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THE IDENTIFICATION OF THE BLUE-GREEN PIGMENT IN THE BLOOD PLASMA OF THE COTTID, *CLINOCOTTUS ANALIS*

Lee-shing Fang

Department of Marine Resources National Sun Yat-sen University Kaohsiung, 80424, Taiwan, Republic of China

ABSTRACT: The pigment responsible for the blue-green blood plasma coloration of *Clinocottus analis* has been isolated by acid hydrolysis and further purified by liquid chromatography. The visible light absorption spectrum of the pigment and the behavior of it on thin layer chromatography was the same as that of authentic biliverdin. Furthermore, it reacted with barbituric acid in a manner similar to that of biliverdin and the pigment was converted to bilirubin through chemical reduction. These results clearly indicated that the blue-green prosthetic group in the plasma of *C. analis* is biliverdin.

Introduction

In a study of fish hemoglobin metabolism, the blood plasma of a small intertidal fish, *Clinocottus analis* (Wodly sculpin), was found to be strikingly green (Fang and Bada, 1982; Fang, 1987). This strange coloration in fish blood was suggested causing by a chromogen (prosthetic group) linked with a protein moiety (Fang and Bada, 1988). The chromogens had been identified as icthyoverdin, bilatriene or biliverdin in different fishes (Tsuchiya and Nomura, 1955; Yamaguchi *et al.,* 1966; Bada, 1970). However, due to the variation of the absorption spectra of these chromoproteins (Kochiyama *et al.,* 1966; Bada, 1970; Low and Bada, 1974), the fluctuation of the absorbance of bilatriene (Tsuchiya and Nomura, 1958) and biliverdin in different solvent conditions (Gray *et al.,* 1961; With, 1968), plus the interference from pigments other than biliverdin in biological fluids (Greenberg *et al.,* 1971), more precise identification of the prosthetic groups in blue-green blood plasma of fish is necessary.

Fortunately, in addition to the conventional absorption spectrum and chromatography methods, Gutteridge and Tickner (1978) and Tickner and Gutteridge (1978) had reported a specific indicator reaction of biliverdin with barbituric or thiobarbituric acid. This greatly increases the confidence of biliverdin identification. Moreover, since in the heme degradation pathway of vertebrates, biliverdin is an intermediate metabolite which is further reduced to bilirubin (Colleran and Heirwegh, 1979), additional identification information can be obtained if the green prosthetic group isolated from fish blood serum is reducible to bilirubin. Therefore, in this study, isolated green chromogen was further treated with reducing agent and its end production was compared with standard bilirubin which reduced from authentic biliverdin.

Materials and Methods

Experimental animal: *Clinocottus analis* specimens average of length from 7.7 to 11.1cm were collected and kept in running sea water aquarium without feeding for 1 to 3 days before blood samples were drawn. Blood samples, obtained by cardiac puncture, were pooled and centrifuged immediately to separate blood serum from erythrocytes. The colored serum was either used fresh or frozen for future study.

The absorption spectrum of crude chromoprotein: Crude blood serum was first visually examined for erythrocytic lysate contamination. If the sample was clear, a visible light absorption spectrum (350 to 800 nm) was obtained using distilled water as a reference, if there was evidence of erythrocyte lysis, the sample was discarded.

Isolation of the prosthetic group from crude serum: Crude serum was first treated with 5 volumes of absolute methanol-HCI (3N) and stirred in a cold (4°C), dark room for at least 60 minutes. Chloroform in an

amount equal to one fifth of the total solution volume was added and the preparation was stirred for another 10 minutes. Distilled water was then gradually added which would cause serum proteins to precipitate into the aqueous phase. This was repeated several times. Finally, the protein precipitate was removed by filtration and the chloroform layer containing the green prosthetic group was collected.

Purification of the prosthetic group by liquid column chromatography: The prosthetic group dissolved in chloroform was usually contaminated by other pigments such as heme or bilirubin which also occur in blood serum. These contaminants could not be removed by the solvent extraction method used by O'Carra and Colleran (1969) because of the limited size of the blood sample obtainable from the fish in this experiment (usually less than 1ml of crude blood serum). Therefore, a micro liquid column chromatographic method was developed.

