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THE NOVEL BILIVERDIN-PROTEIN COMPLEX IN THE BLUE-GREEN SERUM OF WOOLY SCULPIN, *CLINOCOTTUS ANALIS* (PISCES: COTTIDAE).—A BLOOD BILIPROTEIN WITH A DIFFERENT BINDING CHARACTER

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Lee-Shing Fang (1985) The novel biliverdin-protein complex in the blue-green serum of wooly sculpin, *clinocottus analis* (Pisces: cottidae).—A blood biliprotein with a different binding character. *Bull. Inst. Zool., Academia Sinica* **24**(2): 155-164. The binding between the chromogen and the apoprotein of the blue-green biliverdin-protein complex in the blood serum of *Clinocottus analis* was examined by using a dithionite reduction method. Dithionite was found to reduce free as well as albumin bound biliverdin to bilirubin. However, it could not reduce the protein-bound biliverdin in the fish serum while it could reduce biliverdin which had been hydrolyzed from the protein moiety. Authentic biliverdin introduced into the blue-green serum could also be reduced. Dowex-1 resin selectively absorbed endogenous biliverdin from crude blood serum leaving off the decolorized serum which contained the carrier protein moiety. When the depigmented serum was incubated with free biliverdin, the liberated protein moiety did not bind the introduced chromogen.

These information suggest that biliverdin in the blood serum of *C. analis* is bound to its apoprotein in a different manner from those in the serum bilirubin- or biliverdin-albumin complexes which have been previously investigated.

The blood serum of *Clinocottus analis* (wooly sculpin) was found to have a very striking blue-green color (Low & Bada, 1974). The molecule responsible for the color has been identified as the bile pigment, biliverdin, which is bound to a protein moiety (Fang, 1982). Biliverdin is not present in significant concentration in human blood or in the blood of any other animals except in a few fishes (Yamaguchi *et al.*, 1966; With, 1968; Low & Bada, 1974, Fang, 1984) and in humans with severe pathological conditions of biliverdinaemia or green jaundice (Greenberg *et al.*, 1971; Fenech *et al.*, 1967; Lasson *et al.*, 1947).

The reduced form of biliverdin, bilirubin, a potentially toxic metabolic waste of heme degradation (Colleran & Heirwegh, 1979), is the bile pigment normally present in the blood of humans and other vertebrates. When transported in blood, bilirubin is bound to serum albumin which apparently reduces its toxicity (With, 1968; Blanckaert, 1979). Because of the clinical importance of bilirubin in human jaundice and kernicterus, the binding within the bilirubin-albumin complex has been well studied (Jacobsen, 1977; Jacobsen, 1972; Kuenzle *et al.*, 1976). These investigations thus provide a basis for comparing the binding properties of biliverdin-protein complex in the fish serum.

Considering the unique phenomenon of

the normal, continuous existence of biliverdin in the blood of *C. analis*, it is of interest to study the biliverdin-protein complex and compare it to the well studied bilirubin- or biliverdin-albumin complexes in the blood of other animals. From such studies, the mechanism of how the biliverdin in fish blood is maintained may be ascertained.

Two different approaches have been applied to examine and compare the binding character of the fish biliverdin-protein complex. First, a reduction method was established to examine the binding strength between biliverdin and its carrier protein. Then, the chromogen was isolated from the apoprotein by adsorption to an anion exchange resin and the liberated carrier protein moiety was examined for binding with re-introduced biliverdin.

MATERIALS AND METHODS

Experimental fish, reagents and solutions

C. analis, a fish with blue-green blood serum, and *Girella nigricans*, the control fish, were obtained by dip net from tidal pools near Scripps Institution of Oceanography, University of California, San Diego. After being caught, they were kept in running sea water for 24 hrs without feeding. Blood was drawn by cardiac puncture. The heparinized blood was pooled together and centrifuged immediately to obtain the blood serum samples. Serum was stored at 4°C and diluted 3 to 5 times with 0.9% (w/w) NaCl before use.

