STUDIES ON THE BLUE GREEN PIGMENTS OF THE INTEGUMENTS OF FISHES

(1) THE ISOLATION OF ICHTHYOVERDIN FROM THE SCALES OF SAURIES, *COLOLABIS SAIRA* BREVOORT

By

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The integuments of the pelagic fishes appear generally green or blue. Very little is known about the chemical nature of these biochromes and as described later a few investigations have given conflicting results.

The blue pigment of the fin of *Crenilabrus* species, first studied by von Zeynek (1) (2), is related to phycoproteid. Lemberg(3) indicated the similarity between the pigment of *Crenilabrus* species and phycocyan obtained by Kylin in 1910 and by Lemberg in 1930 from certain algae. According to Fontaine (4) (5), the blue and blue green pigment of the serum in labrid fishes and the rose pigment in the serum of certain members of the cyclopteran group are phycochromoproteins, related closely to phycocyan and phycoerythrin respectively. Therefore, he named them ichthyocyan and ichthyoerythrin. These pigments, however, have not yet been definitely established as unique compounds.

Willstaedt(6) reported that the green pigment of the skeleton of the gar fish, *Belone belone*, and eel-pout, *Zoarces viviparus*, was composed of two constituents, one of dark olive green and the other of emerald green, which have benzidine peroxidase action; that the green pigments of the skin and scales of the sea-scorpion, *Cottus scorpius*, were extractable by water as chromoprotein, and that, the pigment of these three species all gave positive Gmelin reaction. Çağlar(7) also investigated the green pigment of the skeleton and integument of the gar fish. As the prosthetic group the pigment extracted with a mixture of equal volumes of 95 per cent alcohol and 10 per cent hydrochloric acid was positive in Gmelin reaction, and was identical with biliverdin in its solubilities. It was found colorimetrically that 2.2 mg of the solid green material obtained

by him contained 0.13 mg of biliverdin.

In spite of many investigations, isolation of the pigment as a crystal and the determination of the chemical structure have not been successful.

The present authors are the first to succeed in the isolation of the blue green pigment in crystalline form from the scales of the saury and have designated tentatively the pigment as "*ichthyoverdin*".

Experimental

1. Extraction and crystallization.

100 kg of fresh scales of sauries were gathered from the holds of the fishing boats, where a vast volume of fallen scales accumlated, at the fish market of Shiogama. The extraction of the pigments were done principally by following Lemberg's method (8).

The scales were washed with water, dipped in acetone and then in ether to remove the fatty materials. Ash removal was made by treating with 5 per cent hydrochloric acid for 30 minutes. The scales were again washed thoroughly with sufficient water until the reaction of Ca^{++} and Cl^{-} were negative and then they were dehydrated by the press. The blue green pigments of the scales were extracted with 10 per cent hydrochloric acid in methyl alcohol.

The optical densities of the solution were measured by the Beckman spectrophotometer model D. U. and the absorption curve obtained is shown in Fig. 1. The absorption maxima are found at 700 mμ and 375 mμ.