The chloroform extract was washed three times with 5 volumes of twice distilled water to remove the remaining methanol and HCl. When this step was omitted, sample resolution would reduce. The extraction was developed in a micro-column packed with silicic acid. The column was first eluded with chloroform until a yellow band passed through the column. Then, a solvent mixture of methanol and chloroform, initially 1:99, v/v with an increasing percentage of methanol was used to develop the column until a green band was finally eluded. Because silica gel has a tendency to dissolve in high concentrations of methanol, the green band was eventually washing out with ethanol: methanol: chloroform (2:1:3, v/v). Adding a few drops of 3N HCl to the elution solvent facilitated the elution.

The absorption spectrum of the purified prosthetic group: The fraction from the column chromatography containing the green band was scanned from 350nm to 800nm against the elution solvent (usually ethanol: methanol: chloroform, 2:1:3, v/v) as a reference to obtain the absorption spectrum of the purified prosthetic group. Standard biliverdin was either obtained from Sigma Chemical Co. (St. Louis, MO, USA) or by oxidation bilirubin following the method used by Tickner and Gutteridge (1978) with a little modifications*. The standard was dissolved in chloroform and carried through the same chromatographic procedure as the fish pigment samples before its absorption spectrum was taken.

Thin layer chromatography: Silica 60 (0.2mm, EM Reagents, Darmstadt, Germany) coated on a plastic sheet was used for thin layer chromatography (TLC). All plates were developed with methanol and then dried in a vacuum desiccator for 24 hours before use. Samples were applied on plates using a TLC applicator, blown dry with N_2, and developed in jar that was equilibrated with the eluant system for at least 30 minutes prior to development. All samples were run at room temperature in the dark. If the result of the first TLC was not definitive, two-dimensional TLC was performed in order to obtain better resolution.

Amount the solvent systems that were tested, the one with the best resolution (n-butanol: ethanol: water; 3.1:1.5, v/v) was chosen and thereafter used for sample vs. standard biliverdin R_f value comparison.

In addition to examining the behavior of the free form prosthetic group on TLC, the esters of the pigment were synthesized and its behavior on TLC was again compared with that of standard biliverdin methyl esters. The esterification of prosthetic group and the standard biliverdin were carried out by two different methods:

(1) The pigment was dissolved in (3N) HCl-absolute methanol (Sigma Chemical Co.) and evaporated to dryness at 75°C.

(2) The pigment was dissolved in 5% H_2SO_4-absolute methanol and placed at 4°C in the dark for 12-16 hours. The ester was then extracted from solution by dilution with water and agitated with chloroform (O'Carra and Colleran, 1970). The extracted chloroform solution was washed 3 times with 5 volume of distilled water and then concentrated by blowing with N_2.

Reduction of the prosthetic group to bilirubin: When Oide and Utida (1967) incubated the green blood serum of *A. japonica* with sodium dithionite, it became yellow within about 10 minutes. The absorption spectrum of the yellow pigment was similar to that of bilirubin. However, other major blood pigments such as hemoglobin or urobilins absorb significantly in the red-yellow color region (Gray, 1953) and could thus have interfered with this analysis. Therefore, the prosthetic group from the plasma of *C. analis* was first isolated and purified by liquid column chromato-

*Instead of heating at 95°C for two hours, the conversing was done in a 70°C water bath overnight. Ascorbic acid was added to the stock solution for stabilization.

graphy as specified above. The purified pigment was then dissolved in a few drops of ethanol, diluted with 0.1M phosphate buffer (pH 7.2) to a volume of 2 to 3ml, and its absorption spectrum was determined. A small amount of sodium dithionite (1mg per ml) was then introduced. The solution was agitated and its color change was observed. Once the color change was completed, the absorption spectrum of the new chromogen was obtained. Exact procedure was used with standard biliverdin and its absorption spectrum was obtained for comparison and identification.

The indicator reaction of biliverdin with barbituric acid: Experiment was performed following the method of Tickner and Gutteridge (1978). Samples in phosphate solution were first acidified by glacial acetic acid, then an equal volume of a solution containing 0.025g of barbituric acid per one ml of 1M NaOH was added. The final solution was stirred for 10 seconds and incubated at 97°C for 10 minutes. If biliverdin was present, the solution would form a conspicuous red chromogen. The chromogen was extracted into n-butanol and its absorption spectrum was obtained. For comparison, standard biliverdin was treated in the same manner as the samples.

