Research Note

Comparison of the total amount of eggshell pigments in Dongxiang brown-shelled eggs and Dongxiang blue-shelled eggs

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ABSTRACT Based on the knowledge of the heme biosynthetic and metabolic pathway and the structures of biliverdin and protoporphyrin, experiments were carried out to compare the difference between the total quality of eggshell pigments in blue-shelled eggs and brown-shelled eggs from the same population (Dongxiang, China) and to analyze the correlation between the quantity of protoporphyrin and biliverdin in the 2 kinds of eggshells. It was found that there was no significant difference between the total quantity of eggshell pigments in Dongxiang blue-shelled eggs and Dongxiang brown-shelled eggs ($P = 0.9006$), and a highly significant positive correlation between the quantity of protoporphyrin and biliverdin in blue eggshells ($P < 0.01$) and a significant positive correlation between the quantity of protoporphyrin and biliverdin in brown eggshells ($P < 0.05$). These results suggested that eggshell protoporphyrin and eggshell biliverdin probably derived from common precursor material.

Key words: chicken, total amount of eggshell pigment, heme, protoporphyrin, biliverdin

2009 Poultry Science 88:1735–1739
doi:10.3382/ps.2008-00434

INTRODUCTION

Bird eggshell color is a kind of intuitionistic and remarkable character, and the mechanism of its formation is very interesting. It has been reported that protoporphyrin gives the brown eggshell its brown color and biliverdin gives the blue eggshell its blue color (Poole, 1965; Kennedy and Vevers, 1973; Ito et al., 1993). The chemical structure of biliverdin in eggshell is the same with that in bile (Lang and Wells, 1987), and in the metabolism process of biliverdin in bile, heme oxygenase-1 (HO-1) is the rate-limiting enzyme in the catabolism of heme to biliverdin, Fe^{2+}, and CO (Maines, 1988). It is assumed in this paper that the biliverdin in eggshell also comes from catabolism of heme. As we know, the final step of the heme biosynthetic pathway is the incorporation of Fe^{2+} into protoporphyrin IX (Hansson et al., 2007) after getting rid of H^+. If the process is reversible, heme will change back into protoporphyrin IX by substituting Fe^{2+} by H^+.

Based on the above knowledge, we put forward a hypothesis that heme was the common precursor material for eggshell protoporphyrin and biliverdin, and there was no significant difference between the quantity of the common precursor material in Dongxiang brown-shelled chickens and Dongxiang blue-shelled chickens. In the formation process of eggshell pigments, heme was transformed into not only eggshell protoporphyrin but also biliverdin, but a greater proportion of heme was converted into eggshell biliverdins in blue-shelled chickens than in brown-shelled chickens, which led to blue eggshell pigmentation. Experiments were carried out to test the postulation.

MATERIALS AND METHODS

Chickens and Eggs

In this study, the hens laying brown-shelled eggs were called Dongxiang brown-shelled chickens, and the hens laying blue-shelled eggs were called Dongxiang blue-shelled chickens. All of the hens were from the same Chinese local population of Dongxiang chicken kept in Jiangxi Donghua Livestock & Poultry Breeding Co. Ltd. (Jiangxi Province, China). Fresh eggs from 45 Dongxiang brown-shelled chickens and 50 Dongxiang blue-shelled chickens were collected for pigment extraction.

Received October 8, 2008.
Accepted April 7, 2009.
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Sample Preparation and Spectroscopic Analysis

Each eggshell was heat-dried and rinsed, 0.25 g was dissolved in 4 mL of solvent [volume of methanol:volume of concentrated HCl ($\mathbf{V_{methanol}}$:$\mathbf{V_{concentrated\ HCl}}$) = 2:1] added beforehand into a 10-mL capped plastic cuvette (Wang et al., 2007). Then, the solution for analysis was placed in darkness for 12 h for complete extraction of shell pigments. After centrifugation at 1,369.55 × *g* for 45 min, the absorbance of the supernatant solution was measured with a spectrophotometer at wavelengths of 412 and 670 nm. Absorbance at 412 nm represents the content of protoporphyrin (Ito et al., 1993), and absorbance at 670 nm represents the content of biliverdin (Zhao et al., 2006).