All solutions were freshly prepared before use. Standard biliverdin and bilirubin solutions (Sigma Chemical Co., St. Louis, MO, USA) were prepared in pH 7.4 phosphate buffer (Singleton and Laster, 1965) and stored in ice and shielded from direct light until use. Bovine serum albumin (Sigma Chemical Co.) was dissolved in distilled water to a concentration of 3 gm per 100 ml resulting in a pH of 7.1. This is the average albumin concentration in the fish blood (Feeney and Brown, 1974). Sodium dithionite (J. Baker Chemical Co., Phillipsberg, NY, USA) was dissolved in double distilled water and the solution was stored in ice. Dowex-1 resin (chloride form, 2% cross-linked, dry mesh size of 100-200, Sigma Chemical Co.) was prewashed with 1N HCl and rinsed with distilled water before use. XAD-2 resin (wet mesh size 20-50, Serva Co., Heidelberg, Germany; donated by Dr. A. Hofmann) was used in the dry form.

Examination of the binding strength between biliverdin and its protein moiety

Identification of the pigment from the serum of *C. analis* was carried out by reduction with sodium dithionite (modified from Oide and Utida, 1967). The reaction took place within seconds if the prosthetic group had first been hydrolytically separated and purified (Fang, 1982). The reaction that occurs is the reduction of green biliverdin to

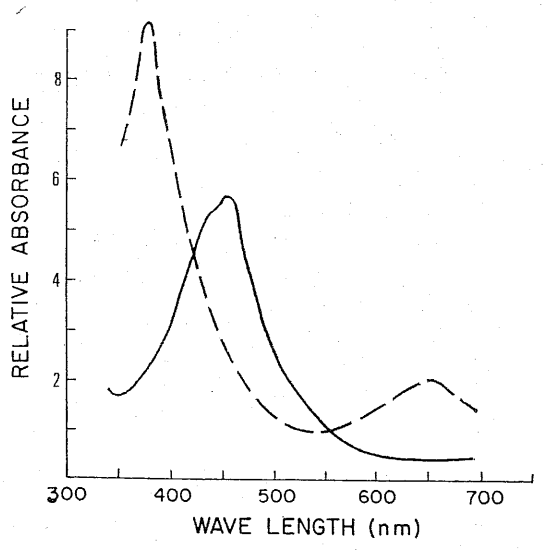

Fig. 1. The absorption spectrum change of a biliverdin-albumin solution after reduction agent (sodium dithionite) was introduced. Biliverdin-albumin (dashed line) solution was reduced to bilirubin-albumin (solid line). The shoulder in 430 nm was due to free bilirubin generated by replacement of bilirubin with dithionite from the bile pigment-albumin complex.