Test for mesobiliverdin: To ensure that the pigment isolated was biliverdin and not its isomer mesobiliverdin, the green pigment was heated with concentrated sulphuric acid. Biliverdin, but not mesobiliverdin, is rapidly destroyed by this treatment (Noir *et al.*, 1965).

Results and Discussion

The absorption spectrum of the clear crude blue-green blood serum is shown in Fig. 1. This is similar to that obtained for the same species by Low and Bada (1974), but quite different from spectrum obtained for parrot fish (Abolin, 1961), eel (Yamaguchi *et al.*, 1966; Oide and Utida, 1967; Fang, 1985), arctic sculpin (Bada, 1970), cabezon (Low and Bada, 1974) and skipjack (Fuke *et al.*, 1974), all species in which blue-green blood chromogen was reported. In fact, none of these spectra are identical, yet in most of these cases the chromogen responsible for the blue-green coloration was believed to be biliverdin.

The purification of the prosthetic group by micro-liquid column chromatography was very efficient. Sample sizes as small as 0.2ml of crude serum can be effectively analyzed. However, in a few cases when the resident time of the green band on the chromato-

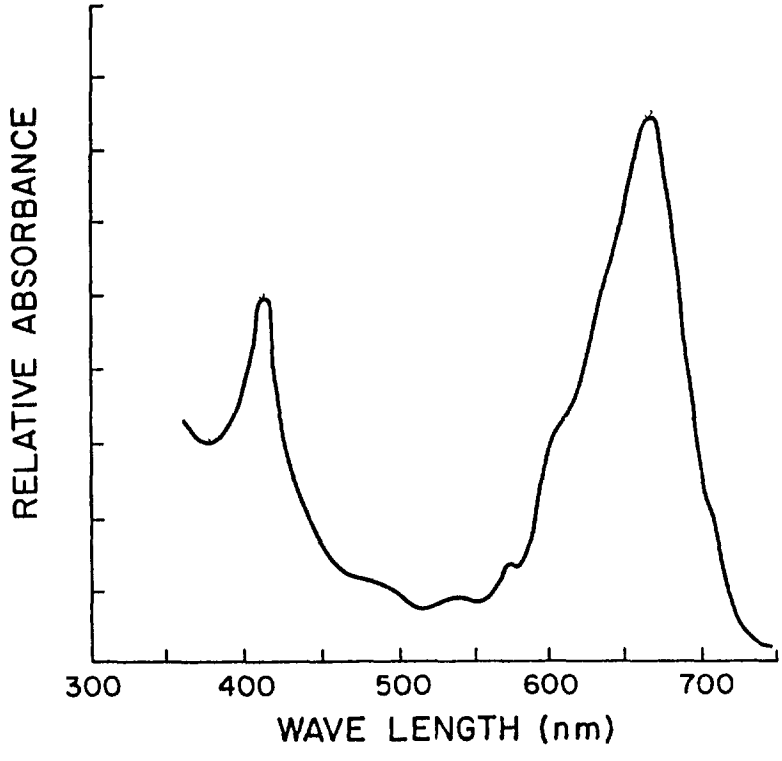

Figure 1. The absorption spectrum of the crude blood serum of *C. analis*.

