Bull. Inst. Zool. Academia Sinica **23**(1): 1-7 (1984)

THE IDENTIFICATION AND OCCURRENCE OF THE CHROMOGEN IN THE BLUE-GREEN BLOOD OF JAPANESE EEL, *ANGUILLA JAPONICA*

LEE-SHING FANG

Institute of Marine Biology, National Sun Yat-sen University, Kaohsiung, Taiwan 800, Republic of China

(Received July 28, 1983)

Lee-Shing Fang (1984) The identification and occurrence of the blue-green chromogen in the blood of Japanese eel, *Anguilla japonica*. *Bull. Inst. Zool., Academia Sinica* **23**(1): 1-7. The blue-green chromogen in the blood of Japanese eel, *Anguilla japonica* Temminck and Schlegel has been isolated and identified to be biliverdin by absorption spectrum, thin layer chromotography, indicator reaction with barbituric acid and converting it to bilirubin. The biliverdin was also demonstrated not esterified using a repetitive TLC isolation techenique.

The eels sampled during December showed a yellow colored plasma while those in April were all blue-green. Two factors, temperature and feeding rate, that might govern the color shifting were examined yet both gave negative results.

Blue-green colored blood plasma was frequently observed in eel, *Anguilla japonica* Temminck and Schlegel. Since almost all of the blood of vertebrates with hemoglobin has a light yellow or pale colored blood plasma, the coloration of the eel is of great interesting. However, because of the localization of this species, the lack of proper investigation methods for this research, and the labile nature of the colored prosthetic group, very few studies have been done in this field. Nevertheless, Yamaguchi *et al.* (1966) and Oide and Utida (1967) had pointed out the blue-green chromogen in *A. japonica* was biliverdin. Yet, due to the variation of the absorption spectra of various blue-green colored fish plasma (Kochiyama *et al.*, 1966; Bada, 1970; Low and Bada, 1974; Fang, 1982), the fluctuation of the absorbance of bilatriene (in which biliverdin is included) (Gray, *et al.*, 1961; With, 1968), and the interference from pigments other than biliverdin in biological fluids (Greenberg, *et al.*, 1971), the identification of the blue-green chromogen in eel plasma should be further verified.

In this study, in addition to the conventionally used spectrum and Thin Layer Chromatography (TLC) methods, three recently developed tests to examine the identity of plasma biliverdin were applied. 1) the indicator reaction of biliverdin with barbituric or thiobarbituric acid (Gutteridge and Tickner, 1978, Tickner and Gutteridge, 1978); 2) the reduction method to convert biliverdin to bilirubin (Fang, 1982,); and 3) A repetitive thin layer chromatography techenique. The last one was specially developed to examine whether the plasma chromogen was an ester or not.

The frequency of the occurrence of the blue colored chromogen in the blood of eel also worths noticing (McDonagh and Plama, 1982). Eel has been reported to have hyperbilirubinaemia (Kawatsu and Sakai, 1980), a phenomenon high concentration of bilirubin is detected in the blood which results to a densely yellow colored plasma. This is contrast to the blue-green colored ones. There is

very little understanding about how and why these completely different physiological phenomena operate in eel. In fact, eel may be the only animal that has been observed to live normally in pathological conditions of either bilirubinaemia or biliverdinaemia.

In this study, a preliminary field survey on the frequency of the blood serum coloration was first performed. Through which, two ecological factors, temperature and feeding rate, were hypothetically correlated with the occurrence of the blue plasma coloration. Then, controlled experiments in laboratory were done to test the hypothesis. The results, although negative, were reported here.

MATERIALS AND METHODS

Experimental fish

A. japonica weighed from 140 to 200 g were obtained from fish farms. Blood was drawn either by cardiac puncture or from caudal artery. The heparinized blood was centrifuged immediately to obtain the plasma, which was then stored in 4°C until using.

Separation and purification of the colored prosthetic group from crude plasma

An absorption spectrum of the crude blue-green plasma from 325 to 800 nm was first scanned by a Hitachi (model 100-60) spectrophotometer. The plasma was then hydrolyzed with HCl-methanol (10%) and the prosthetic group was extracted into chloroform as described in Low and Bada (1974). The chloroform solution was further developed in a silicic acid column by proportionally increasing the concentration of methanol and ethanol in chloroform (starting from 5:5:90, v/v) (Fang, 1982) to purify the isolated prosthetic group. In certain cases, a few drops of 1 N HCl were added to the elution solvent to obtain a quick elution of the green band (the prosthetic group).