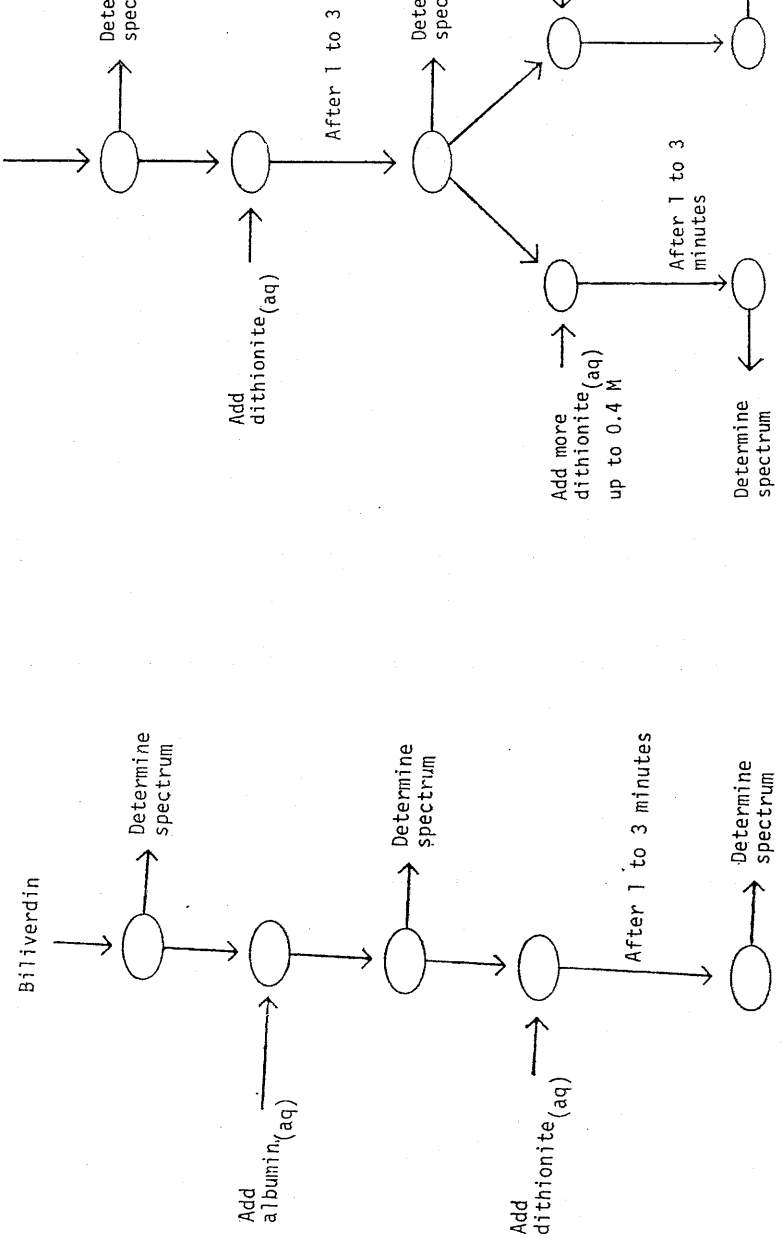

Fig. 2. (A) The test for biliverdin-albumin binding. The final biliverdin: albumin molar ratio was 1:1. The dithionite concentration was 42 mM.

(B) The test for fish serum biliverdin-protein binding. Two ml serum sample was used in each test. The dithionite concentration in aging test was 42 mM.