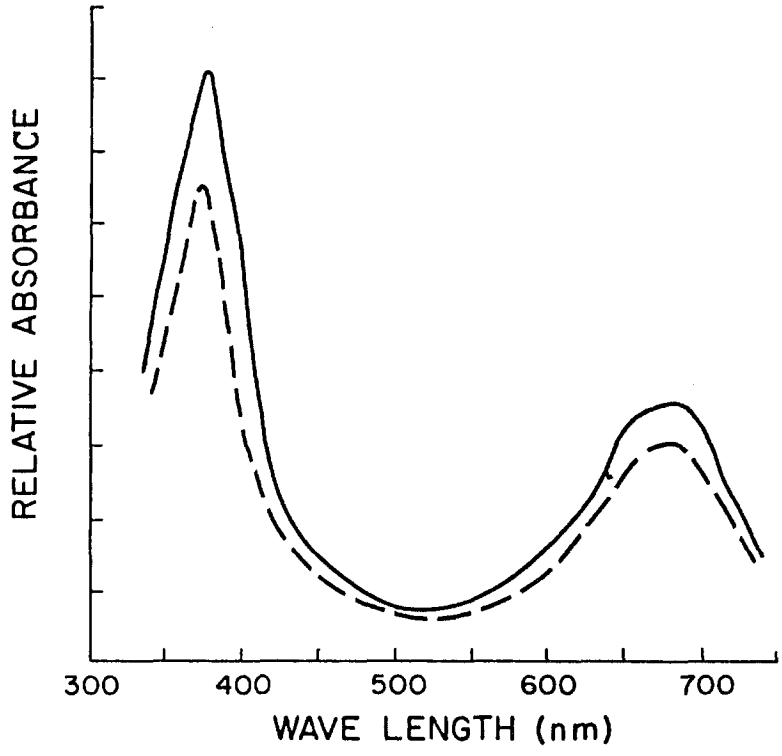

Figure 2. The comparison of the absorption spectrum of the purified prosthetic group from the fish serum with that of the standard biliverdin. The solvent is ethanol: methanol: chloroform, 2:1:3, v/v, with a few drops of HCl. Solid line: standard biliverdin; dashed line: prosthetic group isolated and purified from the blue-green fish serum chromoprotein.

graphy column was long or high concentrations of methanol or ethanol were used for elution, the green band changed to a dark yellow color. Impurities in the elutant and the labile character of bile pigments to redox reactions likely accounted for this observation.

The absorption spectrum of the purified prosthetic group is almost identical to that of the biliverdin standard (Fig. 2), but the absorption maximum of biliverdin at 375 nm was drastically quenched and shifted in the crude blue-green serum when compared with that of the isolated prosthetic group (Fig. 1. 2). Low and Bada (1974) suggested that the coloration of the chromogen in the blood serum of *C. analis* could be changed due to the influence of microenvironments generated by protein. Fang *et al.*, (1986) had further suggested that this was likely resulted from a coiled molecule enveloped by a hydrophobic micelles of the carrier protein.

The result of the TLC analysis of the free form of the prosthetic group is shown in Fig. 3. It is clear that the major green band isolated from the fish serum has the same R_f as that of the standard biliverdin. However, in some plates, one or two minor bands were also observed in the sample. The identity of the minor bands is unknown.

The TLC result for the ester form of the prosthetic group is shown in Fig. 4. The R_f values of the major green band of both the sample and standard were the same. The standard biliverdin showed two major green bands with very similar R_f values which are likely isomers that can be separated after esterification (McDonagh and Palma, 1980; O'Carra and Colleran, 1970). The small bands with low R_f values were not identified but are believed to be partially esterified biliverdin since there are two carboxyl groups in this molecule.

After introducing sodium dithionite into the sample solution, the green color had changed to yellow within seconds (Fig. 5). The color change was the same as that observed in a standard biliverdin experiment, in which standard biliverdin was reduced into bilirubin by dithionite.

The biliverdin indicator reaction of barbituric acid

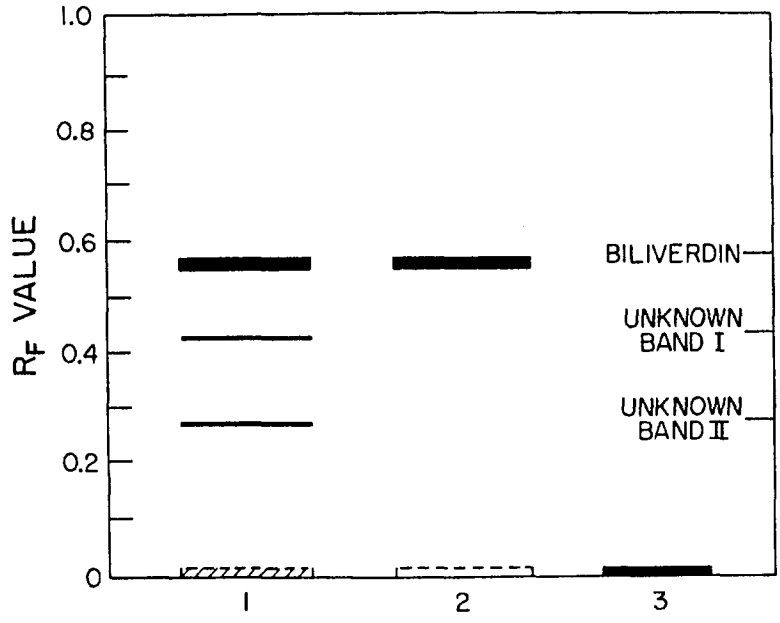

Figure 3. The TLC result of the prosthetic group from the blue-green fish serum and standard biliverdin and bilirubin.
Position 1: prosthetic groups isolated from fish blood serum;
Position 2: authentic biliverdin;
Position 3: authentic bilirubin;
Developing solvent system: n-butanol: ethanol: water (3:1:1.5, v/v), at 25°C in the dark.