Identification of the prosthetic group

Absorption spectrum: Since the plasma prosthetic group is a colored material, it provides an advantage for spectrum identification. Prosthetic group purified from silicic acid liquid column chromatography was used to obtain an absorption spectrum from 320 to 800 nm. Such data were then compared with that obtained from standard biliverdin (dihydrochloride, Sigma, Co., USA) which had been treated the same as the sample.

TLC examination: The purified plasma prosthetic group was applied on silica gel 60 glass plate (0.25 mm, Merck, Germany) and developed in a glass tank pre-equilibirated with $CHCl_3$:CH_3OH:C_2H_5OH(40:15:10, v/v) in dark at room temperature (~25°C). The relative mobility (R_f) of the sample on TLC was then compared with that of standard biliverdin.

The indicator reaction of barbituric acid: This test was a specific reaction for biliverdin primarily followed that described in Tickner and Gutteridge (1978). Purified plasma prosthetic group was first dissolved in pH 7.4 phosphate buffer (Singleton and Laster, 1965). Then, barbituric acid (Sigma, USA) (0.025 g per ml of 1 M NaOH) was added and the solution was incubated at 97°C for 10 minutes. After the reaction, *n*-butanol was introduced and the absorption spectrum of the produced chromogen was taken.

The converting test of the prosthetic group: Since biliverdin is the oxidative form of bilirubin, it will provide strong evidence for the identification of the plasma prosthetic group if the chromogen can be converted back to bilirubin through reductive reaction. A method of this was developed, from an original idea of Oide and Utide (1967), by Fang (1982) who used sodium dithionite to reverse the biological reaction mediated by an enzyme called biliverdin reductase *in vivo* (Colleran and Heiwegh 1979). Similar procedure was applied on the eel plasma prosthetic group.

Examining the existence of ester form of the plasma prosthetic group: Since the prosthetic group of the blue-green plasma was isolated by acid hydrolysis, if it were in ester form (which is very likely because esterification is a common biological detoxification methods),

the ester group could be broken during the hydrolysis process. Thus, a crude plasma TLC isolation (Fang, 1982) with some modification was applied to examine this problem.

Crude plasma was first treated with equal volume of ethanol (99%). Then, the sample was applied on TLC and developed with $CHCl_3$:CH_3OH:C_2H_5OH (40:15:10). However, due to the interference of the proteins in the sample, only small amount of plasma could be applied which resulted in very indecisive R_f of the prosthetic group. To overcome this problem, repetitive TLC on the same plate (Fig. 1) was applied until an appropiate amount of blue chromogen was isolated on the plate. Then, authentic biliverdin was applied to compare its R_f with that of the sample.

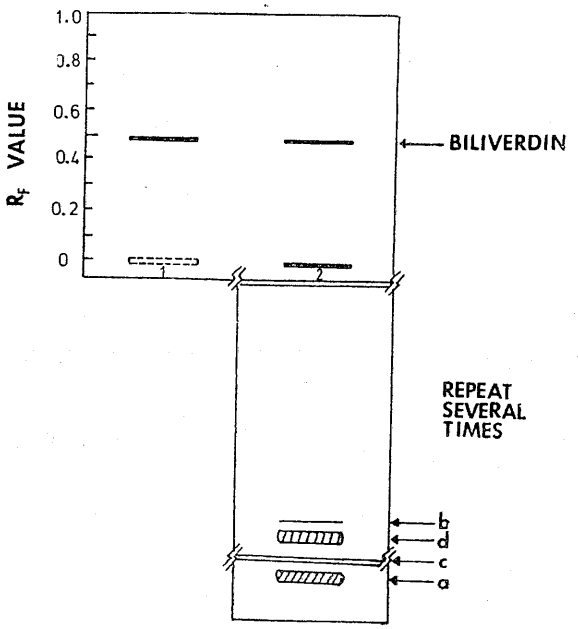

Fig. 1. The repetitive TLC (on the same plate) of the chromoprotein from the blue-green eel plasma to isolate and identify the prosthetic group. a, first applying line of crude plasma; b, green band eluted from a; c, cut off the first applying line after green band moving to a distance; d, apply the serum the second time below the first green band after cutting off the first line. 1, standard biliverdin; 2, prosthetic group isolated from the blue green plasma via repetitive TLC. Developing solvent system, chloroform:methanol:ethanol (40:15:10, v/v) in room temperature at dark.

