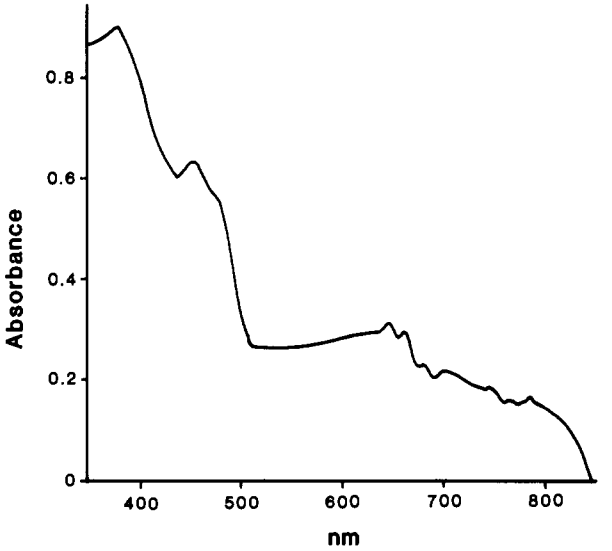

FIGURE 7: Ultraviolet–visible spectrum of the blue and yellow pigments in the Triton-solubilized frog egg yolk platelets. The spectrum was obtained on yolk platelets solubilized in 1% Triton X-100 and passed through Sephadex G-200. The blue-green eluate was analyzed.

the yolk proteins lipovitellin and phosphovitin. By isolation of lipovitellin and phosvitin from platelets of *A. danicolor*, we find that biliverdin is bound to lipovitellin. Furthermore, lipovitellin isolated from yellow eggs is able to bind added biliverdin IXα. By electron microscopy and by optical diffraction analysis we find that the pigment–protein complex occurs in an orthorhombic crystalline array in the yolk platelet.

Biliverdin is important as a pigment in both lepidopterans (Cherbas, 1973) and the Mexican leaf frog. In both cases, a specific biliverdin isomer provides the blue component of the green color, while yellow is provided by lutein which is the major carotenoid present in lepidopterans (Fox, 1979; Bagnara & Hadley, 1973). The utilization of lutein by both species may be based upon the fact that lutein is a principal carotenoid component of lepidoptera (Fox, 1979; Feltwell & Rothchild, 1974) which are a major dietary item of leaf frogs. *A. danicolor* deprived of this lutein source by being fed on crickets lay eggs that are blue. The development of these blue eggs is normal. Some apparent mutants of *A. danicolor* lay eggs which are yellow and lack biliverdin. These frogs may have an enzyme defect in the conversion of hemin to biliverdin.

Acknowledgments

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Registry No. Lutein, 127-40-2; biliverdin IXα, 114-25-0.

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Warfarin Inhibition of Vitamin K 2,3-Epoxide Reductase in Rat Liver Microsomes†

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ABSTRACT: Warfarin is a potent inhibitor of vitamin K 2,3-epoxide reduction to vitamin K in vitro and in vivo. Dithiothreitol, an in vitro reductant for the vitamin K 2,3-epoxide reductase, antagonizes inhibition of the reductase by warfarin via mechanisms that have not been determined [Zimmermann, A., & Matschiner, J. T. (1974) *Biochem. Pharmacol. 23*, 1033–1040]. Experiments with rat hepatic microsomes were undertaken to characterize the interactions that exist between vitamin K 2,3-epoxide, warfarin, and dithiothreitol. Increasing concentrations of dithiothreitol decreased inhibition of the reductase by warfarin. When dithiothreitol was present prior to exposure of the reductase to warfarin, there was less inhibition than when the same concentration of dithiothreitol was present after its exposure to warfarin. Moreover, maximum inhibition of the reductase by warfarin occurred at a much slower rate when dithiothreitol was present initially. Inhibition of the reductase by warfarin was greater when the substrate concentration was 100 μM vitamin K 2,3-epoxide than when it was 10 μM epoxide. On the basis of these data, we conclude that (i) dithiothreitol reduces either directly or indirectly a critical disulfide within the reductase that is reoxidized during reduction of the epoxide substrate, (ii) warfarin and vitamin K 2,3-epoxide are not competitive with respect to one another, and (iii) warfarin binding, which produces inhibition, occurs solely to the disulfide form of the reductase. Once it is bound, warfarin inhibits further reduction of the critical disulfide by dithiothreitol. Dithiothreitol therefore antagonizes warfarin by maintaining the reductase in the reduced state.

The metabolism of vitamin K to vitamin K hydroquinone and vitamin K 2,3-epoxide has been linked to γ-carboxyglutamic acid formation in the coagulation factors II, VII, IX, and X as well as in proteins of other tissues such as lung, placenta, and kidney (Suttie, 1980). Warfarin and other 4-hydroxycoumarin drugs inhibit γ-carboxyglutamate formation and are used extensively for the prevention of a variety of coagulation-related disorders in humans and in the control of wild rodent populations. Most data suggest that a primary function of the 4-hydroxycoumarin anticoagulants is inhibition of the enzyme vitamin K 2,3-epoxide reductase (Matschiner et al., 1970; Sadowski & Suttie, 1974; Bell, 1978), which, in a sulfhydryl-dependent reaction, reduces the epoxide to vitamin K (Zimmermann & Matschiner, 1974). The vitamin K is then reduced to vitamin K hydroquinone, which functions as cofactor for the γ-carboxylation reaction (Friedman & Shia, 1976; Whitlon et al., 1978; Wallin et al., 1978; Fasco & Principe, 1980).

The epoxide reductase is a membrane protein of the endoplasmic reticulum (Zimmerman & Matschiner, 1974). Although it can be solubilized by a variety of detergents (Siegfried, 1978), purification of the enzyme has not been achieved, and thus, little is known about the molecular mechanisms of either epoxide reduction or inhibition of the enzyme by warfarin and the other 4-hydroxycoumarin anticoagulants. Microsomal reduction of vitamin K 2,3-epoxide requires an exogenous sulfhydryl compound such as dithiothreitol; the reductant for the physiological reaction has not been identified. In addition to its role as reductant, dithiothreitol diminishes the extent of reductase inhibition by warfarin (Zimmermann & Matschiner, 1974). Warfarin binding to the reductase in vitro is extremely strong, however, and is not readily reversed

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