Developing Standard Curve to Define Sample Concentration

To make the standard solution, 0.36 mg of protoporphyrin IX (Sigma, St. Louis, MO) was dissolved into 6 mL of solvent ($V_{methanol}$:$V_{concentrated\ HCl}$ = 2:1) added beforehand into a 10-mL capped plastic cuvette, and 0.26 mg of biliverdin hydrochloride (Frontier Scientific) was processed in the same way, the 2 mixtures were vortex-shaked and placed in darkness until dissolved completely, and then 1 × protoporphyrin standard solution (1.06634×10^{-4}moL/L) and 1 × biliverdin standard solution (6.99918×10^{-5} moL/L) were obtained. Three mL of 1 × protoporphyrin standard solution was poured into one of the 10-mL capped plastic cuvette containing 3 mL of solvent ($V_{methanol}$:$V_{concentrated\ HCl}$ = 2:1) and vortex-shaken, so 2^{-1} × protoporphyrin standard solution was obtained. In an analogy of this, 2^{-2}~2^{-9} × protoporphyrin standard solutions were prepared. The 2^{-1}~2^{-9} × biliverdin standard solutions were prepared with the same method as above. The absorbance of the protoporphyrin standard solutions ($\mathbf{X_1}$) was analyzed in a spectrophotometer at a wavelength of 412 nm, and that of biliverdin standard solutions ($\mathbf{X_2}$) was analyzed at a wavelength of 670 nm. Using the standard curve of protoporphyrin and biliverdin, the protoporphyrin concentration of the sample ($\mathbf{Y_1}$) was calculated by substituting X_1 in the linear regression equation for protoporphyrin; the biliverdin concentration of the sample ($\mathbf{Y_2}$) was calculated by substituting X_2 in the linear regression equation for biliverdin.

Total Amount of Eggshell Pigments

The quantity of protoporphyrin and biliverdin in 1 g of eggshell ($\mathbf{Q_p}$ and $\mathbf{Q_b}$, respectively; mol/g) was determined as follows:

$$Q_p = X_1 \times 0.004\ L/0.25\ g$$

$$Q_b = X_2 \times 0.004\ L/0.25\ g,$$

where 0.004 L = the 4 mL of solvent used to dissolve the rinsed eggshell and 0.25 g = the 0.25 g of rinsed eggshell.

The total quantity of pigments in 1 g of eggshell ($\mathbf{Q_T}$; mol/g) is $Q_T = Q_p + Q_b$.

Statistical Analysis

Correlation between the content of protoporphyrin and biliverdin in eggshell was analyzed with the CORR procedure. The absorbances of the protoporphyrin standard solutions or those of the biliverdin standard solutions were subjected to the REG CORR procedure for linear regression analysis. Statistical difference of Q_p, Q_b, or Q_T between the Dongxiang blue-shelled chickens and the Dongxiang brown-shelled chickens was compared, respectively, using unpaired Student's *t*-test procedure. All of the statistical procedures were run in the SAS 8.2 software package (SAS Institute Inc., Cary, NC).

RESULTS

The correlation coefficient between the biliverdin and protoporphyrin content in the eggshells of the Dongxiang blue-shelled chickens is 0.74 ($P < 0.01$), whereas the correlation coefficient between the content of the 2 pigments in the eggshells of the Dongxiang brown-shelled chickens is 0.32 ($P < 0.05$).

Calibration curves of protoporphyrin and biliverdin standard solutions were fitted (equation [1] and equation [2]) using the REG CORR procedure in SAS 8.2 as follows:

$$Y_1 = -2.57231 \times 10^{-7} + 6.14 \times 10^{-6}X_1 \qquad [1]$$

$$Y_2 = 9.48408 \times 10^{-8} + 4.641 \times 10^{-5}X_2, \qquad [2]$$

where Y_1 = the protoporphyrin concentration of the sample; X_1 = the absorbance of the sample at a 412-nm wavelength; Y_2 = the biliverdin concentration of the sample; and X_2 = the absorbance of the sample at a 670-nm wavelength. The regression coefficient for both concentrations was extremely significant ($P < 0.0001$).

The Q_T in individual samples are presented in Figure 1 and the statistical difference of Q_p, Q_b, and Q_T between Dongxiang blue-shelled chickens and Dongxiang brown-shelled chickens is listed in Table 1. As shown in Table 1, no significant difference of Q_T was found between Dongxiang blue-shelled chickens and Dongxiang brown-shelled chickens ($P = 0.9006$), which indicates that the total quantity of pigments, including biliverdin and protoporphyrin, in the eggshells of Dongxiang blue-shelled chickens is in the same level with the Dongxiang brown-shelled chickens, although they have distinguishable eggshell color. Although difference of Q_p was extremely significant between Dongxiang blue-shelled chickens and Dongxiang brown-shelled chickens ($P < 0.0001$), the former only has about half the pro-

Table 1. *t*-test for the statistical difference of Q_p, Q_b, or Q_T between Dongxiang blue-shelled chickens and Dongxiang brown-shelled chickens[1]

Item	Dongxiang blue-shelled chickens (n = 50)	Dongxiang brown-shelled chickens (n = 45)	*t*-value	*P*-value
Mean of the Q_p	4.30E-08 ± 0.27E-08	8.99E-08 ± 0.42E-08	9.45	<0.0001
Mean of the Q_b	5.73E-08 ± 0.32E-08	0.96E-08 ± 0.05E-08	−14.65	<0.0001
Mean of the Q_T	10.03E-08 ± 0.55E-08	9.95E-08 ± 0.44E-08	−0.1253	0.9006

[1]Q_p = the quantity of protoporphyrin in 1 g of eggshell (mol/g); Q_b = the quantity of biliverdin in 1 g of eggshell (mol/g); Q_T = the total quantity of pigments in 1 g of eggshell (moL/g), is equal to Q_p plus Q_b. E = the power with the base of 10.

toporphyrin of the latter; moreover, Q_b of blue-shelled chickens is about 6 times that of brown-shelled chickens ($P < 0.0001$).