The occurrence of the blue-green blood plasma in eel:

Eel blood samples were obtained frequently from early December to April. During which period, the percentage of the appearance of the blue-green blood plasma increasing from 0 to 100 was recorded. The coloration of the plasma was determined to be blue-green either by visual inspection or a spectrum absorbance in the 650 to 680 nm region. The possible environmental factors that might cause the plasma color shift were analyzed. Water temperature seemed to be the condition that varied most drastically during that period. The feeding rate change in accordance with the temperature variation was another factor worth considering since Yamaguchi *et al.* (1968) suggested the quantity of food intake might influence the concentration of the blue colored plasma chromoprotein. These two factors were then examined by laboratory experiments.

Eels with blue-green plasma were selected and the concentrations of the chromogen in their blood were determined by measuring the

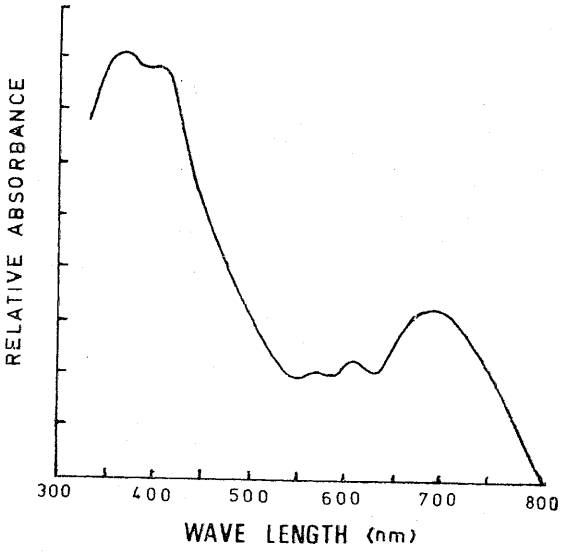

Fig. 2. The absorption spectrum of the crude blood plasma of *Anguilla japonica*.

absorbance of crude plasma at 675 nm using a concentration calibration channel in the spectrophotometer. Then, they were grouped, three per set, and kept either in 16°C or in 28°C (as the control group) temperature controlled aquarium for 60 days. Blood samples were periodically taken so that the concentration change of the blue chromoprotein or any possible coloration shift was monitored. All the fish were fast during the experimental period.

RESULTS AND DISCUSSION

The absorption spectrum of the crude blue-green eel plasma is shown in Fig. 2. When comparing this to that of the similar colored plasma of arctic sculpin (Bada, 1970), several different species of cottids (Low and Bada, 1974), and some labrids (Abolins, 1961), all of them are different. Yet, speaking of the chromogens which were responsible for the strange plasma coloration, most of them were believed to be biliverdin. Thus, in identification of plasma chromogen groups, extensive purification and verification are important.

In addition to pure scientific interesting, the confident identification of the blue-green chromogen in eel plasma to be biliverdin also bring out some possibility of clinical applications. Bilirubin, the reduced form of biliverdin, is the pathogenic molecule in human jaundice and kernicterus when it accumulates in blood (Colleran and Heirwegh, 1979). Biliverdin was also observed accumulating in patient's blood who suffered from liver or gall bladder diseases (Tickner and Gutteridge, 1978; Greenberg *et al.*, 1971; Fenech *et al.*, 1967; Larson *et al.*, 1947). Thus, the existence of the blue-green molecule in eel blood can provide a valuable specimen for future comparative studies in elucidating the mechanism of bile pigment metabolism.