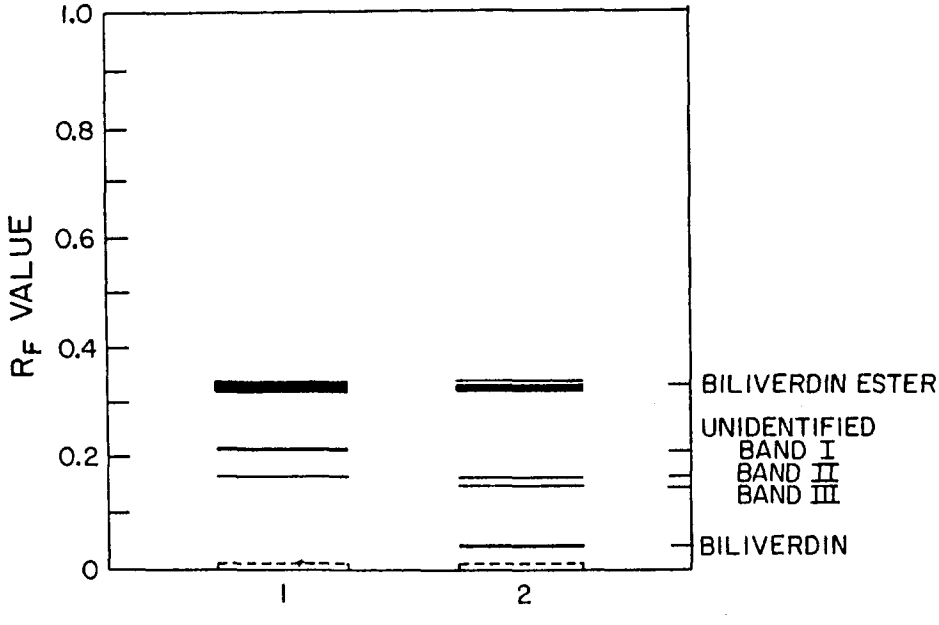

Figure 4. The result of TLC analysis of the prosthetic group after esterification. Spots corresponding to:
Position 1: esterified prosthetic group;
Position 2: standard biliverdin ester.
Developing solvent system: benzene: ethanol (100:8, v/v) at 4°C in the dark.

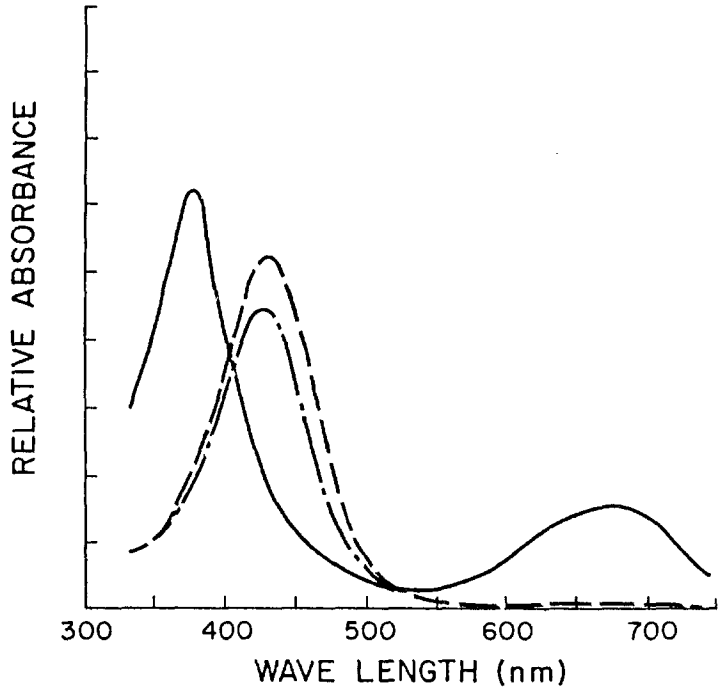

Figure 5. The absorption spectrum change of the prosthetic group of the blue-green fish serum chromoprotein after reduction. The result shows that the prosthetic group can be reduced to bilirubin the same as that of the standard biliverdin did.
(—) Prosthetic group before reduction reaction
(···) Prosthetic group after reduction reaction
(— · —) Standard bilirubin reduced from biliverdin