DISCUSSION

In this experiment, we observed that there was significant correlation not only between biliverdin and protoporphyrin content in the eggshells of the Dongxiang blue-shelled chickens, but also between biliverdin and protoporphyrin content in the eggshells of the Dongxiang brown-shelled chickens, which means that the quantity of eggshell biliverdin and protoporphyrin changed along the same direction. This result supports our partial postulation that eggshell protoporphyrin and biliverdin had a common precursor.

Moreover, it was found that the sum of eggshell biliverdin and protoporphyrin of Dongxiang blue-shelled chickens is not significantly different from that of Dongxiang brown-shelled chickens (Table 1), which indicated that the amount of precursor materials for eggshell pigments of Dongxiang blue-shelled chickens was in the same level with that of Dongxiang blue-shelled chickens. The results provide further proof for our postulation that there was no significant difference between the quantity of the common precursor material for the eggshell pigments in Dongxiang brown-shelleds chicken and Dongxiang blue-shelled chickens but still cannot fully support that the precursor is heme.

In addition, the quantity of biliverdin of Dongxiang blue-shelled chickens was much more than Dongxiang brown-shelled chickens, whereas the quantity of protoporphyrin of Dongxiang blue-shelled chickens was only about half that of Dongxiang brown-shelled chickens (Table 1), which hinted at the probable different transformation from precursor to biliverdin or protoporphyrin between blue-shelled chickens and brown-shelled chickens. We all know that in the pathway of heme (Figure 2; the part linked by solid arrow), heme can transform into biliverdin. When protoporphyrin chelated with Fe^{2+}, it became heme, and it is supposed that heme may turn back into protoporphyrin after substituting Fe^{2+} by H^+ (Figure 2; the part linked by dashed arrow). Therefore, heme is presumed to act as the same precursor material for eggshell protoporphyrin and eggshell biliverdin. If heme was the common precursor material of eggshell protoporphyrin and biliverdin, due to the fact that 1 molecule of heme can only transform into 1 molecule of protoporphyrin, or into 1 molecule of biliverdin, the probable formation mechanism of blue eggshell was that more heme was transformed into biliverdin in the chickens laying blue-shelled eggs than in the chickens laying brown-shelled eggs, and accordingly, less heme was transformed into protoporphyrin in the chickens laying blue-shelled eggs than in the chickens laying brown-shelled eggs. The deduction is logical and in agreement with the experiment results that the proportion of biliverdin to protoporphyrin is different

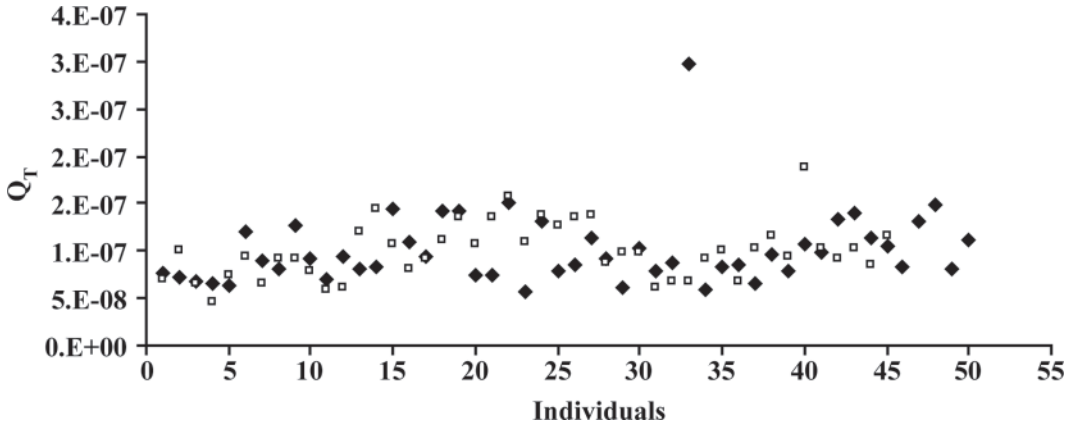

Figure 1. The Q_T of individual samples. Q_T = the total quantity of pigments in 1 g of eggshell of each layer (mol/g), including the blue-shelled chickens (♦) and the brown-shelled chickens (□). Individuals denotes the number of blue-shelled chickens (n = 50) and the number of brown-shelled chickens (n = 45), respectively.