After the chromogen was isolated and purified from the plasma, the absorption spectrum of it was similiar to that of standard biliverdin (Fig. 3). Nevertheless, a small absorption peak around 320 nm of the sample

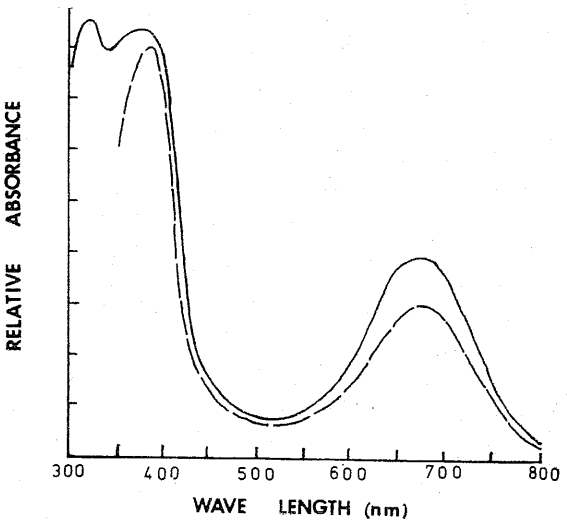

Fig. 3. The comparison of the absorption spectrum of the purified prosthetic group from the eel plasma with that of the standard biliverdin. The solvent is chloroform:methanol:ethanol (5:1:1, v/v) with a few drops of 1N HCl. ——, prosthetic group purified from the eel plasma chromoprotein; ----, standard biliverdin

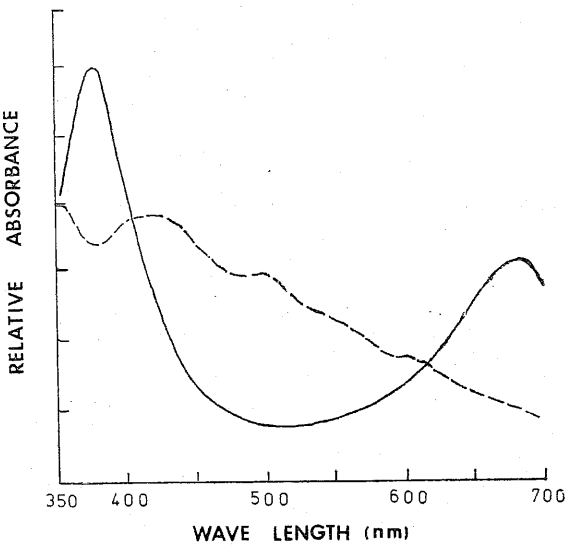

Fig. 4. The absorption spectrum change of the prosthetic group isolated from the blue-green plasma chromoprotein after reaction with barbituric acid. ——, before the reaction; ----, after the reaction.

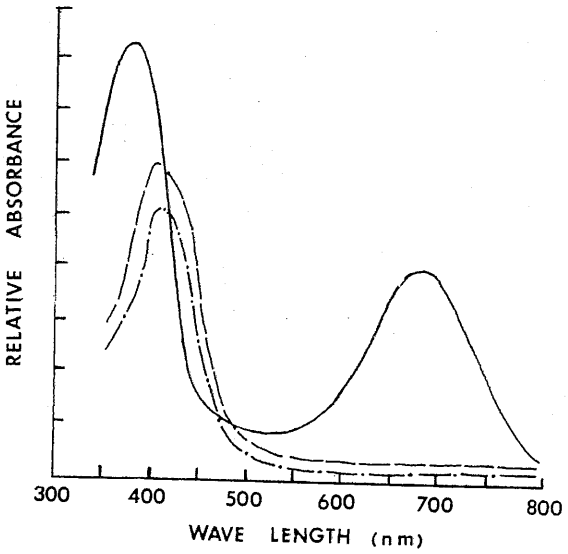

Fig. 5. The absorption spectrum change of the prosthetic group of the eel plasma chromoprotein after reduction by sodium dithionite. ——, prosthetic group before the reduction reaction; ----, prosthetic group after the reduction; —•—, bilirubin reduced from standard biliverdin.

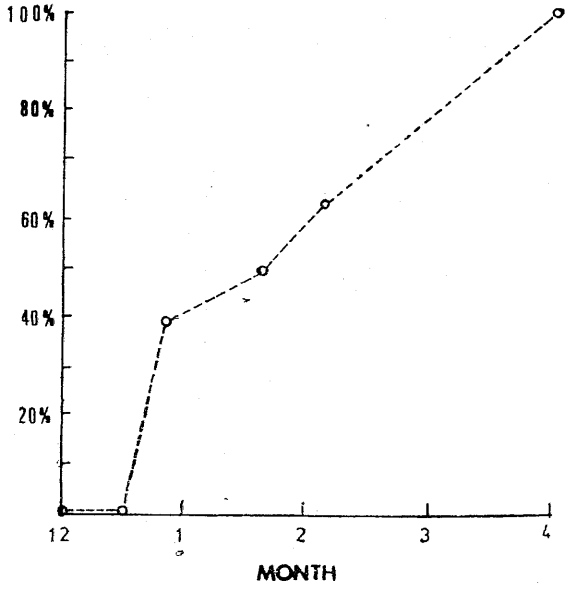

Fig. 6. The percentage of the appearance of the blue-green blood plasma in cultured eel. A total of 41 samples were examined.