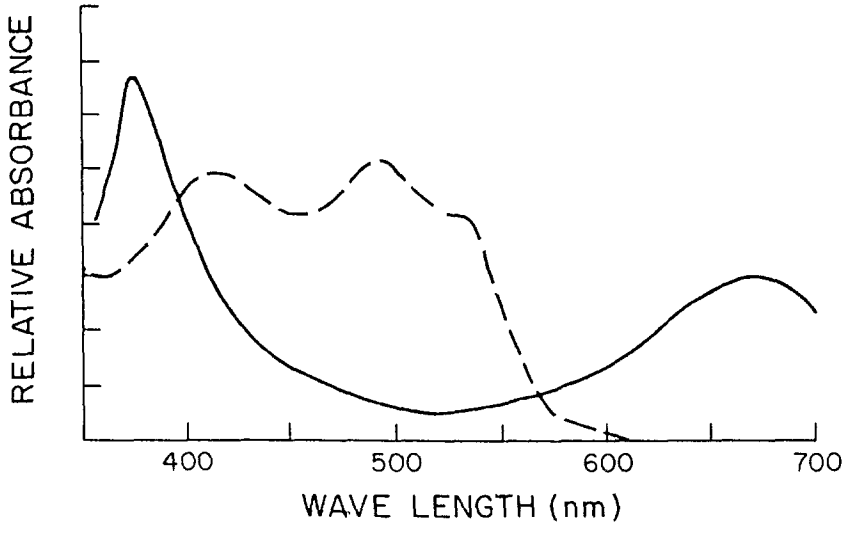

Figure 6. The spectrum change of the prosthetic group isolated from the blue-green serum chromoprotein after a biliverdin indicator reaction. Standard biliverdin has a similar change.
(—) before the reaction
(···) after the reaction

with the prosthetic group was positive. The pink chromogen formed had a absorption spectrum similar to that obtained from the standard biliverdin reaction (Fig. 6).

The sulphuric acid reaction showed that the green pigment was quickly destroyed, indicating that the green coloration was not due to mesobiliverdin.

These various results thus clearly indicated that the blue-green coloration of the blood serum of *C. analis* was due to the occurrence of biliverdin. Tschiya and Nomura (1955) examined a green pigment isolated from the scale of *Cololabis saira* (a saury) and named it ichthyoverdin because considerable differences were found between it and biliverdin IX_{α}. The blue-green pigments in the blood of other fish, although generally believed to be biliverdin, did not present convincing evidences. In this study, however, the prosthetic group of the blue-green pigment in the serum of *C. analis* was precisely identified as biliverdin.

The accumulation of biliverdin in the blood of fish is a strange phenomenon among vertebrates. Similar situation observed only in certain clinical cases in which patients had catarrhal jaundice, biliary atresia or liver cirrhosis (Fenech *et al.*, 1967; Greenberg *et al.*, 1971). This biliverdin came from the degradation of heme and was retained owing to not being able to further metabolize and excrete it. However, despite the extensive review of blue-green blood plasma in fishes by Fang and Bada (1990), there is very little experimental information about where this pigment comes from or how it functions in blood. Fontaine (1941) and Low and Bada (1974) speculated that the pigment could have accumulated from plant chlorophyll or biliproteins from the food chain. Low and Bada (1974) and Yamaguchi *et al.* (1976) also speculated that it could arise from heme degradation within the fish. Preliminary diet control experiments in our laboratory showed that the concentration of the green chromogen in the blood did not decrease significantly after three months of a tetrapyrrol-free diet. This favors the heme degradation hypothesis. However, even that Fang (1987) and Fang and Bada (1988) had proposed the heme catabolic pathway of fish with blue-green blood plasma, more specific study on the originality and physiology role of this strange chromogen in fish is still awaited to be performed.

Conclusion

Using absorption spectrum, column and thin layer chromatography, indicator reaction, and chemical conversion to bilirubin. The pigment responsible for the blue-green coloration of the blood plasma of *C. analis* had been identified as biliverdin.

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