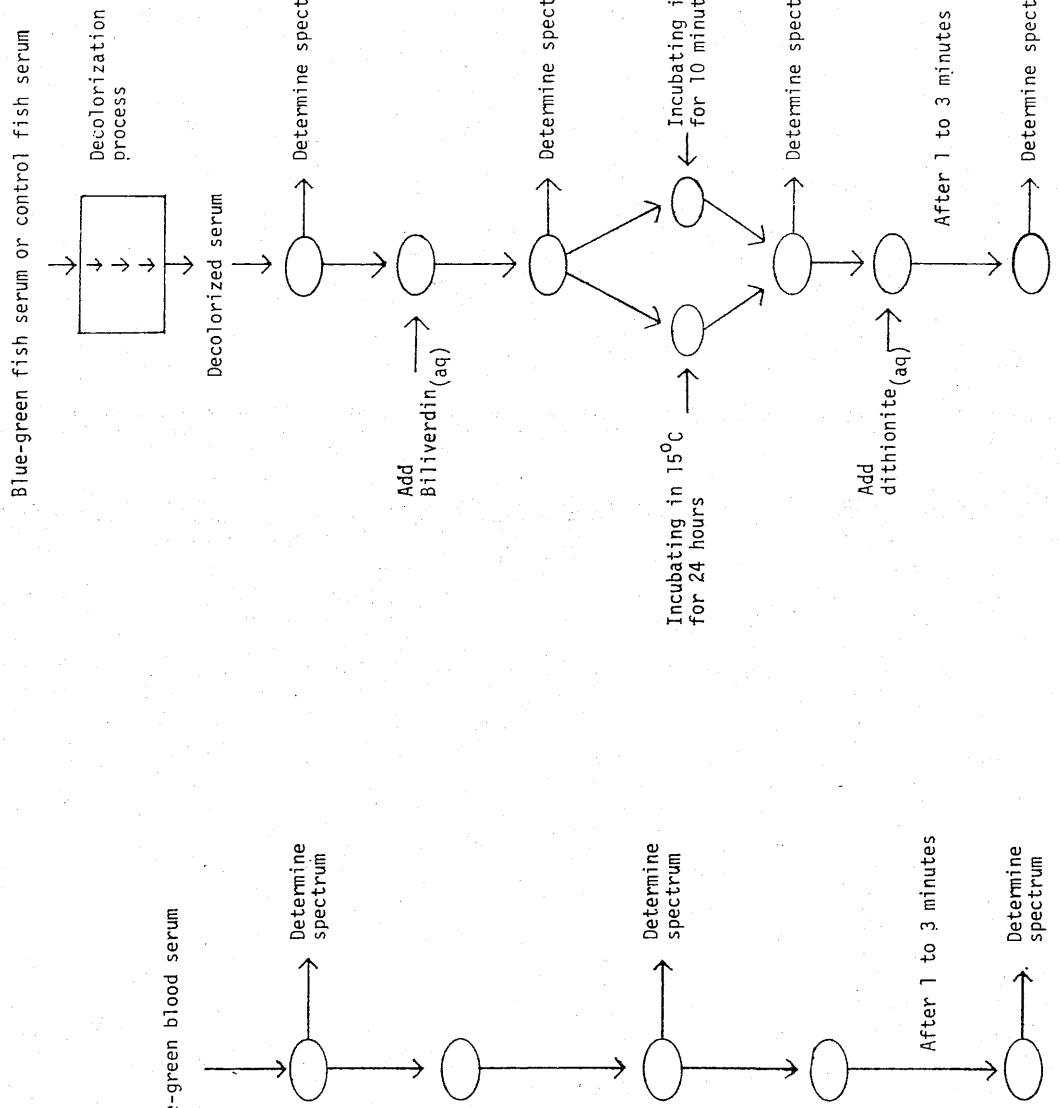

Fig. 2. (C) The test for the binding of the introduced biliverdin by the blue-green fish serum. 0.5 ml of 60 μM biliverdin solution was introduced into 2 ml of crude blood sample.
(D) The test for the binding of the introduced biliverdin by the protein moiety of the blue-green fish serum chromoprotein. Experimental condition was similar to that of scheme (A) except albumin was replaced by decolorized fish serum.

yellow bilirubin, which can be clearly monitored by the change in absorption spectrum (Fig. 1). The binding status of biliverdin in solution was thus detected by introducing a fixed amount of reducing power (sodium dithionite) and measuring the reaction time of the reduction. A similiar principle has been applied to distinguish direct (conjugated) and indirect (unconjugated) serum bilirubin; the reactions of the two types of bilirubin with diazo reagents have different reaction times (With, 1968).

Initially, several concentrations of sodium dithionite were used to determine the quantity of sodium dithionite needed to completely reduce a fixed amount of biliverdin (3 mg per 100 ml of serum, the highest recorded blood biliverdin concentration, Greenberg *et al.*, 1971) in 30 sec at room temperature. A final sodium dithionite concentration of 42 mM was found to be the most suitable. This concentration was thus used in the reduction reactions. The following procedures were used for different purposes:

(a) The binding strength between biliverdin and albumin was examined by the procedure outlined in Fig. 2(A).

(b) The binding strength of biliprotein in the fish serum was examined by the procedure in Fig. 2(B).

(c) To test if the blue-green serum can bind additionally introduced free biliverdin, and to measure the binding strength, the procedure in Fig. 2(C) was used.

(d) To test whether the liberated carrier protein of the fish serum can bind with introduced free biliverdin as strongly as it had bound with the endogenous biliverdin, the procedure in Fig. 2(D) was used.