was a sign of biliverdin ester (Yamaguchi, *et al.*, 1966). This, along with the trailing result of the TLC of the isloated chromogen, made the identification become ambiguous. Could the plasma biliverdin actually be an ester? Or some esters were artificially produced during the acid-methanol hydrolysis which then biased the data. To solve this problem, the repetitive TLC of the crude plasma was performed and its result was in Fig. 1. Crude plasma chromoprotein developed in neutral solvent showed that the chromogen could be isolated from the apoprotein successfully without acid hydrolysis. The subsequent examination revealed it had the same R_f as that of standard biliverdin, demonstrating the two carboxyl groups of the eel plasma biliverdin was in free form. If it were a biliverdin ester, the R_f of it in this elution solvent system would be near 1.

The indicator reaction of barbituric acid was positive (Fig. 4). However, due to a limited sample size, the characteristic absorption of biliverdin reactant at 535 nm of the plasma sample was not clearly presented. This made the distinguish of biliverdin from other verdoglobin-like green pigments become difficult (Tickner and Gutteridge, 1978). Fortunately, the test of converting the prosthetic group into bilirubin was very conclusive (Fig. 5), which, along with other evidences from absorption spectrum and TLC, strongly indicated that the blue-green plasma chromogen of the eel, *A. japonoca*, was a free formed biliverdin.

The percentage of the appearance of the blue-green plasma from December to April was shown in Fig. 6. The possibility that water temperature and feeding rate might control the color change was examined, yet negative results were obtained. The blue chromoprotein in fish either kept in 16°C or fast for 2 months (April to June under nature light-dark rhythm) did not show any signficant decrease or color shift as expected. In fact, those that in the 16°C group even showed a slightly higher concentration than

that of the 28°C group (the control). Yamaguchi *et al.* (1968) found the quantity of the blue-green plasma chromoprotein varied with season and the apoprotein was similar to β_1-lipoprotein which is a lipid carrier in blood of human, thus suggested it was influenced by feeding rate. Our data do not support this hypothesis. However, the data tend to support the speculation proposed by Low and Bada (1974), Yamaguchi, *et al.*, (1976) and Fang (1982) that the chromogen could arise from heme degradation within the fish. Therefore, in spite of the food supply or the ambient temperature change, a continuous inflex of biliverdin from heme catabolism which resulted in a stable chromogen concentration in blood was observed. Another sampling in June showed only 45% of the eel had blue-green plasma, a datum against the temperature or feeding rate controlling hypotheses.

The physiology and biochemistry of heme metabolism in eel, as suggested by the evidences in this study, are unique among vertebrates. Not only its ability of coupling with high concentration of both bilirubin and biliverdin molecules is important to clinical consideration, but also the physiological function of the occurrence of the blue-green blood and the mechanism of the catabolism shift are both interesting subjects. These are very little studied, yet may of significant contributions to the field of comparative physiology and biochemistry.

Acknowledgements: I thank Miss L. H. Chu and Miss S. F. Chiou for many techenical helps. Drs. J. L. Bada and K. H. Chang for critical discussions. This research is supported by National Science Council of the Republic of China (NSC-72-024-B110-01).

REFERENCES

ABOLINS, Ł. (1961) The existences of sexual diamorphism of blood plasma in fishes of the family Labridae. *Arkiv. Zool.* **13**(25): 541-544.

BADA, J. L. (1970) A blue-green pigment isolated from blood plasma of the Arctic sculpin (*Myoxocephalus scorpioides*). *Experientia* **26**: 251-252.

COLLERAN, E. and K. P. M. HEIRWEGH (1979) Comparative aspects of bile pigment formation and excretion. *Comp. Biochem. Physiol.* **64B**: 133-139.