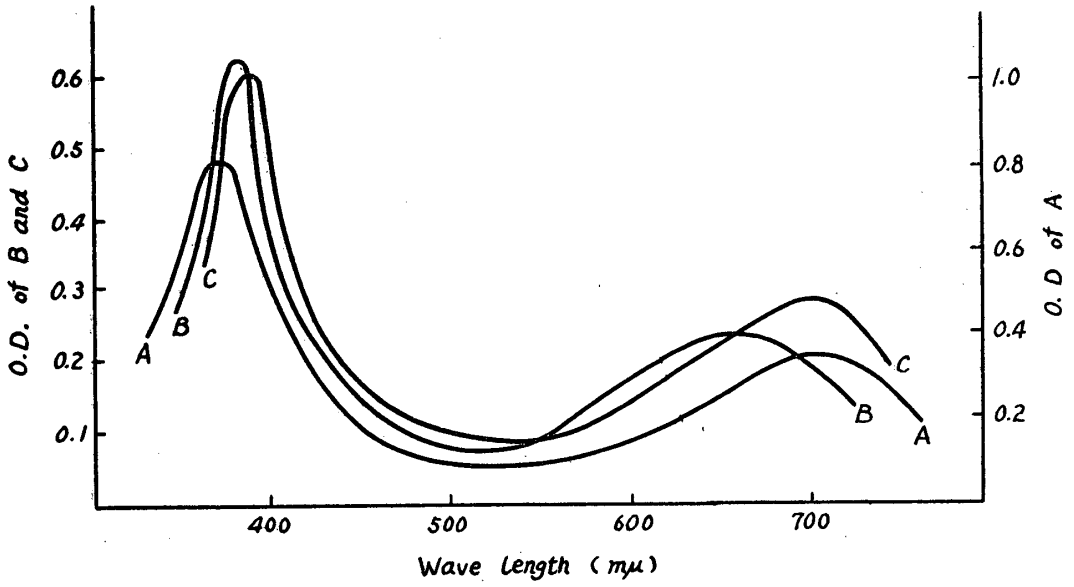

Fig. 1. The absorption curves of the pigment solution.
(A) The solution extracted with 5 per cent HCl-CH_3OH.
(B) The neutral extract solution.
(C) The bluish green needles in 5 per cent HCl-CH_3OH.

Then, the blue green solution was neutralized with saturated sodium carbonate solution and filtered. The absorption maxima of the filtrates shifted to 660 mμ and 377 mμ as shown in Fig. 1. Next, peroxide free ether was added to the solution in the separating funnel and shaked. Saturated sodium acetate solution was added to the mixtures. The pigment passed into the ethereal layer. After washing with water the ethereal solution was treated with an equal volume of one per cent hydrochloric acid. Then the acid solution was washed with ether and stored in the refrigerator.

After a few days, bluish green needles, frequently accompanying with amorphous materials, crystallized out (Plate 1). The yield of this pigment was 65 mg. The needles may be the hydrochloride of the pigment.

The crystals were dissolved in 15 per cent hydrochloric acid in absolute methyl alcohol and allowed to stand at room temperature. After 12 hours the esterified solution of the pigment was evaporated in vacuo. The residue obtained was digested with ether and dissolved in a little amout of methyl alcohol. Then the solution was introduced into the mixture of ether and dilute sodium acetate solution. The pigment passed into the ethereal layer. This ethereal solution was repeatedly washed with two per cent sodium carbonate solution for removing the unesterified pigments, and then immediately with water. The solution was filtered, dehydrated with anhydrous sodium sulphate and evaporated in a stream of nitrogen at low temperature. The residues were digested with petroleum ether and a little ether, taken up in hot absolute methyl alcohol. Thereafter, the solution was concentrated. Only 5 mg of dark green platelets crystallized out. This may be methyl ester of the pigment. The small yield may be due to incomplete esterification. The absorption curve in methyl alcohol solution has its maxima at 690 mμ and 378 mμ. The methyl ester was dissolved in methyl alcohol and chromatographed on magnesium oxide, which gave two fractions. One was the main fraction giving blue green in methyl alcohol and showing the absorption maxima at 675 mμ and 377 mμ. The other was a very small quantity. Its methyl alcohol solution gave a green yellow colour and showed the absorption maxima of 710 mμ and 380 mμ. It is uncertain whether the latter is an artifact or not. These absorption curves are shown in Fig. 2.

Next, the ether was poured into the main fraction of the pigment dissolved in methyl alcohol in the separating funnel. By the addition of water to the mixture the pigment passed into the ethereal layer. The dark green platelets with a violet blue surface colour as shown in Plate 2 crystallized out. Some crystals have oblique faces. The yield was extremely low. The substance was named for the sake of convenience ichthyoverdin methyl ester by the authors. The crystal form is similar to that of oocyan ester (9) and of glaucobilin ester (10).