Liberating the carrier protein from biliverdin in serum: decolorization of the crude blood serum

The protein moiety of the biliprotein was liberated following the method of Willson *et al.* (1972). In this procedure, protein-bound bilirubin in the blood serum was adsorbed and the plasma carrier protein was left intact. Dowex-1 and XAD-2 resins were packed in different mini-columns (8×0.6 cm) and 0.5 ml of the crude fish blood serum was passed through the columns. These two types of absorbent were tested because even though they both are effective in isolating bilirubin from human blood, they have different absorption mechanisms; Dowex-1 is an ion exchange resin while XAD-2 is a hydrophobic resin. Besides these analyses, another method modified from the bile acid isolation procedure of Van Berge Henegouwen and Hofman (1976) was also used. The crude serum and the resin (1:3, v/v) were incubated at room temperature with gentle shaking for 60 to 90 min. Then the whole slurry was packed in a column and eluted with distilled water.

To determine if the carrier protein moiety of the chromoprotein was fractionated by the resin, the blue serum chromoprotein purified by disc polyacrylamide gel electrophoresis (12% gel, Tris-glycine buffer, pH 8.3, temperature 4°C) was further incubated (60 min, 25°C) with Dowex-1 resin as described above. This resin-treated chromoprotein and the purified native blue chromoprotein were again applied to polyacrylamide gel electrophoresis to compare their electrophoretic patterns.

To examine whether only the biliverdin or the whole chromoprotein was adsorbed on the resin, the following test was performed. After the serum protein was eluted from the column, the blue-green colored column was eluted with 42 mM sodium dithionite solution (elution volume equal to bed volume), and the time of the color change on the column was recorded. If only the prosthetic group was adsorbed, the time of the color change will be similar to that of free biliverdin. If the whole chromoprotein is adsorbed on the resin, the color change time will resemble that of the intact fish serum chromoprotein.

RESULTS

Passing the blue serum through a column of either Dowex-1 or XAD-2 resin did not decolorize the blue fish serum as it would when bilirubin or biliverdin-albumin solutions

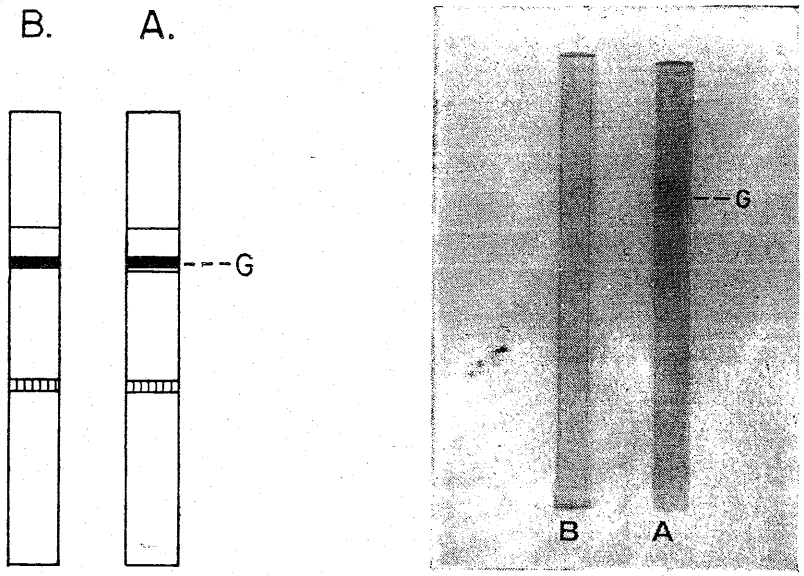

Fig. 3. The electrophoretic patterns of the serum chromoproteins with (gel A) or without (Gel B) the associated green colored chromogen (G). Conditions of electrophoresis are specified in text.

were applied (retention time 10 to 15 min). However, after prolonged incubation (>60 min) with Dowex-1, more than 95% (measured by absorbance decrease at 675 nm) of the chromogen was adsorbed to the resin. The incubation method with XAD-2 resin was not as successful as that of Dowex-1 treatment. The adsorption data thus shows indirectly that the fish serum biliverdin is more tightly bound to its protein moiety than bilirubin or biliverdin to albumin.