FANG, L. S. (1982) The blue-green pigment in the blood serum of a marine fish, *Clinocottus analis*: identification, metablism and biological significance. Ph. D. Thesis. University of California, San Diego. 176 pp.

FENECH, F. F., H. W. BANNISTER and J. L. GRECH (1967) Hepatitis with biliverdinaemia in association with indomethacin therapy. *Br. Med. J.* **3**: 155-156.

GRAY, C. H., A. LICHTAROWICZ-KULCZYCKA, D. C. NICHOLSON and Z. PETRYKA (1961) The chemistry of the bile pigments. Part II. The preparation and spectral properties of biliverdin. *J. Chem. Soc.* 2264-2268.

GREENBERG, A. J., I. BOSSENMAIER and S. SCHWARTZ (1971) A study of serum biliverdin, mesobiliverdin and other green pigments. *Digestive Diseases* **16**(10): 873-880.

GUTTERIDGE, J. M. C. and T. R. TICKNER (1978) The thiobarbituric acid reactivity of bile pigments. *Biochem. Med.* **19**: 127-132.

KAWATSU, H. and T. SAKAI (1980) Hyperbilirubinemia in cultured eel. *Bull. Jap. Soc. Sci. Fish.* **46**: 809-812.

KOCHIYAMA, Y., K. YAMAGUCHI, K. HASHIMOTO and F. MATSUURA (1966) Studies on a blue-green serum pigment of eel-I. Isolation and some physico-chemical properties. *Bull. Jap. Soc. Sci. Fish.* **32**(10): 867-872.

LARSON, E. A., G. T. EVANS and C. J. WATSON (1947) A study of the serum biliverdin concentration in various types of jaundice. *J. Lab. Clin. Med.* **32**: 481-488.

LOW, P. S. and J. L. BADA (1974) Bile pigments in the blood serum of fish from the family Cottidae. *Comp. Biochem. Physiol.* **47A**: 411-418.

MCDONAGH, A. F. and L. A. PALMA (1982) Heme catabolism in fish. Bile pigments in gallbladder bile of the electric torpedo, *Torpedo californicus. Comp. Biochem. Physiol.* **37B**: 501-507.

OIDE, H. and S. UTIDA (1967) Sur la chromoproteine verte du serum de l'Anguille japonaise (*Anguilla japonica*). *Compt. Rend. Soc. Biol.* **161**: 1205-1208.

SINGLETON, J. W., and L. LASTER (1965) Biliverdin reductase of guinea pig liver. *J. Biol. Chem.* **240**(12): 4780-4789.

TICKNER, T. R. and J. M. C. GUTTERIDGE (1978) A simple colorimeteric method for the estimation of plasma biliverdin. *Clin. Chim. Acta.* **85**: 125-129.

WITH, T. K. (1968) *Bile pigments.* Academic Press, N. Y. and London. 830 pp.

YAMAGUCHI, K., Y. KOCHIYAMA, K. HASHIMOTO and F. MATSUURA (1966) Studies on a blue-green serum pigment of eel-II. Identification of prosthetic group. *Bull. Jap. Soc. Sci. Fish.* **32**(10): 873-879.

YAMAGUCHI, K., K. HASHIMOTO and F. MATSUURA (1968) Studies on a blue-green serum pigment of eel-IV. Seasonal variation of concentration of pigment in serum. *Bull. Jap. Soc. Sci. Fish.* **34**(9): 826-835.

YAMAGUCHI, K., K. HASHIMOTO and F. MATSUURA (1976) Identity of blue pigments obtained from different tissue of the sculpin. *Pseudoblennius percoides* GUNTHER. *Comp. Biochem. Physiol.* **55**B: 85-57.

鰻魚血液中藍綠素的出現及鑑定

方　力　行

從日本鰻血液中分離的藍綠色素經用光譜，薄層色層分析，指示反應，及還原成膽紅素等方法，鑑定爲膽綠素。並用連續薄層分離分析技術，證明此血液中的膽綠素不是以酯化形式存在。

在取樣期間鰻魚血漿呈色，部份爲黃色，部份爲藍綠色。經實驗顯示，原本被認爲可能控制此藍綠血漿色素變化的兩種生態因子：「攝食率變化」及「溫度」却不足以造成顯著的血漿顏色改變。