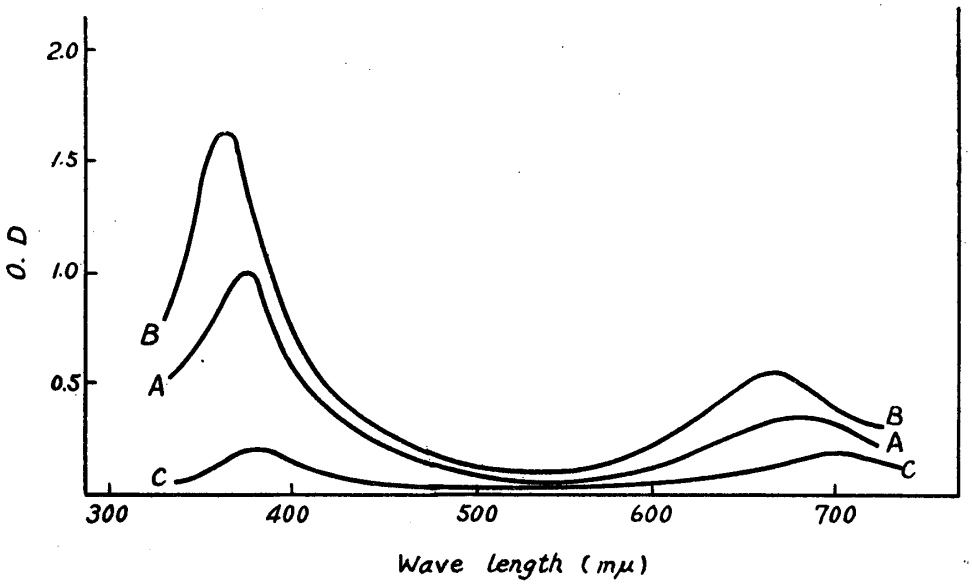

Fig. 2. The absorption curves of the platelets in CH_3OH.
(A) Before chromatography.
(B) The blue green fraction.
(C) The greenish yellow fraction.

2. The reactions and the properties of ichthyoverdin.

When the scales were dipped in the alkaline solution, the blue green pigment changed to a faint greenish yellow. If the scales were put into the acidic condition, the pigment returned again to blue green, but its colour tinge is not very similar to the original one. On treatment with Gmelin reagent the pigment in the scale shows the later stage of the reaction. Ichthyoverdin of the scales could not be extracted with the general organic solvents but were extractable with HCl-CH_3OH or with HCl-CH_3COCH_3. The general properties of ichthyoverdin methyl ester are given in Table 1. The reaction of bilatrienes (11) (12) was made as follows: to the ichthyoverdin methyl ester in ammoniacal alcohol some zinc acetate and very small amounts of iodine were added.

Table 1. The properties of the dark green platelets.
(Ichthyoverdin methyl ester)

Melting point	206–207°C
Absorption maximum	377 mμ, 675 mμ
Gmelin reaction	positive
van den Bergh reaction	negative
Reaction of bilatrienes	positive
Fluorescence	nil
Solubilities (room temperature)	
Water	insoluble
Chloroform	soluble
Methyl alcohol	soluble
Ether	slight soluble
Petroleum ether	insoluble

The blue green solution with intense red fluorescence was found by means of the ultra-violet lamp. Therefore, it is thought that ichthyoverdin is one of bilatrienes.

The optical properties of ichthyoverdin methyl ester were observed by the polarization-microscope. The results are as follows :

the place of polarization is (A) parallel to the long axis of the platelet (X′) : greenish brown, (B) perpendicular to the long axis (Z′) : greenish blue. In the case of crossed Nicol's prism long axis of the crystal is (a) in rectangular position : approximate extinction, (b) in 45° position : greenish blue.

Therefore, the crystal is pleochroic and may belong to either the tetragonal, hexagonal or rhombic system because of the straight extinction. These characters resemble those of uteroverdin ester (8).

3. Gmelin reaction for the blue green pigments in the several fish integuments.

The fresh fish was scaled and small amounts of the reagent were placed on the integuments. The colour reactions were observed. The fishes employed and the results obtained are as follows :

Species	Reaction
Saury (*Cololabis saira* BREVOORT)	+
Bonito (*Katsuwonus vagans* LESSON)	?
Mackerel (*Scomber japonicus* HOUTTUYN)	+
Horse-mackerel (*Trachurus japonicus* TEMMINCK & SCHLEGEL)	?
Herring (*Clupea pallasii* CUVIER & VALEN)	+
Sardine (*Sardinia melanosticta* TEMMINCK & SCHLEGEL)	+

As the reagent is composed of strong acid, the fish integument contracts and is decomposed. Accordingly, the reagent was diluted moderately with water. Although each stages of colour change could not be observed, the change from blue green to red purple could be confirmed. The pigment of the part which is rich in calcareous material, as the skull, reacted well with the reagent.

The dermal blue pigments of ordinary pelagic fishes probably consist of bile pigments, though the observations on bonito and horse-mackerel were doubtful. It was impossible to extract the dermal blue green pigment with HCl-CH_3OH.

Discussion

Ichthyoverdin appears faint sky blue in the dried scales, but deep blue green in wet condition. It occurs mainly in the exposed portion of the scales and in the covered portion the pigment was observed to be extremely faint. The observation coincides with the report of Çağlar(7) who states that if the scales appear green only in one part, the lack of colour in the remaining part

does not indicate a lack of the pigment, but that the hyalodentine layer of the coloured part shows locally a greater thickness. It is an interesting problem why ichthyoverdin appears mostly in the exposed portion of the scales.

In spite of the similarity between ichthyoverdin in saury scales and the blue green pigment in the derm of saury, the latter can not be extracted with HCl-CH_3OH. This is probably because of the difference of combined substance to the pigment.