The decolorized chromoprotein has the same electrophoretic pattern as that of the purified native chromoprotein (Fig. 3). This indicates that the protein moiety was not fractionated by the decolorization process.

The introduction of sodium dithionite reduced the green prosthetic group absorbed on the Dowex-1 resin column to yellow. The reaction happened in seconds after the reagent reached the green pigment. This demonstrated that only the chromogen but not the whole chromoprotein, which cannot be reduced by sodium dithionite, was adsorbed on the column. This result and the electrophoresis experiment indicate that the protein moiety is left unchanged in the serum after the decolorization processes and thus suitable for further studies.

The results of experimental scheme (A) (Fig. 2) are presented in Fig. 1. Both free biliverdin and biliverdin bound to albumin were completely reduced to bilirubin within 2 min after dithionite was introduced. The results of scheme (B) are shown in Fig. 4. The same amount of dithionite that reduced the biliverdin in the biliverdin-albumin complex could not reduce the biliverdin in the biliprotein of the blue-green fish serum. Furthermore, reduction did not occur even the reaction time was increased to half an hour or if the concentration of dithionite was increased up to 10-fold.

The results of scheme (C) are given in Fig. 5. This experiment showed that biliverdin added to the blue-green serum was not bound as tightly as was the endogenous pigment based on following observations: First, the absorption increase at 675 nm was completely decreased after the reducing agent was introduced. Second, the absorption peak at 375 nm, due to the introduced biliverdin, had changed to 430 nm, a peak of bilirubin absorption. The shoulder appeared around 470 nm

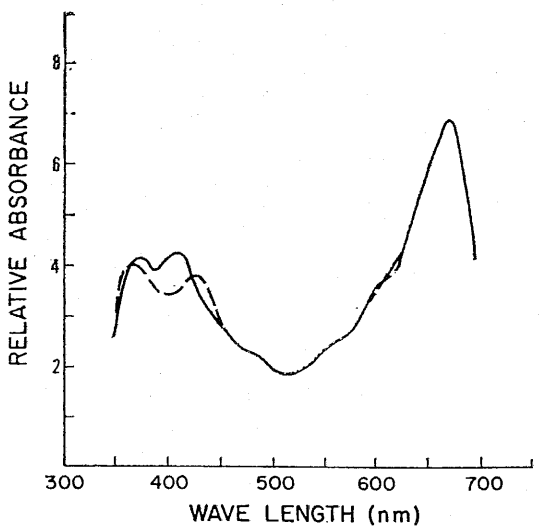

Fig. 4. The absorption spectrum change of the blue-green fish serum after reducing agent (sodium dithionite) was introduced. The new peak around 430 nm (dashline) may be due to the presence of bilirubin which resulted from the reduction of trace amounts of free or nonspecified protein bound biliverdin in the fish serum.

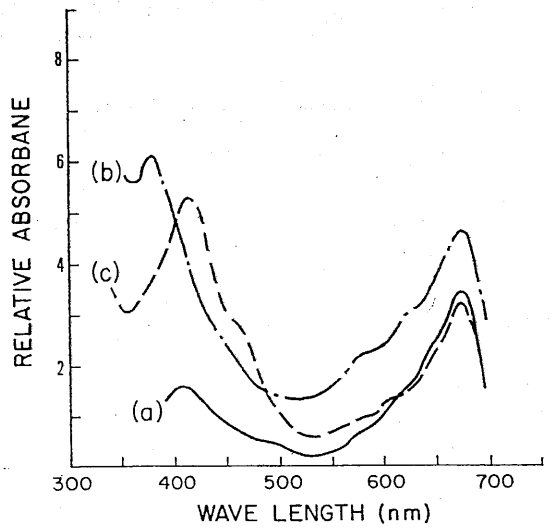

Fig. 5. The result of the test for the binding strength of the introduced biliverdin in the blue-green fish serum.
(a) The spectrum of the original blue serum.
(b) Spectrum after standard biliverdin was introduced to (a).
(c) After dithionite solution was introduced to (b).