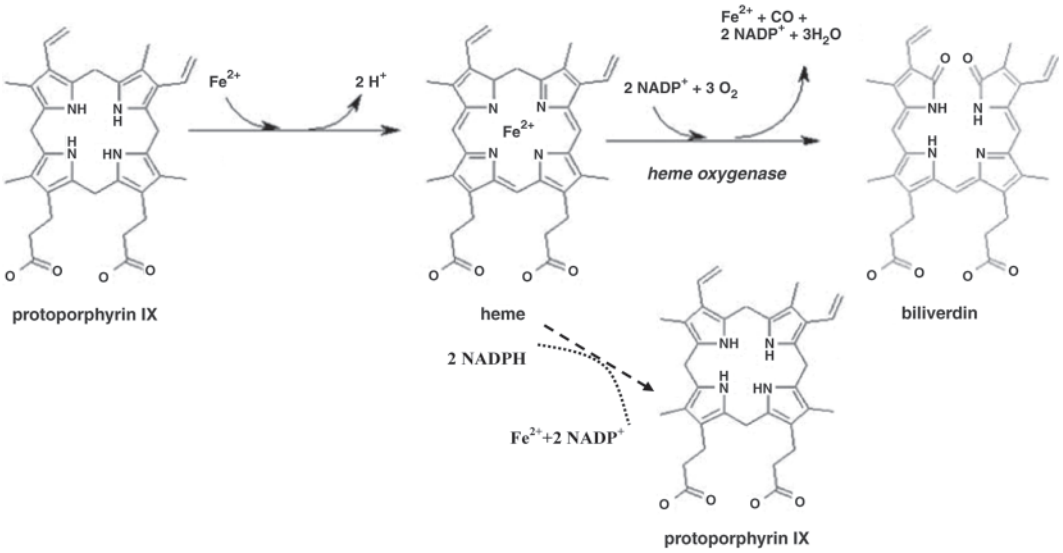

Figure 2. The process of heme biosynthesis and catabolism (linked by solid arrow) and the predicted process of transformation from heme to eggshell protoporphyrin and eggshell biliverdin (linked by dashed arrow).

in blue eggshell and brown eggshell (Table 1), which suggested that heme was probably the precursor for eggshell protoporphyrin and biliverdin.

Yamada (1972) and Baird et al. (1975) supported the hypothesis that porphyrins in eggshell are most likely to be synthesized in the shell gland. Zhao et al. (2006) stated that biliverdin should be synthesized in the shell gland and then deposited onto the eggshell of chickens. Giersberg (1921) thought that eggshell pigments were derived from the disintegration of erythrocytes in the mucous layer of the oviduct and then transported by wandering cells, which penetrate the uterine epithelium; simultaneously, these wandering cells develop dark pigment granules during their migration (Kennedy and Vevers, 1973). If macrophage was the wandering cells, the different viewpoints of the origins of eggshell pigments may be unified for the following reasons: (1) the erythrocytes can be engulfed by macrophage, with the heme ring being opened by heme oxygenase, and heme is oxidized and turned into biliverdin (Prankerd, 2008) and (2) the postulation that heme could act as the common precursor material for eggshell protoporphyrin and eggshell biliverdin was partially supported by our experiment.

Further Opinions

In recent years, many studies (Moreno et al., 2005, 2006; Soler et al., 2005; Morales et al., 2006; Sieffermann et al., 2006) support the viewpoint that eggshell biliverdin reflects female immunocompetence and antioxidant condition (Moreno and Osorno, 2003). During the laying period, the metabolism of oviduct including shell gland was very active and 15.4% of cardiac output went to the oviduct (Boelkins et al., 1973; Scanes et al., 1987); consequently, more reactive oxygen species were produced (Nohl et al., 2003).

Based on the previous reports and our findings, it could be inferred that when blood flowed through the shell gland, the damaged and aging erythrocytes were recognized and engulfed by the macrophage wandering here, then globin and heme were released from the disintegrated erythrocytes, and heme was further catabolized to protoporphyrin or biliverdin, free iron, and CO. When the forming egg entered the shell gland, the cells containing protoporphyrin and biliverdin were stimulated and the pigments began to be deposited to eggshell simultaneously (Wang et al., 2007). The different levels of reactive oxygen species resulted in the different expression levels of heme oxygenase 1 (Schipper, 2004) and the different ratios of protoporphyrin to biliverdin, which led to eggshells different in shade and color.

ACKNOWLEDGMENTS

This work was funded by the Major State Basic Research Development Program of China (2006CB102100) and National Natural Science Foundation of China (30771535).

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