The absorption curves of ichthyoverdin are different from those of biliverdin. Various absorption maxima of biliverdin, however, have been found by many workers (11) (13) (14) (15). These differences might be principally due to the impurities that coexisted with biliverdin. Hence, it may be difficult to identify the pigments by the absorption curves solely. But the absorption maximum of ichthyoverdin methyl ester nearly coincides with that of oocyan methyl ester in acid methyl alcohol solution (680~670 mμ) (9). Moreover, it is an interesting fact that the difference of the absorption maxima between ichthyoverdin in HCl-CH_3OH and that in methyl alcohol (700 mμ − 660 mμ = 40 mμ) is equal to the difference of the absorption maxima of biliverdin in the same conditions (680 mμ − 640 mμ = 40 mμ).

The observation of ichthyoverdin methyl ester under the polarization microscope is similar to that of uteroverdin methyl ester.

The melting point of ichthyoverdin methyl ester lies between those of uteroverdin dimethyl ester and oocyan dimethyl ester. In addition the melting point of ichthyoverdin ester nearly coincides with that of turboglaucobilin(16).

Considering from the above facts ichthyoverdin might be a unique substance. Lemberg (17), however, reported that as the differences of the physical and chemical properties between oocyan and uteroverdin were explained by the dimorphism, uteroverdin, oocyan and biliverdin might be considered as identical. On the other hand, Willstaedt (6) indicated that the pigment of *Belone belone* was not identical with biliverdin.

On the contrary Çağlar (7) (18) (19) believed that the pigment of *Belone belone* was biliverdin. Our experimental date are not sufficient for the identification. The authors, however, can claim that ichthyoverdin belongs to bilatrienes.

The accurate identification is retained for another opportunity.

Summary

(1) For the first time the blue green pigment was isolated in the crystalline form from the scales of the sauries (*Cololabis saira* BREVOORT). This pigment was named tentatively *ichthyoverdin* by the authors.

(2) The absorption maxima of ichthyoverdin methyl ester lie at 377 mμ and 675 mμ in methyl alcohol. The melting point is 206~207°C. The optical

properties by the polarization-microscope and the solubilities for the several solvents were determined. From the results it is believed that ichthyoverdin belongs to bilatrienes.

(3) The dermal blue green pigments of fishes, for instance, saury, sardin, mackerel and herring, gave positive Gmelin reaction, though horse-mackerel and bonito were doubtful. Therefore, the dermal blue green pigment of the pelagic fish contains bile pigment.

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Résumé

Pour la première fois nous les auteurs avons isolé le pigment bleu-vert des écailles du poisson de mer, *Cololabis saira*, sous la forme crystalline. Ainsi, nous lui avons donné à l'essai le nom de *ichthyoverdine*.

Les maxima d'absorption d'ester méthylique de l'ichthyoverdine sont vus sur 675 mμ et 377 mμ dans la solution d'alcool méthylique. Le point de fusion de celui-ci se produit à 206～207°. Les propriétés optiques de l'ichthyoverdine par le microscope polarisant et ses solubilités dans quelques solvants ont été déterminées. En considerant les résultats obtenus, il semble que l'ichthyoverdine appartienne à la catégorie de bilitriènes.

Les pigments des épidermes de certains téléostéens marins, *Cololabis saira*, *Scomber japonicus*, *Clupea pallasii*, *Sardinia melanosticta*, qui sont colorés en vert, en bleu ou en bleu-vert, présentent positivement la réaction de Gmelin. Cependant, chez les *Katsuwonus vagans* et *Trachurus japonicus*, dont les épidermes sont colorés pareillement, la Gmelin ne se présentent pas clairement.

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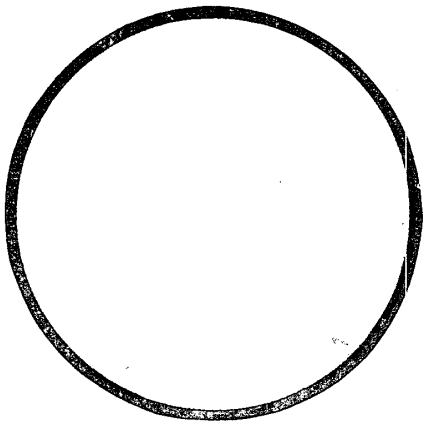

Plate 1. The bluish green needles (Ichthyoverdin hydrochloride).
(×625)

Plate 2. The dark green platelets (Ichthyoverdin methylester).
(×625)