of the dash-line spectrum was due to the spontaneous formation of protein bound albumin which absorbs at 468 nm. In experimental scheme (D) the change in the absorption spectrum was similiar to that shown in Fig. 1. The biliverdin incubated with the decolorized blood serum from either the experimental or control fishes was reduced by dithionite. This suggests that even with the free carrier protein in the serum, introduced biliverdin cannot be bound as the original biliverdin.

All these results strongly suggest that the biliverdin-protein complex in the blood serum of *C. analis* has a much stronger binding between the bile pigment and the carrier protein than serum biliverdin- or bilirubin-albumin complexes found in other animals, and that this binding is of different nature.

DISCUSSION

When biliverdin or bilirubin is introduced into blood serum or an albumin solution, binding between the chromogen and the protein occurs spontaneously (Odell, 1959, Ahlfors, 1981). It was demonstrated in this study that this is not the case with the biliverdin-protein complexes in the fish blood since the biliverdin introduced into the blue-green fish serum was reduced while that of the endogenously bound was not. This result, however, does not exclude the possibility that the added biliverdin was not bound simply because the biliverdin carrier protein in the blue-green fish serum was already sautrated with endogenous biliverdin. This possibility was ruled out by the experiment in which the carrier apoprotein, liberated from biliverdin, was still unable to bind introduced biliverdin.

The results of the electrophoresis studies suggest that, during the liberation process, the protein moiety was not denatured or dis-associated. However, since one electrophoretic band is not necessarily composed of only one protein, this was inconclusive. The whole blue-green biliprotein could possibly have been adsorbed onto the resin and isolated from the

serum, leaving off some other protein(s) which happened to have the same electrophoretic mobility. Fortunately, the reducible character of the adsorbed biliverdin, and its quick reaction time, demonstrated that the separated chromogen was not bound to the protein moiety; therefore only the chromogen and not the whole biliprotein was isolated from the serum. These results, taken together, thus suggest the formation of the fish serum biliverdin-protein complex does not occur spontaneously as it is in other vertebrates, which provides additional evidence for the presence of a different type of binding in the biliprotein complex in the fish serum.

The linkages between bile pigment and protein in biliproteins of marine animals have been classified into two groups: non-covalently and covalently bound (Yamaguchi, 1971). The biliverdin in blue-green fish serum is apparently non-covalently bound since it could be absorbed by resins and was isolated by cold HCl (3%)-methanol treatment (Fang, 1982). This is similar to the biliprotein in the blood of *Anguilla japonica* which was also suggested to bear a non-covalent linkage (Yamaguchi *et al.*, 1966). However, those biliproteins in the muscle of *Cheilinus undulatus* and the scale of *Scarus gibbus* were suggested to be covalent bound (Yamaguchi Matsuura, 1969, Yamaguchi *et al.*, 1977).

The formation of this uniquely inert biliverdin-protein complex in the fish serum may provide a possible explanation for the continuous presence of biliverdin in the blood of some fishes.

CONCLUSION

The binding between biliverdin and its carrier protein moiety in the blue-green blood serum of *C. analis* is much stronger and different in nature when compared with the bindings of previously known serum biliverdin- or bilirubin-albumin complexes. This special linkage may account for the retention of biliverdin in the blood of this fish.

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藍綠色魚血清中膽綠素與其載負蛋白體結合强度之研究

方　力　行

膽綠素之所以在某些血液中長期存在的原因未明。此研究中發展了一種還原測試反應來檢定膽綠素和其載負蛋白體的結合强度。並經由其他分離及再結合過程實驗，指出此種魚類血液中膽綠素一蛋白體之結合强度遠較一般動物血液中之膽色素一蛋白體者爲大。這種特殊的强結合方式，使得膽綠素得以長期留在魚血液中。